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PG-M1: A New Monoclonal Antibody Directed against a Fixative-Resistant Epitope on the Macrophage-Restricted Form of the CD68 Molecule

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A new anti-macrophage monoclonal antibody (PG-M1) was produced by immunizing BALB/c mice with fresh spleen cells from a patient with Gaucher's disease. PG-M1 reacts strongly with a fixative-resistant epitope of an intracytoplasmic molecule, selectively expressed by virtually all macrophages of the human body. Although attempts to immunoprecipitate the molecule recognized by PG-M1 have failed so far, the reactivity of the antibody with COS-1 and WOP cells transfected with a human complementary DNA clone encoding for the CD68 antigen suggests that PG-M1 is a new member of the CD68 cluster. However, unlike other CD68 antibodies (KP1, EBM11, etc.), which react with both macrophages and myeloid cells, PG-M1 detects a fixative-resistant epitope on the macrophage-restricted form of the CD68 antigen. In 957 routinely fixed, paraffin-embedded samples, PG-M1 showed a more restricted reactivity with elements of the monocyte/macrophage lineage than the previously described monoclonal antibodies MAC-387 (anti-calgranulins), KP1 (CD68) and Ki-M1P. Among hematological malignancies, PG-M1 only labels acute leukemias of M4 and M5 type and rare examples of malignant histiocytosis/true histiocytic sarcoma. In contrast, acute leukemias of the M1, M2, M3, M6, M7, and L1-L3 types, non-Hodgkin's lymphomas, and Hodgkin and Reed–Sternberg cells of Hodgkin's disease are consistently PG-M1-negative. In the daily diagnostic practice, PG-M1 seems to be particularly valuable for the diagnosis of myelomonocytic or monocytic leukemia and neoplasms of true histiocytic origin in routine paraffin sections. (Am J Pathol 1993, 142:1359–1372)

In the past, immunohistochemical identification of histiocytes in paraffin sections of normal and pathological human tissues was achieved by using rabbit polyclonal antisera against lysozyme and/or α_1-antitrypsin.[1–5] However, these two markers suffer from several drawbacks. Lysozyme is not expressed by all elements of the monocyte/macrophage system and is also present in normal myeloid elements at various stages of maturation from the promyelocyte to the segmented forms.[1,2] Furthermore, it is a highly diffusible substance that may be passively absorbed by damaged cells, at times making the interpretation of the immunohistological results difficult.[5] In human lymphohemopoietic tissues, α_1-antitrypsin has been detected not only on histiocytes, but also on activated lymphoid cells and related neoplasms, e.g., CD30-positive anaplastic large cell lymphomas.[6] Furthermore, lysozyme and α_1-antitrypsin are expressed in

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several nonlymphohemopoietic tissues (salivary glands, Paneth cells, etc.) and tumors (hepatocarcinoma, germ cell tumors, etc.).[5]

A number of monoclonal antibodies (MAbs) recognizing human monocyte/macrophage-associated antigens (CD11c, CD13, CD16, CD18, CD31, CD32, CD35, CD36, CD68, Ber-MAC3)[7,8] have been generated and used to define different stages of differentiation/activation of monocytes/macrophages in normal and pathological human tissues.[8,9] Unfortunately, these antibodies are of limited value in routine diagnostics, as they can only be applied to frozen sections. Two MAbs (MAC-387 and KP1)[10–12] directed against fixative-resistant epitopes of the calgranulins (or cystic-fibrosis-proteins)[13] and CD68 molecule respectively, have become commercially available and are widely employed reagents for detecting cells of the monocyte/macrophage lineage in paraffin sections. These antibodies, however, suffer from several drawbacks: a) MAC-387 only reacts with a subset of macrophages[10]; b) both MAC-387 and KP1 (CD68) stain at different extent myeloid elements[10–12]; and c) MAC-387 shows a wide reactivity with normal and neoplastic epithelial cells.[10] More recently, Radzun et al[14] reported on a new panmacrophage marker (the Ki-M1P MAb) and claimed that it is more specific than KP1 (CD68), in that it reacts only with macrophages but not with granulocyte precursors.

In this paper, we describe a new MAb (PG-M1) directed against a fixative-resistant epitope of the macrophage-restricted form of the CD68 molecule and provide evidence that, in routinely fixed, paraffin-embedded samples, PG-M1 shows a more restricted reactivity with elements of the monocyte/macrophage lineage than the previously described MAbs MAC-387 (anti-calgranulins),[10] KP1 (CD68),[11] and Ki-M1P.[14]

Materials and Methods

Preparation of the Antigen

The source of the cells for immunization was a large spleen (2 kg wt) from a 20-year-old woman (GR) with Gaucher's disease being treated at the Department of Internal Medicine, Perugia University. The spleen was removed because of severe thrombocytopenia (20×10^3 platelets). The cells were extracted by teasing pieces of splenic tissues through a steel meshwork, suspended in HEPES-buffered (RPMI) 1640 medium and separated on a Lymphoprep density gradient. May-Grünwald-Giemsa-stained cytocentrifuge preparations showed that Gaucher's cells accounted for more than 80% of the total mononuclear cell population. These cells were used for the immunization.

Immunization Schedule and Fusion

BALB/c mice were immunized three times intraperitoneally at 10-day intervals with 20×10^6 spleen cells. A fusion was carried out three days after the last injection, as previously described.[15] Fused cells were plated out into 24-well plates (Costar) containing hypoxouthine-aminopterin-thymidine (HAT) medium. Hybridoma growth was observed in most of the wells after 10 days. The initial screening of supernatants was performed by the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique on cryostat sections from the spleen involved by Gaucher's disease and normal human tonsil. Supernatants from wells showing restricted reactivity with Gaucher's cells and tonsil macrophages were further tested on formalin- and B5-fixed tonsil samples. Cloning of the selected hybridomas was carried out by a limiting dilution technique in flat-bottom 96-well plates. Determination of the Ig subclass was performed by using an INNO-LIA mouse MAb isotyping kit (Dunn Labortechnik GmbH, Asbach, FRG).

Human Cell Lines

A variety of human cell lines (Table 1) were tested for their reactivity with PG-M1 on cytospins prepared from exponentially growing cells. The U-937 cell line was also tested before and after induction of maturation with 12–0-tetradecanoylphorbol-13-acetate (Sigma, Munich, FRG) at a concentration of 80 nmol/L. The cells were incubated throughout the whole experiment with 12–0-tetradecanoylphorbol-13-acetate, i.e., from day 1 to day 10. During the stimulation, samples were taken every day for cytospins and immunostaining.

Cytological Samples

Mononuclear cells from the peripheral blood of 20 normal volunteers were separated by Ficoll-Hypaque gradient centrifugation and suspended at the concentration of 1×10^6/ml. Cells were cytocentrifuged and immunostained with the PG-M1 MAb. Human peripheral blood lymphocytes were stimulated with phytohemagglutinin for 4 days. At hours 0, 72, and 96, cytospins were prepared for

Table 1. *Reactivity of PG-M1 with Established Human Cell Lines**

Cell line	PG-M1	Cell line	PG-M1
T-ALL cell lines		Hodgkin cell lines	
HPB-ALL	–	L-428	–
MOLT-4	–	L-591	–
		L-540	–
HTLV1-transfected T cell line		Cole	–
Hut 102	–	Histiocytoma cell line	
MT-2	–	SU-DHL-1	–
HTLV-2 transfected T cell line	–	Cutaneous T-cell lymphoma cell line	
Mo-T		HUT 78	–
Pre–B cell lines		HUT 102	–
Nalm-12	–		
		Myeloid cell lines	
EBV–transformed B cell lines (non-Burkitt)		K-562	–
B95–8 (monkey)	–	U937	–
BJA-B	–	HL-60	+ (~1%)
Cess	–	KG-1	–
		THP-1	+†
Burkitt's lymphoma		Carcinoma cell line	
Daudi	–	A-431‡	–

* Immunostaining was performed by the APAAP technique in cytospins prepared from exponentially growing cells (see Materials and Methods).
† Weak positivity; ‡ Human epidermoid carcinoma.

immunostaining with the PG-M1 MAb. Native alveolar macrophages were obtained by bronchial lavage, washed in RPMI medium, and cytocentrifuged for immunostaining or prepared for immunoprecipitation.

Tissue Specimens

Samples from a variety of normal human tissues were studied for their reactivity with PG-M1. Human tonsils were obtained from children with recurrent tonsillitis who underwent tonsillectomy at the Ear-Nose-Throat Department, Perugia University Hospital. Normal liver, lymph node, and spleen samples were from patients who underwent exploratory laparotomy for Hodgkin's disease and were found not to be involved by the disease. Normal thymuses were from children who had open heart surgery. Other normal tissues were removed for diagnostic or therapeutic purposes or were obtained at the time of autopsy.

Immunostaining with PG-M1 was also performed on 876 pathological specimens collected at the centers involved in the study and four cases of true histiocytic sarcoma from the Workshop of the Fifth Meeting of the European Association for Haematopathology (Bologna, September 21–25, 1992). The Expert Panel and the participants agreed that these four neoplasms were derived from histiocytes, on the basis of the following morphological features and antigenic markers: diffuse proliferation of large cells with an abundant, often foamy, cytoplasm and irregularly shaped nuclei; positivity of neoplastic cells for lysozyme, CD45 (all cases but one), and KP1 (CD68) and negativity for T-cell and B-cell antigens, the lymphoid-associated antigen CD30, granulopoietic antigens (chloro-acetate-esterase, neutrophilic elastase, myeloperoxidase), and epithelial- and melanoma-associated antigens.

Processing of Specimens

The samples were fixed in formalin, B5, or Bouin-fluid according to standard methods. Jamshidi bone marrow biopsies were fixed according to the following schedules: a) most biopsies were fixed either in B5 (2 hours) or formalin (12 to 24 hours) and decalcified in Decal (2 hours); b) bone marrow biopsies from one of the centers involved in the study (Cologne) were fixed in an aldehyde solution for 12 to 48 hours (2 ml 25% glutaraldehyde, 3 ml 37% formaldehyde, 1.58 g calcium acetate, and distillated water per 100 ml) and decalcified for 3 to 4 days in 10% buffered ethylenediaminotetraacetic acid (EDTA); c) a few bone marrow biopsies underwent fixation/decalcification in Zenker/acetic acid for 18 to 24 hours.[2]

Before immunostaining with PG-M1, 4-μ paraffin sections were dewaxed and hydrated through gradient alcohols. Sections from formalin-fixed tissues were incubated at 37 C either in 0.1% trypsin/0.1% calcium chloride solution (pH 7.8) for 20 to 30 minutes or in 0.05% Protease XIV solution in Tris-buffered saline (TBS pH 7.6) for 5 minutes. No proteolytic treatment was applied to sections cut from B5- and Bouin-fixed material. Fresh samples of normal and pathological tissues were snap-frozen in liquid nitrogen, cut in a cryostat, air-dried overnight

at room temperature, fixed for 10 minutes in acetone, and either stained immediately or stored at −20 C until staining.[16,17]

Monoclonal Antibodies

PG-M1 was used as undiluted culture supernatant. In a number of cases, the reactivity in paraffin of PG-M1 was compared with that of two previously described MAbs (KP1 and Ki-M1P).[11,14]

The KP1 (CD68), Y2/131 (CD68), and anti-calf intestine alkaline phosphatase MAbs were kindly provided by Dr. D.Y. Mason, John Radcliffe Hospital, Oxford, U.K.; the MAb Ki-M1P was a generous gift from Dr. H.J. Radzun, University of Kiel, FRG. The SHCL-3 and Ber-MAC3 MAbs were provided by one of the authors (H. Stein). The rabbit anti-mouse immunoglobulin antibody was purchased from Dako a/s, Glostrup, Denmark.

Immunoenzymatic and Fluorescence-Activated Cell Sorter Studies

Cytospin preparations and tissue sections (frozen and paraffin) were stained by the APAAP technique, as previously described.[18] Endogenous alkaline phosphatase was blocked by adding levamisole to the substrate solution.[18] Slides were then counterstained for 5 minutes in Gill's hematoxylin and mounted in Kaiser gelatin or Apathy's.

Suspensions of native alveolar macrophages obtained from bronchial lavage were incubated (60 minutes at 4 C) with equal amounts of the following antibodies: PG-M1, IOT-8A (as unrelated negative control antibody of the same subclass, IgG3, as PG-M1), SHCL-3 (CD11c), and Ber-MAC3 (as positive control antibodies). After washing, the cells were incubated with fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulins (Sigma, Deisenhofen, Germany) at a dilution of 1:40 and fixed with 1% paraformaldehyde. The cells were analyzed by a fluorescence flow cytometer (EPICS, Profile Analyser).

Characterization of the Antigenic Epitope

Immunoprecipitation Studies

Radiolabeling of cells and immunoprecipitation were carried out according to Wano et al[19] with some modifications. Experiments were performed on activated peripheral blood macrophages,[20] THP-1 cells, and alveolar macrophages. Briefly, 3×10^7 cells were washed twice in methionine-free RPMI 1640 medium (Seromed, Berlin, FRG) and labeled in the same medium supplemented with 10% fetal calf serum and 1 mCi (37 MBq) of L[^{35}S]methionine (NEN, Dreieich, FRG). After 4 hours at 37 C in a humid atmosphere with 5% CO_2, the cells were harvested and washed three times with phosphate-buffered saline containing 2 mg/ml of cold methionine. Cells were treated with phosphate-buffered saline containing 1% Nonidet P-40 (NP-40), 1 mmol/L phenylmethylsulfonylfluoride, and 10 mmol/L EDTA (lysis buffer). Preabsorption was done with 0.2 ml of packed anti-mouse IgG agarose beads (Sigma, Deisenhofen, FRG) for 2 hours at 4 C. Immunoprecipitation was carried out with 10 µl anti-mouse IgG agarose bead (Sigma, Deisenhofen) preincubated with 1 ml of PG-M1 hybridoma supernatant, or with an unrelated control antibody, for 30 minutes at 4 C. After washing six times in lysis buffer, the sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out according to Laemmli's method.[21]

In some experiments, glycoproteins from solubilized alveolar macrophages were purified by retention with Lenti-Lectin-Sepharose 4B. Bound substances were eluted by 1 methyl-alpha-D-mannoside and dialyzed in phosphate-buffed saline containing 1% Nonidet P-40 and protease inhibitors. Glycoproteins were labeled with ^{125}I using Iodo-Beads (Pierce, The Netherlands) and the immunoprecipitation performed as described for the internal labeling procedure.

Transfection of COS-1 and WOP Cells

For transient expression of CD68, monolayers of COS-1[22] and WOP cells[23] were transfected by the DEAE-dextran method[24,25] with CDM8 expression vector[26] containing CD68 complementary (c)DNA insert.[27,28] Cytospins of COS-1 and WOP cells were immunostained (APAAP procedure)[18] with PG-M1 48 hours after transfection. The MAb Y2/131 (CD68) was used as positive transfection control.

Enzymatic Treatment of Tissue Sections

For the characterization of the nature of the epitope recognized by the PG-M1 MAb, cytospins from CD68-transfected COS cells and paraffin sections from macrophage-rich tissues were digested overnight in a humid chamber, before immunostaining, with various enzymes, including: chymotrypsin (1 mg/ml), trypsin (1 mg/ml), pepsin (1 mg/ml), and proteinase (1 mg/ml) in TBS with 2 mg/ml $CaCl_2$.

Cytospins from CD68-transfected COS cells were also incubated overnight at 37 C with the following enzymes: Endo F (2 U per cytospin), Endo H (100 mU per cytospin), and N-glycosidase F (50 U per cytospin); in addition, cytospins were treated with sodium periodate (30 minutes at 4 C in 0.05 mol/L NaOAc, pH 4.5).

Results

Production of the PG-M1 MAb

The supernatant from 1 out of the 500 hybridoma colonies screened on frozen sections from the spleen of patient GR stained Gaucher's cells and reacted strongly with macrophages in formalin- and B5-fixed, paraffin-embedded tonsil samples. This hybridoma was cloned three times to produce the clone PG-M1 (IgG3 subclass) that was used in subsequent studies.

Reactivity of PG-M1 with Human Cells Lines

As shown in Table 1, none of the human cell lines studied was reactive with PG-M1, with the exception of THP-1 and phorbol-stimulated U937 cells. In the latter cells, expression of PG-M1 antigen started on day 4 of stimulation and lasted until the end of experiment (day 10) with a maximum between days 6 and 9.

Effect of Sample Processing on PG-M1 Reactivity

The PG-M1 MAb gave optimal immunocytochemical labeling in paraffin sections from normal and pathological tissue samples fixed in formalin, B5, or Bouin-fluid. In all samples, cells of the monocyte/macrophage lineage showed a strong cytoplasmic (diffuse or granular) staining pattern. Proteolytic treatments usually enhanced the intensity of the staining in formalin-fixed sections. The optimal processing schedule for bone marrow biopsies was fixation in B5 or formalin followed by Decal decalcification. In contrast, overnight fixation/decalcification in Zenker/acetic acid gave inconsistent results due to the high background staining.

In frozen sections, the reactivity pattern of PG-M1 was identical to that observed in paraffin sections but the intensity of staining was weaker.

Fluorescence-activated cell sorter studies with native alveolar macrophages showed no difference between the percentage of cells stained by PG-M1 (about 11% positive cells) and an unrelated negative control antibody (IOT-8A) of the same subclass (IgG3) (about 13% positive cells). The positive control antibodies SHCL-3 and BER-MAC3 stained 88% and 86% of cells respectively. The blank value (performed only with the fluorescein isothiocyanate–conjugated anti-mouse antibody) was 9% positive cells. These findings demonstrate that the antigen recognized by PG-M1 is not expressed on the cell surface.

Table 2. *Reactivity of PG-M1 in Paraffin Sections from Normal Human Tissues**

Tissues	No. Samples 77 (49)*	Reactivity in paraffin
Lymphoid tissues		
Bone marrow	10 (5)	Macrophages, megakaryocytes†
Tonsil	10 (10)	GC and IF macrophages
Lymph node	8 (5)	GC, IF, and sinus macrophages
Spleen	10 (5)	White and red pulp macrophages
Thymus	5 (2)	Cortical and medullary macrophages
Nonlymphoid tissues		
Lung	2 (1)	Alveolar macrophages
Kidney	3 (2)	Interstitial macrophages
Liver	5 (5)	Kupffer cells
Skin	10 (5)	Dermal macrophages
Stomach	2 (2)	Lamina propria macrophages
Gut	2 (2)	Lamina propria macrophages
Pancreas	1	Interstitial macrophages
Thyroid	2 (2)	Interstitial macrophages
Brain	1	Rare glial elements
Skeletal muscle	1 (1)	Rare macrophages
Uterus	1	Scattered macrophages
Testis	2 (2)	Scattered macrophages
Prostate	1	Scattered macrophages
Placenta	1	Scattered macrophages

GC = germinal center; IF = interfollicular.
* The number in parentheses indicates the cases that were available as frozen sections.
† A weak positivity of a percentage of megakaryocytes was observed in about 20% of bone marrow samples.

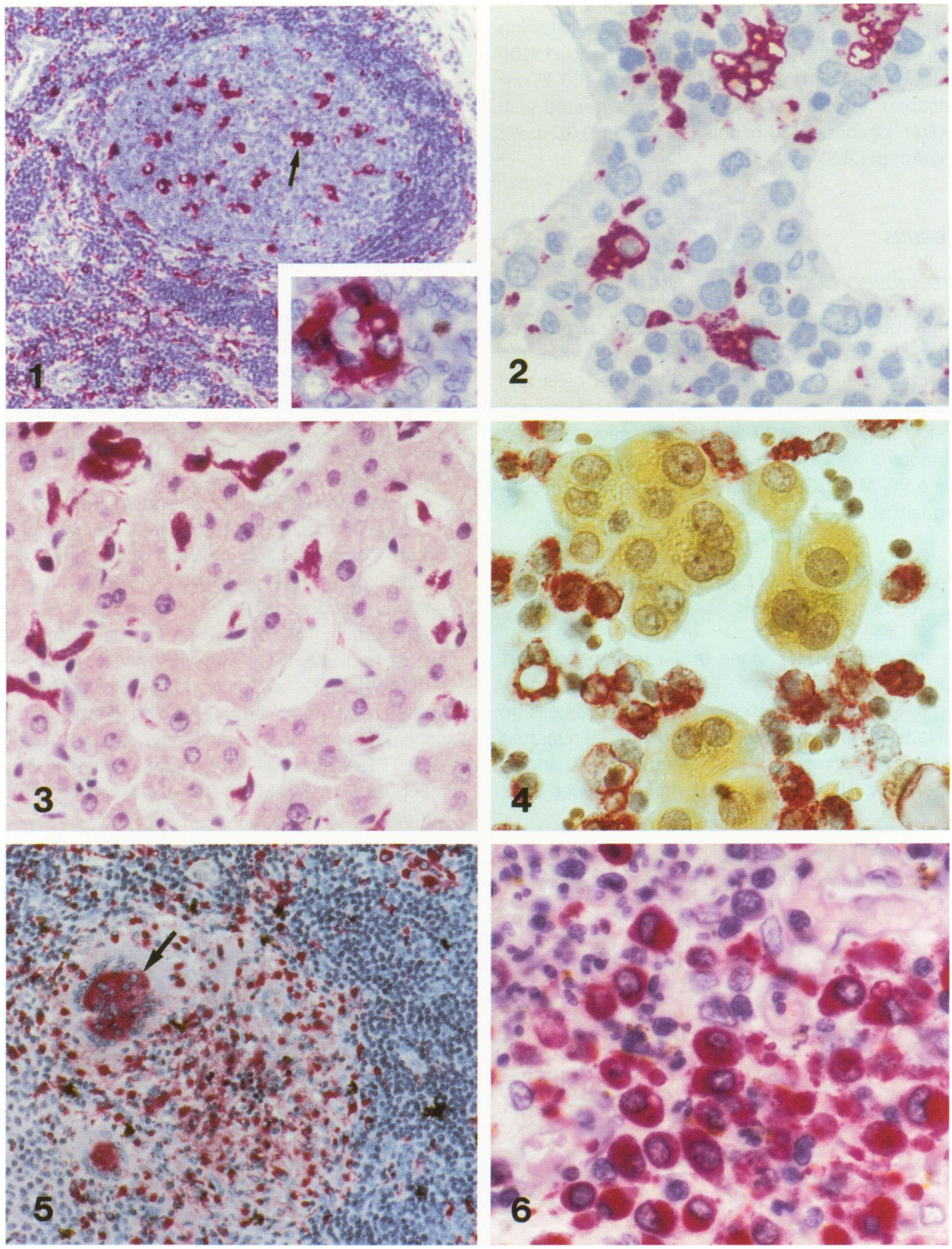

Plate 1. Figure 1. *Reactive lymphadenitis (B5-fixed sample, paraffin section). Many PG-M1-positive macrophages (arrow) are present within the germinal center of a B-cell follicle and in the T-cell area (×125). Inset: higher power view of germinal center macrophages (×800). APAAP technique; hematoxylin counterstain.*

Figure 2. *Normal bone marrow (formalin-fixed/EDTA decalcified bone marrow biopsy, paraffin section). Macrophages are PG-M1-positive, whereas hemopoietic elements are negative. APAAP technique; hematoxylin counterstain; ×500.*

Figure 3. *Normal liver (formalin-fixed sample, paraffin section). PG-M1 strongly stains Kupffer's cells, whereas hepatocytes are negative. APAAP technique; hematoxylin counterstain; ×500.*

Figure 4. *Reactive pleural effusion (alcohol-fixed cytospin). PG-M1 stains only macrophages, whereas lymphocytes and mesothelial cells are PG-M1-negative. APAAP technique; hematoxylin counterstain; ×800.*

Table 3. *Reactivity of PG-M1 with Various Disorders of the Lympho-hemopoietic System*

Tissues	No. Samples 510 (191)*	Reactivity in paraffin
Inflammatory and reactive lymphadenites		
Reactive hyperplasia	30 (15)	Macrophages
Sarcoidosis	10 (3)	Macrophages, granulomas
Tuberculosis	6 (2)	Macrophages, granulomas
Toxoplasmosis	13 (6)	Epithelioid histiocytes
Cat-scratch disease	3 (1)	Macrophages, granulomas
Kikuchi's lymphadenitis	8 (5)	Macrophages, "pl. T cells"
Dermatopathic lymphadenitis	4 (1)	Macrophages
Lymphoproliferative neoplastic diseases		
ALL, Common	14 (3)	Reactive macrophages
B-CLL	13 (6)	Reactive macrophages
B-PLL	2	Reactive macrophages
HCL	15 (10)	Reactive macrophages
Centrocytic l.	23 (3)	Reactive macrophages
Centroblastic/centrocytic l.	30 (15)	Reactive macrophages
Burkitt's l.	8 (5)	Reactive macrophages
Centroblastic l.	50 (20)	Reactive macrophages
Immunoblastic l.	12 (2)	Reactive macrophages
T-lymphoblastic l./T-ALL	12 (5)	Reactive macrophages
Peripheral T-cell l.	23 (8)	Reactive macrophages
Mycosis Fungoides	10 (2)	Reactive macrophages
ALC (CD30+) l.	55 (20)	Reactive macrophages
Hodgkin's disease	80 (26)	Reactive macrophages
True histiocytic sarcoma	5 (5)	Neoplastic cells
Chronic and acute myeloid proliferations		
Chronic myeloid leukemia	15 (5)	Reactive macrophages†
Essential trombocytemia	5 (2)	Reactive macrophages†
Idiopathic myelofibrosis	4 (2)	Reactive macrophages†
Acute myeloid leukemia M1	6 (1)	Reactive macrophages
Acute myeloid leukemia M2	10 (3)	Reactive macrophages
Acute myeloid leukemia M3	4 (1)	Reactive macrophages
Acute myeloid leukemia M4	15 (6)	Leukemic cells
Acute myeloid leukemia M5	6 (2)	Leukemic cells
Acute myeloid leukemia M7	1	Reactive macrophages
Granulocytic sarcoma	1 (1)	Reactive macrophages
Others		
Gaucher's disease	5 (4)	Gaucher cells
Langerhans' cell histiocytosis	5 (1)	Some Langerhans cells
Mastocytosis	7	Mast cells

Abbreviations: l = lymphoma; pl =" plasmacytoid". ALL = acute lymphoblastic leukemia; B-CLL = chronic lymphocytic leukemia, B-cell type; B-PLL = prolymphocytic leukemia, B-cell type; HCL = hairy cell leukemia; ALC = anaplastic large cell.

* The number in parentheses indicates the cases available as frozen sections.

† Weak positivity of megakaryocytes in about 20% of cases.

Normal Tissues

Monocytes were the only cells that reacted with PG-M1 in normal peripheral blood cytospins. Granulocytes and resting as well as phytohemagglutinin-stimulated normal peripheral blood lymphocytes were consistently negative for PG-M1.

As shown in Table 2 and Figures 1 to 6 of Plate 1, PG-M1 stained all macrophages in all tissues investigated. The positivity for PG-M1 was stronger in tissue macrophages than peripheral blood monocytes. PG-M1 stained strongly bone marrow macrophages that showed a typical spider-like aspect with multiple slender processes, (Figure 2, Plate 1); a close association of these elements with erythropoietic islets was frequently seen. Osteoclasts of bone marrow biopsies were also always stained by PG-M1, whereas granulocytes and myeloid precursors were consistently negative (Figure 2, Plate 1). A weak positivity of some megakaryocytes was observed in about 20% of the cases. PG-M1 reacted with the macrophages of germinal centers, interfollicular areas, and sinuses but not with lymphoid elements or accessory cells (follicular dendritic cells, interdigitating reticulum cells, etc.) in lymph nodes and tonsils (Figure 1, Plate 1). The macrophages in the red and white pulp of the spleen, thymic cortex and medulla, and lung stained strongly for PG-M1, as well as

Figure 5. *Cat-scratch disease (B5-fixed lymph node, paraffin section). Epithelioid histiocytes and giant cells (arrowed) constituting a granuloma are stained by PG-M1. APAAP technique; hematoxylin counterstain; ×125.*

Figure 6. *Kikuchi's lymphadenitis (B5-fixed lymph node, paraffin section). A cluster of PG-M1-positive plasmacytoid T-cells is shown. APAAP technique; hematoxylin counterstain; ×800.*

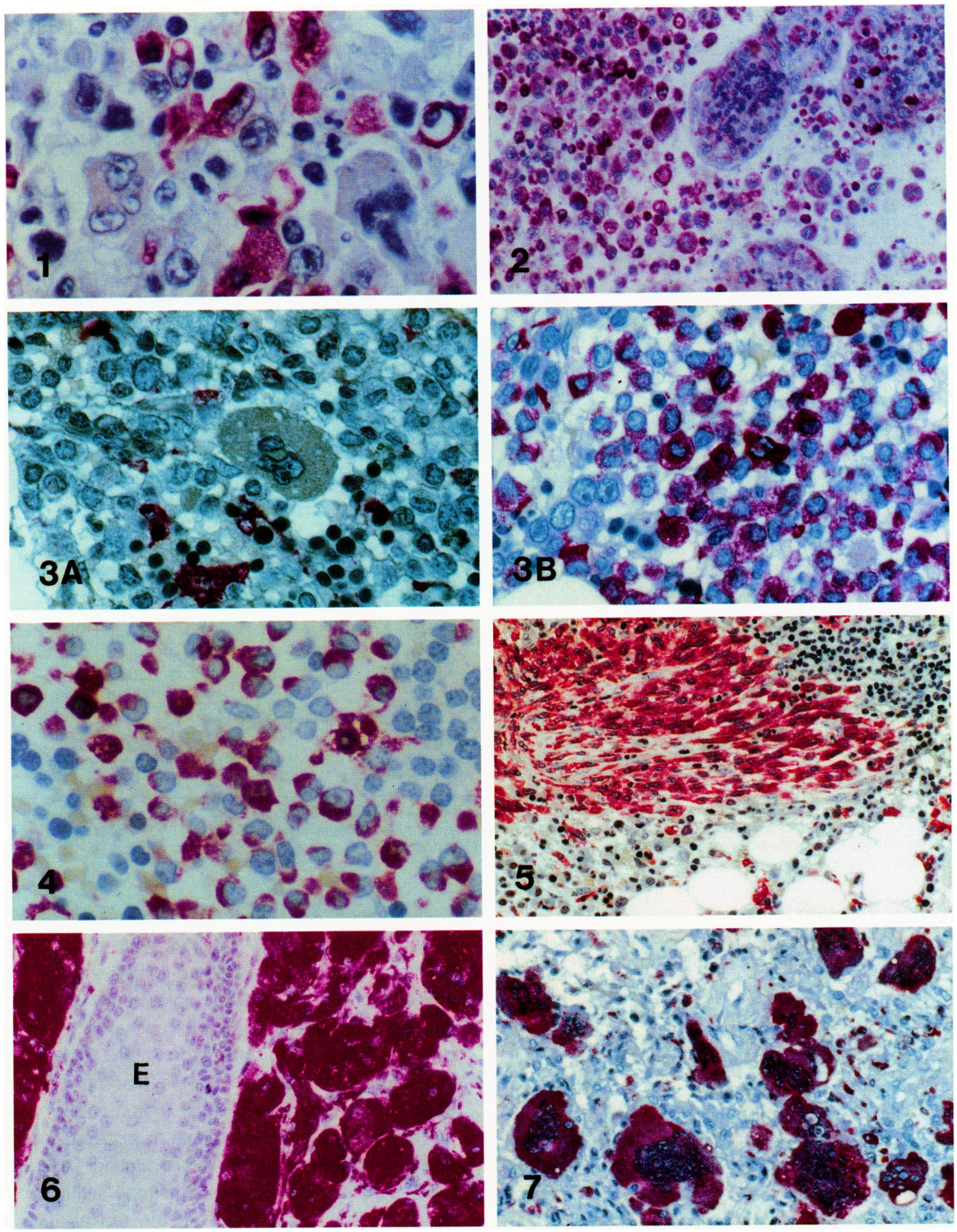

Plate 2. **Figure 1.** *Anaplastic large cell lymphoma, CD30-positive (formalin-fixed lymph node, paraffin section). PG-M1 stains reactive macrophages whereas bizarre neoplastic cells are negative. APAAP technique; hematoxylin counterstain; ×800.*
Figure 2. *True histiocytic lymphoma (B5-fixed lymph node, paraffin section). Malignant cells within sinuses are PG-M1-positive. APAAP technique; hematoxylin counterstain; ×200.*
Figure 3. *Acute myeloid leukemia of M3 type (B5-fixed/Decal decalcified bone marrow biopsy, paraffin section).* **A:** *PG-M1 stains reactive macrophages, whereas leukemic cells and residual erythroid precursors and megakaryocytes are negative.* **B:** *In contrast, leukemic cells are strongly reactive with the KP1 (CD68) MAb. APAAP technique; hematoxylin counterstain; ×600.*
Figure 4. *Acute myeloid leukemia of M5 type (formalin-fixed/EDTA decalcified bone marrow biopsy, paraffin section). Most leukemic cells are labeled by PG-M1. APAAP technique; hematoxylin counterstain; ×600.*

Kuppfer's cells in the liver (Figure 3, Plate 1). Mast cells and synovial cells were the only other PG-M1-positive elements in the human body.

Pathological Lymphohemopoietic Tissues

The reactivity of PG-M1 with a variety of disorders of the lymphohemopoietic system is shown in Table 3. Among inflammatory and reactive lymphadenites, PG-M1 strongly reacted with cells of the monocyte/macrophage lineage, including clusters of epithelioid histiocytes in toxoplasmosis, granulomas, and multinucleated giant cells in sarcoidosis, tuberculosis, and cat-scratch disease (Figure 5, Plate 1). The interdigitating reticulum cells in dermatopathic lymphadenopathy were negative. Most Langerhans' cells in eosinophilic granuloma of the bone were unreactive, although some of them showed a weak dot-like positivity close to the nucleus. In the eight cases of Kikuchi's lymphadenitis examined, PG-M1 strongly stained the vast majority of "plasmacytoid T cells" (Figure 6, Plate 1). As expected, Gaucher's cells of all five cases studied were strongly PG-M1-positive.

Among malignancies of the lymphohemopoietic system (Figures 1 to 5, Plate 2), PG-M1 reactivity was restricted to 5 cases of true histiocytic sarcomas (as defined in the Materials and Methods) (Figure 2, Plate 2), acute leukemias of M4 and M5 type according to the French-American-British (FAB) classification (Figure 4, Plate 2) and mastocytosis (Figure 5, Plate 2). Consistently PG-M1-negative cases included: malignant non-Hodgkin lymphomas of B- and T-cell type (Figure 1, Plate 2), Hodgkin's disease, acute lymphoblastic leukemias, chronic myeloid leukemia, and acute myeloblastic leukemias of M1, M2, and M3 type (Figure 3, Plate 2).

Non-Hematopoietic Inflammatory and Neoplastic Conditions

Most non-hematopoietic tumors turned out to be PG-M1-negative (Table 4), exceptions being 100% of granular cell myoblastomas, about 50% of kidney clear-cell carcinomas, glioblastomas, and meningiomas, and 10% of malignant melanomas. In granular cell myoblastomas (Figure 6, Plate 2), all granular cells were strongly reactive with PG-M1, whereas in the remaining neoplasms, the percentage of stained neoplastic cells ranged from 5% to 100% (Table 4). As expected, giant cell tumors of the bone and xanthogranulomas were PG-M1-positive. In giant cell tumors of the bone (Figure 7, Plate 2), all giant cells, but no mononuclear spindle cells, reacted with PG-M1.

Table 4. *Reactivity of PG-M1 in Paraffin Sections of Nonhemopoietic Tumors*

	No. (370)	Reactivity with tumor cells	% positive tumor cells
Carcinomas			
Skin	15	0/15	0
Lung	20	0/20	0
Breast	6	0/6	0
Stomach	18	0/18	0
Colon	10	0/10	0
Ovary	5	0/5	0
Endometrium	5	0/5	0
Kidney	13	6/13	50%–100%*
Others			
Nevi	15	0/15	0
Melanoma	50	5/50	20%–80*
Chordoma	10	0/10	0
Leiomyoma	6	0/6	0
Leiomyosarcoma	15	0/15	0
Rhabdomyosarcoma	4	0/4	0
Fibrosarcoma	3	0/3	0
Liposarcoma	7	0/7	0
Angiosarcoma	3	0/3	0
Kaposi's sarcoma	8	0/8	0
Mesothelioma	5	0/5	0
Meningioma	18	10/18	20%–60%*
Astrocytoma	5	0/5	0
Glioblastoma	10	4/10	10%–30%*
Neuroblastoma	3	0/3	0
Neurofibroma	20	0/20	0
Neuroepithelioma	1	0/1	0
Schwannoma b.	5	0/5	0
Schwannoma m.	6	0/6	0
Chondrosarcoma	1	0/1	0
MFH	12	10/12	5%–40%†
Giant cell epulis	6	6/6	100%*
Xanthoma	2	0/2	0
Xanthogranuloma	7	7/7	50%–100%*
Dermatofibroma	27	0/27†	0
Dermatofibrosarcoma	7	0/7†	0
Synovial sarcoma	5	0/5	0
Giant cell T. bone	2	2/2	100%‡
Gran. cell myobl.	15	15/15	100%*

B. = benign; m. = malignant; MFH = malignant fibrous histiocytoma; T. = tumor; Gran. cell myobl. = Granular cell myoblastoma.

* The same or even broader reactivity was observed with KP1 and Ki-M1P MAbs. Cases of meningioma and glioblastoma could only be tested with the KP1 but not Ki-M1P MAb.

† Positivity was observed in the histiocytic component.

‡ Positivity of multinucleated cells but not of the mononuclear spindle component.

Figure 5. *Systemic mastocytosis (B5-fixed/Decal decalcified bone marrow biopsy, paraffin section). Mast cells are strongly stained by PG-M1. APAAP technique; hematoxylin counterstain; ×200.*

Figure 6. *Granular cell myoblastoma (formalin-fixed sample, paraffin section). All granular cells are strongly positive for PG-M1. The same staining pattern (not shown) was observed with the KP1 and Ki-M1P MAbs. E indicates epithelium. APAAP technique; hematoxylin counterstain; ×500.*

Figure 7. *Giant cell tumor of the bone (formalin-fixed sample, paraffin section). Giant osteoclasts, but not the mononuclear spindle component, are PG-M1-positive. APAAP technique; hematoxylin counterstain; ×500.*

Comparison of the PG-M1, KP1, and Ki-M1P Reactivities in Paraffin Sections

The results are summarized in Table 5, Figure 3, A and B (Plate 2), and Figures 1 and 2 (Plate 3). The most important finding, from the diagnostic point of view, was the different reactivity of the three MAbs with myeloid elements. In particular, PG-M1 labeled only macrophages, whereas both KP1 and Ki-M1P stained to different extent normal and neoplastic myeloid elements. The latter findings differ from that previously reported for the Ki-M1P MAb (lack of reactivity with cells of granulocyte lineage).[14]

Immunoprecipitation and cDNA Transfectant Studies

Repeated attempts to immunoprecipitate the antigen recognized by PG-M1 failed. About 3% to 5% of the CD68-transfected COS-1 cells and 1% of the CD68-transfected WOP cells were positive with the PG-M1 and Y2/131 (CD68) MAbs 48 hours following transfection. These results indicate that the transfection efficiency of the COS-1 and WOP cells by the CD68 coding vector was 3% to 5% and 1% respectively. All the CD68-transfected cells expressed the PG-M1 epitope. The staining of the transfected cells was weaker with PG-M1 than with the Y2/131 (CD68) control MAb. The fixation of the cytospins was in acetone for 10 minutes. The fixation of the cytospins for 90 seconds with acetone/methanol/formalin (19:19:1) did not affect this result.

Table 5. *Differences in the Reactivity of PG-M1, KP1, and Ki-M1P in Paraffin Sections.**

Reactivity	PG-M1	KP1 (CD68)†	Ki-M1P
Macrophages	+	+	+
Mature granulocytes‡	–	+	–
Myeloid precursors‡	–	+	+§
Myeloid leukemias (M1,M2,M3)	–	+	+
Myeloid leukemias (M4,M5)	+	+	+
Megakaryocytes	–/+	+	+
Hairy cell leukemia cells	–	+	+
Low-grade B cell lymphomas related to follicle mantle	–	nd	+
Melanoma cells	–/+	+	–/+

–/+ occasionally positive; nd = not done.
* Assessed in 100 routine samples from Tables 3 and 4.
† KP1 also showed occasional reactivity with T cells in reactive conditions (two cases); these cases were PG-M1-negative.
‡ Tested on routine bone marrow biopsies of normal bone marrow and chronic myeloid leukemia cases.
§ In eight out of the 10 cases tested.

Epitope Analysis

Following proteolytic digestion with chymotrypsin, trypsin, pepsin, and pronase, there was an increase in PG-M1 reactivity. In contrast, the digestion of cytospins from CD68-transfected COS cells with various carbohydrate-splitting enzymes did not change the PG-M1 reactivity. Unexpectedly, periodate treatment of cytospins increased the PG-M1 reactivity. Thus the above experiments do not allow any conclusion concerning the nature of the epitope recognized by PG-M1.

Discussion

In this paper, we describe the characteristics of a new anti-macrophage MAb (PG-M1) generated by immunizing BALB/c mice with Gaucher's cells. The antigen recognized by PG-M1 is a macrophage-restricted intracytoplasmic molecule, resistant to all commonly used fixatives (formalin, B5, and Bouin) and Decal decalcification.

Unfortunately, no direct information is yet available on the molecular weight of the antigen recognized by PG-M1, because the numerous attempts to immunoprecipitate the molecule from stimulated peripheral blood macrophages, native alveolar macrophages, and THP-1 cells have failed. However, a number of findings point to a relationship of the PG-M1 antigen with the CD68 cluster, which represents a 110-kd glycoprotein probably associated with lysosomal granules. The reasons for this assumption are: a) PG-M1 reacts with COS-1 and WOP cells transfected with a cDNA clone encoding for the CD68 antigen; b) the reactivity pattern of PG-M1 with cells of the monocyte/macrophage lineage, including the plasmacytoid T cells, in paraffin sections is similar to that of the MAb KP1,[11] which is known to recognize a fixative-resistant epitope of the CD68 antigen[27,28]; and c) the reactivity pattern of PG-M1 with cells other than monocytes/macrophages (synovia cells, mast cells, granular cell myoblastoma cells, cells of some renal carcinomas, melanomas, etc.) closely resembles that reported for the antibodies of the CD68 cluster.[29–31]

Unlike many other CD leucocyte antigens, the CD68 molecule (recognized by monoclonal antibodies Y2/131, Y1/82A, EBM11, Ki-M6, Ki-M7, KP1)[27,32–34] is antigenically very heterogeneous.[29] For example, the MAbs KP1 and EBM11 strongly bind to Langerhans' cells as well as to epithelia of the proximal renal tubuli, which are in turn weakly positive for Ki-M6 and Y1/82A; KP1 and EBM11 but not Y1/82A react with hairy cell leukemia cells; KP1

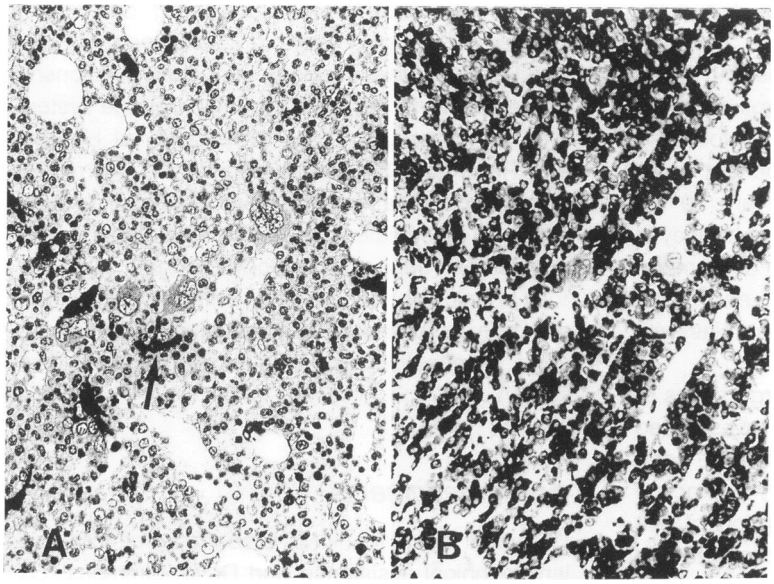

Plate 3. Figure 1. *Chronic myeloid leukemia (B5-fixed/Decal decalcified bone marrow biopsy, paraffin section).* **A:** *PG-M1 labels macrophages (arrow) but not myeloid elements;* **B:** *Ki-M1P stains both macrophages and myeloid precursors. APAAP technique; hematoxylin counterstain; ×500.*

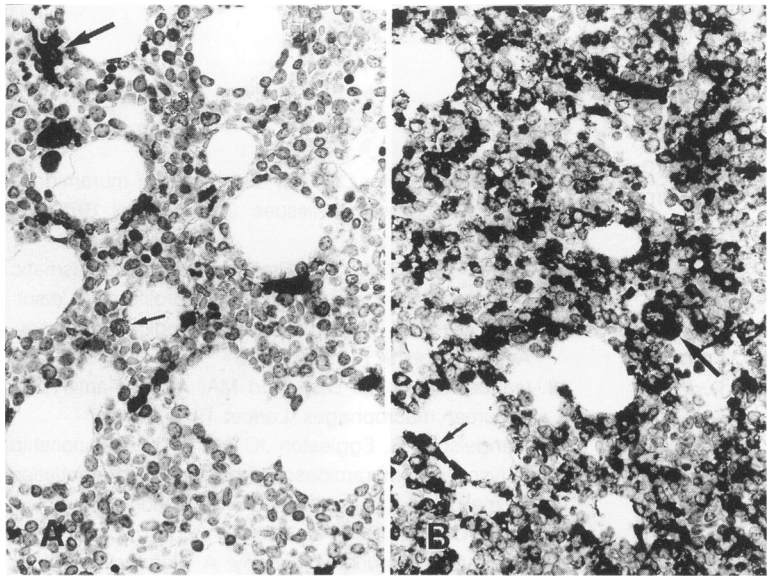

Plate 3. Figure 2. *Acute myeloid leukemia, M2 type (formalin-fixed/EDTA decalcified bone marrow biopsy, paraffin section).* **A:** *PG-M1 stains reactive macrophages (long arrow) and mast cells (short arrow) but not leukemic cells;* **B:** *Most leukemic cells, in addition to macrophages (short arrow), are labeled by Ki-M1P. The long arrow shows a positive megakaryocyte. APAAP technique; hematoxylin counterstain; ×500.*

and EBM11 stain granulocytes and myeloid precursors, whereas Y1/82A usually do not. Thus, among the antibodies of the CD68 cluster, the reactivity pattern of PG-M1 closely resembles that of the antibody Y1/82A (regarded as the most macrophage-specific antibody of the group, although, unlike PG-M1, it recognizes an epitope on the CD68 antigen that is not formol-resistant). It has been suggested that this antigenic heterogeneity within the CD68 cluster could reflect either variations in the carbohydrate side chain of the antigen or differences in the folding of the central peptide core in different cell types.[29] Unfortunately, the experiments we performed to elucidate the nature of the epitope recognized by PG-M1 were inconclusive.

In paraffin sections, the PG-M1 MAb reacts with cells of the monocyte/macrophage lineage more selectively than other reported anti-monocyte/macrophage antibodies (MAC-387, KP1, and Ki-M1P).[10,11,14] In particular, PG-M1 does not stain mature granulocytes (positive for MAC-387 and KP1), myeloid precursors (positive for KP1 and Ki-M1P), acute myeloid leukemias of non-M4 and non-M5 type (positive for KP1 and Ki-M1P), and epithelial cells that are positive for MAC-387. Because of its selective, constant, and strong reactivity with cells of the monocyte/macrophage lineage in paraffin sections (irrespective of the kind and length of fixation and the need for laborious proteolytic procedures, such as proteolysis at 22 C for EBM11),[35]

PG-M1 represents an efficient tool in the daily diagnostic and research work.

Our findings in malignant lymphomas and acute leukemias demonstrate that PG-M1 keeps its selective monocyte/macrophage reactivity even following malignant transformation. The most important applications of PG-M1 in hematopathology are: a) the use of PG-M1 for the diagnosis of true histiocytic sarcomas (possibly in combination with other markers such as CD45, lysozyme, and KP1) and b) the distinction between acute myeloid leukemias with and without monocytic differentiation.

The PG-M1 MAb was tested on a large series of malignant lymphomas that had been investigated with a number of MAbs specific for leukocyte common antigen (CD45), B, T, and monocyte/macrophage antigens. With the exception of five cases of true histiocytic sarcoma, all cases turned out to be PG-M1-negative. This finding further supports the concept that true histiocytic sarcomas are rare[36,37] and that most of the cases, formerly regarded as malignant histiocytosis of the lymph nodes or intestine, belong to newly recognized entities, such as anaplastic large cell lymphomas (CD30-positive),[6,38] Sprue-associated T-cell lymphomas of the intestine,[39] or peripheral T-cell lymphomas associated with hemophagocytic syndrome.[40,41]

PG-M1 was also tested on 42 cases of acute myeloid leukemias typed by means of morphology and established cytochemical techniques according to the FAB system. In all instances, PG-M1 showed the expected reactivity, that is, negativity of acute myeloid leukemia cases showing features of granulocytic differentiation and reactivity with cases that expressed monocyte/macrophage antigens, i.e., M4 and M5-type acute myeloid leukemia. This makes PG-M1 a useful marker for the diagnosis of M4 and M5 leukemias on routinely fixed/decalcified bone marrow biopsies (especially when used in combination with the KP1 and anti-neutrophilic elastase monoclonal antibodies). In this respect, the PG-M1 seems a more reliable marker than the Ki-M1P MAb,[14] which, as shown in this paper, reacts also with most cases of M1, M2, and M3 acute leukemias. Due to its lack of reactivity with cells of myeloid lineage, the PG-M1 MAb also turned out to be an ideal reagent for the study of macrophage populations in both normal bone marrow and myeloproliferative syndromes.[42]

Finally, PG-M1 was used to study the phenotype of reactive and neoplastic cell populations whose histogenesis remain controversial (plasmacytoid T cells, Hodgkin, and Reed–Sternberg cells, etc.). The reactivity of plasmacytoid T cells and mast cells with PG-M1 further supports their close relationship with elements of the monocyte/macrophage system.[43–47] In contrast, the lack of reactivity of Hodgkin and Reed–Sternberg cells with PG-M1 (correlated with the observation that these elements express lymphoid associated antigens),[48] strongly argues against their supposed histiocytic origin.

In conclusion, because of its high specificity and suitability in routine paraffin sections, PG-M1 represents an effective tool in daily diagnostics and research.

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