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Characterization and cellular localization by monoclonal antibodies of the 60 kDa mannose specific lectin of human promyelocytic cells, HL60

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Myelomonocytic lineage cells express an M_r 60 000 mannose specific lectin, MR60 (Pimpaneau *et al.* (1991), *Carbohydr Res* **213**: 95–108). Under non-reducing conditions, this protein migrates as a 120 000 protein. MR60 does not contain any *N*-glycan moiety cleavable by the action of *N*-glycanase. MR60 induces a sugar selective aggregation of beads coated with glycosylated albumin: beads bearing α-D-mannosyl residues are aggregated while beads bearing α-D-glucosyl residues are not. A monoclonal antibody Lec101B, specific for MR60, recognizes a single M_r 60 000 protein by Western blotting. This monoclonal antibody does not label the cell surface of cells expressing MR60, but decorates intracellular vesicles upon permeabilization of these cells.

Keywords: confocal microscopy; macrophage lineage; neoglycoproteins

Introduction

Mature human macrophages express a mannose specific cell surface lectin mediating the endocytosis of mannose-terminated glycoproteins (for review see [1]). This mannose receptor of 175 kDa is not present at the surface of immature cells of the monocytic lineage (HL60 promyelocytes or U937 promonocytes). However, in these cells an intracellular mannose specific receptor has been demonstrated by using fluoresceinylated mannoside substituted bovine serum albumin upon permeabilization [2]. By affinity chromatography on a mannoside substituted column, analogous to that used to isolate the 175 kDa mannose specific receptor from murine macrophages, a 60 kDa protein was isolated from HL60 membranes [2]. In order to characterize more precisely this new mannose specific receptor, the expression of which appears related to the state of maturation, monoclonal antibodies were prepared and used to perform a biochemical analysis of this lectin.

Materials and methods

Affi-Gel 102 was from Bio-Rad (Richmond, CA, USA), methyl α-D-mannopyranoside, Tween-20, 1,4-diazabicyclo-[2-2-2] octane (DABCO), proteinase inhibitors (aprotinin, antipain, leupeptin and pepstatin A) and 4-chloro 1-naphtol reagent were purchased from Sigma (St Louis, MO, USA). Phenylmethylsulfonylfluoride (PMSF) and Coomassie blue were from Serva (Heidelberg, Germany). Peroxide-free Triton X-100 was obtained from Pierce (Oud-Beijerland, The Netherlands). Iodogen was from Bio-Rad (Buckinghamshire, England), $Na^{125}I$ (3.7 GBq ml^{-1}) and X-ray film (hyperfilm-MP) from Amersham (Buckinghamshire, England).

Neoglycoproteins

Man-BSA and Glc-BSA were prepared as previously described [3, 4]. These neoglycoproteins contained 25 ± 3 sugar residues per molecule as determined by the sulfuric acid micromethod [5].

Monoclonal antibodies

MEM59 directed against leukosialin (CD43) was kindly given by V. Horejsi [6]. F21023, an IgM monoclonal antibody directed against an L1210 tumour antigen, was obtained as previously described [7].

Cells

HL60 promyelocytic cells (kindly given by Dr Le Floch, Rhône-Poulenc Rorer, Vitry-sur-Seine, France) and U937 promonocytic cells (kindly given by Dr T. Turtz, Villejuif, France) were grown in RPMI 1640 medium supplemented

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with 10% fetal calf serum, 2 mM L-glutamine and antibiotics. SP2/O/Ag mouse myeloma cells [8], kindly provided by G. Buttin (Institut de Biologie Moléculaire, Paris, France), were grown in MEM 0111 medium with twice the concentration of amino acids and vitamins, supplemented with 2 mM L-glutamine and antibiotics. P388D1, mouse macrophage-like cells were grown as ascites tumour in DBA/2 recipient mice (9).

Purification of the mannose binding protein

Membrane extracts from HL60 or P388D1 cells were prepared as previously described [2]; the solubilized membrane proteins were purified by chromatography on activated agarose beads substituted with 4-isothiocyanatophenyl α-D-mannopyranoside. Proteins specifically eluted with 0.2 M α-methyl mannopyranoside, named MR60, were further analysed. In some preparations, membrane proteins were iodinated before affinity chromatography, using iodogen [10].

Agglutination assay

Neoglycoproteins (Man-BSA and Glc-BSA) were covalently linked to carboxylated polystyrene beads (2μm in diameter) (Polysciences, Eppelheim, Germany), according to the carbodiimide procedure recommended by the manufacturer.

Purified MR60 was solubilized (80 μg ml^{-1}) in agglutination buffer (10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 40 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Triton X-100, 0.2% BSA). A two-fold serial dilution of MR60 solution was performed in a microtitrator U plate (Greiner, Frickenhausen, Germany); 30 μl of each dilution was mixed with 30 μl of the substituted beads suspension (2.5×10^8 beads per ml). After 30 min, agglutination was assessed under a microscope.

Polyacrylamide-gel electrophoresis and isoelectric focusing

Mannose specific proteins were subjected to sodium dodecylsulfate-polyacrylamide (10%) gel electrophoresis (SDS-PAGE) according to Laemmli [11] and stained with Coomassie blue. The isoelectric focusing was carried out in glass tubes (5 mm × 10 cm) packed with a 4% polyacrylamide gel containing 1% Triton X-100, 8 M urea and pH range 3–10 ampholytes (LKB, Bromma, Sweden) under reducing conditions. After overnight isoelectric focusing (300 V), the experiment was stopped. The gel was immediately immersed in 2.5% trichloroacetic acid in distilled water. After washing, the gel was stained with Coomassie blue.

Enzyme digestion

Enzyme digestion was performed by using *N*-glycanase [12, 13]. α1-Acid glycoprotein was used as a control (100 μg ml^{-1}). MR60 was isolated from 2×10^8 cells by affinity chromatography and solubilized in 10 mM Tris/HCl, pH 7.4, buffer containing 150 mM NaCl, 15 mM $CaCl_2$, 1 mM $MgCl_2$, 0.15% Triton X-100, 0.2 M mannose. Proteins were precipitated with TCA (20% final concentration). The pellet was washed with acetone, solubilized in 10 μl 0.2 M Na_2HPO_4 buffer containing 0.5% SDS and 0.1 M β-mercaptoethanol and boiled. Then, 20 μl *N*-glycanase (Genzyme Corp., Boston, MA, USA) solubilized in 0.2 M Na_2HPO_4, pH 8.6, buffer containing 15 mM EDTA and 0.5% Triton X-100, was added (0.02 U final). Incubations were carried out at 37 °C for 12 h. The enzyme reaction was stopped by the addition of 30 μl of Laemmli sample preparation buffer. Samples were boiled and subjected to SDS-PAGE, autoradiography was performed with an intensifying screen.

Monoclonal antibodies, anti MR60

Biozzi's BP strain mice, selected for their high antibody production [14], were immunized twice with 10 μg purified MR60. The first injection was intraperitoneal in the presence of complete Freund's adjuvant and the second was intrasplenic without adjuvant [15] on day 15 (3 days before fusion).

The fusion of splenocytes with SP2/O/Ag mouse myeloma cells and the selection of hybrids were performed as already described [16]. A dot radio-immunological assay on Hybond-C-super membrane (Amersham), adapted from [17], was used for the detection of anti-MR60 producing hybridoma. Briefly, 100 μl of each culture supernatant were poured into a well of a Bio-Dot apparatus (Bio-Rad) covered with a Hybond membrane. After 2 h incubation at room temperature and two washes with 0.02% Tween-20 in PBS, the membranes were incubated for 3 h with ^{125}I-labelled MR60. The membranes were washed once with PBS and twice with distilled water, dried and then autoradiographed for 1 or 3 days at −80 °C with an intensifying screen. A myeloma cell culture supernatant was used as a negative control.

Positive hybridomas were subcultured using the limiting dilution method; 10^6 cells of a positive clone were injected into pristane primed BALB/c × BP hybrid mice or nude mice. Ascites fluid was collected and stored at −20 °C. Isotype determination was based on an immunoenzymatic assay using antigen-coated plates and the 'Mouse typer sub-isotyping kit' from Bio-Rad.

Western blotting

4×10^5 HL60 cells were pelleted, suspended in 16 μl of Tris/HCl buffered saline, pH 7.4, containing 0.5% Triton X-100, 2 mM PMSF, 2 mM EDTA and after centrifugation (10000 × **g**, 15 min), the supernatant was boiled for 5 min. The samples were loaded on SDS-PAGE (10% acrylamide). Following electrophoresis in Laemmli buffer, gels were electrotransferred to nitrocellulose and then filters were soaked for 1 h at 37 °C in 5% BSA-PBS and probed with the appropriate monoclonal antibody (Lec101B, MEM59 or F21023) diluted in 1% BSA-PBS. Lec101B was diluted

1:5, MEM59 1:200 and F21023 1:50. A peroxidase labelled anti-mouse-IgG(H + L) (Bio-Rad) was used as a second antibody and, after washing, strips were developed with 4-chloro-1-naphthol reagent, 15 min at room temperature; the reaction was stopped by a 0.1 N HCl bath.

Immunofluorescence staining

Cells were first fixed at 4 °C for 1 h in PBS containing 1% paraformaldehyde. Cells were then washed twice with complete PBS (PBS supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) and incubated for 1 h at room temperature with 200 µl of the ascites fluid diluted 1:100 or 1:50 in 1% BSA, 0.1% saponin in complete PBS. Cells were further washed twice and incubated for 1 h at room temperature in 200 µl of FITC-conjugated sheep anti-mouse IgM (Immunotech, Marseille, France) diluted 1:100 in 1% BSA, 0.1% saponin in complete PBS. Cells were then washed, put on a microscope slide and blotted to glass by cytocentrifugation. A drop of 1,4-diazabicyclo-[2-2-2] octane (DABCO) (50 mg ml^{-1} in PBS:glycerol 1:1) was added on the cells to avoid photobleaching [18].

Fluorescence confocal laser scanning microscopy

Fluorescence microscopy was performed using a microscope (OPTIPHOT, Nikon) associated with a scanning confocal imaging system (MRC 600 system) from Bio-Rad supplied with a krypton/argon laser and equipped with an excitation filter set (488, 568 and 647 nm). The oil-objective magnification was 60-fold with a 1.4 numerical aperture. Each image was averaged by scans lasting 1 s each. A focal series of eight horizontal planes of 1 µm spaced sections was performed.

Results and discussion

Characterization of the MR60

HL60 and P388D1 membranes were extracted with Triton X-100 in a buffer containing proteinase inhibitors; the unextracted debris was removed by centrifugation (100 000 × **g**, 30 min) and the supernatant subjected to a mannose-agarose column as described previously [2]. A purified preparation of the mannose receptor which exhibits an M_r of 60 000 was specifically eluted by α-methyl-mannoside as shown by SDS-PAGE (Fig. 1); this protein was named MR60. Uncer non-reducing conditions, the MR60 migrates as a 120 000 M_r protein corresponding to a dimer of MR60 (Fig. 2). The weak band corresponding to a protein of 120 000 observed in some preparations on SDS-PAGE under reducing conditions (Fig. 1) was probably due to an incomplete dissociation or to a partial reassociation of the MR60.

Two mannose specific receptors were isolated from P388D1 cells. These cells possessed most of the characteristics of normal macrophages [9], and the 175 000 M_r mannose specific receptor expressed on various primary

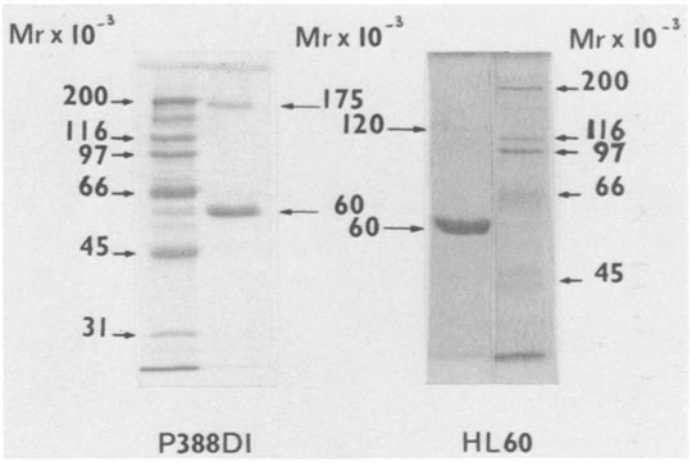

Figure 1. Polyacrylamide gel electrophoresis under reducing condition of HL60 and P388D1 membrane proteins eluted specifically with α-methyl mannose from the mannose-agarose column. Mannose specific proteins were purified from Triton X-100 extracts of 10^6 HL60 and P388D1 cells and subjected to 10% polyacrylamide gel electrophoresis in the presence of SDS under reducing conditions and stained with Coomassie blue. Arrows indicate the MR60 and the 175 kDa macrophage lectin. Standard protein markers were: Myosin heavy chain (200 000 M_r), D-galactosidase (116 000), phosphorylase b (97 000), BSA (66 000), ovalbumin (45 000), carbonic anhydrase (31 000) and soybean trypsin inhibitor (21 000).

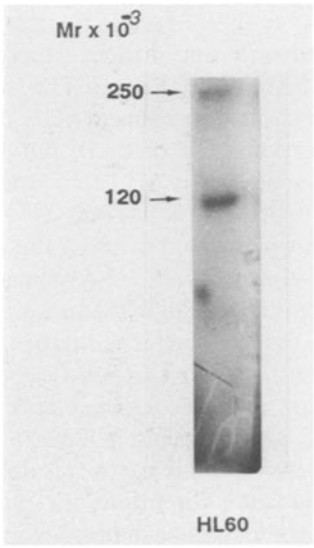

Figure 2. Polyacrylamide gel electrophoresis without reducing agent of the mannose specific receptor, MR60, isolated from HL60 membranes. MR60 was purified from HL60 membranes as above and subjected to a 7% polyacrylamide gel electrophoresis in the absence of any reducing agent. Proteins were stained with Coomassie blue.

macrophages [19, 12] was expected on these cells. However, while murine peritoneal macrophages expressed only this mannose receptor, which is known to mediate endocytosis of mannosylated ligands, the P388D1 tumour cells expressed

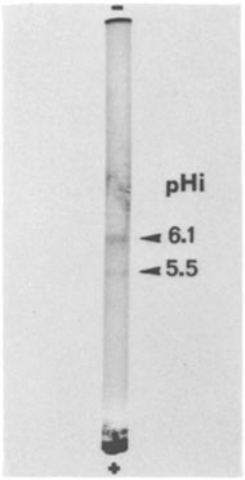

Figure 3. Isoelectric focusing polyacrylamide gel electrophoresis of the HL60 mannose specific lectin, MR60. Isoelectric focusing of MR60 was performed in a 4% polyacrylamide gel containing 1% Triton X-100, 8 M urea and pH range 3–10 ampholytes in glass tubes. Proteins were stained with Coomassie blue after protein precipitation with TCA 2.5%

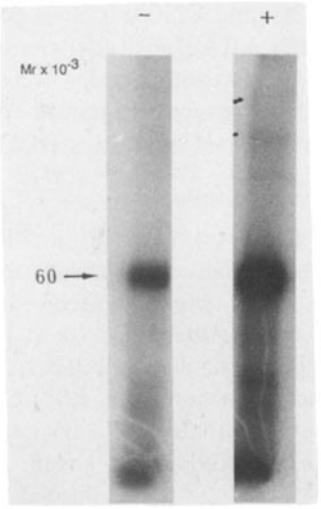

Figure 4. *N*-glycanase treatment of MR60. After TCA precipitation of purified MR60 and solubilization in 0.5% SDS in the presence of 0.1 M dithiothreitol, MR60 was subjected to *N*-glycanase treatment for 12 h at 37 °C. The HL60 mannose specific protein was subjected to SDS-PAGE before (−) and after (+) *N*-glycanase treatment.

in addition, the 60 000 M_r mannose receptor. The presence of MR60 in different cell types and the modulation of its expression are under investigation.

All further experiments were made with MR60 purified from HL60 cells or U937 cells, because elution with mannose produced only one protein which migrates as a single band of 60 000 in SDS-PAGE. The isoelectric point, determined by isoelectric focusing PAGE under reducing conditions, was around 6.1 (Fig. 3), but a minor band corresponding to a isoelectric pH of 5.5 was also visible.

To determine whether the MR60 is a glycoprotein, deglycosylation was performed by using the *Flavobacterium meningoseptum* *N*-glycosidase F (*N*-glycanase) [13], which cleaves both complex and high mannose structures. Deglycosylation was first conducted on native α1-acid glycoprotein, which bears five *N*-glycan moieties (45% of the total weight) and has an apparent molecular weight of 44 000. After *N*-glycanase, two proteins of 25 000 and 23 000 M_r were observed corresponding to two isoforms (data not shown). Under the same conditions the migration of the iodinated MR60 protein was unchanged (Fig. 4), suggesting that this protein does not bear any *N*-glycan moiety.

Agglutination properties of the MR60

According to a common definition [20], a lectin has at least two sugar binding sites and agglutinates cells bearing sugar moieties corresponding to their sugar specificity. The putative agglutinating capacity of the purified MR60, solubilized in 1% Triton X-100, in the presence of divalent cations, was checked using beads substituted with neoglycoproteins. Agglutination was obtained with beads substituted with Man-BSA (Fig. 5) but not with Glc-BSA substituted

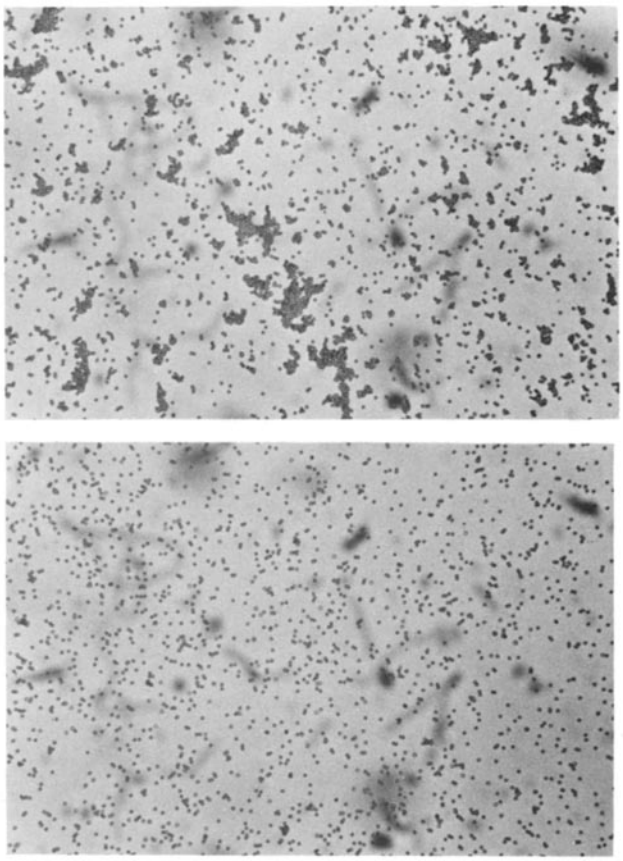

Figure 5. Lectin activity of the HL60 mannose specific protein, MR60. Polystyrene beads substituted with ManBSA (top) were aggregated by the protein MR60 solubilized in 1% Triton X-100. Agglutination was checked 30 min after incubation at room temperature. GlcBSA (bottom) substituted polystyrene beads, used as control, were not agglutinated.

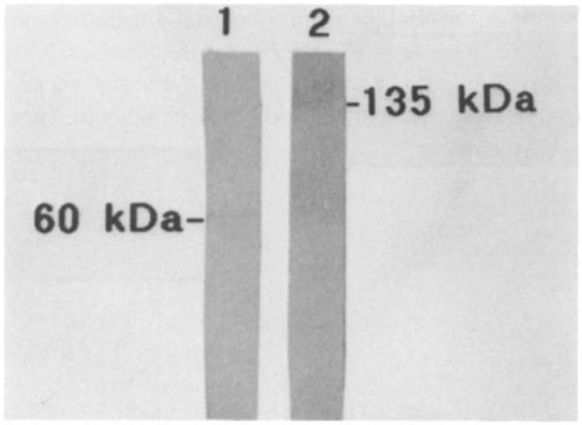

Figure 6. Western blotting of the HL60 mannose specific lectin, MR60. Following polyacrylamide gel electrophoresis of solubilized HL60 membrane proteins, proteins were blotted and stained either with the Lec101B anti-MR60 monoclonal antibody (lane 1) or with the anti-CD43, MEM59 antibodies (control, lane 2). Peroxidase labelled anti-IgG (H + L) was used as a second antibody and visualized by oxidation of 4-chloro-1-naphthol. The position of MR60 (60 kDa) and of leukosialin (135 kDa) are indicated.

beads, indicating that this protein is a lectin and confirming that this lectin is specific for mannose but not for glucose.

Preparation and characterization of anti-MR60 monoclonal antibodies

The MR60 protein obtained from U937 solubilized membrane was checked by SDS-PAGE before injection into mice. Fusion between splenocytes on immunized mouse and SP2 gave 271 growing hybridoma; two were selected for their reactivity with iodinated MR60 isolated from HL60 cells. One of them, named Lec 101B, was further subcultured and characterized; subclone Lec101B belongs to the IgM, κ type. Ascites fluids were produced and the specificity of the monoclonal antibody was checked by Western blotting. After SDS-PAGE of HL60 membrane proteins and electrotransfer on to nitrocellulose, blots were incubated either with Lec101B, or F12023, an IgM used as control, or with MEM59, a monoclonal antibody directed against leukosialin. No band was visible with the unspecific F21023 IgM (data not shown), while a thin band corresponding to a 60000 M_r protein was observed with the blot incubated with Lec101B (Fig. 6). Under the same

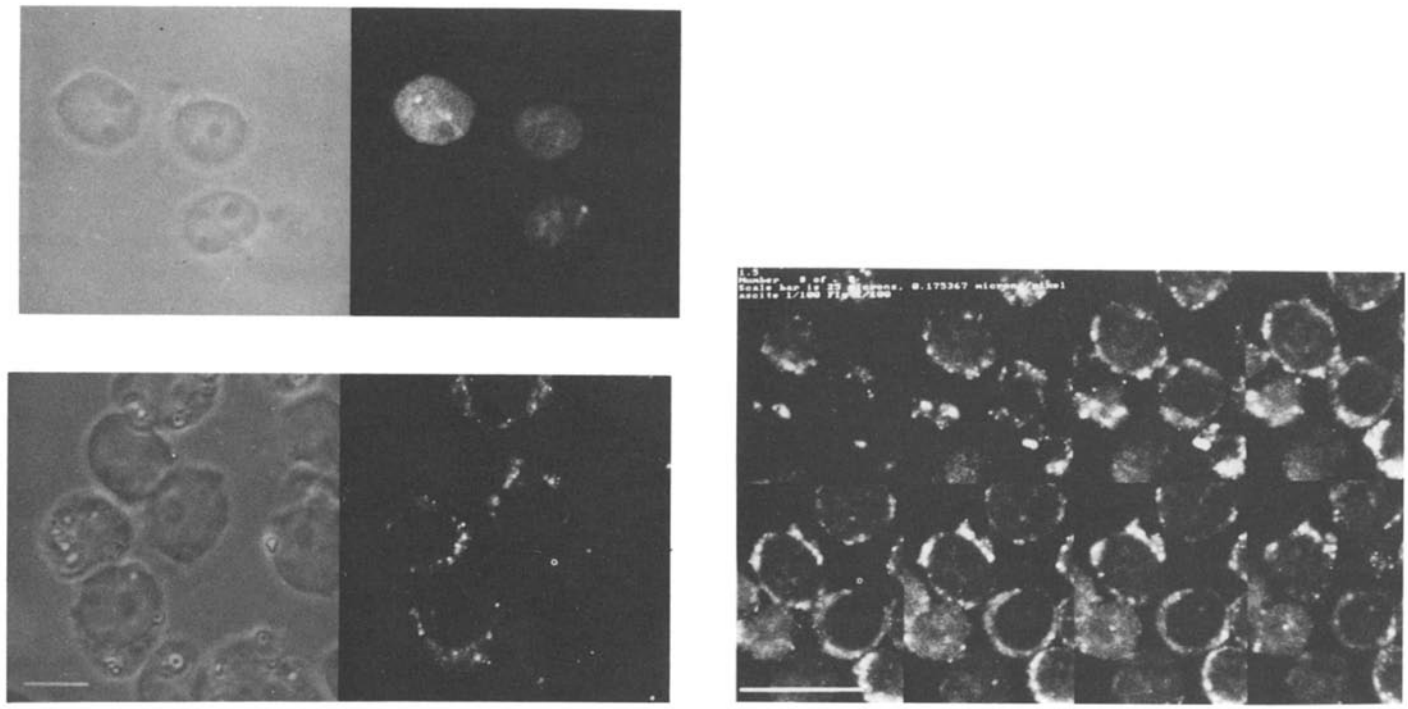

Figure 7. Localization of the HL60 mannose specific lectin, MR60, by confocal microscopy. HL60 cells fixed with 1% paraformaldehyde were permeabilized with saponin (0.1%) and were incubated at 20 °C with ascites fluid from Lec101B antibody (1/100) (b, c) or ascites fluid from F21023, an IgM used as a control (a). After 1 h further incubation with a fluoresceinylated antibody anti-IgG (H + L) (1:100) used as a second antibody, cells were washed and mounted in glycerol:DABCO (v:v). Confocal observations were made after overnight at 4 °C: in (a) and (b) contrast phase images and fluorescence images corresponding to one section are shown; in (c) serial images representing eight sections are shown.

conditions MEM59 revealed a band corresponding to 135000 M_r protein (Fig. 6); leukosialin is present in large amount in these cells [6].

Cellular localization of the MR60 lectin by immunofluorescence

Immunofluorescence experiments were performed with HL60 and U937 cells. After permeabilization with saponin of paraformaldehyde treated HL60 and U937 cells, the monoclonal antibody Lec101B labelled intracellular vesicles around the large nucleus (possessing nucleoli) visible in contrast phase transmission view (left part of photo, Figs 7a and 7b). In Fig. 7c, eight planes of cell section spaced by 1 μm, observed from the top to the bottom of the cells, show the intracellular localization of MR60. Ascites monoclonal antibodies generated against an antigen of the L1210 lymphocytic tumour cell line belonging to the same IgM type [7] were used as a control. In this case, only a weak fluorescent background was observed in the cell (Fig. 7a), indicating that the labelling of intracellular organelles by Lec101B is not due to non-specific recognition of the IgM. Without permeabilization, the cells were not labelled (data not shown).

Conclusions

In conclusion, the mannose receptor isolated from human promyelocytic cells HL60 and from human promonocytic cells U937 is quite different from the mannose receptor isolated from human macrophages [12]. The macrophage mannose receptor is a glycoprotein, the molecular mass of which ranges from 162000 to 180000 depending on the source. Conversely, the mannose receptor isolated from immature monocytic cells is a 60000 M_r protein and does not contain any *N*-glycan moities. Furthermore the MR60 is an intracellular lectin, localized at the level of intracellular vesicles; MR60 is not involved in endocytosis of mannosylated ligands in contrast with the macrophage mannose receptor. The role of this new lectin has not been deciphered. Efforts are underway to clone MR60 cDNA in order to elucidate the differentiation dependent expression of this new mannose receptor.

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