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Ectosomes Released by Human Neutrophils Are Specialized Functional Units[1]

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Here we show that human polymorphonuclear leukocytes (PMN) release ectosomes independently of complement attack during their activation both in vitro and at the site of inflammation in vivo. Patterns of biotinylated proteins on the surface of PMN and on PMN-derived ectosomes indicated a specific sorting of cell surface proteins into and out of ectosomes. Ectosomes expressed clusters of complement receptor 1 (CR1), which allowed them to bind efficiently to opsonized bacteria. Myeloperoxidase and human leukocyte elastase, both stored within the azurophilic granules of PMN, were found to colocalize on ectosomes with CR1. Furthermore, myeloperoxidase colocalized with human leukocyte elastase. In contrast, not present on CR1-expressing ectosomes were CD63, a selective marker for the azurophilic granules, and CD14, which is located within the same granules and the secretory vesicles as CR1. Of the other complement regulatory proteins expressed by PMN, only CD59 colocalized with CR1, while CD55 and CD46 were almost absent. Ectosomes released by activated PMN at the site of inflammation may function as a well organized element (ecto-organelle), designed to focus antimicrobial activity onto opsonized surfaces. ***The Journal of Immunology*****, 1999, 163: 4564–4573.**

Many nucleated cells are known to release cell surface-derived vesicles as an autodefense mechanism during nonlethal complement attack (1–4). These vesicles have been named ectosomes, indicating the regulated cell surface release of vesicles bearing specifically the lytic membrane attack complexes C5b-C9 (5). Similar vesiculation during complement attack has been described for nonnucleated cells, i.e., erythrocytes and platelets (6, 7).

CR1 (CD35, C3b/C4b receptor) is a transmembrane protein expressed on several circulating cells, including erythrocytes, PMN,[3] monocytes, B lymphocytes, and a subset of T lymphocytes (8, 9). On resting PMN CR1 is only weakly expressed, with an estimated number of 3000 receptors/cell. Most of the receptor is stored intracellularly in a specific vesicular compartment, the so-called secretory vesicles (10). Stimulation of PMN leads to a rapid, up to 10-fold increase in CR1 on the cell surface (10, 11) as well as to the shedding of a functional soluble form of CR1 (sCR1) (12–14). A second form of CR1 has recently been found to be released by activated PMN. It has been described as membrane-associated (mCR1), because it is pelleted by ultracentrifugation before, but not after incubation with a membrane detergent. In contrast to sCR1, mCR1 is recognized by Abs against the intracellular portion of the molecule (13), indicating that mCR1 corresponds to the entire transmembrane protein. Based on these observations, this study was undertaken to analyze whether mCR1 might correspond to CR1 released on ectosomes (eCR1) at the time of PMN stimulation.

Materials and Methods

Quantitation of CR1

Concentrations of CR1 were determined with the sandwich ELISA previously described for measuring sCR1 (15). The two mAbs used (3D9 (IgG1) and J3D3 (IgG1), provided by J. P. Atkinson and M. D. Kazatchkine) recognize different epitopes on the extracellular portion of the molecule (16). The ELISA was standardized using recombinant soluble CR1 (rsCR1; provided by T Cell Science, Boston, MA). The limit of sensitivity was 0.02 ng/ml; the intra-assay variability was ±3%.

A second ELISA was used to measure full-size CR1 (12). Briefly, in this full-size CR1 ELISA, a polyclonal rabbit IgG recognizing the intracytoplasmic tail of the CR1 molecule was used as capture Ab, while the second Ab and the detection system were similar to those in the ELISA used for measuring total and soluble CR1. The ELISA was standardized with a known amount of erythrocyte CR1. CR1 remaining in the SN after ultracentrifugation (60 min at 200,000 × *g* at 4°C; sCR1) was not recognized in this full-size CR1 ELISA (12, 13). The limit of sensitivity was 0.1 ng/ml, the intra-assay variability was ±5%. Before all determinations of CR1, samples were centrifuged twice for 15 min each time at 4,000 × *g* at 4°C to remove cells and cell debris.

Quantitation of CD14

Concentrations of CD14 were determined by a sandwich ELISA system as previously described, with the anti-CD14 mAb 63D3 (IgG1) as capture Ab coated on microtiter plates and the peroxidase-labeled mAb 3C10 (IgG2b) used as detection Ab. (Abs provided by R. Landmann). Recombinant soluble CD14 (provided by R. Landmann) was used to standardize the assay (17).

Isolation and stimulation of PMN

PMN (>98% pure, >99% trypan blue exclusion) were isolated from fresh buffy coats of normal donors according to the technique described previously (18). Briefly, a fresh buffy coat obtained from ~400 ml of normal donor blood was diluted 1/1 (v/v) in PBS-EDTA (2 mM), mixed gently with 0.25 vol of 4% Dextran T500 (Amersham Pharmacia Biotech, Dübendorf, Switzerland), and left for 30 min for erythrocyte sedimentation. The leukocyte-rich SN was aspired and centrifuged for 10 min at 200 × *g*. The

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[3] Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; BAL, bronchoalveolar lavage; CR1, complement receptor 1; sCR1, soluble CR1; eCR1, ectosomal CR1; mCR1, membrane-associated CR1; rsCR1, recombinant soluble CR1; HLE, human leukocyte elastase; MPO, myeloperoxidase; NHS, normal human serum; SN, supernatant fluid.

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Table I. *Release of CR1 by PMN within 15 min*[a]

Agent(s) Used to Stimulate PMN	sCR1	eCR1
fMLP (0.1 μM)		
ng/ml	0.5 ± 0.05	0.65 ± 0.1
Molecules sCR1/PMN	274 ± 27	356 ± 55
fMLP (1 μM)		
ng/ml	1.65 ± 0.15	1.85 ± 0.25
Molecules sCR1/PMN	904 ± 82	1013 ± 137
PMA (1 nM)		
ng/ml	0.65 ± 0.15	0.4 ± 0.05
Molecules sCR1/PMN	356 ± 82	219 ± 27
PMA (10 nM)		
ng/ml	1.25 ± 0.1	0.95 ± 0.15
Molecules sCR1/PMN	685 ± 55	521 ± 82
Ionomycin (0.5 μM)		
ng/ml	0.6 ± 0.05	0.55 ± 0.15
Molecules sCR1/PMN	329 ± 27	301 ± 82
Ionomycin (5 μM)		
ng/ml	1.3 ± 0.2	1.05 ± 0.15
Molecules sCR1/PMN	712 ± 110	575 ± 82
PMA (1 nM) and ionomycin (0.5 μM)		
ng/ml	2.85 ± 0.15	0.8 ± 0.1
Molecules sCR1/PMN	1562 ± 82	438 ± 55
None[b]		
ng/ml	0.2 ± 0.05	0.2 ± 0.05
Molecules sCR1/PMN	110 ± 27	110 ± 27

[a] Results are given from four independent experiments, ±SEM.
[b] Temperature change.

pellet was resuspended for 40 s in 9 ml of distilled water to lyse erythrocytes. Isotonicity was restored by addition of 3 ml of KCl (0.6 M) and 40 ml of NaCl (0.15 M). The cells were then centrifuged 10 min at 350 × *g*, and resuspended in 20 ml of PBS-EDTA (2 mM). This suspension was layered over 20 ml of Ficoll-Hypaque (Sigma, St. Louis, MO) and centrifuged for 30 min at 350 × *g*. The PMN-rich pellet was recovered and washed twice in PBS-EDTA (2 mM). All solutions used were determined to be LPS free, and all manipulations were performed under sterile conditions at 4°C, thus minimizing PMN activation.

For stimulation, PMN (5×10^6 cells/ml; final volume, 5 ml) were resuspended in 37°C warm RPMI (Life Technologies, Basel, Switzerland) without (stimulation by temperature change, as shown in Table I) or with fMLP (0.1 μM/1 μM), PMA (1 nM/10 nM), and/or ionomycin (0.5 μM/5 μM; all from Sigma). For kinetic studies of CR1 release, aliquots of 300 μl were removed at different time points, and cell activation was stopped by adding an equal volume of ice-cold RPMI. Trypan blue exclusion after stimulation was the same as that before stimulation (>99%).

For stability studies of eCR1, PMN were removed by centrifugation (twice, 15 min each time, 4000 × *g* at 4°C) after 15-min stimulation with fMLP (0.1 μM). The SN was incubated at 37°C for an additional 30 min. Aliquots of 300 μl were removed after 30 min and analyzed for their total CR1 and soluble CR1 concentrations.

Scanning electron microscopy of PMN

PMN (5×10^6 cells) were fixed in 0.5% glutaraldehyde/3% paraformaldehyde immediately after isolation (unstimulated control) or after 5-min stimulation with fMLP (0.1 μM). They were adsorbed to poly-L-lysine-coated glass coverslips (Sigma) and postfixed in 1% osmium tetroxide (OsO_4). After dehydration through increasing concentrations of ethanol, samples were critical point dried and sputter coated with 12 nm gold. The field emission scanning electron microscope (S-800, Hitachi, Hialeah, FL) was operated at 12 keV.

Transmission electron microscopy

Negative staining. Ectosomes obtained by in vitro stimulation of isolated PMN with fMLP (0.1 μM) for 5 min were fixed with 0.5% glutaraldehyde/3% paraformaldehyde. They were concentrated by ultracentrifugation for 60 min at 200,000 × *g* at 4°C. Ectosomes derived from PMN activated in vivo (BAL) were processed in the same way as ectosomes released by in vitro stimulated PMN. After ultracentrifugation, ectosomes were resuspended in PBS before being adsorbed for 2 min to glow-discharged parlodion carbon-coated grids. For immunogold labeling, grids were incubated for 40 min on a drop of diluted (0.3 μg/μl) anti-CR1 mAb (3D9), washed, and incubated for 40 min on a drop of gold-labeled second Ab (0.1 μg/μl). After washing, all samples were stained with 0.75% uranylformate (pH 4.2).

Ultrathin sections of embedded probes. Ectosomes released by in vitro or in vivo stimulated PMN (as described above) were incubated for 4 h at 4°C with Dynabeads (Dynal, Milan Analytica) covalently coated with mAb (described below). The Abs used included the anti-CR1 mAb 3D9 and as a control the anti-complement factor D mAb 72-96-25 (IgG1) (19). After incubation, Dynabeads were washed five times in PBS, dehydrated, and embedded in Epon 812 resin using standard procedures. Thin sections were cut with an ultramicrotome, and sections were deposited on parlodion carbon-coated grids, stained with 6% uranylacetate for 30 min, and poststained with lead citrate for 1 min. All samples were observed in a Zeiss EM 910 electron microscope (Zeiss, New York, NY) operated at 80 kV acceleration voltage.

Analysis of cell surface and ectosomal biotinylation patterns

Biotinylation of PMN cell surface proteins was performed as previously described (20), using a commercial kit (Amersham Pharmacia Biotech, Dübendorf, Switzerland). Briefly, freshly isolated PMN (5×10^7 PMN/ml) were incubated with biotinylation buffer (diluted 1/10) and 40 μl of biotinylation reagent for 30 min at 4°C. PMN were washed twice with ice-cold PBS-EDTA (2 mM) and resuspended (5×10^6 PMN/ml) for 15 min in prewarmed RPMI (37°C) containing 0.1 μM fMLP. For analysis of biotinylated cell surface proteins, stimulated PMN were collected by centrifugation (10 min, 350 × *g*, 4°C). 2×10^7 PMN were lysed in 500 μl of lysis buffer (PBS, EDTA (1 mM), Triton X-100 (1%), SDS (0.1%), and PMSF (1 mM; Serva Feinbiochemicals, Heidelberg, Germany)) for 60 min at 4°C. Lysed samples were centrifuged twice for 15 min each time at 4000 × *g* at 4°C to remove nonlysed material. To collect ectosomes, SN of activated biotinylated PMN (2×10^7 PMN stimulated with 0.1 μM fMLP for 15 min) were centrifuged twice for 15 min each time at 4000 × *g* at 4°C to remove all cells and cell debris. They were then ultracentrifuged (60 min at 200,000 *g* at 4°C), and the pellet was resuspended in 150 μl of lysis buffer (as described for PMN lysis). Samples were run on 12% polyacrylamide gels with optimal signals obtained by loading the lysate from 4×10^6 PMN/lane and ectosomes derived from 2×10^7 PMN/lane. After SDS-PAGE and Western blotting, proteins were detected with HRP-streptavidin (Milan Analytica, LaRoche, Switzerland) using enhanced chemiluminescence (Amersham Pharmacia Biotech). To control the membrane association of biotinylated proteins found in the pellet after ultracentrifugation, SN from stimulated PMN were incubated for 30 min with Tween-20 (0.2%) to solubilize ectosomes. This led to the loss of biotinylated proteins in the pellet after ultracentrifugation.

Skin blisters

Skin blisters were chemically induced by application of Cantharidin plasters overnight (Adler Apotheke, Masel, Germany) as previously described (21). Briefly, Cantharidin plasters, each 10 × 10 mm, were applied to the ventral forearm of volunteers (after having obtained written informed consent). Within 12 h, Cantharidin induced the formation of blisters, and plasters were removed. At that time a blood sample was taken. Blister fluid was aspired using a thin needle, avoiding rupture of the blister. The amount of fluid that could be collected varied from ~80–250 μl/blister. Three hours after the first collection (i.e., after removal of the plasters) blister fluid had reaccumulated, and fluid was collected again. Cell number and cell type were analyzed using standard procedures. Total protein content determination was performed within all blister fluids; electrophoresis was performed with one sample and its corresponding serum. The albumin/γ-globulin ratio in the serum was 3.2. The albumin/γ-globulin ratio was 2.8 in the blister fluid collected first and 3.0 in the fluid that had reaccumulated 3 h later. This indicated a nonselective accumulation of proteins in blister fluid. Quantitation of CR1 was performed as described above in both blister fluid and serum.

Bronchoalveolar lavage (BAL)

Freshly obtained BAL were analyzed semiquantitatively for their PMN content (from − to +++). PMN-rich BAL were centrifuged (twice, 15 min each time, 4000 × *g*, 4°C) to remove cells and cell debris before further analyzing the SN by electron microscopy and/or CR1 ELISAs as described above.

Rabbit erythrocyte lysis assay

Rabbit erythrocytes (provided by M. Hermle) were incubated for 10 min with human serum (diluted 1/30) in presence of magnesium (2 mM) and EGTA (10 mM) to induce cell lysis (22). The reaction was stopped by

adding EDTA (20 mM). ODs of SN were read at 541 nm. All measurements were made in duplicate or triplicate. The experiments were performed in presence and absence of eCR1. The eCR1 was collected by ultracentrifugation of SN from 15-min fMLP (0.1 μM)-stimulated PMN. The eCR1 was used at a 10-fold higher concentration than that of sCR1 in a pool of normal human serum (NHS; concentration of sCR1 in pooled NHS = 35 ng/ml) (15). The eCR1 was not detectable in NHS (13). A 1000-fold molar excess of a blocking anti-CR1 mAb (3D9) (23) was preincubated with eCR1 to control the specificity of the lysis inhibitory effect. 3D9 alone as well as an isotype-controlled nonrelevant Ab (anti-HLE, AHN10, IgG1, PharMingen, Hamburg, Germany) were used as controls.

Phagocytic bactericidal assay

The phagocytic bactericidal assay was performed according to the technique previously described (21), using *Staphylococcus aureus* strain *Wood46* (Molecular Probes, Juro Supply, Lucerne, Switzerland). To test the effect of eCR1 on phagocytosis of preopsonized bacteria in a serum-free system, bacteria were opsonized by incubation in NHS (diluted 1/10) for 1 or 10 min at room temperature. They were washed twice in ice-cold Dulbecco's PBS, before adding eCR1 (40, 24, and 13 ng/ml) or rsCR1 (120, 60, and 30 ng/ml) plus PMN. The eCR1 used was from PMN autologous to those used in the assay; that is, it was collected from the SN of PMN (stimulated for 15 min with fMLP (0.1 μM)) isolated from the same buffy coat. The killing rate was determined by quantitative cultures from samples (in duplicate/triplicate) drawn at 0 min (i.e., when PMN were added) and 30 min (end point). In addition, in each assay quantitative samples drawn at 0 and 30 min were treated with lysostaphin (10 U/ml) for 5 min. Lysostaphin (Sigma) is known to efficiently kill extracellular *S. aureus* while not being able to enter the cell (24). Thus, the number of bacteria surviving intracellularly could be calculated. For all experimental settings the percentage of intracellularly surviving bacteria was between 0–2%, indicating that the phagocytosis rate equaled the killing rate. Experiments were repeated using heat-inactivated NHS (i.e., NHS incubated for 30 min at 56°C) instead of NHS.

Immunoprecipitation and coimmunoprecipitation

For precipitation of ectosomes, tosyl-activated Dynabeads (Dynal, Milan Analytica) covalently coated with different mouse mAb were used. Abs used for coating included anti-MPO (MPO-7, IgG1, Dako, Zug, Switzerland), anti-HLE (AHN10, IgG1), anti-HLA class I (W6/32, IgG2a, Dako), anti-CD14 (63D3), anti-CR1 (3D9), anti-CD46 (J4–48, IgG1, Sanbio, Bioreba, Rheinach, Switzerland), anti-CD55 (BRIC 110, IgG1, Southern Biotechnology Associates, Birmingham, AL), anti-CD59 (MEM-43/5, IgG2b, Sanbio, Bioreba), anti-CD63 (CLB-gran/12 (CLB-180), IgG1, Caltag Laboratories, Burlingame, CA), anti-HLA class II (TU39, IgG2a, PharMingen), and anti-complement factor D (72-96-25). Blocked uncoated Dynabeads were used as a control. Coating was performed as suggested by Dynal. Briefly 2×10^8 tosyl-activated Dynabeads were washed twice with PBS-BSA 0.1% and resuspended in borate buffer, pH 9.5. Five micrograms of mAb per 10^7 Dynabeads was added. After 10 min, BSA was added to a final concentration of 0.1% for optimal orientation of mAb. After 24 h at 37°C, Dynabeads were washed in PBS-BSA 0.1% before blocking free binding sites in Tris-buffer, pH 8.5, for 4 h.

Freshly isolated PMN (5×10^6 PMN/ml) were stimulated for 15 min with fMLP (0.1 μM). The ectosome-containing SN were incubated with mAb-coated Dynabeads (6×10^7 beads/6 ml of SN) for 4 h at 4°C so as to allow binding of ectosomes bearing the relevant Ag. After incubation Dynabeads were washed five times with PBS/0.1% BSA, then incubated with 200 μl of lysis buffer (as described for *Analysis of biotinylation patterns* above) for 60 min at 4°C to lyse the ectosomes. After removal of Dynabeads, CR1 was measured in the lysates, or MPO activity was determined by use of the assay described below. Controls included pretreatment of SN-containing ectosomes with a membrane detergent (0.2% Tween-20) before incubation with Dynabeads.

When ectosome-containing SN were incubated with Dynabeads coated with anti-CR1 mAb (3D9), the concentration of CD14 was measured in the lysate with the CD14 ELISA described above, or MPO activity was determined (as described below).

The coprecipitation of HLA class I with CR1 was also analyzed using Dynabeads coated with anti-CR1 mAb (3D9) that were incubated with the SN of stimulated PMN as described above. HLA class I was then identified by FACScan analysis of the beads.

MPO activity assay

Enzymatic activity of MPO was determined in a colorimetric assay, where 100 μl of substrate (20 μl of H_2O_2 (30%) in 50 ml of citrate-phosphate buffer (pH 5.0) plus orthophenyldiamine (1 mg/ml)) was added to 60 μl of lysed, pH-adjusted, precipitated ectosomes. All samples were run in triplicate. Purified MPO (Calbiochem-Novabiochem, La Jolla, CA) was used as standard. The reaction was stopped with 2.5 M H_2SO_4, and the absorbance was measured with a microplate reader (Thermo Max, Molecular Devices, Menlo Park, CA) at 490 nm.

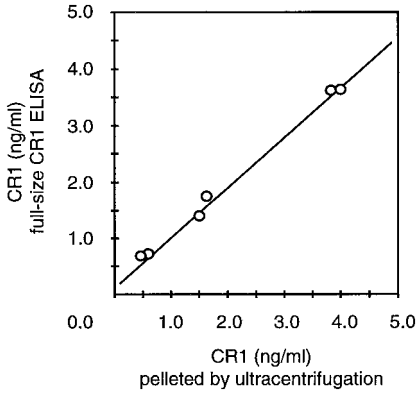

FIGURE 1. Correlation between the two methods to measure eCR1. The *x*-axes show eCR1 measured by the ultracentrifugation method; the *y*-axes show eCR1 measured by the ELISA for full-size CR1. r = 0.99; $p < 0.0005$.

Statistical analysis

Data were analyzed with the StatView SE&Graphics program (SPSS, Chicago, IL).

Results

Quantitation and kinetics of CR1-release by PMN in vitro

We have previously shown that stimulated PMN release soluble CR1 (sCR1). This soluble form corresponds to the extracellular portion of the transmembrane protein (12). A second form of CR1 was released by PMN. This form is recognized by Abs against the intracellular portion of the receptor and thus corresponds to the entire transmembrane molecule (12, 13). As CR1 released as a transmembrane molecule is pelleted by ultracentrifugation before, but not after, treatment with a membrane detergent, it was previously described as membrane-associated CR1 (mCR1; i.e., ectosomal CR1 (eCR1)) (13).

We first performed experiments to define whether transmembrane CR1 in the SN of activated PMN (i.e., ectosomal CR1) corresponded to the fraction of CR1 pelleted by ultracentrifugation (total CR1 − soluble CR1 = pelleted CR1). The method measuring eCR1 by the ELISA specific for full-size CR1 and the ultracentrifugation method provided almost identical results (Fig. 1). Because the ultracentrifugation method was more sensitive, we used it throughout this work.

The kinetics of CR1 shedding by PMN in vitro showed that its release was initiated in minutes (Fig. 2). Stimulation for 15 min with fMLP (0.1 μM/1 μM), PMA (1 nM/10 nM), or ionomycin (0.5 μM/5 μM) induced a dose-dependent release of CR1, with an ~1/1 ratio of eCR1/sCR1 (Table I). However, the ratio of sCR1/eCR1 was dependent on the type of stimulation used, as the combination of PMA (1 nM) with ionomycin (0.5 μM) enhanced the release of sCR1 ~5-fold, with only a slightly enhanced release of eCR1 (Table I and Fig. 2).

Although sCR1 was continuously increasing in the SN after 15-min stimulation, the concentration of eCR1 after 15 min varied. Often the concentration was almost maximal at 15 min, although in other experiments its concentration increased, reaching up to 0.4 ng eCR1/million PMN within 60 min.

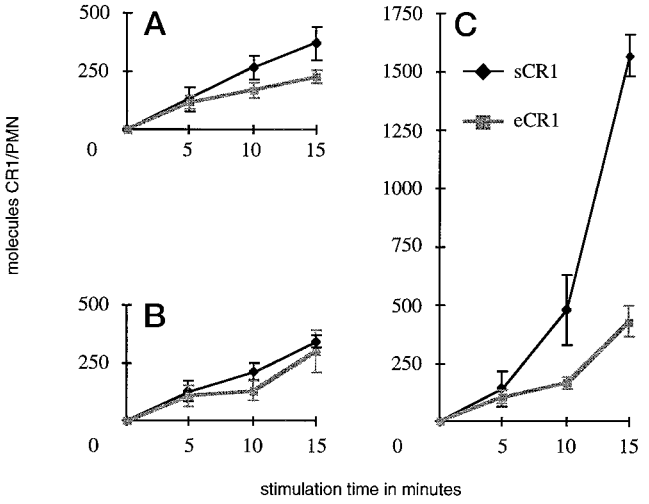

FIGURE 2. Kinetics of the release of CR1 by in vitro stimulated PMN. *A*, PMN stimulated with 1 nM PMA. *B*, PMN stimulated with 0.5 μM ionomycin. *C*, PMN stimulated with 1 nM PMA and 0,5 μM ionomycin. Results are the mean ± SEM from four independent experiments.

Stability of eCR1

To determine whether eCR1 was only transiently associated with ectosomes, SN of stimulated PMN were analyzed (after removal of PMN) over 30 min at 37°C for their content in both sCR1 and eCR1. The eCR1 remained as stable as its soluble counterpart (mean loss (degradation) after 30 min, 8 ± 2% for sCR1, 7 ± 2% for eCR1; mean ± SEM of three independent experiments). Thus, eCR1 did not seem to be a source of sCR1 as there was no increase in sCR1 with a reciprocal loss of eCR1.

Morphological characteristics of PMN at the time of eCR1 release

Having identified a stable, membrane-associated form of CR1 to be released early after activation of PMN, we next analyzed PMN (unstimulated and stimulated for 5 min with 0.1 μM fMLP) by scanning electron microscopy. fMLP was used to stimulate PMN as it more closly mimics a bacterial stimulus than either PMA or ionomycin. As expected, unstimulated control PMN were round with a rather smooth cell surface (Fig. 3*a*). Upon stimulation, known changes in overall morphology with expansion of large pseudopodia occurred rapidly (Fig. 3*b*). In addition, small vesicular protrusions with a size of 70–300 nm appeared on their cell surface (Fig. 3*b*). At that time, the SN of the PMN contained eCR1 at a concentration expected from the kinetic experiments.

Morphological analysis of eCR1 in vitro

In the SN of these PMN, ectosomes were concentrated by ultracentrifugation, resuspended in buffer, and adsorbed to electron microscopy grids. By transmission electron microscopic analysis of immunogold-labeled and negatively stained samples, CR1 was localized on ectosomes (Fig. 3*c*), corresponding in size to the protrusions found on stimulated PMN (70–300 nm). More than one molecule of CR1 was expressed per ectosome, as estimated by the number and distribution of gold particles. Thin sections of embedded ectosomes, precipitated with Dynabeads covalently coated with anti-CR1 mAb, proved these vesicles to be bilamellar structures typical of cell membranes (Fig. 3*d*).

Analysis of biotinylation patterns on PMN and on ectosomes

To study whether other cell surface proteins were released on ectosomes, we biotinylated the cell surface of resting PMN. The biotinylation procedure was performed at 4°C and had no effect on the amount of CR1 released by PMN when stimulated with fMLP

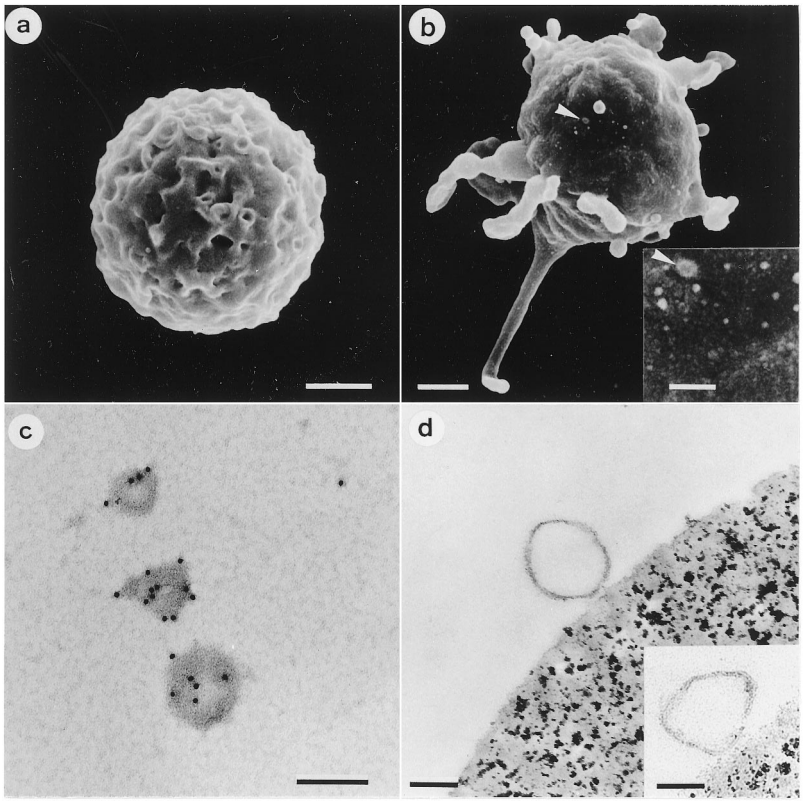

FIGURE 3. PMN and PMN-derived ectosomes analyzed by electron microscope. *a*, Resting PMN, fixed immediately after isolation (scale bar = 1.5 μm). *b–d*, Stimulation with 0.1 μM fMLP for 5 min. *b*, Stimulated PMN (scale bar = 1.5 μm). *Inset*, Ectosomes budding from the cell surface. The arrowhead points to the same structure on overview and inset (scale bar of *inset* = 400 nm). *c*, Immunogold-labeled CR1 on negatively stained ectosomes (scale bar = 150 nm). d, Ectosome precipitated with an anti-CR1 mAb fixed onto Dynabeads (scale bar = 150 nm). *Inset*, The membrane bilayer of an ectosome (scale bar of the *inset* = 100 nm).

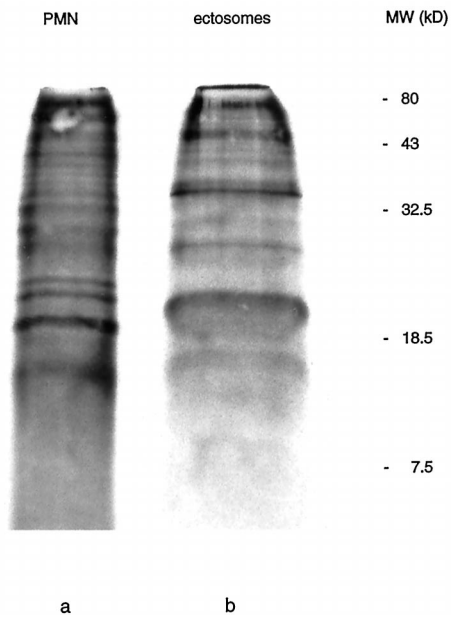

FIGURE 4. Pattern of biotinylated proteins on the surface of PMN and of ectosomes released by these PMN. *a*, Biotinylation pattern of PMN (4×10^6 lysed cells/lane). *b*, Biotinylation pattern of ectosomes (lysed ectosomes derived from 2×10^7 PMN/lane).

(0.1 μM) for 15 min (nonbiotinylated: sCR1, 0.10 $\pm$ 0.01 ng CR1/10^6 PMN; eCR1, 0.13 $\pm$ 0.02 ng CR1/10^6 PMN; biotinylated: sCR1, 0.11 $\pm$ 0.02 ng CR1/10^6 PMN; eCR1, 0.12 $\pm$ 0.02 ng CR1/10^6 PMN; mean $\pm$ SEM from four independent experiments). After stimulation of biotinylated PMN with fMLP (0.1 μM) for 15 min, cells were separated, and ectosomes were concentrated by ultracentrifugation of their SN. Both PMN and ectosomes were incubated in lysis buffer. The biotinylation patterns of PMN cell surface proteins and ectosomal proteins were analyzed by Western blotting.

The pattern of biotinylated proteins on ectosomes showed that many cell surface proteins were released by ectocytosis during the first 15 min of PMN stimulation (Fig. 4*b*). Several proteins were present on both PMN and ectosomes. However, a series of proteins was found predominantly on either PMN or ectosomes, as shown by their different patterns of biotinylation, indicating a specific sorting of proteins into and out of ectosomes at the time of their formation (Fig. 4, *a* and *b*).

Morphological and immunological analyses of eCR1 in vivo

The next step was to determine whether CR1-bearing ectosomes were also found in vivo. We analyzed blister fluid of chemically induced skin blisters and the BAL from patients with (broncho-) pneumonia.

In blister fluid >95% of cells were PMN, both in the fluid collected after application of Cantharidin plasters for 12 h as well as in the blister fluid that reaccumulated within 3 h after removal of the plasters. Total protein content, number of PMN, and concentrations of sCR1 and eCR1 as measured in the blister fluid and blood of three individuals are given in Table II. The amount of eCR1 per million PMN was ~0.6 ng both in the fluid collected after 12 h as well as in the fluid collected 3 h later. This was significantly more than what could be induced by in vitro stimulation with fMLP (1 μM), PMA (10 nM), or ionomycin (5 μM). Correcting the sCR1 concentration in the blister fluid for their total protein content (as accumulation of proteins was nonselective), its concentration became higher than that of sCR1 in the serum of the respective volunteer, indicating local production of sCR1. Interestingly, the amount of (calculated) locally produced sCR1/million PMN in the fluid collected after the blister had refilled was consistently higher than that in the blister fluid collected earlier (~1.5 vs 0.7 ng sCR1/million PMN), although the total protein content was stable.

Within the 18 analyzed BAL qualified as PMN rich (+++ for PMN), 34 $\pm$ 9% of CR1 was membrane associated (mean $\pm$ SD of the 18 BAL analyzed). By ultrastructural analysis using the methods described for characterization of ectosomes released in vitro, the same vesicular structures that were found in the SN of in vitro stimulated PMN were detected (Fig. 5, *a* and *b*).

Function of ectosomal CR1

CR1 is a potent inhibitor of the complement C3 and C5 convertases by binding to C4b/C3b (receptor function) and allowing the factor I cleavage of both molecules (cofactor function) (25).

PMN-derived ectosomes, collected by ultracentrifuging the SN from PMN stimulated for 15 min with fMLP (0.1 μM), were efficient inhibitors of complement-mediated rabbit erythrocyte lysis (22). The specificity of the eCR1 inhibitory effect was demonstrated by adding a 1000-fold molar excess of an mAb known to block CR1 function (3D9) (23). This mAb blocked the lysis inhibitory effect of eCR1. Addition of the same amount of an isotype-matched control mAb as well as Abs alone showed no effect (Fig. 6).

Secondly, eCR1 mediated the inhibition of bacterial uptake by PMN in an opsonization/phagocytosis assay. *S. aureus* strain

Table II. *Analysis of cantharidin-induced blister fluid*[a]

	Volunteer	PMN (10^6/ml)	Total Protein (g/l)	sCR1 (ng/ml)	sCR1/Total Protein [(ng/ml)/(g/l)]	eCR1, ng/ml (molecules eCR1/PMN)
12 h fluid	1	5.9	48	26.6	0.55	3.5 (1465)
	2	5.3	49	30.5	0.62	3.2 (149)
	3	6.1	51	24.9	0.49	3.9 (1579)
12 + 3 h fluid	1	10.9	50	35.8	0.72	6.4 (1450)
	2	10.3	52	41.3	0.79	6.5 (1559)
	3	11.6	51	33.8	0.66	5.9 (1256)
Blood	1	2.5	72	35.6	0.49	0.0 (0)
	2	3.2	70	40.2	0.57	0.0 (0)
	3	2.9	76	32.1	0.42	0.0 (0)

[a] Results are given from three volunteers and six blisters. Two blisters were pooled from each volunteer.

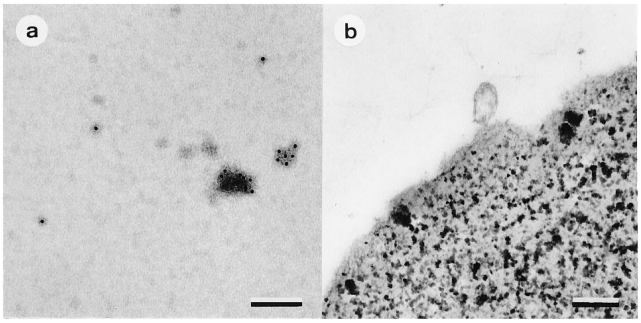

FIGURE 5. Ectosomes in vivo, analyzed by transmission electron microscope. *a* and *b*, Ectosomes found in the SN of BAL from a patient with pneumonia. *a*, Immunogold-labeled CR1 on negatively stained ectosomes analyzed by transmission electron microscope (scale bar = 150 nm). *b*, Ectosome precipitated with an anti-CR1 mAb fixed onto Dynabeads (scale bar = 150 nm).

Wood46 is known to be phagocytosed/killed only after complement-dependent opsonization (26). Using preopsonized *S. aureus Wood46*, the effect of rsCR1/eCR1 on phagocytosis could be tested in a serum-free system, i.e., in the absence of factor I activity. In this way the isolated receptor function of eCR1 could be studied and compared with that of rsCR1. This was important, as cofactor activity of CR1 does not always change in parallel with alterations in its binding properties (27). When bacteria were preopsonized for only 1 min, 120 ng/ml rsCR1 was able to block their uptake/phagocytosis. However, when preopsonized for 10 min, 120 ng/ml rsCR1 did not show any significant effect on bacterial uptake. The same phenomenon was seen with eCR1. Interestingly, eCR1 was more efficient than rsCR1, possibly due to the clustered nature of eCR1; 120 and 60 ng/ml of rsCR1 produced the same inhibition as 40 and 24 ng/ml eCR1, respectively. No inhibition was induced by 30 ng sCR1/ml (Fig. 7).

All phagocytosis experiments were repeated using heat-inactivated NHS instead of NHS. As bacteria were no longer opsonized with C3b/C3bi, they could not be taken up and were able to survive/grow (28). At 30 min the percentage of bacteria was 112 ± 8% compared with the bacterial load at 0 min (100%); (mean ± SEM from six independent experiments).

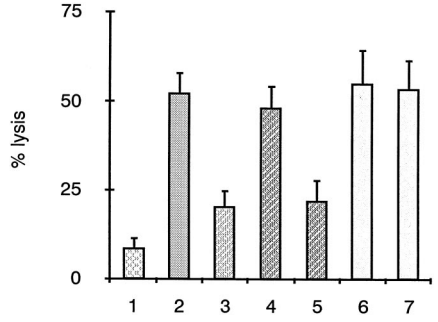

FIGURE 6. Rabbit erythrocyte lysis and its inhibition by ectosomal CR1. *Lane 1*, Lysis in autologous serum. *Lane 2*, Lysis in 3% NHS. *Lane 3*, Lysis in 3% NHS and ectosomal CR1 (350 ng/ml). *Lane 4*, Lysis in 3% NHS, ectosomal CR1 (350 ng/ml), and a 1000-fold molar excess of blocking mAb anti-CR1. *Lane 5*, Lysis in 3% NHS, ectosomal CR1 (350 ng/ml), and a 1000-fold molar excess of control mAb (anti-HLE). *Lane 6*, Lysis in 3% NHS and a 1000-fold molar excess of blocking mAb anti-CR1. *Lane 7*, Lysis in 3% NHS and a 1000-fold molar excess of control mAb (anti-HLE). Results are the mean ± SEM from three individual experiments.

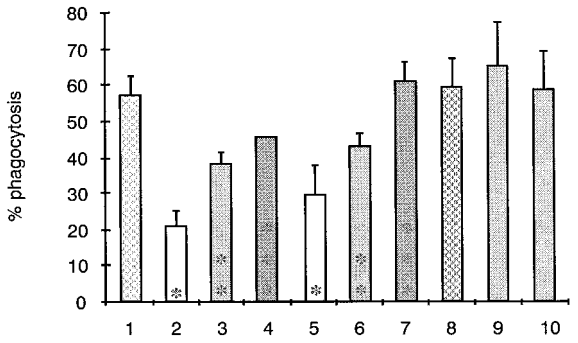

FIGURE 7. Effect of eCR1 and rsCR1 on the uptake of bacteria. *Lane 1*, Percentage of bacteria phagocytosed within 30 min, preopsonized for 1 min. *Lane 2*, Percentage of bacteria phagocytosed within 30 min in the presence of 40 ng/ml eCR1, preopsonized for 1 min. *Lane 3*, Percentage of bacteria phagocytosed within 30 min in the presence of 24 ng/ml eCR1, preopsonized for 1 min. *Lane 4*, Percentage of bacteria phagocytosed within 30 min in the presence of 13 ng/ml eCR1, preopsonized for 1 min. *Lane 5*, Percentage of bacteria phagocytosed within 30 min in the presence of 120 ng/ml rsCR1, preopsonized for 1 min. *Lane 6*, Percentage of bacteria phagocytosed within 30 min in the presence of 60 ng/ml rsCR1, preopsonized for 1 min. *Lane 7*, Percentage of bacteria phagocytosed within 30 min in the presence of 30 ng/ml rsCR1, preopsonized for 1 min. *Lane 8*, Percentage of bacteria phagocytosed within 30 min, preopsonized for 10 min. *Lane 9*, Percentage of bacteria phagocytosed within 30 min in the presence of 40 ng/ml eCR1, preopsonized for 10 min. *Lane 10*, Percentage of bacteria phagocytosed within 30 min in the presence of 120 ng/ml rsCR1, preopsonized for 10 min. Results are the mean ± SEM from three independent experiments, with the exception of the 13 ng eCR1 condition, which represents a single experiment.

Analysis of protein colocalizations on ectosomes

The presence of specific proteins on CR1-bearing vesicles was tested by mAb recognizing HLA class I, CD14, MPO, HLE, CD46, CD55, CD59, and CD63; mAb against HLA class II and factor D were used as controls. The Abs were coated onto Dynabeads, which were then incubated with the ectosome-containing SN of activated PMN. Ectosomes expressing the relevant protein were bound to the beads. After washing the beads, these ectosomes were lysed and tested for the presence of CR1. Thus, the presence of CR1 in the lysate indicated that both molecules localized on the same ectosome. The first molecule to be studied in this way was the cell surface marker HLA class I, because we expected HLA class I to colocalize with eCR1 if not specifically sorted out during ectocytosis. As shown in Fig. 8*a*, CR1 indeed localized on ectosomes isolated with anti-HLA class I mAb. The colocalization was confirmed by exchanging the precipitating mAb with the detecting mAb (data not shown). Next, CR1-expressing ectosomes were tested for the three other complement regulatory proteins expressed by PMN: CD46, CD55, and CD59 (25). Only CD59 mAb coprecipitated a significant amount of CR1 (Fig. 8*a*).

The other proteins found to colocalize with CR1 on ectosomes (MPO, HLE) are part of the antimicrobial arsenal of PMN (Fig. 8*a*). They are both stored within the azurophilic granules (29). PMN activation leads to the release (30) and cell surface up-regulation of both MPO and HLE (31–33). Using the same precipitation procedure, colocalization of MPO and HLE on ectosomes was tested. Instead of CR1, we quantified MPO in the ectosome lysate after immunoprecipitation with anti-HLE mAb and anti-CR1 mAb. These experiments showed that a significant amount of MPO coprecipitated with HLE, although less than with CR1 (Fig. 8*b*).

Selectivity of the ectocytotic release of proteins was further studied by testing the colocalization of CR1 with CD14 and CD63.

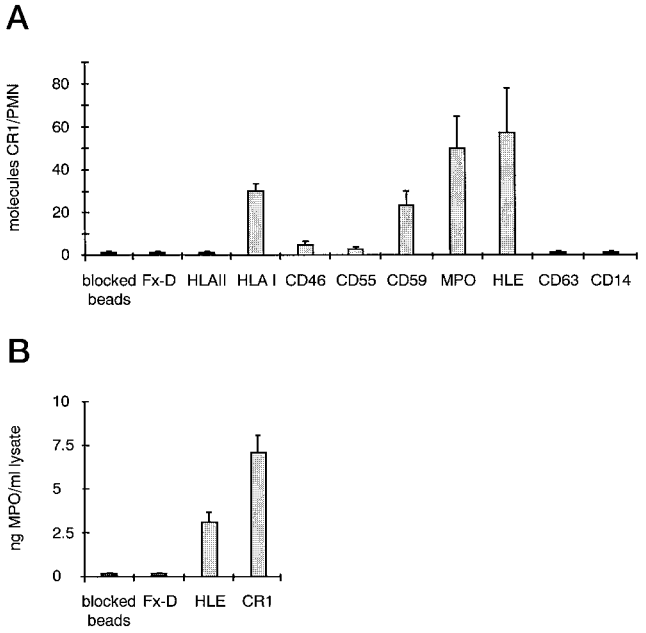

FIGURE 8. Colocalization of different proteins on ectosomes. *A,* Coimmunoprecipitation of CR1 as measured by ELISA in lysates of ectosomes i) after their incubation with blocked uncoated beads, ii) after precipitation with mAb against nonrelevant epitopes (factor D, HLA class II), and iii) after precipitation with mAb against HLA class I, CD46, CD55, CD59, MPO, HLE, CD63, and CD14. Results are the mean ± SEM from at least four independent experiments. *B,* Coimmunoprecipitation of MPO as measured by an activity assay in lysates of ectosomes i) after their incubation with blocked uncoated beads, ii) after precipitation with mAb against a nonrelevant epitope (factor D), and iii) after precipitation with mAb against HLE and CR1. Results are the mean ± SEM from four independent experiments.

CD14 is located both within the secretory vesicles together with CR1 and in the azurophilic granules with MPO and HLE (29, 34, 35). CD63 is a selective marker for the azurophilic granules known to be up-regulated to the cell surface in activated PMN (36). CR1 did not coprecipitate with CD63 or CD14 (Fig. 8*a*). For CD14 the measurements were confirmed by exchanging the detecting and the precipitating Abs. Either way, ELISA signals were below detection limit in four independent experiments.

In the SN of Dynabeads coated with anti-HLA class II and anti-factor D mAb as well as in the SN of blocked uncoated beads the amount of coprecipitated CR1 was below the detection limit of the CR1 and CD14 ELISAs. Addition of Tween-20 (0.2%) to the SN and, thus, lysis of ectosomes before incubation with Dynabeads led to the loss of the signal for all otherwise coprecipitated molecules.

Discussion

In the present work we analyzed the formation and the release of ectosomes by PMN. The major findings were that (i) the formation of ectosomes occurred at the time of cell activation, independently of complement activation, (ii) ectosomes were formed after cell migration in vivo, (iii) proteins were selectively sorted into and out of ectosomes, and (iv) the analysis of proteins colocalized on ectosomes suggests that ectosomes have specific functions.

The release of ectosomes by PMN was analyzed using CR1 (CD35) as a marker. This receptor has been shown to be released in two different forms by activated PMN: a soluble form (sCR1) corresponding to the extracellular part of the molecule (12) and, more intriguing, a membrane-associated form (mCR1) (13). The studies performed here clearly indicated that ectosomes express CR1. Thus, mCR1 is eCR1.

The activation of PMN in vitro showed that the release of ectosomes occurred within minutes and was almost complete after 15 min, although in some experiments more eCR1 was detected at 1 h than at 15 min. As PMN incubated without growth factors in vitro undergo apoptosis within some hours (37), the release of apoptotic bodies might be a source of CR1 associated with membrane. As it was unlikely that the release of a significant amount of apoptotic bodies occurred during the first minutes of incubation, we decided to focus on eCR1 release during the first 15 min of PMN stimulation.

The agents used to stimulate PMN led simultaneously to the shedding of sCR1 and the formation of eCR1, whereby the ratio of sCR1 to eCR1 varied depending on the type of stimulation. Thus, the shedding of sCR1 and ectocytosis of eCR1 were two independent reactions.

Ectosomal CR1 was stable and not cleaved from the surface of ectosomes over 30 min, suggesting that ectosomes are to some extent stable units. The recovery of eCR1 from skin blister fluid and BAL provided similar evidence. Previous data, moreover, indicated the presence of mCR1 (i.e., eCR1) in synovial fluid of patients with arthritis (13).

The electron microscopic studies allowed identifying ectocytosis as a phenomenon that takes place during the first minutes after PMN stimulation. The appearance of small, semivesicular protrusions on the cell surface of activated PMN resembled ectosomes just before release, as they were of the same size as the CR1-bearing, bilamellar vesicles detected in the SN at the same time. From the number and distribution of CR1-marking gold particles, the presence of more than one CR1 molecule per ectosome could be assumed. The ability to release many CR1 molecules on a single ectosome might be an important functional advantage over the monomeric properties of a cleaved molecule and will be discussed further in the context of the performed functional assay.

Most of the observations made in vitro were confirmed by the analysis of skin blister fluid obtained 12 and 12 + 3 h after application of the chemical irritant Cantharidin. Such blister fluids were rich in PMN and exsudated proteins and enriched in sCR1, and they contained measurable amounts of eCR1. The local enrichment in sCR1, compared with plasma, has been reported in synovial fluid (13). It indicates local activation of PMN accompanied by the cleavage of CR1 from their cell surface. The ectosomal CR1 in the blister fluids could not be derived from plasma, because plasma does not contain this form of CR1. Thus, eCR1 was formed by local ectocytosis. Because the leukocytes present in skin blisters were >95% PMN, it was very likely that eCR1 derived from these cells. Interestingly, the ratio of sCR1 to eCR1 varied not only in vitro, but also in vivo. In blister fluid that had reaccumulated after the first collection, the sCR1/eCR1 ratio increased in a manner similar to that when PMA was combined with ionomycin to stimulate PMN in vitro, i.e., more sCR1 than eCR1 was released. From the increasing concentrations of PMN in reaccumulated blister fluid, it could be assumed that the inflammatory response was still increasing. The enhanced release of the more diffusable sCR1 later in the inflammatory process suggests down-regulation of inflammation around the site of complement activation, possibly to minimize nonspecific tissue damage.

The concentration of eCR1 in blister fluid was significantly higher than what could be induced by in vitro stimulation (0.6 ng of eCR1/million PMN in the blister fluid, compared with maximal 0.4 ng of eCR1/million PMN in vitro after 1 h). It may be that we detected more eCR1 in vivo because PMN had more time to release ectosomes. In addition, naturally activated PMN may release

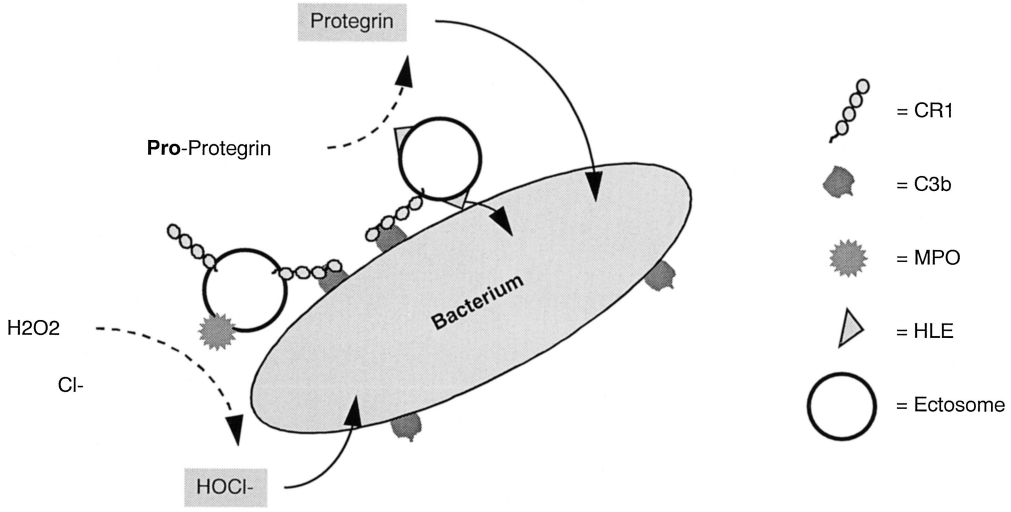

FIGURE 9. Model suggesting ectosomes to play a role in the extracellular antimicrobial activity of PMN. The receptor for opsonin C3b (i.e., CR1) and antimicrobial proteins (i.e., MPO and HLE) were present on the same ectosome and may serve to focus antimicrobial activity onto the surface of opsonized bacteria. This would enhance the efficiency of released antimicrobial proteins and reduce nonspecific autoaggressive potential.

more ectosomes than in vitro activated PMN. However, high concentrations of eCR1 were found within blister fluid that had reaccumulated for only 3 h, where ectosomes probably reached high concentrations because they diffuse less readily than soluble proteins. This biophysical characteristic of ectosomes might allow eCR1 as well as other ectosomal proteins to reach local concentrations at which they become biologically relevant. In the case of CR1 this would be its capacity to bind and participate in the degradation of C4b/C3b.

Ectosomes inhibited rabbit erythrocyte lysis by the alternative pathway of complement. This inhibition was shown to be due to eCR1 specifically, because an mAb-blocking CR1 function abolished the effect of ectosomes (23). The function of eCR1 was further tested in relation to phagocytosis/killing of bacteria by PMN. Bacteria were opsonized for only 1 min with human complement and thus were covered with an only limited amount of C3b. This opsonization was sufficient to allow efficient phagocytosis/killing by PMN (Fig. 7). Such opsonized bacteria were not phagocytosed efficiently when preincubated with rsCR1. This indicated that phagocytosis was C3b/CR1 dependent. The eCR1 had the same effect as rsCR1, confirming its capacity to bind C3b/C4b. Of particular interest was that eCR1 was even more potent than rsCR1, suggesting that it bound more efficiently to the C3b on bacteria. Clusters of CR1 expressed on any one ectosome may have resulted in a multipoint binding to bacteria and thus a more stable binding than monomeric rsCR1. A similar high avidity has been described for immune complexes binding to erythrocytes; CR1 on erythrocytes has been shown to be expressed in clusters, leading to an increased avidity between C3b molecules deposited on the immune complexes and the erythrocyte CR1 (38). In addition to the multivalency discussed above, the proper orientation of the molecules on ectosomes may play a role, where the same molecule might have different properties when fixed onto a vesicle than when soluble. For instance, CD55 (decay-accelerating factor) on proteasomes of seminal fluid has been shown to be more potent than its soluble urinary counterpart (39). The demonstration of a functional difference between soluble CR1 and eCR1 suggests distinct biological roles in vivo.

When bacteria were preopsonized for 10 min, rsCR1 and eCR1 were both incapable of inhibiting phagocytosis/killing of bacteria by PMN. The loss of the CR1-dependent effect was probably due to the much higher number of C3b molecules deposited after 10 min and/or a partial decay of C3b to C3bi, allowing the interaction of C3bi with the complement receptor 3 on PMN (CR3/CD21). CR3 plays an even more important role in phagocytosis than CR (40). It is worth emphasizing that the phagocytosis-blocking effect of eCR1 was observed in a highly artificial setting. In vivo, C3b deposition and degradation are continuous processes, with ectosomal CR1 probably participating in the degradation of C3b. Ectosomes thus are very unlikely to interfere with binding and phagocytosis of opsonized bacteria by PMN in vivo.

Cell surface biotinylation of resting PMN indicated the presence of a specific set of cell surface proteins on ectosomes. A number of proteins were sorted out of ectosomes, whereas others were selectively expressed. That ectocytosis is not a random phenomenon has been shown by others, as the membrane attack complex of complement is removed selectively from the cell surface (3). Furthermore, it is well known that in vitro aging erythrocytes release a selective set of surface proteins by vesiculation (41).

The selectivity of ectocytotic release of proteins was further analyzed by testing the colocalization of MPO and HLE on ectosomes. They were found to colocalize, although the signal was weaker than the one resulting when precipitating with anti-CR1 mAb (Fig. 8*b*). By contrast, CD63, a transmembrane protein used as marker for the azurophilic granules, did not colocalize with CR1 (36). Furthermore, CD14, which is known to be located in the secretory vesicles together with CR1 as well as in MPO- and HLE-positive azurophilic granules, was also absent from CR1-expressing ectosomes (29, 34, 35). Thus, none of the tested transmembrane proteins of the azurophilic granules was released on CR1-expressing ectosomes, whereas the two enzymes, MPO and HLE, which are known to also be released as soluble proteins, were both present on the CR1-expressing ectosomes.

Almost no CD55 was present on CR1-expressing ectosomes, although CD55 is constitutively expressed on the PMN cell surface and is up-regulated from a pool located in the secretory vesicles (29). CD46 and CD59, the two other complement regulatory proteins expressed by PMN, are both constitutively present on the cell surface (25), but only CD59 colocalized with CR1. These findings are interesting, because CD55 and CD59 are both glycosylphosphatidylinositol-linked proteins, and only CD59 is selected into the ectosomes. In addition, the selection of transmembrane proteins

into ectosomes is suggested by the lack of CD46 on CR1-expressing ectosomes (both are present on the cell surface) and the lack of CD55, which is similar to CR1 and is also located within secretory vesicles (29).

Different factors might be responsible for such selections, e.g., the composition of membrane lipids, the cleavage of molecules, the rebinding of others, etc. Further studies are required to define these processes.

MPO and HLE are two important antimicrobial proteins (42–44). MPO is responsible for the production of hypochloric acid ($HOCl^-$) in the presence of H_2O_2 and chloride (44). Hypochloric acid not only has a direct toxic effect on bacteria, but is known to enhance the activity of elastase through inactivation of its main inhibitor, the α1-anti-protease (45). Elastase belongs to the oxygen-independent group of antimicrobial proteins (42, 44) and might act directly through digestion of bacterial proteins or, as suggested recently in a porcine PMN model, indirectly through the cleavage and activation of pro-protegrins (43). In addition to their antimicrobial activity, both enzymes are known to be responsible for nonspecific tissue damage as well (46–48). The observed colocalization of the opsonin receptor CR1 with these two antimicrobial enzymes on ectosomes might represent a new mechanism, increasing the specificity of the extracellular antimicrobial activity of PMN. Both enzymes, when bound to CR1-bearing ectosomes, would be directed to surfaces opsonized with C3b. This focussed antimicrobial defense might be an even more likely physiological role of ectosomes than their complement regulatory capacity, as CR1 is only one regulatory protein among others (CD46, CD55, CD59). A hypothetical model of ectosome anti-microbial function is presented in Fig. 9.

In conclusion, ectosomes are formed by a well-defined process taking place early during PMN activation. Ectosomes express a selected set of proteins on their surface, which at the site of inflammation might represent a functional unit designed for optimal fighting of invading micro-organisms. In addition, ectosomal CR1 might down-regulate complement at the appropriate time point, i.e., after bacteria have been opsonized and PMN have reached the site of infection. Ectosomes might thus be viewed as ecto-organelles, having a specific role to play, a role best played by small vesicles as an intermediate between soluble mediators and cells.

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