In vivo efficacy of systemic tumor targeting of a viral RNA vector with oncolytic properties using a bispecific adapter protein

HUIJIE BIAN[1,3], HOLGER WILDEN[1], PHILIPPE FOURNIER[1], BEN PEETERS[2] and VOLKER SCHIRRMACHER[1]

[1]Division of Cellular Immunology, German Cancer Research Center, D010, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany; [2]Animal Sciences Group, Wageningen UR, Division of Infectious Diseases, P.O. Box 65, 8200 AB Lelystad, The Netherlands; [3]Cell Engineering Research Center, Fourth Military Medical University, Xi'an 710032, P.R. China

Abstract. The aim of the study was: i) to specifically target tumor tissue by Newcastle disease virus (NDV) with oncolytic properties, ii) to improve the delivery system for systemic application of NDV via a bispecific adapter protein and iii) to investigate anti-tumor activity and side-effects. We selected two oncolytic virus strains, one native and the other recombinant, which showed multicyclic replication patterns in tumor cells. In order to reduce normal cell binding, they were modified by preincubation with a recombinant bispecific protein which blocks the viral native cell binding site and introduces a new binding site for a tumor-associated target (in this study, the interleukin-2-receptor, IL-2R). After intravenous transfer to mice, uptake of modified NDV in liver, spleen, kidney and lung was greatly reduced in comparison to unmodified NDV as determined by RRT-PCR of viral M gene copies. In IL-2R+ tumor bearing mice, the same assay revealed a high replication efficiency of the modified virus in the tumor tissue. Tumor therapy experiments showed that the side-effects induced by systemic application were greatly reduced by the adapter protein and that the anti-tumor effects were mostly undiminished. The demonstration of significant systemic anti-tumor activity of this viral vector suggests potential for augmentation by inclusion of one or more therapeutic genes.

Introduction

The field of replicating oncolytic virus therapeutics is rapidly developing (1). Viral therapeutics can be tailored to attack genetic defects commonly found in tumor cells (2). Several oncolytic viruses (either native or genetically engineered), such as replication-competent adenovirus (3), herpes simplex virus (HSV) (4), and vesicular stomatitis virus (VSV) (5) carrying foreign genes have been reported to behave like 'armed' virus vectors in cancer treatment. The use of replication-competent viral vectors instead of traditional replication-defective viruses allows amplification of the potential therapeutic anti-tumor effects (6,7). Of particular relevance are oncolytic effects which destroy tumor tissue, release tumor-associated antigens (TAAs) together with virus-derived danger signals (8) and support post-oncolytic systemic anti-tumor immunity (9). However, a certain number of unresolved problems of cancer therapy with viruses (virotherapy) remain to be solved. They are mostly due to inefficiency of tumor targeting, which consequently results in only weak anti-tumor effects associated with side-effects and toxicity. To improve tumor targeting, adenoviral vectors have been modified by incorporating into their envelope protein an antibody single chain Fv (scFv) fragment directed against carcinoembryonic antigen as a tumor target (10) but this approach has not yet been very successful. An alternative, antibody-mediated targeting of adenoviral vectors by means of bispecific adapter molecules seems to be more feasible. While such reagents demonstrated efficient gene transfer *in vitro* (11), there are as yet only limited data on the *in vivo* efficacy.

The present study employs Newcastle disease virus (NDV), a chicken paramyxovirus with anti-neoplastic properties (12) which is not pathogenic towards humans and for which long-standing clinical experience exists with regard to treatment of cancer patients (13,14). According to their virulence in chickens, NDV strains are subdivided as *lentogenic* (non-virulent), *mesogenic* (mildly virulent) or *velogenic* (higly virulent). NDV is a membrane-enveloped virus with a non-segmented and negative-stranded RNA genome which contains genes encoding the nucleoprotein (NP), phosphoprotein (P), V protein, matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large polymerase protein (L). Viral replication occurs in the cytoplasm of infected cells, thus avoiding potentially deleterious interactions with the genomic DNA, and involves the generation of viral double-strand RNA (dsRNA) molecules which activate innate immune responses via Toll-like receptor 3 (15) and via a cytoplasmic RNA helicase, RIG-I (16).

Previously, we showed that a *lentogenic* recombinant NDV (NDFL-EGFP) carrying the reporter gene coding for

EGFP can be retargeted *in vitro* (17) and *ex vivo* (18,19) to receptor-defined tumor cells by the addition of an adapter molecule. This protein (αHN-IL-2) contains an scFv antibody cloned from a neutralizing HN-specific hybridoma linked to human interleukin-2 (IL-2). It has been shown to block the native cell binding activity of NDV and to simultaneously provide a new binding specificity for the IL-2 receptor α-chain (IL-2R, here used as tumor target). The rationale for the use of the IL-2Rα, p55 (CD25), as a target for immunotherapy has been reviewed by T.A. Waldmann (20). The receptor is not expressed by resting normal cells while it is overexpressed by a proportion of abnormal cells of lymphoid neoplasia (21), including adult T-cell leukemia (22).

In this study, we tested for the first time *in vivo* the concept of retargeting NDV to tumor tissue after systemic intravenous (i.v.) application (systemic tumor targeting). We noticed in previous animal tumor studies that anti-tumor activity of NDV was much better when applied locally (at the tumor site or as a tumor vaccine) than systemically (23). The adapter protein, αHN-IL-2, was employed in this study to test whether blocking of the native cell binding site during transport through the blood could reduce virus uptake in normal tissue. To improve potential anti-tumor activity, we selected more virulent NDV strains than those used before to allow for virus spread within the tumor tissue. For this purpose we used the wild-type *velogenic* strain, NDV *Italien*, and the recombinant *mesogenic* strain, NDFLtag-EGFP (24,25). Viral replication, transgene expression, cytopathic effects, and therapeutic effects were evaluated *in vitro* or *in vivo* in the presence or absence of the bispecific protein, αHN-IL-2.

Materials and methods

Cells and viruses. Eb-M7(IL-2Rα⁺) cells expressing high level of IL-2Rα were obtained from the p11-R-Eb cell line by successive sorting (19). MT-2, a human T-cell leukemia virus-I transformed T cell line, was kindly provided by Dr Masahiko Makino (Department of Microbiology, National Institute of Infectious Disease, Tokyo, Japan). The two cell lines were propagated in RPMI-1640 medium supplemented with 10% fetal-calf serum (FCS) at 37ºC in a humidified atmosphere of 5% CO_2. The human Jurkat CD3 cell line that was sorted from Jurkat cells for CD3-positivity was grown in RPMI-1640 medium supplemented with 5% FCS. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. All reagents were purchased from Gibco Life Technologies (Karlsruhe, Germany).

Recombinant NDV including NDFL *LaSota*, NDFL-EGFP, and NDFLtag-EGFP were generated from a cDNA clone of NDV strain *LaSota* (24,26). NDV-*Ulster 2C* was obtained in 1984 from Dr P.H. Russel (University London, UK). NDV *Italien* was obtained in 1986 from H.D. Klenk (University Giessen, Germany). All viruses were propagated in embryonated chicken eggs, harvested from the allantoic fluid, purified by ultracentrifugation as described previously (27) and cryopreserved in aliquots at -70˚C. The virus was quantified by a hemagglutination assay. One hemagglutination unit (HU) is defined as the smallest virus concentration leading to visible sheep erythrocyte agglutination.

Antibodies and bispecific fusion protein. The mouse anti-F mAb lcii (IgG1) was kindly provided by Dr Iorio (Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachussetts, USA) and was used in flow cytometry to detect NDV viral antigens on host cell surfaces. Polyclonal rabbit anti-NDV serum was prepared by immunizing rabbits four times s.c. with 40,000 HU/ml of NDV-Ulster mixed 1:1 with incomplete Freund's adjuvant. Pre-immune serum and the antiserum were heat-inactivated (for 45 min at 56˚C), aliquoted and stored at -20˚C.

The bispecific fusion protein, αHN-IL-2, was constructed by fusion of a single-chain antibody cloned from a neutralizing HN-specific hybridoma linked to the human cytokine, IL-2, as described previously (17). This protein did compete with anti-HN mAb but not with anti-F mAb (18). Upon binding to NDV, this adapter molecule increased the stability of the virus upon culture at 37˚C (18).

NDV binding and infection of tumor cells. Tumor cell suspensions were washed twice with FCS-free RPMI-1640 medium and $1x10^6$ cells were incubated with native NDV or the same dose of modified NDV/αHN-IL-2 (which was obtained by incubating NDV with an appropriate amount of αHN-IL-2 for 1 h on ice) in a final volume of 1 ml for 1 h at 37˚C in a CO_2 incubator. During the incubation, cells were shaken every 15 min. The cells were then washed twice and either stained with antibodies and analyzed by FACS to measure the bound virus, or further cultured for 24 h to allow for viral replication. Replication of recombinant EGFP-virus in host cells leads to the expression of EGFP that can be measured directly by flow cytometry without antibody staining.

Plaque assay. Vero cells were seeded into a 6-well plate for 16 h before virus infection. When cells just reached confluency, virus dilutions (1.0 ml) were added carefully to the cell mono-layers. After 1 h at 37˚C, unadsorbed virus was removed by aspiration and the monolayers were washed once with serum-free and phenolred-free DMEM. Then the monolayers were overlaid with 2.5 ml of phenolred-free DMEM supplemented with 10% FCS and 2% agar (Invitrogen Gibco Life Technologies, Karsruhe, Germany) and were further incubated in an atmosphere of 5% CO_2 at 37˚C. Three days later, the monolayers were incubated with 0.03% neutral red for 4 h and the virus plaques were observed under the microscope.

Flow cytometry. One million cells/sample were used for analysis by a FACScan flow cytometer (Becton-Dickinson, Heidelberg, Germany). All antibodies were diluted in FACS buffer (PBS containing 5% FCS and 0.1% NaN_3). Cells were washed twice with FACS buffer and then incubated with the first antibody. Subsequently, the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern Bio-technology Associates, Inc., Birmingham, USA) for 30 min on ice in the dark. All FACS data were analyzed with CELLQuest software (Becton-Dickinson).

Determination of HAd and hemolysis activity. The HAd activity of the virus NDFLtag-EGFP was determined by testing its ability to adsorb human erythrocytes. Human erythrocytes were isolated from buffy coats by centrifugation at 600 x g and

then washed with 3 volumes of isotonic PBS. Subsequently, 1×10^8 erythrocytes were co-incubated with either native or modified NDFLtag-EGFP for 1 h using the above described protocol and analysed with flow cytometry after staining the cells with antibodies.

The extent to which erythrocytes are hemolyzed by NDFLtag-EGFP was evaluated by measurement of the released hemoglobin. Briefly, human erythrocytes were incubated with NDFLtag-EGFP for 6 h at 37˚C. The reaction mixtures were then centrifuged at 13,000 rpm for 10 min. The hemoglobin content of supernatants was measured as absorbance by spectrophotometer at a wavelength of 412 nm. In other experiments, NDV *Italien* was used instead of NDFLtag-EGFP.

EGFP expression and IHC in vivo. Pathogen-free DBA/2 mice were obtained from Charles River GmbH (Wiga, Germany) and kept under pathogen-free conditions at the DKFZ animal facilities (Heidelberg, Germany). All procedures involving mice were approved by the Animal Care and Use Committee of the German Cancer Research Center. Mice received s.c. implants of 5×10^6 syngeneic Eb-M7 (IL-2Rα+) cells/100 µl PBS. One week after tumor cell implantation, 5,000 HU NDFLtag-EGFP or an equivalent dose of modified NDFLtag-EGFP/αHN-IL-2 was i.v. injected into mice. To assess EGFP expression and virus distribution in organs, mice were euthanized 24 h after virus injection. Tumor, liver, spleen, kidney, lung, and intestine were harvested and fixed overnight in PBS containing 1.5% paraformaldehyde and 20% sucrose. Six-µm thick sections were prepared from frozen tissues that were previously embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe B.V., The Netherlands). EGFP was visualized by fluorescence microscopy with an EGFP filter. Sections on subbedded slides (coated with 3-triethoxysilyl-propylamine, Sigma, Taufkirchen, Germany) were subjected to IHC using rabbit polyclonal anti-NDV serum (1:800 dilution), followed by chicken anti-rabbit Ig (H+L) Alexa Fluor® 594 (1:400 dilution) (Molecular Probes, Leiden, The Netherlands). IHC sections were counterstained with DAPI.

Quantification of EGFP and M gene expression by real-time reverse transcriptase PCR (RRT-PCR). Before dissection and uptake of the different organs, the mice were perfused transcardially through the aorta with DEPC-treated PBS until lung, liver and kidney turned pale. After the removal of tumor, liver, spleen, kidney, and lung, these tissues were immediately submerged in RNAlater RNA stabilization solution (Qiagen GmbH, Hilden, Germany) in order to protect the RNA. The Qiagen RNeasy procedure for animal tissue was used to extract RNA and the corresponding first-strand cDNA was synthesized using SuperScript™ II RNase H- reverse transcriptase following the instructions of the supplier (Invitrogen, Karlsruhe, Germany).

RRT-PCR was carried out using a GeneAmp® 5700 sequence detection system (Applied Biosystems GmbH, Weiterstadt, Germany). Amplifications were run in separate tubes to illustrate relative quantification of the target gene (EGFP or M) normalized with an endogenous control, mouse ß-actin. Primers for M gene (sense, 5'-AGT GAT GTG CTC GGA CCT TC-3'; antisense, 5'-CCT GAG GAG AGG CAT

TTG CTA-3'), and mouse ß-actin (sense, 5'-ACG GCC AGG TCA TCA CTA TTG-3'; antisense, 5'-AGG ATT CCA TAC CCA AGA AGG AA-3') were synthesized by the German Cancer Research Center. Each RRT-PCR was performed in a 25-µl reaction mixture containing 17.5 µl Master Mix (qPCR™ Core Kit for SYBR® Green I, Eurogentec), 300 nM each corresponding primer for EGFP and for M, 300 nM sense and 900 nM antisense primer for ß-actin. cDNA from PBS-treated mice was used as negative control and was included in each set of experiments. A common threshold was set in the exponential phase of the PCR reactions to determine a specific threshold cycle (C_T) number for each sample. The comparative C_T method was used to quantify the target genes and the amplification efficiencies of the target (EGFP or M). The reference (ß-actin) primer systems were shown to be approximately equal using the standard curve method (slope of log input amount vs. C_T<0.03).
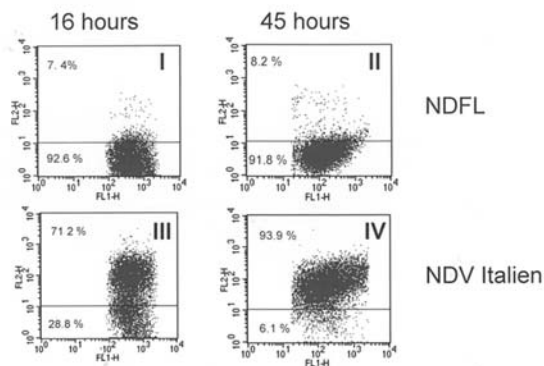
Therapy experiments. DBA/2 mice were irradiated (4.5 Gray) 1 day before the s.c. inoculation of 5×10^6 Eb-M7 (IL-2 Rα+) syngeneic tumor cells. After a desentisization step on day 1, animals received 3 i.v. injections of different virulent NDV preparations. The tumor nodule size was measured in two dimensions with a calliper. The volume of the mass was calculated according to the following formula: Volume = [(greatest diameter) x (smallest diameter)²]/2. At the end of the study, the surviving mice were sacrificed under anesthesia. The animals were subjected to autopsy and different organs were also taken for histology and RRT-PCR analysis.
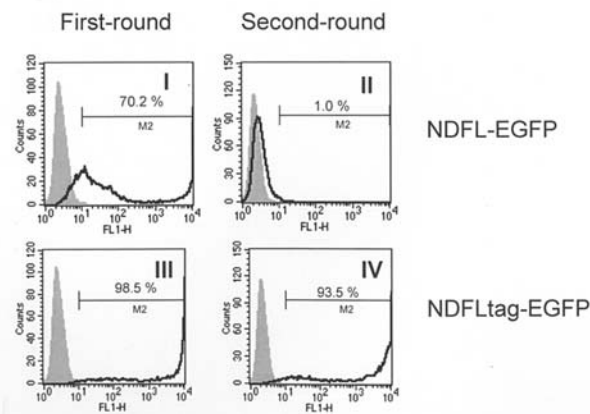
Results

Multicyclic replication and oncolytic properties of virulent NDV strains in vitro. To evaluate the mode of replication in tumor cells of NDV strains with different virulence, we first performed experiments to test for cross-infection (Fig. 1A). For this purpose, virus-infected human MT-2 leukemic-like cells were mixed with a 5-fold excess of non-infected CFSE-labeled MT-2 cells and virus infection of the latter was followed by staining with anti-F mAb and FACS analysis. As shown in Fig. 1A, at 16 h, 71.2% (III) and 7.4% (I) of gated CFSE-labeled cells were infected by virulent NDV *Italien* and avirulent NDFL, respectively. After 45 h, the values were 93.9% (IV) for NDV *Italien* as compared to only 8.2% (II) for NDFL.

To determine whether the two recombinant viruses, NDFL-EGFP and NDFLtag-EGFP (24), which differ only in the cleavage sequence of the fusion protein, have monocyclic or multicyclic replication behavior in tumor cells, we performed sequential infections. MT-2 tumor cells were first infected and analyzed for EGFP expression. Then we transferred the 24-h supernatants of the infected cells onto non-infected MT-2 cells again. After incubation for 24 h, EGFP expression was again analyzed by flow cytometry. Fig. 1B shows that, in the first-round, 70.2% (I) and 98.5% (III) of the cells were infected by NDFL-EGFP and NDFLtag-EGFP, respectively. In the second-round, 1.0% (II) and 93.5% (IV) of the cells were infected by supernatants containing either NDFL-EGFP or NDFLtag-EGFP virions, respectively. Thus, only NDFLtag-EGFP shows multicyclic replication behaviour.
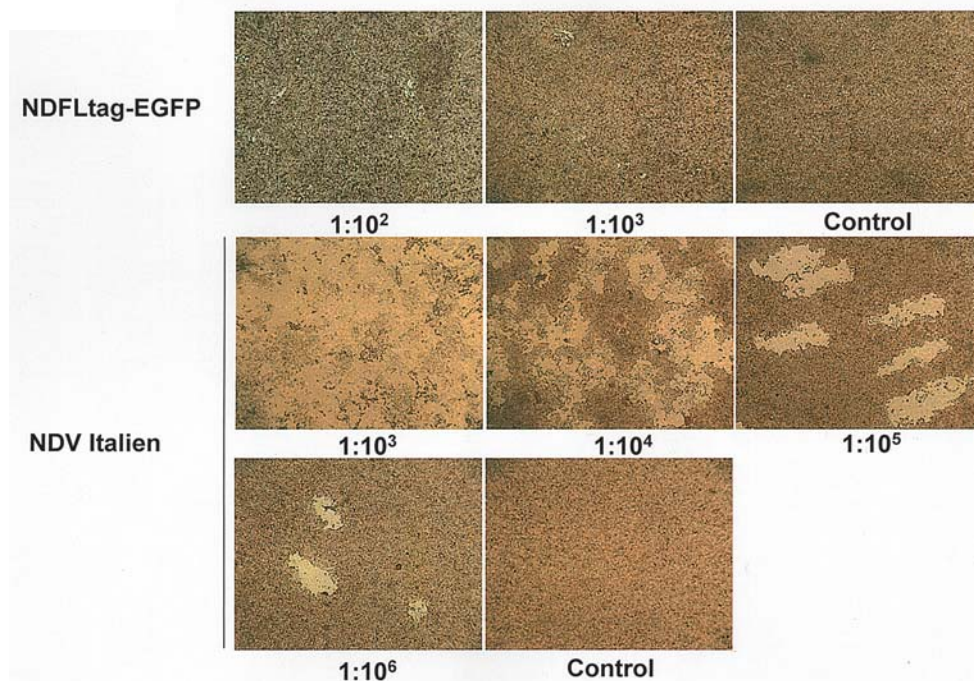
A) Cross infection :

B) Multicyclic replication :

Plaque assays in vero cell monolayers were then performed to test for further differences between the three strains, NDFL-EGFP, NDFLtag-EGFP and NDV *Italien*. The NDFLtag-EGFP strain produced small visible plaques as holes in the vero cell monolayer (Fig. 1C) whereas the NDFL-EGFP did not (data not shown). Large syncytia and plaques were observed in vero cell monolayers after infection with NDV *Italien*. The cells were almost lysed by the higher concentration of this velogenic NDV strain (Fig. 1C).

Inhibition of viral native cell binding by the adapter protein,
αHN-IL-2. We next evaluated whether the bispecific protein, αHN-IL-2, which we employ for retargeting the virus to IL-2 receptor-positive tumor cells, can neutralize the normal cell binding capacity of NDFLtag-EGFP. For this purpose, human erythrocytes were incubated for 1 h with high amounts (1,000 HU) of NDFLtag-EGFP in the absence or presence of different concentrations of the adapter protein, αHN-IL-2. The erythrocytes were then stained with the F-specific mAb before being analyzed by flow cytometry. As shown in Fig. 2A, 100% hemadsorption (HAd) activity was observed with the non-modified virus. With increasing concentrations of αHN-IL-2 (starting at 5 μg/ml), the HAd activity decreased. Fifty percent HAd inhibition was observed at 15 μg/ml and 99% HAd inhibition at 20.7 μg/ml. A similar titration curve was obtained with NDV *Italien* (data not shown).

C) Cytopathic plaque formation :

Figure 1. Virulence and oncolytic properties of the selected NDV strains. (A) Cross infection. MT-2 cells (2x10⁵) were infected with either NDFL (I, II) or NDV *Italien* (III, IV) for 1 h at 37˚C. After washing twice, the cells were co-cultivated with 8x10⁵ CFSE-labeled MT-2 cells. CFSE-positive cells were analyzed by FACS after a two-step staining with anti-F mAb and with goat anti-mouse Ig-RPE at 16 (I, III) and 45 h (II, IV) post-infection, respectively. (B) Multicyclic replication. One million MT-2 cells were infected either with 10 HU NDFL-EGFP (I) or 10 HU NDFLtag-EGFP (III) for 24 h in 1 ml medium. After centrifugation of the cells at 1,200 rpm for 5 min, the supernatants from I and III were then pipetted into wells II and IV respectively, containing 10⁶ MT-2 cells respectively (II and IV). The cells were cultivated for 24 h and then analyzed for EGFP expression by flow cytometry. Cells without virus infection (grey peak) were used as negative controls. (C) Plaque assays. Vero cell monolayers in a 6-well plate were infected for 1 h at 37˚C with 1 ml of serial dilutions of virus (from 1:100 to 1:10⁶) from a virus stock having a titer of 1000 HU/ml. The monolayers were then overlaid with 2.5 ml of phenolred-free DMEM supplemented with 10% FCS and 2% agar for 3 days. The plaques were visualized with 0.03% neutral red for 4 h and observed under the microscope (magnification x25).
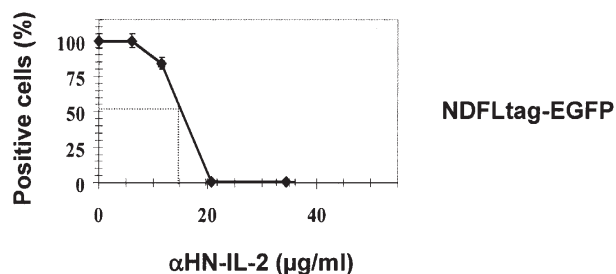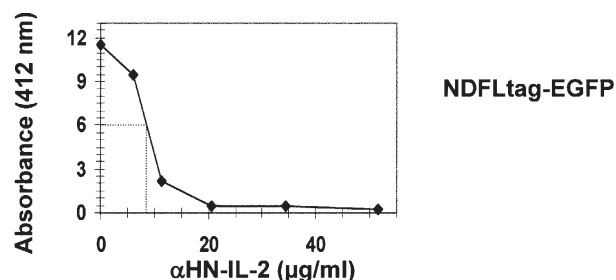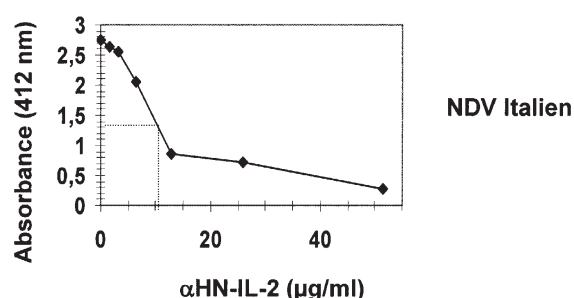
A) Inhibition of erythrocyte binding:

B) Inhibition of hemolysis:

C) Inhibition of hemolysis:

Figure 2. Inhibition of HAd and hemolysis activities of virulent NDV by the adapter protein αHN-IL-2. (A) Inhibition of HAd activity. Approximately 10^8 human erythrocytes were incubated for 1 h at 37˚C with 1,000 HU of NDFLtag-EGFP or NDFLtag-EGFP/αHN-IL-2 modified as in Table I in a final volume of 1 ml. After two-step staining with anti-F mAb and with goat anti-mouse Ig-PE, FACS analysis allowed the quantification of erythrocyte-bound virus. (B and C) Inhibition of hemolysis activity induced by 1,000 HU NDFLtag-EGFP (B) and 62.5 HU NDV *Italien* (C). Erythrocytes were incubated with unmodified or modified virulent viruses for 6 h at 37˚C. After centrifugation at 13,000 rpm for 10 min, the absorbance of hemoglobin in the supernatant was measured by a spectrophotometer at a wavelength of 412 nm. Dotted lines indicate the amounts of αHN-IL-2 at 50% inhibition of hemolysis.

Longer (6-h) incubation of NDV with erythrocytes at 37˚C resulted in hemoglobin release, which could be quantified by measuring the absorbance at 412 nm. Fig. 2B shows that the hemolytic activity of NDFLtag-EGFP virus was blocked by αHN-IL-2 in a dose-dependent manner. Similar observations were made with the velogenic strain *Italien* when using a lower virus amount (62.5 HU) because this strain has the highest virulence. These results demonstrate cytotoxic activity of virulent NDV strains against erythrocytes and successful neutralization of this activity by the adapter protein.

Retargeting virulent NDV via αHN-IL-2 to IL-2R target-positive tumor cells in vitro. We next tested retargeted transgene delivery by NDFLtag-EGFP/αHN-IL-2 using IL-2Rα+

Table I. IL-2 receptor retargeted tumor cell replication of virulent NDV strains *in vitro*.

		% of positive cells tumor target cells	
Group	Virus	IL-2R+	IL-2R-
I	NDFLtag-EGFP	92.4	91.6
II	NDFLtag-EGFP + αHN-IL2	90.5	5.8
III	NDV *Italien* §	9.2	20.8
IV	NDV *Italien* + αHN-IL-2	33.6	6.0

Groups I and II, retargeting of Eb-M7 murine lymphoma cell variants which are either IL-2 receptor positive (IL-2R+) or negative (IL-2R-). For the modification of NDFLtag-EGFP, 1,000 HU of the virus was preincubated with 20.7 µg/ml of αHN-IL-2 for 1 h on ice. NDFLtag-EGFP and the modified NDFLtag-EGFP/αHN-IL-2 were incubated with tumor cells at a concentration of 10 HU/10^6 tumor cells for 1 h at 37˚C. After being washed twice with medium, the two cell lines were further cultured for 24 h to allow for infection and EGFP expression. Cells were then analyzed for EGFP fluorescence by flow cytometry and the percentage of infected cells was calculated based on the FACS results. Cells cultured in parallel without virus were used as negative control. Groups III and IV, retargeting of human tumor cell lines which are either IL-2 receptor-positive (MT2, IL-2R+) or -negative (Jurkat, IL-2R-). Tumor cells (10^6) were co-incubated with 3 HU NDV *Italien* in the presence or absence of 40 µg/ml αHN-IL-2 for 1 h on ice. After being washed twice, the two cell lines were further cultured for 6 h and then stained with anti-F mAb and goat anti-mouse Ig-RPE. They were analyzed in channel two of the flow cytometer.

expressing tumor cells as recipients. For this purpose, Eb-M7 (IL-2Rα+) and Eb-M7 (IL-2Rα-) murine lymphoma cells (19) were infected with NDFLtag-EGFP and modified NDFLtag-EGFP/αHN-IL-2 and followed by FACS analysis to measure EGFP expression. Almost the same amount of transgene EGFP was delivered to Eb-M7 (IL-2Rα+) cells by the modified as by the non-modified virus, while EGFP was hardly observed in Eb-M7 (IL-2Rα-) cells upon infection by the modified virus NDFLtag-EGFP/αHN-IL-2 (Table I, groups I and II).

In a similar way, the retargeted infection and replication of NDV *Italien* was tested in a pair of human tumor cell lines, one line being IL-2R-negative (Jurkat) and the other IL-2R-positive (MT-2). After 6-h incubation, viral replication was evaluated by staining the cells with anti-F mAb and goat anti-mouse Ig-RPE. Viral replication was increased in the target-positive MT-2 cells by a factor >3 when the αHN-IL-2 protein was added (Table I, groups III and IV). In contrast, in target-negative Jurkat cells, virus infection and replication was inhibited in the presence of the adapter protein (Table I, groups III and IV).

These results demonstrate: i) that the native HAd and hemolytic activity of the two virulent strains can be effectively inhibited by modification of the virus with αHN-IL-2, and ii) that the efficiency of gene delivery and viral replication to target positive tumor cells is not diminished when using retargeted modified virus.
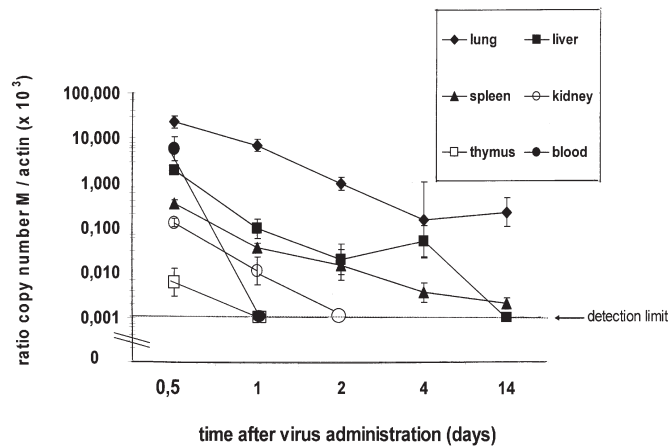
Figure 3. Biodistribution of NDV *Italien* in normal mice. 1,500 HU NDV *Italien* was injected i.v. into DBA/2 mice. Different organs (lung, liver, spleen, kidney, thymus and blood) were collected at different time-points (2 mice for each analysis) after virus administration. Virus copy number was detected by quantitative PCR based on the M specific mRNA (triplicates of each measurement) and normalized to ß-actin. Bars indicate standard deviations.

Biodistribution of virulent NDV strains in normal and tumor-bearing mice after systemic application. To test the bio-distribution of virulent NDV in normal mice, 6-week old DBA/2 mice were i.v. injected with 1,500 HU NDV *Italien* and different organs were harvested at different time-points after virus application. To determine the sites of viral genome replication, RNA was isolated from the different samples and transcribed into cDNA. Quantitative RRT-PCR allowed the determination of the amount of M and ß-actin mRNA. The detection limit was 7 copies for the M gene and 32 copies for the ß-actin gene. When calculating the ratio copy number M/ ß-actin (x10^3), the detection limit for the virus was at 0.001 for the input amount of cDNA used in the experiment. As shown in Fig. 3, NDV *Italien* was detected at 0.5 h after i.v. injection mainly in the lung, blood, liver and spleen of normal mice. Only a few M mRNA copies were found in the thymus. The amount of virus decreased rapidly over time and reached the detection limit at less than 1 day (blood and thymus), 2 days (kidney) and around 14 days (lung, liver, spleen).

We then tested the biodistribution of unmodified or αHN-IL-2 modified NDFLtag-EGFP virus in tumor bearing mice. DBA/2 mice bearing Eb-M7 (IL-2Rα$^+$) s.c. tumors (0.3-0.4 cm diameter) at the right flank were injected i.v. with 5,000 HU of native or modified NDFLtag-EGFP. After 12 and 24 h, tumors and organs of interest were analyzed for the presence of the mRNA corresponding to viral M gene, transgene EGFP and ß-actin by RRT-PCR. We calculated the M- and EGFP-gene copies relative to the ß-actin copies. Table II shows the ratio of copy number M to ß-actin for native or modified virus. Upon modification with the adapter protein, there was much less virus at the different sites. Twelve hours after inoculation, the ratio of M copy number of modified to unmodified virus at the tumor site was 17%, in the lung, 12%; in the liver, 26%; in the spleen, 9%; and in the kidney, 12%. Twenty-four hours after inoculation, the M-gene copy numbers had either decreased or increased compared to the 12-h value as indicated by the arrow. The

Table II. Effect of the adapter protein on the biodistribution of NDV after intravenous injection into IL-2R$^+$ tumor bearing mice.

	NDFLtag-EGFP Ratio copy number M/ß-actin (x10^3)					
	Native			αHN-IL2 modified		
	12 h	24 h	Change factor	12 h	24 h	Change factor
Tumor	2.54	12.52	x4.93 ↑	0.44	4.33	x9.84 ↑
Lung	13.32	21.05	x1.58 ↑	1.59	5.23	x3.29 ↑
Liver	25.21	20.05	x1.26 ↓	6.66	0.99	x6.73 ↓
Spleen	24.52	8.73	x2.81 ↓	2.18	4.61	x2.11 ↑
Kidney	7.92	1.94	x4.08 ↓	0.98	Neg.	↓

DBA/2 mice bearing Eb-M7 (IL-2Rα$^+$) tumors were injected i.v. with 5,000 HU of either native NDFLtag-EGFP or NDFLtag-EGFP/αHN-IL-2 modified as in Table I. After 12 and 24 h, mice in each group were perfused transcardially through the aorta with DEPC-treated PBS until lung, liver and kidney turned pale. RNA was extracted from tumors and from organs of interest (lung, liver, spleen and kidney). It was then transcribed into cDNA and used for the quantification of the mRNA corresponding to the viral M gene and to the transgene EGFP by RRT-PCR. The copy number ratio of the M gene and of the ß-actin gene was calculated as $1/\{2 \exp [\Delta C_T \text{(matrix-ß-actin)}]\}$. The factor describes the change of the M/ß-actin ratio between 12 and 24 h after virus infection. Its direction is indicated by the arrow.

factor of increase or decrease revealed that the highest increase was observed with modified virus at the tumor site. The second highest increase was seen in tumor tissue by unmodified virus. With the latter, there was only a slight increase in lung tissue while, in all other organs, there was a decrease. In contrast, with modified virus, we saw a clear-cut decrease in the liver but an increase in the lung and spleen.

After 12 h, the ratio for EGFP expression of modified to unmodified virus (data not shown) was 16.3% in tumor tissue and 2-4.5% in normal tissue, where EGFP expression was reduced by 94-100% by αHN-IL-2. After 24 h, the above ratio was 13.2% in tumor tissue and 1.6% or less in normal tissue, except for the spleen. EGFP mRNA copies in spleens of tumor bearing mice treated with modified virus increased from 1.8% (12 h) to 5.5% (24 h). Since, in normal mice, the M mRNA copies in the spleen decreased during the first 24 h after NDV injection (Fig. 3), the findings from tumor bearing mice suggest that it were disseminated Eb-M7 tumor cells in the spleen which were targeted and infected *in vivo*.

We then performed a similar experiment with NDV *Italien*. DBA/2 mice bearing Eb-M7 (IL-2Rα$^+$) tumors were injected i.v. 3 times with 1,000 HU of NDV *Italien* or NDV *Italien*/αHN-IL-2 at days 1, 3, and 5. Mice were sacrificed on day 9. The tissue distribution of virus, was analyzed by immuno-histochemistry (IHC), as before for avirulent NDV (19). Compared to unmodified virus, the amount of modified virus particles was high in tumor and low in all the tested normal
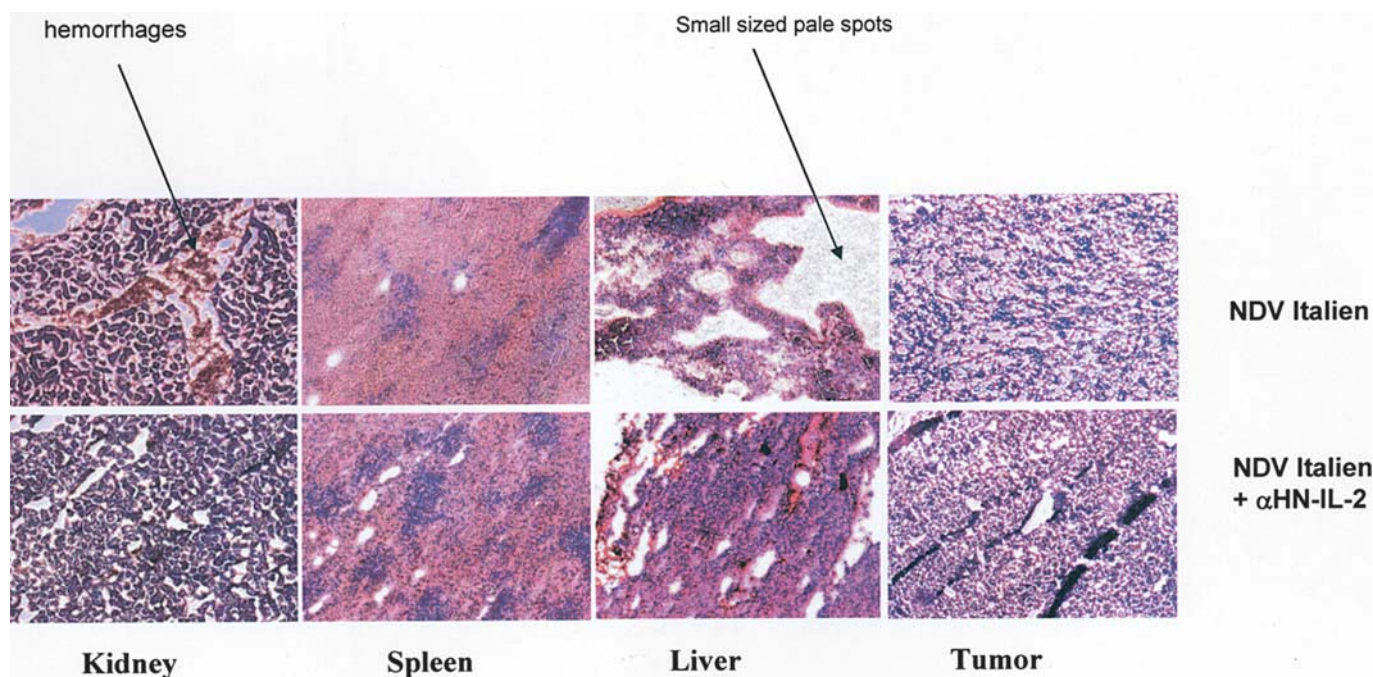
Figure 4. Viral cytopathic effects. DBA/2 mice bearing Eb-M7 (IL-2Rα⁺) tumors on their right flanks received i.v. 2,000 HU of either native NDV *Italien* or modified NDV *Italien*/αHN-IL-2. The latter was obtained by incubation of 2,000 HU of the virus with 250 μg αHN-IL-2 in a final volume of 300 μl for 1 h on ice. Mice were sacrificed 24 h after virus injection. 6-μm sections of fixed tumors and organs were stained by H&E technique before microscopic inspection. Arrows point to cytopathic effects in the liver and to hemorrhages in the kidney.

tissues, including liver, spleen, kidney and thymus (data not shown). We conclude that there was a tumor versus normal tissue enrichment with modified NDV *Italien*.

Cytopathic side-effects mediated by virulent NDV strains in vivo. Mice which received i.v. 5,000 HU of NDFLtag-EGFP or 2,000 HU NDV *Italien* had signs of severe acute cytopathic side-effects. Histology (Fig. 4) revealed small-sized pale spots on the liver (see arrow), an enlarged spleen, and black content in the large intestine which indicated bleeding in the digestive tract. In contrast, all organs from mice injected i.v. with the same dose of modified virulent virus appeared normal. H&E staining revealed that necrosis had occurred in tumors treated by either unmodified or modified virus. However, much more severe damage occurred to liver and spleen and hemorrhages were observed in the kidney (see arrow) when mice were treated with unmodified virulent NDV (Fig. 4).

Side-effects of systemically applied virulent NDV were also observed by following the body weight of treated animals. DBA/2 mice bearing Eb-M7 tumors received i.v. a desensitization dose of 500 HU NDV *Ulster* on day 1. This was followed on days 2, 3 and 4 by injection of 1,000 HU native NDV *Italien* (NAT MED group) or 1,000 HU modified NDV *Italien* (MOD MED group) or 2,000 HU modified NDV *Italien* (MOD HIGH group) (Fig. 5A). As shown in Fig. 5B, the mean body weight decreased on day 2 by 3-5% (ca. 1g) in the virus-treated groups in comparison to the PBS control. On days 4 and 6, there were significant differences of body weights between the NAT MED group and the MOD MED group. These findings thus corroborate the histological findings and demonstrate a clear benefit of the retargeting approach with the bispecific adapter protein.

Anti-tumor effects of native and modified virulent NDV strains after systemic application. Next we evaluated the effects of systemic virus application with or without bispecific adapter protein on the growth of a local tumor following s.c. injection of $5x10^6$ Eb-M7 (IL-2Rα⁺) cells into DBA/2 mice. The treatment protocol was that of Fig. 5A. In the first experiment (Fig. 5C), a reduction in tumor growth was observed after 6 days in the MOD HIGH group. In another group that was treated with the same high dose of native NDV *Italien*, 4 out of 6 mice died and the effect on tumor growth could not be evaluated (data not shown). This clearly shows that there is a maximal tolerable dose (MTD) in mice in the range of 2000 HU NDV *Italien*.

In the repeat experiment (Fig. 5D), we used 10 animals per group and used only a medium dose of native NDV *Italien*. Nine days after treatment, a significant effect was seen on the local tumor volume in all three groups of virus-treated animals. These results demonstrate that oncolytic NDV can be systemically applied to achieve an effect on a locally growing tumor. When using the bispecific adapter protein, side-effects can be reduced without substantially diminishing the anti-tumor effect.

Discussion

Oncolytic virus therapeutics are moving fast from the laboratory into preclinical and clinical trials (26,27). This translational research, however, has revealed hurdles to such cancer therapy that can be overcome only by using multidisciplinary approaches. We demonstrate with oncolytic NDV that a specially designed bispecific adapter protein can efficiently neutralize normal cell binding of the virus *in vitro* and *in vivo*, thus reducing unwanted side-effects. Biodistribution data

A)

Time (days)

B)

time after challenge (days)

C)

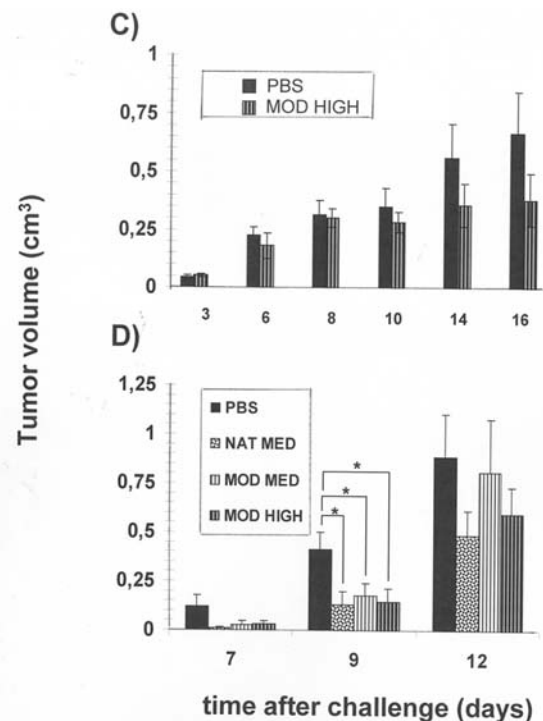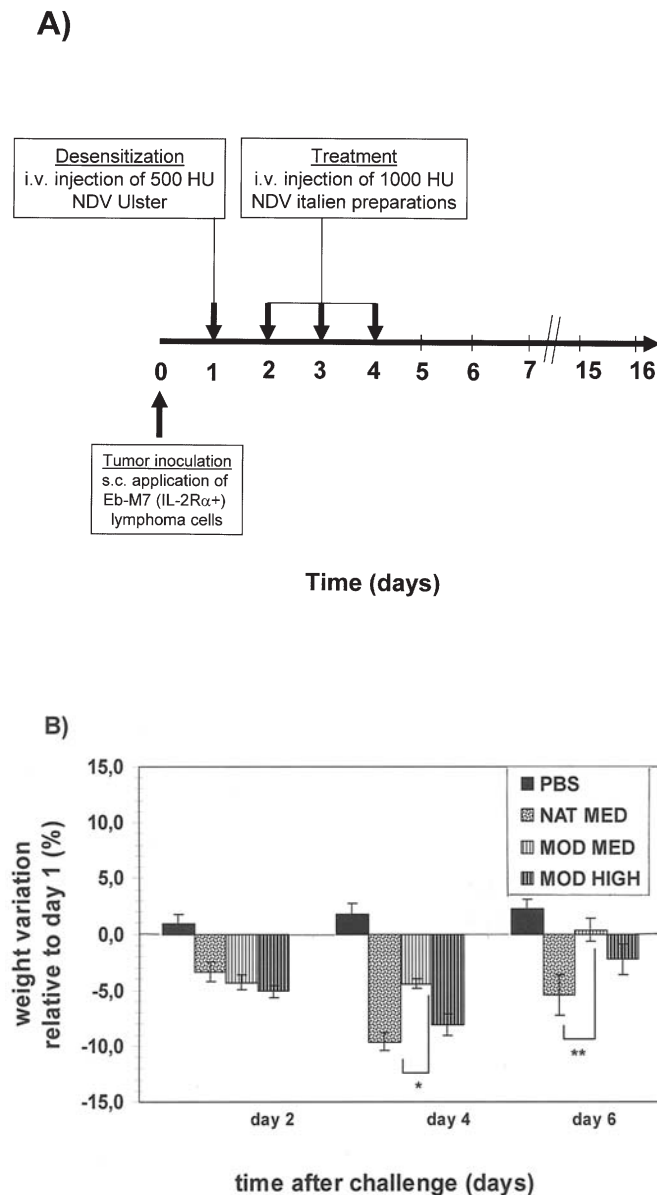**D)**

time after challenge (days)

Figure 5. Anti-tumor effects and side-effects after systemic NDV application. (A) Experimental design. Irradiated DBA/2 mice were s.c. inoculated with 5×10^6 Eb-M7 (IL-2Rα^+) cells. One day later, they received 500 HU of NDV *Ulster* i.v. as a means of desensitization. For therapy, the animals received three i.v. injections of different NDV *Italien* preparations at days 2, 3 and 4. PBS was added to the virus preparation to a final volume of 300 μl. The modification was performed as described in the legend to Fig. 4. (B) Body weights. Following the scheme in Fig. 5A, mouse body weights were measured every other day. The mean body weight change was calculated as a percent of the body weight $\pm$ standard error of the mean (SEM) among a cohort of 10 mice in each group. NAT MED, native NDV *Italien* 1,000 HU per injection; MOD MED, modified NDV *Italien*/αHN-IL-2 1,000 HU per injection; MOD HIGH, modified NDV *Italien*/αHN-IL-2 2,000 HU per injection. The P-values were calculated: *P<0.0006, **P=0.03. (C and D) Anti-tumor effects. Following the scheme in A, two therapy experiments were performed. Groups in C and D contained 6 and 10 mice, respectively. The tumor volumes were determined at the time-points indicated. The P-values were corrected for multiple testing. *P<0.05.

revealed tumor versus normal tissue enrichment. The intravenous delivery of oncolytic NDV strain *Italien* into tumor bearing mice is shown to be safe and efficient when the virus is first modified by a virus neutralizing bispecific fusion protein with a tumor retargeting specificity.

When an oncolytic virus is given systemically to a patient, there are many barriers that prevent it from reaching the tumor and infecting cancer cells (27). Within minutes, most of the initial virus inoculum is absorbed by the lung and liver. Virus that escapes these organs, can enter the circulation where it can be quickly neutralized through absorption by blood cells, through the complement cascade or by neutralizing antibodies. For a virion to access the tumor, it must leave the circulation, traversing or leaking through the vascular endothelium against a gradient of interstitial fluid pressure. Additionally, resident or infiltrating leukocytes may limit cell to cell spread of the virus, either directly through antiviral activity or indirectly by the release of soluble inflammatory mediators, including interferons and other cytokines.

In Fig. 6 we present the concept of our approach of systemic anticancer treatment by retargeting NDV to tumor cells via a bispecific adapter protein. It is a two-step process in which the first step involves transport of the virus through the blood to the tumor site. αHN-IL-2 prevents interaction of the virus with blood cells and might protect it from immune responses. Once the virus has reached the tumor microenvironment by leaving the blood circulation through tumor-vascular endothelium, it can make specific contacts via the adapter protein to target IL-2 receptor-positive tumor cells. This contact will then allow an initial round of viral replication. The second step involves the spread of virus at the tumor site and oncolytic and immunostimulatory events.

The efficiency of the second step is largely dependent on the virulence of the strain of virus used. In the past, we have been working with lentogenic strains such as *Ulster* and *LaSota* to infect patient derived tumor cells *ex vivo* for production of the immunogenic virus modified autologous tumor vaccine, ATV-NDV. We studied the immunological effects of this tumor-cell modification (28-31) and reported clinical benefits upon application of this vaccine (Reichard *et al*, Proc Am Assoc Cancer Res 33: abs. 521, 1992; 32-35). In the present study, we investigated systemic tumor targeting
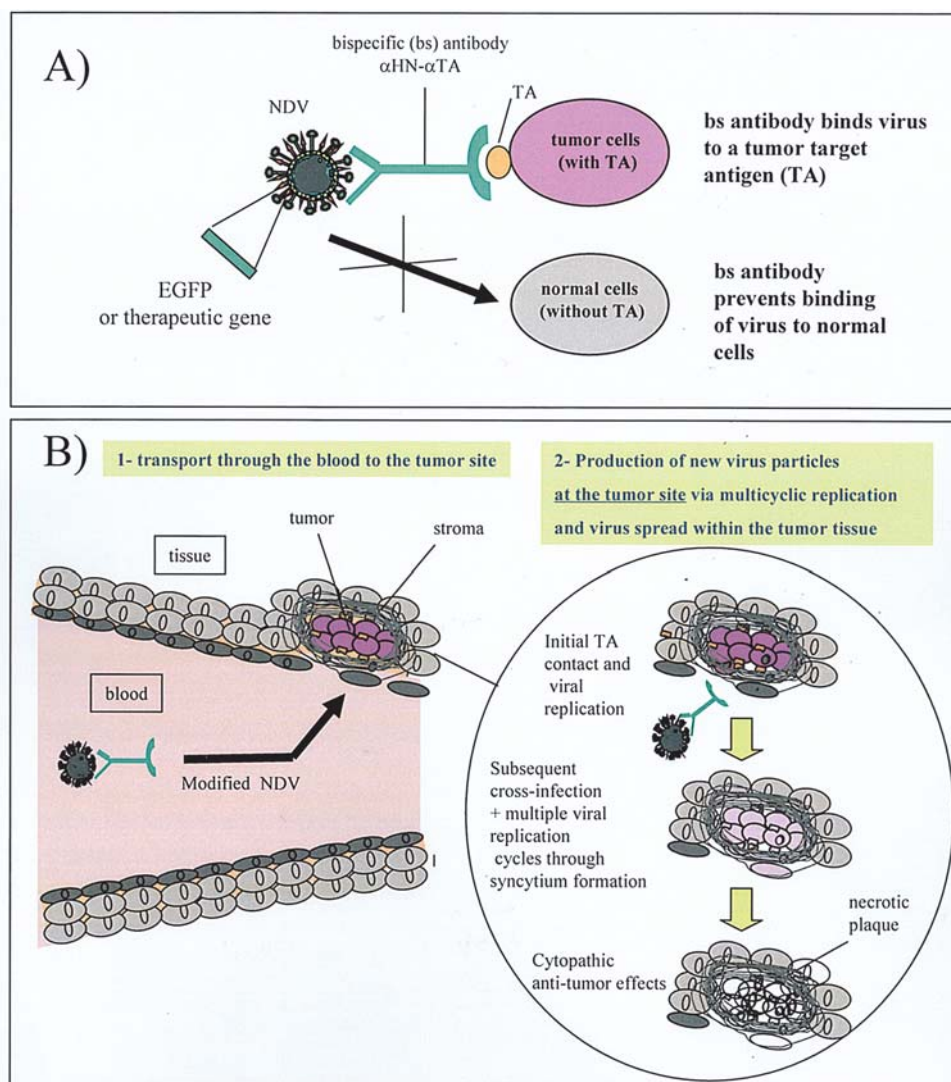
Figure 6. Concept of systemic anti-cancer treatment by targeting oncolytic NDV to tumor cells via a bispecific adapter protein. (A) A recombinant oncolytic NDV carrying a reporter gene, EGFP or a therapeutic gene, is modified by addition of a bispecific adapter protein. This bispecific protein neutralizes the binding of the virus to normal cells and redirects the virus to cells bearing a defined tumor antigen. (B) The prevention of virus binding to normal cells will contribute to reduced uptake in normal tissue and to accumulation of virus at the tumor site. Once the oncolytic viruses reach the tumor, they will replicate locally, leading to the production of new virus particles which are not bound to bispecific proteins. The virulent viruses will then show multicyclic replications within neighbouring tumor cells, thereby inducing oncolytic effects. Further therapeutic effects can be expected from the inclusion of therapeutic genes in such a viral RNA vector.

of NDV and used for this purpose either a recombinant mesogenic strain derived from *LaSota* (NDFL) or the native velogenic strain *Italien*. Both strains have a furin cleavage site in their F protein which allows its activation in a proteolytic environment such as the tumor microenvironment (25,36). Pathogenicity tests in one-day old chickens showed that the parental NDFL-EGFP strain without the furin cleavage site was completely non-virulent [intracerebral pathogenicity index (ICPI) = 0.00) whereas NDFLtag-EGFP was virulent (ICPI=1.28 out of a possible maximum of 2.0]. The ICPI of strain *Italien* is 1.85.

We first demonstrated *in vitro* in quantitative assays the increase of virulence of the two strains in comparison to the non-virulent strains, NDFL or NDFL-EGFP. Results of cross infection, multicyclic replication and plaque assays demonstrated the following order of increasing virulence: NDFL-EGFP < NDFLtag-EGFP < NDV *Italien*. A plaque assay in tumor monolayers showed that NDV *Italien* had

the highest oncolytic capacity. The virulent strains also had a high capacity to lyse normal cells such as erythrocytes. This is particularly relevant because oncolytic NDV strains are already being used in clinical trials for systemic intravenous delivery (37-39). Although the oncolytic strains PV701 (37,38) (Wellstat Biologics Corporation, USA) or the Hungarian veterinary attenuated *velogenic Herfordshire* NDV fowl plaque vaccine (renamed MTH-68/H) (39), were found to be well tolerated when given i.v. and although some patients had antitumor responses (39), data from this study, both from *in vitro* and from *in vivo* analyses, reveal clear-cut cytopathic effects on normal cells. Clinicians using oncolytic NDV strains for systemic treatment of cancer patients should thus be cautious, especially when giving velogenic strains, high doses and multiple repeated injection schedules.

We not only demonstrate cytopathic effects for the first time but we also show how they can be circumvented. The addition of the bispecific fusion protein, αHN-IL-2, was shown

to be capable of inhibiting erythrocyte binding of the virus as well as causing inhibition of hemolysis.

This study is the first with oncolytic NDV in which gene transfer vector biodistribution was examined. Such studies are of pivotal importance for safety assessment in clinical gene therapy development (40). According to RRT-PCR data, on day one after i.v. administration of NDFLtag-EGFP virus to normal mice, viral M-gene copies were no longer detectable in the blood while, after 12 h, there were still high copy numbers. The virus was found in the lung, liver, spleen and kidney. From these organ sites, it disappeared more slowly than from the blood. After 14 days there was only a low copy number left in lung tissue. Similar results were obtained in mice bearing IL-2 receptor-positive syngeneic local tumors. The main difference was the tumor tissue, in which we observed 12 to 24 h after i.v. virus administration a 5-fold increase of viral copy numbers. When using NDFLtag-EGFP virus which had been modified with αHN-IL-2, we saw a nearly 10-fold increase of viral copy numbers in the tumor tissue while there was a clear-cut decrease in the liver and kidney. In the spleen, there was a 2-fold increase following injection of the modified virus while, with the non-modified virus, there was a decrease by a factor of nearly 3. Since NDV does not replicate in normal cells (41,42), this difference suggests that the spleen contained IL-2 receptor-positive disseminated tumor cells which could be retargeted.

It is important to emphasize that, in our concept, the bispecific fusion protein is mainly used to get a better transport of the virus through the blood before the latter reaches the tumor site. Once it has reached the tumor microenvironment, the further spread of virus does not require the adapter molecule anymore. The adapter protein, produced by us as described (17) was found to be capable of neutralizing the binding to normal cells of all the different strains of NDV that we tested. It can thus be easily combined with NDV strains of different virulence. The fusion protein reduced by 90-100% the uptake of systemically applied recombinant NDFLtag-EGFP virus in normal tissue. Also the cytopathic side-effects seen in liver, spleen and intestine with native virulent virus were not seen with the modified virus. A clear benefit of the retargeting approach with a bispecific protein could also be demonstrated when following the body weight of treated animals.

Once the modified virus has reached a tumor target cell it will infect it and start a replication cycle. We demonstrated previously that retargeted tumor cell infection depends on specific recognition of IL-2 receptor by virus bound αHN-IL-2 and on the viral F protein (18). We also showed that NDV replication is tumor selective (41) and associated with defects of tumor cells in antiviral defence (42). A prerequisite for viral spread in the targeted tumor tissue is that progeny virus particles from the first infected tumor cell are infectious and capable of cross-infecting other tumor cells. We showed in the present study *in vitro* that this process requires *mesogenic* or *velogenic* NDV strains with an active F protein. Hydrophobic fusion peptides within the viral envelope of these strains promote syncytium formation between neighbouring tumor cells (36,37), whereby the virus spreads without an extracellular phase. This is perhaps a reason why, in a phase I clinical trial, neutralizing antibodies apparently did not interfere with viral delivery to the tumor (37). By contrast, neutralizing antibodies

are a significant hurdle to systemic delivery of other oncolytic viral vectors, such as VSV or reovirus (27,43). We demonstrate that the virulent NDV strains used were capable: i) of cross-infecting other tumor cells, ii) of multicyclic replication and iii) of forming syncytia and necrotic plaques. From these *in vitro* findings, it is likely that the virulent strains used are also capable of spreading within the tumor microenvironment. Such spreading, however, is unlikely to be uniform; it might be limited by tumor intestitial fluid pressure and perhaps by other factors within the tumor microenvironment.

Finally, we were able to demonstrate anti-tumor effects after systemic virus application. The treatment protocol included a first injection with a low-dose of non-virulent NDV to induce a desensitization reaction as described in a clinical study (37), followed on day 2-4 with daily injections of 1,000 HU of virulent NDV *Italien* with or without modification. Nine days after tumor inoculation, a significant effect was seen on the local tumor volume in all three groups of virus-treated animals. The modified virus was as effective as the non-modified one. Although the anti-tumor effects were apparently transient, this is the first time that we have observed significant and reproducible anti-tumor effects following systemic virus application. One has to consider that three virus inoculations within a short time interval, as in this protocol, are much less than what has been performed in clinical studies (37-39). We also have to keep in mind that intra- or peritumoral application of NDV or NDV-infected tumor cells showed more pronounced anti-tumor activity than systemic application (23). This suggests that it is important to further improve on the delivery aspect to achieve more pronounced and longer-lasting antitumor activity. Virus encapsulation in liposomes might further improve the efficiency of systemic delivery; it has been successful with another viral vector, *Semliki-Forest-Virus* (44).

Further developments for the systemic use of NDV as an anticancer agent are necessary before one can consider clinical applications of NDV as a recombinant gene therapy vector. Construction of a fully retargeted oncolytic NDV virus by reverse genetics and by engineering of the HN protein is another promising strategy. Enhanced gene delivery vectors have been obtained for adeno-associated virus by high-throughput selection from a large mutant capsid library (45). Once the delivery system is optimized, the efficiency of anti-tumor activity of a recombinant NDV vector can of course be improved by the incorporation of therapeutic genes, as has been accomplished with other viral gene therapy vectors (9,26,27).

Acknowledgements

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