LYMPHOCYTIC CHORIOMENINGITIS VIRUS MX STRAIN DOES NOT INDUCE THE EXPRESSION OF TUMOR-ASSOCIATED CARBONIC ANHYDRASE IX IN PERSISTENTLY INFECTED HELA CELLS

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Summary. – Lymphocytic choriomeningitis virus (LCMV) is an arenavirus that readily causes persistent infections, in which it does not interfere with vital functions of the cells, but can affect expression of "luxurious" genes. MX strain of LCMV (MX LCMV) has been identified as an agent transmissible by cell-to-cell contact in the human carcinoma MaTu cells grown in a mixed culture with HeLa cells. When compared to uninfected HeLa, the MaTu-MX-infected HeLa cells, to which the virus was transmitted via co-cultivation with mitomycin C-treated MaTu cells, showed an elevated expression of a protein called MN, suggesting that MN can be induced by MX LCMV. MN protein was later identified as the carbonic anhydrase isoform IX (CA IX), whose expression has been predominantly associated with hypoxic tumors of poor prognosis. Since the proposal that MX LCMV can induce such a cancer-related protein could substantially change our view on the biology of LCMV-host interaction we undertook its verification. Instead of co-cultivation, we used MaTu cell-free extracts to transmit MX LCMV to HeLa cells. These cells were then grown for more than 30 passages, but the level of MN/CA IX did not increase throughout the whole cultivation period as compared to uninfected HeLa cells. Moreover, a treatment of MaTu-MX-infected HeLa cells with ribavirin eliminated the virus, but did not reduce the MN/CA IX expression. Our results clearly showed that MN/CA IX is independent of MX LCMV and that the virus itself does not influence the MN/CA IX level in HeLa cells.

Key words: lymphocytic choriomeningitis virus; MX strain; persistent infection; HeLa cells; MaTu cells; carbonic anhydrase IX

Introduction

LCMV (the species *Lymphocytic choriomeningitis virus*, the genus *Arenavirus*) is a rodent-borne agent causing life-long persistent infection of its major host *Mus musculus*. Humans are infected mainly by inhalation of aerosolized rodent excreta, or through ingestion or direct contact with rodent urine, faces and saliva (Childs and Wilson, 1994). LCMV can cause different human diseases, ranging in severity from flu-like illness to meningitis and encephalitis (Buchmeier and Zajac, 1999). Intrauterine infection can result in choriomeningitis, hydrocephalus and fetal death (Jährling and Peters, 1992; Barton and Mets, 2001).

LCMV, like other arenaviruses, is an enveloped virus containing bisegmented single-stranded RNA genome with an ambisense arrangement (Southern, 1996). The small (S) segment encodes major structural proteins: a nucleoprotein (NP) at the 3'-end in negative sense and a glycoprotein precursor (GPC) at the 5'-end in positive sense. The large (L) segment encodes RNA-dependent RNA polymerase at the 3'-end in negative sense and a zinc-binding ring finger Z protein at the 5'-end in positive sense. Unique replication strategy of LCMV involves independent expression of NP

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Abbreviations: CA = carbonic anhydrase; FCS = fetal calf serum; GH = growth hormone; GPC = glycoprotein precursor; LCMV = lymphocytic choriomeningitis virus; MAb = monoclonal antibody; NP =nucleoprotein and GPC thereby allowing for establishment of viral persistence with high expression of NP and low or undetectable expression of GPC (Oldstone and Buchmeier, 1982; Bruns *et al.*, 1990). Persistent LCMV infection readily occurs both *in vivo* and *in vitro*. Since the virus replicates in a wide variety of cell types without any signs of cytolysis or cytopathic effect, it clearly does not interfere with vital "housekeeping" cell functions. However, there are several examples suggesting that LCMV is able to modulate expression of so called "luxurious" genes whose products contribute to differentiated cell functions. Persistent LCMV infection in C3H/ST mice is associated with a growth hormone (GH) deficiency due to markedly reduced transcription initiation of *GH* gene in the absence of cellular injury (Oldstone *et al.*, 1982; Klavinskis and Oldstone, 1989). A decreased level of GH mRNA is also expressed in a rat pituitary cell line persistently infected with LCMV (de la Torre *et al.*, 1992). LCMV persistence in thyroid epithelial cells can perturb thyroid hormone production (Klavinskis and Oldstone, 1987). Furthermore, persistent infection of mouse neurons with LCMV results in a downregulation of GAP43, a protein involved in neuronal plasticity associated with learning and memory (Cao *et al.*, 1997).

In addition, persisting LCMV, which was isolated from the human carcinoma MaTu cell line grown in a mixed culture with HeLa cells and named MX, was proposed to induce expression of at that time novel protein MN (Pastoreková *et al.*, 1992; Závada *et al.*, 1993; Reiserová *et al.*, 1999; Gibadulinová *et al.*, 1998). In those experiments, MaTu cells of presumably mammary tumor origin (Widmaier *et al.*, 1974) were treated with mitomycin C and co-cultivated with cervical carcinoma HeLa cells. During the co-cultivation, MX LCMV was transmitted from MaTu to HeLa cells via direct cell-to-cell contact and the infection spread in HeLa cells throughout several subsequent passages (Závada and Závadová, 1991). These MaTu-MX-infected HeLa cells were then found to contain a highly elevated level of MN protein whose expression correlated with high cell density, tumorigenic phenotype of HeLa x fibroblast hybrids and human tumors *in vivo* (Pastoreková *et al.*, 1992; Závada *et al.*, 1993). The MN cDNA and MN gene were subsequently cloned and, based on the presence of a conserved carbonic anhydrase (CA) domain, MN was identified as the transmembrane CA IX isoform (Pastorek *et al.*, 1994; Opavský *et al.*, 1996). In the following years, expression of MN/CA IX has been found clearly associated with various types of carcinomas (especially those of poor prognosis) mostly due to its induction by tumor hypoxia (Wykoff *et al.*, 2000; Potter and Harris, 2003). Moreover, MN/CA IX has been causally implicated in tumor progression via its capacity to modulate cell adhesion and to control pH in hypoxic cells (Švastová *et al.*, 2003, 2004; Závada *et al.*, 2000; Robertson *et al.*, 2004).

Therefore, the original proposal that MX LCMV increases the expression of MN/CA IX could gain a new dimension, as the virus would then potentially represent an unexpected risk factor when persistently infecting a tumor patient. That was the reason, why we decided to verify the relationship between MX LCMV and MN/CA IX. Our data presented here argue against this proposal and support the view that the expression of MN/CA IX is independent of MX LCMV.

Materials and Methods

Cell culture. MaTu cells, transfer of MX LCMV to HeLa cells by co-cultivation and resulting MaTu-MX-infected HeLa cells have been described earlier (Závada and Závadová, 1991). The cells were cultured in DMEM medium (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) at 37°C in the presence of 5% CO_2. The cultures were maintained at high cell density to allow for easier virus transmission via cell-to-cell contact. MaTu cell-free extract prepared by disruption of the cells in a hypotonic buffer followed by sonication and three cycles of freezing-thawing (van der Zeijst *et al.*, 1983) was used for infection of HeLa cells (Reiserová *et al.,* 1999).

Monoclonal antibodies (MAbs) were produced and characterized by Pastoreková *et al.* (1992). While MAbs M16 and M67 were used for detection of LCMV NP, MAb M75 was specific for human MN/CA IX protein.

Ribavirin treatment. MaTu-MX-infected and uninfected HeLa cells were plated at a density of 1 x 10^5 cells per 3 cm dish in duplicate and allowed to adhere for 8 hrs. An antiviral drug, ribavirin (Sigma) that can cure LCMV infection was dissolved in dimethyl sulphoxide (DMSO) and added to one parallel of each culture at final concentration of 50 μg/ml for 72 hrs according to de la Torre *et al.* (1992). Another parallel of each culture was treated with the corresponding concentration of DMSO (negative control). The cells were allowed to recover for 24 hrs and the same treatment was repeated twice more. Presence of viral NP as an indicator of MX LCMV infection was monitored by immunofluorescence test.

Immunofluorescence analysis. Cells were fixed with methanol at -20°C for 5 mins, washed and treated for 1 hr at 37°C with the respective MAb in undiluted hybridoma medium followed by a FITC-conjugated swine anti-mouse IgG (Vector Laboratories, USA) diluted 1:300. The cells were washed, mounted in an antibleach medium containing Citifluor (Agar Scientific, UK) and analyzed by a Nikon E400 epifluorescence microscope.

Western blot analysis. Cells were lysed in cold RIPA buffer as described by Pastoreková *et al.* (1992). Total protein was assayed in all extracts using the BCA kit (Pierce, USA). Aliquots containing 125 μg of total cell protein per sample were subjected to SDS-PAGE (10% gel) and the resolved proteins were blotted to PVDF membrane. The blots were incubated in 5% milk for 30 mins followed by MAb M75 in hybridoma medium diluted 1:2 for 1 hr, washed, incubated with a HRP-conjugated anti-mouse IgG diluted 1:2,000 (Sevapharma, Czech Republic) and developed using the enhanced chemiluminiscence ECL kit (Amersham Pharmacia Biotech, UK).

ELISA. Microplate wells were coated overnight at 37°C with the corresponding aliquots of cell extracts diluted in PBS. After blocking with 10% FCS in PBS, the coated wells were incubated with the respective MAb in undiluted hybridoma medium for 1 hr at room temperature, washed and treated with a HRP-conjugated swine anti-mouse IgG diluted 1:5,000 in 10% FCS in PBS for 1 hr at room temperature. After washing, a substrate solution (10 ml of Mc Ilwaine buffer pH 5.5 contaning100 mmol/l Na_2HPO_4 and 40 mmol/l citric acid, 10 mg of *ortho*-phenylenediamine and 10 µl of 30% H_2O_2) was added to each well and the plates were incubated for 5–10 mins in dark. The reaction was stopped by adding of 2 mmol/l H_2SO_4 and A_{492} was read in ELISA reader (Labsystems, Finland).

Flow cytometry. Cells were washed with PBS, scraped and fixed in 70% ethanol at -20°C for 30 mins. After rehydration, the cells were incubated with the respective MAb in hybridoma medium diluted 1:10 in PBS containing 10% FCS for 30 mins at 4°C, followed by a FITC-conjugated secondary antibody (Vector Laboratories, USA) diluted 1:200. The analysis was performed using a Becton-Dickinson FACScan flow cytometer. A total of 10^4 cells were analyzed per sample.

Results and Discussion

Expression of MN/CA IX in HeLa cells infected with MX LCMV

MX LCMV has been suggested to induce MN/CA IX protein on the basis of the data obtained using MaTu-MX-infected HeLa cells, which were generated by co-cultivation of HeLa cells with mitomycin C-treated MaTu cells (Závada and Závadová, 1991; Pastoreková *et al.*, 1992; Závada *et al.*, 1993). These MaTu-MX-infected HeLa cells consistently showed a highly increased level of MN/CA IX irrespective of whether they were plated at low or high density. In contrast, the expression of MN/CA IX was absent from the sparse uninfected HeLa cells and was only detectable at intermediate level in dense HeLa cells. Thus, high expression of MN/CA IX seemingly correlated with the presence of MX LCMV-specific protein corresponding to the NP of persisting LCMV.

However, our later experiments suggested that the treatment of MaTu cells with 5 µg/ml mitomycin C for 3 hrs as used by Závada and Závadová (1991) might not be sufficient to achieve an irreversible mitotic arrest of MaTu cells and their subsequent elimination from the co-culture. This raised a possibility that a part of original MaTu cells could survive the treatment and that these cells could in fact become a source of an increased level of MN/CA IX found in MaTu-MX-infected HeLa cells in our previous studies. Therefore, we decided to accomplish a MX LCMV infection using MaTu cell-free extracts. This transfer of MX LCMV to uninfected HeLa cells is feasible and the virus can be detected in the culture approximately after two passages (Reiserová *et al.*, 1999). In this study, we enabled the MX LCMV to spread thoroughly in HeLa cells throughout 10 passages and then we followed its effect on the expression of MN/CA IX protein. At passage 10, we used flow cytometry to show that the percentage of MN/CA IX-positive cells was high in MaTu-MX-infected HeLa cells (obtained by co-cultivation), whereas it was low in uninfected as well as MX LCMV-infected HeLa cells (obtained by the cell-free extract) in spite of high prevalence of cells expressing viral NP in both kinds of cell cultures (Fig. 1A). The ELISA analysis of expression of MN/CA IX and viral NP at different passages revealed that the prolonged presence of the virus did not have any significant positive effect on the level of MN/CA IX in MX LCMV-infected HeLa cells. In contrast, MaTu-MX-infected HeLa cells showed again a higher level of MN/CA IX despite a somewhat lower expression of viral NP compared to MX-infected HeLa cells (Fig. 1B). Western blot analysis of MN/CA IX corroborated the data obtained by ELISA (Fig. 1C). Finally, immunofluorescence analysis of the cells at passage 50 using specific MAbs clearly showed that the membrane MN/CA IX staining did not correspond to the presence of cytoplasmic viral NP staining, suggesting that the infection with MX LCMV is not related to MN/CA IX expression (Fig. 1D).

Expression of MN/CA IX in MaTu-MX-infected HeLa cells treated with ribavirin

In an alternative approach, we used an antiviral drug, ribavirin to eliminate persistent MX LCMV infection from MaTu-MX-infected HeLa cells in order to see whether the absence of the virus can reduce the level of MN/CA IX. The cells were subjected to three cycles of treatment for 72 hrs and the virus elimination was monitored by ELISA with MAb M16 (data not shown). The same treatment was applied to uninfected HeLa cells in order to check for the effect of the drug *per se* on the expression of MN/CA IX. The treatment with ribavirin led to a complete removal of the virus from the infected cells, but it did not influence the expression of MN/CA IX as demonstrated by ELISA analysis (Fig. 2A. The same results were obtained by Western blot and immunofluorescence analyses (Fig. 2B, C). The lack of viral NP affected neither the distribution nor the intensity of the staining signal for MN/CA IX (Fig. 2C). Hence, these results are in accord with the above suggestion that MX LCMV is not involved in the regulation of MN/CA IX expression.

MN/CA IX is independent of MX LCMV

Altogether, the experiments performed in this work clearly do not conform with the previous conclusion that MX LCMV is a potent inducer of MN/CA IX, but rather suggest that both entities exist independently of each other.

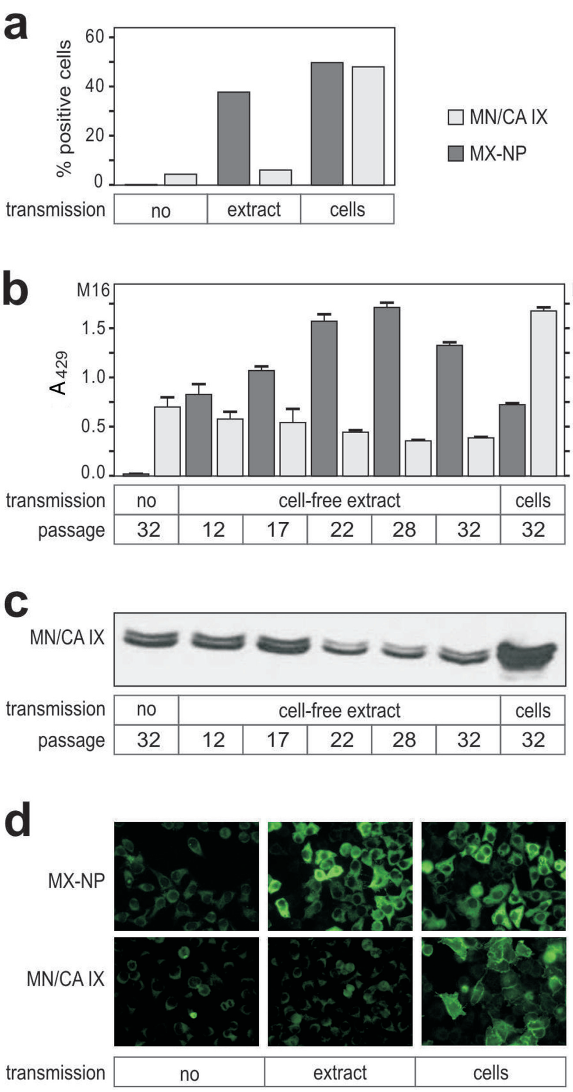

Fig. 1

MN/CA IX expression in HeLa cells infected with MX LCMV via MaTu cell-free extract

Expression of MN/CA IX was examined in uninfected HeLa cells (no transmission), MX-infected HeLa (transmission by MaTu cell-free extract) and MaTu-MX-infected-HeLa cells (transmission by co-cultivation with MaTu cells) at different passages by various immunodetection methods: flow cytometry at passage 10 following transmission (a), ELISA (b) and Western blot analysis (c) at several passages ranging from 12 to 32, and immunofluorescence analysis at passage 50 (d).

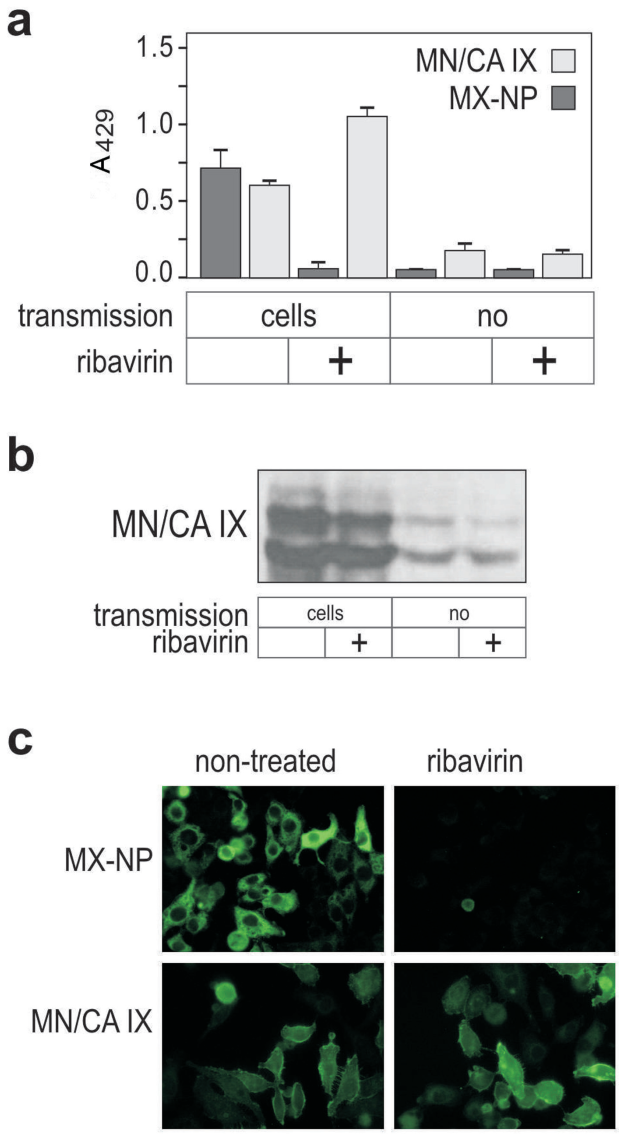

Fig. 2

MN/CA IX expression of MaTu-MX-infected HeLa cells treated with ribavirin

Ribavirin-treated and untreated sparse MaTu-MX-infected HeLa cells were examined for the presence of viral NP and MN/CA IX by ELISA (a), Western blot (b) and immunofluorescence analyses (c). Uninfected HeLa cells (no virus transmission) were used as negative control (a, b).

This is supported also by the earlier data showing that MX LCMV can be transmitted to cells that do not express MN/CA IX (including human fibroblasts and non-tumorigenic HeLa x fibroblast hybrids) without any inducing effect and that MN/CA IX is expressed in a number of cell lines in the absence of LCMV (Závada *et al.*, 1993).

Nevertheless, the original idea was not inconceivable, taking into account several pieces of existing information concerning contribution of viruses to carcinogenesis (zur Hausen, 1991) and effects of LCMV on expression of some non-essential genes in persistently infected cells and tissues (Buchmeier and Zajac, 1999). It seemed quite plausible that a long-lasting LCMV infection of tumor cells could have some inapparent consequences leading to modulation of their behavior. Although the direct effect of MX LCMV on expression of the tumor-associated MN/CA IX protein has been now disproved, it is still possible that the virus has other important targets that remain to be uncovered. However, currently available data do not provide any supporting evidence for the involvement of persisting LCMV in induction of any cancer-related genes.

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