Clin. exp. Immunol. (1991) **84**, 170–174

Internalization of MHC class I molecules is a prerequisite for endocytosis of endorphin by lymphocytes

A. M. MOMMAAS, M. C. WIJSMAN, W. VERDUIJN*, B. J. VERMEER & F. M. J. CLAAS* *Department of Dermatology and Laboratory for Electron Microscopy, and *Immunochemistry and Blood Bank, University of Leiden, Leiden, The Netherlands*

(Accepted for publication 26 November 1990)

SUMMARY

The nature of the interaction between γ-type endorphins and the HLA class I molecules was studied by immunoelectronmicroscopy. The HLA molecules were not involved in the actual binding of endorphin to the cell. In contrast, for the endocytosis of γ-endorphin, co-internalization of the HLA class I molecules is essential. The internalization process starts with clustering of γ-endorphin and HLA class I molecules in coated pits. Cells that do not carry HLA class I molecules (Daudi) or do not internalize HLA class I molecules (EBV-transformed B cells) bind but do not internalize γ-endorphin. On the basis of these observations, we suggest that the MHC class I molecules may function as transport molecules. Whether it is a general phenomenon that non-immunological ligands use the HLA class I molecules to get into the cell and immunological ligands (viral proteins) to reach the cell surface, remains to be established.

Keywords HLA endorphin endocytosis immunoelectromicroscopy

INTRODUCTION

Recent data show that MHC-encoded class I molecules are not only involved in immunological recognition processes but also in the interaction between non-immunological ligands, i.e. endoperoxidase–thromboxane (Curry, Messner & Johnson, 1984), insulin (Due, Simonsen & Olsson, 1986; Kittur *et al.*, 1987), γ-endorphin (Claas *et al.*, 1986) and their receptors. In T lymphocytes and blood monocytes/macrophages, MHC class I molecules are internalized via coated pits (Machy *et al.*, 1987; Dasgupta *et al.*, 1988) and show spontaneous recycling to the cell membrane (Tse & Pernis, 1984) as has been found for several membrane receptors (Ciechanover, Schwartz & Lodish, 1983; Goldstein *et al.*, 1985). The function of this internalization of class I molecules is not known yet. One could hypothesize that this process is functionally related to the uptake of peptides via receptor-mediated endocytosis. To test this hypothesis we have extended our studies on the interaction between γ-endorphin and HLA class I molecules (Claas *et al.*, 1986) with pre-embedding electron microscopy and immunoelectron microscopy.

Correspondence A. M. Mommaas, PhD, Department of Dermatology, University Hospital Leiden, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.

MATERIALS AND METHODS

Cells and antibodies

Peripheral blood mononuclear cells were obtained from heparinized blood by Ficoll–Isopaque gradient centrifugation. Epstein–Barr virus (EBV) transformed B cell lines were made according to our standard protocol (Mensink *et al.*, 1986): peripheral blood mononuclear cells were incubated with supernatant from an EBV-infected marmoset cell line (B 95·8) for 1 h at 37°C. The mixture was transferred to a 24-well Costar plate (Costar, Cambridge, MA) and 2 ml of culture medium were added. Medium was refreshed every 3 days. The cell lines were tested after 3–4 weeks of culture.

Phytohaemagglutinin (PHA) blasts were prepared in 24-well Costar plates by incubating 10^6 peripheral blood mononuclear cells with 1% of PHA (Wellcome Diagnostics, Beckenham, UK) in 2 ml of culture medium for 4 days. *Mycobacterium leprae*-specific T cell clones were obtained by limiting dilution as described by Ottenhof *et al.* (1986).

For the detection of the HLA class I antigens, the monoclonal antibodies W6/32, B.9.12.1 and B.1.1G6. were used (Rebai & Malissen, 1983).

Conjugation of des-Tyr-γ-endorphin to colloidal gold

Colloidal gold particles with a diameter of 10 nm were prepared according to Slot & Geuze (1985). The endorphin (kind gift of

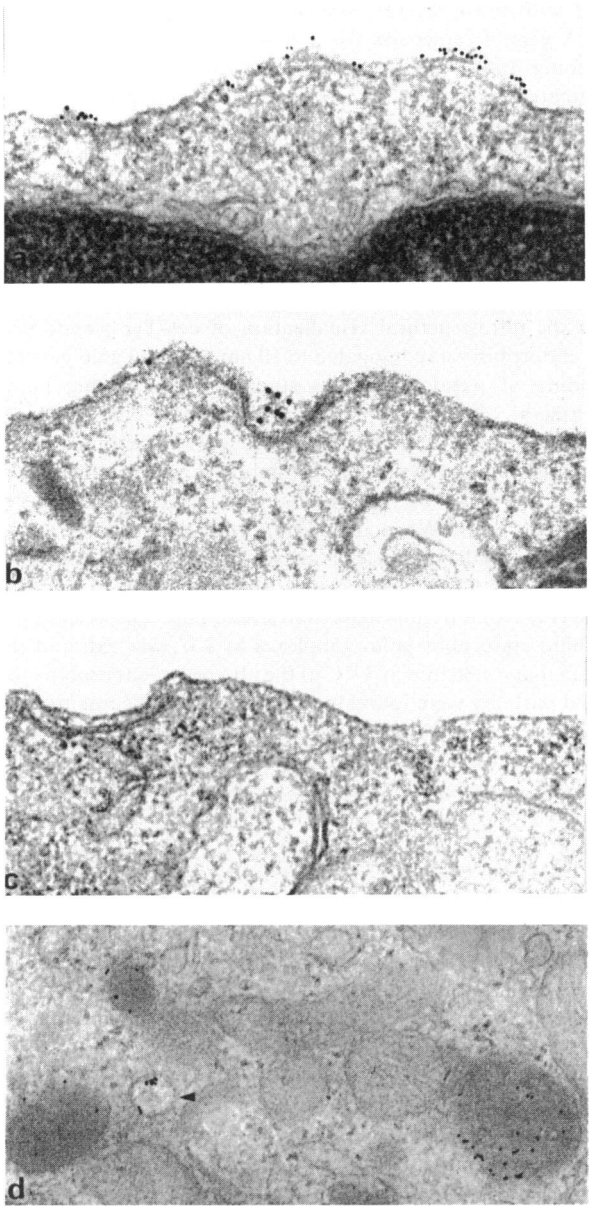

Fig. 1. Electron micrographs of the binding and uptake of des-Tyr-γ-endorphin by freshly isolated human lymphocytes. The cells were incubated with endorphin–gold and processed for electron microscopy as described in Materials and Methods. (a) Binding of endorphin–gold complexes in a heterogeneous fashion along the plasma membrane of a lymphocyte, incubated for 1 h at 8°C (magnification ×47 000); (b) detail of the plasma membrane, showing a coated pit containing endorphin–gold complexes (magnification ×77 000); (c) competition experiment (control) in which no gold particles were found along the plasma membrane (magnification ×47 000); (d) internalized endorphin–gold particles are found in electron lucent endosomes (arrowhead) or in dense structures, probably lysosomes (magnification ×38 000).

Dr J. M. van Ree) was conjugated to colloidal gold as described for low-density lipoproteins (Mommaas-Kienhuis *et al.*, 1985) at a concentration of 1 μg/ml colloidal gold. The excess of unlabelled endorphin was removed by centrifugation for 1 h at 25 000 ***g***.

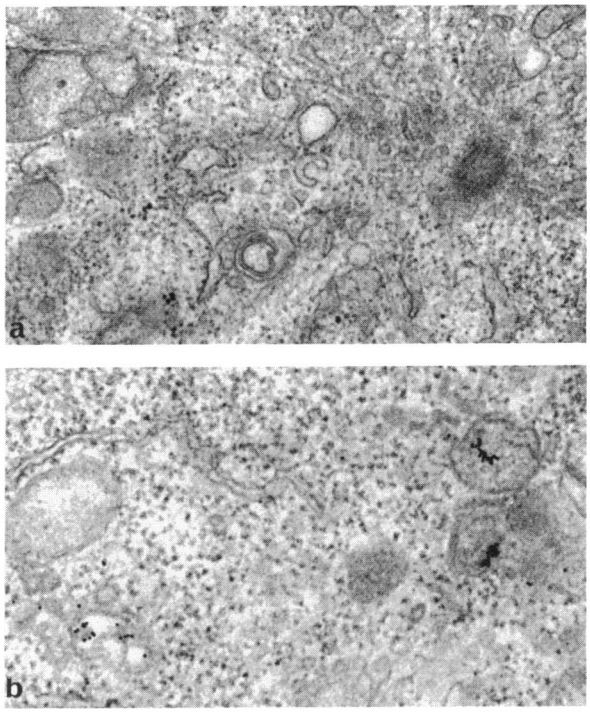

Fig. 2. Electron micrographs of the interaction of endorphin–gold with B and T lymphocytes. The cells were incubated with endorphin–gold and processed for electron microscopy as described in Materials and Methods. (a) Detail of a B lymphocyte showing no internalization of endorphin–gold, when incubated at 37°C for 30 min (magnification ×40 000); (b) detail of a T lymphocyte in which endorphin–gold particles are found in endosomes and dense bodies (magnification ×40 000).

Incubation procedures

The cells were incubated with endorphin–gold at a final concentration of 4 μg/ml, for 1 h at 8°C, for binding studies. In internalization experiments the cells were allowed to bind endorphin–gold for 1 h at 8°C, washed with phosphate-buffered saline (PBS) and warmed to 37°C for 30 min. In control experiments the cells were incubated with a 100-fold excess of unlabelled endorphin, added to the endorphin–gold complexes.

Electron microscopy

Nine peripheral blood mononuclear cell samples from seven healthy donors and four samples of both T and B cell lines were processed for electron microscopy. After incubation procedures the cells were washed twice with PBS, fixed with 1·5% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4, for 10 min at room temperature, post-fixed with 1% osmiumtetroxide with 0·05 M potassium hexacyanoferrate, dehydrated in a graded ethanol series up to 70% and embedded in epon. Ultra-thin sections were stained with uranyl acetate and lead hydroxide.

Immunoelectron microscopy

Eight peripheral blood mononuclear cell samples were studied using the immunoelectron microscopy technique. After incubation with endorphin the cells were fixed with 1% paraformaldehyde in 0·1 M phosphate buffer. Specimen processing and the preparation of ultracryosections were performed according to Tokoyasu (1973). The ultracryosections were incubated 'on

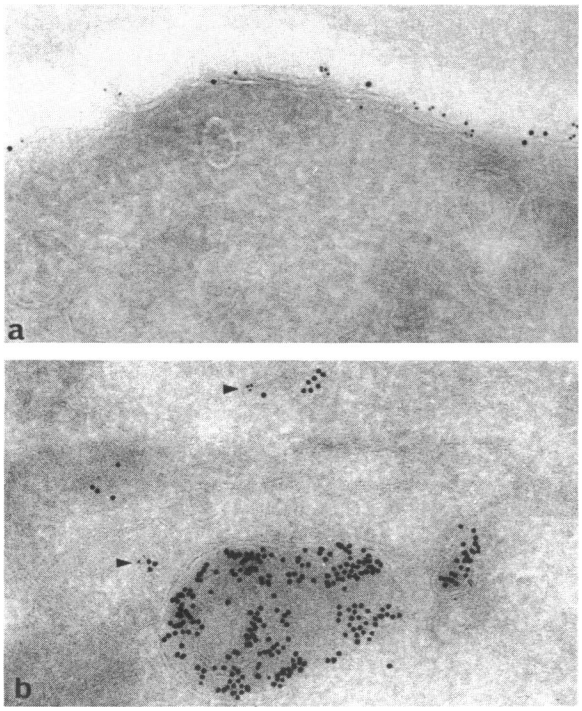

Fig. 3. Ultracryosections of freshly isolated human lymphocytes with a double labelling of endorphin (10 nm gold) and HLA class I molecules (5 nm gold). Cells were incubated with endorphin conjugated to 10 nm for 10 min at 37°C prior to fixation. Fixation, prepartion of ultracryosections and incubation with antibodies, were performed as described in Materials and Methods. (a) Along the plasma membrane the endorphin (indicated by 10 nm gold) and the HLA class I molecules (5 nm gold) are distributed in no specific correlation (magnification × 68 000); (b) the endorphin–gold-containing endosomal structures are often associated with small vesicles containing HLA class I molecules (arrowheads) (magnification × 68 000).

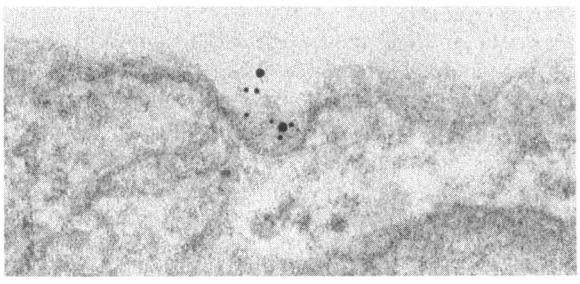

Fig. 4. Detail of a cultured human umbilical endothelial cell, showing a coated pit with endorphin (10 nm gold) and HLA class I molecules (5 nm gold). Cells were incubated with endorphin–gold for 1 h at 8°C. After washing with PBS, they were further incubated with a monoclonal antibody against HLA class I molecules and a goat anti-mouse antibody conjugated to 5 nm coloidal gold. The cells were then fixed and processed for electron microscopy as described in Materials and Methods (original magnification × 13 700).

grid' with one of several monoclonal antibodies directed against HLA class I molecules for 1 h at room temperature. After washing three times with PBS–glycine, the sections were incubated with a goat anti-mouse antibody conjugated to 5 nm colloidal gold (Janssens Life Science Products) for 1 h at room temperature. After washing with distilled water the sections were embedded and stained with methylcellulose and uranylacetate.

RESULTS

For the ultrastructural visualization of des-Tyr-γ-endorphin, the endorphin was conjugated to 10 nm colloidal gold particles. Binding of γ-endorphin was studied by incubating human peripheral blood lymphocytes at 8°C with endorphin–gold complexes. Many gold particles were found distributed heterogeneously along the plasma membrane (Fig. 1a), sometimes in coated pits (Fig. 1b). No colloidal gold particles were observed inside the cells. When incubation with endorphin–gold was performed in the presence of an excess of unlabelled endorphin, binding of colloidal gold particles to the cell surface was rarely seen (Fig. 1c). For internalization studies, the cells were allowed to bind endorphin–gold complexes at 8°C, washed, and then incubated for 30 min at 37°C in the absence of endorphin–gold. Gold particles were found in various endocytic compartments, i.e. small vesicles, larger, electron lucent endosomes and dense bodies, probably lysosomes (Fig. 1d). In all the preparations that were screened, these features were observed. However, each sample also contained a subset of cells that did not react with γ-endorphin–gold.

When EBV-transformed B cells and PHA- or antigen (*M. leprae*) stimulated T cells were incubated in a similar fashion, both cell types showed binding of endorphin–gold. However, no uptake of gold particles took place by the B cells (Fig. 2a), whereas the T cells internalized the endorphin–gold in a way similar to that found for the peripheral blood lymphocytes (Fig. 2b; compare with Fig. 1d).

On ultracryosections of freshly isolated human lymphocytes that were incubated with endorphin and anti-MHC class I antibodies, both endorphin and MHC class I molecules were found to be distributed heterogeneously along the plasma membrane. No specific co-localization of the two molecules was observed (Fig. 3a). When the endorphin was internalized into endosomes and dense bodies, these endocytic structures were often associated with small vesicles containing MHC class I molecules (Fig. 3b).

In binding experiments of endorphin and MHC class I molecules to cultured human endothelial cells, co-localization of the two molecules was found in coated pits (Fig. 4).

DISCUSSION

Using colloidal gold as an ultrastructural marker we were able to visualize binding and internalization of des-Tyr-γ-endorphin by human peripheral blood lymphocytes (Fig. 1).

In all these binding and internalization experiments, however, a subset of the investigated lymphocytes showed hardly any conjunction with the endorphin–gold complexes. Recently, it was demonstrated that B and T lymphocytes differ in the processing of MHC class I molecules. Activated T lymphocytes recycle MHC class I molecules spontaneously, without cross-

linking by antibodies or other agents (Tse & Pernis, 1984; Pernis, 1985). Electron microscopy studies showed that internalization of MHC class I molecules in these cells takes place via coated pits (Machy *et al.*, 1987). The same phenomenon was found for monocytes (Dasgupta *et al.*, 1988). B lymphocytes, on the other hand, do not internalize the MHC class I molecules they have on their membrane (Pernis, 1985). In order to investigate whether the diversity in endorphin–gold labelling on peripheral blood lymphocytes reflects a difference of reactivity of B and T cells, we applied the same gold labelling methods on B and T lymphoid cell lines. EBV-transformed B cells showed a little binding but no uptake of gold particles when incubated with endorphin–gold at 8°C or at 37°C (Fig. 2a). In contrast, PHA- or antigen (*M. leprae*) stimulated T cells showed significant internalization of endorphin–gold (Fig. 2b). To elucidate further the role of MHC class I molecules in the receptor-mediated uptake of endorphin by T lymphocytes, a combined pre-embedding and immunoelectron microscopical technique was used. Peripheral blood lymphocytes were incubated with endorphin–gold (10 nm) as described above. After fixation, ultracryosections were prepared and subsequently labelled with monoclonal antibodies directed against MHC class I molecules. These antibodies were visualized with a second antibody conjugated to 5 nm colloidal gold. This approach excludes a possible induction of internalization of the MHC class I molecules by cross-linkage with antibody. Both the binding to plasma membrane and the internalization of endorphin were investigated in this way. At the cell surface both endorphin and MHC class I molecules were found distributed heterogeneously in no specific connection (Fig. 3a). However, in internalization experiments, when the cells were incubated with endorphin–gold for 10 min at 37°C, these endorphin–gold particles were found in dense bodies. These dense bodies were generally associated with small vesicles containing MHC class I molecules labelled with 5 nm gold particles (Fig. 3b). In other experiments on cultured human umbilical endothelial cells, we found co-localization of γ-endorphin and HLA class I molecules in coated pits (Fig. 4). This suggests that the role of MHC class I molecules in endocytosis starts when the ligand–receptor complexes cluster in coated pits, an early stage in the internalization process. Then, after co-internalization, MHC class I molecules are shielded in endosomes from transport to and degradation in lysosomes, and recycled to the plasma membrane. This phenomenon of shielding and recycling has been described for various ligand–receptor interactions (Anderson *et al.*, 1982; Geuze *et al.*, 1983), where the receptors are recycled to the cell surface for re-use. In those systems the acidic environment of endocytic vesicles plays an important role in the uncoupling of ligand and receptors (and the subsequent recycling of the receptor). Tse & Pernis (1984) described intracytoplasmic vesicles with MHC class I molecules that are formed by internalization from the plasmamembrane. Recycling of these molecules to the cell surface is sensible to agents which inhibit the acidification of endocytic vesicles.

In conclusion, our data suggest that the recycling process of HLA class I molecules is related to a transport function of these molecules. After binding of γ-endorphin to its receptor, this complex is co-internalized with HLA molecules. B cells, which do not internalize MHC class I molecules (Pernis, 1985), are not able to internalize endorphin. In addition, Daudi, a cell line lacking expression of HLA class I molecules, showed no internalization of endorphin (unpublished data). It remains to be established whether the recycling process of the class I molecules is also functionally related to observed interaction with other non-immunological (insulin) and immunological (viral antigens) ligands. One could speculate that the class I molecules are involved in the internalization of several hormone receptor complexes into the cell, whereas their function in relation to immunological ligands is the transport from the intracellular compartments to the cell membrane. Whether the polymorphism of the class I molecules is also reflected in these processes is being studied.

ACKNOWLEDGMENTS

We thank Mrs G. W. Roest, Mr L. D. C. Verschragen and Mr J. J. Beentjes for expert technical assistance, and Prof. J. J. van Rood for his critical reading of the manuscript.

REFERENCES

ANDERSON, R.G.W., BROWN, M.S., BEISIEGEL, U. & GOLDSTEIN, J.L. (1982) Surface distribution and recycling of the low density lipoprotein receptor as visualized with anti-receptor antibodies. *J. Cell Biol.* **93,** 523.

CIECHANOVER, A., SCHWARTZ, A.L. & LODISH, H.F. (1983) The asialoglycoprotein receptor internalizes and recycles independently of the transferrin and insulin receptors. *Cell*, **32,** 267.

CLAAS, F.H.J., VAN REE, J.M., VAN DER POEL, J.J., VERDUYN, W., DE WIED, D. & VAN ROOD, J.J. (1986) The interaction between gamma-type endorphin and HLA class I antigens. *Hum. Immunol.* **15,** 347.

CURRY, R.A., MESSNER, R.P. & JOHNSON, G.J. (1984) Inhibition of platelet aggregation by monoclonal antibody reactive with β_2-microglobulin chain of HLA. *Science*, **224,** 509.

DASGUPTA, J.D., WATKINS, S., SLAYTER, H. & YUNIS, F.J. (1988) Receptor-like nature of class-I HLA: endocytosis via coated pits. *J. Immunol.* **141,** 2577.

DUE, D., SIMONSEN, M. & OLSSON, L. (1986) The major histocompatability complex class I heavy chain as a structural subunit of the human cell membrane insulin receptor: implications for the range of biological functions of histocompatibility antigens. *Proc. natl Acad. Sci. USA*, **83,** 6007.

GEUZE, H.J., SLOT, J.W., STROUS, G.J.A.M., LODISH, H.F. & SCHWARTZ, A.L. (1983) Intracellular site of asialogylcoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. *Cell*, **32,** 277.

GOLDSTEIN, J.L., BROWN, M.S., ANDERSON, R.G.W., RUSSELL, D.W. & SCHNEIDER, W.J. (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Ann. Rev. Cell Biol.* **1,** 1.

KITTUR, D., SHIMIZU, Y., DE MARS, R. & EDIDIN, M. (1987) Insulin binding to human B lymphoblasts is a function of HLA haplotypes. *Proc. natl Acad. Sci. USA*, **84,** 1351.

MACHY, P., TRUNEK, A., GRANNARO, D. & HOFFSTEIN, S. (1987) Major histocompatibility complex class I molecules internalized via coated pits in T lymphocytes. *Nature*, **328,** 724.

MENSINK, E.J.B.M., SCHUURMAN, R.K.B., SCHOT, S.D.L., THOMPSON, A. & ALT, F.W. (1986) Immunoglobulin heavy chain rearrangements in X-linked agamma-globulinemia. *Eur. J. Immunol.* **16,** 963.

MOMMAAS-KIENHUIS, A.M., KRIJBOLDER, L.H., VAN HINSBERGH, V.W.M., DAEMS, W.T. & VERMEER, B.J. (1985) Visualization of binding and receptor-mediated uptake of low density lipoproteins by human endothelial cells. *Eur. J. Cell Biol.* **36,** 201.

OTTENHOF, T.H.M., KLATSER, P.R., IVANY, J., ELFERINK, L., DE WIT, M.Y.L. & DE VRIES, R.R.P. (1986) Mycobacterium leprae specific protein antigens defined by cloned human helper T cells. *Nature*, **319,** 66.

PERNIS, B. (1985) Internalization of lymphocyte membrane components. *Immunol. Today*, **6,** 45.

REBAI, N. & MALISSEN, B. (1983) Structural and genetic analysis of HLA class I molecules using monoclonal antibodies. *Tissue Antigens*, **22,** 107.

SLOT, J.W. & GEUZE, H.J. (1985) A new method for preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* **38,** 87.

TOKOYASU, K.T. (1973) A technique for ultracryotomy of cell suspensions and tissues. *J. Cell Biol.* **57,** 551.

TSE, D.B. & PERNIS, B. (1984) Spontaneous internalization of class I major histocompatibility complex molecules in T lymphoid cells. *J. exp. Med.* **159,** 193.