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Immunology Study for NDV Treatment in Mice Bearing Mammary Adenocarcinoma Tumor

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Abstract :

Newcastle disease virus selectively kills human cancer cells versus normal cells.Newcastle disease virus has pleiotropic immune stimulatory properties.

This study was conducted to investigate the ability of Iraqi strain of NDV to induce immune stimulation when used as oncolytic agent by intratumoral injection, IL-2 and IFN-gamm as well as CD8 and CD56 were used as parameters to determine of immune stimulation properties. The results showed high immune stimulatory effect for Iraqi strain of NDV.

Introduction:

Neoplastic transformation, results from a series of genetic alterations, some of which may result in the expression of cell surface antigens that are seen as non self by the immune system. Immune-mediated recognition of autologous tumor cells may be a positive mechanism capable of eliminating transformed cells (1).

Immune system play a major role in rejecting transformed cells, it is apparent that the tumor-bearing host has sustained a failure of the immune system to control the proliferation of malignant cells, so the augmentation of the hosts natural immune defenses will provide effective modality of anticancer therapy (2).

The concept of using viruses to treat cancer dates back to 1912 when regression of cervical carcinoma was observed (3). Until 1970, 38 of total 53 viruses tried have been shown to exert an anti-neoplastic effect in either man or animals (4).

Today virotherapy is asserting itself as a formidable treatment option alongside surgery, chemo- and radiation therapy (5).Virotherapy uses replication competent oncolytic viruses to kill tumor cells (6). Newcastle disease is a highly contagious and fatal viral disease that affects all species of birds (7, 8). Newcastle Disease Virus has a single stranded negative-sense RNA genome (9). All aspects of replication of NDV occur in the cytoplasm (7). Newcastle disease virus selectively kills human cancer cells versus normal cells (10). Newcastle disease virus has pleiotropic immune stimulatory properties. Ito et al. (11) reported that virulent and avirulent strain of NDV stimulates Interferon production in mouse fibroblasts. Washburn and Schirrmacher (12) demonstrated that NDV infection to human tumor cell lines induce IFN-α and IFN-β as well as RANTS and IP-10.

In addition, NDV can stimulate human peripheral blood mononuclear cells and rat splenocytes to produce TNF-α in *in vitro*. NDV infection of human tumor cells can markedly increase their sensitivity to lysis by TNF-α (13, 14 and 12). NDV also induces nitric oxide synthase and which is known to be associated with increased macrophage antitumoral activity (15, 16 and 17). Tumor-specific antigens may be better recognized by the immune system if they are associated with virus antigens (virus proteins that have been inserted into the plasma membrane of host cells) (18). If this enhanced recognition takes place, then it may increase the chance that cancer cells, whether they are virus infected or not, will be recognized as foreign by the immune system and be destroyed (19). Additional *in vitro*

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studies have shown that NDV-infected human cancer cells are better at activating human cytotoxic T cells, helper T cells, and natural killer cells than uninfected cancer cells (20).

The NDV protein hemagglutinin-neuraminidase, which is present in the plasma membrane of virus-infected cells, appears to play a role in the enhancement of T cell activation. There is evidence that this protein makes infected cells more adhesive, thereby promoting the interaction between virus-infected cells and immune system cells (21 and 22). Also it found that NDV induce B7-B1-2 independent T-cell costimulatory activity in human cancer cells (23).

The interaction between NDV-infected cancer cells and T cells can be improved if monoclonal antibodies that bind the hemagglutinin-neuraminidase protein on the cancer cells and either the CD3 protein or the CD28 protein on T cells (i.e., bispecific monoclonal antibodies) (24). It has been proposed that vaccines consisting of NDV-infected cancer cells and bispecific monoclonal antibodies be tested in humans (25 and 26). Development of cancer vaccine using NDV was done by two ways: 1-The use of oncolysates, i.e., preparations containing plasma membrane fragments from NDV-infected cancer calls, as anticancer vaccines, as active specific immunotherapy (27; 28 and 29). 2- The use of intact UV irradiated cancer cells infected with a nonlytic strain of NDV as whole cell vaccines. whole cell vaccines can stimulate the immune system better than oncolysates, and that cells infected with a nonlytic strain of NDV will remain intact in the body long enough to generate these more effective immune responses, the cancer cells used are treated with enough gamma radiation to prevent further cell division, but not enough to cause cell death, either before or after they are infected with the nonlytic virus (30 and 31).

NDV vaccine inoculation to bovine papillomas were effective to resolve it in five cows, this was due to stimulation of an antibody response with limited increase in TNF- activity (32). Ismail (33) used BCG and NDV as immunotherapy for transplanted tumors in mice with encouraging results.

Materials and Methods:

1. Newcastle disease virus propagation:

Isolated Newcastle disease virus was purified by ultracentrifugation (50000xg, 60min, 4C˚) by using (sorval ultracentrifuge, USA) the sediment was resuspended in PBS and purified over cushin Density gradient with 35% sucrose (BDH, England) was used with 97000 xg at 60min, 4C˚. This was done twice. The virus was resuspended in PBS and stored at -196 C˚ (5). Newcastle disease virus was quantified in which one hemagglutination unit (HAU) is defined as the smallest virus concentration leading to visible chicken erythrocyte agglutination.

2. Ahmed Majeed-2003 (AM3) Transplantable mammary adenocarcinoma line:

This transplantable tumor line was established from Spontaneous murine mammary adenocarcinoma of aged female mouse that transplanted into immunosuppressed mice and successfully adapted for grown in immunocompetent mice for more than 38 passages *in vivo*. And used as animal tumor model in the development and testing of new anticancer agents in ICCMGR (31).

3. Experimental Animals:

Inbred female mice (8-10) weeks old, (20-25g) weight housed and maintained in ICCMGR animal house, with controlled conditions of temperature (23 ± 5ºC). The animals were fed on special formula food pellets and given water ad libitum. Throughout the experiments, each five animals were housed in a plastic cage containing hard-wood chip as bedding. The bedding was changed weekly to ensure a clean environment.

4. Transplantation of AM3 mammary adenocarcinoma Tumor Cells:

A method established by Al-Shamery (31): Mice were anesthetized by intraperitonial (I.P) injections of zylazine (40mg/kg) (laboratories Calier, Barcelona, Spain). The tumor mass region was well disinfected with 70% ethanol. Implantations of tumor tissue were carried out by aseptically aspirating the subcutaneous tumors using needle gage 18. The tissue fragments were placed immediately in sterile PBS and the tumor cells were allowed to settle down and the supernatant was discarded, and then the tumor fragments were resuspended in PBS at appropriate volume. Single cell suspension was made through mechanical disaggregation of the cells by vigorous pipetting. Tumor suspension aspirated by syringe with needle gage 18 and inoculated with S/C injection of 10 x 106 viable cells in 0.1ml cell suspension into shoulder region through puncture in thigh region.

5. In vivo experiment:

Tumor bearing mice was divided to 5 animals per group starting at 10 days after tumor transplantation, day 1 was the day which measurements started which is 2 days before first injection, Group One recieved Intratumoral injection (IT) with NDV (2 x 107HAU) alone for a week, 2 days interval (4 IT injections). Group 2 was control injected with PBS only.

6. Quantitative Determination of Mouse IL-2 and IFN-γ in serum of treated mice by Enzyme Linked Immunosorbent Assay (ELISA):

The IL-2 and IFN-γ ELISA kits (Mabtech, Sweden) were performed according to the manufacturer's instructions in short: microtiter plate was coated with 100 µl / well of monoclonal antibody, for each IL-2 and IFN-γ (IL-2 mAb diluted to 2 µg /ml in PBS, IFN-γ mAb diluted to 1µg / ml in PBS). The plate was incubated overnight at 4-8 ºC. then washed and blocked then washed. Stock solutions of the recombinant mouse (IL-2 and IFN-γ) standard were prepared. The dilutions of standards were prepared freshly in incubation buffer. A serial dilution (ten fold dilution) was prepared from the stock solution. The preparation

was done from the high concentration, suggested in the leaflet of the kit, to the low one as follows: (IL-2 standard dilution: 1000, 100, 10, 1, 0.1 ρg/ml). (IFN-γ standard dilution: 10000, 1000, 100, 10, 1, 0.1 ρg/ml). A triplicate was performed for each standard dilution and the mean was calculated. The plate was washed, and then 100 µl/well of diluted biotinylated monoclonal antibody were added: (IL-2 biotinylated mAb was diluted to 1µg/ml in incubation buffer, IFN-γ biotinylated mAb was diluted to 0.5 µg/ml in incubation buffer). The plate was incubated for 1 hour at room temperature (20-25 ºC). then 100 µl/well of streptavidin-ALP diluted 1:1000 in incubation buffer were added to the wells. The plate was incubated for 1 hour at room temperature and 100 µl/well of p-nitrophenyl phosphate substrate solution were added to the wells. The plate was incubated for 1 hour at room temperature in dark. The optical density (O.D.) was measured at 405 nm in an ELISA reader (Asyshitech, UK). A standard curve was generated by plotting the (O.D.) of known cytokine concentration. After the preparation of standard curve, the concentration of each cytokine in mouse serum and cell culture supernatant was determined by plotting the (O.D.) of the sample on the standard curve and determining the value accordingly.

7. Histopathological samples processing:

The steps were followed according to (34) for tissue preparation, paraffin sections and carried out in Shandon automated histokinate system (Thermo, USA), they cut and marked and put in plastic box. Dehydration, embedding, sectioning and staining were done as described by Luna (34). The slides used for Immunohistochemistry were coated with gelatin, all slides kept in clean dray place until stained.

8. Cellular immunity:

To characterize the tumor infiltrating lymphocytes (TILs) and cytokine expression, a panel of purified monoclonal antibodies were used. The following primary and secondary antibodies were used: Rat anti-mouse CD8 (isotype IgG2a, concentration 0.5mg/ml)(Chemicon International). Its secondary Ab is Mouse anti-rat IgG2a, Heavy chain (Biotin). (concentration 1mg/ml) (USBiological, USA). Rat anti-mouse CD56 for Natural killer (NCAM-1 neural cell adhesion molecule) (isotype IgG2a, concentration 0.2mg/ml) (USBiological, USA). Its secondary Ab is Mouse anti-rat IgG2a, Heavy chain (Biotin). (concentration 1mg/ml) (USBiological, USA). Rabbit anti-mouse tumor necrosis factor alpha (TNF-γ)(isotype IgG, concentration 0.1mg/ml)(USBiological, USA). Its secondary Ab is Goat anti-rabbit IgG, H & L, X- adsorbed (Biotin). (concentration 2mg/ml) (USBiological, USA).

9. Immunohistochemistry Procedure:

Immunohistochemistry was performed according to USbiological recommended procedure as follows: Paraffin embedded section for each monoclonal antibody test of the treated and control mice were cut into 5 µm thickness, then placed on positive charged slides and left overnight to dry at room temperature. The slides were deparaffinised and rehydrated. Antigen retrieval protocol for the tissue sections immunostained for CD8, CD56 and TNF-α was performed by putting the slides in a closed jar containing antigen retrieval solution (10mM citrate buffer pH 6.0) in order to stabilize the temperature and avoid evaporation. Antigen retrieval was done by autoclaving at 121 ºC for 2-5 minutes. The slides were allowed to cool in the antigen retrieval solution for 20-30 minutes at room temperature (20-25 ºC).

Then the slides were removed and placed in the washing buffer jar for 5 minutes. The sections were blocked for endogenous peroxidase and for unspecific binding of proteins. Treatment with primary antibody by adding 30-40 µl of diluted primary antibodies onto each section. The slides were incubated in humid chamber for 2 hours at room temperature (20-25 ºC). Then the slides were washed and treated with biotinylated secondary antibody by adding 30-40 µl of diluted biotinylated secondary antibody onto each section. The slides were incubated in humid chamber for 1 hour at room temperature (20-25 ºC). Then washed and Avidin-HRP was added (Thirteen -40µl of diluted Avidin-HRP) (1:500 in PBS) on each section. The slides were placed in the humid chamber and incubated at room temperature for 30 minutes. Then the slides were washed. Then freshly prepared DAB substrate mixture were applied to cover the tissue section.

The slides were incubated in the humid chamber in dark for 20 minutes at room temperature (20-25 ºC). Then the slides were rinsed gently with distilled water from a washing bottle and Counterstained by Mayer's hematoxylin stain and dehydrated and mounted. The slides were examined under light microscope. Cell counts were determined at x400 magnification in 10 randomly chosen fields/ sample. Results were expressed as mean ± SE positive cells per field. The expression of cytokine (TNF-α) was based on the mean number of positive cells per field (Table 3-2).

10. Indirect immunoperoxidase study for staining tissues infected with Newcastle disease virus:

Indirect immunoperoxidase kit for Newcastle disease virus is designed to demonstrate the presence of Newcastle disease virus in tissue mounted on histological slides (TropBio Pty Ltd, James Cook University, Townsville Queensland, Australia).. The detecting antibody is a mixture of three monoclonal antibodies produced to Newcastle disease virus. The mixture of this antibodies recognized most strains of Newcastle disease virus. The reaction carried out on histological slides and the results assessed using a conventional light microscope. Antigen in infected cells stains red-brown. Uninfected cells do not stain. Cell counts were determined at x400 magnification in 10 randomly chosen fields/ sample. Results were expressed as mean ± SE immunopositive cells per field.

3-3: Statistical Analysis

Repeated measures multivariate ANOVA test was used within the SPSS (2007) program to analysis of data to

study the effect of group, time and concentration in the difference traits.

Results:

1. Quantitative Determination of Mouse IL-2 and IFN-γ in Serum of treated mice by Enzyme Linked Immunosorbent Assay (ELISA):

As shown in Figure-1, there was no detectable IL-2 (less than 4 pg/ml) in the serum of NDV treated group (G1) within 7 or 14 days of treatment, whereas at the end of experiment values were elevated (P<0•05) at the day 31(3.05 pg/ml).On the other hand, there was an increase in the IFN-γ concentration from day 7 (44.5 pg/ml) to (4550 pg/ml) at day 14 but no observed concentration (Figure-4.117) (less than 2 pg/ml) at day 31.

In the control untreated group (G2) there was 0.1pg/ml of IL-2 (less than detectable levels 4 pg/ml) in the serum within day 7, while at day 14 it was 4.3 but there was no detectable (0.1pg/ml) of IL-2 at day 31 of treatment. IFN-γ concentration increased from day 7 where it was undetectable to significant increase (405 pg/ml) at day 14 which decreased but still significant increase in concentration at day 31 (103pg/ml).

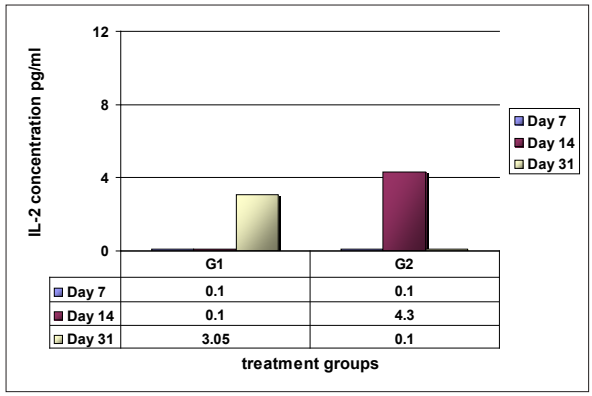

Figure-1, showing the variation in IL-2 concentration in treated groups and control

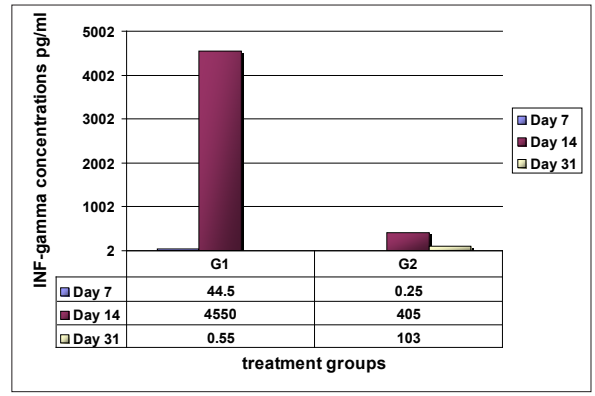

Figure-2 represents differences in IFN-γ concentration in treated groups and control.

2. Immunohistochemical Analysis of NDV HN antigen in tumor and normal organs tissue sections:

Immunohistochemical analysis was performed to determine whether NDV antigen was found in the mammary adenocarcinoma tissues mediate the tumor growth inhibition observed after treatment. A specific mAb for NDV HN antigen was used on the paraffin sections of tumor tissues. Group 1 examined after 7 days of treatment for NDV HN antigen presence in tissue sections, the virus show highly significant presence for immunopositive cells to HN antigen (95%) which illustrated in figure-3.

After 14 and 31 days of NDV treatment, tumor sections revealed the same high percentage of HN NDV positive cells (figure-4 and figure-5). Untreated control group show no immunopositive cells in all tissue sections examined (figure-6).

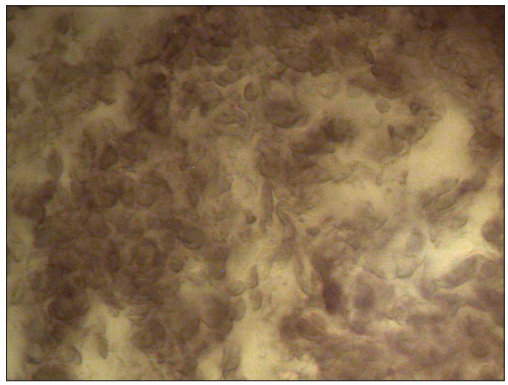

Figure-3 show presence of immunopositive cells to HN antigen (95%) in G1

Figure-4 represent G1 at day 14 which show high percentage of HN NDV positive cells

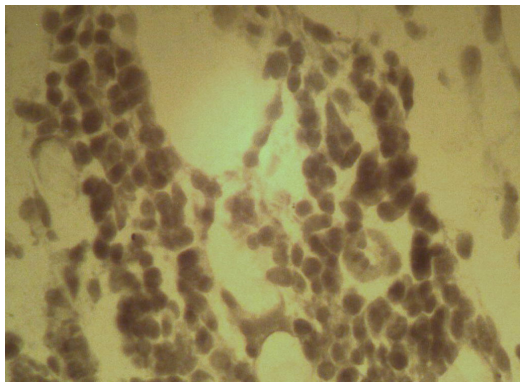

Figure-5 show day 31 in G1 where there was an increase in virus immunopositive cells which reached to 75%

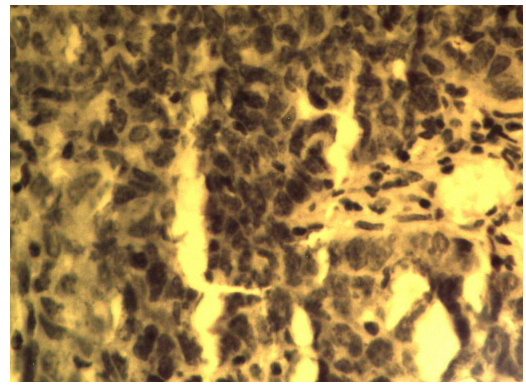

Figure-6 represent untreated control group which show no immunopositive cells in all tissue sections examined

3. Immunohistochemical Analysis of Tumor Infiltrating Lymphocytes in Mammary Adenocarcinoma Tissue in Treated and Control Groups

Immunohistochemical analysis was performed to determine whether anti-tumor immunologic events in the mammary adenocarcinoma tissues mediate the tumor growth inhibition observed after treatment. Anti-CD8 and anti-CD56 mAb were tested on the paraffin sections of tumor tissues from treated and control groups.

Immunohistochemical analysis (table-1) and figure-7 showed significant ($p<0.05$) increase in the mean number of CD8+ cells in the group treated with NDV (G1) compared with the untreated control group at day 7. NDV treated group CD8 mean number was significant at day 14 and day31 of treatment when compared to control untreated group. Immunopositive cells can bee seen in figure-8.

Table-1: Mean and standard deviation of tumor-infiltrating CD8 lymphocyte in mammary adenocarcinoma tissues of different treatment groups.

Type of treatment	CD8 7 days mean	CD8 14 days mean	CD8 30 days mean
G1 µ±SD	10±3.16 A,b	20.7±19.14 A,b	26±15.03 A,b
G2 µ±SD	3.5±1.915 A,a	2.5±1.732 A,a	3.25±1.5 A,a

Different small letter represents significant differences (P≤0.05) between means of the same column. Different capital letters represent significant differences (P≤0.05) between means of the same rows.

The analysis also revealed a significant increase ($p<0.05$) in the infiltration of cells immunostaining positive for a natural killer cells (NK)-surface marker CD56 (table-2 and figure-9) in the group treated with NDV (G1) compared with the untreated control group at 7 days of treatment.

This increased insignificantly to the end of the experiment. Immunopositive cells can bee seen in figure-10.

Table-2: Represent mean and standard deviation of tumor-infiltrating CD56 NK lymphocyte in mammary adenocarcinoma tissues of different treatment groups.

Type of treatment	NK 7 days mean	NK 14 days mean	NK 30 days mean
G1 µ±SD	4.5±2.08 A,a	5.5±1.732 A,a	7±4.243 A,a
G2 µ±SD	2±2.309 A,ab	4.7±5.5 A,a	5.5±5.8 A,a

Different capital letter represents significant differences (P≤0.05) between means of the same column. Different small letters represent significant differences (P≤0.05) between means of the same rows.

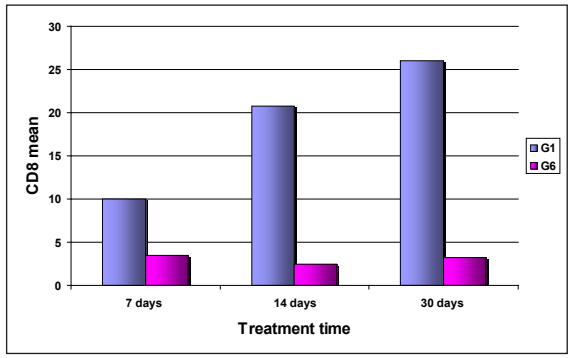

Figure-7, Revealed increased mean number of CD8 positive cells in NDV treated group.

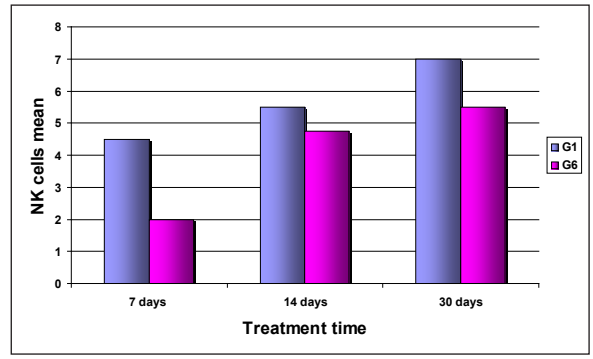

Figure-9, Revealed increased mean number of NK positive cells in NDV treated group at day 7 which continue to increase to the end of the experiment.

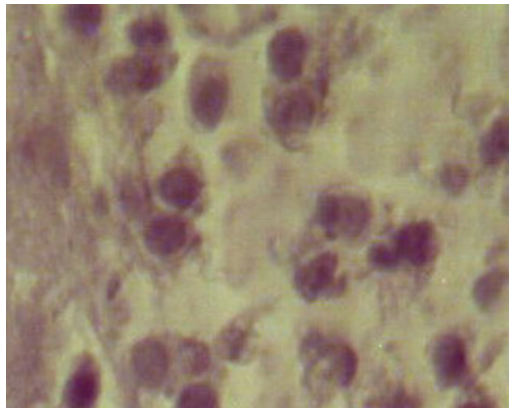

Figure-8 Tumor sections from G1 at day 31 represent CD8 (white arrow) (C) x400, x400 magnification.

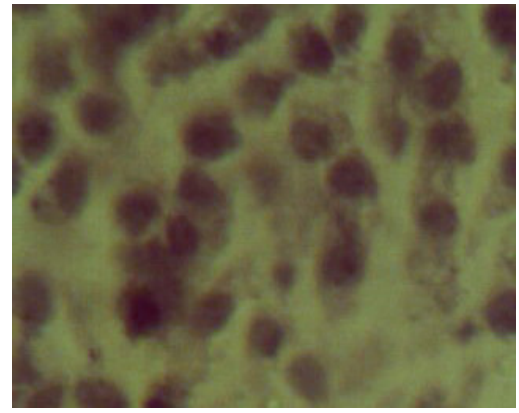

Figure-10 Tumor sections from G1 at day 31 represent CD56 Nk immunopositive cells (white arrow) x400 magnification.

4. Immunohistochemical Analysis of the Expression of TNF-α in Mammary Adenocarcinoma Tissue in Treated and Control Groups:

The mean expressions of TNF-α and the frequency distribution of the expression in mammary adenocarcinoma tissue (IHC assay) in the treated groups are shown in figure-11 and table (3).

The expression of TNF-α at day 7 was significantly ($p<0.05$) higher in the NDV treated group (G1) compared with the untreated control group-6; where it have no significant expression when compared to G6. But the value of TNF-α expression in the G1 was significantly ($p<0.05$) higher. NDV treated group was the most significant group to induce TNF expression in compare to control group.

Newcastle disease virus treatment alone continue to be the most effective inducer for TNF alpha at the end of the experiment followed. The general observations that appeared from the experiment were NDV treatment is good TNF alpha inducer. Immunopositive cells can be seen in figure-12 and 13.

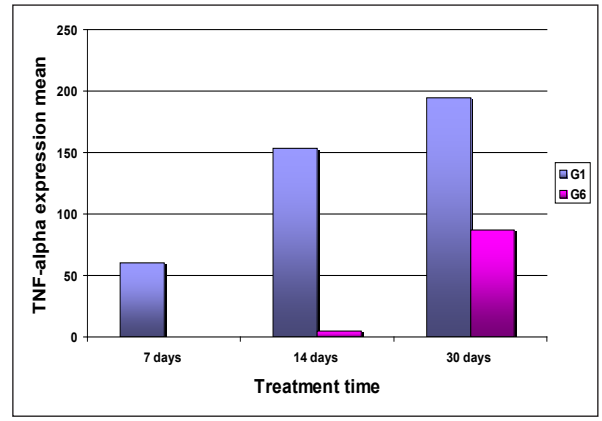

Figure-4.11, Mean expression of TNF-α in treated and untreated mice tissue sections.

Table (3): Represent the mean values of TNF-α expression and the frequency distribution of expression in mammary adenocarcinoma tissues in treated groups.

Type of treatment	TNF-α 7 days mean	TNF-α 14 days mean	TNF-α 30 days mean
G1 μ±SD	60±8.16 A,a	153.07±41.1B,a	194.75±8.81 B,a
G2μ±SD	0±0.0 A,b	5±0.816 A,b	87±8.165 B,c

Different small letter represents significant differences (P≤0.05) between means of the same column. Different capital letters represent significant differences (P≤0.05) between means of the same rows.

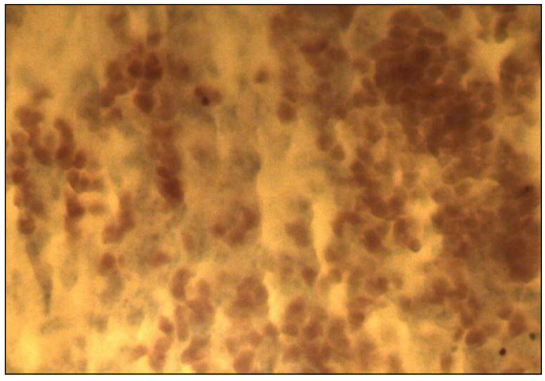

Figure-12 TNF + cells in G1 after 14 days (red arrow) x200 magnification.

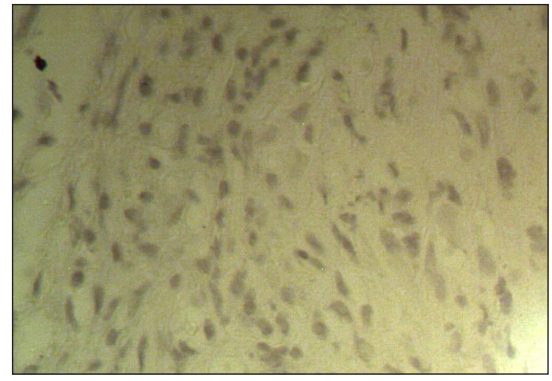

Figure-13 TNF + cells in G2 after 14 days (white arrow) x200 magnification.

Discussion:

Determination of IL-2 and IFN-γ in Serum of treated mice:

The results showed that Newcastle Disease Virus treatment Induce increase in IL-2 when virus still replicate and shed from tumor site within 2 weeks of treatment. Furthermore IFN-γ concentration in NDV treatment group increased from last intratumoral injection at 44.5 pg/ml, to reach higher concentration among groups (4550 pg/ml) after 7 days from last injection, But no observed concentration (less than 2 pg/ml) at day 31 (after 24 days from last injection).

Haas et al. (25) reported that infection of tumor cells with NDV stimulate T cells to increase production of IL-2 and IFN-γ. Present results were in agreement with Pecora et al. (35) who treated human tumor patients with NDV and found detectable increase in IFN-γ was first seen by 6 hours after dosing and reached peak levels at 20hrs after dosing and returned to base line after 2-3 days. Bhagat et al. (36) found that chicken vaccinated with a virulent NDV strain induced significantly higher IFN-γ mRNA expression than control group. Yin et al. (37) recorded efficiency of IFN-γ when coupled with HN of NDV in induce immunity in chicken from NDV infection, which explain the important role for the two elements. Vigil et al. (38) reported the important role for IL-2 in combination with NDV to eradicate tumors in mice, in their experiment they produce recombinant NDV secret IL-2 when infect tumor cells as well as other recombinant with IFN-γ , GM-CSF and TNF-α, their results indicate that NDV-IL-2 was the most efficient treatment to induce complete regression in 6 of 10 mice where the virus alone induce regression in 2 of 10 in his experiment, while other groups was similar to NDV alone effect. This experiment showed the important role for IL-2 in virotherapy for treatment cancer.

In control group IFN-γ and IL-2 concentrations were low. As development of many tumors may reflect a failure of immunosurveillance and the absence of an immune response during early tumor growth. Many potential mechanisms permitting escape from immune destruction have been identified; Selective out growth of antigen negative cells which are poorly immunogenic (39). Reduce the MHC-1 expression which make the tumor cell escape from cytotoxic T lymphocyte (40). Tumor might actively suppress the immune response through immunosuppressive factors such as IL-10 (41) and transforming growth factor

beta (TGF-β) (42). The role of TGF-β as an antiproliferative for T-cells appears to work primarily through inhibition of IL-2 production and inhibition of intracellular activators of T cell differentiation pathways (43). Moreover AlShamery (31) found that transplanted mammary adenocarcinoma secrete unidentified immunosuppressant factor which reduce lymphocyte mitotic index. In present study this transplanted mammary adenocarcinoma was used as the model, combination treatment found to overcome the tumor cell escapee mechanisms and induce immune response as well as marked growth inhibition.

Immunohistochemical Analysis of NDV HN antigen in tumor and normal organs tissue sections:

Immunohistochemical analysis revealed presence of NDV antigen in the treated mammary adenocarcinoma tissues mediated the tumor growth inhibition observed after treatment. Group one with NDV alone showed the highest percentage of virus presence. Wagner et al. (44) conducted an immunohistochemical study with an anti-NDV HN antibody displayed the abundant presence of viral antigen in the cytoplasm of apoptotic and pre-apoptotic tumor cells in NDV treated patient with glioma. Freeman et al. (45) mentioned that the recovery of infectious NDV particles from the tumor's cyst fluid and the tumor tissue obtained by biopsy performed about 130 days after the start of virotherapy in human tumor patients indicated that the NDV-HUJ reached the extracellular space of the tumor.

Al-Shamery (31) described presence of NDV particles and budding from infected mammary adenocarcinoma treated by IT NDV injection in transmission electron microscope sections which more confirmed by our present results that showed presence of HN antigen of NDV in infected tumor cells which indicate virus replication inside tumor cells even after weeks from the end of the treatment. Presence of NDV HN antigen in tumor cells surface have special importance. It will modified tumor cell surface and increase its immunogenicity. Making tumor cell more recognizable from the immune system cells. This proved in *in vitro* lymphocyte attachment assay (31), where the infected tumor cells were exhibited marked lymphocyte attachment while uninfected cells showed no attachment. This will mediate tumor cells destruction through immunological stimulation as mentioned by Schirrmacher et al. (46). Another important result can be achieved from virus replication inside tumor cells which is direct oncolytic activity, secondary to virus replication and cytolysis after virus budding from the tumor cell membrane. Furthermore, Al-Shamry, 31 experiment finding showed that replication of NDV inside tumor cells will induce apoptosis in infected tumor cells as showed in *in vitro* increased mitochondrial permeability transition event test which indicate early apoptosis pathway.

As well as increased in caspase-12 expression in tumor sections of NDV treated group and combination groups. This indicates induction of endoplasmic reticulum apoptosis pathway, which showed by presence of immunopositive cell for caspase-12 (31) .

pecora et al. (35) described presence of NDV budding from tumor cell membrane in patient treated with i.v NDV before 11 months and was replicating in tumor despite presence of neutralizing antibodies. Wu et al. (47) used oncolytic adenovirus for infection of metastatic bladder cancer model which detected the virus protein immunohistochemically after systemic treatment.

Immunohistochemical study of Tumor Infiltrating Lymphocytes:

Newcastle disease virus treated group tissue sections showed highest increased in CD8 and NK cells infiltration in the tumor tissue where the CD8 and NK number increased significantly in compare to control group all day tested (7, 14 and 31 after treatment). Newcastle disease virus was found in the present experiment to be a potent activator of CTL and to significantly expand NK cells and macrophages as confirmed by other study (48). Increases in relative CTL numbers were observed significantly in NDV treated group (G1).

Our results suggested that immunogenicity of tumor cells could be increased by viral infection and oncolysis supported by *in vitro* experiments Schirrmacher et al. (48). The underlying mechanisms include the release of proinflammatory cytokines after NDV infection into tumor cells or cells of the immune system (including APCs and macrophages), the ability to release cryptic antigens as apoptotic bodies, and the coexpression of viral proteins in tumor cells which are potent immunogens (CTL specific to NDV proteins might help liberate tumor antigens for cross-priming of APCs).

The changes in levels of NK cells and IFN-γ within or after NDV infection suggest these responses as critical participants of a cascade of innate immune responses to the virus. Described as circulating cells attracted by viral infection of a host, NK cells start the immune process at least partly by releasing IFN-γ before exiting the site of inflammation, giving way to secondary responses. NK cell-derived IFN- γ is the prototypic monocyte+ macrophage activator, without which monocytes+ macrophages cannot clear intracellular organisms as mentioned by Cooper et al. (49). Wu et al. (47) described Natural killer cells as a major component of the innate immune system.

These cells represent a distinct population of cytotoxic lymphocytes, characterized by the CD16+ and/or CD56+ phenotype, NK cells are activated during viral infections and they mediate direct lysis of target cells by releasing cytotoxic granules containing lytic enzymes, or by binding to apoptosis-inducing receptors on the target cell. Fulci et al. (50) *in vitro* data showed that pretreatment with CPA inhibits production of IFN- γ by NK cells but does not affect their viability within 48 h, probably because of the slow replication cycle of these cells. Thus, the *in vivo* mechanism of CPA modulation of innate immunity against oncolytic virus HSV may be related to the inhibition of IFN- γ production by NK and monocytic cells in response

to the virus and to the decrease of intratumoral density of HSV-stimulated macrophages. When making IFN- γ, NK cells make a plethora of cytokines and chemokines, many of which may serve as chemoattractants, decreasing viral load, and are likely to be similarly affected by CPA.

Immunohistochemical Analysis of the Expression of TNF-α in Mammary Adenocarcinoma Tissue in Treated and Control Groups

The general results that obtained from this experiment were in correlation with previous experiments. NDV treatment is the best TNF alpha inducer in compare with control untreated group, Haas et al. (25) reported that infection of tumor cells with NDV stimulate T cells to release high amount of TNF-α. Zeng et al. (14) refer to HN antigen of NDV as main protein of NDV who responsible on this induction.

Hotte et al. (51) reported detectable TNF-α in the serum of NDV treated human tumor patients after 20hrs post dosing. Tos et al. (52) indicated that TNF-α may play a regulatory role in the proliferation of human tumor cells and suggest potential role for it as antitumor therapeutic strategy by restores an apoptotic cell death program and induce a bystander-killing effect. Saenger et al. (53) mentioned that TNF has direct antiproliferative and cytotoxic effects on cells, with some selectivity for tumor cells. It also reduces tumor blood flow and causes tumor vascular damage. TNF may also modulate the immune response by stimulating macrophage and NK cell activity.

Combination treatment of NDV and CPA is second to NDV alone. Combination with lower dose of CPA is more effective in TNF expression induction which explained by CPA high dose may suppress immune response induced by NDV.

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دراسة مناعية لعلاج فايروس النيوكاسل (NDV) في فئران مصابة باورام الثدي الغدية

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الخلاصة:

يقتل فايروس النيوكاسل الخلايا السرطانية ولايقتل الخلايا الطبيعية, ويمتلك خواص تحفيز مناعية مثيرة للاهتمام. اجريت هذه الدراسة للتحقق من قدرة العترة العراقية من فايروس النيوكاسل لاستحداث تحفيز مناعي عندما تستعمل كعامل حال للورم بالحقن داخل الكتله الورمية.

تم تقدير مستوى التحفيز المناعي بقياس مستوى الوسائط الخلوية IL-2 و IFN gamma و قياس مستوى الخلايا التائية السامة والخلايا القاتله الطبيعية. اضهرت النتائج وجود مستوى تحفيز مناعي عالي لفايروس النيوكاسل العترة العراقية مما يرشحها للمزيد من الدراسات.