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In-vivo and *In-vitro* Oncolytic Effect of Newcastle Disease Virus

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Abstract: Newcastle disease virus (NDV) clone 30 was propagated on Vero cells. Plaque forming test and minimal cytotoxic concentration$_{50}$ at multiplicities of infection (MOI)of 0.01, 0.1 and 1were performed to use minimal concentration of the virus. HeLa cells were infected with an MOI of 1.0. At 1, 3, 5 and 7 days post infection. On the other hand the oncolytic activity of NDV was tested on induced tumor in mice. The minimal lethal concentration$_{50}$ was firstly determined to use minimal safe concentration of the virus. Then 40 mice were divided into two groups The first group consist of 30 mice IP injected with 5×10^6 lymphoma cells to induce generalized tumor. Fifteen mice of this group received 5×10^6pfu of NDV in 100 µl PBS, either as a single treatment (seven mice) or as multiple treatments: days 1 (two mice), 4 (two mice) and 7 (two mice) after tumor challenge. The other 15 mice of this group received 100 µl PBS. All mice were sacrificed 52 days after tumor challenge or until death occurs in very sick mice. All abdominal organs were observed. On tissue culture level NDV at an MOI of 1, more than 80% of HeLa cells were killed at 5 days. Even at the lowest MOI of 0.01 over 40% of the cells had been killed by day 7. While in-vivo mice received only the tumor cells showed on PM finding enlarged internal organs specially the liver. On the other hand, there were no gross tumors in 6 NDV-treated mice (43.3%). The mice received NDV as a single treatment showed 30% regression of the tumors. While mice received NDV as two treatments showed 40% regression of tumors and 60% in three NDV treatments.

Key words: Newcastle Disease · Newcastle Disease Virus · Clone 30 · Oncolytic Anti-Cancer · Antitumor · Antineoplastic

INTRODUCTION

Newcastle disease virus(NDV) is a negative-sense, non-segmented,single-stranded RNA virus belonging to *Paramyxoviridae* Family and genus*Avulavirus*. It has been shown to possess oncolytic activity, causing specific lysis of cancerous but not normal cells [1, 2]. Naturally occurring NDV has been reported to be an effective oncolytic agent in a variety of animal tumor models [3, 4]. NDV has been recorded as an antineoplastic agent in animal and human and the interest in using this virus has increased steadily in recent years [5, 6]. The virus is able to propagate in the cytoplasm of tumor cells [7], which may be attributed to defective interferon (IFN) signaling pathways in tumor cells [8]. Normal cells, with an effective antiviral response, inhibit viral replication, thereby allowing use an attenuated strain of NDV as a safe and effective anticancer therapeutic agent. The known sensitivity of NDV to IFN and its inability to replicate in normal mammalian cells are likely to contribute to its safety in humans [9]. In addition, NDV can be used against a variety of tumors, as it is known to enter cells by binding to sialic acid residues, which are present on a wide distribution of human and murine cancer cell lines [10]. NDV infection of tumor cells improves T-cell responses [11]. Little is known about the NDV mediated activation of Natural killer (NK) cells. The cytolytic activity of NK cells against virus-infected or tumor cells is regulated by the engagement of activating or inhibitory NK cell surface receptors, the actions of cytokines and cross talk with other immune cells [12, 13].

The aim of this study is the first to monitor the *in-vitro* and *in-vivo* oncolytic activities of NDV clone 30, which is genetically improved strain and consider safe and show very minor effect on exposure.

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MATERIALS AND METHODS

This work was performed in the National Research Center (NRC) in the period of July-November 2012.

Cell Lines: HeLa cervix carcinoma and Vero cells (green monkey kidney cell) were used. Both cells were obtained from Vaccine and sera institute (VACSERA) Dokki, Cairo, Egypt. Cells were maintained in Roswell ParkMemorial Institute (RPMI) 1640 media. All media contained 10% fetal calf serum, 100 µg/ml penicillin and 100µg/ml streptomycin. Cells were maintained in a 5% CO_2 humidified incubator at 37°C.

Virus: NobilisND clone 30 was obtained from *Intervet*® Company. NDV clone 30 is a freeze dried vaccine against ND each dose contains at least 10 $^6EID_{50}$ of the ND strain clone 30.

Virus Propagation: NDV was propagated on Vero cells supplemented with RPMI 1640 media, 10% fetal calf serum, 100 µg/ml penicillin and 100µg/ml streptomycin in a 5% CO_2 humidified incubator at 37°C. Plaque forming test was performed according to Sambrook *et al.* [14].Virus titers were determined by inoculation of serial virus dilutions into Vero cells. The stored aliquots were taken from the-80°Cfreezer, thawed on ice and reconstituted to the appropriate volume with cold phosphate buffered saline (PBS).5×10^4 cells/well in 96-well plates (Costar, Corning, NY, USA) and infected 6 h later at multiplicities of infection (MOIs,virus particles per tumor cell) of 0.01, 0.1 and 1. Cells were infected in 96-well plates for1, 3, 5 and 7 days and the minimal cytotoxic concentration $_{50}$ was calculated.

***In vitro* Testing of Oncolytic Activity of NDV on Tumor Cell Line:** In 96-well tissue culture plate5×10^4HeLacells/well was inoculated. HeLa cells were infected 6 h later with an MOI of 1.0 of NDV. At 1, 3, 5 and 7 days post infection, the supernatants were collected and the virus titers determined by serial dilution in Vero cells. Where, the day before infection, Vero cells were plated to confluence in a 96-well plate. Vero cells were infected with serial supernatant dilutions ina total volume of 20 µl. The titer of the virus was determined by using Reed and Muench method[15].

***In-vivo* Assay:** 40 mice were obtained from animal house NRC. Mice permitted food and water ad libitum. Anesthesia of animals was induced with chloroform and sacrificed by increased dose of anesthesia.

Estimation of Minimal Lethal Concentration$_{50}$(MLC_{50}): Eight-to-10-week-old mice underwent intra-peritoneal (IP)injection of100µl PBS or 2×10^6, 5×10^6, or 1×10^7Plaque forming unit (pfu) of NDV. The mice were monitored for behavioral changes including failure to eat, drink, or groom normally. $MLC_{50}$was calculated using Reed and Muench method[15].

***In vivo* Testing Of oncolytic Activity Test:** The mice were divided into two groups. The first group consist of 30 mice IP injected with $5X10^6$ lymphoma cells to induce generalized tumor. Fifteen mice of this group received 5×10^6pfu of NDV in 100 µl PBS, either as a single treatment (seven mice) or as multiple treatments: days 1 (two mice), 4 (two mice) and 7 (two mice) after tumor challenge. The other 15 mice of this group received 100 µl PBS. The ten control animals were treated with IP injection of PBS. For the duration of the study, all animals were observed every day, examined for cachexia and monitored for signs of behavioral change, including abnormal eating, drinking and grooming. All animals were sacrificed 52 days after tumor challenge or until death occurs in very sick mice. All abdominal organs were observed according to Song *et al.,* [16].

RESULTS AND DISCUSSION

NDV, a *Paramyxoviridae* family member, known as an anticancer the rapeutic agent more than 50 years ago as a result of its potent oncolytic and limited toxicity to normal cells. NDV selectively replicates within tumor cells, making it an attractive anticancer therapeutic with a low toxicity profile [6].

Virus Propagation and Minimal Cytotoxic Concentration$_{50}$ (MTC_{50}): NDV was propagated on Vero cell and the viral cultural morphology was monitored. Cells showed syncytia formation and vaculation this may be attributed to fusogenic action the f protein of NDV (Fig. 1) this result matches with the results of Zamarin *et al*. [16] and Song *et al*. [17]. The MTC_{50} was estimated to use minimal concentrations on cells.The ability of NDV to kill Vero cell line was tested *in vitro* at MOIs of 0.01, 0.1 and 1 (Fig. 2). The virus effectively killed HeLa cells and a Dose response was observed. At an MOI of 1, more than 80% of the cells were killed at 5 days.Even at the lowest MOI of 0.01 over 40% of the cells had been killed by day 7(Fig. 3). The best result obtained was at MOI 1 which was used for HeLa cell this fact matches with Song *et al.,* [18] on MKN-74 human gastric cancer cells.

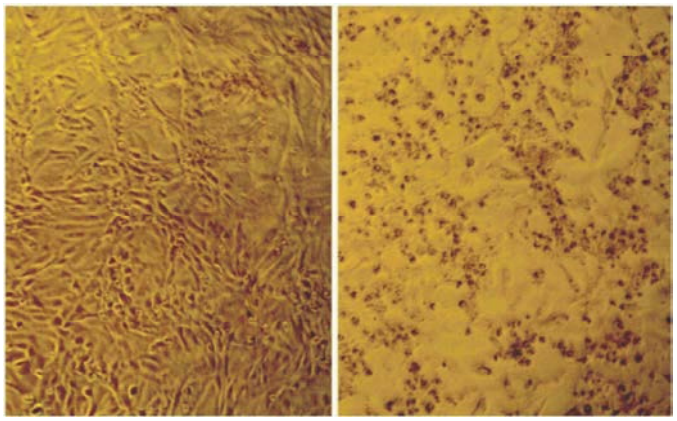

Fig. 1: Photo on left shows normal Vero cells. While on the right shows Vero cells four days post infection.

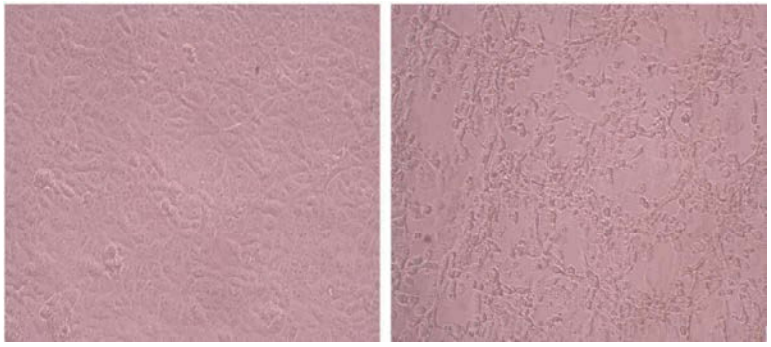

Fig. 2: Photo on the left shows normal HeLa cells. Photo on the right shows the cells four days post infection which shows syncytia formation and vaculation

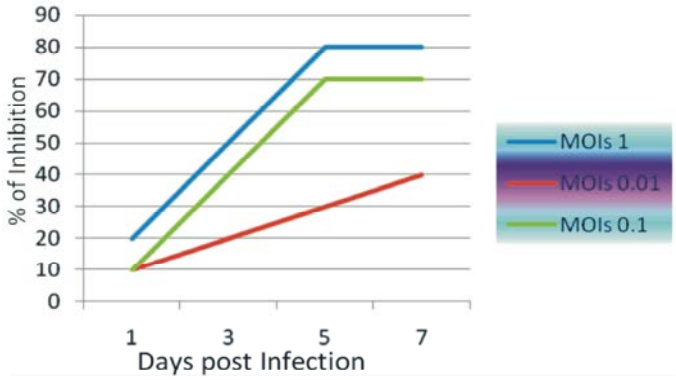

Fig. 3: Chart shows the percent of Vero cell destruction by NDV at MOIs 1, 0.1 and 0.01 after 1, 3, 5and 7 days post infection. Best result obtained is at MOI 1.0.

Other researches found that the best result obtain was at MOI 0.1 on panel of human cancer cells included human pancreatic, breast, thyroid, head and neck and gastric cancers, as well as human and murine malignant melanoma cell lines [17].

***In vitro* Testing of Oncolytic Activity of NDV on Tumor Cell Line:** The efficiency of viral replication was monitored by collecting supernatants of infected HeLa cells at different time points (At 1, 3, 5 and 7 days post infection). The highest virus titer on Vero cells (10^4) was detected 5 day after infection of HeLa cells(Fig. 4).

***In-vivo* Assay:** The best safe result was obtained by the concentration of 5×10^6pfu of NDV so this concentration was used. To induce generalized tumor 5×10^6 of lymphoma cell line was injected IP into mice. Both concentrations of NDV and Lymphoma cell show no

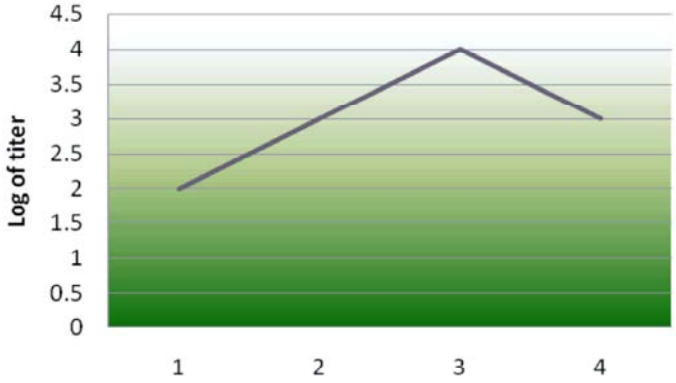

Fig. 4: *In vitro* NDV replication in HeLa cell line after *in vitro* infection with an MOI of 1.0. Supernatants were collected 1, 3, 5 and 7 days post-infection and titered by serial dilution and on Vero cells. Peak viral titers measured 10^4 at 5 days after infection

Fig. 5: Mice received 5×10^6 of lymphoma cell line IP which caused generalized tumor in the abdomen. The abdomen became large and firm.

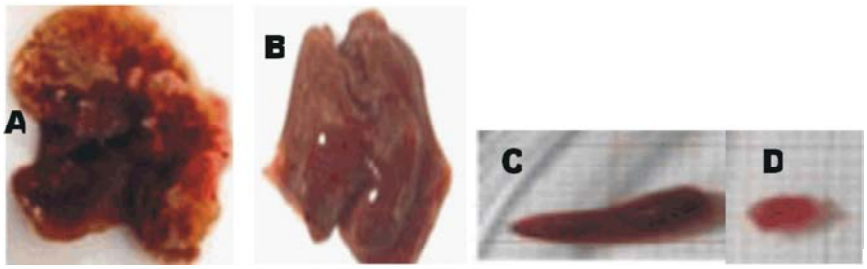

Fig. 6: Liver with tumor due to injection of mice with Lymphoma cells in photo 6A. Photo 6B shows the liver after injection with Lymphoma cells and NDV. Photo 6B shows spleen of mice injected with Lymphoma cell. Photo 6D shows spleen of mice injected with both Lymphoma cells and NDV.

toxicity in mice. After 3 weeks of tumor cell injection generalized swelling of the abdomen of each mouse was observed (Fig. 5). All mice were sacrificed at 52 days or earlier after lymphoma cell line injection and the mice showed poor grooming and firm large abdomen. Control positive mice (received only the tumor cells) showed these above mentioned finding and on PM finding there were enlarged internal organs specially the liver. On the other hand, there were no gross tumors in 6 NDV-treated mice (43.3%). The mice received NDV as a single treatment showed 30% regression of the tumors. While mice received NDV as two treatments showed 40% regression of tumors and 60% in three NDV treatments (Fig. 5). The regression of tumor remains to be clarified and may show variation regarding the strains of NDV used and which type of cancer is targeted. NDV triggers apoptosis in a

wide range of cancer cell types via the mitochondrial/intrinsic pathway, through loss of membrane potential and thereby inducing release of cytochrome in the tumor cell. The results also indicate the extrinsic pathway is activated and NDV-mediated apoptosis in a late stage [19].The results of this study seems to be promising and the frequency of NDV treatment shows no significant results. Also these results suggested that the NDVmay be an attractive candidate for oncolyticviro therapy of a varietyof tumors and malignancy.

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