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SC5 mAb Represents a Unique Tool for the Detection of Extracellular Vimentin as a Specific Marker of Sézary Cells

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Circulating malignant Sézary lymphocytes result from a clonal proliferation of memory/activated CD4+CD45RO+ T lymphocytes primarily involving the skin. Recently, the CD158k/KIR3DL2 cell surface receptor has been identified to phenotypically characterize these cells. We previously described a mAb termed SC5 that identifies an unknown early activation cell membrane molecule. It is expressed selectively by T lymphocytes isolated from healthy individuals upon activation, and by circulating Sézary syndrome lymphocytes. In addition, we found that SC5 mAb was reactive with all resting T lymphocytes once permeabilized, indicating that SC5 mAb-reactive molecule might present distinct cellular localization according to the T cell activation status. In this study, we show for the first time that SC5 mAb recognizes the intermediate filament protein vimentin when exported to the extracellular side of the plasma membrane of viable Sézary malignant cells. We demonstrate that SC5 mAb is unique as it reacts with both viable malignant lymphocytes and apoptotic T cells. As vimentin is also detected rapidly at the cell membrane surface after normal T lymphocyte activation, it suggests that its extracellular detection on Sézary cells could be a consequence of their constitutive activation status. Finally, as a probable outcome of vimentin cell surface expression, autoantibodies against vimentin were found in the sera of Sézary syndrome patients. The Journal of Immunology, 2006, 176: 652–559.

utaneous T cell lymphomas (CTCL) are a heterogeneous group of lymphomas primarily involving the skin (1). Mycosis fungoides (MF) and Sézary syndrome (SS) are the two major clinical variants of CTCL. MF, the most common form, is characterized by erythematous patches, plaques, or tumors of the skin constituted of clonally derived malignant T lymphocytes that phenotypically resemble mature/memory T cells. SS, a more aggressive leukemic and erythremic variant of CTCL, is characterized by the presence of a clonal CD4+CD45RO+ T lymphocyte population with atypical cerebriform nuclei in the skin, lymph nodes, and peripheral blood (2). To understand the pathogenesis and to facilitate the molecular diagnosis of Sézary cells, a gene expression analysis approach was undertaken and revealed the selective expression of the tyrosine kinase receptor EphA4 and of the transcription factor Twist (3), an overexpression of JunB (4), and a complete loss of Bcl2 (5). Furthermore, using a modified representational difference analysis, Su et al. (6) reported the aberrant mRNA and protein expression of T-plastin in Sézary cells. A different approach to better understand the pathogenesis of Sézary cells, and to distinguish them from normal reactive CD4+ lymphocytes, was to identify characteristic cell membrane receptors. Thus, the immunophenotypic analysis of peripheral blood T cells from patients with SS has revealed a frequent loss of T lymphocyte receptor expression, such as CD7 or CD25, when compared with normal T lymphocytes (7–12). Recently, we reported the presence of the CD158k/KIR3DL2 inhibitory cell membrane receptor for HLA-A alleles on different CTCL lines established from the skin or the blood of patients with SS (13). Furthermore, we found in a large group of patients with SS a strong positive correlation between the percentage of CD158k+ blood lymphocytes, detected by flow cytometry, and the percentage of Sézary cells evaluated by cytomorphology (14). Interestingly, in situ analysis revealed that CD158k is expressed by cutaneous malignant lymphocytes from SS patients, but not from patients with early MF (15). This identified CD158k as the first specific cell surface marker available for the evaluation of the circulating tumoral burden, and for the follow-up of patients with SS. In addition to CD158k, we further evidenced the presence, on the cell surface of circulating Sézary cells, of a structure that was recognized by a mAb that we called SC5 (16, 17). However, in contrast to CD158k, which expression is highly restricted to a minor circulating NK and CD8+ T cell subset, the SC5-reactive molecule was found to be expressed in the cytoplasm of all circulating lymphocytes, and at the cell surface of normal T lymphocytes following activation. Thus, we postulated that this nonidentified protein might correspond to an intracellular structure in normal resting T lymphocytes, and that its surface membrane expression increased rapidly after activation, as reported for CD152 (18, 19), and for a heterogeneous family of lysosome-associated membrane proteins (20).

In this study, we report that the SC5 mAb-reactive molecule, located on the extracellular side of the plasma membrane of activated normal T lymphocytes and of viable malignant SS cells, corresponds to the class III intermediate filament protein vimentin. More importantly, we identified SC5 mAb as a unique tool for the detection

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4 Abbreviations used in this paper: CTCL, cutaneous T cell lymphoma; LSP1, lymphocyte-specific protein 1; MF, mycosis fungoides; PI, propidium iodide; SS, Sézary syndrome.
of vimentin at the cell membrane surface of viable lymphocytes, and therefore discuss whether this intermediate filament protein could be targeted as a tumor or an inflammatory Ag for mAb therapy.

Materials and Methods

Patients

After informed consent and approval by an ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Henri Mondor), sera and PBMCs were isolated from blood samples of 10 patients with CTCL. The patients had not been previously treated with chemotherapy.

Cells and cell lines

PBMCs were isolated by the technique of Ficoll-Isoopaque (Pharmacia) density gradient centrifugation. To obtain T cell lines, cells were maintained in RPMI 1640 supplemented with 2 mM l-glutamine, 100 μg/ml penicillin/streptomycin (Invitrogen Life Technologies), 10% heat-inactivated human serum (Jacques Boy Institute), and 100 IU/ml human rIL-2 (produced by Sanofi-Aventis). The mycoplasma-free HUT78 cell line was grown in RPMI 1640 containing 10% FCS (Perbio Science Europe) and antibiotics.

Antibodies

The mouse SC5 mAb (IgM) was raised in our laboratory (Institut National de la Santé et de la Recherche Médicale Unité 659) against the leukemic NK cell line YTindi, as described elsewhere (17). SC5 hybridoma culture supernatant was used for Western blot analysis. The purified anti-vimentin monoclonal antibody SC5 hybridoma culture supernatant was used for Western blot analysis. The purified anti-vimentin monoclonal antibody, goat polyclonal Abs (C-20) were generated against a peptide located in the C terminus part of human vimentin (sc-7557; Santa Cruz Biotechnology), and an ESI-QTOF instrument (Waters). Mascot (Matrix Sciences) and ProteinLynx Global server (Matrix Sciences) softwares were used together for Swiss- and National Registar-National Center for Biotechnology Information-protein data bank searches.

Generation of Flag-tagged vimentin constructs

The cDNA coding for a C-terminal Flag-tagged vimentin was generated by PCR amplification of the full-length coding region of vimentin. The resulting PCR product was purified and ligated into the pcDNA3.1 vector, according to the manufacturer’s recommendations (Invitrogen Life Technologies). Similarly, the vimentin-deleted constructs were raised by PCR amplification of the coding regions corresponding to aa 101–466 (Δ1–100), 139–466 (Δ1–138), and 228–466 (Δ1–227), fused to a C-terminal Flag tag. All cDNA constructs were further inserted into pcDNA3.1 expression vector.

Generation of GST-vimentin fusion protein

Total RNAs were extracted from the human CTCL cell line HUT78, and vimentin cDNA was generated by RT-PCR. The PCR product was cloned into the pGEX-4T1 vector (Amersham Biosciences) at BamHI and EcoRI sites. The cDNA sequence was confirmed by double-stranded sequencing analysis.

Expression and purification of GST-vimentin protein were essentially performed according to the manufacturer’s procedure and as previously described (22). Where indicated, the fusion protein was subjected to digestion with thrombin before analysis by SDS-PAGE and Western blotting.

Immunoprecipitation and immunoblotting

Cells (2 × 10^7 per sample) were lysed in 1% Nonidet P-40 lysis buffer and processed as described above. After preclearing on protein A-Sepharose beads, lysates were incubated with SC5, anti-vimentin (clone V9), or isotype-matched irrelevant mAb. Immunoprecipitates were collected on protein G-Sepharose beads and separated by SDS-8% PAGE under nonreducing conditions. Proteins were electrotransferred on nitrocellulose membrane and subjected to immunoblotting using SC5 or anti-vimentin Abs and HRP-conjugated secondary Abs. Detection was realized with an ECL detection system, according to the manufacturer’s recommendations (Amersham Biosciences).

For the mapping of SC5 mAb recognition site, COS7 cells were transfected with the indicated plasmid using a DEAE-dextran transfection method (23). After 48 h of culture, cells were harvested and lysed. Recombinant proteins were immunoprecipitated using an anti-Flag mAb and protein G-Sepharose beads, and resolved by SDS-12% PAGE under nonreducing conditions. Immunoblotting was performed as described above.

Confocal immunofluorescence microscopy

HUT 78 cells were placed in V-shaped 96-well plates (3 × 10^5 cells/well). After a washing step in PBS, cells were fixed in PBS/4% paraformaldehyde. For intracellular staining, cells were permeabilized with PBS/0.1% Triton X-100. After quenching with 50 mM NH4Cl, and saturation of unspecific sites with PBS/1% BSA, cells were incubated with SC5 and anti-vimentin (C-20) Abs. After washes, cells were subsequently incubated with goat anti-mouse PE (Beckman Coulter) and donkey anti-goat FITC (Jackson ImmunoResearch Laboratories) secondary Abs. Cells were transferred onto coverslips, and slides were mounted using Moviol (Calbiochem). Analysis of cell labeling was performed on a Zeiss confocal microscope (LSM510; Zeiss).

Results

SC5 mAb stains circulating SS lymphocytes and the Sézary cell line HUT78

The SC5 mAb raised against the functional cytotoxic NK tumor cell line YTindi was initially selected for its reactivity with both the immunizing tumor cell line and a minor population of normal PBLs (Fig. 1A, left panel). We previously reported that the PBMCs reactive with SC5 mAb, corresponding to 5–10% of gated lymphocytes, included CD4^+^, CD8^+^, as well as CD56^+^ cells (17). Most of these SC5^+^ circulating lymphocytes belonged to the activation/memory cell subset coexpressing CD56 and CD16. The expression of the SC5 mAb-reactive molecule was highly increased in peripheral blood T cells from patients with SS (16, 17), as shown in Fig. 1A (right panel), with PBMC from a representative patient presenting >90% of malignant CD4^+^.
cells. Furthermore, we found that the Sézary cell line HUT78, which was weakly recognized by an anti-TCRα mAb, was also dimly labeled by SC5 mAb (Fig. 1B). Thus, circulating SS T lymphocytes and the HUT78 cell line both presented an increased reactivity toward SC5 mAb when compared with normal resting T cells.

Identification of vimentin as the Ag recognized by the SC5 mAb

We previously reported that stimulation of PBMCs with PHA resulted in an increased cell staining with SC5 mAb when compared with resting cells, a maximum detection level being observed after 72–96 h of activation (17). To identify SC5 mAb-reactive protein, large-scale SC5 immunoprecipitates were obtained from lysates of PHA-treated cells. Immunoprecipitations with an isotype-matched control revealed that the Ab recognizes a protein complex in PHA-activated cells that at least encompasses α-actinin 4, LSP1, and vimentin. To further assess which of these polypeptides was specifically recognized by SC5 mAb, immunoprecipitates were prepared from HUT78 cell lysates using commercially available Abs directed against each of the identified proteins. An anti-vimentin Ab (C-20), raised toward the C-terminal moiety of the protein, was first tested (Fig. 3A). Immunoprecipitations with a control irrelevant Ab, and with SC5 mAb, were done in parallel. Immunoblot analysis with SC5 mAb allowed the detection of two protein bands at 53 and 60 kDa in the anti-vimentin precipitate (C-20). After stripping and reprobing of the blot with the anti-vimentin Ab (C-20), an identical protein pattern was obtained. Conversely, Western blot analysis of SC5 mAb immune complex with SC5 or anti-vimentin Ab resulted in the visualization of a broad signal in the 50- to 60-kDa range (Fig. 3A). In contrast, no signal was obtained when probing an anti-LSP1 or α-actinin 4 immunoprecipitate with SC5 mAb (Fig. 3B).

To definitely assess the specificity of SC5 mAb for vimentin, a cDNA construct coding for a GST-vimentin fusion protein was generated. Following expression and purification, a thrombin digestion was performed and the resulting cleaved polypeptide was separated by means of SDS-PAGE. Immunoblot analyses were then realized using SC5 mAb or two anti-vimentin Abs of distinct origin (Fig. 3C). An identical recognition profile, with the detection of a 50- to 60-kDa protein, was obtained independently of the Ab used for revelation. Altogether, these results demonstrated that SC5 mAb was directed against vimentin.

SC5 mAb specifically recognized vimentin when located on the outer side of the plasma membrane

The identification of SC5 mAb as an anti-vimentin Ab, combined with our previous observation that SC5 immunostaining was positive on malignant Sézary cells (16, 17), suggested that vimentin might present an atypical location at the plasma membrane in these cells. To further investigate this possibility, immunofluorescence confocal microscopy was performed on the HUT78 cell line (Fig. 4A). In a first set of experiments, cells were fixed and double stained with SC5 (red) and anti-vimentin (green) Abs (Fig. 4A, left panel). An overlay of the two labelings (yellow) showed that both Abs exerted a similar reactivity toward vimentin. Indeed, an immunostaining polarized at one edge of the cell, and which appeared to be tightly associated with the plasma membrane, was constantly

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**FIGURE 1.** Sézary cells present an increased reactivity toward SC5 mAb. A, PBMCs were isolated by density gradient centrifugation from the blood of a healthy donor (left panel) or of a representative SS patient (right panel). Double labeling was performed by incubation of the cells with SC5 and anti-TCRα mAb, followed by goat anti-mouse IgM FITC and IgG1 PE secondary Abs, or anti-TCRVβ23 PE when indicated. Isotype-matched controls were used for delimiting the positive regions in the two color histograms. B, Double staining of the Sézary cell line HUT78 was performed as in A.

**FIGURE 2.** Purification of proteins coprecipitated by SC5 mAb. Lysates from PHA-activated PBMCs were subjected to SC5 precipitation. Proteins were separated by two-dimensional gel electrophoresis and detected by silver nitrate staining of the gel. The protein spots of interest were cut out from the gel and further processed for peptide sequencing. The identity of each polypeptide, as well as the position of the IgM H and L chains, and of the nonspecifically precipitated murine serum albumin are indicated.
detected. An underneath diffuse intracellular labeling, linked to the one detected at the plasma membrane, was also observed.

A second set of experiments was done, in which a step of permeabilization of the cells was added after fixation and before immunostaining (Fig. 4A, right panel). A complete and homogeneous staining of the plasma membrane was observed with both Abs under these conditions. This membrane-bound staining was associated to a deeper cytoplasmic labeling, when compared with the one observed in fixed and nonpermeabilized cells. These observations demonstrated that, according to the cell treatment (fixation alone vs fixation and permeabilization), one could distinguish a membrane-bound vimentin, presenting a polarized location, from the total pool of cellular vimentin.

One limitation of the preparation of cells for their observation by confocal microscopy relies on the use of a fixative agent before immunostaining. This therefore did not allow us to establish whether the immunofluorescent signal was associated with the outer or the inner face of the plasma membrane. To overcome this difficulty, flow cytometry analyses were conducted using SC5 mAb as well as two distinct anti-vimentin Abs (Fig. 4B). In agreement with the confocal microscopy data, all three Abs were found

FIGURE 3. SC5 mAb is directed against vimentin. A, Immunoprecipitations were performed on HUT78 cell lysates using an irrelevant Ab (control), SC5 mAb, or purified goat anti-vimentin Ab C-20. After separation by SDS-PAGE, proteins were first detected by Western blotting using SC5 mAb. The nitrocellulose membrane was then stripped and subjected to a second step of revelation with the anti-vimentin Abs C-20. B, Immunoprecipitations were performed on lysates of PHA-activated normal PBMCs using purified SC5, anti-LSP1, or anti-α-actinin 4 mAb. Immune complexes were separated by SDS-PAGE, and proteins were detected by Western blotting using SC5 mAb. C, Vimentin was expressed as a GST fusion protein and digested with thrombin. The cut protein was then immunoblotted using SC5 mAb or the anti-vimentin Abs V9 or C-20. Protein detection was made with the appropriate HRP-conjugated secondary Ab and an ECL detection system.

FIGURE 4. SC5 mAb recognizes a pool of vimentin located on the outer face of the plasma membrane. A, The Sézary cell line HUT78 was either fixed (left panel) or fixed and permeabilized (right panel) before immunostaining. Double labeling was performed using SC5 mAb (red) and the anti-vimentin Abs C-20 (green). An overlay of both staining is shown (yellow) as well as a light microscopy view of the cell field. B, Flow cytometry analyses were performed on HUT78 cells that were subjected to a step of fixation alone (upper panel), fixation and permeabilization (middle panel), or left untreated (lower panel) before labeling. Cell staining was then performed with SC5 mAb or the commercial anti-vimentin Abs V9 or C-20, followed by the appropriate FITC-conjugated secondary Abs, as indicated. Mouse (for SC5 and V9) or goat (for C-20) control Igs were used as negative controls. C, Cell surface immunolabeling was performed on resting or PHA-activated lymphocytes from a healthy donor, or alternatively on Sézary cells isolated from a representative SS patient. Cell staining was performed without any pretreatment of the cells, with SC5 mAb or the commercial anti-vimentin Abs V9 or C-20, as described in B. Mouse (for SC5 and V9) or goat (for C-20) control Igs were used as negative controls.
to similarly label HUT78 cells when previously fixed, or fixed and permeabilized (Fig. 4B, upper and middle panels). However, a striking difference was observed when the immunostainings were performed on nontreated cells. Thus, while the cell labeling remained negative with both anti-vimentin Abs, a positive signal was still obtained with SC5 mAb (Fig. 4B, lower panel). A similar set of experiments performed on nontreated or PHA-activated normal PBMC, and on lymphocytes isolated from a representative SS patient (Fig. 4C), definitely confirmed the unique specificity of SC5 mAb. Indeed, as expected, the V9 (middle panel) and C-20 (right panel) anti-vimentin Abs remained unreactive with the cell types tested when not subjected to a fixation step. In contrast, SC5 mAb exhibited almost no stain with freshly isolated PBMCs from a healthy donor, but it showed an increased reactivity toward their PHA-activated counterparts, and more importantly with the Sézary malignant cells obtained from a SS patient (Fig. 4C, left panel). Altogether, these results demonstrated that, unlike commercially available Abs, SC5 mAb specifically reacts with a peculiar fraction of vimentin. The observation that this pool of vimentin was concentrated at one edge of the cell, and its association with the extracellular leaflet of the plasma membrane, strongly suggested that vimentin was exported from the intracellular to the extracellular environment in both normal activated T cells and malignant SS lymphocytes.

Mapping of SC5 mAb recognition site

The observation that the anti-vimentin Abs used in this study apparently displayed different binding abilities toward the intracellular and extracellular fractions of vimentin prompted us to map their corresponding recognition site. As the commercially available Ab C-20 was generated against a peptide located within the C-terminal portion of the protein, we focused our attention on the specificity of recognition of V9 and SC5 mAb. cDNA constructs coding for full-length vimentin or deletion mutants, fused to a C-terminal Flag tag, were generated (Fig. 5A). Anti-Flag immunoprecipitates were prepared from lysates of transiently transfected COS cells and further analyzed by immunoblotting using V9 or SC5 mAb (Fig. 5B). We observed that the vimentin mutants presenting a deletion of the N-terminal head (mutant Δ1–100) alone or together with the 1A domain (mutant Δ1–138) were still detected by V9 or SC5 mAb. Moreover, the truncation of the full N-terminal half of the protein (mutant Δ1–227) resulted in a molecule recognized by V9 mAb, but which showed no more reactivity with SC5 mAb. We therefore concluded that both C-20 and V9 Abs are directed against the C-terminal moiety of vimentin, while SC5 mAb recognition site is part of the N-terminal half of the protein, and more precisely corresponds to a region located in the 1B domain.

SC5 mAb is reactive with apoptotic T lymphocyte cell line

One of the earliest cellular events upon apoptosis is the appearance of phosphatidylinerine residues at the cell membrane, which can be revealed by the binding of annexin V. To determine whether the presence of vimentin at the cell surface of SS lymphocytes is a consequence of an apoptotic cell status, we simultaneously tested annexin V and SC5 binding on the HUT78 cell line. The PI uptake, reflecting the necrotic cell status, was also estimated. The results shown in Fig. 6A indicated that the HUT78 cells were negative for annexin V binding, and for PI uptake, whereas they presented a significant amount of extracellular vimentin as identified by SC5 mAb. These data demonstrated that vimentin was detected at the cell surface of viable Sézary cells, and that this location did not derive from a process of cell death.

We next investigated whether the relocation of vimentin from the intracellular compartment to the extracellular matrix could be an inducible event specific to apoptotic cells. A T lymphocyte cell line, developed by long-term growth of normal peripheral blood T lymphocyte in the presence of IL-2, was tested for annexin V and SC5 labeling. We observed that this cell line showed no reactivity with SC5 mAb under basal conditions of cell growth (Fig. 6B). In contrast, a high number of cells was found to be positively labeled with SC5 mAb when cells were deprived of IL-2 for 4 days (Fig. 6C). Importantly, 45% of the SC5-positive cells were also stained by annexin V. These results indicate that besides its reactivity with viable Sézary malignant cells and activated T lymphocytes, the anti-vimentin SC5 mAb might serve as an apoptotic marker as efficiently as annexin V.

Autoantibodies against vimentin are present in the serum of SS patients

The unusual location of vimentin on the extracellular side of the plasma membrane of SS lymphocytes led to the possibility of an autoantibodies production by Sézary patients. This hypothesis was tested by performing Western blot analysis on vimentin using the serum corresponding to 10 Sézary patients. The serum of two patients was found to recognize vimentin, at a dilution of 1/100 (Fig. 7). In contrast, no signal was obtained when using the serum of healthy donors (Fig. 7, control lane), inferring the specificity of the detection. In addition, Abs against vimentin were detected for a third patient when the serum was used at a 1/50 dilution for immunoblotting (data not shown).

Discussion

Sézary cells correspond, in most patients, to lymphocytes exhibiting a CD4+CD45RO+ phenotype. It is generally admitted that
the lack of CD7 and CD26 expression characterizes the malignant lymphocytes, although controversial reports were made concerning the former receptor (14). Until now, and apart from the identification of specific TCRVβ rearrangements, only the KIR3DL2/CD158k cell membrane structure was described to phenotypically identify skin and blood malignant Sézary cells, its expression currently allowing their efficient isolation from the patients’ blood (14). To further identify cell surface receptors expressed by the malignant CD4+ CTCL cells, we developed a mAb called SC5. We previously established that it recognized a plasma membrane Ag, which detection was increased on PBMCs from patients with SS, when compared with PBMCs isolated from healthy individuals (16, 17). In addition, we observed that the SC5 mAb-reactive molecule was mainly located in the cytoplasm of normal resting T lymphocytes, and that it rapidly became detectable at the cell surface upon T cell activation (16, 17). Our first attempts to characterize the SC5 mAb-corresponding molecule, by performing immunoprecipitation on lysates of cell surface biotinylated activated T lymphocytes, led to the detection of a 96-kDa protein (17). In this study, we report further biochemical studies indicating that SC5 mAb precipitates a protein complex containing a 96-kDa and two 60-kDa proteins from PHA-activated lymphocytes (Fig. 2). These polypeptides were identified by peptide sequencing as α-actinin 4, LSP-1, and vimentin, respectively. In contrast to what we initially thought, we established that the SC5 mAb is in fact directed against the 60-kDa molecule, exerting an isoelectric point of 5.1 and corresponding to vimentin. In light of these new data, the previously detected 96-kDa protein most likely corresponded to the coprecipitated α-actinin 4. The generation of a GST-vimentin fusion protein provides definitive evidence for SC5 mAb specificity toward vimentin. Indeed, the use of SC5 mAb in Western blot analysis allows the specific detection of vimentin as efficiently as known anti-vimentin Abs (Fig. 3).

Unlike the commercial anti-vimentin Abs tested, SC5 mAb exerted a unique ability of Ag recognition. Thus, identical patterns of immunolabeling were obtained on the Sézary cell line HUT78 when fixed and/or permeabilized, regardless of the anti-vimentin Ab used for detection (Fig. 4). However, a striking difference was observed when cells were not subjected to any treatment before immunostaining. Under such conditions, SC5 mAb was the only Ab able to interact with a pool of vimentin otherwise not detected by the commercial Abs (Fig. 4, B and C). We therefore established that, while recognizing intracellular vimentin, SC5 mAb could also specifically target a fraction of the protein located on the outer face of the plasma membrane. By generating vimentin deletion mutants, we established that, while V9 and C-20 Abs were directed against regions present within the C-terminal moiety of the protein, SC5 mAb recognition site was located in the N-terminal half of the molecule (Fig. 5). This therefore suggests that the protein sequence recognized by SC5 mAb was exposed in both the intra- and extracellular form of the protein, while the one targeted by the commercial Abs became unaccessible in the soluble form, most likely as a consequence of conformational modifications. Because SC5 mAb was found to specifically react with viable Sézary cells or activated T lymphocytes (16, 17), the detection of extracellular vimentin on living cells using this Ab might represent a powerful tool for the identification of malignant transformed lymphocytes.

It is well established that vimentin is an intermediate filament protein that participates to the formation of the intracellular cytoskeletal structure. The comparison of the confocal microscopy data obtained on fixed cells vs fixed and permeabilized cells evidenced the existence of a vimentin pool that is polarized at one edge of the Sézary cells (Fig. 4A). Because this immunofluorescent signal was observed with both SC5 mAb and the purified polyclonal goat anti-vimentin Abs C-20, it cannot only reflect the presence of vimentin on the external side of the cell membrane. Rather, it might also correspond to a pool of protein undergoing a translocation from the intracellular to the extracellular compartment. The detection of vimentin on the outer leaflet of the plasma membrane of Sézary cells might thus result from a dynamic and induced process of exocytosis. Interestingly, it has been recently reported that vimentin, which is highly abundant in human activated macrophages, is secreted from ex vivo long-term cultured monocyte-derived macrophages (26). The pool of secreted vimentin was then involved in the settings of two major activated macrophage functions, namely bacterial killing and the generation of oxidative metabolites. Furthermore, the release of vimentin in the

FIGURE 6. SC5 mAb labels apoptotic T lymphocytes. A, HUT78 cells were double labeled with SC5 mAb and either PI (left panel) or annexin V FITC (right panel). The percentages of single- and double-labeled cells are indicated for each cell group. B and C, An IL-2-dependent T cell line was obtained by long-term culture of PBMCs in the presence of IL-2. Immunolabeling was then performed with SC5 mAb and annexin V FITC on cells grown with IL-2 (B) or after induction of apoptosis by deprivation of IL-2 for 4 days (C).

FIGURE 7. Presence of anti-vimentin autoantibodies in the serum of SS patients. Purified GST-vimentin was subjected to digestion with thrombin and separated by means of SDS-PAGE. After transfer onto a nitrocellulose membrane, samples were subjected to immunoblotting using SC5 mAb or sera prepared from the blood of SS patients (serum 1 and 2). The control corresponds to the use of the serum obtained from a healthy individual, and is representative of 10 tested. Protein revelation was performed with peroxidase-conjugated secondary Abs and an ECL detection system.
extracellular environment was found to be associated to its location on the extracellular side of the membrane of monocytederived macrophages. More recently, Xu et al. identified the endothelial-cell-specific Ab PAL-E as an anti-vimentin Ab (27). PAL-E mAb shares some common features with SC5 mAb. Indeed, PAL-E was found to stain live endothelial cells (28), and this labeling was polarized along the luminal endothelial surface (29, 30), thus resembling what we observed upon SC5 labeling of Sézary cells. Further investigation established that blood endothelial cells exerted an unexpected vimentin metabolism, because the protein was expressed and secreted as a dimer. Strikingly, PAL-E was found to be reactive with dimeric vimentin, but hardly detected the protein in its monomeric form. In addition, further investigation demonstrated that the commercial V9 mAb was also able to detect the vimentin dimers expressed by endothelial cells, or following secretion. At this point, it is important to mention that we never detected vimentin as a dimeric protein following immunoprecipitation with SC5, V9, or C-20 Abs on lysates from Sézary cells or from activated normal T lymphocytes. In addition, only soluble monomeric vimentin was detected from the culture medium of HUT78 Sézary cell line after immunoprecipitation (data not shown). Thus, while PAL-E specifically allows the detection of a dimeric pool of vimentin processed by blood endothelial cells, SC5 mAb remains the only mAb allowing the detection of extracellular vimentin at the surface of viable Sézary cells or of activated normal T lymphocytes. Further studies will now be needed to determine the role of secreted vimentin in the pathophysiology of SS.

Several hypotheses were made to explain how vimentin could be secreted, although it lacks a signal sequence. It was proposed that its highly positively charged N-terminal sequence could react with the endoplasmic reticulum hydrophobic core of the lipid bilayer and direct the protein into membranes (26). In addition, the C terminus moiety of vimentin contains a di-acidic motif preceded by a Y-X-X-ϕ motif (in which ϕ is a hydrophobic residue), known to be required for the selective export of some proteins from the endoplasmic reticulum into the Golgi apparatus and found in many membrane-associated proteins (26). Although allowing the detection of the entire intracellular vimentin pool by confocal microscopy or biochemical approaches, the conventional anti-vimentin Abs did not highlight the presence of extracellular vimentin on Sézary cells. Similarly, the quantification of vimentin transcripts within the cells would not have answered the question of vimentin relocation from the cytoplasm to the outer side of the plasma membrane. Because of its original specificity, SC5 mAb currently represents a unique tool for the detection of vimentin translocation in Sézary cells. It should be mentioned that another cytoplasmic protein, T-plastin, involved in the regulation of actin assembly and cellular motility, has also been identified as a potential Sézary cell-specific marker (6), pointing to a strong involvement of cytoskeleton-related proteins in Sézary cells.

Finally, we found that, apart from its ability to react with extracellular vimentin on viable Sézary cells, SC5 mAb is also capable of detecting cell surface vimentin on apoptotic T lymphocytes. Apoptosis is characterized by cellular and nuclear shrinkage, cytoplasmic blebbing, condensation of nuclear chromatin, and fragmentation of nuclear DNA (31). Thus, besides the analysis of apoptosis-associated proteins (32), the detection of vimentin at the cell surface could represent an additional tool to evaluate an early stage of cell death. It is important to emphasize that the reactivity of SC5 mAb with Sézary cells is not a consequence of their apoptotic status. Indeed, we demonstrated that these cells were negative for annexin V binding and PI uptake (Fig. 6A), and that they failed to react with Abs directed against cytoplasmic molecules, such as α-actinin 4 (data not shown).

In the past few years, the serological identification of recombinantly expressed genes has increasingly been used to screen new tumor Ags. This serological identification of recombinantly expressed genes method has been demonstrated to be a powerful tool to identify Ags such as tumor-suppressor genes, oncogenes, cancer-testis genes, and differentiation Ags (33, 34). Interestingly, vimentin has been isolated as a potential CTCL-associated Ag (35). In this study, our findings clearly confirm that vimentin represents a tumor Ag in SS patients. Interestingly, they also suggest that the presence of anti-vimentin autoantibodies in the serum of these patients might result from the presence of the protein at the Sézary cell surface rather than from its overexpression, as reported for prostate carcinoma (36) and endometrial neoplasms (37). However, further studies are needed to determine whether the presence of autoantibodies is concomitantly associated with the detection of soluble vimentin in the serum of Sézary patients.

In conclusion, we report for the first time that vimentin is located at the cell membrane surface of malignant Sézary cells and apoptotic T lymphocytes. This observation was rendered possible by using the unique specificity of the anti-vimentin SC5 mAb. We further suggest that anti-vimentin autoantibodies are present in the serum of SS patients, which might represent a useful marker for diagnosis or prognostic purposes.

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Disclosures
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