Mast Cell-Dependent B and T Lymphocyte Activation Is Mediated by the Secretion of Immunologically Active Exosomes

Dimitris Skokos, Sophie Le Panse, Irène Villa, Jean-Claude Roussel, Roger Peronet, Bernard David, Abdelkader Namane and Salaheddine Mécheri

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Mast cells are ubiquitously distributed among tissues including bone marrow and lymphoid tissue. These cells are known to play a central role in allergic responses due to the synthesis of proinflammatory mediators. The discovery that mast cells can produce a large panel of cytokines (1) suggested that they could influence T and B cell activation and development. We and others have demonstrated that, in addition to their role in inflammatory reactions, mast cells can contribute to the regulation of specific immune responses (2). It has been shown that mast cells from different species were able to present immunogenic peptides and superantigens to specific T cell clones and T cell hybridomas (3–5). Ag-presenting species were able to present immunogenic peptides and superantigens to specific T cell clones and T cell hybridomas (3–5). Ag presentation by bone-marrow-derived mast cells (BMMC) is strictly controlled by cytokines; IL-4 and GM-CSF were potent inducers of the Ag-presenting capacity, whereas IFN-γ completely abrogated this function. With respect to interactions between mast cells and B cells, human mast and basophilic cell lines as well as purified human lung mast cells and blood basophils can provide the cell contact signals to purified B cells to produce IgE (6). This B cell-mast cell interaction occurred in the absence of T cells and the human cell lines HMC-1 (mast) and KU812 (basophilic), used as B cell counterparts, both have been shown to express the ligand for CD40, which is known in conjunction with IL-4 to be responsible for IgE production.

In a previous study, we provided evidence indicating that mast cells have a direct effect on B cell stimulation through a T cell-independent mechanism (7). Mast cell-dependent B cell activation resulted, within 48 h of incubation, in blast formation, proliferation, and IgM production. More recently, we have shown that T cells that cannot be directly activated by mast cells get stimulated when B cells were present in the coculture. This Ag-independent stimulation that occurs when mast cells are cocultured with spleen cells resulted in large clusters of T cell blasts as well as IL-2, IL-12, and IFN-γ production. This mast cell-mediated heterotypic aggregation as well as cytokine production was completely inhibited by anti-LFA-1 and anti-ICAM-1 mAb (8). Other investigators have shown that activated mast cells were able to form heterotypic aggregates with activated but not with resting T lymphocytes. This coculture, which resulted in histamine release, was adhesion-dependent because the addition of anti-LFA-1 and anti-ICAM-1 inhibited the adhesion-induced mast cell degranulation (9). Based on our previous demonstration that mast cell supernatant could replace the cell-to-cell contacts to generate B and T lymphocyte activation, we sought in the present report to characterize this mast cell mediator. Combining biochemistry and immunoelectron microscopy methods, we found that the mast cell-derived B and T lymphocyte stimulatory activity was constitutively secreted and consisted of membrane vesicles termed exosomes, originally stored in the mast cell cytoplasmic granules. In addition to MHC class II molecules, several proteins including adhesion and costimulatory molecules were found to be associated with exosomes. A characteristic feature of mast cell-derived exosomes is their potential to induce in vitro and in vivo lymphocyte activation with IL-2 and IFN-γ production, and no detectable IL-4.

These results reveal a previously unrecognized mechanism by which mast cells express their inflammatory and immunoregulatory functions. Mast cells may, in addition to cell-to-cell contacts

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Dimitris Skokos,* Sophie Le Panse,† Irène Villa,* Jean-Claude Rousselle,‡ Roger Peronet,* Bernard David,* Abdelkader Namané,§ and Salaheddine Mécheri²

Mitogenic activity of bone marrow-derived mouse mast cells and mast cell lines P815 and MC/9 on B and T lymphocytes is present in their culture supernatants. To identify this activity, mast cells were incubated in serum-free medium and the supernatant was subjected to differential centrifugation, which resulted in two fractions, the hypodense and dense fraction (pellet). When analyzed for their mitogenic activity on spleen cells, all activity was found to be associated with the dense fraction. Electron microscopy studies revealed the presence in this fraction of small vesicles called exosomes with a heterogeneous size from 60 to 100 nm of diameter. When cocultured with spleen cells, purified exosomes induced blast formation, proliferation, as well as IL-2 and IFN-γ production, but no detectable IL-4. Similar data were obtained by injecting exosomes into naive mice. In contrast to mast cell lines, a pretreatment with IL-4 is required for bone marrow-derived mast cells to secrete active exosomes. Structurally, exosomes were found to harbor immunologically relevant molecules such as MHC class II, CD86, LFA-1, and ICAM-1. These findings indicate that mast cells can represent a critical component of the immunoregulatory network through secreted exosomes that display mitogenic activity on B and T lymphocytes both in vitro and in vivo. The Journal of Immunology, 2001, 166: 868–876.
and cytokine release, use a third intercellular communication vector that consists of secreted exosomes.

Materials and Methods

**Mice**

DBA/2 (6–8 wk old) were purchased from Janvier (Laval, France). STAT6-knockout (KO) and p47phox-KO mice were kindly provided by J. Ihle (St Jude Children’s Hospital, Memphis, TN) and S. Holland (National Institutes of Health, Bethesda, MD), respectively.

**Reagents and Abs**

BSA was purchased from Sigma (St. Louis, MO). Mouse recombinant IL-3, IL-4, IFN-γ and GM-CSF were purchased from Immugenex (Los Angeles, CA). Unlabeled anti-IFN-γ (clone AN18) and biotinylated anti-IFN-γ (clone R46A2) were purchased from Pharmingen (San Francisco, CA). Rat anti-mouse CD86 (clone 1G10) and anti-CD86 (clone 2D10) mAbs were obtained from Dr. Gordon Powers (Hoffmann-La Roche, Nutley, NJ). Rat anti-mouse LFA-1 and anti-ICAM-1 mAbs were kindly provided by Dr. Genevieve Milon (Institut Pasteur, Paris, France). Mouse anti-mouse I-A<sup>BL</sup> mAb was prepared from clone 25-9-17S (American Type Culture Collection, Manassas, VA). Rabbit anti-mouse CD40 and CD40 ligand (CD40L) were kindly provided by Dr. Elaine K. Thomas (Immunex, Seattle, WA). Rabbit anti-mouse CD40 and CD40 ligand (CD40L) were kindly provided by Dr. Elaine K. Thomas (Immunex, Seattle, WA). Rabbit anti-mouse CD40 and CD40 ligand (CD40L) were kindly provided by Dr. Elaine K. Thomas (Immunex, Seattle, WA). Rabbit anti-mouse CD40 and CD40 ligand (CD40L) were kindly provided by Dr. Elaine K. Thomas (Immunex, Seattle, WA).

**Culture**

P815 is a mastocytoma cell line from DBA/2 mice. The IL-3-dependent mast cell line MC/9, derived from the A/J × C57Bl/6 F<sub>1</sub> mouse, was kindly provided by Dr. T. Hara (DNAX, Palo Alto, CA).

**Preparation of BMMCs**

BMMCs from DBA/2, STAT6-KO, and p47phox-KO mice were prepared as described by Rapin (10) and modified by us. After 3 wk of culture using RPMI 1640 supplemented with 10% FCS (American Type Culture Collection) and in the presence of 3 U/ml of rIL-3 (Diaclone, Besançon, France), the cells were harvested after 21 days of culture and consisted of 98% pure mast cells as assessed by toluidine blue staining. Consistent with our previous reports, nonspecific esterase staining and immunofluorescence staining for Mac-1, NLDC-145, and B220 cell surface Ag indicated that mast cell preparations were not contaminated with macrophages, dendritic cells, or B cells, respectively. BMMCs were cultured for the last 48 h before harvest in the presence of 3 U/ml rIL-3 and 100 U/ml IL-4 in insulin-transferrin-sodium selenite supplement (ITS; Boehringer Mannheim, Indianapolis, IN)-complemented RPMI 1640 in the absence of FCS. Because FCS has been reported to contain exosomes, this culture procedure provides us with supernatants that contain exosomes exclusively from mast cells again.

BMMCs and cell lines were tested for contamination by mycoplasma using a highly sensitive mycoplasma PCR ELISA performed according to the manufacturer procedure (Roche Diagnostics, Meylan, France).

**Exosome purification**

Exosomes were prepared from the supernatant of 3-wk-old BMMC cultures. During the last 48 h, BMMCs were cultured at 3 × 10<sup>5</sup> cells/ml in IL-3- and IL-4-containing RPMI 1640 supplemented with 1/1000 dilution of ITS in the absence of FCS. Supernatants were then subjected to two successive centrifugations at 300 × g for 5 min and at 1,200 × g for 20 min to eliminate cells and debris, followed by a centrifugation for 2 h at 70,000 × g. Two fractions were obtained: a high-density (pellet) and a low-density (hypodense) fraction. The exosomes concentrated in the pellet were washed twice in a large volume of PBS centrifuged at 70,000 × g for 1.5 h. The amount of exosomal proteins recovered was measured by Bradford assay (Bio-Rad, Richmond, CA). Immunoassay was conducted by injecting rabbits with 200 μg exosomes in 1 ml PBS emulsified in an equal volume of CFA (Difco, Detroit, MI). Three booster immunizations with the same dose were given at 3-wk intervals in incomplete Freund’s adjuvant, and the rabbits were bled 1 wk after the last injection. All immunizations were administered in multiple 100-μl intradermal and s.c. injections. The purification of the rabbit anti-exosome Abs was conducted by using protein A-SepharoseCL4B immunosorbent column (Pharmacia, Piscataway, NJ). Exosomes were specifically recognized by the rabbit anti-exosome Abs using solid phase ELISA.

**Splenocyte activation and IL-2 assay**

Spleen cells harvested from DBA/2 mice were incubated at 10<sup>6</sup> cells/ml for 4 min at 4°C in Gey’s solution, which allowed the lysis of RBC. After two washes, cells were resuspended in RPMI 1640 medium supplemented with 10% FCS. The spleen cell stimulation assay was conducted in flat-bottom 96-, or 24-well plates (Life Technologies, Cergy-Sontoise, France) using RPMI 1640 supplemented with 10% (v/v) FCS. To a final volume of 200 μl/well (5 × 10<sup>5</sup> splenocytes) or 1 ml/well (3 × 10<sup>5</sup> splenocytes), mast cells were added at a mast cell/spleen cell ratio of 1/100 previously established as the optimal mast cell concentration for lymphocyte activation. For a strict comparison, unfraccionated and fractionated supernatants harvested from a 3 × 10<sup>6</sup> cells/ml were routinely tested for their activity at 1/100 dilution. Pellets containing purified exosomes were also used at 1/100 dilution after reconstitution to the initial volume before separation. After 48 h of incubation, 50-μl aliquots of supernatants were collected and assayed for IL-2. IL-2 was titrated using the IL-2-dependent CTLL-2 cell line. Supernatants were incubated for 18 h with 10<sup>5</sup> CTLL-2 cells and 0.25 μCi [methyl-<sup>3</sup>H]Thid/well was added 8 h before cell harvest. Spleen cell proliferation was measured by thymidine uptake following the same procedure. The results are given as the mean cpm of duplicate cultures.

**Detection of IL-4 and IFN-γ**

Flat-bottom 96-well plates (Nunc, Copenhagen, Denmark) were coated for 2 h at 37°C with anti-IL-4 mAb (Interchim, Ann Arbor, MI) at 1 μg/ml or with anti-IFN-γ mAb AN18 at 3 μg/ml. After 3 washes in 0.1% PBS-Tween 20, plates were saturated for 1 h at 37°C with 0.1% PBS-BSA. After 3 washes, standards and culture supernatants were then incubated overnight at 4°C. Plates were then incubated for 2 h at 37°C with 1 μg/ml of biotinylated anti-IL-4 (Interchim) or anti-IFN-γ (R46A2) mAbs, followed by a further 2-h incubation with streptavidin-peroxydase conjugate. After washing, O-phenylenediamine (Sigma) was added and absorbance was determined by OD at 490 nm.

**FACS analysis**

After 48 h of spleen cell culture in the presence of mast cells or mast cell-derived exosomes, cells were collected and centrifuged at 300 × g. Cell pellets were resuspended in 15 μl of PBS and added in a FACSscan cytofluorograph (Becton Dickinson, San Jose, CA). Blast cells refer to activated cells exhibiting large size, and the percentage was determined by an arbitrary gate that distinguished resting cells from activated cells. The percentage of blast cells was calculated as follows: the percent of blast cells = (number of large cells/number of total cells) × 100.

**Electron microscopy and immunogold labeling**

Formvar-coated copper grids were incubated with bacitracin for 2 min. Vesicular preparation (15 μl at 100 μg/ml) were dropped on grids for 10 min and fixed with 0.5% glutaraldehyde (Euromedex, Souffleweyersheim, France) and then washed with ammonium acetate and negatively stained in 1% uranyl acetate (Merck, West Point, PA) for 5 min. Excess stain was removed and the grids were air dried before visualization. For negative stain immunogold labeling, 15 μl of the preparation was placed on formvar-coated nickel grids wet with bacitracin for 2 min and then fixed with 3% paraformaldehyde for 5 min. Grids were washed in PBS, blocked with 0.5% PBS-BSA for 30 min and then incubated with anti-exosomes rabbit Abs (1/200) in 0.1% PBS BSA for 45 min. Grids were washed again and incubated with protein A conjugated to 10-nm gold particles (purchased from Dr. Slot, Medical School, Utrecht University, Utrecht, The Netherlands) diluted in 0.1% PBS-BSA for 45 min at room temperature. Grids were washed with PBS, fixed with 0.5% glutaraldehyde, rinsed with ammonium acetate 0.2 M and then negatively stained with uranyl acetate and examined under a Philips EM410 electron microscope. In addition, normal rabbit Iggs were used for immunocytochemistry in control experiments.

**Electron microscopy and immunocytochemistry**

Mast cells were fixed with 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Cells were prepared as previously described (11–13), embedded in 10% gelatin for 30 min on ice and cut into small gelatin blocks that were infiltrated with 2.3 M sucrose and frozen in liquid nitrogen. Ultrathin cryosections were performed in a Leica (Deerfield, IL) Ultracut E ultramicrotome with a model RMC cryochamber at −80°C. The sections were then incubated with protein A and 10 nm colloidal gold for 45 min. The sections were washed with PBS, fixed with 1% glutaraldehyde (pH 7.4) and stained with 0.5% PBS for 5 min, rinsed with PBS and then water, and stained...
FIGURE 1. IL-4 treatment of mast cells is prerequisite for the generation of the lymphocyte-stimulating activity. BMMC treated in different cytokine conditions, IL-3, IL-3 and IFN-γ, or IL-3 and IL-4 were incubated at a mast cell/spleen cell ratio of 1/100 for 48 h at 37°C. As control, spleen cells were cultured alone in the absence of mast cells. Lymphocyte activation was assessed by thymidine uptake for proliferation and by forward scatter for blast formation. Data are expressed as mean values ± SEM from three different experiments. * Significant differences from cells treated with IL-3 alone or with IL-3/IFN-γ (p < 0.01).

Detection of Ags associated with exosomes

Flat-bottom 96-well plates (Nunc) were coated overnight at 4°C with 10 μg/ml of exosome solution. After three washes in 0.1% PBS-Tween, plates were saturated for 1 h at 37°C with 0.1% PBS-BSA followed by the addition of different mAbs including anti-LFA-1, anti-ICAM-1, anti-IAββ, anti-CD80, anti-CD86, anti-CD-40, anti-CD-40L and control Abs from rabbit, mouse, and rat. After 2 h of incubation at 37°C, plates were washed and incubated for 2 h at 37°C with peroxidase-labeled anti-mouse, anti-rabbit, or anti-rat IgG. After washing, O-phenylenediamine (Sigma) was added. Absorbance was determined by OD at 490 nm.

Labeling of cells and exosomes

For starvation, BMMC were incubated at 4 × 10⁵/ml for 1 h at 37°C in cysteine/methionine-free medium supplemented with 0.1% ITS. The cells were then incubated overnight in the same medium containing 10 μCi/ml [³⁵S]methionine/cysteine (Promix®; ICN Pharmaceuticals, Costa Mesa, CA). The cells were washed and reincubated further for 24 h at 37°C in fresh complete medium. Exosomes were purified from the supernatant and their protein content analyzed by a 5–15% SDS-PAGE. The gel was silver stained, dried, and autoradiographed.

Analysis of exosomal proteins by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS)

Exosome preparation (50 μg) was diluted in reducing sample buffer and boiled for 5 min. Separation of exosomal proteins was performed by electrophoresis using a 5–15% SDS-PAGE. For in-gel digestion, sample preparation was performed as described by Shevchenko (15). Briefly the band was excised from the gel, washed, in-gel reduced, S-alkylated with iodoacetamide, and in-gel digested with bovine trypsin (sequencing grade, Roche Molecular Biochemicals, Meylan, France) at 37°C overnight. Peptides were extracted, dried with SpeedVac and resolubilized in 8 μl of 0.1% trifluoroacetic acid. ZipTips (Millipore, Bedford, MA) were used to desalt samples. Mass peptide mapping was performed using 1 μl of the tryptic digest mixture using α-cyano-4-hydroxy cinnamic acid (Sigma). The samples were analyzed by MALDI-TOF-MS on a Voyager DE STR (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). The instrument was operated in the delayed extraction mode and the delay time was 150 ns. Each mass spectrum was an average of 250 laser shots. Calibration was performed with four proteins.

For the database search on NCBInr, monoisotopic masses were assigned, using MS-FIT from Protein Prospector. The parameters were set as follows: no restriction on the isoelectric point of proteins, 100 ppm were allowed as the maximum mass error, mass protein was adapted to each apparent molecular mass ± 25% for each band according to the SDS-PAGE analysis, and one incomplete cleavage per peptide was considered.

Statistical analysis

Results are expressed as the means ± SEM from two to four experiments. The Mann-Whitney U test was used to compare the mean values between different groups, and the significance was set at a p value of <0.05.

Results

Role of cytokines in the acquisition by mast cells of the lymphocyte-stimulating activity

To investigate the optimal conditions for mast cells to induce lymphocyte activation, mouse BMMC were cultured 19 days in the presence of IL-3 followed by treatment with different cytokine
combinations for the last 48 h, including IL-4 and IFN-γ. Mast cells were washed before their coculture with the spleen cells to eliminate any residual IL-4 or IFN-γ, and during this incubation period no cytokine was added. Lymphocyte activation that occurred after 48 h of culture at the optimal mast cell/spleen cell ratio of 1/100 gave rise to a characteristic heterotypic adhesion of B and T lymphocytes (as shown by specific labeling with anti-B220 and anti-CD4 mAbs, respectively; data not shown) that underwent blast formation and proliferation. As shown in Fig. 1, IL-4 was found to be the only cytokine that was able to prime BMMC before their coculture with spleen cells to provide them with lymphocyte-stimulating activity. IL-3 alone, or in combination with IFN-γ, or GM-CSF (not shown) did not induce any activity. Subsequently, in the next experiments and throughout the manuscript the same experimental set up was used, which consisted of the pretreatment of BMMC with IL-4 48 h before their coculture with spleen cells.

The mast cell-derived lymphocyte-stimulating activity is associated with a high-density fraction present in their culture supernatant

Earlier evidence (7) indicated that physical contact between mast cells and spleen cells was not required and that mast cell supernatants were fully active. To isolate the bioactive material, supernatant from 48-h cultures of IL-4-treated BMMC was subjected to differential centrifugation. After two centrifugation steps at 300 \( \times \) g and 1200 \( \times \) g to eliminate cells and debris followed by a final 70,000 \( \times \) g ultracentrifugation, a dense fraction was pelleted and the resulting hypodense fractions were separated and tested for their capacity to induce lymphocyte stimulation. As shown in Fig. 2, most of the activity of the initial supernatant was recovered into the pellet while the hypodense fraction lacked activity. This dense fraction was able to induce both blast formation and proliferation of splenocytes and was as effective as the initial unfraccionated supernatant. Unfraccionated supernatants as well as 70,000 \( \times \) g pellets obtained from two mast cell lines, P815 and MC/9, were similarly effective in inducing splenocyte activation. However, in contrast to BMMC, the lymphocyte-stimulating activity induced by the two mast cell lines is constitutively expressed and did not require pretreatment with IL-4 (Fig. 2).

The high-density fraction of the mast cell supernatant contains membrane vesicles called exosomes.

To characterize the structural features of the biologically active high-density fraction of mast cell supernatant, the 70,000 \( \times \) g pellet was analyzed by negative staining. As shown in Fig. 3, the pellet consisted of a population of 60- to 100-nm vesicles called exosomes released by mast cells. Exosomes were absorbed to carbon-coated grids and negatively stained with uranyl acetate. The insert shows exosomes immunogold-labeled with rabbit anti-exosome Ab. Bar = 100 nm.

FIGURE 3. The lymphocyte-stimulating activity is associated with exosomes released by mast cells. Exosomes were absorbed to carbon-coated grids and negatively stained with uranyl acetate. The insert shows exosomes immunogold-labeled with rabbit anti-exosome Ab. Bar = 100 nm.

FIGURE 4. Mast cell exosomes are localized in the intracytoplasmic granules and can be released in the extracellular environment. Ultrathin cryosections of BMMC were immunogold labeled with rabbit anti-exosome Ab. A, BMMC show numerous anti-exosome-positive granules (Bar = 200 nm). B, A cluster of anti-exosome-positive vesicles appeared at the outer leaflet of the plasma membrane indicated by the arrow (Bar = 100 nm).
exosomes some of them with an electron-dense core. The term exosome was originally used to describe small membrane vesicles (50–100 nm of diameter) released by reticulocytes during their final stage of maturation into RBC (16). To determine the intracellular localization of exosomes, we used Abs raised in rabbits immunized with purified exosomes. The specificity of these Abs was assessed by the lack of reactivity with exosomes purified from human mast cells. BMMC were fixed and ultrathin cryosections were immunogold labeled with anti-exosome Abs coupled to 10-nm gold particles. Fig. 4A shows that almost all gold particles accumulate into the intracytoplasmic granules, suggesting that these compartments are the storage site of exosomes. As shown in Fig. 4B, exosomes appeared at the external leaflet of the plasma membrane indicating that exosomes may be externalized from mast cells most likely through an exocytic profile based on the fusion of the limiting membrane of granules with the plasma membrane. Control preparations incubated with preimmune rabbit Ig did not show any immunogold labeling (not shown). Positive labeling of secreted exosomes demonstrates that these vesicles are similar to those found in the granules. Based on the protein content of exosomes, we consistently obtained 0.5–0.8 mg/10^6 cells of exosomal proteins from the 48-h culture supernatants of 3 × 10^6 BMMC/ml. Altogether, these data demonstrate that the mast cell-dependent lymphocyte-stimulating activity as shown in Fig. 2 is very likely mediated by intracytoplasmic granule-associated exosomes, which are constitutively released into the extracellular medium.

**Purified exosomes induce splenocytes to proliferate and to produce cytokines.**

We have previously reported that mast cells cultured with splen cells not only induced B and T lymphocyte proliferation but also resulted in IL-2 and IFN-γ production (8). To investigate whether exosomes purified from BMMC-derived supernatant were endowed with similar biological activities as BMMC, spleen cells were incubated in the presence of unfractionated BMMC supernatant (1/100), exosome-depleted supernatant (hypodense fraction) (1/100), exosomes (15 ng/ml), and BMMC (at a BMMC/spleen cell ratio of 1/100). After 48 h of culture, cell proliferation and cytokine production were used as a read-out for lymphocyte stimulation. As shown in Fig. 5, unfractionated mast cell supernatant was able to induce lymphocyte proliferation and IL-2 and IFN-γ production. More interestingly, the whole biological activity of the unfractionated supernatant was found to be associated with exosomes.

**FIGURE 5.** Capacity of BMMC-derived exosomes to induce lymphocyte proliferation and cytokine production. Exosomes, unfractionated, and exosome-depleted supernatants (hypodense fraction) obtained from IL-4-treated BMMC and cultured for 48 h in ITS-supplemented RPMI 1640 were added at 1/100 dilution to spleen cells. After 48 h of culture, lymphocyte proliferation was assessed by thymidine uptake. In parallel, culture supernatants were tested for their content in IL-2 and IFN-γ as measured by CTLL-2 proliferation assay and by ELISA, respectively. Positive control consisted in incubating BMMC with spleen cells at the ratio of 1/100. As negative control, spleen cells were cultured alone. Values are presented as means ± SEM from three separate experiments.

**FIGURE 6.** Exosomes from IL-4-treated BMMC are able to induce lymphocyte activation in vivo. DBA/2 mice were injected i.p. with 10^7 (•) and 5 × 10^6 (■) IL-4-treated BMMC, or with 5 μg (●) and 0.5 μg of exosomes (■). Control mice (•) were injected with PBS. Six days later, spleen and lymph node cells were harvested and cultured at indicated concentrations in the absence of any stimulus. After 72 h of incubation, cell proliferation (A) was monitored by thymidine uptake, and culture supernatants were tested for their content in IL-2 (■) and IFN-γ (■) (B) using CTLL-2 proliferation assay and ELISA, respectively. Results are presented as mean values ± SEM from two different experiments.
somes, whereas the exosome-depleted supernatant completely lost its activity. None of the cultures contained detectable IL-4 as assessed by ELISA (not shown). These data demonstrate that mouse BMMC constitutively release exosomes that are endowed with a full biological activity similar to that obtained by intact mast cells and preferentially induce Th1-type responses.

Occurrence of lymphocyte-stimulating activity by exosomes in vivo

Based on previous demonstration (8) that IL-4-treated BMMC induce in vivo B and T lymphocyte activation and cytokine production and that this activity is mediated by exosomes, mice were injected i.p. with 5 μg, or 0.5 μg of BMMC-derived exosomes. Control mice were injected with 10^7 BMMC, 5 × 10^6 BMMC, or with PBS alone. Spleen and lymph node cells were harvested 6 days later and cultured for 48 h after which lymphocyte activation was assessed. Fig. 6 shows that, in addition to a characteristic heterotypic cell aggregation (data not shown), mice injected with exosomes displayed cell proliferation and IL-2 and IFN-γ production. Data also indicate that exosomes were as potent as mast cells in inducing lymphocyte activation. These results suggest that exosome-induced lymphocyte activation in vivo may have physiologic significance.

Detection of Ags associated with exosomes

We have previously reported that membrane vesicles or exosomes present in type I and type II mast cell granules contain lysosomal-associated protein (lamp)1 and 2 as well as invariant chain and MHC class II molecules (17). Here, we extended the investigation to other immunologically relevant molecules. As shown in Fig. 7, using a sandwich ELISA method, we demonstrate that, in addition to MHC class II molecules, CD86, CD40, CD40L, LFA-1, and ICAM-1 molecules were found to be associated with exosomes. These results may ascribe to exosomes a possible Ag presenting function or a capacity to transfer this property to other APCs.

To further examine the protein composition of exosomes, exosomal proteins were separated by a 5–15% SDS-PAGE gradient. Analytical electrophoresis was first performed on exosomes from metabolically labeled BMMC, and the patterns of bands obtained on silver stained gel and after autoradiography were similar (Fig. 8A), demonstrating that all the proteins in the exosomes are of mast cell origin. As shown in Fig. 8B, preparative electrophoresis of exosomes displayed about 14 major bands ranging from about 150 to 6 kDa. To identify these proteins, 10 major bands indicated by arrows in Fig. 8 were excised.
and trypsin-digested, and the resulting peptide fragments were analyzed by MALDI-TOF-MS. The profiles of the generated tryptic peptides were compared with the theoretical profiles of tryptic digests from known proteins available in the databases (18). As shown in Table I, of 10 bands analyzed, 7 proteins associated with exosomes were identified as follows: CD13, ribosomal protein S6 kinase, annexin VI, CDC25, γ-actin-like protein, γ-actin, and cytoplasmic γ-actin. According to the reported functions of these proteins, one possible candidate for the exosome-related lymphocyte-stimulating activity could be the CDC25 molecule, a phosphatase directly involved in the progression of the cell cycle. It should be pointed out that other components among several minor proteins contained in the exosome extract that could not be picked up by the MALDI-TOF-MS analysis may well be responsible for such biological activity.

Table I. Identification of exosomal proteins by MALDI-TOF-MS

<table>
<thead>
<tr>
<th>Band</th>
<th>Total No. of Peptides</th>
<th>Protein Identified (National Center for Biotechnology Information accession number)</th>
<th>% of the Protein Covered</th>
<th>Estimated Protein Molecular Mass (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>CD13/animopeptidase N (NP032512)</td>
<td>13%</td>
<td>109.6</td>
</tr>
<tr>
<td>2</td>
<td>NI</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>Ribosomal protein S6 kinase (NP033123)</td>
<td>13%</td>
<td>81.6</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>Annexin VI (lipocortin VI) (P68)(P14824)</td>
<td>34%</td>
<td>75.7</td>
</tr>
<tr>
<td>5</td>
<td>NI</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>CDC25 (P48967)</td>
<td>14%</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>γ-actin-like protein (AAF08293)</td>
<td>25%</td>
<td>43.6</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>γ-actin (CAA31455)</td>
<td>23%</td>
<td>41</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>Cytoplasmic β-actin (CA27396)</td>
<td>18%</td>
<td>39.1</td>
</tr>
<tr>
<td>10</td>
<td>NI</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*As shown in Fig. 8, bands indicated by arrows were excised and trypsin-digested, and the resulting peptides were analyzed by MALDI-TOF-MS as described in Materials and Methods.

*Total number of peptides used for comparison with the database. NI, Nonidentified.

*Sequence of amino acids matching the identified protein.
involved as well. The IL-4R α-chain was found to be associated with p47phox, an activator of the NADPH oxidase in B cells (22). To investigate the possible implication of these two IL-4R-dependent signaling pathways, STAT6− (23) and (22). To investigate the possible implication of these two IL-4R-dependent signaling pathways, STAT6− (23) and (22).

To identify the molecular mechanism by which exosomes from IL-4-treated BMMC induce B and T lymphocyte activation, among several molecules we have examined including MHC class II, CD86, CD40, CD40L, LFA-1, and ICAM-1, only Abs against LFA-1 and ICAM-1 were able to completely block cell activation and cytokine production (data not shown). These data are in agreement with those obtained when stimulation was conducted by using BMMC (8). However, LFA-1- and ICAM-1-dependent cell activation may participate only at a distal level and is a consequence of proximal events initiated by distinct molecules. One of the possible candidates among proteins associated with exosomes that could be involved in the lymphoproliferative response is a member of the mouse CDC25 gene family expressed in mammalian cells. CDC25 gene encodes a Thr/Tyr phosphatase, which activates cyclin-dependent kinase directly involved in the mitosis process (25, 26). However, CDC25 should not be the exclusive molecule that mediates this biological effect. Considering the relatively wide spectrum of exosomal proteins, other mast cell exosome-associated molecules, unidentified as yet, may well be potentially immunostimulatory. Alternatively, heat shock proteins that have not been picked up by our MALDI-TOF-MS analysis, a member of which (hsc73) was recently found to be selectively associated with exosomes derived from dendritic cells (21) may account for the immunostimulatory activity observed in our system. Indeed, heat shock proteins are known to be potentiators of Ag-dependent (27) and Ag-independent (28) T cell responses. Based on previous work (17), accumulation of some proteins in mast cell exosomes did not occur fortuitously because MHC class II molecules, mannose phosphate receptor, lysosomal membrane proteins (lamp1 and lamp2) are colocalized and enriched in these vesicles. Whether this preferential localization applies to all exosomal proteins, this remains to be investigated. Besides cell-to-cell contacts, and soluble mediators such as cytokines, exchange of exosomes may be considered as an alternative for intercellular communication. Such a mechanism of protein transfer to a naive cell via exosomes has been proposed to explain transfer of MHC molecules between different cells of the immune system. Follicular dendritic cells in tonsil germinal centers do not synthesize MHC II molecules but express them at the cell surface and act as APCs once exposed to supernatants of B cells that contain MHC II-bearing exosomes (29). Recently, it has been demonstrated that MHC II-bearing exosomes derived from MHC II-positive cells were found attached to the follicular dendritic cell surface (30).

What could be the pathophysiological relevance of exosome release by mast cells? The ubiquitous tissue distribution of mast cells and their potential to release several inflammatory and immunoregulatory cytokines provide these cells with a unique role in host defense mechanisms (31). Several pathophysiologic conditions indicate that mast cells and T lymphocytes are found in close apposition in inflammatory sites (32, 33). Based on their dual potential to release cytokines and immunologically active exosomes, mast cells may play a role in the recruitment and activation of B and T lymphocytes in inflamed tissues. Evidence that exosomes were active in vivo was provided by the ability of BMMC-derived exosomes to induce lymphocyte activation and cytokine release from lymph nodes and spleen cells obtained from exosome-injected mice. What remains to be investigated is whether exosome secretion by normal tissue mast cells occurs constitutively or is under the control of a regulatory process. It is consistently observed that mast cells and mast cell-derived exosomes preferentially induce Th1-type responses as evidenced by the production of IL-2, IFN-γ, and IL-12 (8). In support of these findings, we recently demonstrated that in vivo transfer of Ag-pulsed mast cells to syngeneic mice induced Ag-specific IgGl and IgG2a Abs but no IgE response could be elicited (Villa et al., unpublished results). It can be postulated that mast cells, directly or through exosomes, may down-regulate allergic responses. The unique property of exosomes from mast cells and mast cell lines to activate B and T lymphocytes suggest that mast cells may contribute to the development and the amplification of specific and nonspecific inflammatory responses.

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