

B Lymphocytes Secrete Antigen-presenting Vesicles

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Summary

Antigen-presenting cells contain a specialized late endocytic compartment, MIIC (major histocompatibility complex [MHC] class II-enriched compartment), that harbors newly synthesized MHC class II molecules in transit to the plasma membrane. MIICs have a limiting membrane enclosing characteristic internal membrane vesicles. Both the limiting membrane and the internal vesicles contain MHC class II. In this study on B lymphoblastoid cells, we demonstrate by immunoelectron microscopy that the limiting membrane of MIICs can fuse directly with the plasma membrane, resulting in release from the cells of internal MHC class II-containing vesicles. These secreted vesicles, named exosomes, were isolated from the cell culture media by differential centrifugation followed by flotation on sucrose density gradients. The overall surface protein composition of exosomes differed significantly from that of the plasma membrane. Exosome-bound MHC class II was in a compact, peptide-bound conformation. Metabolically labeled MHC class II was released into the extracellular medium with relatively slow kinetics, $10 \pm 4\%$ in 24 h, indicating that direct fusion of MIICs with the plasma membrane is not the major pathway by which MHC class II reaches the plasma membrane. Exosomes derived from both human and murine B lymphocytes induced antigen-specific MHC class II-restricted T cell responses. These data suggest a role for exosomes in antigen presentation in vivo.

Helper T lymphocytes recognize exogenous antigens bound to MHC class II molecules, displayed at the surface of a variety of APC such as macrophages, B lymphocytes, and dendritic cells including Langerhans cells of the epidermis. MHC class II molecules are heterodimers of two transmembrane proteins, an α and β subunit with molecular masses of 33–35 and 25–30 kD, respectively. After biosynthesis, α and β subunits assemble in the endoplasmic reticulum with trimers of a nonpolymorphic type II transmembrane polypeptide, the invariant (I) chain (1, 2). After transport to the Golgi complex, most of the class II molecules are targeted to endocytic compartments (3, 4), where the I chains are dissociated from the α/β dimers, and degraded, leaving MHC class II molecules ready to bind peptides derived from endocytosed proteins (5–7). Recent evidence has shown that I chain dissociation and peptide binding are facilitated by HLA-DM molecules (8).

We have recently demonstrated that in human B cells (9) and in dendritic cells, including Langerhans cells (10–12) and macrophages (13), most of the intracellular class II molecules reside in MHC class II-enriched compartments

(MIICs).¹ MIICs have lysosomal characteristics: they contain lysosomal marker molecules (LAMP1, CD63, β -hexosaminidase, and cathepsin D), are positioned late in the endocytic pathway, and are acidic (14). A further characterization of MIICs was accomplished with their purification from murine macrophages (15), human B cells (16), murine B cells (17, 18), and melanoma cells (19). Interestingly, it has recently been shown by immunoelectron microscopy that the majority of HLA-DM molecules also resides in MIICs (20). Together, these studies indicate that MIICs are involved in antigen processing and peptide binding to class II molecules. However, functionally different subclasses of MIICs may exist (16, 18). In all APC studied so far, MIICs and related compartments contain internal membranes (9, 14, 16). These membranes probably originate from inward vesiculation of the limiting membrane of MIICs (21). Two types of MIICs could be distinguished by morphological criteria: those displaying numer-

¹Abbreviations used in this paper: BSAG, gold particles derivitized with BSA; MIIC, MHC class II-enriched compartment; TIR, transferrin receptor.

ous internal vesicles and those containing membrane sheets (10, 16, 22). Whether these two types of MIICs have differential functions in antigen processing and presentation remains to be established.

A major unresolved question in MHC class II trafficking concerns the pathway via which intracellular class II molecules are transferred to the cell surface. In macrophages, vesicles emanating from class II-enriched phagolysosomes have been suggested to provide for transport of MHC class II molecules to the plasma membrane (13). However, so far such vesicles have not been identified in other APC. MIICs in B cells and Langerhans cells are spherical structures without attached vesicles and tubules (9–12). Alternatively, MHC class II molecules may be transported to the plasma membrane by *trans*-Golgi network-derived vesicles (1, 23). So far no evidence has been presented for either of these pathways.

Using immunoelectron microscopy, biochemistry, and antigen presentation assays, we now show that in B cells MIICs are exocytic compartments. After fusion with the plasma membrane, small vesicles contained within the lumen of the MIICs are released into the extracellular milieu. The externalized vesicles, termed exosomes analogous to vesicles released by reticulocytes (24–26), exhibited abundant MHC class II molecules at their surface, and specifically presented antigenic peptides to T cells. Possible roles of exosomes *in vivo* are discussed.

Materials and Methods

Cell Lines and Antibodies. The EBV-transformed human B cell lines RN (HLA-DR15⁺) and JY (HLA-DR15⁻) were cultured in RPMI medium (Gibco Laboratories, Paisley, Scotland) supplemented with 10% FCS (Hyclone Laboratories, Inc., Logan, UT) and 100 IU penicillin. The T cell clone 2F10 recognizing the peptide 418–427 from the HSP65 antigen of *Mycobacterium leprae* in the context of HLA-DR15 was generated as described (27, 28) and cultured in IMDM (Gibco Laboratories) supplemented with 10% heat-inactivated pooled human serum. The murine B cell line TA3 (H-2^{k,d}) (29) and the ribonuclease (90–105)-I-E^f-specific T cell hybridoma WA.23 (30) were maintained in DMEM with 10% FCS. The antibodies used in this study were rabbit anti-HLA DR (3, 9), mouse monoclonal anti-DR DA6.231 (31), mouse monoclonal anti-DR Tü-36 (32); mouse monoclonal anti-HLA DR B.8.11.2, and mouse monoclonal anti-HLA-DP B7.21 (gift of Dr. A. Mulder, Department of Immunohematology and Blood Bank, University Hospital, Leiden, The Netherlands); mouse mAb directed against the lysosomal protein CD63 (33), rabbit anti-human LAMP1 (34), and rabbit anti-human transferrin receptor (TFR) (gift of Dr. Alan Schwartz; Washington University, St. Louis, MO).

Isolation and Purification of Exosomes. RN cells were washed by centrifugation and recultured in fresh medium for 2 d. Cell culture media (35 ml) containing 2–5 × 10⁷ cells were centrifuged for 10 min at 300 g to remove the cells. After a second centrifugation at 300 g, the medium was centrifuged for 10 min at 1,200 g (2×), 30 min at 10,000 g, 60 min at 70,000 g, and 60 min at 100,000 g sequentially, using a rotor (SW27; Beckman Instruments, Inc., Fullerton, CA). The pellets were solubilized in non-reducing SDS sample buffer at room temperature for analysis by

SDS-PAGE and Western blotting as described previously using ¹²⁵I-protein A (35). When indicated, aliquots of the samples were incubated at 100°C in the presence of β-ME. For electron microscopy, the 70,000-g pellet was resuspended in 100 μl of RPMI medium. For further purification of exosomes, the 70,000-g pellet was resuspended in 5 ml of 2.5 M sucrose, 20 mM Hepes/NaOH, pH 7.2. A linear sucrose gradient (2–0.25 M sucrose, 20 mM Hepes/NaOH, pH 7.2) was layered on top of the exosome suspension in a tube (SW27; Beckman Instruments, Inc.), and the sample was centrifuged at 100,000 g for 15 h. Gradient fractions (18 × 2 ml) were collected from the bottom of the tube, diluted with 3 ml PBS, and ultracentrifuged for 1 h at 200,000 g using a rotor (SW50; Beckman Instruments, Inc.). The pellets were solubilized in SDS sample buffer lacking β-ME at room temperature and analyzed by SDS-PAGE and Western blotting. Protein concentrations were determined using the BCA assay from Pierce Chemical Co. (Rockford, IL). Quantitation of MHC class II detected by Western blotting was realized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Biotinylation. 10⁸ RN cells were washed three times with PBS at 0°C by centrifugation and incubated for 30 min at 0°C in the presence of 1 mg/ml Sulfo-NHS-biotin (Pierce Chemical Co.). Nonreacted biotin was then quenched for 30 min with 15 mM NH₄Cl. The cells were washed with PBS at 0°C and lysed in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5% NP-40, 5 mM MgCl₂, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 20 μg/ml aprotinin, 0.1 mM PMSF). Nuclei were removed from the lysate by centrifugation. Exosomes secreted by 2 × 10⁸ cells were isolated by differential centrifugation followed by flotation on sucrose gradients as described above. Exosome pellets obtained from pooled sucrose gradient fractions were resuspended in PBS and biotinylated as described above for cells. Biotinylated exosomes were then pelleted through 0.3 M sucrose, 20 mM Hepes/NaOH, pH 7.2, and solubilized in lysis buffer. MHC class II was immunoprecipitated from samples containing lysed biotinylated cells and exosomes using the anti-class II mAbs DA6.231 or Tü-36. The mAbs were adsorbed from hybridoma culture supernatants using protein A-Sepharose beads (Pharmacia Biotech Inc., Piscataway, NJ). Antibody-coated beads were washed with immunomix (50 mM Tris-HCl, pH 7.5, 0.5% NP-40, 5 mM EDTA, 150 mM NaCl, 0.1 mM PMSF) and added to the lysates that had been precleared with protein A-Sepharose. After a 16-h incubation at 4°C, the beads were washed extensively with immunomix. Immunoprecipitated MHC class II and samples of the total cell and exosome lysates were eluted from the beads in SDS sample buffer at reducing conditions at 95°C and analyzed by SDS-PAGE and Western blotting. After the transfer to Immobilon-P membranes (Millipore Corp., Milford, MA), the membranes were blocked in PBS containing 0.1% gelatin, 0.05 Triton X-100, and 0.01% anti-foam (Sigma Chemical Co., St. Louis, MO). Biotinylated proteins were labeled using ¹²⁵I-streptavidin (0.1 μg/ml, 2 × 10⁶ cpm/μg) and detected using a PhosphorImager (Molecular Dynamics).

Metabolic Labeling. Cells were washed three times with MEM lacking methionine and cysteine and preincubated for 45 min at 37°C in a 5% CO₂ atmosphere. Cells were then pulsed for 45 min with 50 Mbq/ml [³⁵S]methionine (Tran-S-label; ICN, Irvine, CA) and chased for different periods of time in RPMI supplemented with 10% FCS, 1 mM methionine, and 1 mM cysteine (5 × 10⁷ cells/time point). After pulse-chase labeling, the cells were pelleted by centrifugation for 10 min at 300 g. The supernatants were collected and centrifuged for 5 min at 10,000 g followed by 30 min at 200,000 g in a rotor (model SW60; Beckman Instruments, Inc.). Cells and the 200,000-g pellets were lysed in

lysis buffer as described above. Nuclei were removed from the cell lysates by centrifugation for 5 min at 10,000 g. MHC class II was immunoprecipitated as described above. TfR was immunoprecipitated as described previously (35). Precipitated MHC class II was eluted from the beads in SDS sample buffer at nonreducing conditions at room temperature. Precipitated TfR was eluted from the beads in SDS-sample buffer at reducing conditions at 95°C. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. MHC class II was quantified using a PhosphoImager.

Immunoelectron Microscopy. The RN B cell line and the T2-DR3 cell line were pulse incubated with 5-nm gold particles derivatized with BSA (BSAG OD₅₂₀ = 5 in RPMI medium lacking serum) for 10 min at 37°C. The cells were washed extensively at 4°C with RPMI medium by centrifugation and chase incubated at 37°C for 10, 20, 50 or 80 min in the absence of BSAG. The cells were fixed in a mixture of 2% paraformaldehyde–0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and processed for ultracryomicrotomy and immunolabeling (36). To improve ultrastructure and visualization of membranes, the ultrathin cryosections were collected as described by Liou and Slot (37). For electron microscopy of the isolated exosomes, droplets of RPMI with suspended membranes from 70,000-g pellets (see above) were put on Formvar-carbon-coated electron microscopy grids, fixed as above, immunolabeled, and stained using the method described for ultrathin cryosections.

Antigen Presentation Assays. The EBV-B cell lines RN (HLA-DR 15⁺) and JY (HLA-DR 15⁻) were incubated in the presence or absence of purified HSP 65 protein from *M. leprae* (50 µg/ml) (38) for 4 h in 10 ml serum-free RPMI at 2 × 10⁶ cells/ml. Then 30 ml of RPMI supplemented with 10% FCS was added, and the cells were incubated for 20 h at 37°C. The cells were then washed to remove free antigen and incubated further for 24 h in RPMI/10% FCS medium at 37°C. Exosomes were prepared by differential centrifugation as described above. The efficiency of HSP 65 antigen presentation was measured by culturing 10,000 cells of the T cell clone 2F10 with irradiated (6,000 rad) EBV cells. As indicated in the legend to Fig. 6, increasing numbers of cells or dilutions of exosome preparations were used. The highest concentration of exosomes corresponded to material that was secreted by 1.6 × 10⁶ cells in 24 h. B cells or exosomes resuspended in 100 µl IMDM/10% pooled human serum were added to the T cell clone (50 µl IMDM/10% pooled human serum per well) in 96-well flat-bottom microtiter plates (Costar Corp., Badhoevedorp, The Netherlands) for 4 d at 37°C, 5% CO₂ in humidified air. Antibodies to HLA class II molecules were added at 10 µl/well in a final concentration of 10 µg/ml. When indicated, 5 µg/ml of HLA-DR15-restricted epitope of HSP65 (peptide 418–427) was added to the exosomes. 16 h before termination, 0.5 µCi of [³H]thymidine was added to the wells. The cells were then harvested on glass fiber filters using an automatic cell harvester, and the [³H]thymidine incorporation into cell DNA was determined by liquid scintillation counting. The results are expressed as the mean of triplicate measurements. Exosomes were prepared similarly from TA3 cells and incubated together with ribonuclease (90–105) peptide and WA.23 T hybridoma cells. After 24 h, supernatants were harvested, and the level of IL-2 secretion by WA.23 cells was determined by CTL line bioassay (39).

Results

MIICs Can Fuse with the Plasma Membrane. MIICs containing abundant MHC class II molecules are a general fea-

ture of human and murine B lymphoblastoid cell lines (3, 9, 16, 22). In this study we analyzed immunogold-labeled ultrathin cryosections of the human B cell line RN by immunoelectron microscopy and found typical MIICs with internal membranes abundantly labeled for MHC class II (Fig. 1). Both multivesicular and multilaminar MIICs were present.

The RN cells displayed many exocytic profiles suggesting fusion of the limiting membrane of MIICs with the plasma membrane. These profiles contained vesicles reminiscent of those present in the multivesicular MIICs (Fig. 2). Membrane sheets were not found in the fusion profiles, indicating that predominantly multivesicular MIICs fused with the plasma membrane. We named the externalized MIIC-derived vesicles exosomes analogous to vesicles that are released by reticulocytes as a consequence of fusion of multivesicular bodies with the plasma membrane (25, 26). Both exosomes and the limiting membranes of the exocytic profiles were immunolabeled for MHC class II (Fig. 2) and for the lysosomal membrane proteins LAMP1 (Fig. 2 B) and CD63 (not shown).

To further demonstrate that the contents of MIICs can be exocytosed, we tested whether previously endocytosed BSAG particles were reexternalized and could be found in exocytic profiles. RN cells were allowed to internalize BSAG particles for 10 min at 37°C, washed extensively at 4°C to remove extracellular BSAG, and chased in the absence of BSAG for varying periods of time at 37°C. BSAG was detected in MIICs after 20 min of chase (Fig. 1), consistent with previous observations on dendritic cells (10–12), the JY B cell line (9), and other B cells (16, 22). MIICs containing internal vesicles (early MIICs) were reached by the BSAG before those accommodating membrane sheets (late MIICs) (Fig. 1). BSAG began to appear in exocytic profiles after 20 min of chase (Fig. 2 A) and was abundant after a 50-min chase (Fig. 2 B). The observation that BSAG was not associated with the plasma membrane after 10 min of chase indicated that BSAG recovered in exocytic profiles after prolonged chase times indeed resulted from reexternalization.

Isolation of Exosomes. Exosomes were isolated from the culture media of RN cells by differential centrifugation (25). Pelleted membranes were analyzed by Western blotting. The majority of MHC class II-containing membranes sedimented at 70,000 g (Fig. 3 A, lane 6). To test whether secreted MHC class II molecules were membrane bound, membranes from the 70,000-g pellets were floated into linear sucrose gradients. Western blot analysis of nonboiled and nonreduced gradient fractions showed that compact, peptide-bound MHC class II molecules floated to an equilibrium density of 1.13 g/ml, confirming their association with membrane vesicles (Fig. 3 B). Next, membranes from the 70,000-g pellet were analyzed morphologically. As can be seen in Fig. 3 C, the 70,000-g pellet was composed of a homogeneous population of vesicles of 60–80 nm that labeled for MHC class II. The vesicles were morphologically similar to those found in sections of MIICs (Fig. 1) and exocytic profiles (Fig. 2). We conclude that the secreted

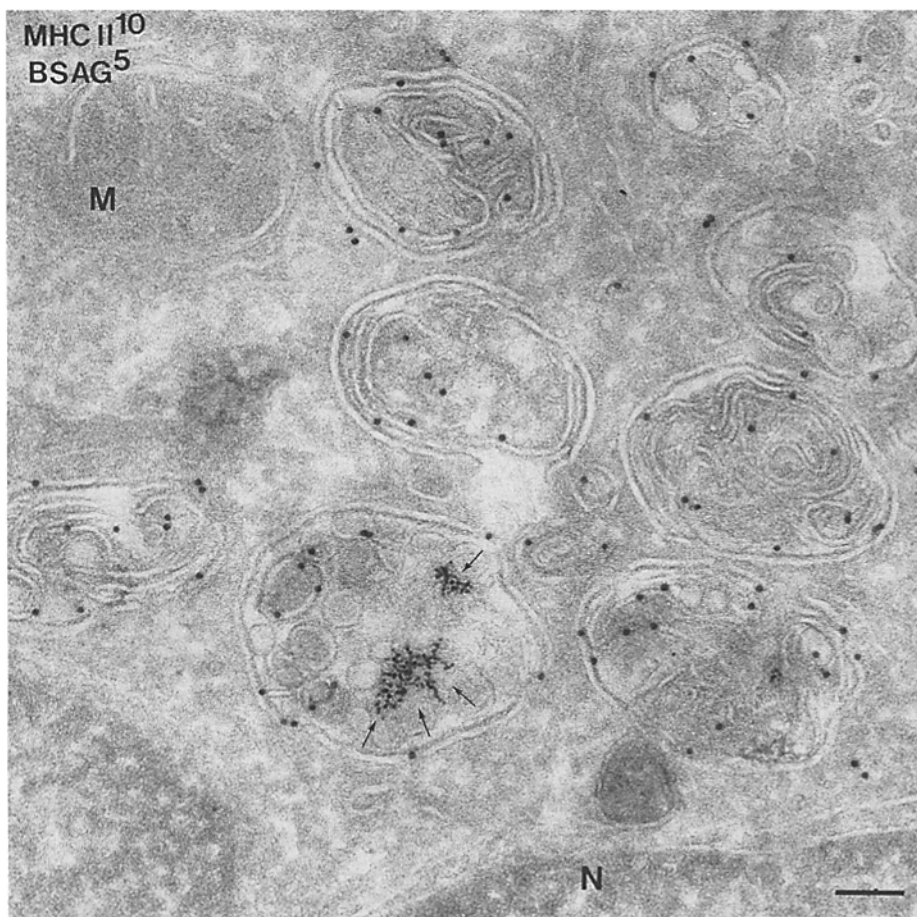


Figure 1. RN B cells contain typical MIICs. Ultrathin cryosection of an RN cell immunolabeled for MHC class II with 10-nm gold particles as indicated in the figure. Cells had internalized 5 nm BSAG (BSAG⁵) for 10 min and were chased in the absence of BSAG for 20 min before fixation. MHC class II molecules are localized in multivesicular and multilaminar MIICs. Two types of MIICs containing either vesicles or membrane sheets are shown. Note that after 30 min of internalization, BSAG is present only in multivesicular MIICs (arrows). N, nucleus; M, mitochondrion. Bar, 0.1 μ m.

MHC class II molecules were complexed with peptides and associated with membrane vesicles.

Exosome Membranes Are Distinct from the Plasma Membrane.

To investigate the possibility that the MHC class II-containing vesicles that sedimented at 70,000 g represent shed plasma membrane fragments, the presence of the TfR was monitored by Western blotting. TfRs are absent from MIICs (9) and from class II-enriched cell fractions in B cells (16). In most cell types, including B cells, TfRs are predominantly present at the plasma membrane and in early endosomes. TfRs have been localized in the limiting membrane of multivesicular bodies in most cell types, but not in their internal vesicles (40). Fig. 4 A shows that TfRs were only detectable in the cell lysate (lane 3) but not in the 70,000-g pellet (lane 4). The significant enrichment of MHC class II over TfR in the 70,000-g pellet compared with the cell lysate (compare lanes 1 and 2 with lanes 3 and 4, respectively) suggests that little if any shed plasma membrane contaminated the exosome preparation. To further analyze differences in protein composition of the plasma membrane and exosomes, plasma membrane proteins and exosomal membrane proteins were biotinylated. Probing Western blots with ¹²⁵I-streptavidin revealed at least four proteins that are highly enriched in plasma membranes and four proteins that are highly enriched in exosomes (Fig. 4 B). MHC class II was identified among the enriched bio-

tinylated proteins in plasma membrane (lanes 3–5) and in exosomes (lanes 6–8) by immunoprecipitation. The differential membrane protein composition of the plasma membrane and exosomes again strongly indicates that exosomes did not derive from shed plasma membrane.

Release of Newly Synthesized Class II Molecules. To determine the kinetics and the extent to which newly synthesized MHC class II molecules are released into the medium, RN cells were metabolically pulse labeled with [³⁵S]methionine for 45 min and chased for different periods of time as indicated in Fig. 5. Cells and exosomes were collected by centrifugation, lysed, and MHC class II was immunoprecipitated. After pulse labeling, the majority of ³⁵S-MHC class II in the cells was immunoprecipitated as SDS-unstable α/β -I chain complexes (Fig. 5 A). These complexes were converted to SDS-stable, α/β -peptide complexes \sim 3 h after synthesis (not shown), consistent with the kinetics reported for other human B cell lines (3, 41). After 6 h of chase, ³⁵S-MHC class II appeared in the compact configuration. Only very little ³⁵S-MHC class II was detected in the exosome fraction at this time. Increasing amounts of ³⁵S-compact MHC class II were recovered from the exosomes after 12 and 24 h of chase. After 24 h of chase, $10 \pm 4\%$ ($n = 5$ from three independent experiments) of the total newly synthesized MHC class II was recovered from exosomes. As a control, newly synthesized ³⁵S-TfR was immunoprecipitated and

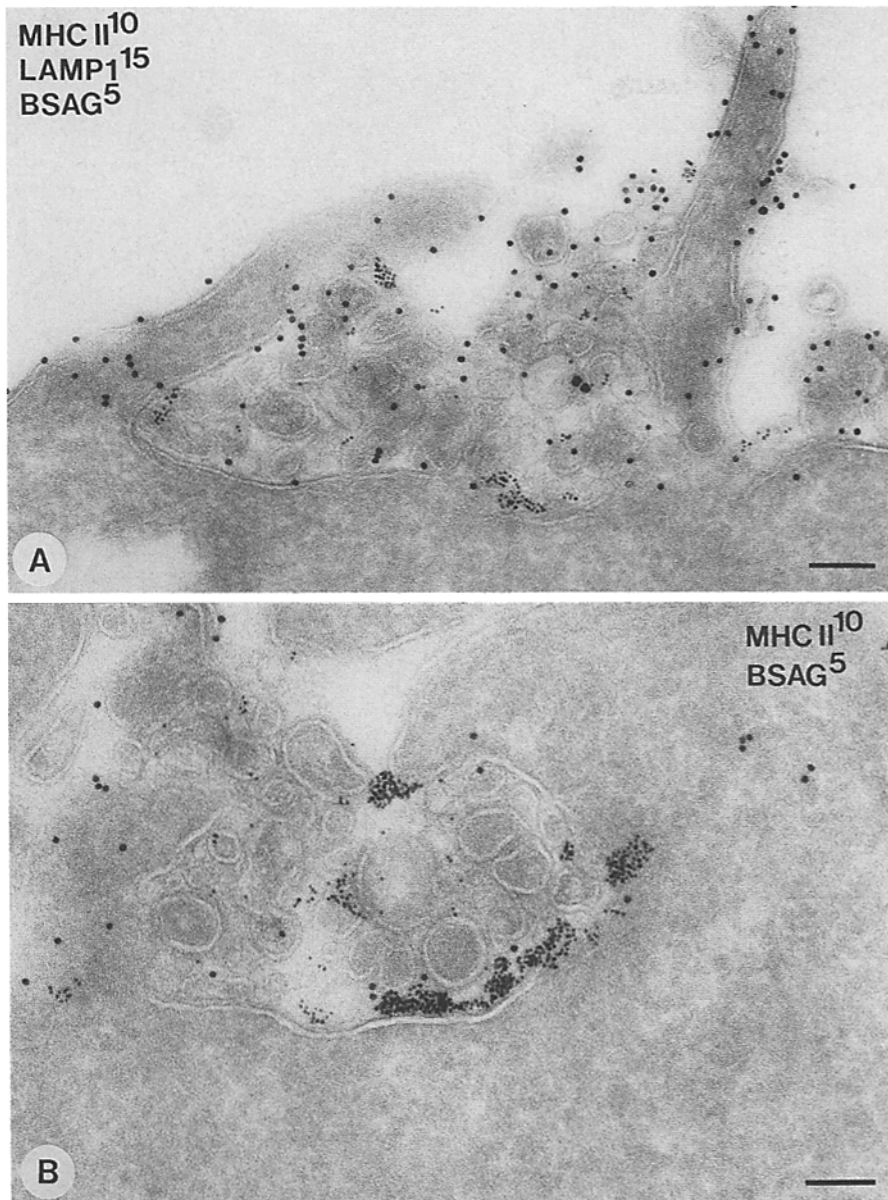


Figure 2. MIICs can fuse with the plasma membrane. RN B cells were allowed to internalize 5 nm BSAG particles for 10 min and chased for 20 min (A) or 50 min (B) in the absence of BSAG. (A) Ultrathin cryosections immunogold labeled for MHC class II and LAMP1 as indicated in the figure. The fusion profile is defined by the presence of externalized BSAG that had previously been endocytosed. In addition to BSAG, the exocytic profile contains MHC class II-labeled vesicles (exosomes). (B) Exocytic profile containing BSAG and exosomes labeled for MHC class II. Bars, 0.1 μ m.

analyzed by SDS-PAGE (Fig. 5 B). After pulse labeling, the precursor form of the T β R was detected. Complex glycosylated 35 S-T β R, migrating at a slightly lower mobility, was detected after chasing the cells. In contrast to 35 S-MHC class II, no 35 S-T β R was found in the exosome fraction, even after 24 h of chase, again illustrating the selective incorporation of MHC class II in exosomes.

Exosomes Can Stimulate T Cells. The recovery of SDS-stable MHC class II molecules from exosomes suggested their association with peptides (41, 42). Thus exosomes may be able to present antigens to T cells. We tested this possibility using antigen presentation assays. HLA-DR15-positive RN cells and exosomes isolated from culture media of RN cells were allowed to bind peptide 418-427 from the model antigen HSP 65 of *M. leprae*. The cells and exosome preparations were then added to the T cell clone

2F10, which recognizes this peptide in the context of HLA-DR15 (28, 29). In a parallel experiment, RN cells were allowed to endocytose the intact HSP 65 protein continuously for 24 h, washed, and incubated in the absence of antigen for another 24 h. Cells and exosomes isolated from the chase medium were then used to stimulate 2F10 cells as above. Both cells and exosomes incubated with antigenic peptide (Fig. 6, A and C) and cells and exosomes derived from media of cells that were preincubated with antigen (Fig. 6, B and D) were able to induce specific T cell responses. As a control, exosomes were prepared from culture media of an equivalent number of DR15-negative JY cells that had been incubated either in the presence or absence of the antigen. Although JY cells secreted an equivalent amount of exosomes, these were ineffective in stimulating 2F10 T cell proliferation (not shown). For exosomes derived from

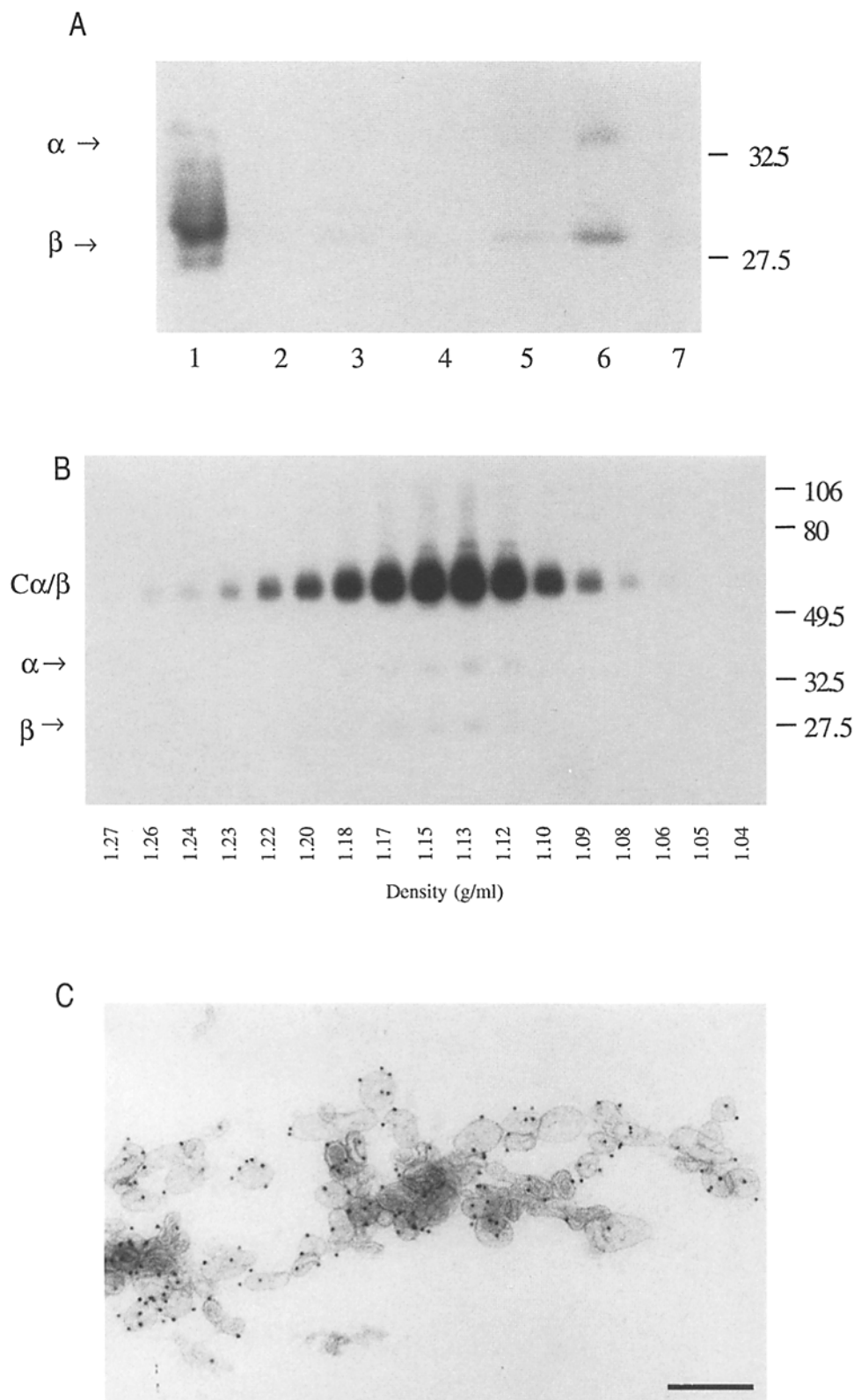
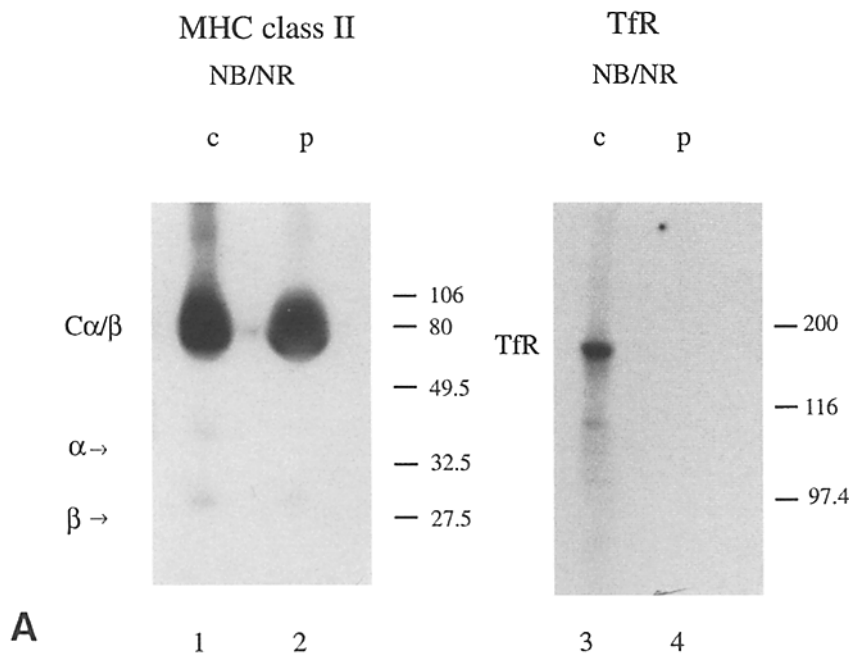


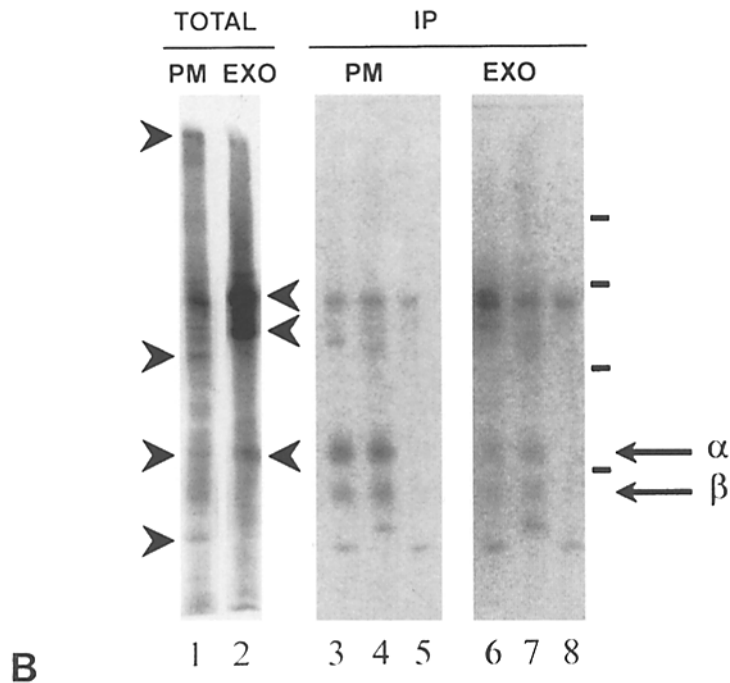
Figure 3. Analysis of exosomes isolated from RN cell culture media. (A) Membranes obtained by differential centrifugation of cell culture media were analyzed for the presence of MHC class II by Western blotting. Cells were pelleted in two sequential centrifugation steps at 300 g (lane 1, first run; lane 2, second run); lane 1 contains material from 0.6×10^6 cells. Membranes in the culture medium from $20\text{--}50 \times 10^6$ cells were pelleted by sequential centrifugation steps: twice at 1,200 g (lanes 3 and 4) and once at 10,000 g (lane 5), 70,000 g (lane 6), and 100,000 g (lane 7). The pellets were solubilized at 100°C under reducing conditions and analyzed by SDS-PAGE. Per-lane samples equivalent to 10^6 cells were loaded. Class II α and β chains were recovered mainly from the cells (lane 1) and from the 70,000-g pellet (lane 6). (B) Membranes pelleted from culture media at 70,000 g were fractionated by floatation on sucrose gradients. MHC class II from gradient fractions was solubilized at room temperature at nonreducing conditions and detected by Western blotting. Class II molecules were recovered in fractions 5–12 corresponding to densities of 1.22–1.08 g/ml. The majority of MHC class II was in the SDS-stable compact form with a molecular mass of $\sim 56\text{--}60$ kD ($C\alpha/\beta$). (C) Whole-mount electron microscopy of the 70,000-g pellet immunogold labeled for MHC class II. The pellet is composed of 60–80-nm vesicles showing abundant MHC class II labeling at their surface. Bar, 0.2 μm .

RN cells that had been preincubated with antigen, a half-maximal T cell response was obtained with an amount of exosomes secreted by 3×10^5 cells in 24 h (Fig. 6 D). For RN cells that had been preincubated with antigen, 2×10^4

cells were required to achieve the half-maximal T cell response (Fig. 6 B). To test whether these responses were associated with MHC class II, exosomes to which peptide had been added were incubated in the presence of mAbs



A



B

Figure 4. Differential protein composition of exosomes and plasma membranes. (A) Exosomes contain compact MHC class II but lack TfR. Samples corresponding to 3×10^6 cells were applied in lanes 1 and 3. Samples corresponding to exosome preparations from the media of 15×10^6 cells were applied in lanes 2 and 4. The left panel shows Western blot analysis of MHC class II from cells (lane 1) and 70,000-g pellets (lane 2). The samples were incubated at 20°C at non-reducing conditions (NB/NR) before SDS-PAGE. Compact α/β dimers (C α/β) are detected in both the cell samples (lane 1) and the 70,000-g pellet (lane 2). The right panel shows a Western blot for TfR from cells (lane 3) and the 70,000-g pellet (lane 4). A major band with a molecular mass of 180 kD (TfR dimer) is present in the cell lysate but is absent in the 70,000-g pellet. (B) Biotinylated proteins on the plasma membrane and on exosomes. Cells (lanes 1 and 3–5) and exosomes (lanes 2 and 6–8) were biotinylated and lysed. Biotinylated proteins from either total lysates (lanes 1 and 2) or immunoprecipitates from the lysates (lanes 3–8) were assayed by Western blotting using ^{125}I -streptavidin. Clear examples of biotinylated proteins selectively enriched in either the plasma membrane (lane 1, derived from 3×10^5 cells) or exosomes (lane 2, derived from the medium of 3×10^7 cells) are indicated by arrowheads. MHC class II complexes were immunoprecipitated from cell lysates (lanes 3–5, 3×10^6 cells) and lysed exosomes (from the medium of 3×10^7 cells) using either Tü36 (lanes 3 and 6) or DA6.231 (lanes 4 and 7). In the control lanes (5 and 8), an irrelevant mAb was used. The precipitated samples were eluted at 95°C, resulting in the dissociation of the α and β chains (arrows). Biotinylated proteins were detected on Western blots using ^{125}I -streptavidin. For the detection of total biotinylated proteins (lanes 1 and 2), the setting of the PhosphorImager was six times less sensitive compared with the setting for the immunoprecipitates. Molecular mass markers (106, 80, 49.5, and 32.5 kD) are indicated at the right.

directed against HLA-DR (B.8.11.2) or HLA-DP (B7.21) and then added to the T cells. Anti-HLA-DR blocked T cell proliferation completely, whereas anti-HLA-DP was ineffective (Fig. 6, triangles and circles, respectively). Thus, stimulation of T cells by exosomes was DR restricted. We also tested whether exosomes derived from a murine B cell

line were able to generate a T cell response. Exosomes were isolated from the culture medium of the murine B cell line (I-E⁺) TA3 and incubated in the presence or absence of a RNase-derived peptide (aa 90-105). Exosomes and cells were used to stimulate WA.23 hybridoma cells that specifically recognize 90-105 peptide-IE^k complexes. Fig. 7 shows that

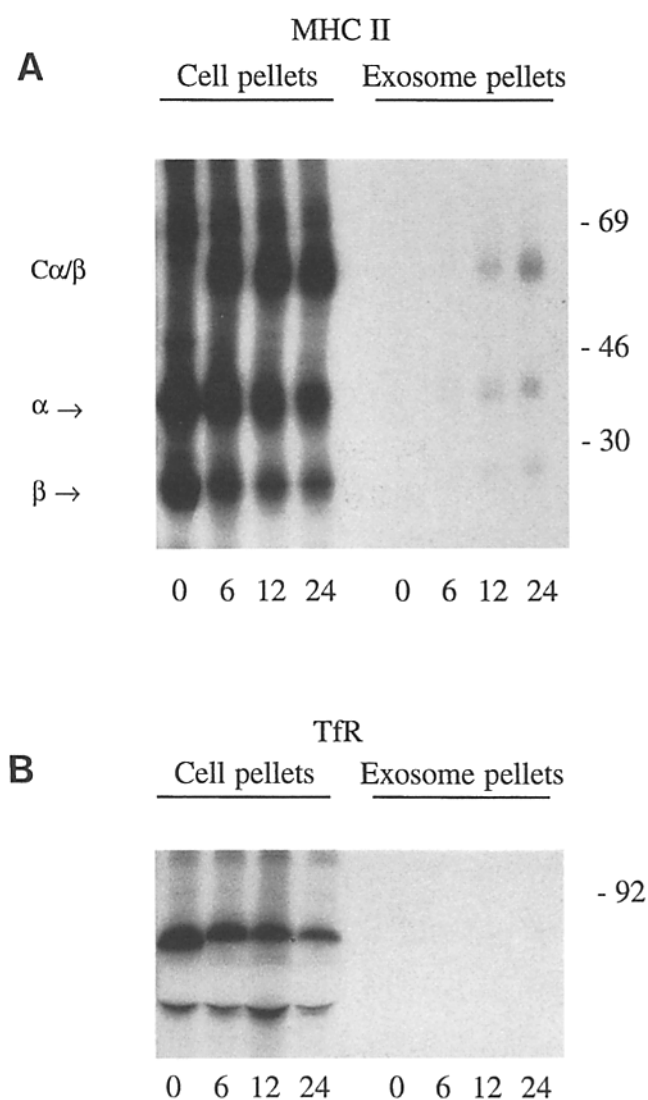


Figure 5. Release of newly synthesized class II molecules. RN cells were pulse labeled with [³⁵S]methionine for 45 min (lane 0) followed by a chase in the absence of label for 6, 12, or 24 h. (A) MHC class II molecules were immunoprecipitated from lysates of the cells and pelleted exosomes, dissociated from the beads at nonreducing conditions at room temperature, and analyzed by SDS-PAGE and fluorography. MHC class II immunoprecipitated as SDS-unstable complexes of α/β I chain. SDS-stable α/β dimers were recovered from the cells but not from the medium after 6 h of chase. In the medium, SDS-stable α/β dimers are detected after 12 h of chase, and this signal is considerably increased at 24 h. (B) The TfR was immunoprecipitated from lysates of the cells and pelleted exosomes. No TfR was detected in the exosomal fraction after 24 h of chase.

exosomes released by 2×10^7 TA3 cells over 24 h exposed to 10 μM RNase (90–105) peptide were capable of stimulating IL-2 secretion by WA.23 cells. Control culture medium and the supernatant obtained after ultracentrifugation of exosomes did not stimulate IL-2 secretion. Lower peptide concentrations (0.1–1 μM) produced stimulation of WA.23 cells when presented by intact TA3 cells but not when presented by exosomes (data not shown). In conclusion, our observations show that these human and murine

B cell lines secrete exosomes able to specifically stimulate T cells.

Discussion

In this study we show that, in B cells, the limiting membrane of MIICs can fuse with the plasma membrane in an exocytic fashion, resulting in the release of small membrane vesicles (exosomes) into the culture media. Exosome release as a consequence of fusion of multivesicular bodies with the plasma membrane has been documented for reticulocytes (25, 26). Although our observations suggest that this phenomenon is common to several B cell lines, it has not been described previously. Biochemical data of more than a decade ago on so-called shedding of class II molecules by murine B cell lines, however, may be related to the observations we describe here. These studies document the release of intact MHC class II molecules (43) recovered in 100,000-g fractions from B cell culture media (44). The MHC class II molecules were part of supramolecular particles containing membrane lipids (45). The release was interpreted as shedding of plasma membrane. Our present observations throw new light on these data and suggest that the released MHC class II-containing particles in fact were exosomes. Other early studies demonstrated the presence of class II molecules associated with membrane lipids in serum (46), which suggests the existence of exosomes *in vivo*. However, a possible relationship between exosomes and circulating forms of MHC class II needs further analysis.

Our conclusion that MIICs in B cells are exocytic compartments is based on the following observations: (a) The plasma membrane showed indentations reminiscent of fused MIICs that contained vesicles similar in size and morphology to those present in intracellular MIICs (Fig. 2). (b) The vesicles present in MIICs and at the cell surface expressed the same markers, MHC class II (Fig. 2), LAMP1 (Fig. 2 B), and CD63 (not shown). LAMP1 and CD63 were not detected at other regions of the plasma membrane. (c) Internalized tracer particles were reexternalized and appeared in the MIIC fusion profiles (Fig. 2). (d) Membrane vesicles similar in morphology to exosomes could be isolated from the culture media by differential centrifugation and flotation on sucrose gradients (Fig. 3 C). (e) Purified exosomes did not contain TfRs, a marker for the plasma membrane and endosomes (Figs. 4 A and 5 B). Furthermore, the total surface protein composition of exosomes differed from that of the plasma membrane as revealed by surface biotinylation (Fig. 4 B). Together, these observations strongly argue against the idea that MHC class II-containing membranes in the media resulted from plasma membrane shedding. The release of MHC class II molecules from the cells via exosomes is significant. When the cells were metabolically labeled with [³⁵S]methionine, within 24 h $10 \pm 4\%$ of the newly synthesized MHC class II was found in exosomes recovered from the media.

Cell fractionation studies from several laboratories have indicated that antigen processing and peptide loading of MHC class II molecules probably occurs in MIICs or re-

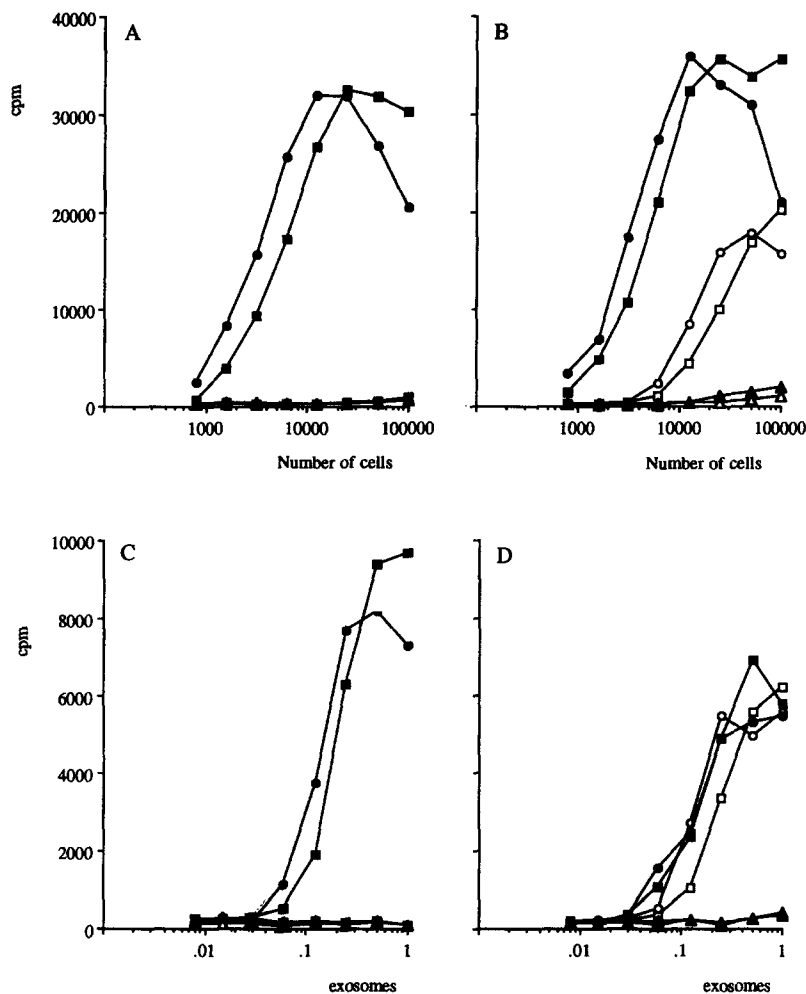


Figure 6. Presentation of HSP 65 antigen by HLA-DR15⁺ RN B cells and exosomes to the CD4⁺ T cell clone 2F10. Proliferative responses to naive cells (A), to cells preincubated with antigen (B), to exosomes derived from naive cells (C), and to exosomes derived from cells preincubated with antigen (D). The solid symbols show proliferation measurements after addition of HSP 65-derived peptide (418-427); the open symbols show where peptide was not added. HLA class II restriction was determined by adding 10 $\mu\text{g}/\text{ml}$ anti-DR antibody (triangles), anti-DP (circles), or no antibody (squares). The exosomes at the highest concentration were derived from media of 1.6×10^6 cells. All assays were performed in triplicate, and results are expressed in cpm [³H]thymidine incorporated into T cells. The SEM for triplicate cpm measurements was <10%. Results shown form a representative example of experiments performed in duplicate.

lated endocytic compartments (15–19). The pathway(s) via which intracellular MHC class II molecules are transported from the MIICs to the cell surface are presently unknown. Since the limiting membrane of MIICs contains MHC class II molecules and is incorporated in the plasma membrane during exocytic fusion, this pathway may contribute to MHC class II transport to the plasma membrane. However fusion of MIICs with the plasma membrane is not likely to be the only, or even the major, pathway of MHC class II delivery to the cell surface. First, our metabolic labeling data show that the release of newly synthesized MHC class II into the medium (Fig. 5 A) occurred with kinetics much slower than transport of newly synthesized MHC class II molecules from MIIC to the plasma membrane (3, 41). A possible caveat of this interpretation is that sticking of released exosomes to the plasma membrane may slow down their recovery from the medium. Second, we found that predominantly multivesicular MIICs fused with the plasma membrane, whereas electron microscopic and cell fractionation data (16) suggest that significant amounts of MHC class II are transported to other types of MIICs. We have identified two types of MIICs: multivesicular ones that received endocy-

tosed tracer first (early MIICs) and multilaminar MIICs positioned later in the endocytic pathway (late MIICs). The exocytic profiles, and the 70,000-g pellets obtained from the culture media, contained vesicles (exosomes) rather than membrane sheets, suggesting that secretion is mainly restricted to the multivesicular MIICs. The internal MIIC vesicles are formed by inward budding of the limiting membrane of MIICs (see reference 21, Figs. 16 and 17) similar to those described for multivesicular bodies in other cell types (47). Therefore the exosomes were expected to expose the luminal domain of MHC class II molecules at their surface. This orientation was confirmed by immunogold labeling on cryosections of exosomes and by immunogold labeling of isolated intact exosomes.

In multivesicular endosomes, which share many features with MIICs, internal vesicles contain receptors destined for degradation, whereas recycling receptors are sorted to the limiting membrane (40, 48). MIICs in APC may represent a similar type of sorting device to spatially segregate MHC class II molecules to the internal vesicles, in order to discharge them from the cells through exocytosis. Thus, fusion of MIICs with the plasma membrane may allow cells

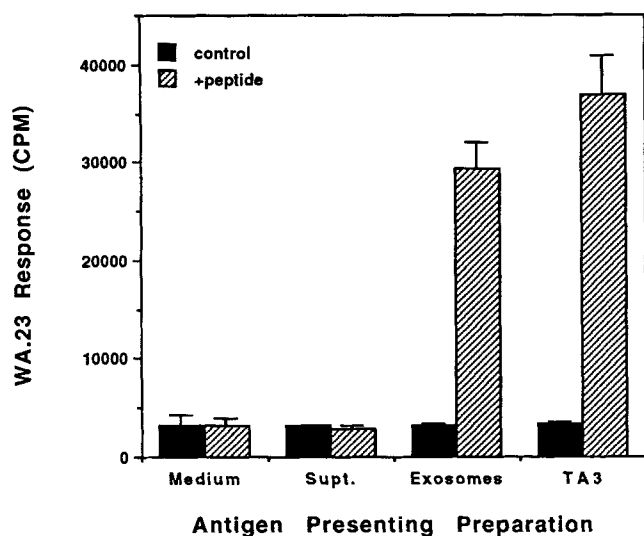


Figure 7. Peptide presentation by the exosomes isolated from the culture medium of the murine B cell line TA3. WA.23 T hybridoma cells (10^5 cells/well) were incubated with $10 \mu\text{M}$ ribonuclease (90-105) peptide in the presence of intact TA3 cells (10^5 cells/well), exosomes (70,000-g pellet), supernatant from the 70,000-g spin, or normal medium. The wells with exosomes contained material isolated from medium containing 2×10^7 cells. T cell response was determined by IL-2 secretion, measured using a CTLL cell proliferation and [^3H]methyl thymidine incorporation bioassay. Lower concentrations of peptide ($0.1\text{--}1 \mu\text{M}$) produced stimulation with TA3 cells but not with exosomes (data not shown).

to discard MHC molecules via exosomes. However, we now document the presence of presentable peptide-MHC class II complexes at the surface of exosomes. Peptide generated and bound to MHC class II intracellularly, as well as those bound to MHC class II on exosomes *in vitro*, produced a strong, peptide-specific and MHC class II-restricted stimulation of T cells. Exosomes therefore fulfill the re-

quirements to induce T cell responses, including the expression of ubiquitous accessory molecules (49). Preliminary observations have indeed indicated that several of these molecules (B7, ICAM, LFA-3) are present in exosome preparations (our unpublished data). Our study does not provide conclusive information about the efficiency of antigen presentation by exosomes. From the data presented in Fig. 6, it can be estimated that exosomes are $\sim 10\text{--}20$ times less efficient in antigen presentation than cells. However, it should be considered that in antigen presentation assays, contact between B and T cells may be optimized selectively because of sedimentation of cells but not of exosomes.

Secretion of exosomes by B lymphocytes is reminiscent of that of the internal vesicles in cytolytic granules of CTL (50, 51). Both MIICs and cytolytic granules are lysosome-like compartments. The internal vesicles of cytolytic granules are exocytosed by the CTL upon CTL-target cell interaction, and presumably mediate the killing of target cells (50). Whether B cell exosomes also have an extracellular physiological role *in vivo* remains to be established. It has been suggested that follicular dendritic cells are able to pick up MHC class II molecules released by surrounding B cells by an unknown mechanism (52). The possibility that exosomes provide for transfer units of MHC class II-peptide complexes between different cells of the immune system is worth studying. Preliminary observations on physiological APC like dendritic cells, monocytes, and macrophages, which all contain multivesicular MIICs, have indicated the presence of exosomes at their surface. It can be speculated that *in vivo*, in the circulation, exosomes may function as transport vehicles for MHC class II-peptide complexes responsible for maintenance of long-term T cell memory or T cell tolerance. Further investigations are required to explore the usefulness of exosomes, in particular as biological vehicles, in immunotherapy.

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