Measles Virus Infection Induces Terminal Differentiation of Human Thymic Epithelial Cells

HÉLÈNE VALENTIN,1* OLGA AZOCAR,1 BRANKA HORVAT,1 REJANE WILLIEMS,2 ROBERT GARRONE,2 ALEXEI EVLASHEV,1,3 MARIA L. TORIBIO,4 AND CHANTAL RABOURDIN-COMBE1

Laboratoire d’Immunobiologie Fondamentale et Clinique, INSERM U503, ENS de Lyon, 69364 Lyon Cedex 07,1 and Institut de Biologie et Chimie des Protéines, UPR 412-CNRS, 69367 Lyon Cedex 07.2 France; Institute of Experimental Medicine, RAMS, 197376 Saint Petersburg, Russia;3 and Centro de Biológica Molecular Severo Ochoa, Universidad Autónoma de Madrid, Facultad de Ciencias, Campus de Cantoblanco, 28049 Madrid, Spain4

Received 3 August 1998/Accepted 7 December 1998

Measles virus infection induces a profound immunosuppression that may lead to serious secondary infections and mortality. In this report, we show that the human cortical thymic epithelial cell line is highly susceptible to measles virus infection in vitro, resulting in infectious viral particle production and syncytium formation. Measles virus inhibits thymic epithelial cell growth and induces an arrest in the G0/G1 phases of the cell cycle. Moreover, we show that measles virus induces a progressive thymic epithelial cell differentiation process: attached measles virus-infected endothelial cells correspond to an intermediate state of differentiation while floating cells, recovered from cell culture supernatants, are fully differentiated. Measles virus-induced thymic epithelial cell differentiation is characterized by morphological and phenotypic changes. Measles virus-infected attached cells present fusiform and stellate shapes followed by a loss of cell-cell contacts and a shift from low- to high-molecular-weight keratin expression. Measles virus infection induces thymic epithelial cell apoptosis in terminally differentiated cells, revealed by the condensation and degradation of DNA in measles virus-infected floating thymic epithelial cells. Because thymic epithelial cells are required for the generation of immunocompetent T lymphocytes, our results suggest that measles virus-induced terminal differentiation of thymic epithelial cells may contribute to immunosuppression, particularly in children, in whom the thymic microenvironment is of critical importance for the development and maturation of a functional immune system.

The primary causes of infant death in developing countries are associated with measles virus (MV) and human immunodeficiency virus (HIV) infection. Both infections result in the development of profound immunosuppression that contributes to secondary infections and mortality (4, 11, 18, 27). The dysfunction of the immune system in HIV patients is dramatic and ultimately fatal, whereas MV-induced immunosuppression is transitory. The physiological mechanisms involved in MV-induced immunosuppression are still not well understood. The first indication of MV-induced immunosuppression is a transient unresponsiveness to tuberculosis that is observed for several weeks after recovery from MV infection (47). Furthermore, in vitro stimulation of lymphocytes by mitogens (26, 35) or alloseantigens (15) is suppressed after MV infection, and cytokine production has been shown to be abnormal as well (14, 22, 39, 48). Suppression of antibody synthesis as well as natural killer cell activity was reported in different in vitro and in vivo models (7, 19, 43, 50). Infection of dendritic cells with MV leads to a rapid up-regulation of activation markers, indicating their functional maturation in vitro (39). As a result, MV-infected dendritic cells suppressed mitogen-dependent proliferation of uninfected peripheral blood lymphocytes or T cells (14, 20, 39). Finally, it has been demonstrated that MV could induce apoptosis of infected cells and noninfected lymphocytes after their contact with infected cells in vitro (1, 9, 14).

Measles virus, belonging to the Paramyxoviridae family and the Morbillivirus genus, is an enveloped nonsegmented-strand RNA virus. Infection with MV is initiated by interaction of viral hemagglutinin (H) protein with the cellular receptor, the CD46 molecule, initially described as the membrane cofactor protein (8, 16, 30). This attachment is followed by virus-cell fusion, release of nucleocapsids into the cytoplasm, and expression of H and fusion (F) glycoproteins at the cell surface. During infection, CD46 is down-regulated concomitantly with the appearance of MV H protein on the cell surface (24, 30, 37). MV initially replicates in the respiratory tract and then spreads to local lymphoid tissue, where virus replication can occur in macrophages and/or in dendritic cells (10, 14, 20). This secondary viremia allows the spreading of the virus to other lymphoid organs, such as the thymus, liver, skin, and conjunctive tissues (6, 29, 49). Thymic stromal cells have been described as a target for MV in the SCID-hu mouse model after direct intrathymic inoculation of MV. As a result, MV replication in the thymic epithelial cells (TEC) as well as monocytes/macrophages leads to induction of thymocyte apoptosis (3, 45). Similarly, TEC were shown to be prominent targets of HIV and human cytomegalovirus infection, where infection of thymic epithelium is associated with profound thymic injury (2, 5, 28, 40, 41).

TEC play a critical role in the development and maturation of immunocompetent T cells, providing a microenvironment with a unique capacity to generate a functional and diverse T-cell repertoire (21). Thus, the interaction of virus with the thymic microenvironment may result in an alteration in the
development of functional T cells. In this study, we demonstrate that MV infection induces terminal differentiation of TEC which is characterized by the arrest of proliferation and by morphological and phenotypic changes typical of epithelial cell maturation, followed by apoptosis of fully differentiated TEC. These results suggest that MV-induced terminal differentiation of TEC may contribute to a long-term immune dysfunction, particularly in infants, in whom ontogenesis of the immune system requires a functional thymic microenvironment.

MATERIALS AND METHODS

Cell culture, MV infection, and viral titration. The previously described (12) P1.A4.D6 cortical TEC clone from human postnatal thymus was used. TEC were cultured in RPMI 1640 (Gibco Life Technologies, Inc., Grand Island, N.Y.) supplemented with 2 mM L-glutamine (Gibco Life Technologies), 10 mM HEPES (Gibco Life Technologies), 40 μg of gentamicin (Scherger/Plough), and 1% fetal calf serum (Gibco Life Technologies). TEC were subcultivated at low density before infection, and attached TEC (3 × 10^5 cells/cm² in 25 ml of culture medium) were infected with 1 PFU/cell of Vero-cell-derived infectious MV Hadle strain over a 2-h period, followed by three extensive washes. (These cells will be referred to hereafter as MV-TEC.) As a control, medium or MV inactivated by UV light over 30 min at 254 nm (UV-MV-TEC) was used. This MV strain has been classified as the vaccine MV Edmonston-like strain (34).

Cytofluorometry analysis. The monoclonal antibodies (MAbs) used were clone MC20.6 (CD46; immunoglobulin G1 [IgG1] isotype [31]), clone 55 (anti-H; IgG2b isotype [31]), clone 120 (anti-nuclear protein [NP]; IgG2b isotype [31]), clone S503 (anti-F) (kindly provided by F. Wild [Lyon, France]), CAM5.2 (IgG2b isotype [Becton Dickinson, Mountain View, Calif.]), and AE3 (IgG1 isotype [Diagnostic Biosystems, Fremont, Calif.]). These latter two MAbs recognize keratin polypeptides of low (43 and 50 kDa) and high (58 and 65 to 67 kDa) molecular mass, respectively. Mouse IgG1 and IgG2b isotypes (Immunotech, Marseille, France) were used as negative controls of immunofluorescence.

For the detection of cell surface H and F viral proteins and CD46 molecules, immunofluorescence assays were performed as previously described (44), using biotinylated mouse antibody followed by streptavidin conjugated to rhodamine or fluorescein isothiocyanate (FITC)-conjugated anti mouse Ig (Jackson ImmunoResearch Laboratories, West Grove, Pa.). FITC-labeled cells were then stained with mouse biotinylated clone 120 (anti-NP). The cells were incubated with biotinylated anti-NP antibody and were labeled with streptavidin conjugated to rhodamine or fluorescein isothiocyanate.

For cell cycle analysis, the attached P1.A4.D6 cells (5 × 10^5) were collected and fixed with 70% ethanol in phosphate-buffered saline. Fixed cells were incubated for 30 min at room temperature with 100 μg of RNase (Sigma Chemical Co., St. Louis, Mo.) at 4°C for 30 min. Permeabilized cells were incubated with labeled anti-NP antibody followed by streptavidin conjugated to rhodamine or fluorescein isothiocyanate.

For cell cycle analysis, the attached P1.A4.D6 cells (5 × 10^5) were collected and fixed with 70% ethanol in phosphate-buffered saline. Fixed cells were incubated for 30 min at room temperature with 100 μg of RNase (Sigma Chemical Co., St. Louis, Mo.) at 4°C for 30 min. Permeabilized cells were incubated with labeled anti-NP antibody followed by streptavidin conjugated to rhodamine or fluorescein isothiocyanate.

Results

RESULTS

1. MV are susceptible to MV infection in vitro. The ability of human TEC to produce infectious viral particles was measured by assaying the culture supernatant for PFU at different time points after infection (Fig. 1). MV infection of TEC was productive and reached a peak at day 5 postinfection (4.09 ± 0.04 log10 PFU/ml) (Fig. 1A) with syncytium formation in less than 10% of infected TEC layers (see Fig. 5B and data not shown).

2. MV production or syncytium formation was detected in cultures of TEC exposed to UV-inactivated MV (see Fig. 5B).

3. The ability of infected cells to produce F, H, and NP viral proteins and to down-regulate CD46 from the cell surface was analyzed by flow cytometry (Fig. 1B and C). Synthesis of all three viral proteins was detectable by 48 h after MV infection and increased gradually with time (Fig. 2B). More than 95% of TEC were infected by days 5 and 7 postinfection (data not shown). Down-regulation of MV receptor CD46 was observed (Fig. 1C) concomitantly with the appearance of viral H protein on the cell surface. In contrast, no modification of CD46 cell surface expression was detected in UV-MV-TEC compared to the uninfected TEC at day 2 postinfection (data not shown).

4. Impaired growth of MV-TEC. The consequence of MV replication on TEC growth was then analyzed by evaluating the number of viable cells by trypsin blue exclusion (Fig. 2). The number of viable MV-TEC was lower than those of UV-MV-TEC and uninfected TEC, suggesting that arrest of cell growth and/or cell death occurred in MV-TEC (Fig. 2A). The absence of thymidine incorporation from the MV-TEC cultures indicated that a cell growth arrest occurred (data not shown).

5. We next analyzed the cell cycle distribution of infected and uninfected TEC at different time intervals from 1 to 7 days postinfection. Our results demonstrated a significant decrease of MV-TEC in the S/G2/M phases of the cell cycle from 50.1% ± 6.8% by day 1 to 21.8% ± 4.8% by day 7 and subsequently an accumulation in the G0/G1 phases of the cell cycle (Fig. 2B). Cell cycle arrest in the G0/G1 phases was linked to the replication of MV in TEC, since cell cycle distribution did not change during the culture of uninfected cells (data not shown) and UV-MV-TEC (remaining at between 44.9% ± 3.7% to 47.7% ± 4.4% in the S/G2/M phases) (Fig. 2B).

6. MV replication induced TEC differentiation. UV-MV-TEC monolayers were highly packed and formed regular clones characteristic of typical epithelial cells in culture (Fig. 3A). Significant morphological differences in MV-TEC monolayers were observed (Fig. 3D). MV-TEC lost classical epithelial-like morphology, decreased cell-cell contacts, and acquired the
squamous phenotype of highly differentiated TEC with fusiform to stellate shapes of dispersed single cells (Fig. 3D).

Induction of TEC differentiation by MV was examined by analyzing keratin expression in attached infected cells at days 5 and 7. Previous reports indicated that keratin isoforms are specific to epithelial stage differentiation with loss of low-molecular-weight keratin and acquisition of high-molecular-weight keratin during differentiation (13, 42). Therefore, we performed staining with either anti-CAM5.2 MAb (specific for low-molecular-weight keratin) or anti-AE3 MAb (specific for high-molecular-weight keratin) on cytopsins of TEC cultures. We showed that UV-MV-TEC expressed low-molecular-weight keratin in the cytoplasm (Fig. 3B), but a lower level of high-molecular-weight keratin (Fig. 3C). In contrast, MV-TEC displayed a very low level of low-molecular-weight keratin in the cytoplasm (Fig. 3E). In addition, MV-TEC strongly expressed the high-molecular-weight keratin characteristic of differentiated cells (Fig. 3F). MV replication is required for this phenotype shift, as noninfected (data not shown) and MV-UV-TEC did not differ in the pattern of anti-keratin staining (Fig. 3B and C).

The percentage of differentiated MV-TEC was quantified by double staining with anti-NP and anti-keratin MAbs. After MV infection, 54% ± 6.6% of the floating TEC population expressing NP with a strong disappearance of low-molecular-weight keratins was fully differentiated (Fig. 4C). An intermediate state of differentiation for attached MV-TEC was observed, with a moderate expression of low-molecular-weight keratin (Fig. 4B) compared to UV-MV-TEC (Fig. 4A) and floating MV-TEC (Fig. 4C). Attached and floating MV-TEC (Fig. 4E and F) but not UV-MV-TEC (Fig. 4D) highly coexpressed high-molecular-weight keratin and NP, indicating that differentiation is associated with viral replication.

MV-TEC died by apoptosis. Trypan blue staining indicated that all floating MV-TEC were dead (data not shown). In order to understand the mechanisms of cellular death induced by MV, DNA fragmentation in attached and floating cells was evaluated. A weak degradation of genomic DNA was observed in attached MV-TEC at days 3 to 7 (Fig. 5A). A typical internucleosomal fragmentation of DNA was observed in floating MV-TEC from days 3 to 7 postinfection (Fig. 5A). In contrast, no degradation in attached controls such as uninfected TEC (data not shown) and UV-MV-TEC (Fig. 5A) was detected. These data were confirmed and expanded by the study of the nuclear morphology in attached and floating MV-TEC by Hoechst 33342 staining. Less than 10% of attached MV-TEC showed nuclear fragmentation, and these apoptotic cells were found only in syncytia (Fig. 5B). In contrast, more than 90% of floating MV-TEC showed nuclear condensation characteristic of apoptosis (Fig. 5B).

MV-induced mitochondrial swelling and hyperpolarization of attached TEC. To further investigate the initial steps of MV-TEC apoptosis, we studied the early alterations of mitochondria by electron microscopy analysis and DIOC₆(3) staining, specific for the changes in the ΔΨₘ (Fig. 6). Electron-microscopic observations revealed that uninfected and UV-MV-TEC contained an irregularly shaped nucleus, large bundles of cytoplasmic intermediate filaments, and numerous clustered mitochondria (data not shown). In contrast, MV-TEC showed some swelling mitochondria with an electron-dense matrix (Fig. 6B) or with a disruption of the outer membrane (Fig. 6C). Some MV-TEC presented normal mitochondria (Fig. 6A). The accumulation of DIOC₆(3), measuring an increase in ΔΨₘ, was observed in attached MV-TEC by flow cytometry at day 7 postinfection. A consistent decrease

FIG. 1. In vitro MV replication in human TEC. TEC were infected with MV Hallé strain (1 PFU per cell). (A) Kinetics of viral particle production in cell-free supernatants of MV-TEC. The number of PFU is expressed as the mean ± the standard deviation of five individual experiments. (B) Kinetics of MV proteins expression using cytofluorometry analysis. TEC cultures were analyzed at different time points after infection for the expression of membrane (H and F) or intracellular (NP) MV-specific proteins. Results are representative of five distinct experiments in which the standard deviations were less than 15%. (C) Down-regulation of CD46 molecule on MV-TEC at day 5 postinfection. ——, uninfected TEC; ——, UV-MV-TEC; ——, MV-TEC. (Histogram profiles for uninfected cells and UV-MV-TEC are totally superimposed and therefore indistinguishable [right].) Fluorescence profiles are shown as histograms of cells labeled with MAbs. The dark histogram at left shows results of a negative control corresponding to the staining by an irrelevant MAb of an isotype identical to that of the specific MAb. Results are representative of six different experiments.
Mechanisms of pathogen-induced cell differentiation, particularly in the thymic epithelium, are still largely unknown. We showed here that TEC are highly susceptible to MV infection in vitro, resulting in massive viral replication and syncytium formation. These data are in agreement with previous findings based on in vitro and in vivo analyses and suggest that the thymic epithelium may be a target for MV and may indirectly provoke thymic injury by causing thymocyte depletion (3, 6, 29, 33, 45, 49). However, the alteration in cell functions induced by MV replication was not determined in these studies. We used a human cell clone derived from cortical thymic epithelium (12) to analyze a direct effect of MV on this cell type, without the potential interference and/or interaction of other cell populations present in thymic organ cultures. The present study demonstrates for the first time that MV replication in thymic epithelial cells induces terminal cell differentiation followed by apoptosis.

Consequent to MV replication, the ability of TEC to proliferate was profoundly affected, with an arrest in the G\textsubscript{0}/G\textsubscript{1} phases of the cell cycle. An induction of growth arrest by MV was previously shown in other cell types, and various mechanisms were proposed. MV-induced impairment of uninfected peripheral blood lymphocyte proliferation did not require viral replication but was dependent on the coexpression of MV H and F glycoproteins by the MV-infected and irradiated cells (36, 38). Our study indicates that viral replication is critical for the TEC growth arrest since UV-MV, containing H and F membrane proteins, does not have any effect. However, we do not exclude the role of H and F proteins in the inhibition of TEC proliferation. Under the conditions of our study, the amount of H and F expressed early in UV-MV-TEC may be insufficient to trigger differentiation signals. In addition to H and F viral proteins, the expression of other viral protein(s) may be required as well. The role of this protein(s) remains to be elucidated.

In addition to cell growth arrest, our study demonstrates that when replicating MV, cortical thymic epithelium undergoes major changes in morphology, associated with acquisition of fusiform and stellate cellular shapes, squamous morphology, and a loss of cell-cell contacts. These findings strongly suggest that MV replication in TEC induced cell differentiation. We confirmed this observation by performing immunostaining to analyze phenotypic changes characteristic of differentiated TEC. We demonstrated that MV promotes a shift from low- to high-molecular-weight keratin expression, characteristic of differentiated epithelial cells (13, 42). This MV-induced differentiation mechanism corresponds to a progressive process correlating with the impairment of TEC growth beginning at day 3 after infection. The down-regulation of low-molecular-weight keratin expression at day 5 is followed by the acquisition of high-molecular-weight keratin and by morphological changes at day 7, ending with a complete destruction of in vitro cortical thymic epithelium layers. This observation is in agreement with previous studies demonstrating that differentiated TEC contain specific cytoskeletal proteins which are associated with late stages of epidermal keratinocyte maturation (25). How-
FIG. 3. Differentiation of MV-TEC characterized by morphological and phenotypical changes. Left and right panels show UV-MV-TEC and MV-TEC, respectively. Phase-contrast photomicrographs of UV-MV-TEC (A) and MV-TEC (D) at day 7 postinfection. Morphology of representative TEC monolayers was documented by light microscopy (Olympus) using Hoffman optics. The photomicrographs were taken at the same original magnification (×10). Phenotypic changes were evaluated after...
ever, very little is known about the cortical TEC differentiation process in vitro and in vivo. Therefore, we may hypothesize that cortical and medullary TEC could share a common TEC differentiation pathway.

MV replication in cortical TEC is required to induce the differentiation process ending with apoptosis of fully differentiated TEC in vitro. Our observation is in agreement with other studies, in which autopsies of patients with fatal measles infec-

FIG. 4. Representative flow cytometry analyses of fully differentiated floating MV-TEC. TEC were analyzed for the coexpression of NP MV and low-molecular-weight keratin (A through C) and for coexpression of NP MV and high-molecular-weight keratin (D through F). Dual fluorescence of attached UV-MV-TEC (A and D), MV-TEC (B and E) and floating MV-TEC (C and F) by day 7 postinfection are shown. Results are presented as the means of three different experiments. Quadrant limits were positioned on the negative control (not shown) for the percentages as well as the means of fluorescence intensity (MFI) determination of NP low- and NP high-molecular weight keratin coexpression, respectively. The risk of error was evaluated at the levels of $P < 0.05$ (*), $P < 0.001$ (**), or $P < 0.0001$ (***) in comparison with UV-MV-TEC.

immunostaining of low (revealed by CAM5.2 MAb at day 5 - B, E) - or high (revealed by AE3 MAb at day 7 - C, F) - molecular-weight keratins. Labeling was performed on attached UV-MV-TEC (B and C) and in MV-TEC (E and F) after cytocentrifugation. TEC were examined using an epifluorescence microscope (Axioplan 2, Zeiss; original magnification, ×63 with an oil immersion lens). Results are representative of five different experiments.
FIG. 5. MV infection induced apoptosis in TEC cultures. In all experiments, more than 90% of attached TEC excluded propidium iodide, and more than 90% of floating cells incorporated it in MV-TEC cultures, as determined by cytofluorometry. (A) Electrophoresis of low-molecular-weight DNA from attached and floating TEC cultures at different time points. DNA from TEC cultures were extracted as described in Materials and Methods. (B) Nuclear fragmentation in syncytia of MV-TEC monolayers and condensation in differentiated floating MV-TEC cultures at day 5 postinfection, after Hoechst staining. Data are representative of four different experiments. The white arrow indicates syncytia, and the white scale bar represents 15 μm.
tions demonstrated lesions in the thymus with degenerative and/or necrotic changes associated to a rapid and predominant loss of the thymic cortex (49). A marked involution of the thymic medulla was also observed, and MV antigens were found in Hassall’s corpuscles and in adjacent MV-infected thymic stromal cells (6, 29, 45, 49). We demonstrated that MV-induced apoptosis was seen in fully differentiated TEC but also in syncytia. The ability of MV to induce apoptosis has been observed in some cell lines (9), infected peripheral blood lymphocytes (1), and dendritic cells (14), as well as in T cells in contact with infected dendritic or epithelial cells (3, 14). Our data showing progressive mitochondrial swelling and disruption of the outer mitochondrial membrane indicate that differentiated MV-TEC are committed to apoptosis before they detach from matrix. These characteristics have been previously defined to be specific of early steps of apoptosis (46). In addition, late stages of apoptosis were detected in fully differentiated floating MV-TEC as well as in syncytia of attached MV-TEC. The characteristic of large syncytial multinucleated giant cells, the so-called Warthin-Finkeldey cells, resulting from fusion of more than 100 nuclei, have been described in lymphoid tissues, such as that of the thymus, during the early phase of measles (32). In addition to differentiation-induced apoptosis, it is possible that the cytopathic effect of MV leads also to apoptosis of syncytium-forming TEC.

In addition, MV replication in TEC leads to CD46 down-regulation. A correlation between CD46 internalization and in vitro susceptibility to complement-mediated lysis has been reported (37). Therefore, MV-induced CD46 down-regulation may render TEC susceptible to complement-mediated lysis, which could contribute to thymic damage observed during MV infection.

Recent reports have shown that in vivo MV infection of TEC leads to induction of thymocyte apoptosis, which may contribute to a long-term alteration of the immune system (3, 45). As thymocyte survival depends on the signal provided by

**FIG. 6.** Early apoptotic process occurred in attached MV-TEC by day 7. Ultrastructure of mitochondria in MV-TEC (×35,000). Shown are normal mitochondria (A); swollen mitochondria with a dense matrix, suggesting alteration of permeability (B); and a large swollen mitochondrion with a rupture of the outer membrane (C). Scale bars, 0.5 μm. (D) DIOC₆(3) fluorescence of attached and floating TEC cultures was analyzed by flow cytometry.
stromal cells, it remains to be determined if the destruction of cortical thymic epithelium observed in vitro is induced directly, by MV replication, or indirectly, by complement-mediated lysis, and whether it could deliver abnormal signal to thymocytes and/or affects positive selection of thymocytes. As MV infection most often occurs in childhood, when the functioning of the thymus is required, defects in TEC function and survival may contribute to the immune dysfunction and to the development of immune suppression.

ACKNOWLEDGMENTS

We particularly thank J.-F. Nicolas and J. Marvel for their scientific advice during this work. We are grateful to J. Maryanski and V. Lotteau for reading the manuscript and to S. Manier, D. Gerlier, and P. Juridic for helpful suggestions. We also thank Y. Leverrier, A. Cheff, A. Thomas-Cachard, M.-T. Nugeyre, C. Domenger, and M. Chamoux for valuable technical assistance. The useful comments of C. Servet-Delprat, A. Astier, M.-C. Trescol-Biémont, S. Guerret, and P.-O. Vidalin are greatly appreciated.

This work was supported in part by institutional grants from the Centre National de la Recherche Scientifique, the Ministère de l'Enseignement supérieur et de la Recherche, the Institut National de la Santé et de la Recherche Médicale and by additional support from Association pour la Recherche sur le Cancer and l'Education National, de l'Enseignement Supérieur et de la Recherche. A.E. is a recipient of a grant from Fondation pour la Recherche Médicale.

REFERENCES


