0022-1767/92/1493-0747$02.00/0
THE JOURNAL OF IMMUNOLOGY
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Vol. 149, 747-753, No. 3, August 1, 1992
Printed in U.S.A.

SHEDDING OF THE CD44 ADHESION MOLECULE FROM LEUKOCYTES INDUCED BY ANTI-CD44 MONOCLONAL ANTIBODY SIMULATING THE EFFECT OF A NATURAL RECEPTOR LIGAND[1]

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The CD44 adhesion molecule, playing an important role in leukocyte extravasation, was down-regulated by PMA and ionomycin on granulocytes and by an immobilized or soluble anti-CD44 mAb both on granulocytes and lymphocytes. Soluble labeled CD44 molecules of lower apparent molecular mass as compared to their membrane counterparts were isolated from culture supernatants of stimulated surface iodinated cells. Shedding rather than internalization is the mechanism found to be responsible for the loss of CD44 from the cell surface. The size of the soluble CD44 shed from the cells stimulated in vitro corresponds to soluble CD44 isolated from human serum. These data suggest that shedding, induced by anti-CD44 antibody simulating the effect of a natural CD44 ligand, is an important regulatory mechanism controlling surface CD44 expression on leukocytes in vivo.

The extravasation of leukocytes is an exquisitely regulated process critical for homeostasis and for effective host responses to infectious organisms and tumors. Lymphocytes continuously recirculate from the blood into various lymphoid organs providing immunologic surveillance. During diverse inflammatory events, other hemopoietic cells, such as neutrophils and monocytes, also migrate into lymphoid and nonlymphoid tissues to perform various effector functions.

Leukocyte migration is controlled by specific interactions with vascular endothelium using a number of surface receptors, such as CD44, leukocyte function-associated Ag-1 (CD11a/CD18), CD11b/CD18, LECAM-1[3] (LAM-1, Leu8), VLA-4 (CD49/CD29) etc., expressed distinctly on various leukocyte subpopulations. Particular attention has focused on CD44 because this molecule expressed broadly on leukocytes seems not only to play a key role in lymphocyte homing but also participates in multiple other cellular functions. CD44 has been implicated in lymphocyte binding to high endothelial venules (1), in T cell activation (2-4), in monocyte stimulation to IL-1 production (4), and in lymphopoiesis (5). It has been shown that CD44 glycoproteins form a family of molecules that differ both in their polypeptide backbones and posttranslational modifications. Complementary DNA coding for various CD44 molecular species have been cloned and sequenced by several groups (6-8). It is suggested that individual CD44 glycoproteins with distinct structure and cellular expression exhibit different binding capacities for their specific ligands, including hyaluronic acid (9, 10), fibronectin (11), collagen types I and VI (11), and a mucosal vascular endothelium surface molecule (12). In addition, a new CD44 variant produced by alternative splicing has been recently described to confer metastatic potential to carcinoma cells (13, 14).

Despite the great effort devoted to the studies on CD44 molecules, their exact role in leukocyte functions is still far from being fully understood. One of the important questions to be answered is: what are the regulatory mechanisms of CD44 surface expression that are responsible for proper timing of CD44 participation in the sequence of adhesion and other cellular processes. We describe rapid CD44 shedding from cell surface induced by cell stimulation that might be just one of these mechanisms controlling CD44 surface expression in vivo.

MATERIALS AND METHODS

Reagents. PMA, ionomycin, protein A, Nonidet P-40, and affinity purified goat anti-mouse Ig were purchased from Sigma Chemicals Co. (St. Louis, MO), FITC-conjugated swine anti-mouse Ig from Institute of Sera and Vaccines (Praha, Czechoslovakia), Ficoll-Paque and CNBr-Sepharose 4B from Pharmacia LKB Biotechnology (Uppsala, Sweden), verografin from Spofa (Praha, Czechoslovakia), V8 protease from Serva (Heidelberg, FRG), and [^{125}I]NaI from NEN Products (Boston, MA). RPMI 1640 medium containing 10% FCS, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml) was used for cell culture.

Antibodies. The reactivity of anti-CD44 mAb MEM-85 (15), anti-CD45 mAb MEM-28, -55, and -56 (16), anti-CD18 MEM-48 (17), and AFP-02 mAb, recognizing human α-fetoprotein (18), has been described. Anti-CD44 mAb 84-4C4 and 106-4D5 were kindly provided by Dr. R. Villela (Hospital Clinic, Barcelona, Spain) and HEB-03 by Dr. M. Němec (Institute of Molecular Genetics, Praha, Czechoslovakia). Anti-CD18 mAb M232 was obtained from a mAb panel of IVth International Workshop on Human Leukocyte Differentiation Antigens, Vienna, 1989.

Isolation of PBL and granulocytes. Lymphocytes were isolated from blood of healthy donors by Ficoll-Paque density gradient centrifugation. Monocytes were removed by adherence to plastic.

Granulocytes were prepared from blood according to Lochmanová and Lochman (personal communication) as follows: heparinized blood mixed (1:4) with 6% Dextran T500 in PBS was incubated at room temperature for 45 min to sediment E. Supernatant obtained was layered on a discontinuous gradient prepared from a solution A (10 parts of 34% verografin mixed with 24 parts of 14.6% Ficoll) and solution B (10 parts of 34% verografin mixed with 24 parts of 9% Ficoll) and sedimented at 1650 × g for 20 min at 4°C. Granulocytes

Received for publication February 20, 1992.
Accepted for publication May 4, 1992.
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[1] This work was supported by a collaborative grant from the Austrian Academy of Sciences "Ost-West-Programm" and by Grants 55 215 and 75 224 from the Czechoslovak Academy of Sciences.
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[3] Abbreviation used in this paper: LECAM-1, leukocyte-endothelial cell adhesion molecule.

sedimented at the interphase of the solution A and B were harvested and washed in the medium (granulocytes prepared were of >95% purity).

Down-regulation of CD44 by immobilized MEM-85. Lymphocytes (granulocytes) were cultured in medium in the presence of MEM-85 or irrelevant AFP-02 mAb, immobilized to a plastic carrier, at 37°C for 8 h (3 h, respectively). The incubation time that was necessary for maximum effect of MEM-85 on CD44 expression and that differed for lymphocytes and granulocytes, was selected. After mAb treatment, the cells were collected, washed in medium, and analyzed by flow cytometry.

The carrier with immobilized mAb was prepared as follows. The bottom of a well of a 24-well culture plate (Nunclon) was coated with goat anti-mouse Ig (protein A, respectively), dissolved in PBS (0.2 mg/ml), by 1 h incubation at 37°C. MEM-85 or AFP-02 (ascitic fluid diluted 1/100 in PBS) was then added to the well with immobilized goat anti-mouse Ig (3 h, 4°C). Antibodies attached in this way to plastic were cross-linked by 0.1 mM disuccinimidyl suberate (30 min, room temperature). The carrier with cross-linked immobilized antibodies was finally incubated with PBS containing 0.1 M glycine to quench residual reactive groups and washed in PBS.

Flow cytometry analysis. Cells were resuspended in PBS containing 0.5% BSA and 0.1% NaN_3 with mAb added (ascitic fluid diluted 1/100) and incubated on ice for 1 h. After washing, FITC-conjugated swine anti-mouse Ig diluted according to manufacturer's instructions in PBS containing 0.5% BSA and 0.1% NaN_3, was added. The cell suspension was incubated on ice for 1 h. The cells washed in cold PBS were fixed in 2.5% formaldehyde at room temperature for 10 min. The analysis was performed on FACScan (Becton Dickinson, Mountain View, CA). Surface expression of CD44, CD11b, and CD18 was measured using MEM-85, Leu 15 (Becton Dickinson), and MEM-48, respectively. Background antibody binding was estimated by isotype-matched negative control mAb.

Surface iodination and lysis of cell and iodination of isolated proteins. Cells were surface ^{125}I-labeled by the lactoperoxidase method (19). After extensive washing surface labeled cells were lysed in a solution (30 mM Tris-HCl, 0.14 M NaCl, 1% Nonidet P-40) on ice for 30 min; cell debris was then removed by sedimentation (12,000 × g; 5 min). Isolated proteins were iodinated by the chloramine T method (20).

Solid-phase immunoisolation technique. ^{125}I-labeled proteins were isolated from supernatants of surface iodinated cell cultures or cell detergent lysates by a modified solid-phase immunoisolation technique (21). Briefly, 96-well U-bottom plastic plates (Falcon Labware, Oxnard, CA) were coated with goat anti-mouse Ig (0.1 mg/ml) at 37°C for 1 h. After washing with PBS, 50 μl of mAb solution (ascitic fluid diluted 1/100 in PBS) were added and incubated at 4°C for 3 h. Residual unoccupied binding sites on the plastic were blocked by 0.2% gelatin in PBS at 37°C for 90 min. Then the solution containing labeled Ag was added and incubated on ice for 6 h. After washing with PBS, the contents of wells were eluted with SDS-PAGE sample buffer. Ag/mAb complexes were isolated by the solid-phase immunoisolation technique using the wells coated with immobilized goat anti-mouse Ig only.

SDS-PAGE. Electrophoresis in the presence of SDS was performed on polyacrylamide gels in the discontinuous buffer system of Laemmli (22). Bands of ^{125}I-labeled proteins were visualized by autoradiography. Molecular mass of proteins was estimated by comparison with the positions of M_r standards (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Isolation of soluble CD44 from human serum. One ml of human serum was passed through a minicolumn with 0.1 ml of an immunosorbent prepared by coupling of MEM-85 mAb to cyanogen bromide-activated-Sepharose 4B. After washing with PBS, the material adsorbed was eluted with 0.2 ml of 0.1 M 2-aminoethanol. The eluate neutralized with acetic acid was ^{125}I-labeled by the chloramine T method. ^{125}I-labeled soluble CD44 was isolated from this solution by the solid-phase immunoisolation technique using MEM-85. The high and the low M_r soluble CD44 were separated from each other by SDS-PAGE. The zones corresponding to the particular forms, visualized by brief autoradiography, were cut out of the gel. The separated CD44 forms were then eluted from the crushed gel by overnight incubation in a solution containing 0.1 M NH_4HCO_3, 0.1% SDS, 1 mM PMSF, and 5 mM iodoacetate, pH 9.5. The eluates obtained were supplemented with BSA (0.2%), lyophilized, and extracted by methanol.

Limited V8 protease cleavage. The separated ^{125}I-labeled high and low M_r soluble CD44 forms were solubilized in 50 μl of 0.1 M Tris-HCl buffer, pH 6.8, incubated with 0.5 μg of V8 protease at 37°C for 3 h and analyzed by SDS-PAGE.

RESULTS

PMA induced down-regulation of CD44 on granulocytes. CD44 surface expression was significantly down-regulated in granulocytes after 30 min incubation of these cells in culture medium in the presence of PMA (Fig. 1). Surface CD44 was completely lost from the cell surface after PMA treatment lasting 2 h (Fig. 1). In contrast, surface expression of other granulocyte Ag, used as controls, was either up-regulated (CD11b/CD18, Fig. 1) or remained unchanged (CD59, not shown). A similar effect on CD44 expression, even though much less profound, was exhibited by ionomycin (Fig. 1).

Neither PMA (100 ng/ml) alone nor in combination with ionomycin (10 μg/ml) induced surface CD44 down-regulation in lymphocytes (not shown).

Surface CD44 down-regulation induced by anti-CD44 mAb. Surface CD44 was down-regulated on lymphocytes after 8 h incubation of these cells in culture medium in the presence of MEM-85 immobilized to plastic surface via goat anti-mouse Ig (the antibodies immobilized to plastic were cross-linked by DSS to minimize their release from the carrier; Fig. 2*a*). Surface CD44 expression on nonstimulated lymphocytes displays a bimodal character reflecting the existence of two $CD44^+$ cell populations: the major population consists of $CD44^{high}$ cells, the minor one is formed by $CD44^{low}$ cells (Fig. 2*a*, control mAb-treated cells). CD44 expression pattern did not change during 24 h incubation of lymphocytes both in the presence of an irrelevant mAb and in culture medium alone (not shown). In contrast, after MEM-85 mAb treatment most of $CD44^{high}$ cells became $CD44^{low}$ (Fig. 2*a*, MEM-85-treated cells). The CD44 surface expression pattern obtained after 8 h incubation with MEM-85 remained the same even after 24 h treatment of lymphocytes under the same conditions (not shown). At the same time, the expression of another surface Ag, CD18, did not change (Fig. 2*a*). MEM-85 added to culture medium in a soluble form also down-regulated surface CD44 on these cells in the qualitatively same manner after 24 h incubation (not shown), however, the effect of the soluble mAb compared to the immobilized mAb (measured after 8 h incubation) was much less profound (Fig. 3). However, mixture of anti-CD44 mAb MEM-85, 84–4C4, HEB-03, and 106–4D5 used in the soluble form brought about rapid CD44 down-regulation comparable to immobilized MEM-85 (Fig. 4*a*). All these soluble anti-CD44 mAb tested separately down-regulated surface CD44, however, their mixture was most effective (not shown).

The same mixture of soluble anti-CD44 mAb used for the lymphocyte CD44 down-regulation induced a rapid loss of CD44 from the surface of granulocytes similar to PMA-triggered CD44 down-regulation (Fig. 4*b*, compare to Fig. 1). CD44 homogeneous expression on granulocytes was markedly decreased after anti-CD44 mAb treatment. To exclude the possibility that the signal for CD44 down-regulation on granulocytes delivered by anti-CD44 mAb was FcR dependent, granulocytes were incubated with MEM-85 immobilized to plastic via protein A. Also in this case, CD44 surface expression was markedly decreased after 3 h treatment demonstrating that the effect of the anti-CD44 mAb was specific and FcR independent (Fig. 2*b*).

Shedding of CD44 from cell surface. To decide

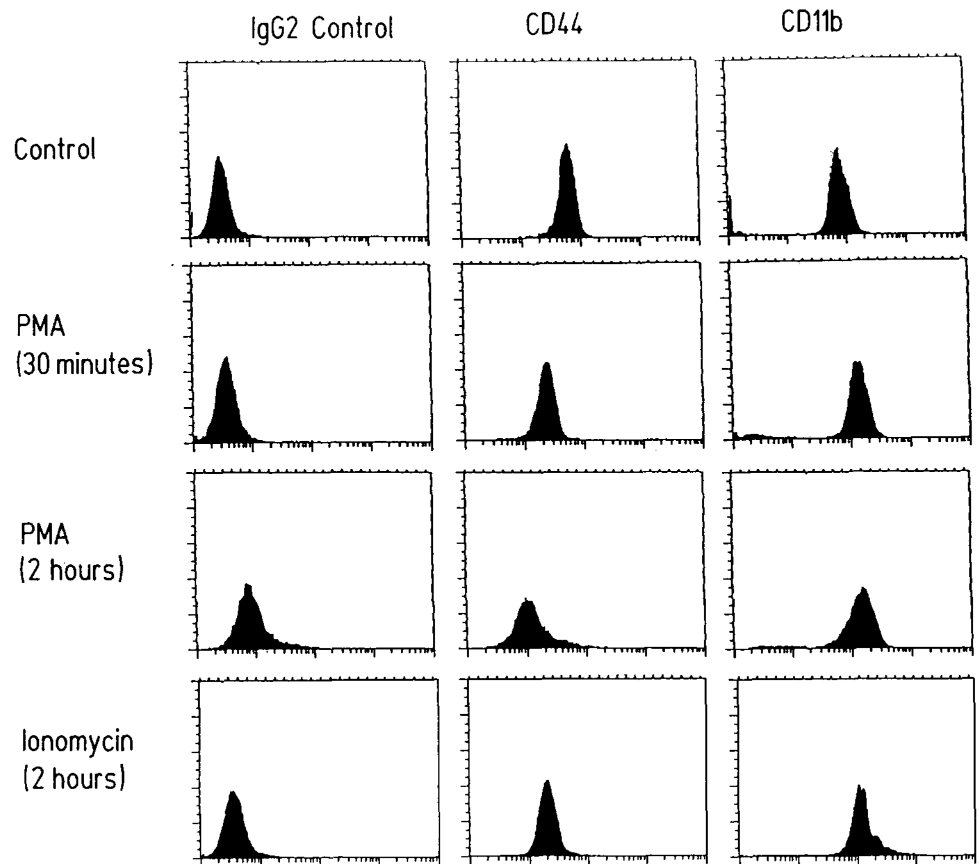

Figure 1. Down-regulation of CD44 on stimulated granulocytes. Granulocytes were incubated in culture medium at 37°C for indicated period of time with PMA (20 ng/ml), ionomycin (10 μg/ml) or in the absence of any stimulant. Surface expression of CD44 and CD11b was measured by flow cytometry. The *x-axis* shows logarithm of fluorescence and the *y-axis* shows cell number.

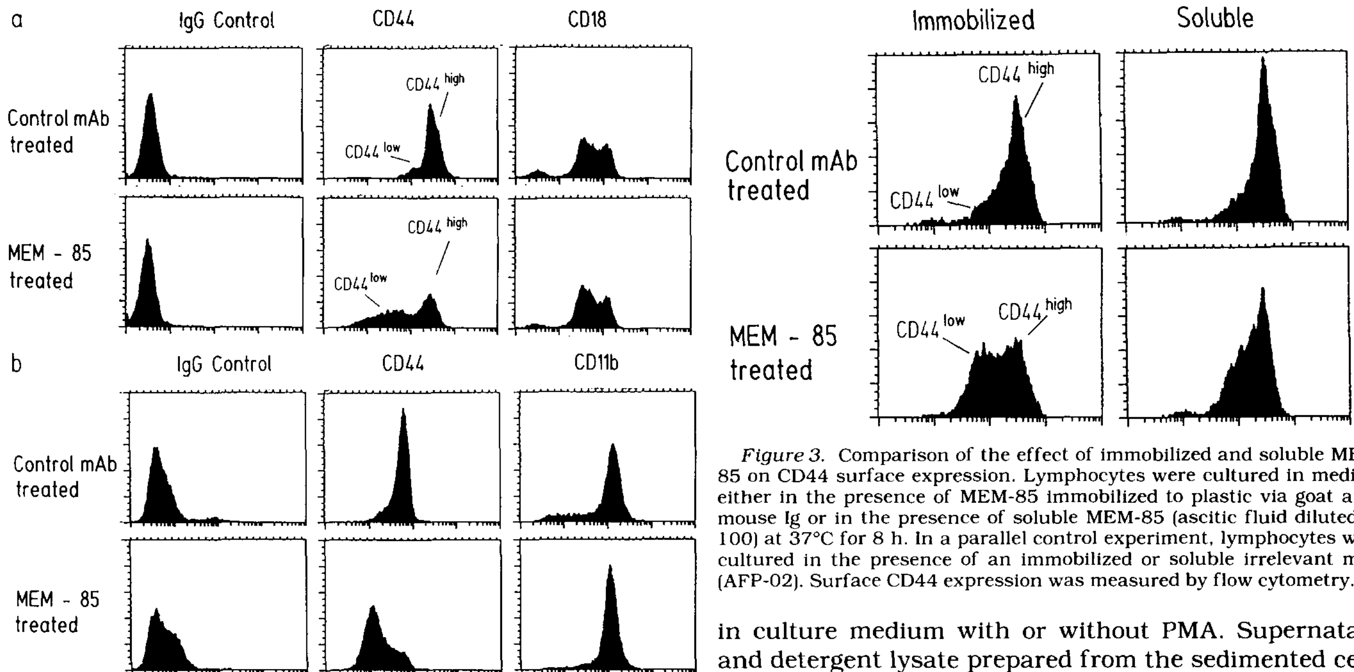

Figure 2. Down-regulation of CD44 induced by immobilized MEM-85. Lymphocytes (*a*) and granulocytes (*b*) were cultured in medium in the presence of MEM-85 immobilized to plastic via goat anti-mouse Ig (*a*), protein A (*b*), or in the presence of immobilized irrelevant AFP-02 mAb used as a negative control. Surface expression of CD44 and CD18 (*a*), CD11b (*b*) was measured by flow cytometry.

Figure 3. Comparison of the effect of immobilized and soluble MEM-85 on CD44 surface expression. Lymphocytes were cultured in medium either in the presence of MEM-85 immobilized to plastic via goat anti-mouse Ig or in the presence of soluble MEM-85 (ascitic fluid diluted 1/100) at 37°C for 8 h. In a parallel control experiment, lymphocytes were cultured in the presence of an immobilized or soluble irrelevant mAb (AFP-02). Surface CD44 expression was measured by flow cytometry.

whether shedding or internalization is the mechanism responsible for the induced CD44 down-regulation, a search was made for soluble CD44 released from the cell surface. Surface ^{125}I-labeled granulocytes were incubated in culture medium with or without PMA. Supernatant and detergent lysate prepared from the sedimented cells were analyzed for the presence of CD44 using the solid-phase immunoisolation technique and SDS-PAGE. A soluble-labeled CD44 was isolated from the culture supernatant of PMA-treated granulocytes (Fig. 5, *right panel*) although no labeled CD44 was detected in the lysate of these cells (Fig. 5, *left panel*). In contrast, membrane CD44 was isolated from the lysate of control cells (Fig. 5, *left panel*). The supernatant of control cells also contained a detectable amount of soluble CD44 that was, however, much lower than in the supernatant of PMA-

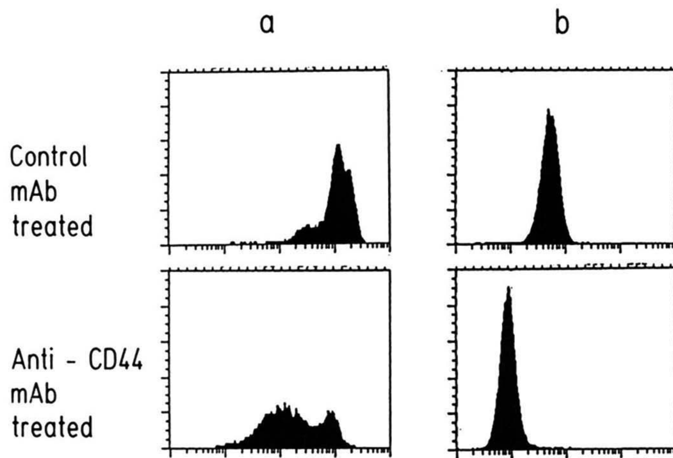

Figure 4. Effect of the mixture of soluble anti-CD44 mAb on CD44 surface expression. Lymphocytes (*a*) and granulocytes (*b*) were cultured in the presence of soluble MEM-85, 84-4C4, HEB-03, and 106-4D5 (ascitic fluids diluted 1/500) or in the presence of irrelevant AFP-02 mAb (ascitic fluid diluted 1/100, used as a negative control), at 37°C for 8 h (*a*) and 4 h (*b*). Surface expression of CD44 was measured by flow cytometry using the same mixture of anti-CD44 mAb used for the treatment of cells.

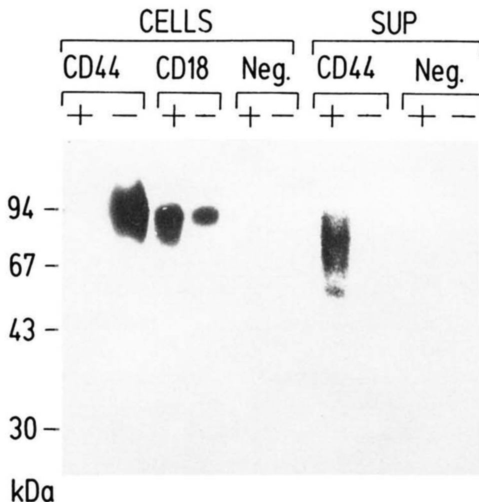

Figure 5. Shedding of CD44 from PMA stimulated granulocytes. Surface ^{125}I-labeled granulocytes were incubated in medium with (+) or without (−) PMA (20 ng/ml) at 37°C for 2 h. CD44 was isolated from the detergent lysate of cells and the supernatant by the solid-phase immunoisolation technique. CD18 isolated from the detergent cell lysate by means of MEM-48 was used as a positive control, irrelevant AFP-02 mAb was used as a negative control. The eluates obtained were analyzed by SDS-PAGE followed by autoradiography.

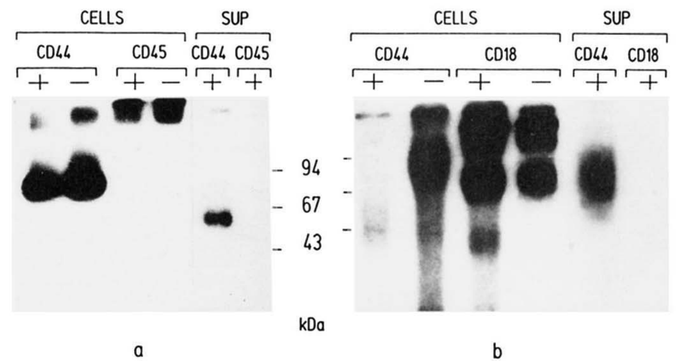

Figure 6. Shedding of CD44 induced by anti-CD44 mAb. Surface ^{125}I-labeled lymphocytes (*a*) and granulocytes (*b*), preincubated with the mixture of soluble anti-CD44 mAb MEM-85, 84-4C4, HEB-03, and 106-4D5 were after intensive washing either lysed immediately in the detergent lysis buffer or cultured in medium at 37°C for 4 h. After the incubation, Ag/mAb complexes were isolated by the solid-phase immunoisolation technique from the detergent lysates and the supernatants of the cells incubated at 37°C (+) and from the lysates of the cells prepared immediately after the preincubation with mAb (−). Mixture of anti-CD45 mAb MEM-28, MEM-55, and MEM-56 (*a*), and mixture of anti-CD18 mAb MEM-48 and M232 (*b*) was used as a control. All mAb were used in the form of 1/500 diluted ascitic fluids. The eluates obtained were analyzed by SDS-PAGE followed by autoradiography.

treated cells and that is hardly seen in Figure 5 (a "passive" shedding). The apparent molecular mass of the soluble CD44 was slightly lower compared to that of the membrane form (Fig. 5, *right panel*; compare to the *left panel*). No soluble forms of CD18, CD53, and CD45 (used as controls) were detected in the supernatants of PMA-treated or control cells (not shown); the same amounts of these Ag were detected in the lysates of PMA-treated and control cells (Fig. 5, *left panel*, data shown only for CD18).

To find out whether shedding is also the mechanism responsible for CD44 down-regulation induced by anti-CD44 mAb, surface ^{125}I-labeled lymphocytes (or granulocytes, respectively) were saturated with the mixture of MEM-85, 84-4C4, HEB-03, and 106-4D5 mAb at 4°C. After extensive washing, the cells were cultured in medium at 37°C. The supernatant and the cell lysate, prepared from the sedimented cells, were analyzed for the presence of CD44/anti-CD44 mAb complexes using the solid-phase immunoisolation technique and SDS-PAGE. Soluble CD44/anti-CD44 mAb complexes were detected in the supernatants both of lymphocytes (Fig. 6*a*) and granulocytes (Fig. 6*b*) preincubated with anti-CD44 mAb demonstrating that these mAb also induced CD44 shedding.

Soluble forms of CD44 isolated from human serum. Soluble CD44 was isolated from normal human serum by the immunoaffinity chromatography on MEM-85 immobilized to Sepharose 4B. The eluate obtained was ^{125}I-labeled and analyzed for the presence of soluble CD44 by the solid-phase immunoisolation technique employing MEM-85, followed by SDS-PAGE. The material, isolated by this mAb, migrated on SDS-PAGE as a very diffuse zone consisting of two major molecular species with different size: a low molecular mass form corresponding to 60 to 80 kDa and a high molecular mass form corresponding to 100 to 150 kDa (Fig. 7*A*). The same results were obtained using another anti-CD44 mAb HEB-03 (not shown).

To exclude the possibility that the high M_r protein is an associated molecule unrelated to CD44, the ^{125}I-labeled high and the low M_r forms were separated by SDS-PAGE (Fig. 7*B*), cut out and eluted from the gel, and analyzed by the solid-phase immunoisolation technique using MEM-85. Both forms were specifically recognized by MEM-85 (Fig. 7*C*) demonstrating that they represent CD44 molecules. To show that these two soluble CD44 forms are different molecules rather than that the high M_r form is a dimer of the low M_r form, they were separated by SDS-PAGE, cut out of the gel, and subjected to the limited proteolytic cleavage using V8 protease. The peptide maps of these two forms obtained (analyzed by SDS-PAGE) clearly differed from each other (Fig. 7*D*) indicating that these two forms represent different CD44 molecular species.

Sera of five healthy donors were analyzed for the presence of the two major soluble CD44 forms using the method described above. Both CD44 forms were identified in four of them (Fig. 8, results shown only for one of

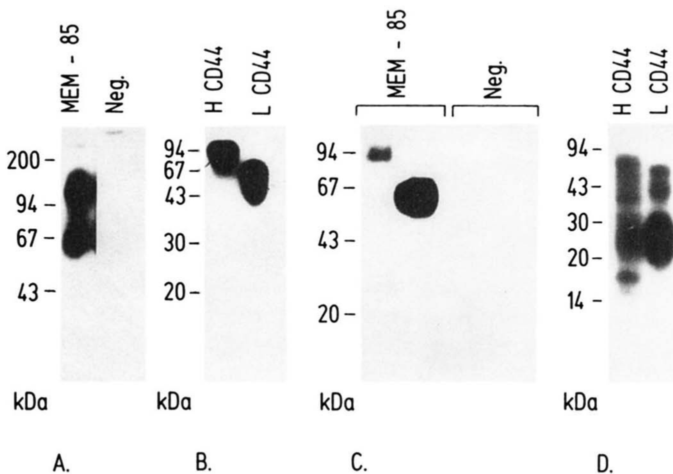

Figure 7. Characterization of soluble CD44 isolated from human serum. *A*, Analysis of soluble CD44 from serum. Soluble CD44 was isolated by the solid-phase immunoisolation technique from the ^{125}I-labeled material obtained by the affinity chromatography of serum (as described in *Materials and Methods*). MEM-85 was used as anti-CD44 mAb, irrelevant AFP-02 mAb was used as a negative control. The eluates prepared were analyzed by SDS-PAGE followed by autoradiography. *B*, Separation of the high (*H*) and the low (*L*) M_r soluble CD44 forms. The ^{125}I-labeled soluble CD44 forms separated from each other as described in *Materials and Methods* were analyzed by SDS-PAGE followed by autoradiography. *C*, Reactivity of the separated high and the low M_r soluble CD44 forms with MEM-85. Reactivity of the separated CD44 forms, solubilized in PBS containing 1% Nonidet P-40, with MEM-85 was tested by the solid-phase immunoisolation technique using MEM-85 and irrelevant AFP-02 mAb as a negative control, followed by SDS-PAGE and autoradiography. *D*, Comparison of peptide maps of the high (*H*) and the low (*L*) M_r CD44 forms. The separated ^{125}I-labeled CD44 forms were subjected to limited cleavage with V8 protease and analyzed by SDS-PAGE followed by autoradiography.

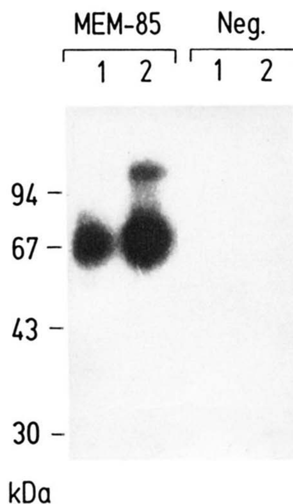

Figure 8. ^{125}I-labeled soluble CD44 isolated from sera of two different donors (1 and 2), analyzed by SDS-PAGE and autoradiography.

these donors) whereas one serum contained only the low M_r form (Fig. 8).

Comparison of apparent molecular masses of various CD44 soluble forms. The apparent molecular masses of soluble CD44 forms isolated from the culture supernatants of PMA-treated granulocytes and anti-CD44 mAb-treated lymphocytes were compared to the M_r of the CD44 forms isolated from human serum using SDS-PAGE (Fig. 9). The soluble CD44 shed from lymphocytes had the same size as the low M_r CD44 isolated from serum corresponding to 60 to 70 kDa (Fig. 9, *lanes 1* and *2*). The soluble CD44 shed from granulocytes migrated as a diffuse zone corresponding to 60 to 100 kDa (Fig. 9, *lane* 3). The size of soluble CD44 shed from PMA-stimulated granulocytes was identical to that of soluble CD44 shed from the same cells stimulated with anti-CD44 mAb (not shown).

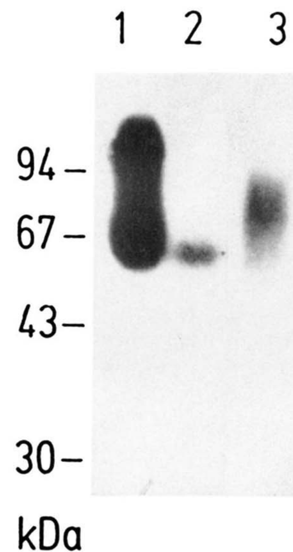

Figure 9. Comparison of M_r of various ^{125}I-labeled soluble CD44 forms analyzed by SDS-PAGE and autoradiography. Soluble CD44 isolated from serum (*lane 1*), shed from lymphocytes (*lane 2*), and shed from granulocytes (*lane 3*).

DISCUSSION

Surface CD44 was rapidly down-regulated on granulocytes by PMA or ionomycin. Shedding rather than internalization was found to be the mechanism responsible for this process as ^{125}I-labeled CD44, lost from cell surface during PMA treatment, was isolated in a soluble form from the culture supernatant. An endogenous protease seems to be involved in CD44 shedding as: 1) M_r of soluble CD44 isolated from the culture supernatant was lower compared to membrane CD44 indicating that membrane CD44 was cleaved, 2) PMA-induced down-regulation of surface CD44 from granulocytes was blocked by protease inhibitors as shown by others (23), and 3) shedding of CD44 from the cell surface was induced by PMA in culture medium even in the absence of serum (not shown) excluding the possibility that an exogenous protease present in serum caused shedding. Neither PMA alone nor in combination with ionomycin was able to induce a rapid loss of CD44 from the surface of lymphocytes.

To look for a natural stimulus initiating rapid CD44 shedding from granulocytes that may be simulated in these cells by PMA, the effect of granulocyte-macrophage-CSF, FMLP chemotactic peptide, C5a complement fragment, and LPS on CD44 expression was tested. None of these reagents induced CD44 down-regulation after 2 h treatment as measured by flow cytometry analysis (data not shown). In contrast, in the same experiment another surface molecule with a very similar cell distribution and physiologic function, LECAM-1 homing receptor, was down-regulated by all these reagents (not shown) which is in accordance with the data previously published (24, 25). These results clearly indicate that CD44 shedding from granulocytes is induced by a stimulus different from that triggering LECAM-1 down-regulation. In T lympho-

cytes, surface LECAM-1 is known to be also down-regulated after activation via TCR/CD3 complex (26) or by PMA (27) whereas CD44 is up-regulated after this type of activation (28) suggesting that the regulation of surface CD44 and LECAM-1 expression differs in these cells too. The data obtained indicated that rapid CD44 shedding may be induced by another natural stimulus, such as CD44 interaction with its ligand. This hypothesis seems to be correct as anti-CD44 mAb simulating the effect of a natural CD44 ligand brought about rapid CD44 shedding both from granulocytes and from lymphocytes. The pattern of CD44 down-regulation in granulocytes initiated by mAb was, however, quite different from lymphocytes. CD44 homogeneous expression on granulocytes was almost entirely lost after mAb stimulation in the similar manner as after PMA treatment. However, lymphocytes comprise two CD44 positive populations: majority of the cells have high CD44 expression ($CD44^{high}$), whereas the smaller population includes $CD44^{low}$ cells. After mAb stimulation, $CD44^{high}$ cells largely disappeared and became $CD44^{low}$. These results suggest that either a homogenous set of CD44 molecules was only partially lost from the surface of lymphocytes or that two CD44 forms that can be distinguished from each other by their sensitivity to mAb-induced shedding, may exist on lymphocyte cell surface. The latter possibility is more likely as at least two CD44 forms have been detected on peripheral mononuclear cells (29). Moreover, one of these forms contains a unique polypeptide stretch located in its extracellular part close to plasma membrane in which a cleavage site for serine proteases has been identified (29).

There is another piece of evidence supporting the idea that CD44 shedding is induced by CD44 interaction with its natural ligand. CD44 surface expression has been tested separately on lymphocytes entering vs leaving secondary lymphoid organs (28). Lymphocytes trafficking into these organs have been found to consist of a major population of $CD44^{high}$ cells whereas only a minor subpopulation was $CD44^{low}$. In contrast, lymphocytes leaving the secondary lymphoid organs, where they are supposed to interact with high endothelium expressing natural CD44 ligands, were largely $CD44^{low}$. Thus, the changes in surface CD44 profile on lymphocytes going through these organs follow exactly the same pattern observed by us during anti-CD44 mAb stimulation of peripheral lymphocytes in vitro. In addition, MEM-85 causing CD44 down-regulation was found to be far more effective in the immobilized form compared to the soluble antibody. The immobilized antibody very likely better simulates the action of natural CD44 ligand expressed on high endothelial venules (in the case of lymphocytes) and on an extracellular matrix (in the case of granulocytes) than the soluble antibody.

Finally, presence of the soluble CD44 in serum with M_r corresponding to M_r of the soluble CD44 shed from lymphocytes and possibly from granulocytes in vitro suggests that CD44 shedding takes place in vivo. CD44 shed from lymphocytes seems to represent one of the two major components of the soluble CD44 isolated from serum, namely the low M_r form. At present, it is hard to say whether the soluble CD44 isolated from serum contains also CD44 shed from granulocytes as both the high and the low M_r forms of the serum CD44 migrated on SDS-PAGE as diffuse zones. However, if it does, CD44 shed from granulocytes would represent only a minor component of the soluble CD44 isolated from the sera used. It is quite possible that granulocyte soluble CD44 may be produced in a higher quantity only under some pathologic circumstances (e.g., during inflammation). Thus, it would be interesting to test the soluble CD44 profile in sera of various patients. Isolation of soluble CD44 from serum with M_r reminiscent of the low M_r soluble CD44 form obtained by us, has been published previously (30). However, the authors of this report did not detect the high M_r soluble CD44. An explanation of that could be that either anti-CD44 mAb used by these authors did not recognize the high M_r form or the serum used for CD44 isolation did not contain the second form (this form was also absent in the serum of one of five healthy donors tested by us). The high M_r soluble CD44 (as judged by its size) may be a product of shedding of epithelial CD44 that has been discovered as a second major CD44 form besides hematopoietic CD44 (8). However, it is also possible that this form is released as a product of an alternatively spliced mRNA.

Down-regulation of CD44 on granulocytes induced by PMA, calcium ionophore, TNF-α, and FMLP has been described (23). However, the authors have failed to detect the soluble CD44 produced in response to this stimulation. Nevertheless, their data suggest that TNF-α may be, apart from CD44 ligand, an additional natural stimulant inducing CD44 down-regulation on granulocytes. As mentioned above, FMLP, in our work, did not down-regulate CD44 expression on these cells.

Taken together, shedding seems to be an important regulatory mechanism of surface CD44 expression on trafficking leukocytes. It seems that peripheral lymphocytes that are largely $CD44^{high}$ are seeking secondary lymphoid organs where they interact physically with high endothelium. This interaction may induce CD44 shedding of a unique CD44 form, sensitive to a protease cleavage, transforming the $CD44^{high}$ cells to $CD44^{low}$ cells that afterward leave these organs. Similar events may take place during extravasation of granulocytes leading to the loss of surface CD44 on these cells after their interaction with a matrix. Still, there are two important questions left to be answered: 1) What are the natural ligands turning on CD44 shedding in vivo? and 2) What is the mutual cooperation between CD44 and other adhesion molecules, such as LECAM-1 and some integrins during leukocyte trafficking?

Recently, a number of functionally important surface leukocyte molecules have been shown to be down-regulated by induced enzymatic cleavage (shedding) in response to external stimuli, e.g., TNF receptor (31), CD16 (32, 33), CD14 (34), LECAM-1 (24), intercellular adhesion molecule-1 (35), CD23 (36), CD32 (37), etc. In all these cases, shedding functions as a powerful regulatory mechanism of Ag surface expression that seems to be of a general significance in vivo. So far, no details are known about the enzymes responsible for shedding of these molecules from cell surface. Some data already available (33, 34, 38) suggest that endogenous proteases play a key role in these processes. In addition, apart from the fact that shedding seems to regulate Ag expression under "normal" conditions there is an evidence growing that increased shedding of some surface molecules may be associated with cancer (39). As the expression of some

CD44 forms has been shown to correlate with tumor formation and metastatic proclivity of lymphoma cells (13, 14), it is tempting to speculate that shedding of CD44 may also be, under some circumstances, involved in the metastatic process regulating invasiveness of neoplastic cells.

Acknowledgments. We are grateful to Dr. I. Hilgert for critical reading the manuscript and for his interest and support. We also thank Dr. M. Němec and R. Vilella for providing us with HEB-03, 84–4C4, and 106–4D5 mAb, Sandoz Forschungsinstitut (Vienna, Austria) for GM-CSF, Dr. P. Kružík for performing flow cytometry analysis, A. Řeřichová and E. Konopásková for technical assistance, and J. Josek for preparing the photographs and E. Tvrzníková for typing the manuscript.

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