Ki-M8 Monoclonal Antibody Reactive With an Intracytoplasmic Antigen of Monocyte/Macrophage Lineage

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A monoclonal antibody (MoAb), Ki-M8, that reacts specifically with cells of the monocyte/macrophage system is described. On light and electron microscopic immunohistochemistry, Ki-M8 recognizes intracytoplasmatically localized antigens of mol wt 30,000 and 32,000, increasingly expressed during differentiation of monocytes into macrophages. Ki-M8 antigen is detectable on almost all known tissue macrophages and monocyte/macrophage-related cell lines after appropriate stimulation. In functional terms Ki-M8 significantly impairs the generation of oxygen radicals during an induced respiratory burst. Applied to acute nonlymphoblastic leukemias, a clear-cut differentiation of the monocytic phenotype and differentiation is possible on the basis of Ki-M8 immunoreactivity. Ki-M8 represents a reagent specific for the monocyte/macrophage system with regard to antigen distribution in normal and neoplastic cells as well as with regard to its influence on a typical monocyte/macrophage-related function.

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Materials and Methods

Generation of Monoclonal Antibody

Macrophages were obtained by glass adherence from fresh minced human lymph node specimens, which were removed because of enlargement due to chronic lymphadenitis and used as immunogen. Splenic cells of Balb/c mice were fused with the nonsecreting myeloma cell line X63-Ag8.653 at a ratio of 1:1.6 After suspension in HAT-supplemented culture medium (1 x 10^-4 mol/L hypoxanthine, 4 x 10^-3 mol/L amphotericin, 6 x 10^-5 mol/L thymidine; Boehringer, Mannheim, West Germany), cells were dispersed in Greiner 24-well fusion plates (Greiner, Nütingen, West Germany) at a concentration of 1 x 10^6 cells/well using 1 x 10^8 syngeneic peritoneal macrophages as well as feeder layer. After about ten days, when viable cell clusters were visible, supernatants were screened for immunohistochemical reactivity using cryostat sections of human tonsils (see below). The hybridoma colony showing the Ki-M8 specificity as described in this study was subjected to a limiting dilution procedure and processed for mass culturing and ascites production. On immunodiffusion, MoAb Ki-M8 was shown to be of IgG1 type.

Specificity Tests of the MoAb Ki-M8

Light microscopic immunohistochemistry. Immunohistochemical staining was performed on 8-µm cryostat sections of surgically removed tissue specimens. Special attention was given to those organs known to be populated by cells of the monocyte/macrophage system (n = 4 for each analyzed organ). Furthermore, cytopsin preparations of normal human granulocytes (n = 5), T lymphocytes (n = 5) and B lymphocytes (n = 5), monocytes (n = 5), peritoneal macrophages (n = 4) and alveolar macrophages (n = 4) separated as described elsewhere,10,18 and smears of bone marrow (n = 3) were subjected to immunohistochemical testing. Because of the close association of Ki-M8 reactive cells to T cell areas of lymphoid tissue (see results), special attention was given to the purification of T cell populations by passage of nonadherent cells of the interphase through an antihuman immunoglobulin column packed with beads coupled with human IgG, antihuman rabbit IgG, and human C3, as described in detail elsewhere.17 In addition, Ki-M8 reactivity was studied on monocytes cultured over five days by prolonged glass adherence (n = 3) as well as stimulated over five days with lymphokine-conditioned media (n = 3) obtained by addition of concanavalin A (Con A; Serva, Heidelberg, West Germany; 30 µg/mL) to separated normal blood lymphocytes over three days.14 The following cell lines were tested: Unstimulated and 12-O-tetradecanoylphorbol-13-acetate stimulated U-937 cells (TPA; Sigma, Munich, West Germany; 1.6 x 10^-4 mol/L over three days); unstimulated; dimethyl sulfoxide (DMSO)-stimulated (1.2% over five days); and TPA-stimulated HL-60 cells (1.6 x 10^-4 mol/L over three days). These cell lines represent promyelocytes (HL-60), monocyte precursors (U-937), and, following stimulation, mature granulocytes (DMSO stimulation of HL-60) or monocyte/macrophage differentiation forms (TPA stimulation of HL-60 and U-937).18 In addition, cell lines BALM4, NALM6, BALL-1 (considered as B cell leukemia lines), cell lines CCRF-CEM, SKW-3, TALL-1 (considered as T cell leukemia lines), cell lines KG-1, MLI, (considered as myelomonocytic lines), and cells of human tonsil were tested. Using monoclonal antibodies, several cell lines of the monocyte/macrophage system were shown to be Ki-M8 positive, as were normal human lymphoid tissues, which are positive for Ki-M8. Normal human blood lymphocytes and tissues were negative for Ki-M8.
Ki-M8 REACTIVE WITH MONOCYTES/MACROPHAGES

Reactions with Monocytes/Macrophages

The immunoperoxidase method was applied as described in detail elsewhere using a three-step incubation procedure with MoAb Ki-M8 (undiluted culture supernatants or 1:2,000 diluted ascites fluid). To avoid endogenous peroxidase activity and to increase sensitivity, the immune alkaline phosphatase technique was applied in some cases.22 To exclude surface binding of MoAb antibody Ki-M8, cell suspensions of unstimulated monocytes and monocytes stimulated by lymphokine-conditioned media and zymosan A (see below) were stained by indirect immunofluorescence. After incubation with MoAb Ki-M8 (60 minutes, 4°C) and intensive washing, rhodamine-conjugated goat antimouse IgG (Medac, Hamburg, West Germany) was added, (60 minutes, 4°C) and, following two washing steps, cells were evaluated by fluorescence microscopy.

Electron microscopic immunohistochemistry. Electron microscopic immunohistochemistry was performed on 1- to 3-mm slices of lymph node specimens fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) for one hour and snap frozen in liquid nitrogen. Cryostat sections were then used for the immunoperoxidase method as described above followed by postfixation in 1% osmium tetroxide in 0.1 mol/L phosphate buffer, pH 7.2.27 As positive controls we used the commercially available MoAb antimonocyte 1 (Mono 1; Bethesda Research Laboratory, Gaithersburg, MD). As negative controls, supernatants of unfused myeloma cells and sera of nonimmunized mice (instead of the primary MoAb Ki-M8) were applied, which did not render any immunoreactivity pattern and thus excluded nonspecific binding during the immunohistochemical procedures.

Functional and Biochemical Analyses

Measurement of chemiluminescence was performed as described by Kato et al.26 Twenty micrograms luminol (Sigma; dissolved in 10 µL PBS) was added to 500 µL monocyte suspension (2 x 10⁷ cells/mL RPMI 1640). After preincubation for 10 minutes at 37°C, 1 mg zymosan A (Sigma) dissolved in 20 µL medium was added and the chemiluminescence response was recorded over 40 minutes using the Berthold Biolumate Counter model LB 9505 (Wildbad, West Germany). Chemiluminescence was measured in untreated monocytes and monocytes to which monoclonal antibody Ki-M8 was added in a dilution of 1:100, 1:500, 1:1,000, and 1:2,000 for 2.5 hours. In addition, chemiluminescence measurement was performed for untreated monocytes and monocytes treated with MoAbs Ki-M8 and antimonocyte 1 (diluted 1:100) for 2.5 hours and 24 hours. Furthermore, chemiluminescence response of granulocytes was checked over 40 minutes after incubation with MoAb Ki-M8 (diluted 1:100) in comparison to untreated granulocytes.

For the estimation of the mol wt of the Ki-M8-reacted antigen, proteins were extracted from freshly ectomized human tonsils (n = 3) with Triton-X-100 (1 vol% in aqua dest) and solubilized in sample buffer (0.125 mol/L Tris buffer, pH 6.8, containing 4% sodium dodecyl sulfate (SDS) and 20% vol% glycerin). After incubation for two hours at room temperature, electrophoresis was performed under nonreducing conditions on a 0.3-mm thick 8% SDS-polyacrylamide gel over 7-cm separation distance according to Laemmli.29 Proteins were blotted overnight at 4°C on nitrocellulose membranes (Schleicher and Schüll, Dassel, West Germany). Membranes were immunostained applying the immune alkaline phosphatase method as described above using a mouse MoAb against alkaline phosphatase of calf intestine as tertiary antiserum after covering of unspecific binding sites. Immunostained protein bands were compared with the whole tonsillar protein pattern and a mol wt standard stained with Amido black.

Analysis of Leukemias

Fresh heparinized venous blood of 40 patients with acute leukemia was subjected to Ficoll-Urografin density gradient centrifugation at d = 1.077 g/mL.30 Cytocentrifuge preparations of the interphase consisting of leukemic blasts, myeloid precursors, as well as normal lymphocytes and monocytes, were subjected to Pappenheim staining and enzymocytochemical staining for peroxidase,23 naphthol AS-D chloroacetate esterase,53 and nonspecific acid esterase.24 Immunophenotyping was performed with the immune alkaline phosphatase method as described above. In addition to Ki-M8, the following MoAbs were applied: Vim 2 specific for granulocytes and myelomonocytic precursors and Vim D5 specific for granulocytes.55,56 My 7-recognizing granulocytes, monocytes, and myelomonocytic precursors; 3C4 described by Schienle et al.21 specific for granulocytes and granulocytic precursors; Ki-M1 specific for monocytes.47 The lymphoblastic nature of the analyzed leukemias was excluded by their negative reaction for the MoAbs To13 and HD37 used as pan-B cell reagents60 as well as by their negative reaction for the MoAb Leu-4 used as pan-T cell reagent.23 In addition, Ki-M8 was applied to 30 cases of acute and chronic lymphocytic leukemias diagnosed on the basis of morphological and enzymocytochemical criteria and on the basis of their reactivity with the lymphoid-associated MoAbs mentioned above.

RESULTS

Reactivity Pattern

Light microscopy. On light microscopic immunohistochemical testing, Ki-M8 reacted with an intracytoplasmatically localized antigen selectively expressed on cells of the monocyte/macrophage system. The failure of Ki-M8 to recognize surface structures could also be confirmed by indirect immunofluorescence staining of viable unstimulated monocytes and monocytes stimulated with lymphokine-conditioned media, which did not show any surface labeling. Considering separated cell populations, blood monocytes displayed the lowest degree of reactivity, becoming faintly visible only after applying the immune alkaline phosphatase technique. On the contrary, in stimulated blood monocytes as well as peritoneal and alveolar macrophages, reactive cytoplasmic structures could only hardly be discriminated because of the high degree of reactivity (Fig 1). Granulocytes as well as T and B lymphocytes did not reveal any positive immunohistochemical staining by counting 500 cells in each sample. Special attention was given to granulocytes that did not render any positive staining of either intracytoplasmic or surface structures. The exclusive reactivity with the monocyte/macrophage system could also be shown by testing permanent cell lines. Only those that were induced to differentiate along the monocyte/macrophage differentiation pathway, such as TPA stimulated HL-60 and U-937 cells, proved to be positive for Ki-M8. Unstimulated myelomonocytic leukemia lines as well as B cell, T cell, and non-T, non-B cell leukemia lines were clearly negative for Ki-M8 (Table 1).

Studying different tissues all known macrophage populations, with the exceptions of starry sky macrophages of lymphoid follicles (Fig 2) and macrophages of central nervous tissue, reacted with Ki-M8. The reactive cell populations included macrophages of lymph node sinuses and

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Fig 1. Ki-M8 reactivity in separated normal human monocytes (A), lymphokine-stimulated monocytes (B; for details see text), and alveolar macrophages (C). There is a stepwise increase of immunoreactivity from unstimulated blood monocytes (faint granular pattern; arrow) to alveolar macrophages (strong diffuse reactivity). Cytospin preparations, immune alkaline phosphatase reactions, original magnification x 560.

Table 1. Reactivity Pattern of Ki-M8 as Tested by Immunohistochemistry on Separated Blood Cells, Cell Lines, and Tissue Samples

| Granulocytes | B and T lymphocytes | Monocytes, unstimulated | Monocytes, stimulated* | Myelomonocytic leukemia lines* | HL-60 and U-937, stimulated* | B cell, T cell, and non-T, non-B leukemia lines* | M of lymphoid follicles (starry sky M) | M of lymphatic T zones | M of lymph node sinuses | M of bone marrow | M of splenic red pulp | M of liver (von Kupffer cells) | Peritoneal and alveolar M | M of connective tissue (histiocytes) | M of gastrointestinal tract | M of placenta (Hofbauer cells) | M of renal interstitium | M of brain (glial cells) | Dendritic reticulum cells | Interdigitating reticulum cells | Langerhans' cells |
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|              |                     |                        |                        |                                |                               |                                               |                            |                 |                 |                |                 |                                |                            |                                   |                        |                                |                 |                                |                            |                                |

Abbreviations: -, negative; (+), weakly positive; +, positive; M macrophages.

*See text for details.

interfollicular macrophages of lymphoid tissue (lymphatic T-zones), monocytoid-differentiated mononuclear cells and macrophages of bone marrow, macrophages of splenic red pulp, von Kupffer cells of the liver, macrophages of connective tissue known as histiocytes, macrophages of the gastrointestinal tract and interstitium of the kidney, as well as Hofbauer cells as the macrophage population of the placenta (Table 1). Dendritic reticulum cells (follicular dendritic cells) of lymphoid follicles (Fig 2) as well as interdigitating reticulum cells of lymphatic T-zones and Langerhans' cells of the epidermis, known as accessory cells of B cell and T cell immune response, respectively, and differently claimed to belong to the monocyte/macrophage system, were clearly negative for Ki-M8 (Table 1). The reactivity of Ki-M8 with natural killer (NK) cells seemed to be unlikely because immunohistochemical staining of lymphoid tissue with Leu-743 (Becton Dickinson, Mountain View, CA) specific for NK cells clearly revealed a different staining pattern of tonsils with the bulk of reactive cells within the follicle. Cross-reactions with other tissue structures could not be observed, such as those of epithelial, endothelial, neural, and mesenchymal origin analyzed in organs mentioned in Table 1, in endocrine organs, in male and female genitals, in salivary glands, and in lung, heart, and striated muscle.

Electron microscopy. On electron microscopic evaluation, the reactivity of Ki-M8 was mainly confined to the bulk of lysosomal structures as well as phagosomes. Within these organelles Ki-M8 stained the surrounding walls as well as part of the interior. Other cell structures, especially the surface membrane and nuclei, did not show any immunoreactivity (Fig 3).
K-M8 REACTIVE WITH MONOCYTES/MACROPHAGES

Results of Functional and Biochemical Analyses

Blood monocytes treated for 2.5 hours with K-M8 showed a significantly decreased chemiluminescence response (Fig 4A). Whereas dilutions of K-M8 of 1:2,000 did not show any significant effects, dilutions of down to 1:100 lead to a dose-dependent decrease of chemiluminescence response amounting to about 30% in case of 1:100 dilution as compared with untreated and antimonocyte 1-treated monocyte samples (Fig 4B and C). These differences could not be due to variations or undesired stimulation during monocyte separation because one and the same blood sample was used for these experiments. After prolonged culture over 24 hours, no further differences could be observed between the untreated and K-M8 as well as antimonocyte 1-treated monocytes (Fig 4C). Particle ingestion was not influenced by either K-M8 or antimonocyte 1 (data not shown). In addition, as shown by light microscopic immunohistochemistry and immunofluorescence staining, phagocytosis of zymosan A did not induce surface expression of the K-M8 antigen.
Fig 4. (A) Chemiluminescence response (cpm × 10^3) of blood monocytes treated with MoAb Ki-M8 over 2.5 hours. Ki-M8 significantly inhibits the generation of oxygen radicals measured over 40 minutes in comparison to untreated blood monocytes (standard = ST). (B) Chemiluminescence response of blood monocytes treated with various dilutions of MoAb Ki-M8 over 2.5 hours (mean values, n = 4). Chemiluminescence of untreated blood monocytes corresponds to 100%. A dilution of 1:100 of MoAb Ki-M8 leads to a decrease of chemiluminescence amounting to about 30%. (C) Chemiluminescence response (cpm × 10^3) of blood monocytes treated with MoAbs Ki-M8 and antimonocyte 1 (Mono 1) over 2.5 and 24 hours (n = 4) in comparison to untreated blood monocytes (standard = ST = 100%). A significant inhibition of chemiluminescence response can be observed only in case of monocytes treated with Ki-M8 over 2.5 hours. ■ Ki-M8; □ Mono 1; □ ST. (D) Chemiluminescence response (cpm × 10^3) of untreated (standard = ST) and Ki-M8-treated granulocytes measured over 40 minutes. A significant difference cannot be detected.

Chemiluminescence responses of granulocytes, untreated or treated with Ki-M8, did not show significant differences (Fig 4D).

Ki-M8 immunostained two protein bands of 30,000 and 32,000 daltons under nonreducing conditions (Fig 5). These protein bands could be observed in all three samples of tonsilar extracts analyzed, although with different degrees of intensity. Control stainings applying physiologic saline or supernatants of unfused myeloma cells instead of MoAb Ki-M8 did not show any positively stained protein band (data not shown).

**Ki-M8 in Myelomonocytic Leukemias**

The number of blasts in the analyzed cases ranged from 29% to 97%. Blasts were considered as reactive with a given MoAb antibody if the number of positive blasts exceeded 10%. Ki-M8 showed a positive immunostaining in eight of the analyzed cases (20%), which were also reactive with the MoAbs My7- and Ki-M1-recognizing monocyes but not with the MoAbs Vim2-, VimD5-, and 3C4-recognizing granulocytes and myelomonocytic precursors. Based on these data a clear distinction between the monocytic, on one hand, and the pure granulocytic and myelomonocytic leukemias, on the other hand, could be obtained. Enzymecytochemically Ki-M8 reactive cases were positive for peroxidase, naphthol AS-D chloroacetate esterase, and nonspecific acid esterase in eight (100%), four (50%), and eight (100%) cases, respectively. Thus a direct relationship between the immunohistochemical analysis and the French-American-British (FAB) classification applying morphological and enzymecytochemical criteria could not be found, as shown also by other authors. The incidence of cases with a positive reaction to

**Fig 5.** Antigens immunostained by the MoAb Ki-M8 in comparison to the whole tonsillar protein pattern and a mol wt standard stained with Amido black (from left to right; for details see text). The antigens display mol wt of 30,000 and 32,000 daltons.
these enzymecytochemical stainings among the remaining 32 patients amounted to 19 (60%), eight (25%), and 31 (97%), respectively. The analyzed cases of acute and chronic lymphocytic leukemias did not show any immunoreactivity with MoAb Ki-M8.

**DISCUSSION**

The significance of monocytes and macrophages for the immune response and the multitude of disorders caused by abnormalities of these cells underline the importance of reliable reagents characterizing this cell system. Monocytes and macrophages have been defined by morphological, cytochemical, immunologic, and functional methods; of such analyses, however, have been performed in vitro using easily accessible members of this cell system such as blood monocytes and macrophages of serous cavities in addition to permanent monocyte/macrophage-related cell lines subjected to different stimulation procedures. This issue also holds true for most of the MoAbs established so far to characterize the monocyte/macrophage system. The majority of such reagents did not prove entirely specific due to their concomitant reactivity with granulocytes, T lymphocyte subpopulations, including NK cells, or platelets.

The MoAb Ki-M8 described in this study fulfills the criteria of a restricted occurrence within the monocyte/macrophage system applying samples of separated cell populations, of human tissue sections, and of permanent human cell lines. On immunohistochemical staining of frozen tissue sections and smears of bone marrow, only monocytes and macrophages with the typical distribution pattern in lymphatic and nonlymphatic organs were selectively recognized (Figs 1 and 2). The only exception was made by the so-called starry sky macrophages of the lymphoid follicles and macrophages of the central nervous tissue. Antigen-presenting cells (accessory cells of B cell and T cell immune response) such as dendritic reticulum cells (folicular dendritic cells) of lymphatic B zones as well as Langerhans' cells of the skin and interdigitating reticulum cells of lymphatic T zones were not immunostained by Ki-M8, although their relationship to the monocyte/macrophage system has variously been claimed.

Considering permanent human cell lines, the promyelocytic cell line HL-60 and the histiocytic cell line U-937 revealed positive only after adequate stimulation to evolve into mature macrophages (Table 1). Epithelial, endothelial, and neural cells as well as mesenchymal cells not related to macrophages, including lymphocytes and granulocytes, invariably showed a negative reaction.

Considering the fundamental capabilities of monocytes/macrophages and related cells beyond their ability to differentiate from bone marrow precursors over monocytes into tissue macrophages and beyond their regulatory function during immune response, mainly expressed in antigen presentation, the effector function in connection with non-specific defense against microorganisms has been extensively investigated. Tests covering these aspects of macrophage function measure phagocytosis and release of lysosomal contents. The ultrastructural localization of Ki-M8 reactive antigen in lysosomal structures (Fig 3) motivated us to study the effect of Ki-M8 on such effector functions usually considered to be bound to lysosome integrity. The results showed that Ki-M8 significantly impaired the generation of oxygen radicals during the respiratory burst primed by ingestion of zymosan particles, whereas particle incorporation was not influenced (Fig 4A and B). Other monocyte/macrophage specific MoAbs of the same immunoglobulin class recognizing exclusively surface antigens such as antimonocyte 1 did not show any effect on this function (Fig 4C). The observation that Ki-M8 impaired respiratory burst leaving particle incorporation unaltered may suggest a direct effect of Ki-M8 on oxygen-radical generating mechanisms rather than on phagocytosis activity or on modulation of membrane receptors. It remains to be established, however, by which mechanism Ki-M8 is internalized in monocytes during incubation to exert effects on chemiluminescence and why this effect is abolished after 24 hours.

In a further step we applied the MoAb Ki-M8 to acute nonlymphoblastic leukemias. In the FAB classification of monocytic and granulocytic leukemias in addition to the pure granulocytic (M1-M3) and pure monocytic differentiation patterns (M5), a further group of leukemias having granulocytic as well as monocytic properties in common (M4) has been separated. These myelomonocytic leukemias (M4) probably reflect frozen differentiation stages of precursor cells with a bimodal differentiation potency to evolve either in granulocytes or monocytes. Considering the fact that myelomonocytic precursors may express nonspecific acid esterase, there is a need for a more specific marker to clearly separate the monocyte lineage. The high specificity of Ki-M8 for monocytic cells and specifically its negativity in all three types of granulocytes render this antibody appropriate for the application under this aspect. In 40 cases of acute nonlymphoblastic leukemias, eight cases were found to possess the monocytic phenotype due to their characteristic pattern of reactivity being positive for Ki-M8 and being negative for MoAbs recognizing granulocytes and myelomonocytic precursors.

There seems to be no evidence that the Ki-M8 reactivity pattern could be covered by one of the hitherto described MoAbs directed against the monocyte/macrophage system, since antibodies with mol wt of 30,000 and 32,000 (Fig 5) and with an intracytoplasmatic localization (Fig 3) have not been described so far for this cell system. Some similarity might exist to the MoAb recently established by Zwaal et al. In contrast to this reagent the Ki-M8 antigen is weakly expressed in blood monocytes and increases mainly during the late stages of monocyte/macrophage differentiation. To our knowledge Ki-M8 comprises one of the first MoAbs specific for monocytes and macrophages not only on the basis of antigen distribution under normal and neoplastic conditions but also considering its effect on a typical monocyte/macrophage-related function. Further studies will show whether the inhibitor potency of Ki-M8 will also apply for other functions being probably dependent on lysosomal integrity such as other secretory activities or lysis of tumor cells.
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Ki-M8 monoclonal antibody reactive with an intracytoplasmic antigen of monocyte/macrophage lineage

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