Review

Establishment of equine and murine monocytic cell lines and their susceptibility to West Nile virus

Katsuyuki Kadoi[1], Rossella Lelli[1], Vincenzo Caporale[1], Takeshi Onodera[2]

[1]Institute G. Caporale, Via Campo Boario, 64100 Teramo, Italy.
[2]School of Agricultural and Life Sciences, University of Tokyo, Tokyo, 113-8657, Japan.

Two monocytic cell lines, temporary named HsMo (horse monocyte) and MsMo (mouse monocyte) were established from blood samples of horse and Balb/C mouse according to the culture method previously informed (Kadoi, 2011). Tests were performed to certify the viral susceptibility of these cells in comparison with that of Vero cells for West Nile virus (WNV). Two strains of WNV adapted to grow in Vero cells were titrated for infectivity in the monocytic cells and Vero cells in a routine microplate assay system. The highest titer was obtained in the assay system with HsMo cells followed by the system with MsMo cells. These monocytic cells were sensitive enough for detecting WNV, however, WNV infected monocytes produced a strong acidic substance in which the acidity is much higher grade than that produced in Vero cells. In microplate culture the color of medium changes from redden neutral color to yellow indicated by phenol red dye included in cell culture medium, in parallel with CPE occurrence. This is a color test for WNV assay in monocytic cells

**Keywords**: Horse monocytes, mouse monocytes, WNV susceptibility.

INTRDUCTION

The invasion of West Nile virus (WNV) became a worldwide serious problem since the causative pathogenic virus has readily spread in many countries, Africa, the Middle East, Europe, USA, USSR, and Asia. (Dauphin et al., 2004; Zeller and Schuffenecker, 2004; Hayes et al., 2005). WNV is an arbovirus transmitted by various mosquito species (Anderson et al., 1999). This is the main reason for the elimination of the disease once introduced to virgin area. Several animal species are known to be susceptible hosts (Lanciotti et al., 1999; Weingartle et al., 2004) and birds are suspected to be amplifying host (Anderson et al., 1999; Kramer and Bernard, 2001; McLean et al., 2001; Weingartle et al., 2004). The virus was isolated from samples of humans followed by horses in the beginning of the history. However, it was now understood that the virus is widely distributed in animal world since the virus was isolated not only clinically affected animals, but was also isolated from asymptomatic animals as camel and grass mouse,

frugivorous bat, and mosquitoes. The isolation of causative virus from field materials is an important task for reference laboratories and a sensitive assay method is desired even though several diagnostic protocols have been established. Accumulating information indicate mononuclear phagocytes (monocyties, macrophages, and dendritic cells) are sensitive enough not only for WNV (Mather et al., 2003; Rios et al., 2006) but also for Dengue virus (Wang et al., 2002; Blackley et al., 2007; Kou et al., 2008), for human immunodeficiency virus (Cassol et al., 2006; Shen et al., 2011), for Crimean Congo hemorrhagic fever virus (Connolly et al., 2009), for Visna/Maedi virus (Narayan et al., 1982; Crespo et al., 2011), for Tanapox virus (Nazarian et al., 2007), for Measles virus (Lemon et al., 2011), and for Aujeszky disease virus (Kadoi, 2002). We have succeeded to establish a horse monocytic cell line (HsMo) and a mouse monocyrtic cell line (MsMo) and their susceptibility to WNV was examined in comparison with Vero cells.

Primary monocyte cultures were prepared in the manner mentioned previously (Kadoi, 2011). Briefly, small amount of heparinized blood was respectively

*Corresponding Author E-mail: katsuyukikadoi@yahoo.co.jp
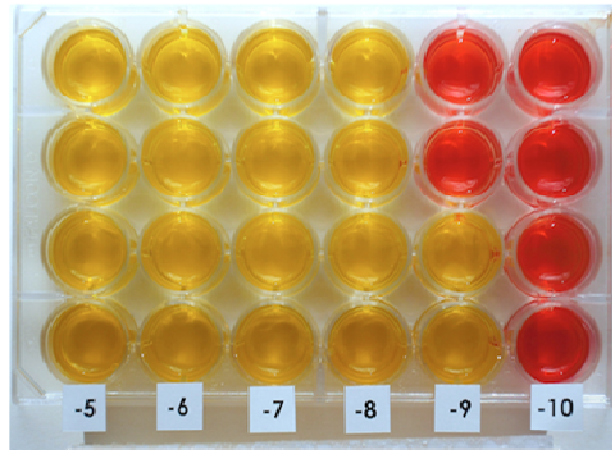
WNV (Italy08)/HsMo/7DPI/37°C
Figure1. (a) HsMo cells, non-infected control culture. Slip culture was methanol fixed and Giemsa stained. Bar = 100 μm

collected from a healthy horse and four of mice, 4-week old of Balb/C (Charles River, Co. USA). The heparinized blood was centrifuged at 200 G for 15 minutes and the part of plasma was separately harvested. The plasma was centrifuged again at 300 G for 15 minutes for clarification followed by filtration with 220 nm membrane (Millipore Co. USA) for primary culture. The part of precipitated cells were diluted with an equal amount of PBS and overlaid on Lymphoprep (density = 1.077, AXIS-SHIELD PoC AS, Norway) for the separation of monocytes by the density centrifugation mentioned previously (Kadoi, 2011). The autologous plasma was supplemented in the primary culture stage of mouse monocyte culture and autologous horse serum was prepared in advance was supplemented in the primary culture of horse monocyte culture. Both mouse plasma and horse serum were filtered by 220 nm Millipore membrane and then used without heating. Primary monocyte cultures were grown on the feeder cells of chicken embryonic cell line (KCEK) (Kadoi, 2010) as mentioned (Kadoi, 2011).

Both HsMo and MsMo cell lines were established by limited dilution method routinely made in microplate culture (48 well plate, Nunc A/S, Denmark) after their adaptation in subcultures in vitro. The passage level at $34^{th} – 37^{th}$ of HsMo cells and at $18^{th} – 20^{th}$ of MsMo cells were used in the present experiment.

Karyotyping was routinely made on young actively growing cells by adding a small amount of colchicine (0.06 mcg per ml) as mentioned previously (Kadoi, 2000). Karyotyping of HsMo cells indicated 64 (2n) and that of MsMo cells was proved to be 40 (2n) by a usual manner (Kadoi, 2000).

Slip culture of cells were examined cytochemically for non-specific esterase and acid phosphatase by the kits (Muto chemicals, Japan) in the same manner as mentioned (Kadoi, 2000). In our experiences the cells positive for both enzymes were judged as monocytes.

Vero E6 (Vero) cells were originally supplied from Pasteur institute, France. The passage level at $10^{th} – 20^{th}$ in our hands were used for present study.

Cell growth medium (GM) used for monocytic cells consisted of a modified RPM-1640 (mRPMI) (Kadoi, 2011) supplemented with 10% FBS (Biowhittacker, Belgium). Sodium bicarbonate solution was separately prepared at 7.5% aqueous solution and autoclaved. It was added at 2.0 ml per 100 ml GM and 3.5 ml per 100 ml of cell maintenance medium (MM). MM for monocytic cells was mRPMI supplemented with BSA (Sigma-Aldrich Co. Germany) at 0.02% in final concentration. The MM is also used as diluent for the preparation of 10-fold virus dilution in infectivity assay. GM for Vero cells consisted of Eagle-MEM (MEM) (Sigma-Aldrich Co. Germany) supplemented with 10% FCS. Vero cells were well maintained under MM above. Antibiotics incorporated in these media were kanamycin sulfate, Na ampicillin, and streptomycin sulfate at 200 mcg per ml as usual.

Two strains of WNV were examined. One is strain Eg 101 (Melnick et al., 1951) and the other virus strain used was one of Italian isolates made in 2008 from horse material (Italy08) (Savini et al., 2008). Eg 101 strain is highly passaged virus in Vero cells and Ita08 strain is a low passaged virus in Vero cells (5th passage level in Vero cells). Both virus strains are proved to be included in lineage-1 in phylogenetic analysis (Savini et al., 2008).

Fresh seed virus was prepared in Vero cells in flask cultures by infecting with the virus at ca. 0.5 $TCID_{50}$ per cell. When CPE was occurred more than 50 % of infected cells at 37°C incubation (day 3 post infection), the cultures were freeze-thawed once and centrifuged

Table 1. Comparison of infective titers of WNV measured in 3 types of cells.

Cells	Infectivity of Eg 101	Infectivity of Italy08
Vero	$10^{7.33*}$	$10^{7.91}$
HsMo	$10^{7.91}$	$10^{9.16}$
TMMO	10^{791}	$10^{8.50}$

for clarification. The supernatant was dispensed into small vials, stored at -80°C, and tested within 2 months of storage.

Virus infective assay was performed in microplates. Three types of cells, Vero, HsMo, and MsMo, were seeded in plates (24 well type plate, Falcon, Becton Dickinson and Co., USA). The cell suspension adjusted at the viable cell number 150,000 per ml of GM was dispensed to the plate at 1 ml per well. More than 95% confluent cell layer at 36-40 hours incubation at 37°C was used for infectivity assay. Ten-fold dilution of seed virus was prepared in tubes respectively for Eg 101 and Italy08 strains. The part of GM was sucked off from the plate cell culture and the virus dilution was inoculated at 1 ml per well. Four wells were used per virus dilution. The plates were inserted in plastic envelope and incubated at 37°C. Daily observation under an inverted microscope was carried out for CPE occurrence and the final CPE reading was made on day 6 postinfection (PI). Infectivity in $TCID_{50}$ per ml was calculated according to Kerber's method (Kerber, 1931). The measurement of infectivity was made 3 times in different occasion.

The infectivity measure in 3 types of cells was summarized in Table 1. According to the infective titers obtained, HsMo was judged to be more sensitive than others since significantly higher infective titers were demonstrated in every occasion. MsMo cells were also sensitive cells, though a slightly less infective titer than that in HsMo cells were obtained. The occurrence of CPE appeared earlier in HsMo cells than those in other cells. The characteristic of the CPE on HsMo and MsMo cells, clearly observed on 2 PI, in starting focal cytopathic effects as shrinkage and rounding of cells, pyknotic nuclei, and appearance of hole in the cell monolayer. A formation of fused cells is detected in an early incubation. More than 80% cell layers are destroyed at 3 PI. Clear CPE also appeared in infected Vero cells on 2 DPI and more than 80% cells were destructed on 4 DPI. Fusion formation was not evident in Vero cells. CPE positive well in monocytic cells indicated an evident acidic pH in which easily observed by phenol red dye included in MM. This phenomenon is applicable as a color test.

Instead of the same seed of WNVs prepared in Vero cells was examined, the infectivity titers estimated in 3 types of cells were not in the same degree. The highest titer was always obtained in HsMo cells followed by MsMo cells. This is worth to note that what the titer measured in Vero cells was not indicating the real number of infective virion produced in Vero cells. The data clearly proved that there is a difference of viral susceptibility among 3 types of cells. Also it was re-confirmed that monocytic cell lines have higher sensitivity for WNV. The production of acidic substance in WNV infected cells was already indicated in 1984 (Barnard, 1984), however, monocytic cell lines as HsMo and or MsMo produced a much stronger acidity than other cells when infected with WNV, but not with other flaviviruses (Kadoi's unpublished data). On the other hands, Vero cells are, African green monkey kidney origin, widely used for many viruses as good host cells. We have not studied yet any cytokine production capability of the monocytic cells yet. However, these cells are well cryopreserved as other cell lines. Fundamental cytological studies have not been completed on the cell lines, the most interesting work remained to study a possibility of differentiation to macrophage-like cells and fibrocytes since monocytes may be multipotent progenitors (Seta and Kuwana, 2010). The culture of monocytic cells has really prospective future to open a new field of bioscience (Kuwana et al., 2004). Our preliminary study on the monocytes indicated a rapid morphological diversity occurrence depending on culture conditions. The present data proved a superior sensitivity of two monocytic cell lines for WNV, however, the sensitivity (or susceptibility) of monocytes for WNV is not equivalent to the cellular capability of progeny virus production. Monocytes may detect smaller number of WNV than Vero cells do, however, monocytes may have a scavenger-like function, in which trapped virus may be destroyed in parallel with viral replication. In fact the production of a strong acidic substance is harmful for maintenance of viral progeny. The acidity is much severe than those produced in epithelial cells in deed. The CPE figure in infected monocytes indicates an impression of severe bombing to destroy the infected cells to very fine fragments. In a routine infectivity assay, plates are left for 6 – 7 days in incubator for final reading and a color test may be recorded as well. However, such long days incubation are not convenient for the recovery of progeny virus. For higher recovery of progeny virus a freezing cultures in early incubation stage, at 20 – 30% CPE, and or a small amount of extra addition of sodium bicarbonate solution may be beneficial. N -Nitro-L-arginine (NNA) (Sigma-Aldrich, Germany)
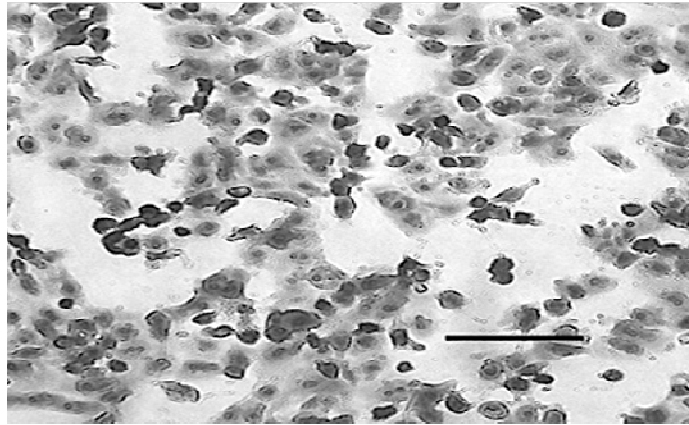
Figure 1. (b) HsMo cells, infected with WNV (Italy08) day 2 post infection. CPE figure is similar to those in infected Vero cells, however it shows more evidently. Slip culture was methanol fixed and Giemsa stained.Bar = 100 μm
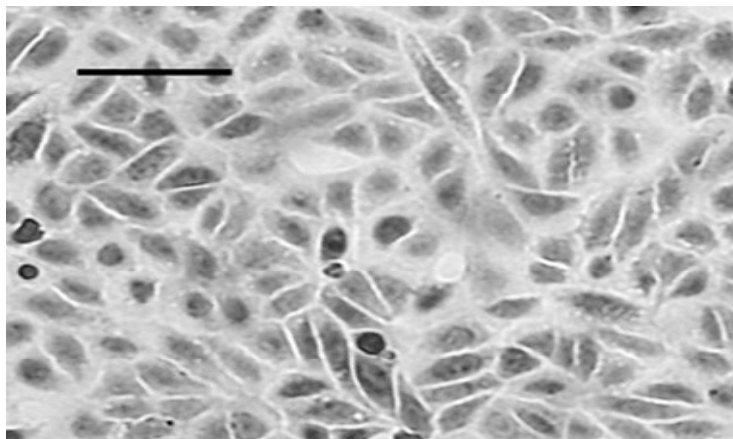

Figure1. (c) The infectivity assay of WNV (Italy08) made on HsMo cells. CPE positive well became acidic pH and CPE negative well remained in neutral pH according to the phenol red dye inclusion. The plate was fixed with 3% formalin/saline for 10 minutes.

known as an irreversible inhibitor of constitutive nictric oxide synthase and a reversible inhibitor of inducible nitric oxide synthase was not effective to inhibit acidity produced by WNV infected cells when NNA was added at 10-25 mM in MM. In our preliminary tests indicated the monocytic cell lines grow constantly with GM mentioned above. Eagle-MEM, supplemented with the FCS was not adequate for monocyte culture, even though the same amount of glucose, galactose, and fructose were added as in mRPMI.

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