Localization of the Epstein–Barr virus protein LMP 1 to exosomes

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The Epstein–Barr virus latent membrane protein (LMP 1) functions as a constitutively active signalling molecule and associates in lipid rafts clustered with other signalling molecules. Using immunofluorescent confocal microscopy, LMP 1 was shown to have an heterogeneous distribution among individual cells which was not related to the cell cycle stage. LMP 1 was shown to localize to intracellular compartments in cells other than the plasma membrane. Co-labelling of cells with both an LMP 1 antibody and an antibody to the Golgi protein GS15 revealed that the intracellular LMP 1 partly co-localized with the Golgi apparatus. Further confirmation of intracellular LMP 1 localization was obtained by immunoelectron microscopy with rabbit polyclonal LMP 1 antibodies and cryosectioning. As well as being present in intracellular foci, LMP 1 co-localized in part with MHC-II and was present on exosomes derived from a lymphoblastoid cell line. Preparations of LMP 1 containing exosomes were shown to inhibit the proliferation of peripheral blood mononuclear cells, suggesting that LMP 1 could be involved in immune regulation. This may be of particular relevance in EBV-associated tumours such as nasopharyngeal carcinoma and Hodgkin’s disease, as LMP 1-containing exosomes may be taken up by infiltrating T-lymphocytes, where LMP 1 could exert an anti-proliferative effect, allowing the tumour cells to evade the immune system.

INTRODUCTION

LMP 1 is a 63 kDa membrane protein that very closely resembles a constitutively active membrane receptor. LMP 1 has a hydrophilic amino terminus, six highly hydrophobic 20 aa transmembrane domains separated by five less hydrophobic 8 aa segments and a long hydrophilic cytoplasmic tail containing two active domains (Liebowitz et al., 1986). An intronic start codon results in a smaller 45 kDa truncated LMP 1 protein, termed ‘lytic LMP 1’, which is expressed only in some EBV strains, specifically during virus replication (Baichwal & Sugden, 1987; Hudson et al., 1985). The lytic LMP 1 protein shares little or no functional similarity to the full-length form, in that it does not transform rodent fibroblasts, it does not alter the B-lymphocyte phenotype and is dispensable for transformation (Wang et al., 1985, 1988).

LMP 1 protein has been shown to associate with the cytoskeleton and aggregates to form patches on the cell membrane, a function that, like other cellular receptors, is necessary for its activation of NF-κB (Gires et al., 1997; Mann et al., 1985). The domains involved in forming these aggregated patches have been defined as the 25 aa cytoplasmic amino terminus and the first two membrane-spanning domains (Bloss et al., 1999). A significant proportion of LMP 1 is detergent insoluble, and as such, was originally defined as cytoskeleton associated (Liebowitz et al., 1986). More specifically, it binds to the cytoskeleton protein vimentin and can up-regulate expression of this protein (Liebowitz & Kieff, 1989; Liebowitz et al., 1987). Glycosphingolipid-rich domains or lipid rafts are discrete domains within the plasma membrane that are detergent insoluble and provide a clustering point for signal transduction proteins such as G-proteins as well as LMP 1 and its interacting protein, TRAF3 (Ardila-Osorio et al., 1999; Clausse et al., 1997). One study has proposed that LMP 1 may also accumulate in the lysosomal compartment (Laszlo et al., 1991). Lysosomes are thought to form by fusion of pinocytotic vesicles to yield endosomes which, in turn, transform first into multivesicular bodies (MVB) and then into mature lysosomes (Morales et al., 1999). Haematopoietic cells have been shown to use lysosomal compartments to store and release their secretory products. The direct fusion of lysosome-related compartments with the plasma membrane in haematopoietic cells has been associated with the release of exosomes (Andrews, 2000).

Non-structured intracellular aggregation of LMP 1 in a fraction of lymphoblastoid cells of different origin was noted...
by others (Boos et al., 1987; Rowe et al., 1987) and was suggested to relate to the cell cycle stage of these cells, but this was not explored any further up till now. LMP1 expression levels vary considerably among different EBV-carrying cell lines (Meij et al., 2000). Recently, LMP1 was shown to be released by lymphoblastoid cells into the extracellular space, possibly associated with exosomes (Dukers et al., 2000). Exosomes are internal vesicles of MVBs formed by inward budding of the vesicular membrane creating a membrane-enclosed compartment containing cytoplasm. Exocytosis of MVBs and the release of 40–100 nm exosomes has been described for reticulocytes, B- and T-lymphocytes, mast cells, platelets and macrophages (reviewed in Denzer et al., 2000).

Exosomes derived from antigen-presenting cells contain MHC class I and II (antigen-presenting proteins), CD86 (co-stimulatory protein) and ICAM-1 (adhesion molecule), as well as various tetraspan proteins such as CD9, CD37, CD53, CD63, CD81 and CD82, which are all involved in signal transduction, adhesion and complex formation with MHC. Furthermore, peptide-loaded MHC II-carrying exosomes can stimulate CD4<sup>+</sup> T-lymphocyte proliferation, dendritic cell-derived MHC I-carrying exosomes can activate specific CD8<sup>+</sup> CTL responses and mast cell-derived exosomes can stimulate both B- and T-lymphocyte proliferation (Raposo et al., 1996; Skokos et al., 2001; Zitvogel et al., 1998). This suggests an important role for exosomes in the immune system for antigen presentation and B- and T-lymphocyte activation. One previous report has suggested that LMP1 may be secreted in exosomes and that an immunosuppressive domain within the conserved first transmembrane helix might influence T-cell activation and proliferation leading to T-cell anergy (Dukers et al., 2000). In this study we have used immunofluorescence and immunoelectron microscopy to demonstrate that LMP1 localizes to an intracellular compartment and that LMP1 can be found in extracellular exosomes. We propose that LMP1-containing exosomes may be involved in suppression of the immune response against LMP1-expressing EBV-associated tumours.

METHODS

Cell lines. The EBV-negative Burkitt’s lymphoma (BL) cell line DG75 and the A-type lymphoblastoid cell lines (LCLs) QIMR LMB95-8, QIMR CW-B95-8, QIMR NK Wil, QIMR SS Wil, QIMR AL Wil, QIMR NB-B95-8 as well as the B-type LCL QIMR-AL AG876 were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) and incubated in a humid incubator at 37 °C in 5% (v/v) CO₂. The cell line DG75-EBO-ERNA 3,4,6 was also used in this study and was cultured as above with the addition of 600 μg hygromycin B ml⁻¹ to the medium.

Antibodies. The LMP1 antibodies used in this study were: mouse monoclonal antibody OT21C, specific for aa 290–318; mouse monoclonal antibody OT22CN, specific for aa 1–13; rabbit polyclonal antiserum 96-121, specific for the carboxy terminus of LMP1 aa 186–386; the rabbit polyclonal antiserum 97-48, specific for two epitopes in the carboxy terminus of LMP1 aa 365–386; and the commercially available CS1-4 antibodies, which contain a cocktail of four monoclonal antibodies specific for the carboxy terminus of LMP1 aa 186–386 (Dako). Other antibodies used in this study included rabbit polyclonal antiserum GS15 (Xu et al., 1997), raised against a Golgi-specific protein (a kind gift from Professor David James, University of Queensland, Australia) and the mouse monoclonal anti-HLA DR antibody (kind gift from Rajiv Khanna, Queensland Institute of Medical Research, Australia). Secondary antibodies used were HRP-conjugated anti-mouse, FITC-conjugated anti-rabbit and PE-conjugated anti-mouse (Silenus). 10 nm gold-labelled Protein A (PAG; a gift from Jan Slot, Utrecht, The Netherlands) and 15 nm gold-labelled anti-mouse IgG (British Biocell International) were used for immunogold labelling.

Colorimetric (MTT) proliferation assay. For the T-lymphocyte proliferation assay, PBMCs were cultured in a 96-well round-bottom culture plate at a concentration of 5 × 10⁴ cells per well in RPMI 1640 medium supplemented with 10% FCS. The mitogen phytohaemagglutinin (PHA) was added to a final concentration of 20 μg ml⁻¹. Cells were cultured for 3 days at 37 °C in a 5% CO₂ atmosphere (volume 200 μl per well). MTT was added to a final concentration of 1 mg ml⁻¹ and the cells were cultured for a further 4 h while formazan crystals formed. The crystals were resuspended in DMSO and the absorbance was read at 560 nm with a Tecan Spectra ELISA plate reader using Biolase software. The percentage proliferation was calculated by the following formula: [TEST(Abs. 560 nm) / PHA positive control(Abs. 560 nm)] × 100.

DNA cell cycle analysis. Cell cycle profiles were obtained by washing cells once in PBS and then resuspending the cells in a nuclear DNA stain (50 μg propidium iodide ml⁻¹, 1 mg RNase A ml⁻¹ and 0-02% Triton X-100 in PBS). Cells were analysed on a FACSCalibur (Becton Dickson) using CellQuest and Modfit data analysis software. For cell cycle analyses the cell lines QIMR NB-B95-8 LCL and DG75 were incubated for 48 h with drugs that induce cell cycle checkpoints. The drugs used were Amphidicolin (5 μg ml⁻¹; Calbiochem), Mimosine (100 μM; Sigma), Colcemid (100 ng ml⁻¹; Gibco-BRL), Nocodazole (0.5 μg ml⁻¹; Calbiochem) and ICRF (0.5 μg ml⁻¹; Sigma). Cells were harvested following drug treatment and were analysed by flow cytometry for cell cycle profiles and by immunofluorescence for LMP1 localization.

Immunofluorescence assay and confocal microscopy. Cells were washed in PBS and spotted onto a 12-well multiwell slide (ICN Biomedicals); they were then air-dried, fixed in 100% acetone and blocked for 30 min in 20% FCS in PBS at room temperature. They were then incubated with primary antibody (CS1-4, diluted 1:5 in 20% FCS/PBS) for 1 h at room temperature. Slides were then washed three times in PBS and incubated with PE-conjugated antimouse antibody (diluted 1:160 in 20% FCS/PBS). Co-localization of LMP with the Golgi protein GS15 was examined using immunofluorescence, as above, except slides were sequentially incubated with antibodies: firstly with CS1-4 anti-LMP (1:50), then PE-conjugated anti-mouse (1:100), then anti-GS15 antibody (1:100), and finally FITC anti-rabbit (1:100), with three washes in PBS after each incubation. The slides were then mounted in Vectashield mounting medium and were scanned using a Bio-Rad MRC 600 confocal microscope. Images were acquired using COMOS (Confocal Microscope Operating Software V6.03) and were analysed using CAS (Confocal Assistant Software V3.10).

Immunoelectron microscopy. LMP1 was detected by electron microscopy using the following method. Cells were washed in PBS, fixed in 4% paraformaldehyde/PBS for 30 min, and then further washed with PBS. The cells were enrobed in 10% gelatin, cooled to 4 °C and cut into small gelatin blocks that were infiltrated with polyvinylpyrrolidone/sucrose overnight at 4 °C. The cells were mounted on copper pins and frozen in liquid nitrogen.
Cyrosectioning was done with a Leica Ultracut S ultramicrotome fitted with the Leica FCS cryosectioning system. Ultrathin sections were collected using 1-3 M sucrose and attached to Formvar-coated copper grids. For immunolabelling, the grids were blocked for 5 min in blocking buffer (20 mM glycine, 0-2% fish skin gelatin, 0-02% BSA in PBS), incubated for 30 min with rabbit polyclonal anti-LMP1 96-121 antiserum diluted 1:20 in blocking buffer, and then washed four times in blocking buffer. The grids were incubated for 30 min with 10 nm PAG diluted 1:85 and were washed four times in blocking buffer. For double labelling with MHC class II the grids were sequentially incubated with antibodies: firstly with mouse anti-LMP1 96-121 antiserum diluted 1:20 in blocking buffer, and then rabbit anti-LMP 96-121 antiserum (1:20) and finally 10 nm-gold-labelled anti-mouse IgG. grids were looped and reduced to a thin film of methylcellulose and air dried for 10 min. The sections were examined under a JEOL 1010 transmission electron microscope. Images were captured on Kodak SO-163 film and digitized using a Leafscan 45 scanner.

Isolation of exosomes. Approximately 5 × 10^7 DG75 and QIMR NB-B95-8 cells were washed in RPMI 1640 supplemented with 10% FCS and were re-cultured for 48 h. Exosomes were then purified from 35 ml of the cell culture medium using differential centrifugation (Raposo et al., 1996). Firstly, the cells were removed by centrifugation at 300 g for 10 min. The supernatant was then sequentially centrifuged at 300 g for 10 min, 500 g for 10 min (twice), 2000 g for 30 min (twice), 10 000 g for 30 min and finally 70 000 g for 60 min. For immunoelectron microscopy the 70 000 g pellet containing the exosomes was resuspended in 20–50 μl of growth medium and then spotted onto Formvar-coated copper grids and processed for immunolabelling as described above. For the T-lymphocyte proliferation assay exosomes were resuspended in 180 μl of growth medium.

RESULTS

Intracellular localization of LMP 1

To determine if LMP 1 localizes to an intracellular compartment in B-lymphocytes, an EBV-positive LCL, QIMR NB-B95-8, was analysed by immunofluorescence for LMP 1 expression using the LMP 1 antibody CS1-4 (Fig. 1). The cell line DG75 was also analysed by immunofluorescence as a negative control. The cells were harvested and spotted onto a slide, dried, fixed and incubated with the LMP 1 antibody followed by the PE-conjugated secondary antibody. The slides were analysed using a confocal microscope and images were collected from a series of focal planes, representing digital serial sections (Z-stack) (Fig. 1). The DG75 cells showed no LMP 1 expression while the QIMR NB-B95-8 LCL displayed LMP 1 as punctate patches on the cell membrane. However, in addition approximately 30% of the cells (72/227 cells over nine fields) contained a large proportion of LMP 1 within the cell in a large cytoplasmic aggregate.

Many EBV-positive cell lines contain an intracellular LMP 1 fraction

To ensure that this intracellular localization of LMP 1 was not restricted to the QIMR NB-B95-8 cell line, other EBV-infected cell lines were analysed for LMP 1 expression by immunofluorescence and confocal microscopy. The EBV-negative BL cell line DG75, EBV-positive BL cell lines Mutu I and Mutu III, the A-type EBV-positive LCLs NK Wil, SS Wil, AL Wil, CW-B95-8 and QIMR NB-B95-8 and the B-type EBV LCL AL Ag876 were all analysed. Each cell line was harvested and spotted onto a slide, dried, fixed and incubated with the LMP 1 antibody followed by the PE-conjugated secondary antibody. The slides were analysed using a confocal microscope as before (data not shown). In the EBV-negative cell line DG75, no LMP 1 expression was detected. In the BL cell line Mutu I, which represents a group I phenotype, most cells showed no LMP 1 expression; however approximately 1% of cells did appear to express low levels of LMP 1, indicating that a small percentage of these cells may have drifted to a group II/III phenotype. In the group III phenotype BL cell line Mutu III, expression of LMP 1 was detected, albeit at much lower levels than the LCLs. In each of the LCLs, LMP 1 was detected on the cell membrane in patches and as an intracellular aggregate showing a heterogeneous size and distribution among individual cells. These results confirm that LMP1 has distinct plasma membrane and intracellular localizations in different EBV-positive cell lines and that the heterogeneous distribution is an inherent feature. To compare the level of expression of LMP 1 in each of these cell lines the cells were harvested and analysed by Western blot with the LMP 1

Fig. 1. Immunofluorescence analysis of LMP 1 expression in an LCL. The LCL NB-B95-8 and the EBV-negative cell line DG75 were dried onto a slide, fixed and incubated with mouse monoclonal anti-LMP 1 (CS1-4) followed by PE-conjugated anti-mouse secondary antibody. The cells were then analysed by confocal microscopy. Representative images of NB-B95-8 and DG75 cells that were digitally serial sectioned using the Z-stack function of the confocal microscope are shown. The white arrows show cells that contain a large proportion of intracellular LMP 1.
antibody (CS1-4). The level of expression detected by Western blot correlated with the level of expression as detected by immunofluorescence (data not shown).

**Intracellular LMP 1 levels are not cell cycle dependent**

Since only 30% of cells contained intracellular LMP 1 we investigated the possibility that the intracellular LMP 1 may be produced in a cell cycle-dependent manner. The LCL QIMR NB-B95-8 was cultured for 48 h in the presence of the drugs Amphidicolin or Mimosine, which block the cells in the $G_1$ phase of the cell cycle, ICRF or Nocodazole, which block cells in the $G_2$ phase, or Colcemid, which blocks cells in the M phase of the cell cycle. Following drug treatment the cells were harvested, dried onto slides and LMP 1 expression was detected using immunofluorescence and confocal microscopy. After treatment with each of the drugs the percentage of cells containing an intracellular portion of LMP 1 was counted over four to six fields, and remained at between 19–39% following each of the treatments (Table 1). The cell cycle profile demonstrated that the drugs blocked the cells at the appropriate stage of the cell cycle (values in bold type in Table 1) and this treatment did not significantly affect the percentage of cells containing intracellular LMP 1. These results suggest that the intracellular localization of LMP 1 was not cell cycle dependent.

**Partial co-localization of LMP 1 with the Golgi protein GS15**

If LMP 1 is associated with membrane-bound organelles, one possibility was an association with the trans-Golgi network. To determine whether the intracellular LMP 1 localized to the Golgi network, QIMR NB-B95-8 cells were analysed by immunofluorescence using an anti-LMP 1 antibody and an antibody to the Golgi protein GS15 (Xu et al., 1997). A series of images was captured for a number of fields for both the QIMR NB-B95-8 and DG75 cell lines (Fig. 2). Expression of GS15 in the DG75 cell line was confined to the perinuclear region in the cytoplasm and these cells did not show any LMP 1 expression. Expression of GS15 in the QIMR NB-B95-8 cell line also surrounded the nucleus and in most instances was localized to one side of the cell. LMP 1 expression was detected in the LCL as both plasma membrane patches and intracellular foci. In approximately 50% of cells that contained intracellular LMP 1 partial co-localization with GS15 was observed. This indicated that a proportion of intracellular LMP 1 co-localized to structures within the Golgi network.

### Table 1. Percentage of cells containing intracellular LMP 1 in cells treated with drugs that block the cells in various stages of the cell cycle (values in bold)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intracellular LMP 1</th>
<th>$G_1$</th>
<th>$S$</th>
<th>$G_2/M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31-7% (72/227, 9 fields)</td>
<td>52-03</td>
<td>35-21</td>
<td>12-76</td>
</tr>
<tr>
<td>Amphidicolin</td>
<td>38-9% (21/54, 6 fields)</td>
<td>72-43</td>
<td>27-57</td>
<td>0-00</td>
</tr>
<tr>
<td>Mimosine</td>
<td>31-3% (15/48, 4 fields)</td>
<td>79-70</td>
<td>20-30</td>
<td>0-00</td>
</tr>
<tr>
<td>ICRF</td>
<td>31-0% (35/113, 5 fields)</td>
<td>15-90</td>
<td>6-15</td>
<td>77-95</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>29-5% (18/61, 5 fields)</td>
<td>20-35</td>
<td>2-63</td>
<td>77-02</td>
</tr>
<tr>
<td>Colcemid</td>
<td>19-4% (13/67, 6 fields)</td>
<td>24-1</td>
<td>6-72</td>
<td>69-18</td>
</tr>
</tbody>
</table>

### Fig. 2. Immunofluorescence analysis of LMP 1 and GS15 localization in an LCL. The LCL NB-B95-8 and DG75 were analysed by immunofluorescence for expression of LMP 1 and the Golgi protein GS15. The cells were dried onto a slide, fixed, and sequentially incubated with anti-LMP 1 antibody, PE-conjugated anti-mouse secondary antibody, anti-GS15 antibody and finally FITC-conjugated anti-rabbit antibody. The slide was mounted and analysed by confocal microscopy. (A) Representative image from the DG75 control cell line and three representative cells from the NB-B95-8 cells. LMP1 staining appears red, GS15 staining appears green and areas where co-localization occurs appear as yellow. (B) Montage of merged images from the NB-B95-8 cell line showing images 4–10 out of 17 in the Z-stack series.
Intracellular localization of LMP 1 by IEM

The LCL QIMR NB-B95-8 and the EBV-negative cell line DG75 were cryosectioned and ultrathin sections were collected onto Formvar-coated copper grids which were then immunolabelled with the rabbit polyclonal LMP 1 antibody 96-121. Cryosectioning of cells fixed with 4% paraformaldehyde was used to increase the antigenicity. A grid from each cell line, DG75 and QIMR NB-B95-8, was labelled with PAG alone and showed that the 10 nm PAG resulted in minimal background (data not shown). The DG75 cell line labelled with both the rabbit anti-LMP 1 and the 10 nm PAG showed only background staining (Fig. 3E) while in the QIMR NB-B95-8 cell line (Fig. 3A–C) LMP 1 was observed on the cell membrane as well as in intracellular foci. Images from 15 QIMR NB-B95-8 cells were captured and in nine of these cells at least one intracellular focus of LMP 1 was observed, varying from 4 to 34 gold particles per focus. These results show that the LMP 1 protein localized to intracellular compartments that ranged in size from approximately 50 to 200 nm.

Co-localization of intracellular LMP 1 with MHC class II

Exosomes derived from B-lymphocytes contain MHC class II and are formed in specialized multivesicular bodies, called MHC class II compartments (MIICs) (Raposo et al., 1996). To determine if intracellular LMP 1 might localize to MIICs, a double immunolabelling with LMP 1 and MHC class II (HLA DR) antibodies was performed. The LCL QIMR NB-B95-8 was cryosectioned and ultrathin sections were collected on Formvar-coated copper grids. The grids were sequentially immunolabelled with mouse anti-HLA DR, 15 nm gold-conjugated goat anti-mouse (15 nm GAM), rabbit anti-LMP 1 and finally 10 nm PAG in that order. As a negative control a grid was stained with 15 nm GAM alone and showed no background staining for this antibody (data not shown). The QIMR NB-B95-8 cell line labelled positive for MHC II, and in some cells the MHC II co-localized with LMP 1 (Fig. 4). Of the cells that contained intracellular foci of LMP 1, three out of ten LMP 1 foci also contained 15 nm MHC II labelling, which was mostly associated with membrane structures in the cytoplasm. While this was only a small proportion, it indicated that some of the intracellular LMP 1 was associated with MHC II.

Extracellular localization of LMP 1

Cells that overexpress LMP 1 were analysed by immunoelectron microscopy to determine if overexpression would lead to increased plasma membrane or intracellular localization of LMP 1. The cell line DG75-EBO-EBNA 3,4,6 was used to overexpress LMP 1. This cell line was transiently transfected with the plasmid pMEP-LMP and was treated with 5 µM CdCl₂ to induce expression of LMP 1. The cells were harvested and cryosectioned and sections were labelled with rabbit anti-LMP 1 antibody and 10 nm PAG. Five out of 20 cells observed were positive for LMP 1, reflecting the transfection efficiency of approximately 20%. Of the LMP 1-positive cells, four contained either two or three intracellular foci of LMP 1 similar in structure and size.

Fig. 3. Immunoelectron micrograph of NB-B95-8 cell labelled with LMP 1 antibody. NB-B95-8 cells were cryosectioned and sections collected on Formvar-coated copper grids and labelled with the polyclonal rabbit anti-LMP antibody and 10 nm PAG. (A) Typical cell with specific LMP 1 staining; bar represents 500 nm. The nucleus, cytoplasm and plasma membrane (PM) are shown. B) 3 × magnification of the cell membrane showing LMP 1 staining along the cell membrane; bar represents 150 nm. (C, D) 4 × magnification of intracellular foci of LMP 1 staining; bar represents 125 nm. DG75 cells were cryosectioned and sections collected on Formvar-coated copper grids and labelled with the polyclonal rabbit anti-LMP antibody and 10 nm PAG. (E) Typical micrograph of a DG75 cell indicating the amount of background staining with the rabbit LMP 1 antibody in this LMP 1-negative cell line. (F) 4 × magnification: the nucleus, cytoplasm and plasma membrane (PM) are shown; bar represents 50 nm.
to those observed in the QIMR NB-B95-8 cell line and one cell contained LMP 1 only on the cell membrane (data not shown). In addition to this, LMP 1 was also localized to an extracellular vesicle similar to that seen in the QIMR NB-B95-8 LCL cell population (Fig. 5A). These results suggested that overexpression of LMP 1 may lead to an increase in the intracellular localization of LMP 1 and that LMP 1 may be present on extracellular vesicles resembling exosomes.

LMP 1 is present on isolated exosomes

To determine if LMP 1 was secreted on exosomes, immunoelectron microscopy was carried out on preparations of purified exosomes. Using differential centrifugation (Raposo et al., 1996), a standard method for isolating exosomes, exosomes were isolated from both DG75 and QIMR NB B95-8 cell lines, spotted onto Formvar-coated copper grids and labelled with a mouse monoclonal anti HLA-DR (MHC II), the polyclonal rabbit anti-LMP antibody and 15 nm gold-conjugated anti mouse IgG and 10 nm PAG. (A) Micrograph of two cells, one that contains specific 10 nm LMP 1 foci colocalizing with 15 nm MHC II. Bar represents 200 nm. The nucleus, cytoplasm and plasma membrane borders of the two cells are shown. (B) 4 × magnification; bar represents 50 nm.

Exosomes derived from an LCL inhibit proliferation of T cells

Previous work from Dukers et al. (2000) has shown that peptides derived from LMP 1 were capable of inhibiting the proliferation of T cells. Since we have shown that LMP 1 is present on exosomes the possibility that LMP 1 containing exosomes might be able to inhibit T cells was evaluated. To assess this an in vitro T cell proliferation assay was performed in the presence of exosomes derived from an LCL. PBMCs were isolated from three healthy donors and were seeded at 5 × 10⁴ cells per well in triplicate and proliferation was stimulated with the mitogen PHA. Cells were treated with or without PHA as controls while test cells were incubated with exosomes derived from either the EBV-negative cell line DG75 or the EBV-positive LCL QIMR NB-B95-8. Cells were incubated for 3 days and proliferation was assessed using the MTT assay. The results presented in Fig. 5(C) are an average of three experiments. The proliferation of the cells treated with DG75 exosomes was reduced somewhat (80 % of that observed with the positive control) whereas cells incubated with QIMR NB-B95-8-derived exosomes showed a significant reduction in proliferation (40 % of that observed with the positive control). These results show a significant reduction in the T-lymphocyte proliferation when cultured in the presence of LCL-derived exosomes compared to the DG75 derived exosomes (P=0.00046) and indicate that LMP 1-containing exosomes may be capable of reducing T cell proliferation.

DISCUSSION

Analysis of LMP 1 expression in the LCLs QIMR NB-B95-8, CW-B95-8, NK Wil, SS Wil, AL Wil and AL Ag876, using immunofluorescence, revealed that LMP 1 localized to an intracellular compartment in approximately 30 % of cells while all cells contained LMP 1 at the plasma membrane. This heterogeneous distribution among individual cells was noted before (Boos et al., 1987; Rowe et al., 1987) but has received very little attention thus far and remains unexplained, although Rowe et al. (1987) suggested a possible relation with cell cycle stage in individual cells. Biochemical fractionation of an LCL revealed that almost all of the LMP 1 in cells was located in the membrane fraction, indicating that the intracellular LMP 1 was likely to be bound to intracellular membranes. Analysis of cells treated with cell cycle-blocking drugs showed that the distribution of LMP 1 within the cell was not changed throughout the

Fig. 4. Immunoelectron micrograph of NB-B95-8 cells labelled with MHC II and LMP 1 antibodies. NB-B95-8 cells were cryosectioned and the sections were collected on Formvar-coated copper grids and labelled with a mouse monoclonal anti HLA-DR (MHC II), the polyclonal rabbit anti-LMP antibody and 15 nm gold-conjugated anti mouse IgG and 10 nm PAG. (A) Micrograph of two cells, one that contains specific 10 nm LMP 1 foci colocalizing with 15 nm MHC II. Bar represents 200 nm. The nucleus, cytoplasm and plasma membrane borders of the two cells are shown. (B) 4 × magnification; bar represents 50 nm.
cell cycle. Co-labelling of cells with both the LMP 1 antibody and an antibody to the Golgi protein GS15 revealed that LMP 1 partly co-localized with the Golgi apparatus in a proportion of cells.

Immunoelectron microscopy was used to examine the ultrastructure of the regions that contained intracellular LMP 1 foci. The new LMP 1 rabbit polyclonal antibodies are an excellent tool for analysis of LMP 1 expression by immunoelectron microscopy. The only previous studies of LMP 1 by electron microscopy used monoclonal antibodies that were specific for a single epitope and employed fixed Araldite-embedded cells, which dramatically decreases the antigenicity of epitopes available for antibody binding (Laszlo et al., 1991). Using rabbit polyclonal LMP 1 antibodies and cryosectioning we have been able to detect far more LMP 1 in each cell, which has allowed a more detailed analysis of the intracellular staining pattern. Cryosections from the QIMR NB-B95-8 LCL immunolabelled with the rabbit polyclonal LMP 1 antibody revealed that in approximately two-thirds (9/15) of the cells LMP 1 localized to an intracellular compartment. The higher percentage of cells with intracellular LMP 1 detected using immunoelectron microscopy than was observed by immunofluorescence was likely due to the increased sensitivity of immunoelectron microscopy.

One possibility for intracellular localization of LMP 1 is as a reservoir. The intracellular LMP 1 aggregate resembles an intracellular reservoir previously described for the MHC costimulatory molecules CD80 and CD86 (Smyth et al., 1998). As LMP 1 mimics a constitutively active signalling molecule a reservoir of LMP 1 might allow it to be shuttled to the cell surface membrane when LMP 1 signalling is required in high amounts and then transported back to the reservoir when not required. Overexpression of LMP 1 in the DG75-EB0-EBNA 3,4,6 cell line resulted in an apparent increase in the percentage of cells containing intracellular LMP 1, supporting the possibility of the intracellular LMP 1 being a reservoir. Alternative to acting as a storage reservoir, LMP1 aggregates may represent ubiquinated clusters of proteins awaiting proteasomal digestion (Dantuma & Massucci,)

![Fig. 5. Immunoelectron micrograph of isolated exosomes from NB-B95-8 and DG75 cells. (A) Extracellular LMP 1-positive vesicles detected in cryosections. The DG75 EBO-EBNA 3,4,6 cell line was transfected with pMEP-LMP 1 and induced for 48 h. These cells and the NB-B95-8 LCL were then cryosectioned and labelled with the polyclonal rabbit anti-LMP antibody and 10 nm PAG. These micrographs show specific 10 nm LMP 1 localizing to 100 nm extracellular vesicles in both the LMP 1-expressing DG75-EB0-EBNA 3,4,6 cells (top panel) and NB-B95-8 LCL (bottom panel) cell populations. Bars represent 200 nm. (B) Exosomes that were isolated using differential centrifugation from the cell lines NB-B95-8 and DG75, spotted onto Formvar-coated copper grids and labelled with the polyclonal rabbit anti-LMP antibody and 10 nm PAG. LMP 1 localized to approximately 14% of the 40–100 nm exosomes derived from the NB-B95-8 LCL. Magnifications of some LMP 1-positive exosomes are shown; bar represents 200 nm. (C) T-lymphocyte proliferation assay with exosomes. PBMCs were isolated and seeded at 5 x 10^5 cells per well and stimulated with 20 µg PHA ml^-1. Cells were cultured without PHA (−PHA) as negative controls. Exosomes derived from DG75 or NB-B95-8 LCL were added to the cells, which were cultured for 3 days. Cell proliferation was analysed using an MTT assay. Results shown are an average of triplicate wells from three experiments with PBMCs isolated from three different donors. The average absorbance at 560 nm is shown (± SD).](http://vir.sgmjournals.org)
The intracellular LMP 1 foci also closely resemble, in size and structure, the intracellular localization of MHC class I and class II in the endocytic pathway (Benaroch et al., 1995; Chiu et al., 1999). MHC class II localizes to a specialized multivesicular body known as MHC class II compartments (MIICs). MIICs closely resemble lysosomes in that they contain lysosomal markers (LAMP1, CD63, β-hexosaminidase and cathepsin D), are positioned in the late endocytic pathway and are acidic (Sanderson et al., 1994). Previous data that suggested that LMP 1 may localize to lysosomes could in fact have detected MIICs. It is from these MIICs that MHC II-loaded exosomes are produced and on fusion of the MIIC with the plasma membrane these exosomes are released (Raposo et al., 1996). Thus there is the possibility that the intracellular LMP 1 pool may traffic together with the MHC molecules to these MIICs to be packaged and secreted in exosomes. Analysis of MHC II expression in the LCL showed co-localization with LMP 1 in 3/10 LMP 1 foci, which suggested that some of the intracellular LMP 1 localized to MIICs. The fact that not all LMP 1 foci co-localized with MHC class II could be due to either differences in the efficiency of binding of the monoclonal HLA DR antibody compared to the rabbit polyclonal LMP 1 antibody or differences in expression levels of LMP 1 and MHC class II.

LMP 1 has been shown to activate the Rho GTPase cdc42 (Puls et al., 1999). While this function has been suggested to be involved in the cytostatic effect of LMP 1 it may also be involved in the secretion of exosomes, as cdc42 has been shown to regulate the generation of different populations of transport vesicles from the trans-Golgi network (Musch et al., 2001). Thus, LMP 1 may activate cdc42 resulting in upregulation of the production of vesicles allowing LMP 1 to be exported.

In addition, LMP 1 was found to be associated with extracellular vesicles in cryosections and to be present in preparations of purified exosomes. Utilizing the serial dissection function of the confocal microscope, we demonstrated that a large proportion of LMP 1 localized to an intracellular membrane-bound compartment that extends through the cytoplasm and partially co-localizes with the Golgi network. Previous work utilizing Western blot analysis of differentially centrifuged cell supernatant suggested that LMP 1 may be associated with exosomes (Dukers et al., 2000). Electron microscopy data showed the presence of extracellular LMP 1-positive vesicles attached to the cell surface in cryosections of an EBV-positive LCL, and LMP 1 was found to be present in exosomes isolated from the culture fluid of an LCL.

Exosomes derived from antigen-presenting cells have been shown to stimulate the proliferation of both T- and B-lymphocytes while peptides derived from LMP 1 have been shown to inhibit the proliferation of T-lymphocytes (Dukers et al., 2000). EBV-positive cells may release exosomes, containing LMP 1, which could then inhibit the proliferation of T-lymphocytes in the surrounding microenvironment. Indeed exosomes, which were shown by electron microscopy to contain LMP 1, were isolated from the LCL QIMR NB-B95-8 and were capable of inhibiting the mitogen-stimulated proliferation of the PBMCs from three donors by 40% compared to exosomes derived from the DG75 cell line.

EBV-infected cells need to evade the immune system and remain viable for latent persistence or lytic replication. The data presented indicate that LMP 1 may be excreted from cells in exosomes and these exosomes could be involved in suppressing the immune system to allow the virus to survive. This might be relevant in the case of EBV-positive Hodgkin’s disease and nasopharyngeal carcinoma where tumour cells express LMP 1 and while LMP 1 encodes CTL epitopes these tumour cells are not killed by the infiltrating T-lymphocytes (Oudejans et al., 1997, 2002).

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REFERENCES


Localization of LMP 1 to exosomes


