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TCR Activation of Human T Cells Induces the Production of Exosomes Bearing the TCR/CD3/ζ Complex[1]

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We show in this study that human T cells purified from peripheral blood, T cell clones, and Jurkat T cells release microvesicles in the culture medium. These microvesicles have a diameter of 50–100 nm, are delimited by a lipidic bilayer membrane, and bear TCR β, CD3ε, and ζ. This microvesicle production is regulated because it is highly increased upon TCR activation, whereas another mitogenic signal, such as PMA and ionomycin, does not induce any release. T cell-derived microvesicles also contain the tetraspan protein CD63, suggesting that they originate from endocytic compartments. They contain adhesion molecules such as CD2 and LFA-1, MHC class I and class II, and the chemokine receptor CXCR4. These transmembrane proteins are selectively sorted in microvesicles because CD28 and CD45, which are highly expressed at the plasma membrane, are not found. The presence of phosphorylated ζ in these microvesicles suggests that the CD3/TCR found in the microvesicles come from the pool of complexes that have been activated. Proteins of the transduction machinery, tyrosine kinases of the Src family, and c-Cbl are also observed in the T cell-derived microvesicles. Our data demonstrate that T lymphocytes produce, upon TCR triggering, vesicles whose morphology and phenotype are reminiscent of vesicles of endocytic origin produced by many cell types and called exosomes. Although the exact content of T cell-derived exosomes remains to be determined, we suggest that the presence of TCR/CD3 at their surface makes them powerful vehicles to specifically deliver signals to cells bearing the right combination of peptide/MHC complexes. ***The Journal of Immunology,*** **2002, 168: 3235–3241.**

Eukaryotic cells secrete proteins from the biosynthetic pathway by constitutive exocytosis of secretory vesicles or by regulated release of secretory granules upon appropriate triggering. Several groups have shown that the endocytic pathway is involved in an alternative pathway of secretion. Exosomes are membrane vesicles homogeneous in size, which originate from exocytosis of vesicles that have formed by inward budding from the limiting membrane into the lumen of endosomes. These endosomes are then called multivesicular bodies (MVBs)[3] (reviewed in Ref. 1). This phenomenon has been shown in particular in cells from the hemopoietic lineage, B cells (2), cytotoxic T cells (3), dendritic cells (4), mastocytes (5), reticulocytes (6), and platelets (7).

Exosomes have been involved in specific functions, although some are still speculative. For example, in reticulocytes, exosomes are enriched in molecules that decrease or disappear during maturation (6, 8) and therefore may serve to clear these molecules. Exosomes have also been shown to mediate Ag presentation (2, 4) and may be generally implicated in transfer of material from one cell to another. Recently, it has been shown that exosomes of B cell origin are present in abundance on the cell surface of follicular dendritic cells of human tonsil tissue (9). These exosomes mediate the presence at the surface of these cells of MHC class II molecules, which are not synthesized by follicular dendritic cells (9). It has also been shown that the chemokine receptor CCR5 may be transferred by microvesicles from $CCR5^+$ cells to $CCR5^-$ cells (10).

$CD8^+$ CTL have been shown to release microvesicles from endocytic origin that have been implicated in killing of target cells (3, 11). Cytolytic granules of CTL are secretory lysosomes with MVB-like appearance. Immunoelectronmicroscopy has shown that the internal vesicles of CTL MVBs contain granzyme and perforin (11) as well as TCR, CD3, and CD8 (3). The presence at the surface of the microvesicles of TCR/CD3 complexes, CD8, and other molecules, such as adhesion molecules (12, 13), probably plays an important role in the specific delivery of lytic substances to the target cells, avoiding damage to bystander cells.

We show in this study that upon TCR stimulation, Jurkat cells, T cell blasts derived from peripheral blood of control donors, and T cell clones release small membrane vesicles. These microvesicles have the morphological characteristics of exosomes and they bear markers specific of exosomes. The potential role of these microvesicles will be discussed.

Materials and Methods

Cell lines and preparation of T cell blasts and rosette-forming cells

Jurkat T cells were maintained in RPMI 1640 containing Glutamax (Life Technologies, Rockville, MD), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (European grade; Biological Industries, Beit Haemek, Israel). PBMC from control donors were prepared by centrifugation on

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[3] Abbreviations used in this paper: MVB, multivesicular body; AICD, activation-induced cell death; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; EM, electron microscopy; MART-1, melanoma Ag recognized by T cells.

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Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ). E^+ cells (enriched in T and NK cells) were obtained from the PBMC by rosette formation with neuraminidase-treated SRBCs. To prepare T cell blasts, PBMC were activated for 3 days with PHA-P (1/700; Difco, Detroit, MI), washed, and maintained in a medium consisting of 45% AIMV (Life Technologies), 45% RPMI 1640, and 10% FCS supplemented with 2 mM glutamine, penicillin, and streptomycin. Human rIL-2 (Chiron, Emeryville, CA) was added at a concentration of 100 U/ml 2 days later. Blasts were used after 14 days of activation. The MelanA/melanoma Ag recognized by T cells (MART-1)-specific $CD8^+$ T cell clone and the melanoma cell line used in this study have been described elsewhere and cultured as described (14).

Preparation of microvesicles

To get rid of the microvesicles contained in FCS, medium, i.e., RPMI 1640 plus Glutamax containing 10% FCS, was ultracentrifuged overnight at 100,000 × *g*. The supernatant was then filtered on 0.22-μm filters (Millipore, Bedford, MA) and referred to as depleted medium. Jurkat T cells, T cell blasts, or E^+ cells were resuspended in depleted medium at 2×10^6 cells/ml. They were incubated, for the time indicated, on plastic petri dishes (Greiner, Nurtingen, Germany) coated with the anti-CD3ϵ mAb UCHT1 (ascitic fluid diluted at 1/500). Alternatively, they were incubated with 10 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) plus 5×10^{-7} M ionomycin (Calbiochem, La Jolla, CA) or 10 μg/ml puromycin (Sigma-Aldrich). For the experiments with the $CD8^+$ T cell clone, 15×10^6 T cells were incubated overnight, alone or in the presence of 3×10^6 cells from the autologous melanoma cell line, in depleted medium. Cells from the tumor cell line were incubated in the same condition without T cells as a control. After activation, cells and medium were centrifuged for 6 min at 300 × *g*, and supernatants were filtered on 0.22-μm filters and ultracentrifuged for 1 h at 4°C and 100,000 × *g*. Pellets were then resuspended in 50 ml PBS containing 1 mM PMSF (Sigma-Aldrich), 0.5% aprotinin (Sigma-Aldrich), and 1 mM sodium orthovanadate (Sigma-Aldrich), and centrifuged again for 1 h at 100,000 × *g*; these washed pellets were centrifuged one more time for 1 h at 100,000 × *g* in 3.5 ml PBS supplemented with protease inhibitors. Pellets suspended in 40 μl PBS containing protease inhibitors were then considered as microvesicle preparation. Protein concentration in each sample was measured by a Bradford assay using a kit according to the manufacturer's instruction (Bio-Rad, Richmond, CA).

Abs and reagents

The following Abs were used in this study: UCHT1 (murine IgG1), an anti-CD3ϵ mAb (obtained from a hybridoma provided by P. Beverley, University College, London, U.K.); *Apa*I.1 (murine Ig), an anti-CD3ϵ mAb given by A. Alcover (Institut Pasteur, Paris, France); an anti-ζ mAb (murine IgG1; Santa Cruz Biotechnology, Santa Cruz, CA); a polyclonal goat anti-TCR β Ab (Santa Cruz Biotechnology); FITC-conjugated anti-$\alpha\beta$ TCR (murine IgM; BD PharMingen, San Diego, CA); a PE-conjugated anti-CXCR4 mAb (murine IgG2a; BD PharMingen); a PE-conjugated anti-CD2 mAb (murine IgG1; BD PharMingen); a PE-conjugated anti-CD40L mAb (murine IgG1; BD PharMingen); a PE-conjugated anti-Fas ligand (murine IgG1; BD PharMingen); a FITC-conjugated anti-CD28 mAb (murine IgG1; BD Biosciences, Mountain View, CA); a FITC-conjugated anti-CD18 (murine IgG1; BD Biosciences); PE-conjugated anti-CD45RA (murine IgG2b; BD PharMingen); a PE-conjugated anti-HLA A, B, C mAb (murine IgG1; BD PharMingen); a FITC-conjugated anti-CD63 mAb (murine IgG1; Immunotech, Westbrook, ME); L243, a PE-conjugated anti-HLA-DR mAb (murine IgG2a; BD PharMingen); a PE-conjugated $F(ab')_2$ donkey anti-mouse IgG (H and L chains; Jackson ImmunoResearch Laboratories, West Grove, PA); a polyclonal rabbit anti-Cbl Ab (Santa Cruz Biotechnology); a polyclonal rabbit anti-$p59^{fyn}$ Ab (Santa Cruz Biotechnology); an anti-$p56^{lck}$ mAb (murine IgG2b; Santa Cruz Biotechnology); 4G10, an anti-phosphotyrosine mAb (murine IgG2b; Upstate Biotechnology, Lake Placid, NY); and HRP-conjugated donkey anti-rabbit IgG or goat anti-mouse IgG (Pierce, Rockford, IL).

Immunoelectron microscopy

For electron microscopy (EM) observation of whole-mount microvesicles, pellets prepared as described earlier were fixed in phosphate buffer containing 2% paraformaldehyde (Carlo Erba, Rodano, Italy) and loaded on Formwar/carbon-coated EM grids to be processed for immunogold labeling.

Indirect immunogold labeling of vesicles was performed at room temperature with the anti-CD3ϵ mAb UCHT1 or an anti-TCR β described earlier, followed by rabbit anti-mouse or rabbit anti-goat (DAKO, Carpinteria, CA). Bound Abs were detected by protein A coupled to 10-nm gold particles (purchased from Department of Cell Biology, Utrecht University, Utrecht, The Netherlands).

All samples were then postfixed in 1% glutaraldehyde, contrasted in a mixture of methylcellulose/uranyl acetate, and viewed with a CM20 Twin Philips electron microscope (Philips Electronic Instruments, Mahwah, NJ).

Western blot analysis, separation, and labeling of proteins from microvesicles

For immunoprecipitation and Western blot analysis, cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.4); 140 mM NaCl; 2 mM EDTA; 50 mM NaF; 1% Nonidet P-40; 0.5% Na deoxycholate; 0.1% SDS; 100 μM Na_3VO_4; 2 μg/ml antipain, pepstatin, leupeptin; 1% aprotinin; and 1 mM PMSF) for 20 min at 4°C. Nuclei and cell debris were removed by centrifugation. Microvesicles solubilized in lysis buffer or postnuclear lysates were then either immunoprecipitated with 2 μg anti-ζ mAb, followed by 50 μl 50% protein G-Sepharose, or directly analyzed under reducing conditions by SDS-PAGE and electroblotted on Immobilon P membrane (Millipore). The Ab/Ag complexes were visualized by an ECL detection system according to the manufacturer's instructions (Amersham Pharmacia Biotech).

FACS analysis of microvesicles or cells

For FACS analysis, microvesicles prepared from cell supernatant or medium (used as negative control) were incubated with 5 μl 4-μm-diameter aldehyde/sulfate latex beads (Interfacial Dynamics, Portland, OR) in a final volume of 20 μl for 15 min at room temperature; 10 μg BSA was then added in each sample, and the incubation was prolonged for 15 min. This was followed by a 75-min incubation in 1 ml PBS with gentle shaking; reaction was stopped by incubation for 30 min in PBS supplemented with 100 mM glycine. Microvesicle-coated beads were washed twice in FACS wash (3% FCS and 0.1% NaN_3 in PBS) and resuspended in 400 μl FACS wash. In parallel, cells used for the preparation of microvesicles were washed twice in FACS wash. Cells or 20 μl microvesicle-coated beads were incubated for 30 min with each primary Ab, followed when necessary by incubation with a PE-conjugated secondary Ab, and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Inhibition of the AICD and detection of T cell apoptosis

To block the activation-induced cell death (AICD) induced by anti-CD3 activation, we pretreated the T cells before activation with 50 μM of the cysteine protease inhibitor z-Val-Ala-Asp-CH_2F (zVAD-FMK; Bachem, Torrance, CA) for 3 h at 37°C; cells were then activated, as previously described, in the presence of 50 μM zVAD-FMK. The potentiometric fluorescent dye 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes, Eugene, OR) can be used to measure mitochondrial membrane depolarization, which is an early marker of apoptosis (15). Cells were incubated for 15 min at 37°C with 40 nM $DiOC_6$, followed by a 5-min incubation with 5 μg/ml propidium iodide on ice (Sigma-Aldrich). Apoptotic cells were evidenced by their reduced uptake of $DiOC_6$ measured by using the FL1 channel of a FACSCalibur flow cytometer.

Results and Discussion

We first examined the potential presence of microvesicles in supernatants of Jurkat T cells collected after 2 h of activation with immobilized anti-CD3 UCHT1 mAb or after 2 h in medium only. These supernatants were filtered on 0.22-μm filters and ultracentrifuged at 100,000 × *g*; after two washes in PBS, pellets were prepared for whole-mount EM analysis. Supernatants from UCHT1-activated Jurkat T cells contained microvesicles, which had the morphological characteristics of exosomes as shown in Fig. 1*A*. Their size ranged from 50 to 100 nm, and they were delimited by a lipidic bilayer. We then analyzed the potential presence of TCR/CD3 complexes at the surface of these microvesicles by immunolabeling of the whole-mount preparations with Abs against the TCR β-chain or the CD3ϵ-chain. Addition to the microvesicle preparations of protein A gold only did not reveal any labeling of the vesicles (data not shown). However, a positive labeling of the vesicles obtained from UCHT1-activated Jurkat with the secondary Ab anti-mouse Ig, followed by protein A-gold was observed and most probably reflected the presence at the surface of vesicles of the UCHT1 mAb used to activate the Jurkat cells. In

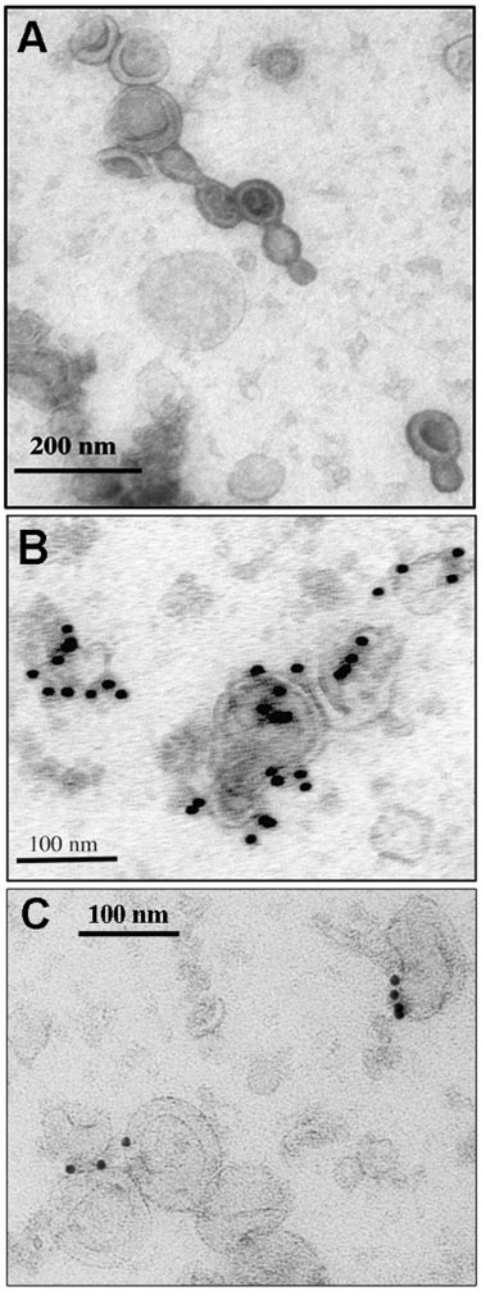

FIGURE 1. EM characterization of Jurkat-derived microvesicles. Microvesicles have been purified from the supernatant of Jurkat cells activated by an immobilized anti-CD3ϵ (UCHT1) by differential centrifugation and analyzed by whole-mount immunoelectron microscopy. *A*, The vesicles are not labeled. *B*, Vesicles were immunolabeled with UCHT1. *C*, Vesicles were immunolabeled with goat polyclonal Abs against TCR β.

fact, when anti-goat Ig were used, no labeling was detected; moreover, vesicles prepared from unactivated Jurkat cells did not show any labeling with anti-mouse Ig followed by protein A-gold (data not shown). As shown in Fig. 1, *B* and *C*, microvesicles produced by activated Jurkat cells bear the TCR β-chain as well as the CD3ϵ-chain.

These results show that Jurkat T cells can produce vesicles that bear TCR/CD3. We then studied whether normal T lymphocytes were also able to produce microvesicles with the same characteristics.

We thus prepared microvesicles from T lymphoblasts obtained from peripheral blood of control donors. To do so, PHA/IL-2 blasts were incubated in the absence or presence of immobilized anti-CD3 mAbs for 2 h at 37°C, and microvesicles were prepared as previously described. Microvesicle preparations coming from Jurkat cells or T blasts were analyzed by SDS-PAGE and Western blot. For the same number of cells, TCR-activated T lymphoblasts or Jurkat cells produced more microvesicles than unactivated cells, as reflected by protein concentrations in the different samples (data not shown). CD3ϵ- and ζ-chains were detected by Western blot analysis in the microvesicle preparations from T lymphoblasts and Jurkat cells (Fig. 2*A*), confirming the results obtained by immunoelectronmicroscopy. For the same amount of protein run, the

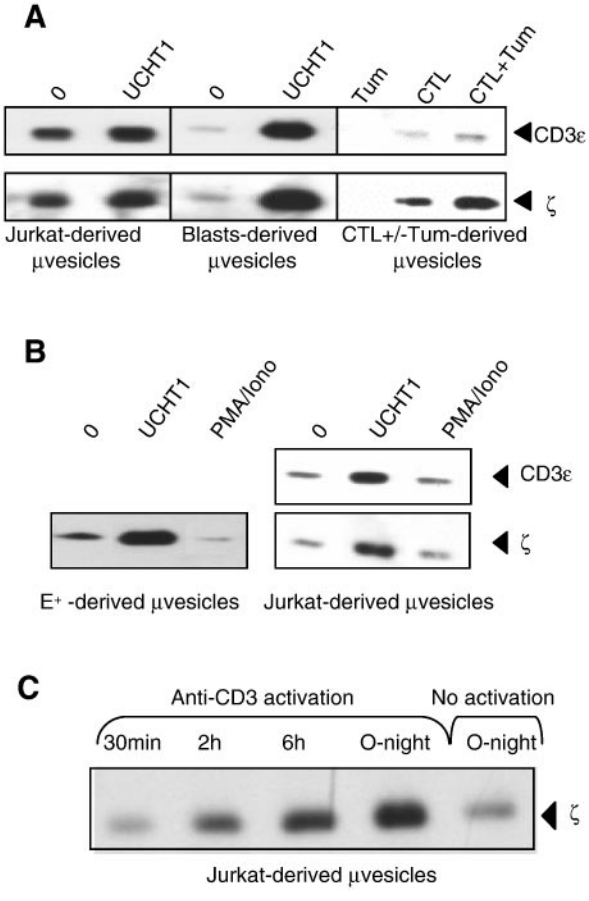

FIGURE 2. The CD3/ζ complex is present on the microvesicles of human $CD4^+$ T cells and $CD8^+$ T cell clones. TCR, but not PMA plus ionomycin activation, leads to an increased microvesicle production. *A*, Jurkat cells (*left panel*) and PHA/IL-2 blasts obtained from a normal donor (*middle panel*) were activated, overnight or for 2 h, respectively, by immobilized anti-CD3ϵ mAbs (UCHT1), whereas MART-1-specific $CD8^+$ T cell clones (*right panel*) were incubated overnight, alone or in the presence of the autologous melanoma cell line (ratio 5:1). Microvesicles prepared from all these cells and from the tumor only as a control for the third experiment were submitted to SDS-PAGE and Western blotted with anti-ζ and anti-CD3ϵ mAbs. *B*, PBMC-derived E^+ cells from a normal donor and Jurkat cells were activated for 2 h or overnight, respectively, either by immobilized UCHT1 or by PMA plus ionomycin. Microvesicles were purified, analyzed by SDS-PAGE, and Western blotted with the anti-ζ mAb (E^+-derived microvesicles, *left panel*) or the anti-ζ and anti-CD3ϵ mAbs (Jurkat-derived microvesicles, *right panel*). *C*, Jurkat cells were activated from 30 min to one night, and a kinetic analysis of the ζ protein content in the microvesicles derived from these cells was performed by Western blot. In *A* (only Jurkat and blast-derived microvesicles) and *B*, microvesicles produced by the same number of cells were loaded in each lane. In *A* (only CTL-derived microvesicles) and *C*, the same amount of proteins (10 μg/lane) was loaded.

intensity of the signal for CD3ε and ζ was stronger in the preparations coming from activated cells, showing an enrichment of CD3/ζ material in the microvesicles of activated T cells. These results were confirmed by immunoelectronmicroscopy analysis, as demonstrated by the higher number of CD3ε and TCR β molecules per vesicle in the preparations coming from activated T cells (data not shown).

We then studied the potential production of microvesicles bearing the TCR/CD3 complexes by Ag-activated T cell clones. We performed this study in the LT12 $CD8^+$ T cell clone specific for the tumor Ag MART-1 described elsewhere (14). $CD8^+$ clonal T cells were left inactivated or were cocultured overnight with an autologous melanoma cell line expressing MART-1; microvesicles were prepared from the tumor cell line alone or the $CD8^+$ T cells cultured in the absence or presence of the tumor cell line. As shown in Fig. 2*A*, microvesicles prepared from the LT12 cells activated in the presence of the Ag-expressing tumor cell line are enriched in ζ and CD3ε expression. As expected, microvesicles prepared from the tumor cell line did not express any chain of the TCR/CD3/ζ complex.

We then wanted to know whether production of exosomes was specifically induced by TCR activation. We thus incubated T lymphoblasts or Jurkat cells with PMA plus ionomycin at concentrations that induce NF-AT activation and IL-2 production (data not shown). Supernatants of PMA plus ionomycin-activated T lymphoblasts and Jurkat T cells were not enriched in microvesicles, as revealed by immunoelectronmicroscopy on whole-mount microvesicle preparations or measurement of the protein concentration in the samples (data not shown). Moreover, the microvesicles produced by T cells activated by PMA plus ionomycin, as opposed to the ones prepared from UCHT1-activated cells, were not enriched in CD3ε- and ζ-chains, as revealed by Western blot analysis (Fig. 2*B*). These results demonstrate that the mere activation of T cells is not sufficient to induce the production of microvesicles bearing the TCR/CD3/ζ complex.

Kinetic analysis of the accumulation of vesicles in the supernatants of UCHT1-activated Jurkat cells was performed. We did not detect any enrichment of ζ in the vesicles prepared from supernatants of Jurkat activated for 30 min (Fig. 2*C*). However, an enrichment of ζ was observed after 2 h of activation. Thereafter, for the same quantity of protein analyzed, the intensity of the ζ band revealed by Western blot analysis increased with time, showing an enrichment of ζ in the vesicles during activation (Fig. 2*C*).

Apoptotic cells produce microvesicles, also called apoptotic blebs, budding directly from the plasma membrane and carrying a number of transmembrane and intracellular proteins (13, 16, 17). We thus wanted to know whether the microvesicles produced by TCR-activated T cells were apoptotic blebs or true exosomes. Therefore, we treated Jurkat cells overnight with the immobilized anti-CD3 UCHT1 mAb in the presence or absence of zVAD-FMK, a cysteine protease inhibitor, which has been shown to inhibit cell death induced by anti-CD3 activation (18) and prepared microvesicles from the supernatants. The number of apoptotic cells was checked in each population by labeling with $DiOC_6$, a potentiometric fluorescent dye, which measures the mitochondrial membrane depolarization, considered as an early marker of apoptosis (15), and propidium iodide, which measures the cell viability. Fig. 3*A* shows that, as previously described (18), anti-CD3 activation induced some apoptosis of the Jurkat cells, 24% of T cells with a lower mitochondrial potential in the nontreated cells vs 31% in the UCHT1-activated cells. Treatment of the cells with zVAD-FMK partially inhibited apoptosis in both the nonactivated and UCHT1-activated T cells, as shown by the reduction in the percentage of T cells presenting a lower mitochondrial potential, respectively 20%

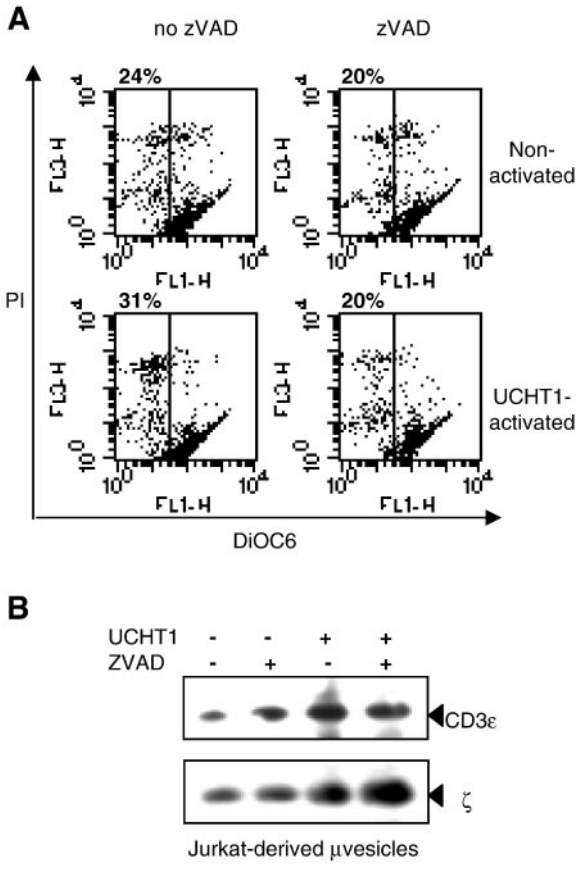

FIGURE 3. Inhibition of AICD does not prevent the release of microvesicles and their enrichment in CD3/ζ. After a 1-h pretreatment with the caspase inhibitor zVAD-FMK at 50 μM, Jurkat cells were activated overnight with immobilized UCHT1 still in the presence of z-VAD. Microvesicles were then prepared from supernatants of cells treated with the inhibitor or left untreated as a control. *A*, Rate of apoptosis in the four conditions is checked by labeling the cells with propidium iodide and $DiOC_6$. Cell fragments were excluded by gating on the forward light scatter/side light scatter (FSC/SSC) dot plot. Percentages of events located in the *left part* of the dot plot (corresponding to a reduced uptake of $DiOC_6$) are shown. *B*, Detection of ζ and CD3ε by SDS-PAGE and Western blot analysis in microvesicles produced by the same number of cells in the four conditions.

for the nonactivated cells and 20% for the UCHT1-activated T cells. The microvesicle samples were submitted to SDS-PAGE, and proteins were revealed with anti-ζ and anti-CD3ε mAbs. Fig. 3*B* shows that treatment of the cells with zVAD-FMK did not reduce the enrichment of ζ and CD3ε in the microvesicles prepared from UCHT1-activated Jurkat cells, although, as previously shown, percentages of apoptotic cells were the same in nonactivated and UCHT1-activated T cells. Same results were obtained with T cells derived from peripheral blood (data not shown). These results show that the microvesicles produced by TCR-activated cells are not apoptotic blebs, and that the enrichment of CD3/ζ material in these microvesicles is not due to apoptosis of the T cells.

Altogether, these results demonstrate that T lymphocytes produce microvesicles, which present some characteristics of exosomes. Production of these vesicles seems to be regulated because T cells activated through the TCR release these exosomes more abundantly than unactivated cells. The presence of TCR molecules at their surface is noteworthy because it may confer to these exosomes a specificity and a directionality, making them powerful

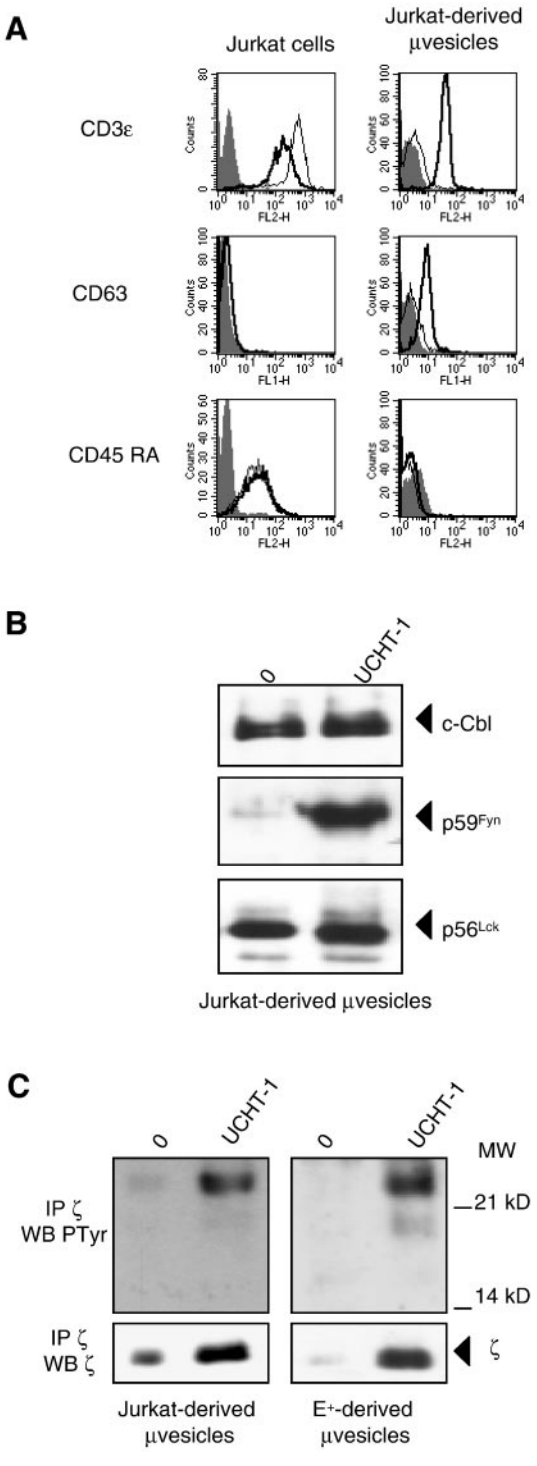

FIGURE 4. Characterization of surface markers present on Jurkat-derived microvesicles by FACS analysis. Signaling proteins and phosphorylated ζ are present on these microvesicles. *A*, A total of 5 μg microvesicles prepared from supernatants of nonactivated or overnight immobilized UCHT1-activated Jurkat cells were coated on preactivated latex beads and labeled by Abs detectable by FACS analysis. Shown are histograms of three representative stainings (*top*, CD3ε; *middle*, CD63; *bottom*, CD45 RA) performed on cells (*left panels*) as well as on microvesicles (*right panels*). Thin line, Nonactivated cells; thick line, activated cells. Of note for the microvesicles, the secondary anti-mouse Ig alone were used. This detected the UCHT1 mAb used to activate the cells and present on microvesicles of activated cells, making thus consistent the negative labeling on microvesicles from resting cells. Controls (filled histograms) were performed with isotype controls or directly coupled secondary Abs. *B*, Microvesicles coming from an equivalent number of resting or UCHT1-activated Jurkat cells were analyzed by SDS-PAGE and Western blotted with Abs directed against c-Cbl (*upper panel*), $p59^{fyn}$ (*middle panel*), or $p56^{lck}$ (*lower panel*). *C*, Microvesicles produced by an equivalent number of resting or UCHT1-activated Jurkat cells (*left panel*) and resting or UCHT1-activated E^+ cells from a normal donor (*right panel*) were immunoprecipitated with 2 μg anti-ζ mAb. To assess the phosphorylation status of ζ, samples were submitted to SDS-PAGE and blotted first with an anti-phosphotyrosine mAb and then with the anti-ζ mAb.

Table I. *Surface screening of PHA/IL-2 blast-derived microvesicles by FACS analysis*[a]

Surface Labeling	Mean Fluorescence Intensity	
	Resting blast-derived microvesicles	Activated blast-derived microvesicles
Anti-mouse	8.2	78.1
TCR	11.4	28.1
CD3ϵ	23.3	86.6
CXCR4	10.1	46.9
CD2	22.9	310.0
CD18	12.5	37.4
MHC class I	48.3	718.8
MHC class II	13.1	28.6
CD63	12.6	99.3
CD28	11.9	10.7
CD40L	14.3	9.2
CD45	11.0	13.4

[a] Microvesicles were prepared from PHA/IL-2 blasts obtained from healthy donors activated or not for 2 h by the immobilized anti-CD3ϵ mAb UCHT1. For both conditions, an amount of microvesicles produced by the same number of cells was coated on latex beads and then labeled by a panel of directly coupled Abs against various T cell markers. As a control we used a microvesicle preparation coming from depleted medium only; mean fluorescence intensity for each Ab was normalized to 10. Presented here is a summary of the normalized mean fluorescence intensity results.

vehicles to deliver signals specifically to a population of target cells bearing the right MHC-peptide combination. Previous studies have shown that $CD8^+$ cytotoxic T cells produce small vesicles bearing the TCR, CD3, and CD8 proteins at their surface. These vesicles are released by cytolytic granules in the synaptic space; they contain granzyme and perforin and mediate lysis of the target cells (3, 11). It has been proposed that the presence of TCR and CD8 complex would ensure the unidirectional delivery of the lethal compounds to the target cells and avoid any bystander damage (19).

To further characterize the microvesicles released by Jurkat cells and T lymphoblasts, we coated them on preactivated latex beads, which were then labeled with a panel of mAbs against various surface markers of T lymphocytes and analyzed by flow cytometry (13). We confirmed that exosomes of CD3-activated Jurkat T cells or T lymphoblasts bear CD3ϵ, TCR, and CD63 (Fig. 4*A* and Table I). On the one hand, some transmembrane proteins, such as CD45 and CD28, although highly expressed by T lymphocytes, were absent from the surface of T cell-derived microvesicles. The CD40 ligand was not detected either in the preparations. On the other hand, CD2, CD18, CXCR4, and MHC class I and to a lesser extent MHC class II molecules were detected on the microvesicles prepared from CD3-activated Jurkat cells and lymphoblasts.

Our results demonstrate that these microvesicles are not mere fragments of broken plasma membranes because they do not contain proteins, which are among the most abundantly represented on the plasma membrane. For example, CD45 is absent from the microvesicles.

Several of the proteins found in the microvesicles of T cell origin we described in this study have been shown to be associated with endosomes and lysosomes: CD63, MHC class II (20), MHC

class I (4), CD2 (21), and CD18 (13), and for most of them except CD2, they have been shown to be present on exosomes from diverse cellular origin (4, 7, 12, 22). The microvesicles we described are thus most probably from endocytic origin, which together with their morphology make them look like exosomes. Exosomes form by inward budding from the limiting membrane into the lumen of endosomes, which are then called MVBs (23). MVBs seem to follow two distinct pathways: either they fuse with lysosomes (24, 25) or they fuse to plasma membrane, resulting in the exocytosis of the internal vesicles into the extracellular space. Membrane proteins and lipids are selectively recruited from the limiting membrane of MVBs to inwardly budding vesicles. Incorporated membrane proteins are often destined for lysosomal degradation; for example, the epidermal growth factor receptor is rapidly endocytosed upon ligand binding, sorted into the luminal vesicles of MVBs, and ultimately targeted to lysosomes and degraded (26). It has also been shown since the early 1980s that activation of T cells with Ag-pulsed APCs or with mAbs directed against the TCR/CD3 complex induces the endocytosis and degradation of the TCR/CD3/ζ complexes, resulting in down-modulation of its surface expression (Refs. 27 and 28 and reviewed in Ref. 29). This down-regulation may contribute to several features of the T cell response. First, by reducing the number of receptors at the cell surface, down-regulation of the complexes leads to extinction of sustained signaling in T-APC conjugates and affects T cell responsiveness to further antigenic stimulation (27, 30). Second, TCR down-modulation may permit the serial engagement of many TCRs by a small number of peptide-MHC complexes (31), allowing the T cell to reach a threshold of stimulation necessary for engagement in the full program of activation. The TCR/CD3/ζ complexes endocytosed after recognition of the peptide-MHC class II complexes, like the epidermal growth factor receptors, are targeted to lysosomal compartments (32); they may thus also transit through MVBs. It is worth noting that at least part of the CD3ϵ found in the microvesicle preparations comes from the pool that has been endocytosed, because it is still bound to the anti-CD3 mAb used to activate the T cells. The chemokine receptor CXCR4, which is present on the exosomes of activated T lymphoblasts, has also been shown to be down-modulated in response to TCR activation (33); thus, during its endocytic journey it may be targeted to MVBs and partly secreted in exosomes.

The endocytic origin of the molecules we described in this study is reinforced by the presence of proteins that are not transmembrane receptors. As shown in Fig. 4*B*, the two Src-related tyrosine kinases, Fyn and Lck, were specifically enriched in the microvesicles from UCHT1-activated Jurkat cells. The adaptor protein c-Cbl, which is implicated in the regulation of T cell activation (34) and has been shown to be a novel type of E3 ubiquitin ligase (35, 36), was also present in these samples. The tyrosine kinase $p56^{lck}$ has already been detected in the endosomal fraction of activated T cells (37), and c-Cbl has been shown to remain associated with the epidermal growth factor receptor through its journey to the lysosomes and was detected in MVBs (38). The presence of these signaling proteins in the exosomes may sign their role in the biogenesis of these vesicles and/or the sorting of proteins into the endocytic pathway. On this line, it has recently been shown that ubiquitination serves as a signal for sorting proteins into the vesicles that invaginate into the MVB (39); thus, c-Cbl is perhaps the E3 ubiquitin ligase responsible for these sorting events. The fact that we found signaling proteins in the microvesicles of TCR-activated T lymphocytes may be due to the presence in these vesicles of a pool of TCR/CD3 complexes, which has been triggered and is thus still associated with the signaling machinery. Indeed, activation of T cells by the TCR has been shown to induce the formation of multifunctional complexes (40); all or part of this transduction machinery may remain associated with the TCR/CD3/ζ complex throughout the endocytic pathway. To further confirm that the TCR/CD3/ζ complexes found in the microvesicles prepared from UCHT1-activated T cells came from the pool of TCR, which has been triggered, we checked the phosphorylation status of ζ in the microvesicles. We thus immunoprecipitated ζ in the solubilized microvesicles and performed a Western blot analysis revealed with anti-phosphotyrosine mAb. As shown in Fig. 4*C*, ζ was phosphorylated on tyrosine in UCHT1-activated Jurkat cells or E^+ T cells.

Whereas production of exosomes by many cell types has been demonstrated only in vitro, their production in vivo has yet to be demonstrated. Moreover, their physiological role is still hypothetical. We can, however, speculate on potential functions of the exosomes that we described in this work.

Reticulocytes have been shown to clear transferrin receptors by exosome release (6, 8); we wondered whether exosomes may also mediate the clearance of the TCR/CD3/ζ complexes down-modulated upon TCR activation. A quantitative study of the ζ-chain present in the exosome preparations revealed that between 0.1 and 1% of the total ζ was targeted to the exosomes, whereas 50–60% of ζ disappeared after activation (data not shown), suggesting that most of the down-modulated TCR/CD3/ζ complexes are degraded and not secreted.

Besides direct cell-cell contact and the secretion of soluble proteins, exosomes may represent a new way of communication between cells. They could deliver integrated signals through different surface receptors on target cells and, if exosomes fuse with acceptor cells, they may transfer membrane and cytosolic proteins between different cells. The $CD4^+$ T cell-derived exosomes we described in this study bear the TCR/CD3 complex as well as molecules, such as CD2 and CD18, which are implicated in cell adhesion. These features may confer on them the ability to specifically target a signal to cells bearing the right MHC-peptide combination. What kind of signal may be delivered? Some authors have reported that activated human T cells release bioactive Fas ligand and APO_2 ligand in microvesicles (41); these two molecules have been implicated in apoptosis (42, 43); thus, such microvesicles may induce rapid autocrine or paracrine cell death. However, we were not able to detect Fas ligand in our exosome preparations either by Western blot analysis or by flow cytometry. This discrepancy may be due to differences in the protocol used to activate T cells.

Transfer of chemokine receptors through vesicles released by peripheral mononuclear cells has already been reported (10); the exosomes we described bear the chemokine receptor CXCR4, and they may thus transfer this molecule to cells negative for this marker. CXCR4 is a receptor predominantly expressed on naive subset of T cells (44) and is the only known receptor for stromal cell-derived factor-1 (45), a chemokine implicated in T cell migration (46). CXCR4 has also been shown to serve, together with CD4, as an accessory factor for cell entry of T cell-tropic HIV isolates (47–49). Transfer of CXCR4 through the $CD4^+$-derived exosomes may confer on negative cells the ability to migrate to tissues in response to stromal cell-derived factor-1. It may also have implication in the spreading of HIV-1 infection by increasing the target cell repertoire of HIV-1. This hypothesis remains to be demonstrated.

During the past few years, it has been shown that sorting events of proteins in MVBs are tightly regulated and result in selective recruitment of proteins to the internal vesicles (50). These proteins are either destined to be degraded in the lysosome or externalized as exosomes. Our study on protein composition of $CD4^+$ T cell-

derived exosomes may reveal some clues to the biogenesis of MVBs and to the sorting events of receptors found in these vesicles.

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