Ranjit S. Bhardwaj, Claudia Zotz, Gabriele Zwadlo-Klarwasser▲, Johannes Roth, Matthias Goebeler, Karsten Mahnke, Matthias Falk, Georg Meinardus-Hager and Clemens Sorg

Institute of Experimental Dermatology, University of Münster, Münster and Institute of Pharmacology▲, RWTH Aachen, Aachen

The calcium-binding proteins MRP8 and MRP14 form a membrane-associated heterodimer in a subset of monocytes/macrophages present in acute but absent in chronic inflammatory lesions*

Monocytes/macrophages expressing an epitope recognized by a monoclonal antibody 27E10 are present in acute but are absent in chronic inflammatory disorders. This report shows that the 27E10 antigen is formed by noncovalent association of the two Ca^{2+}-binding proteins MRP8 and MRP14 which belong to the S100 protein family. Identification has been confirmed immunochemically, by matrix-assisted UV-laser desorption/ionization spectrometry and by partial amino acid sequencing. Surface expression of the MRP8/MRP14 complex on a subset of monocytes is reported for the first time and shown to be up-regulated in a Ca^{2+}-dependent manner. The 27E10 surface-positive monocytes isolated by cell separation techniques release high amounts of tumor necrosis factor-α and interleukin-1β in contrast to their 27E10 surface-negative counterparts thus emphasizing their role in inflammation.

1 Introduction

Circulating blood monocytes are recruited under physiological and inflammatory conditions to tissue sites where they differentiate to various macrophage phenotypes which exert a wide spectrum of functions. To study different macrophage subsets *in situ*, we have developed mAb against human macrophages [1–3]. One of these, the mAb designated 27E10, detects a surface antigen that is found on a subset of peripheral blood monocytes and granulocytes. It is increasingly expressed on monocytes during culture reaching a maximum between days 2 and 3. Its cell surface expression is enhanced by IFN-γ, PMA and LPS [3]. The epitope is absent in platelets, lymphocytes and all tested human cell lines. In immunohistological studies $27E10^+$ macrophages were found to dominate in acute inflammatory lesions (*e.g.* periodontitis [4]), while they are widely absent under chronic inflammatory conditions (*e.g.* BCG granuloma, rheumatoid arthritis, sarcoidosis) and in normal tissues [3]. From this it has been concluded that the $27E10^+$ macrophage phenotype is characteristic for the acute stage of inflammation.

In the present report we characterize the epitope defined by mAb 27E10. It is formed by noncovalent association of the two Ca^{2+}-binding proteins MRP8 and MRP14, which had been sequenced and cloned by our group before [3, 5, 6]. Localization of both proteins in monocytes and granulocytes has previously been supposed to be restricted to intracellular sites [7]. Calgranulin A/B, cystic fibrosis antigen and L1 antigen light/heavy chain are synonyms used in the literature for MRP8 and MRP14, respectively [8]. Each of these two proteins contains two EF-hands assigning them to the S100 protein family, which includes S100-α/β calcyclin, intestinal calcium-binding protein and p11 [9, 10]. Although no definite function has been determined for these proteins so far there is evidence that they are involved in cell cycle progression, cell differentiation, cytoskelet-membrane interactions, and phosphorylation events [11–13]. Recent studies have shown an inhibitory activity of a complex form of MRP8 and MRP14 for casein kinases I and II [11].

We further demonstrate that the assembly to a membrane-associated complex of Ca^{2+}-binding proteins is a distinct differentiation step which may not only be induced by LPS, IFN-γ, and TNF-α but also by Ca^{2+} ionophores. In cell separation experiments it is shown that monocytes expressing the 27E10 epitope produce high amounts of TNF-α and IL-1β in contrast to their $27E10^-$ counterparts.

[I 10397]

* This work was supported by funds of the Bundesministerium für Forschung und Technologie.

Correspondence: Clemens Sorg, Institute of Experimental Dermatology, von-Esmarch-Str. 56, W-4400 Münster, FRG

2 Materials and methods

2.1 Reagents

Immunochemicals were purchased from Dianova (Hamburg, FRG), FITC-conjugated mAb 27E10, mAb against rMRP8 and rMRP14 were obtained from BMA (Augst, Switzerland) affinity-purified polyclonal monospecific rabbit antisera against rMRP8 and rMRP14 were kindly provided by Dr. J. Brüggen (CIBA-GEIGY, Basel, Switzerland).

2.2 Cells and culture conditions

Human monocytes from buffy coats (Blutbank Münster, FRG) were isolated and cultivated as described [4]. For the evaluation of modulation of 27E10 surface expression human monocytes in culture (days 1–3) were exposed to various agents for 1 h. The following compounds were added: endotoxin (LPS) from *Escherichia coli*, serotype 055:B5 (1 μg/ml, Sigma, Deisenhofen, FRG); the phorbol ester PMA ($1{,}6 \times 10^{-7}$M, Sigma); human rIFN-γ (100 U/ml, Boehringer Mannheim, Mannheim, FRG); the

 0014-2980/92/0707-1891$3.50+.25/0

ionophores A23187 (10 μM, Calbiochem, Bad Soden, FRG), ionomycin (5 μM Calbiochem) and monensin (10 μM, Sigma); the Ca^{2+} channel blocker verapamil (100 μM, Sigma); the calmodulin antagonist trifluoperazine (10 μM, Calbiochem); and $CaCl_2$ (4 mM, Sigma).

2.3 Cell separation and determination of cytokine release

Monocytes cultivated for 1 or 2 days were stained with mAb 27E10 (2 μg/ml), washed and then co-incubated at 4 °C for 1 h with 2×10^6 sheep anti-mouse IgG-coated Dynal beads per 10^6 cells. After separation by a magnet, the beads were detached from the cells by treatment with papain (0.01 mg/ml PBS at 37 °C for 4 min). Purity of $27E10^+$ cells was >97 % in all experiments as verified by staining of cytopreparations with mAb 27E10 in the immunoperoxidase technique. The cells were cultivated at a density of 10^6 cells/ml for 24 h. Supernatants were harvested, centrifuged and analyzed for cytokine release. IL-1β and TNF-α were determined using ELISA kits (Biermann, Bad Nauheim, FRG).

2.4 Preparation of monocyte and granulocyte extracts and immunoaffinity chromatography

Monocyte and granulocyte extracts were prepared as described [3, 14]. The protein content was 4–5 mg/ml, as determined by the Bradford method [15].

Protein G (Pharmacia, Freiburg, FRG) purified mAb 27E10 was coupled to CNBr-activated Sepharose (20 mg/3 ml), according to the manufacturers instructions (Pharmacia). Cell lysates of day-2 monocytes (10^9 monocytes in PBS containing 1 mM PMSF) were subjected to immunoaffinity chromatography as described [14].

2.5 SDS-PAGE, silver staining, Western blot and dot blot

Aliquots of 10 μg protein of lyophilized affinity eluate were separated under reducing (5 % 2-ME) or nonreducing conditions by SDS-PAGE on 15 % gels [16]. Proteins were visualized by silver staining [17] or were electrotransferred and immunostained as described [14] using mAb 27E10 (1 μg/ml), anti-rMRP8 or anti-rMRP14 (0.5 μg/ml). Controls were performed with mouse IgG_1 (isotype control) and rabbit IgG, respectively.

For dot blots extracts from granulocytes and monocytes (500 μg/ml) were treated with 6 M urea containing 10 mM DTT. Parallel samples were desalted using a Sephadex G-25 column (PD-10, Pharmacia) equilibrated with PBS and 0.01 mM Ca^{2+}. Subsequently, protein content was determined. In another set of experiments equal amounts of rMRP8 and rMRP14 were mixed under reducing and nonreducing conditions with or without Ca^{2+} (2 h, 37 °C). Dot blots of cell extracts (10 μg/dot) and recombinant proteins (1–2 μg/dot) were developed similarly as Western blots using mAb 27E10, anti-rMRP8, anti-rMRP14 as well as isotype control and rabbit IgG of irrelevant specificity.

2.6 Flow cytometric analysis

Cell surface expression of 27E10 antigen on monocytes was analyzed with an EPICS-V cell sorter (Coulter Electronics, Hialeah, FL) as described [3]. The panel of antibodies included mAb 27E10 [3], RM3/1 [2], 910D7 for HLA-DR [18], CD14, CD16, CD18 and isotype controls, anti-rMRP8, anti-rMRP14 and control rabbit IgG of irrelevant specificity. The mAb were used at a concentration of 2 μg/ml IgG, polyclonal antisera at 1 μg/ml. Percentage of positive cells was determined by a least square algorithm method (Immuno-Software; Coulter). Data are given as percentage of specific antibody-stained cells in comparison to isotype control staining.

To investigate the effect of Ca^{2+} on 27E10 surface expression in a time-dependent manner day-2 or day-3 monocytes were harvested and washed three times with PBS containing 1 % BSA. One million cells per ml RPMI medium conditioned with 1 % FCS were incubated with either FITC-conjugated mAb 27E10 or isotype control (2.5 μg/ml) at room temperature. Fluorescence measurements were performed with a FACScan (Becton Dickinson, Mountain View, CA). Data acquisation and analysis was obtained by using Chronys software (Becton Dickinson). Dead cells were gated out after addition of propidium iodide (100 mM) to the cell suspension. Flow rate was approx. 10^4 cells/min. Cells treated with mAb 27E10 or isotype control were measured the latter showing constant baseline levels for 1 h. In parallel, specimens showing a constant fluorescence for 5 min were incubated with or without immediate addition of A23187 (10 μM) plus $CaCl_2$, 1 mM) and fluorescence was measured for a further 56 min.

For flow cytometric determination of either mAb 27E10- or A23187-induced Ca^{2+} mobilization, monocytes (10^6 cells/ml) were first loaded with 1 μM of fluo-3-AM (Calbiochem) according to the protocol of Becton Dickinson. Cells were then analyzed by FACScan (Becton Dickinson). After addition of either mAb 27E10 or A23187 (10 μM plus $CaCl_2$, 1 mM) approx. 1×10^4 cells were analyzed per min. Fluorescence intensity as measure of increase in cytoplasmic Ca^{2+} level was then analyzed [19, 20] by using the Chronys program (Becton Dickinson).

2.7 Matrix-assisted UV laser desorption/ionization (LDI) mass spectrometry and amino acid sequence determination

The molecular weight (mol. wt.) of the 27E10 antigen was determined by using LDI mass spectrometry. LDI analysis of the 27E10 purified eluate was carried out with or without reduction by DTT (10 mM, 15 min at 95 °C) as described by Karas et al. [21]. Affinity-purified material was separated on SDS-PAGE. The $27E10^+$ protein band of 25 kDa was cut out, reduced, loaded on a second gel and electrotransferred to fluorotransmembrane (Pall, Dreieich, FRG). Components of 10 and 14 kDa were visualized after staining with amido black. After Edman degradation the 10-kDa protein could directly be analyzed with an automated gas-phase protein sequencer (477A, Applied Biosystems, Weiterstadt, FRG). Because of N-terminal blockage the 14-kDa component was first subjected to direct cleavage with CNBr as described [22]. Fragments were separated in the absence

of urea on SDS-polyacrylamide gradient gels (9%–26%) as described [23]. Electroblots were stained with amido black and subjected to Edman degradation and protein sequencing.

3 Results

3.1 Characterization of the 27E10 antigen

Monocyte lysates (day-2 culture) were separated on an affinity column with mAb 27E10. Eluates were analyzed by SDS-PAGE and silver staining which revealed a pattern of five protein bands of 28, 25, 21, 14 and 10 kDa under nonreducing conditions. Only the two bands at 14 and 10 kDa were detectable under reducing conditions (Fig. 1A lane a and b).

As this pattern was reminiscent of that obtained earlier with the Ca^{2+}-binding proteins MRP8 and MRP14 [3, 24], Western blots of nonreduced eluate were developed with mAb 27E10, anti-rMRP8 and anti-rMRP14. Under nonreducing conditions the mAb 27E10 only reacted with the 25-kDa band whereas anti-rMRP14 detected proteins of 28, 25 and 14 kDa (Fig. 1B lane a and b). On the other hand, anti-rMRP8 stained the 25-, 21-, and 10-kDa bands (Fig. 1B lane c). Mouse IgG_1 and rabbit IgG of irrelevant specificity did not show any immunoreactivity (data not shown).

These data suggest that the larger molecules represent complexes of MRP8 and MRP14 monomers. To analyze the composition of the different complexes single protein bands were cut out after separation on SDS-PAGE (Fig. 2, top) and re-electrophoresed on a second gel under reducing conditions (Fig. 2A). The 25-kDa protein detected originally by mAb 27E10 dissociated into 14- and 10-kDa molecules (Fig. 2A, silver staining) which were reactive with anti-rMRP14 and anti-rMRP8, respectively (Fig. 2B, Western blot, lane c and d). The 28- and 21-kDa molecules were shown to be homodimers of MRP14 and MRP8 (Fig. 2B, Western blot, lane a and e).

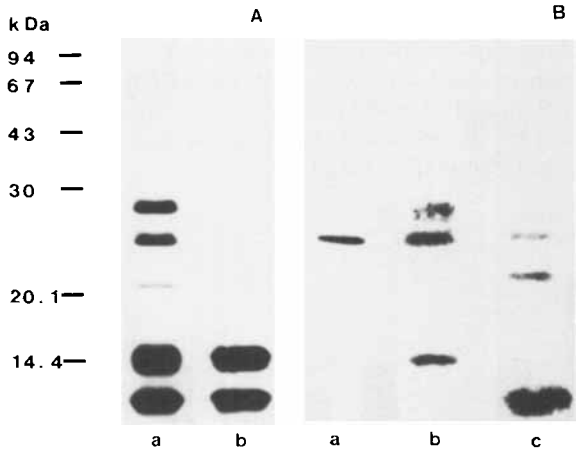

Figure 1. Analysis of proteins absorbed by mAb 27E10 from lysates of monocytes. Supernatants of disrupted monocytes were subjected to affinity chromatography with mAb 27E10. The eluated material was separated by SDS-PAGE (15% acrylamide). (A) Silver staining: nonreducing (lane a) and reducing (lane b) conditions. (B) Western blotting: mAb 27E10 (lane a), anti-rMRP14 (lane b) and anti-rMRP8 (lane c) were visualized by goat antibodies specific for mouse or rabbit IgG conjugated with alkaline phosphatase. M_r markers (at the left side) used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carboanhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa).

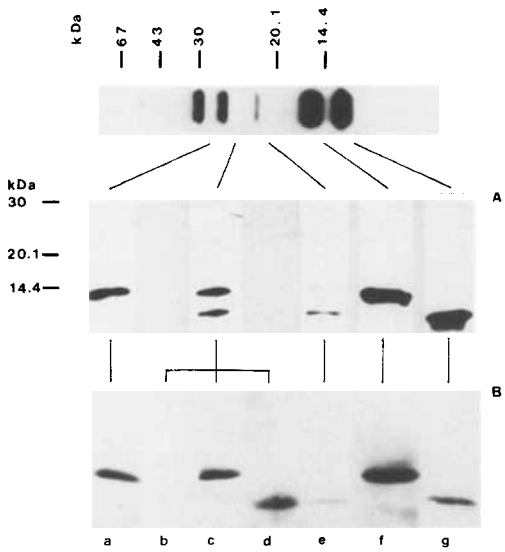

Figure 2. Identification of components of protein complexes specifically eluted with mAb 27E10. After separation by SDS-PAGE (15% acrylamide) under non-reducing conditions (silver staining on top), gel pieces corresponding to distinct protein bands were excised from neighboring lanes, treated with 2-ME (10 min at 95°C) and re-electrophoresed under reducing conditions. (A) Silver staining. (B) Western blotting: anti-rMRP14 (lanes a, c and f), anti-rMRP8 (lanes d, e and g) and mAb 27E10 (lane b). Note that mAb 27E10 did not detect any of the dissociated protein components.

To further verify that the 27E10 epitope is constituted of MRP8 and MRP14, exact mol. wt. determinations were performed using laser mass spectrometry. This method has recently been reported to allow mol. wt. determinations of proteins with a deviation of less than 1% [21]. The mol. wt. of the monomers (10975 and 13335) coincide with those calculated for the deduced amino acid sequence of MRP8 (10835) and MRP14 (13242), respectively (Fig. 3). Under reducing conditions (Fig. 3B) only the molecules corresponding to MRP8 and MRP14 could be detected. Under nonreducing conditions (Fig. 3A) the mol. wt. for the complexes eluted from the 27E10 affinity column were determined at 22077, 24320 and 26459 which correspond to the theoretical mol. wt. of $(MRP8)_2$, (MRP14, MRP8) and $(MRP14)_2$. The MRP14 peak showed a shoulder representing another isoform of the mol. wt. 12860 [14].

For further identification of the constituents of the 25-kDa complex partial amino acid sequences were determined. Only the 10-kDa molecule could be directly sequenced. The N terminus of the 14-kDa molecule was found to be blocked as shown by our previous data [6, 25] as well as by recent results reported by Edgeworth et al. [26]. Therefore, the 14-kDa molecule was digested with CNBr, fragments were electroblotted and subsequently sequenced. The amino acid sequence of the 10-kDa molecule was identical with the amino acids 2–10 of MRP8 and the two peptide

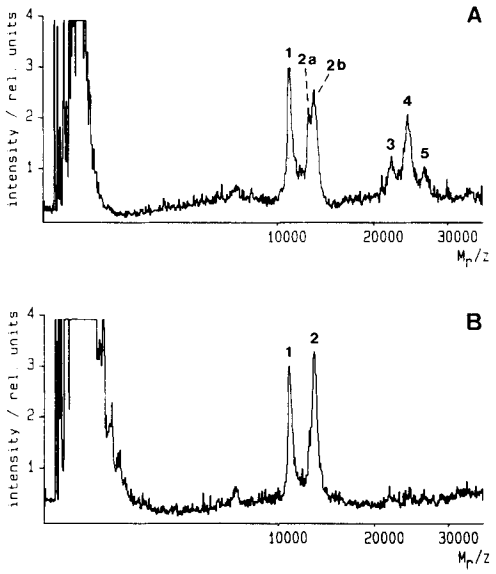

Figure 3. Mol. wt. determination by matrix-assisted UV LDI mass spectrometry of the affinity-purified 27E10 antigen under nonreducing conditions revealed: (A) six molecules with the mol. wt. 1 = 10975; 2a = 12860; 2b = 13335; 3 = 22077; 4 = 24320; 5 = 26459 as determined by the peak centroids; and under reducing conditions (B) two molecules with mol. wt. 1 = 10929; 2 = 13267.

fragments of the 14-kDa molecule corresponded to amino acid position 6–22 and 96–110 of MRP14, respectively.

To exclude the possibility that a third, so far undetected molecule could be involved in the formation of the 27E10 epitope, purified rMRP8 and rMRP14 were incubated with PBS containing Ca^{2+}. As shown in dot blots (Fig. 4B, a–c), the individual proteins did not react with mAb 27E10, whereas the epitope was readily formed in a mixture of the two proteins. The epitope maintained its reactivity in the presence of DTT. It was destroyed by urea, yet restored after removal of urea by desalting (Fig. 4A, a–c). The possibility that urea could obstruct the binding of the proteins to the hydrophobic blot membrane was excluded since anti-rMRP8 and anti-rMRP14 showed positive reactivity in these experiments (data not shown).

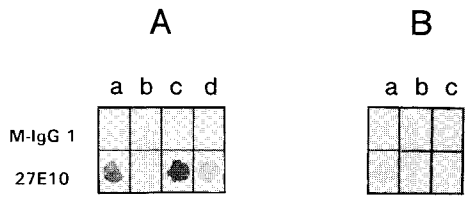

Figure 4. Reconstitution of the 27E10 epitope. (A) Cytoplasmic protein (10 μg) from granulocyte or monocyte extracts were dotted and stained with isotype control and mAb 27E10, respectively. Lysate in PBS containing 0.01 mM CA^{2+} (a), treated with 6 M urea containing 10 mM DTT (b), and subsequently renatured (desalted) in PBS (c). Treatment solely with DTT is shown in lane d. (B) rMRP8 and rMRP14 (2 μg each in PBS containing 0.01 mM Ca^{2+}) were mixed and incubated for 2 h at 37 °C, 2 μg of this protein mixture (a), or solely 2 μg of rMRP8 (b) and 2 μg of rMRP14 (c) were dot blotted. The immunostaining with mAb 27E10 and isotype and control antibody was performed according to the procedure described for Fig. 1B.

3.2 Surface expression of the 27E10 epitope

Previous flow cytometric analysis revealed surface expression of 27E10 which is up-regulated by LPS, IFN-γ and PMA [3]. In addition, we have recently shown that the Ca^{2+}-binding proteins MRP8 and MRP14 form complexes in a Ca^{2+}-dependent manner [14]. We, therefore, attempted to analyze the influence of Ca^{2+} on 27E10 surface expression.

For these experiments day-1, -2 and -3 cultured monocytes were incubated with Ca^{2+}-ionophores A23187 and ionomycin, calmodulin antagonist trifluoperazine and Ca^{2+} channel blocker verapamil prior to flow cytometry (Fig. 5). A23187 and ionomycin increased the number of $27E10^+$ monocytes 2–3-fold compared to control at day 1. At days 2 and 3 of culture 27E10 was also inducible, yet with a weaker effect. Simultaneous incubation with A23187 and Ca^{2+} lead to an enhanced surface expression. Elevated concentrations of Ca^{2+} alone had no effect. Similarly, no effects were seen with verapamil and trifluoperazine. In addition, up-regulation of 27E10 could be observed with monensin, a monovalent ionophore which complexes Na^+ ions but almost no Ca^{2+} and blocks the transport of secretory proteins within the Golgi complex. No effect of these agents was seen on other surface constituents like HLA-DR antigen, CD14, CD16 and CD18, which were determined in parallel (data not shown). To investigate the time course of intracellular calcium increase and subsequent surface expression of 27E10 the following experiments were performed. First, the rise of intracellular Ca^{2+} concentration was determined by flow cytometry in cultured monocytes after stimulation with A23187 using the Chromys Software program (Becton Dickinson). A 1.5-fold rise was recorded within 30 s (Fig. 6A). In a parallel specimen 27E10 surface expression was measured. Monocytes were incubated with excess of either fluoresceinated mAb 27E10 or isotype control. The incubation with antibodies did neither lead to a rise in Ca^{2+} level nor to up-regulation of 27E10 surface expression since fluorescence intensity remained constant for more than 30 min. As early as 2 min after addition of A23187 an augmented expression of 27E10 was detectable which further increased over the next hour (Fig. 6B).

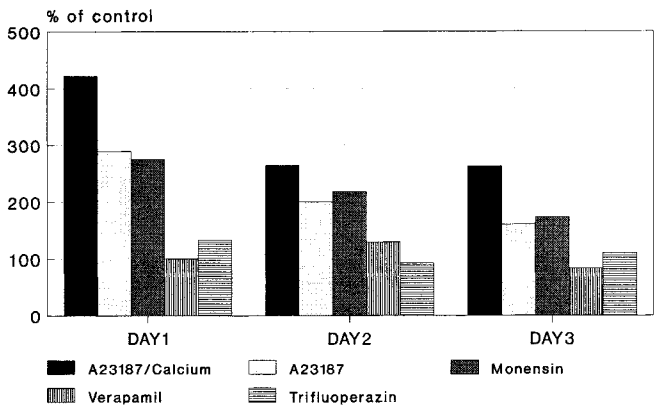

Figure 5. Modulation of 27E10 cell surface expression on monocytes in culture. Cells were harvested at days 1, 2 and 3 after 1 h of stimulation with agents as described. Flow cytometric analysis was performed from 30000 cells. Reactivity of mAb 27E10 was compared to isotype control. Data are given as percentage of stimulated cells compared to unstimulated control cells.

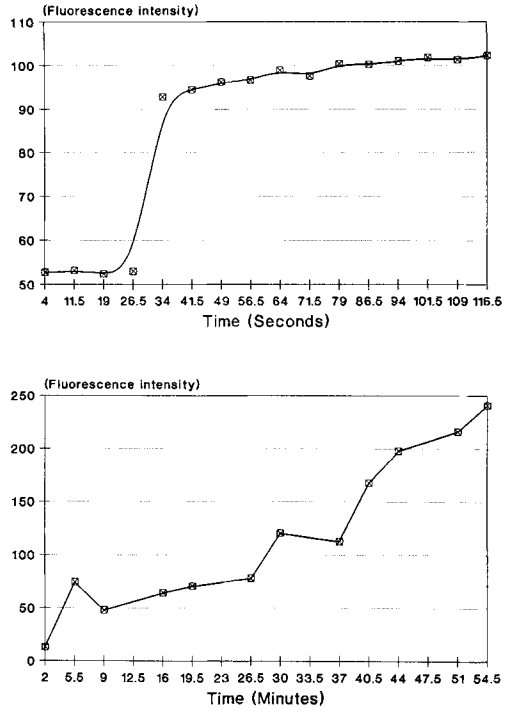

Figure 6. Influx of Ca^{2+} and cell surface expression of 27E10 antigen by monocytes induced with ionophore A23187 as measured by flow cytometry. Increase in the fluorescence intensity of the dye fluo-3 is shown as a measure of the increase of cytoplasmic Ca^{2+} levels (A). Rise in 27E10 surface expression is detected as increase of fluorescence of FITC-conjugated mAb 27E10 after addition of A23187 to cells as compared to unstimulated cells (B).

3.3 Characterization of the 27E10$^+$ phenotype

To characterize monocytes of the 27E10$^+$ phenotype, the cells of 1- and 2-d culture were separated with antibody-coated magnetic beads and analyzed for their secretory activity. IL-1β and TNF-α content was determined in the supernatant of the 24-h cultures. 27E10$^+$ monocytes released significantly higher amounts of TNF-α and IL-1β at both days of culture than their 27E10$^-$ counterparts and unfractionated cells (Table 1). Standard deviations reflect the high variance in secretory activity within individual donors. However, 27E10$^+$ monocytes always released 4- to 100-fold higher concentrations of these mediators than 27E10$^-$ cells. The possibility of unspecific induction of cytokine production could be excluded since neither addition of mAb 27E10 or beads to unfractionated cells nor papain treatment lead to any increase of cytokine release (data not shown). In contrast to unfractionated cells only isolated 27E10$^+$ monocytes produced high amounts of cytokines.

Table 1. Secretion of TNF-α, IL-1β by isolated 27E10$^+$ monocytes[a)]

Monocytes after: A. 1 day of culture	TNF-α (ng/ml +/− SD)	IL-1β (ng/ml +/− SD)
Unfractionated	0.55 +/− 0.81	1.38 +/− 1.26
27E10[b)]	0.87 +/− 0.87	1.88 +/− 1.19
27E10$^+$	4.79 +/− 4.74	5.74 +/− 4.40
Monocyte after: B. 2 days of culture	**TNF-α (ng/ml +/− SD)**	**IL-1β (ng/ml +/− SD)**
Unfractionated	0.12 +/− 0.23	0.32 +/− 0.59
27E10$^-$	0.06 +/− 0.10	0.43 +/− 0.86
27E10$^+$	1.08 +/− 1.00	1.49 +/− 2.15

a) See Sect. 2.3.
b) Values obtained from 27E10$^+$ cells are significantly different from those shown by unfractionated or 27E10$^-$ cells as determined by the Wilcoxon test ($p < 0.05$)

4 Discussion

We have previously described an mAb designated 27E10 which reacts with a subset of monocytes/macrophages in acutely inflamed tissues but is absent in normal tissues. The epitope is also found in PMN and a subpopulation of blood monocytes [3].

In the present study we have characterized the epitope defined by mAb 27E10. We could demonstrate that this epitope is constituted by complexation of two low mol. wt. proteins. These molecules were identified as the Ca^{2+}-binding proteins MRP8 and MRP14 by three different approaches, *i.e.* immunological identification (Western blotting), exact mol. wt. determination by matrix-assisted LDI mass spectrometry [21] and partial amino acid sequencing.

Neither recombinant nor native individual MRP8 or MRP14 from cellular lysates are recognized by mAb 27E10. However, incubation of both recombinant proteins leads to formation of the 27E10 epitope thus ruling out the participation of a third component. Denaturation of native 27E10 antigen with urea and subsequent renaturation as well as DTT treatment proved that the epitope is formed by noncovalently bound MRP8 and MRP14 without involvement of disulfide bridging.

MRP8 and MRP14 are identical with the so-called light and heavy chains of the L-1 antigen complex [27]. This protein complex of 36 kDa has been demonstrated immunohistologically in granulocytes, monocytes and squamous epithelial cells [28].

The affinity-purified proteins were separated as dimers (28, 25 and 21 kDa) and monomers of MRP8 (8 kDa) and MRP14 (14 kDa) on SDS-PAGE under nonreducing conditions. Under reducing conditions only monomers were seen. This pattern was identical to that of purified MRP reported previously by our group [14]. MRP8 and MRP14 were shown to exhibit a strong tendency to form complexes. By use of chemical cross-linkers *in vivo* formation of three different MRP8/MRP14 complexes was demonstrated: a 48.5-kDa tetramer ($(MRP8)_2(MRP14)_2$), a 35.0-kDa trimer ($(MRP8)_2(MRP14)$) corresponding to the L-1 complex [27] and a 24.5-kDa heterodimer of MRP8/MRP14 could be detected. The complexes were shown to be noncovalently linked and therefore disintegrate during SDS-PAGE [14].

The tetramer and trimer complexes were not unequivocally recognized by mAb 27E10 on Western blots which is apparently due to high load of cross-linker resulting in the modification of the epitope (data not shown). Disulfide linkage of MRP8 and MRP14 could be shown to be an artefact since no covalently linked dimers were detectable after blocking of -SH groups in crude cell extracts by iodoacetamide [14]. Assembly of the MRP8 and MRP14 complex has been described to be a Ca^{2+}-regulated process [14]. In the present study we further demonstrate that the surface expression of the 27E10 antigen is influenced by Ca^{2+}. Increasing intracellular Ca^{2+} levels by ionophores as A23187 and ionomycin lead to an up-regulation of 27E10 surface expression. As shown by flow cytometry, surface expression of $27E10^+$ MRP8/MRP14 complex is a rapid response to an increase of intracellular Ca^{2+} levels. It is currently unknown how Ca^{2+} mobilization and subsequent up-regulation of 27E10 antigen are governed. Calmodulin is probably not involved since its inhibitor trifluoperazine did not show any effect. The Ca^{2+} channel blocker verpamil did not influence 27E10 surface expression either.

How transport and plasma membrane association of MRP8/MRP14 complexes are mediated is not known so far. The Ca^{2+}-binding proteins MRP8 and MRP14 do not contain a signal sequence or a transmembrane region [7]. This places these proteins into a similar category as PD-ECGF [29], bFGF [30] and IL-1β [31] which also lack a signal sequence. So far, neither MRP8 or MRP14 nor their complexes have been demonstrated to be released by viable cells (data not shown) [26]. Instead, the $27E10^+$ surface expression as shown here corroborate the plasma membrane association of the MRP8/MRP14 complex. A vesicle transport as reported for membrane glycoproteins [32] is unlikely since monensin, which blocks intracellular transport at the level of the Golgi apparatus [33], rather leads to enhancement than to inhibition of 27E10 surface expression. This may be explained by a rise of Ca^{2+} concentration secondary to monensin-induced movement of Na^+ ions into cellular compartments [34]. Thus, the MRP8/MRP14 complex seems to be translocated to the membrane by an alternative, obviously Ca^{2+}-dependent pathway. A pathway apart from the endoplasmic reticulum (ER)-Golgi route which might be suggestive due to the lack of a signal sequence, may additionally protect free thiol residues present in MRP from being oxidized in the ER. However, the exact mechanism for translocation to, as well as association with the plasma membrane needs to be elucidated.

As suggested for annexins Ca^{2+} facilitates association of hydrophobic regions with membrane phospholipids [35], a similar mechanism may be responsible for 27E10-antigen/membrane interactions. The molecular orientation of the MRP8/MRP14 complex embedded in the membrane is currently unknown. Using different mAb against MRP8 and MRP14 only intracellular localized epitopes have been detected so far (own unpublished data, [36, 37]). Thus, with mAb 27E10 surface expression of MRP is demonstrated for the first time. MRP14 has been shown to be phosphorylated in a calcium-dependent way [38]. Our data clearly demonstrate that 27E10 epitope formation does not require phosphorylation. The question of whether phosphorylation is a prerequisite for complex formation or membrane association is currently under investigation. In further experiments $27E10^+$ monocytes were characterized functionally. Isolated $27E10^+$ monocytes spontaneously released substantial amounts of IL-1 and TNF-α in contrast to the $27E10^-$ population. These data point to the previously reported prevalence of early infiltrating $27E10^+$ monocytes/macrophages in acute inflammatory disorders [3, 4] which now have been demonstrated to exhibit the property to secrete inflammatory cytokines. From many inflammatory tissues investigated so far it appears that the influx of monocytes/granulocytes expressing MRP8 and MRP14 is most characteristic. In acute inflammatory lesions, the monocytes seem to differentiate further and to assemble the heterodimer bearing the 27E10 epitope. This observation has been confirmed by the results obtained from psoriasis vulgaris [3], periodontitis [4] and cardiac allografts [39]. In a recent study on a series of rejections of renal allografts (Goebeler et al., manuscript in preparation), in chronic rejections as well as in other chronic conditions (colitis ulcerosa, theumatoid arthritis, sarcoidosis, multiple sclerosis, Alzheimer's disease, own unpublished data), one also observes the influx of $MRP8^+$ and $MRP14^+$ cells, yet, the 27E10 epitope is rarely or not at all formed. Furthermore, a dissociation of cells into an $MRP8^+$ and $MRP14^+$ phenotype seems also to occur, as indicated by our previous report [7] and which is in agreement with recent studies shown by Delabie at al. [40]. While the significance of this phenomenon for the pathomechanism of chronic diseases is not clear, though it indicates that differentiation of monocytes to macrophages seems to deviate from the pathway observed in acute inflammation. The question is whether this deviation can be corrected and whether it would be beneficial for the patient.

We thank Anne Erpenbeck, Claudia Fliegner, Karin Fischer and Bruno Overwien for excellent technical assistance.

Received February 12, 1992; in revised form April 7, 1992.

5 References

1 Zwadlo, G., Bröcker, E. B., von Bassewitz, D. B., Feige, U. and Sorg, C., *J. Immunol.* 1985. *134:* 1487.
2 Zwadlo, G., Voegeli, R., Schulze Osthoff, K. and Sorg, C., *Exp. Cell. Biol.* 1987. *55:* 295.
3 Zwadlo, G., Schlegel, R. and Sorg, C., *J. Immunol.* 1986. *137:* 512.
4 Topoll, H., Zwadlo, G., Lange, D. E. and Sorg, C., *J. Periodont. Res.* 1989. *24:* 106.
5 Lagasse, E. and Clerc, R. G., *Mol. Cell. Biol.* 1988. *8:* 2402.
6 Odink, K., Cerletti, N., Brüggen, J., Clerc, R. G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R. and Sorg, C., *Nature* 1987. *330:* 80.
7 Zwadlo, G., Brüggen, J., Gerhards, G., Schlegel, R. and Sorg, C., *Clin. Exp. Immunol.* 1988. *72:* 510.
8 Longbottom, D., von Heyningen, V. in Heizmann, C. V (Ed) *Novel Calcium-Binding Proteins.* Springer-Verlag, Berlin 1991, p. 191.
9 Kligman, D. and Hilt, D. C., *Trends Biochem. Sci.* 1988. *13:* 437.
10 Persechini, A., Moncrief, N. D. and Kretsinger, R. H., *Trends Neurosci.* 1989. *12:* 462.
11 Murao, S., Collart, F. R. and Huberman, E., *J. Biol. Chem.* 1989. *264:* 8356.
12 Glenney, J. R. and Glenney, P., *J. Cell Biol.* 1985. *100:* 754.
13 Fan, K., *Brain Res.* 1982. *237:* 498.
14 Teigelkamp, S., Bhardwaj, R. S., Roth, J., Meinardus-Hager, G., Karas, M. and Sorg, C., *J. Biol. Chem.* 1991. *266:* 13462.

15 Bradford, M. M., *Anal. Biochem.* 1976. *72:* 248.
16 Laemmli, U. K., *Nature* 1970. *227:* 680.
17 Ansorge, W., *J. Biochem. Biophys. Methods* 1985. *11:* 13.
18 Sorg, C., Bröcker, E. B., Zwadlo, G., Redmann, K., Feige, U., Ax, W. and Feller, A. C., *Transplantation* 1985. *39:* 90.
19 Sei, Y. and Arora, P. K., *J. Immunol. Methods* 1991. *137:* 237.
20 Tsien, R. Y., *Methods Cell Biol.* 1989. *30:* 127.
21 Karas, M. and Bahr, U., *Trends Anal. Chem.* 1990. *9:* 321.
22 Zingde, S. M., Shirsat, N. V. and Gothoskar, B. P., *Anal. Biochem.* 1986. *155:* 10.
23 DeWald, D. B., Adams, L. D. and Pearson, J. D., *Anal. Biochem.* 1986. *154:* 502.
24 Burmeister, G., Tarcsay, L. and Sorg, C., *Immunobiology* 1986. *171:* 461.
25 Brüggen, J., Tarcsay, L., Cerletti, N., Odink, K., Rutishauser, M., Holländer, G. and Sorg, C., *Nature* 1988. *331:* 570.
26 Edgeworth, J., Gorman, M., Bennett, R., Freemont, P. and Hogg, N., *J. Biol. Chem.* 1991. *266:* 7706.
27 Berner Berntzen, H. and Fagerhol, M. K., *Scand. J. Clin. Lab. Invest.* 1990. *50:* 769.
28 Brandtzaeg, P., Dale, I. and Fagerhol, M. K., *Am. J. Clin. Pathol.* 1987. *87:* 681.
29 Ishikawa, F., Miyazano, K., Hellman, U., Drexler, H., Wernstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W. and Heldin, C. H., *Nature* 1989. *338:* 557.
30 Abraham, J. A., Mergia, A., Whang, J. L., Tumolo, A., Friedman, J., Hjerrild, K. A., Gospodarowicz, D. and Fiddes, J. C., *Science* 1986. *233:* 545.
31 Rubartelli, A., Cozzolino, F., Talio, M. and Sitia, R., *EMBO J.* 1990. *9:* 1503.
32 Kääriäinen, L., Hashimoti, K., Saraste, J., Virtanen, I. and Penttinen, K., *J. Cell Biol.* 1980. *87:* 783.
33 Tartakoff, A. M., *Cell* 1983. *32:* 1026.
34 Mollenhauer, H. H., Morre, D. J. and Rowe, L. D., *Biochim. Biophys. Acta* 1990. *1031:* 225.
35 Blackwood, R. A. and Ernst, J. D., *Biochem. J.* 1990. *266:* 195.
36 Hogg, N., Allen, C. and Edgeworth, J., *Eur. J. Immunol.* 1989. *19:* 1053.
37 Brandtzaeg, P., Jones, D. B., Favell, D. J. and Fagerhol, M. K., *J. Clin. Pathol.* 1988. *41:* 963.
38 Edgeworth, J., Freemont, P. and Hogg, N., *Nature* 1989. *342:* 189.
39 Mues, B., Brisse, B., Steinhoff, G., Lynn, T., Hewett, T., Sorg, C., Zuhdi, N. and Robbins, G., *Eur. Heart J.* 1991. *12:* 32.
40 Delabie, J., de Wolf-Peeters, C., van den Oord, J. J. and Desmet, V. J., *Clin. Exp. Immunol.* 1990. *81:* 123.