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Ultrastructural Localization of the CD68 Macrophage-associated Antigen in Human Blood Neutrophils and Monocytes

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The ultrastructural localization of the CD68 antigen, a 110-kd intracellular glycoprotein associated with myeloid cells and with monocytes/macrophages, was investigated in human neutrophil granulocytes by postembedding immunogold staining, using monoclonal antibody KP1. The antigen was found in the primary granules of neutrophils, although not all primary granules were labeled. It was absent from the plasma membrane. In monocytes, it was also detected within cytoplasmic granules, colocalized with lysozyme and myeloperoxidase. This observation confirms and completes results obtained by immunofluorescence and other light-microscopic methods. Moreover this study shows that the CD68 epitope recognized by antibody KP1 is able to resist fixation and embedment and therefore emphasizes the value of using KP1 as a marker for this macrophage-associated molecule. (Am J Pathol 1991, 139:1053–1059)

It was reported at the Fourth Workshop on Human Leucocyte Differentiation Antigens 1 that a glycoprotein (molecular weight approximately 110 kd) is detected in monocytes/macrophages and granulocytes by a number of monoclonal antibodies.[1] This antigen, designated CD68 by the Workshop, constitutes one of the best immunocytochemical markers of macrophages in tissue sections.[2–5]

One of the reagents that defined the CD68 antigen was monoclonal antibody KP1.[1,3,6] This antibody, raised against a lysosomal fraction of human lung macrophages, recognizes a wide spectrum of tissue macrophages (eg, germinal center macrophages, alveolar macrophages, macrophages from human tonsils, splenic red pulp, connective tissue of the dermis, Kupffer cells of liver, blood monocytes). In the present study, we have used the immunogold staining technique to investigate the intracellular localization of the CD68 antigen in both mature granulocytes and monocytes from human blood. We report that this molecule is localized to the primary granules of neutrophils and also to the cytoplasmic granules of blood monocytes.

Material and Methods

Cells

Neutrophils were isolated from heparinized peripheral blood by dextran-Radioselectan sedimentation as previously reported.[7] Briefly, blood was layered over an equal volume of a mixture comprising 24 volumes 9% dextran T500 (Pharmacia, Uppsala, Sweden) in 0.9% NaCl and 10 volumes of 38% Radioselectan (Sherring Lab, Lys-lez-lannoy, France). After sedimentation of red blood cells for 40 minutes at 20°C, the leukocyte-rich supernatant was removed and centrifuged at 300*g* for 8 minutes. The cell pellet was resuspended and washed three times in Hank's balanced salt solution. The cells then were divided into two aliquots, one sample being analyzed by immunofluoresence, and the other by immunoelectron microscopy.

Monocytes were prepared by Ficoll-Hypaque density gradient centrifugation.[8] Briefly, heparinized human peripheral blood was layered onto Ficoll (Seromed) (Blood:Ficoll = 10:7.5) and centrifuged for 30 minutes. The mononuclear cells in the supernatant then were washed three times.

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Pre-embedding Immunogold Staining and Processing for Electon Microscopy

To determine whether CD68 antigen was present on the cell surface membrane, unfixed cells were incubated in antibody KP1 for 1 hour, followed by incubation for 1 hour in 5 nm gold-labeled goat anti-mouse IgG (Janssen Pharmaceutica Beerse, Belgium) at 4°C. Antibody KP1 was used at a dilution of 1:2 in Hank's medium, and the goat anti-mouse IgG was diluted twofold in buffer containing 50 mmol/l (millimolar) Na_2HPO_4 and 0.3 mg/ml polyethylene glycol 20,000 (Serva, Heidelberg, West Germany).

Cells then were fixed with 0.5% glutaraldehyde in 0.1 mol/l (molar) phosphate buffer (pH 7.4) for 1 hour at 4°C and embedded in glycol methacrylate (GMA, Rohm and Haas, Darmstadt, West Germany) according to Leduc and Bernhard.[9] Briefly, the cells were infiltrated after fixation with increasing concentrations of GMA. The final embedding medium was composed of seven parts 97% GMA in 3% distilled water and three parts nondestabilized butyl methacrylate containing 1% benzoyl peroxide (Merck, Darmstadt, West Germany). The mixture was partially polymerized by heat before use. Polymerization was performed at 4°C by ultraviolet irradiation type A 405 lamp, P. W. Allen and Co, London, containing Philips 6-watt fluorescent tubes, color type 05).

Granule fractions were purified by subcellular fractionation.[10] After fixation with 1% glutaraldehyde in 0.1 mol/l phosphate buffer for 1 hour at room temperature, granules were embedded in GMA as described above.

Postembedding Immunogold Staining

Thin sections of cells that had been exposed to KP1 and immunogold before embedding in GMA were collected on nickel grids coated with formvar. Grids were floated, with the sections facing downward, on the surface of antibodies or other reagents in microtiter wells according to De Mey.[11] Grids were treated with 10% hydrogen peroxide for 10 minutes at room temperature. After washing with distilled water, grids were rinsed in 0.02 mol/l TRIS-buffered saline (TBS) containing 1% normal goat serum. They then were incubated with antibody KP1 at 4°C for 24 hours, and washed three times in TBS containing 0.1% bovine serum albumin (BSA) for 90 minutes. Grids then were incubated with the secondary antibody, goat anti-mouse IgG labeled with 5-nm colloidal gold particles, at a 1/10 dilution in 0.02 mol/l TBS (pH 8.2) containing 1% BSA, for 1 hour at room temperature. Sections were counterstained with uranyl acetate and lead citrate and observed on a Philips CM10 electron microscope at 60 kV.

Some grids were double immunostained by first labeling for CD68 with antibody KP1 as described above, followed by incubation for 2 hours at room temperature with rabbit anti-lactoferrin (1:200, Cappel Downington, Pennsylvania) or with rabbit anti-myeloperoxidase (1:200, Dakopatts, Denmark) or with rabbit anti-lysozyme (1:500, Dakopatts, Denmark) and then with 15 nm gold-labeled goat anti-rabbit IgG (1:10 Janssen Pharmaceutica, Belgium).

As a control, normal goat serum or monoclonal antibody against platelet glycoprotein Ib (antibody AN51, Dakopatts) or a pool of monoclonal antibodies against von Willebrand factor (provided by Dr. D. Meyer, Kremlin-Bicêtre, France) was used in place of antibody KP1.

Results

Neutrophil PMN

On ultrastructural immunogold staining, antibody KP1 labeled the large polymorphonuclear neutrophils (PMN) granules. Those KP1-positive granules lacked a dense matrix and were identified as primary granules on the basis of their size, number, and appearance, according to our own and others' observations (Figure 1).[12–14] Some of these primary granules showed little or no labeling. The smaller, denser, more numerous secondary granules did not display any labeling. The plasma membrane, nucleus, and mitochondria also were free of gold particles.

Double staining for KP1 and myeloperoxidase confirmed that these two antigens were colocalized in the same type of granules, ie, primary granules (Figure 2a). Some primary granules that expressed myeloperoxidase did not exhibit labeling for CD68, confirming that KP1 did not react with all primary granules. When double staining KP1 in conjunction with lactoferrin (a specific marker for secondary granules), it was consistently found that antibody KP1 labeled lactoferrin-negative granules (Figure 2b).

Finally fractions containing primary and secondary neutrophil granules that had been isolated by subcellular fractionation were immunolabeled for CD68. This experiment also showed staining only of primary granules (Figure 3a) without evidence of labeling of the secondary granule fraction (Figure 3b). The proportion of labeled granules, however, was not superior to what had been observed on intact cells.

The antibody did not react with the cell surface membrane of neutrophil polymorphs by the pre-embedding staining technique. No other PMN organelles were labeled, including mitochondria, cytoplasmic matrix, and nucleus.

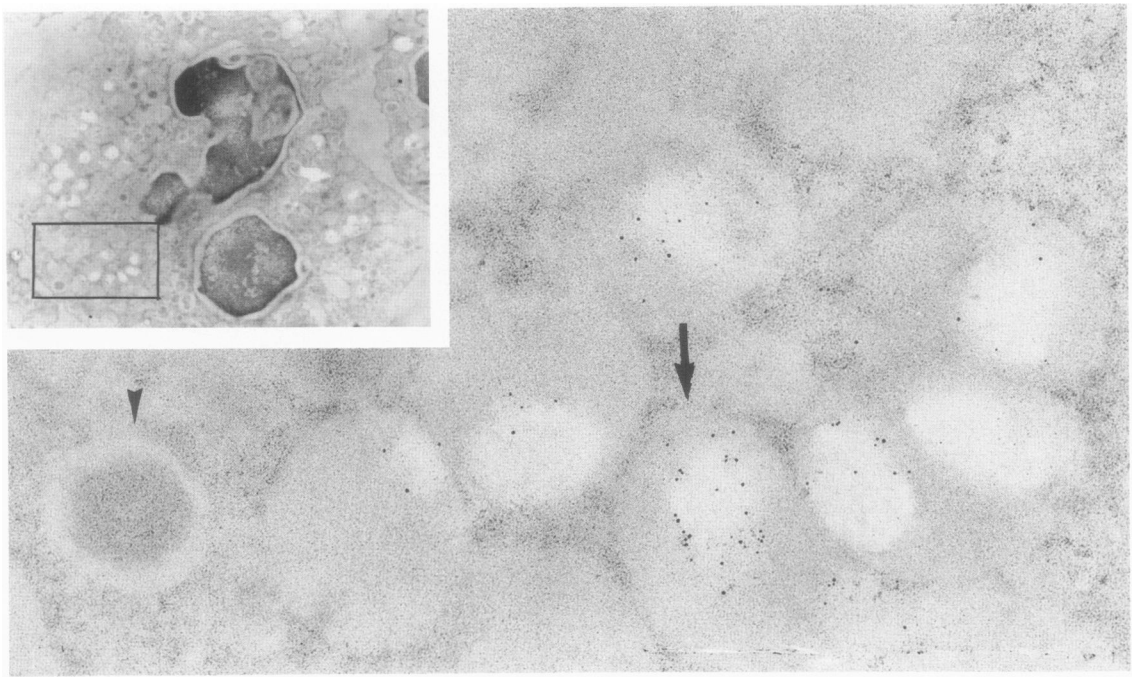

Figure 1. *Section of a blood PMN labeled for CD68 antigen with antibody KP1 by the immunogold technique. Labeling is distributed evenly over the large pale granules of neutrophils (arrow). The smaller dense secondary granules (arrowhead) display no labeling; M × 52,000.*

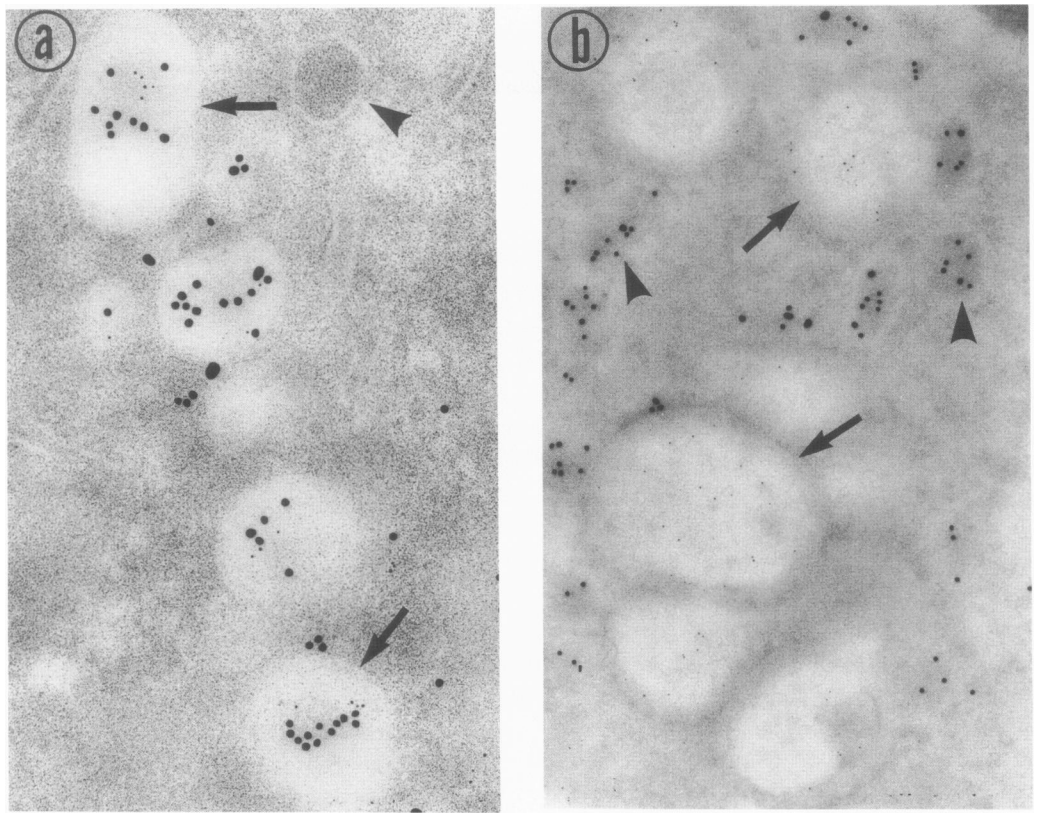

Figure 2. *Part of a PMN double labeled for* (a) *myeloperoxidase (15 immunogold particles) and CD68 (5 nm gold particles), both antigens are found in the matrix of the large primary granules (arrows). The dense specific granules are negative for both markers (arrowheads), M × 56,700;* (b) *lactoferrin (15 nm gold particles) and CD68 (5 nm gold particles). Secondary granules, identified by their lactoferrin content, do not stain for CD68 (arrowheads), whose labeling is found to be associated with the distinct population of primary granules (arrows), M × 40,000.*

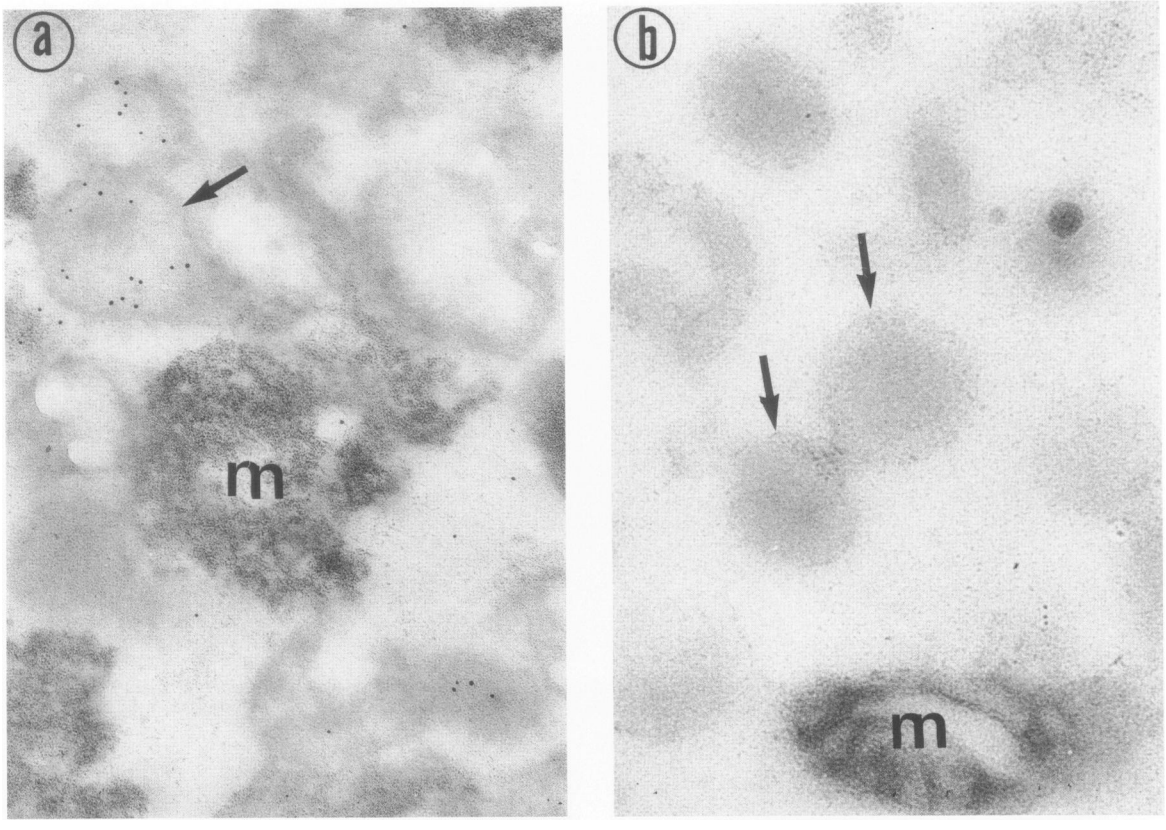

Figure 3. *Immunolabeling for CD68 of the isolated primary and secondary granules.* **a**: *Gold particles label the matrix of the primary granule (arrow). As a control, mitochondria (m) are free of any labeling, M × 71,250.* **b**: *Secondary granules are not labeled by KP1 (arrows), M × 71,300.*

Monocytes

In monocytes, antibody KP1 labeled some of the large pale granules as well as some of the smaller and more electron-dense granules (Figure 4). Double-immunolabeling showed that these positive granules were also positive for myeloperoxidase (Figure 5a), as well as for lysozyme (Figure 5b).

Other organelles such as mitochondria, nucleus, cytoplasmic background did not react with KP1.

Control

As a specificity control, the platelet alpha-granules were always negative for KP1. Also no labeling was observed in the control sections, when antibody KP1 was replaced by irrelevant monoclonal antibody (anti-platelet glycoprotein Ib and anti–von Willebrand factor).

Discussion

Although numerous subpopulations of granules have been described iteratively,[15–17] it is well known that neutrophil granulocytes contain two types of granules, designated primary and secondary.[18–20] Secondary granules are smaller than primary granules, have a denser matrix by electron microscopy, and contain lactoferrin.[12,21–23] We have confirmed by immunoelectron microscopy that the CD68 antigen, detected by antibody KP1, is localized in a population of large pale granules in neutrophil granulocytes, which could be identified as primary granules because double staining showed that they lacked lactoferrin. In immunostained purified granule fractions, CD68 also was detected in the primary granules and not in secondary granules. CD68 therefore can be added to the list of antigens (eg, elastase, myeloperoxidase, etc.) that are localized in neutrophil primary granules. To rule out the possibility of recognition of other cross-reacting epitopes by KP1, it would have been useful to study other anti-CD68 antibodies; however this was not possible because no other antibodies are suitable for use on embedded material.

CD68 was not detected in all the primary granules of neutrophilic granulocytes or in all the granules within monocytes. Immunolabeling subcellular granule fractions confirmed this observation (although our previous

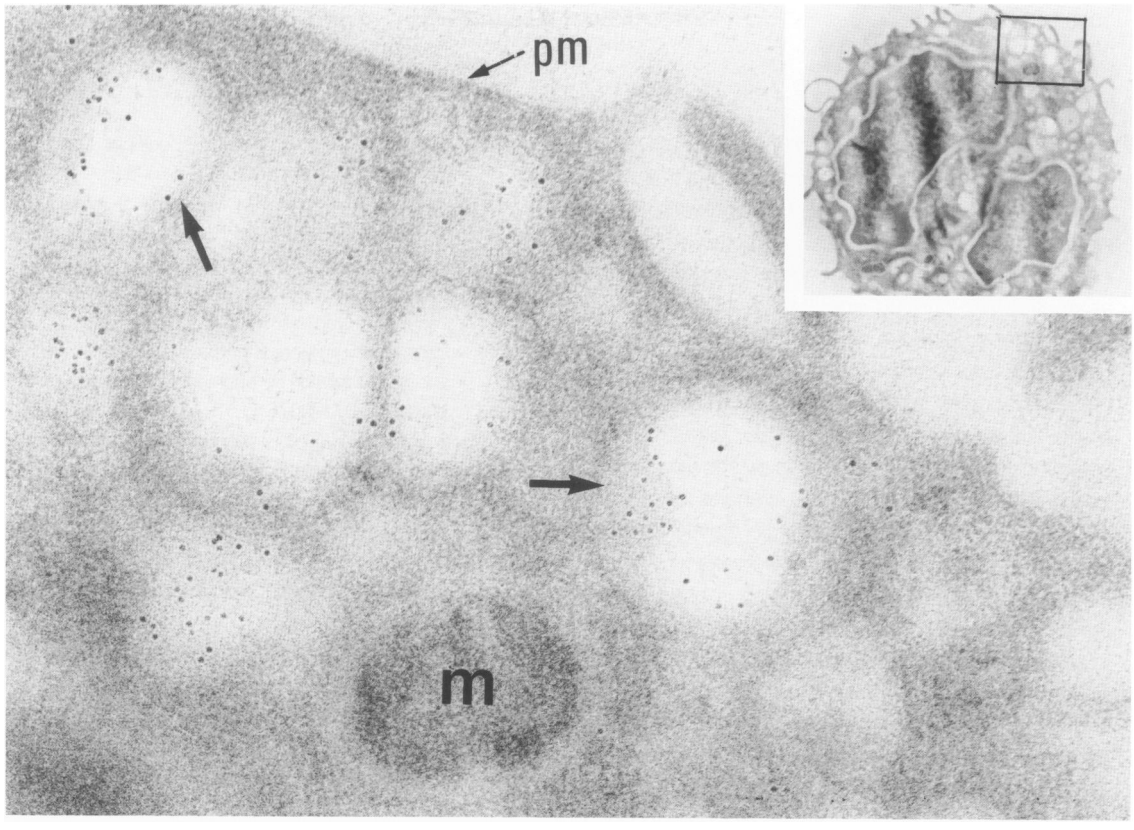

Figure 4. *Section of a monocyte labeled for CD68 antigen with KP1 antibody by the immunogold technique. Labeling is found within most of the cytoplasmic granules (arrows). The plasma membrane (pm) and mitochondria (m) are devoid of any labeling, M × 60,700.*

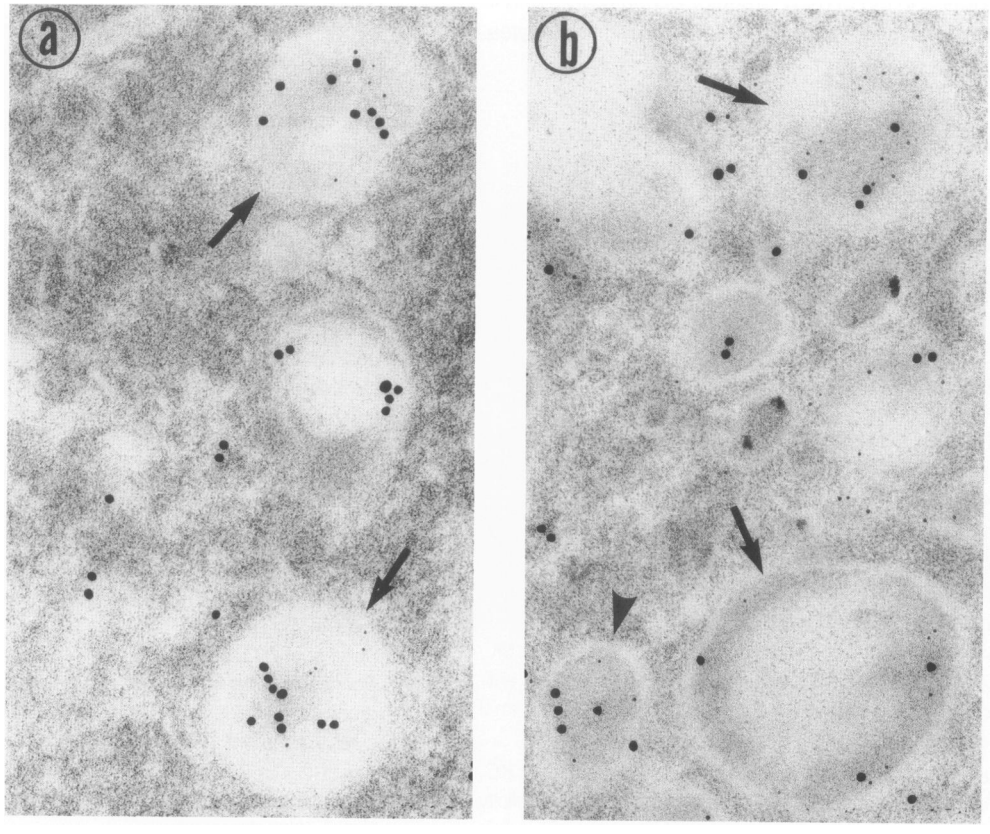

Figure 5. *Part of a blood monocyte double labeled* (a) *for myeloperoxidase (15 nm gold particles) and CD68 (5 nm gold particles); both antigens are found in the same granules (arrows), M × 55,000;* (b) *for lysozyme (15 nm) and CD68 (5 nm). The two proteins are colocalized and found in the large (arrows) and small (arrowheads) cytoplasmic granules, M × 55,700.*

findings had shown a better immunolabeling of lysozyme in specific granules from purified fractions than in intact cells).[13] It is conceivable that the antigenicity of CD68 was partially lost during fixation and after embedding.[24] Alternatively the CD68 antigen may not be contained in all neutrophils, primary granules, and monocyte granules; further studies are needed to resolve this point.

Previous studies reported CD68 to be associated with blood monocytes, and its localization was described as being diffuse intracytoplasmic.[25] Our ultrastructural observations extend this observation by showing that CD68 is a component of cytoplasmic granules of monocytes and is not found in the cytoplasmic matrix. This finding can be related to the recent observation by light and electron microscopy that CD68 also is associated with the cytoplasmic granules of mast cells.[26] It also will be interesting to study CD68 antigen localization in macrophages, because it was first described in this cell type.[6]

In conclusion, the monoclonal anti-CD68 antibody KP1 has been demonstrated to be useful in many instances, including for diagnostic pathology. The present study, based on CD68 in neutrophils and monocytes, emphasizes its value by confirming that KP1 can react with its epitope on the CD68 antigen after fixation and plastic embedding, and also demonstrates that this antigen is found in the primary granules of neutrophils and in both types of cytoplasmic granules in blood monocytes.

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