Preface

This review offers a scholarly analysis of some of the most topical aspects of cancer therapy using viruses. The authors begin their review by recounting the early events that sparked interest in the research of cancer-killing viruses. The meticulous narrative reveals and corrects many common misconceptions and misuses of published material circulating in the literature. For those who have not already had the chance, I strongly recommend reading at least some of the papers referenced by Drs. Sinkovics and Horvath in their historical introduction; perusing these early reports I cannot help but wonder if we are not constantly reinventing the wheel.

The authors provide an extensive list of viruses, taking the time to describe each one adequately and putting them in the context of the immune system. The viruses may kill the cancer cells they infect, but more often than not the pattern of infection in a tumor is incomplete (Vähä-Koskela et al. [3]). The conjecture is then that the remaining cancer cells, whether infected or not, are subject to recognition and purging by the immune system. While appealing, this hypothesis needs to be corroborated both experimentally and in the clinic. The evasive, often downright immunosuppressive, power of cancer cells cannot be underestimated, and we know too little about the capacity of cancer-initiating cells to escape bulk tumor immunity to draw firm conclusions (Rescigno et al. [2]).

The authors discuss the requirement of leaving components of the innate immune system untouched in order to maximize oncolytic efficacy. However, generalizations may not be possible; whereas virotherapy with many viruses may benefit from innate immune responses, other viruses rely more on oncolytic power and are seemingly hampered by innate immunity (Wu et al. [4]). The authors also discuss the newly discovered capacity of bacteriophages to interfere with tumorigenesis. While it is not surprising that xenogenic particles (bacteriophages) may attach to cancer cells in a seemingly selective fashion (cancer cells upregulate many receptors), it was surprising to learn this binding interfered with the metastasizing capacity and facilitated opsonization of the cancer cells. Certainly these findings warrant further scrutiny, and the corollary to use bacteriophages as therapeutics is equally tantalizing. Still it must be kept in mind that the human body is bombarded by a spectrum of foreign entities from birth, phages included, and thus phage immune auditing is likely an evolutionarily conserved process to which host cells (at least in the colon) have adapted. It may be that the appropriate countermeasures against phage binding are already in place (in a small but ultimately regrowing subset of cancer cells).

Throughout the review, the authors provide intermittent comments and raise important questions that cancer immunologists and virotherapists alike would do well to consider. I would like to add that while the blind enthusiasm spurred by near miraculous discoveries in mice has been replaced by a more sober attitude and moderated expectations as a result of many not so astounding clinical trials, data from these trials are still vital and will continually help in defining and redefining treatment modalities and regimens (Liu et al. [1]). We may not be able to fully cure the majority of patients during the coming decades, but I think we can expect a steady increase in statistical patient survival coupled with a decline in mortalities as a result of evidence-based adaptation of the tools at hand. The review by Drs. Sinkovics and Horvath clearly underscores that true progress has been made and that more is still to come.

I am still new to the field of research, yet I consider myself very fortunate to have already had the chance to help fawn a new player in the field (attenuated Semliki Forest virus) and to have seen it transgress the boundaries of its cradle and being put to the test alongside a panel of other potent oncolytic candidates. While I have not had the pleasure of meeting either Dr. Sinkovics or Dr. Horvath having immersed myself in this splendid narrative I perceive a closeness to the authors akin to that of a student to his professor. The authors have had the privilege to partake in the birth of oncolytic virus research, and so I have learned much from both this review and many others written by the authors. From the extent of the review at hand, I can only guess at the wealth that still remains unwritten.

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Commentary

Bloch was the first to show that phages can accumulate in cancer tissue and eventually inhibit tumor growth [1]. Kańtoch and Mordarski [3] further showed that cancer cells bind phages both *in vitro* and *in vivo*. We demonstrated that the wild-type phage T4 and its substrain HAP1 markedly inhibit experimental lung metastases of murine B16 melanoma and proposed that the mechanism is mediated by the beta3 integrin signaling pathway [2]. These data were recently confirmed by Szczaurska-Nowak at our Institute (personal communication, manuscript in preparation). Moreover, *in vitro* interactions between T4 phages and bone marrow dendritic cells followed by tumor antigen activation caused augmentation of the anti-tumor effect (the percentage of tumor growth inhibition can be as high as 76% compared with untreated control mice [4]). Thus it may well be that in the future phages will find some role as a weapon to combat cancer. A short chapter dedicated to this perspective is therefore justified.

Andrzej Górski
Editor

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REVIEW

Arch. Immunol. Ther. Exp., 2008, **56**, 3s–59s
PL ISSN 0004-069X

DOI 10.1007/s00005-008-0047-9

Natural and genetically engineered viral agents for oncolysis and gene therapy of human cancers

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Received: 2008.07.07, **Accepted:** 2008.09.22, **Published online first:** 2008.12.23

Abstract

Based on personal acquaintances and experience dating back to the early 1950s, the senior author reviews the history of viral therapy of cancer. He points out the difficulties encountered in the treatment of human cancers, as opposed by the highly successful viral therapy of experimentally maintained tumors in laboratory animals, especially that of ascites carcinomas in mice. A detailed account of viral therapy of human tumors with naturally oncolytic viruses follows, emphasizing the first clinical trials with viral oncolysates. The discrepancy between the high success rates, culminating in cures, in the treatment of tumors of laboratory animals, and the moderate results, such as stabilizations of disease, partial responses, very rare complete remissions, and frequent relapses with virally treated human tumors is recognized. The preclinical laboratory testing against established human tumor cell lines that were maintained in tissue cultures for decades, and against human tumors extricated from their natural habitat and grown in xenografts, may not yield valid results predictive of the viral therapy applied against human tumors growing in their natural environment, the human host. Since the recent discovery of the oncosuppressive efficacy of bacteriophages, the colon could be regarded as the battlefield, where incipient tumor cells and bacteriophages vie for dominance. The inner environment of the colon will be the teaching ground providing new knowledge on the value of the anti-tumor efficacy of phage-induced innate anti-tumor immune reactions. Genetically engineered oncolytic viruses are reviewed next. The molecular biology of viral oncolysis is explained in details. Elaborate efforts are presented to elucidate how gene product proteins of oncolytic viruses switch off the oncogenic cascades of cancer cells. The facts strongly support the conclusion that viral therapy of human cancers will remain in the front lines of modern cancer therapeutics. It may be a combination of naturally oncolytic viruses and wild-type viruses rendered oncolytic and harmless by genetic engineering, that will induce complete remissions of human tumors. It may be necessary to co-administer certain chemotherapeutic agents, advanced cancer vaccines, or even immune lymphocytes, and targeted therapeuticals, to ascertain, that remissions induced by the viral agents will remain complete and durable; will co-operate with anti-tumor host immune reactions, and eventually will result in cures of advanced metastatic human cancers.

Key words: naturally oncolytic viruses, viral oncolysates, human cancer immunity, interaction of resident viral flora with the oncolytic virus, oncosuppressive bacteriophages, genetically engineered oncolytic viruses.

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[**]The entire manuscript (text, literature, tables, figures) was designed and written by the senior author, who consulted the co-author frequently. The co-author contributed to data collection and interpretation. The co-author prepared Newcastle disease virus (NDV) oncolysates and immune lymphocytes for immunotherapy of patients. Both authors share equal responsibility for the contents in the rest of this publication. The co-author is available at joseph.horvath@hemispherx.net

A BRIEF NARRATIVE HISTORICAL INTRODUCTION

The beginnings

The Italian clinician. N. G. De Pace presented at the International Cancer Congress in 1910 in Paris the case history of one of his patients who experienced remission of her enormously large vegetating uterine cervical carcinoma consequentially to receiving the Pasteur-Roux live attenuated rabies vaccine. The vaccine was that of an "emulsione di midollo rabido attenuato secondo il metodo di Pasteur." The patient was bitten by a rabid dog ("era stata morsicata da un cane idrofobo") and was therefore not treated with the intention to induce oncolysis. The doctor attributed the regression of the cancer ("il tumore non esisteva piú") to oncolysis by the virus: "eliminazione di grossi pezzi di tumore" due to cancer cell death caused by viral cytolysis: "un azione citolitica sulle cellule neoplastiche" [73]. Thereafter, several other patients with uterine cancers were treated in Italy with the rabies vaccine, now with the intention to induce oncolysis. Some of these patients responded to the treatment with temporary regression of their tumors, but not as dramatically as the first patient. Eventually these patients relapsed and died ("l'esito letale"). The first patient achieved a complete remission (by gynecological examinations) lasting from 1904 to 1912, then she either relapsed or developed a second new primary gynecologic carcinoma with inguinal lymph node metastases ("con metastasi alle ghiandule inguinali"). In the latter case, she was a patient who was actually cured of a cancer by viral therapy, and the cancer could have been virally (human papillomavirus, HPV) induced; however, antitumoral immunity, if any, thus acquired failed to protect her against the same type of cancer occurring so many years later. This ground-breaking report would not be readily published today in the era of "evidence-based medicine"; the rabies virus was not reisolated from the dying tumor cells and there is no mention of Negri bodies within the virally infected tumor cells. The presence of rabies virus cytoplasmic inclusion bodies would be proof for viral replication in the tumor cells. There is extensive literature on Negri bodies in PubMed from Texas to Maiduguri Teaching Hospital, Nigeria [4, 311].

There are most disturbing citations in several USA publications of a paper by G. Dock from 1904 that was allegedly entitled "Rabies virus vaccination of a patient with cervical cancer" [96, 263, 291, 452]. We traced the first such citation back to the Journal Clinical Investigation's volume 105, page 837, 2000 issue (cited in [151]). However, those citations must be wrong; the real Dock paper with the volume, year, and page numbers as cited is entitled "Influence of complicating diseases upon leukemia" [79], and there is no mention in it of a patient who would have received rabies vaccine. These repeated false citations are perpetuated by authors who do not read the literature they cite in the original. In the real 1904 Dock paper [79], leukemic patients are listed whose blood counts temporarily improved after natural infections, which were not proven to be viral but were very likely bacterial (including tuberculosis). Astute and detailed descriptions are provided of leukemic patients who improved clinically, but only temporarily, coincidentally to an unidentified viral ("morbilli", "influenza") or other "septic" infections (Kovacs' case, Koermoeczi's case, as cited in [79]).

In 1912, De Pace mentioned "Coley" (Dr. William B. Coley of New York City), who induced remissions in tumors (sarcomas) with direct injections of bacterial filtrates ("inoculando culture virulente filtrate di streptococco erisipelatoso e di bacillo prodigioso") [73], but had no knowledge of Dock (Dr. George Dock of Ann Arbor, Michigan). De Pace is the one who deserves the credit for observing viral oncolysis of a human tumor very early in the last century and for initiating further clinical efforts to induce the same results with viral therapy in other patients.

Thereafter, no interest in the viral therapy of human cancer was expressed, such as to conduct clinical trials, for over thirty years. In the late 1940s, G. T. Pack of the Memorial Hospital in New York City inoculated live attenuated rabies vaccine into human melanoma tumors and reported the first remissions induced in that entity of human malignancy [283]. Brief discussions of the histological features of the virally infected dying melanoma cells followed, but without mentioning Negri bodies (see above) in the tumor cells [158, 159]. The matter of remissions of leukemia due to coincidental infections, especially viral infections, was investigated in 1964, some 60 years after the publication of the original 1904 Dock paper [79], by actually infecting a patient suffering from acute myelogenous leukemia with live viruses (arboviruses, influenza A/B viruses, Newcastle disease virus, NDV), thus inducing short-lasting partial remissions. The patient kept relapsing after short remissions, but when he eventually died of staphylococcal septicemia, his postmortem bone marrow was devoid of leukemic cells [447].

Oncolytic viruses cure tumors in the laboratory

Institut Pasteur, Paris, France. Instead of clinical trials in patients, C. Levaditi and S. Nicolau in the 1920s and thereafter at the Pasteur Institute in Paris tested a large number of viruses against a large number of tumors in laboratory animals (mice, rats, and rabbits) and reported tumor regressions, sometimes durable complete resolutions, of these experimentally induced tumors (cited in reference [348]). In Sinkovics' textbook "Die Grundlagen der Virusforschung" [348], the citations from 732 to 784 (and more) are the publications of Levaditi et al. written in French from 1921 to 1953 and published in the Annales de l'Institut Pasteur and Comptes rendus de l'Académie des Sciences.

Overwhelming evidence was provided from laboratories that naturally oncolytic viruses existed and could

replicate in tumor cells *in vitro* and *in vivo*, preferentially limiting their replication within tumors and only occasionally harming the tumor-bearing host. In the USA, the most prominent of these laboratories was that of Alice Moore at the Sloan-Kettering Cancer Center in New York City [258–260].

Sloan-Kettering and M. D. Anderson pay attention. The Wistar Institute follows suit

The M. D. Anderson Hospital conference. The young M. D. Anderson Hospital in Houston, Texas, organized a most remarkable conference in 1957 on oncogenic and oncolytic viruses. Oncolytic viruses were discussed by Alice Moore, Hilary Koprowski, the newly appointed director of the Wistar Institute in Philadelphia, and Jerome Syverton. Moore announced that her clinical associate, Chester Southam, was already administering viral therapy to patients at Sloan-Kettering [259]. The following viruses were inoculated into tumor-bearing patients: Egypt 101 strain of West Nile encephalitis virus, Bunyamvera virus, Semliki Forest virus, Newcastle disease, and mumps and vaccinia viruses. Temporary partial remissions were observed (sometimes in viremic and very ill patients) [259, 381]. Albert Sabin commented: "Dr. Moore mentioned some very disappointing aspects of the possible use of viruses as oncolytic agents in human beings. The most disappointing aspect is the fact that even when a virus is oncolytic, and it punches a hole in a tumor, the immune response of the individual to the multiplication of the virus occurs so fast that the effects are quickly wiped out, and the tumor continues to grow." Thereafter, Sabin went ahead to propose that human tumors should be treated with sequential inoculations of different oncolytic viruses [321]. Koprowski reported that he cured highly malignant mouse tumors (Ehrlich's ascitic carcinoma, Krebs 2B carcinoma) with Bunyamvera and other viruses. He described "release of antigenic material from virus--infected ascites tumors." When the animals, after the regression of virally lysed tumors (West Nile virus lysing Ehrlich ascites carcinoma), were inoculated with live tumor cells (not virally infected native cells from the same tumor), they resisted the challenge [213]. However, the Ackermann and Kurtz article [1] of 1953 on the cure of mouse ascites tumors by intraperitoneally inoculated neurotropic influenza A virus was not mentioned. Syverton and McLaren showed that the HeLa cell line recently established from human carcinoma of the uterine cervix supported the multiplication of polioviruses, herpes simplex virus, and vaccinia virus as the viruses were cytopathic to the tumor cells [398].

Just a year earlier (1956), the senior author of this review listed over 20 oncolytic viruses and over 50 interfering viruses in his monograph Die Grundlagen der Virusforschung [348]. Among those citations was an article by Syverton and Berry [397] in which the replication of various viruses in virally induced Shope rabbit papilloma was listed. The virally induced Shope papillomas carried the co-infections of Sabin's simian virus B and vaccinia virus; together with these viruses, the highly virulent Brazilian Sanarelli strain of rabbit myxoma virus also replicated in Shope rabbit papillomas, but virus III (*Herpesvirus cuniculi*) and other herpes viruses failed to establish themselves in the Shope papilloma tumors. Syverton did not mention any of these observations in his lecture in Houston.

The senior author of this review visited Chairman of the Department of Microbiology, Dr. Jerome Syverton, in Minneapolis in 1957 to present his paper on oncostatic-oncolytic NDV in mouse ascites carcinoma [349–351]. These NDV strains were isolated by the senior author from patients with the "oculoglandular syndrome" of the ophthalmologist Magda Radnót and were adapted to the brain of suckling mice. This work was done in 1955 in Budapest, Hungary, contemporaneously with, but unaware of, the seminal work of A. D. Flanagan, R. Love, and W. Tesar. This work was published much belatedly due to the Hungarian national uprising of 1956. The Flanagan et al. article is given credit in the belated publication [351] and is cited in reference [358]. At this time, Syverton made this remark about rabbit tumor viruses transferred to rats and/or grown in cultured tumor cells *in vitro* (of which no written record could so far be found): "Here is an oncogenic virus" (meaning rabbit myxoma virus) "which can be oncolytic when it replicates in another tumor in another species." Dr. Syverton offered a position to this author at his department, but soon died unexpectedly of a massive heart attack; since then, this author regrets that he missed the opportunity to work with Dr. Syverton.

The co-discoverer of interferon prepares viral oncolysates. In 1967, ten years after the M. D. Anderson Conference, J. Lindenmann and P. A. Klein summarized the immunological consequences of neurotropic influenza viral and reoviral oncolysis manifesting in resistance to challenge with tumor cells in mice that survived viral oncolysis. The same (or better) resistance to challenge with tumor cells could be induced in mice actively immunized with influenza viral lysates of tumor cells (the Ehrlich ascites carcinoma "viral oncolysates"; Table 13 in the monograph [234]). Way ahead of its time, but unexplained, is Fig. 16c in the monograph; there, lymphocytes surround and lyse a virally infected Ehrlich ascites carcinoma cell [234]. These pioneering authors ask: "How did the antibody induce the aggregation of host cells around the tumor cells" [234]? In contrast, intact Ehrlich carcinoma cells serially passed in their hosts coexist with, and are ignored by, host lymphocytes (Fig. 42 in [348]). The monograph comments on the first human trials of oncolytic viral therapy (with direct inoculations of arboviruses at Sloan-Kettering by Southam): "results that were not encouraging" [234].

Viral oncolysates at M. D. Anderson. In the same year (1967), the Surveillance Committee of M. D. Anderson Hospital received an application for approval; it contained protocols for the viral therapy (direct intratumoral inoculations of viruses, active "tumor-specific"

vaccination with influenza A viral oncolysates) of selected patients with melanoma and sarcomas. The projects included tests for quantitative and serial measurements of antibody- and lymphocyte-mediated tumor-specific immune reactions [352]. The Hospital's Surveillance Committee approved the project in 1968, but the National Cancer Institute (NCI, Bethesda, MD), working soon with Mr. Nixon's expanded budget for the "Conquest of Cancer", denied support, but with "approval" (issuing their much despised wording: "approved without funding due to low priority"). Newly appointed project site visitors of the granting agency determined the level of priority of the grant applications (many times, without a project site visit). While viral therapy of cancer was doomed to failure by the highest ranking experts of the US NCI, this author, undaunted (but not supported), continued to cite data supporting the value of viruses in cancer therapy [354, 367], whether the viruses were oncolytic to cancer cells or interfered with oncogenic viruses, and especially if the cited articles were published in Cancer Research or in the Journal of the National Cancer Institute (JNCI) [26, 37, 134].

Mouse leukemia viruses teach lessons in anti-tumor immunity. Paradoxically, malignant cells (rat tumors) became antigenic when superinfected with leukemia viruses (the Friend virus) budding from the cell membrane [210]. Thus an oncogenic virus, or its attenuated subclones, could act as "xenogenizing" immunogens. Observations of and explanations for the "spontaneous regressions" of virally induced tumors emerged [90, 401]. Was it the genetic background of the host recognizing Gross virus-carrier leukemic lymphocytes for immune rejection [90, 401] or were there passenger viruses co-existing with oncogenic viruses that induced direct oncolysis, or host immunity leading to the neutralization of the oncogenic virus or to the elimination of the virally infected tumor cells? Thus viruses, even oncogenic retroviruses, could act against tumors not by direct oncolysis, but by inducing antitumor host immunity [25, 59, 317]. However, in some systems, antagonism might have become operational between humoral and cell-mediated immunity. Anti-NDV immune globulins covering viral epitopes on the surface of NDV--infected mouse tumor cells enhanced tumor growth, probably by blinding immune T cells (and unexpectedly failing to induce natural killer cells or macrophages) [374]. In a human sarcoma cell system, cytosine arabinoside suppressed such antibodies and allowed increased activity of cytolytic immune T cells [362]. Should viral therapy of a tumor-bearing host be combined with cytosine arabinoside?

The National Cancer Institute shaken awake

NDV lyses human tumor xenografts. The attitude of the highest authorities of the NCI changed for the first time many years later, in 1994, when an editorial appeared in the JNCI giving reverence to the paper by R. M. Lorence et al. on oncolysis of human tumor (neuroblastoma, fibrosarcoma) cells induced by the 73-T (Cassel) strain of NDV *in vitro* and in xenografts *in* vivo [200, 239]. In this article, in a subsequent article [240], and in the JNCI editorial there were citations of a report published in Hungary claiming that a live attenuated veterinary poultry vaccine, NDV-H, induced stabilization of disease and remissions in patients with advanced cancers [63].

Hungary licenses NDV for the viral therapy of human cancers. Hungarian clinicians reported remissions induced by them with the attenuated veterinary NDV-H (referred to as MTH-68/H) virus in patients with metastatic cancers [63]. The work was initiated and financed by a general practitioner in Alexandria, Virginia, USA, who arrived in the USA from Hungary in 1957. He closed his private practice and took up residence in Hungary in the mid-1980s. He assumed the first authorship of the multi-authored text; the co-authors of this report were affiliated with bona fide academic Hungarian institutions [63]. Indeed, this clinical trial reflected a meritorious effort considering the circumstances and timing.

The JNCI article and its editorial [200, 239] resulted in the first ever national license and financial state support in Hungary for an oncolytic virus ("the MTH-68/H virus") for human cancer therapy. The availability of this virus for human cancer therapy in Hungary (with payment for service) was advertised on the Internet from the 1980s on for foreigners traveling to Budapest for NDV-H therapy of cancer. The web site http://www.healthy.net/library/articles/canclinic/csatary.htm is still active, but its contents have since been updated and the price of the treatment has been omitted (printed copies of the above-mentioned previous advertisement remain available). Treatment of cancer with the virus NDV-H (later MTH-68/H) was promoted by the prominent unconventional cancer treatment advocate Ralph W. Moss, who in Hungary is a most popular author of the biography of the Hungarian Nobel Prize winner Albert Szent-Györgyi, published under the title "Free Radical" (Paragon House Publishers, 1988, New York) [261]. The promoters claimed the recovery of a Hungarian poultry farmer from metastatic stomach cancer in the 1950s who was exposed to and/or consumed NDV--infected chickens during an undocumented "fowl plague" outbreak: http://www.ralphmoss.com/newcastle.html (1994). However, this individual has no name, no hospital records, and no pathology report of his alleged stomach cancer. Nevertheless, this colorful anecdote has been widely cited in the literature (even in the era of evidence-based medicine) [96, 239, 240].

The promoters, complying with the demand of the Hungarian public health authorities (officers of the State Institute of Public Health, Budapest), contracted experts of the Phylaxia-Sanofi company in Budapest to purify and stabilize the attenuated veterinary NDV-H vaccine virus, or its derivative, the H(Ph/80) strain newly imported from England, which was accom-

plished. The promoters patented the principle and the technology of viral therapy for human cancers and the use of all purified veterinary and human viral vaccines for the purpose of treating common (genital herpes, viral hepatitis, AIDS) and oncogenic-virally induced (malignant) human diseases (Csatary L. USA Patent 5,602,023 1997, Csatary L. and Massey R. J. USA Patent 5,215,745 1993, cited in [240]). However, neither the applicants nor the patent agencies mentioned the extensive studies (and limited clinical trials) carried out (but not patented) with attenuated or tissue culture-adapted veterinary viruses for human tumor cell lysis in the 1960s at the Roswell Park Memorial Institute, in Buffalo, NY [144, 461].

By the early 1990s, the purified and stabilized poultry NDV-H, or its further English derivative, the newly imported H(Ph/80) NDV-H vaccine virus [64, 65], was licensed in Hungary for intravenous use in cancer patients. Its egg-passaged derivative was renamed "more than hope" MTH-68/H virus for human use. 1968 was the year when the veterinary NDV-H vaccine might have been used for the treatment of the first human cancer. At that time, the virus used was the veterinary virus vaccine; it was not purified and it was not referred to as MTH-68/H. It is not known if the NDV veterinary viruses used in 1968 and in the 1980s and thereafter, when the name MTH-68/H was first used, are the same. The international clinical use of the MTH-68/H virus has been administered by the United Cancer Research Institute of Alexandria, Virginia, but it was not revealed which clinics or research laboratories and institutions, if any, "united" to form the private agency so named and presided over by the sponsor of the project [63, 261]. However, the Hungarian state license for the use of the MTH-68/H virus for human cancer therapy was withdrawn in 1998 consequentially to allegations that the MTH-68/H inoculations were done without an approved and licensed protocol, that the viral vaccine was not prepared according to World Health Organization regulations, and that the records of approximately 4000 cancer patients treated with the MTH-68/H virus in Hungary were not completed, were not reliable, or were claimed to have been "lost"; thus the efficacy of the treatment could not be accepted as proven or documented ("nem bizonyított" "nem igazolt") [153]. The authorities closed down the clinic where the MTH-68/H virus was administered to patients. These reviewers (J. G. Sinkovics and J. C. Horvath) were not involved in any capacity whatsoever in the promotion of the MTH-68/H virus(es) for human cancer therapy or in the support or withdrawal of support of its licensing, and refrained from being interviewed on this subject. This report is based on published factual data for the illustration of an exceptional chapter in the history of the viral therapy of human cancer. Hungary was the first state to issue such a license [240].

The unexplained origin of the MTH-68/H virus. The velogenic (higly virulent) Hertfordshire native virulent virus was isolated in England in 1933 (Herts'33) and remained virulent in bird passages, but appeared to yield by egg passages the attenuated mesogenic (moderately virulent) H virus strain (NDV-H). Mesogenic NDV strains retaining an intracerebral pathogenicity index ≥0.7 for baby chicks are precluded from use as vaccines [64, 65]. Lentogenic NDV strains are avirulent and are the preferred vaccine strains. The nucleotides 47 to 420 form the variable region of the F (fusion) viral protein (see below); the amino-acid (AA) sequences of this region determine virulence or loss of it [64, 65, 302].

The original attenuated Hertfordshire (H) vaccine strain was brought to Hungary from Weybridge, England, in 1943 and maintained by egg passages. By the 1950s it practically eradicated "fowl plague" caused by virulent NDV in Hungary (personal communications in 1949 to the senior author by Prof. Károly Bamberger). Genetic analysis of the attenuated virus (NDV-H) revealed a genotype III, whereas the original Herts'33 virulent isolate was of genotype IV. For this reason, NDV-H could not have been derived from the original virulent Herts'33 virus. The possibility is now entertained that a genetically unrelated genotype III mesogenic field virus was mixed with the original virulent Hertfordshire isolate (Herts'33) and egg passages favored the selection of the former. A new Hertfordshire virus strain was purchased from the Veterinary Laboratory Agency, Weybridge, UK, in 1999 and it was received at the Institute for Veterinary Medical Products (IVMP) in Budapest. This is the Herts'33/56(IVMP) strain. The Hungarian Phylaxia factory received the first attenuated NDV-H derivative H(Ph/80) strain in 1980 and the second H(Ph/02) strain in 2002. The first passage of the H(Ph/02) strain was seeded at Phylaxia on August 29, 2002. This NDV strain is identical to the Herts'33/56 strain; it is referred to at Phylaxia as MTH-68/H [19, 64, 65].

The viral strain that the Phylaxia firm identifies as the MTH-68/H was brought to the country in 2002 as H(Ph/02), being identical with Herts'33/56(IVMP) [64, 65]. Were these two or three MTH-68/H viruses, the original NDV-H virus used before the 1980s and retroactively referred to sometime in the 1980s as MTH-68/H and the newly imported H(Ph/80), or H(Ph02) (Herts'33/56IVMP), the same? H(Ph02) was used after 2002, but it is also designated as MTH-68/H [63]. This very much sought after virus strain under the name MTH-68/H is still available in Budapest for a price (so it is widely known in the USA, Germany, Israel, and Tijuana, Mexico) [240, 261]. If these attenuated NDV strains were imported to Hungary from England, why are they advertised as if they were "developed" or "created" in Hungary [261]? Whatever its origin, the NDV--H/MTH-68/H viral strain(s) would deserve enlistment in a comprehensive clinical trial, preferably conducted in a controlled academic environment (and provided free--of-charge to the enrolled investigational patients). If its oncolytic efficacy is proven to be as extraordinary as it has been claimed [63, 95, 235, 240, 261, 432], it should be

licensed for the viral therapy of human cancers; then proper credit should be due its promoters [63, 261].

The continuation

Natural viral infections result in human tumor remissions. In the meantime there appeared short concise reports of individual patients whose leukemias, lymphomas, or other tumors remitted (partially or completely, but usually only temporarily) coincidentally or consequentially to a natural viral infection. The most dramatic of these events were the regressions of African Burkitt's lymphoma, or Hodgkin's disease, in children infected naturally by the measles virus [36, 135, 329, 408]. Such events gave the impetus (much belatedly, only recently) to the Mayo Clinic team to genetically engineer the measles virus for the therapy of human cancers [320].

The clinical use of new attenuated NDV strains. When Lorence and his associates started using NDV therapy for human cancers in the USA (see below) [240], it was neither Cassel's 73-T nor the Hungarian MTH-68/H NDV strains that they administered to patients, but a new NDV strain referred to as PV701 [293]. At the Krebsforschungszentrum in Heidelberg, Germany, V. Schirrmacher enlisted for human cancer therapy the highly attenuated Ulster strain of NDV [334]. In Israel, the OV001/HUJ attenuated NDV strains have been used for the treatment of patients with glioblastoma multiforme [108]. In China, the LaSota attenuated NDV strain was used to treat patients with adenocarcinomas of the gastrointestinal tract in combination with a cancer cell-derived vaccine [230]. All attenuated NDV strains (D26, LaSota, Hitchner, HUJ, MTH-68/H, PV701, 73-T, Ulster) should be investigated for any contaminating virus: in embryonated eggs avian anemia circovirus (see below) [42, 99] and reticuloendothelial virus [97, 405], in Ehrlich ascites-passed NDV strains minute murine parvovirus [123, 368] and lymphocytic choriomeningitis virus [295], and possibly for other viruses that may coexist in the dominant NDV stock preparations.

Behind the Iron Curtain. While viral therapy of cancer remained officially an unfavorable subject matter in the USA, at the August Kirchenstein Institute in Riga, Latvia, then under Soviet rule, Aina Ya. Muceniece published her monograph on the virotherapy of human cancers in as early as 1972. Enteroviruses were favored there (and in Russia) for oncolysis induction in the late 1960s. This superb monograph [262] is descriptive and critical and contains chapters such as "On the question of stimulating the growth of tumors by virus infection" ("stimulyacii rosta opukholi pod virusnoy infekcii"), "Some questions arising when verifying ….virus oncolysis", and "Problems connected with the selection of viruses oncolytic for human tumors." Muceniece elaborates on the "Development of anticancer immunity following virus oncolysis" ("razvitie protiborakobovo immuniteta v processe virusovo onkoliza"). She published a black and white picture (between pages 288 and 289) of a human angiosarcoma cell infected with cocksackie virus B-5. Nuclear damage and cytoplasmic vacuolization are evident, as the tumor cell is being lysed; the dying cell is surrounded by host lymphocytes attached to the dying cell (but these are not commented on in the caption).

After stagnation, molecular virologists explode the field. With the eventual discontinuation of institutionally approved and supported, but not NCI funded, human virus-therapy protocols, first at Sloan-Kettering [381] and M. D. Anderson Cancer Centers [355, 356] and later, more recently, at Emory University [28], several attempts at individualized viral therapy of human cancers in private practices by private practitioners were initiated [63, 93] or intensified. As meritorious as some of these adventures might have been, these exercises are not covered in this article, which is devoted to "academic and evidence-based medicine". The most prominent of these claims is the response of patients with glioblastoma multiforme (GMF) to treatment with the NDV strain MTH-68/H (see below). These patients were treated individually in various private practices and their case histories were collected for publication from several different physicians' clinics. These case histories were presented orally in a scholarly lecture by G. Gosztonyi et al. at the Third International Meeting on Oncolytic Viruses as Cancer Therapeutics, Banff, Canada, 2005, and the lecture is cited in [432]. These are claims that need to be confirmed by clinical trials conducted in an academic environment. In this review, academically, or even privately, supported Hospital Surveillance Committee-, Hospital Board of Clinical Research-, or FDA-approved protocol-based clinical trials for the viral therapy of human cancers are preferentially evaluated.

Studies on viral therapy of human cancers are now carried out by teams of experienced virologists, immunologists, geneticists, clinical pathologists, and medical/surgical oncologists-hematologists [94, 291, 315, 424]. The work is being conducted either in academic institutions affiliated with universities (both laboratory work and clinical trials) or in the laboratories of private companies, which contracted principal investigators for the clinical trials of their products. The rules and regulations of "best medical practice" are to be observed. However, just any reported positive results should not be favored for citation without professional questioning and criticism, and not before the final evaluation of prospectively randomized clinical trials [235]. Well-documented individual cases of complete remission should be reported as a guideline for the design of controlled clinical trials. The purpose of this article is the presentation of a balanced and critical account of clinical trials, imperfect as they may be, but conducted and evaluated in a controlled academic environment.

The state of the art. Viral therapy of human cancers is widely supported by the media and is readily accepted by desperate cancer patients and their oncologists. The promoters of this treatment modality, be they represen-

tatives of academic medicine or of industrial and private enterprise, enthusiastically endorse it. In peer-reviewed reports, highly positive results are emphasized [63, 96, 235, 240], even in the era of evidence-based medicine. In contrast, one can hear of the rare, unprofessionally expressed, opposing opinion in which the failure of the opponent's comprehension of the fine immuno-molecular mechanisms, and thus the eminent promise, of viral oncolysis is concealed by the flat declaration that the "field of human cancer virotherapy is in chaos" (statement by an unidentified commentator on an application). The authors of the present article wish to provide proof for the fact that viral therapy of cancer is well within the realm of academic medicine and is in observance of all its established rules that regulate the conduct of laboratory and clinical research. The admitted problem is that the extraordinary success rate of the viral therapy of cancer in experimental animals has not yet been realized in clinical trials for human patients. However, the approach to the problem is well organized and orderly, especially when undertaken by the academia. This article reflects the order in the field, as it lists the naturally oncolytic viruses and viruses rendered oncolytic by genetic engineering. To this effect, a brief introductory review is hereby submitted.

The molecules and their pathways (e.g. Rb/E2F/p16, p53, IFNs, ILs, PKR, EGF-R, Ras, Wnt) targeted by naturally oncolytic or genetically rendered oncolytic adenoviruses, herpesviruses, influenza virus, NDV, parvoviruses, poxviruses, reovirus, vesicular stomatitis virus (Ad, HSV, IV, NDV, VSV) in tumor cells and in the tumors' microenvironment are clearly defined [137]. Interventions helpful to the oncolytic virus to perform its actions (vascular leakage in the tumor bed induced by interleukin (IL)-2, depletion of regulatory T (T_{REG}) cells, antagonists of immune T cell and NK cell activation) are combined with cancer virotherapy [41, 214, 218, 291, 314]. While the apoptosis-inducing oncolytic virus prevents the full maturation of its progeny, it provides dying tumor cells for processing in dendritic cells (DCs). As mature DCs express tumor antigens not in the tolerogenic manner tumor cells do, but in an immunogenic manner, host anti-tumor immune reactions for the attack on intact tumor cells will be induced [226]. While the cell-free oncolytic virus is subjected to the innate and adaptive immune reactions of the tumor-bearing host (stromal barriers, inflammatory reactions, some cytokines which may even promote the tumor, virus-neutralizing antibodies), the anti-viral immune reactions elicited by the virally infected tumor cells (NK cells, immune T cells) destroy the tumor cells [55]. Healthy and transformed cells infected by a virus produce more or less interferon (IFN). Of the IFN-induced genes, the dsRNA-dependent protein kinase (PKR) shuts down the cell's own protein synthesis by phosphorylating the α-subunit of the eukaryotic translation initiation factor-2 (eIF2) [18]. Amino-acid deprivation and the production of misfolded proteins result in the phenomenon of endoplasmic reticulum stress (see below). While cellular protein synthesis is thus disrupted, the cell remains permissive to the commands of the viral genome and allows the full maturation of new viral progenies, leading to cytoplasmic lysis [18].

Yet the mechanisms of viral oncolysis vary. Previously, two major mechanisms of oncolysis were readily recognized: 1) cytolysis, that is dissolution of the cytoplasm by a replicating oncolytic virus as new viral progenies burst out of the cell, and 2) "nuclear clumping," a form of programmed cell death, suicide of the infected cell, occurring before full maturation of a new viral progeny takes place. Other forms of virally induced cell death recently elaborated on are 3) the morphological features of autophagy [184, 193], 4) apoptosis, initiated either intrinsically by mitochondria or extrinsically by a death domain [368], 5) the generation of tumor cell "syncytiosomes" by fusogenic viruses, their resistance to IFNs, and their ultimate lysis [364, 429], 6) the mitotic catastrophe [169], and 7) p53-independent endoplasmic reticulum stress [89, 95]. Beneath these morphological features lie the highly variable oncogenic cascades that the oncolytic virus specifically antagonizes. Many of these are clearly recognized and, consequentially, the genetically engineered oncolytic viruses are specifically directed to inhibit selected oncogenic pathways.

The authors of this manuscript presented a poster (Horvath et al.) [169] entitled "Oncolytic viruses kill tumor cells directly and indirectly" at the Third International Meeting on Oncolytic Viruses as Cancer Therapeutics at Banff, Alberta, Canada, in 2005. In text and microphotographs they showed in virally infected tumor cells death domain-dependent extrinsic and mitochondria-initiated intrinsic apoptotic deaths without the release of mature viral progenies, autophagy in virally infected Kaposi's sarcoma cells (and in rhabdomyosarcoma cells attacked by the patients's autologous lymphocytes), events of mitotic catastrophe, and cell membrane ruptures and cytoplasmic bursts with the release of mature viral progenies from tumor cells (melanoma and sarcoma cells). The viral lysates of these tumor cells were used as the first viral oncolysates for active tumor-specific immunizations of patients in the early 1970s [167, 370]. Documented were the formations of tumor cell syncytia, the "syncytiosomes", by fusogenic oncolytic viruses and the lysis of these fused tumor cell conglomerates with the release of mature oncolytic viral particles. The recently discovered phenomenon of p53-independent endoplasmic reticulum stress induced by some naturally oncolytic viruses (see above) [95] was omitted from those discussions. Instead, immune reactions of the host to virally infected tumor cells which were rendered neo-antigenic by the expression of viral structural proteins were shown. The participating elements of these immune reactions consisted of immune T cells, NK cells, macrophages (practicing the antibody-directed cytotoxicity reaction), and antibodies and complement. These reactions could be quantitatively augmented by actively immunizing the human host with autologous or allogeneic viral oncolysates or by infusing adoptively autologous

"immune lymphocyte" preparations [353, 357, 363, 371, 375]. The conclusions of the poster discussion were complex. The same virus could induce different forms of cell death in individual cells of a cloned tumor cell culture or in different tumor cell types. Antibodies could both antagonize (acting against immune T cells) or augment cell-mediated cytotoxicity (co-operating with NK cells and macrophages) [371]. Tumor cells maintained for decades in established cultures reacted differently to oncolytic viruses than native fresh tumor cells obtained from the patients. It was concluded that in a tumor-bearing patient, the factors of immune surveillance were de facto already compromised. Finally, xenografted human tumors represented a subclone of the original tumor selected for growth in an environment different from the native tumor bed of the host; thus their reactions to therapeutic interventions, including those of oncolytic viruses, may differ from those of a tumor in its native environment [359, 361].

The genetically engineeed viruses are designed to be tumor selective and target clearly defined oncogenic cascades. Good examples, out of many, are the Ar6pAE2fF and Ar6pAE2fE3F and E2F and E2F-E3 adenoviral vectors, which kill Hep3B human hepatocellular carcinoma xenografts based on defects in the tumor suppressor retinoblastoma (Rb) protein in these tumor cells [187]. The viral genomic sequences are necessary to encode the early transactivator E1A viral proteins for the replication of the virus; adenoviral particles with deleted E1A sequences can serve as non-replicating vectors of transgenes. In the healthy cell, hypophosphorylated Rb proteins bind E2F cellular proteins and thus inhibit the activation of E2F-responsive genes. Rb proteins phosphorylated at multiple sites cannot bind E2F proteins; thus genes with an E2F-responsive promoter, among them the cyclin kinases, could be activated and the cell cycle progresses. In the infected or adenovirally transformed cells, the E1A proteins bind and inactivate the hypophosphorylated Rb proteins at the regions where Rb proteins bind E2F proteins, thus allowing inappropriate entry of the cell cycle into its S phase. Accumulated free E2F proteins stimulate the genes for the synthesis of DNA replication initiating proteins. Tumor cells, and host cells infected by oncogenic adenoviruses, readily utilize this pathway for cell survival and replication. There is an element in this pathway which is essential for the accumulation of free E2F proteins; the E2F gene itself has an E2F-responsive element in its promoter, enhancing its own expression. Consequently, Rb pathway-defective tumor cells overexpress E2F. In the E2F- and E2F-E3-carrier adenoviral vectors, the viral genomic fragments of the E1A promoter were replaced by a 270-bp fragment of the human E2F-1 promoter, thus restricting E1A expression to Rb pathway-defective E2F-overexpressing tumor cells and curtailing the replication of these vectors in healthy cells.

These authors endeavor to present a well-organized article conducive not only for the design of simplistic infections of a tumor or its host by an oncolytic virus, but rather for the combination of two or more oncolytic viruses acting against different oncogenic cascades while suppressing antiviral immunity, allowing the viruses to act and enhancing the pre-existing but unexpressed antitumoral immunity of the host. The elements of this evasive anti-tumor immunity now reinforced, are to remain preserved in memory cells even after the rejection of the targeted tumor. There is order, and no "chaos," in the field of oncolytic virotherapy of human cancers. The problems are recognized, confronted, and resolved or, if for the time being unresolved, the gathering of better factual information is being persued for the resolution of the intricate unsettled questions.

NATURALLY ONCOLYTIC VIRUSES

Paramyxo- (rubula-) viruses (mumps and NDV)

The viral genomes. The 15-kb-long negative-sense single-stranded RNA genome of the *Mononegavirales* order NDV cannot be directly translated into viral structural or other proteins (a total of six). Due to the lack of cellular enzymes, mRNAs are not generated for the reaction. Instead, the viral enzyme RNA-dependent RNA polymerase transcribes the negative viral RNA genomic strand into a positive RNA strand. The negative viral genomic RNA strand ("anti-genome") can be a template of, and be copied into, full-length positive RNA strands; the positive RNA strands copied into negative single-stranded viral genomes are packaged into the new viral particles. The paramyxoviral genome in the viral particle is nonsegmented [71]. In medical oncology, after W. Cassel's first declaration, NDV is regarded as an "antineoplastic agent" [46] (reviewed in references [50, 165, 369]).

Fresh isolates. These were derived from patients infected by mumps virus or NDV in the 1950s. The fresh isolates shared antigens and these were considered to be related [88, 350]. There is amino-acid homology in the hemagglutinin-neuraminidase proteins (see below) of simian virus 5, NDV, and mumps viruses [215]. The NDV L protein shares amino-acid sequences with that of the Sendai and vesicular stomatitis viruses (VSV) [466], either because the ancestor paramyxoviruses and rhabdoviruses were related or because horizontal gene acquisitions occurred in host cells co-infected with ancient paramyxo- and rhabdoviruses. Virus-neutralizing antibodies bind epitopes of the NDV fusion (F) protein [467].

Human mumps virus-induced parotitis (rubula inflans, red swelling) is not always confined to the salivary glands; it may involve the testicles (orchitis), ovaries (oophoritis), or pancreas (pancreatitis). It was an extremely tempting idea to use attenuated mumps virus for the viral therapy of human adenocarcinomas. In Japan, partial durable tumor regressions were reported in tumor-bearing patients (including patients with ascitic ovarian carcinomas, brain gliomas) treated with

mumps virus [16, 345]. However, Japanese authorities in the 1970s disallowed the mumps vaccine for the treatment of human cancers (T. Asada, personal communication in a letter to the senior author, 1994).

Cellular receptors. Paramyxoviruses use sialic acids, signaling lymphocyte-activating molecules (SLAM CD150) [32], and glycose-aminoglycans and asialo-glycoproteins for their attachment to the cell surface and entry to the cytoplasm. Paramyxoviruses (including NDV) express fusion (F) proteins and host cells with virus particles attached may fuse into syncytia; the negative fusion regulatory protein was also identified [185, 280]. Some antibodies enhance mononuclear giant cell formations in NDV-infected HeLa and other tumor cell lines [185]. Paramyxoviruses replicate in the cytoplasm of their host cells. Infected host cells may die apoptotic deaths or lyse when the new viral progenies burst out of the cell. It is frequently stated that the NDV genome is very stable. However, wild-type NDV strains can gain extraordinary virulence as sequence differences occur at the fusion protein cleavage site and in the matrix protein nuclear localization signal. Such wild-type NDV strains originated from the avian paramyxovirus type 1 strains and emerged in California in 1972 and in Canada in 1990–1992 [336]. Highly virulent versus apathogenic NDV strains did not arise as a result of genetic recombinations; point mutations in the viral genome bring about these changes in virulence [412].

Apoptosis. The MTH-68/H NDV strain kills by apoptosis induction rat pheochromocytoma cells and human cancer cells established and maintained in tissue culture [96, 399]. Results of elaborate experiments obtained in these systems are considered valid in explaining how NDV strains (the MTH-68/H virus) kill human cancer cells in patients. However, tumor cell lines established and maintained for decades in the laboratory are unnatural artificial creations that do not exist in nature [74]. For this reason they are not comparable to native cancer cells metabolizing and masquerading as "self" in their natural environment, i.e. the tumor-bearing host [359]. It has not been investigated properly how native human cancer cells in the human host *in vivo* acquire resistance to NDV (including resistance to the MTH-68/H strain). It is not known how fresh human cancer cells differ from artificially *in vitro* grown colonies of established human cancer cell lines in their reactions to NDV infection.

Genetically modified NDV strains constructed from the moderately pathogenic Beaudette and the avirulent LaSota viruses exert intensive damage in human tumor cells, first by the intrinsic mitochondria-mediated pathway by releasing cytochrome c and activating caspases 9 and 3 and, secondly through the extrinsic death receptor pathway in which caspase 8 is activated through the TNF-related apoptosis-inducing ligand (TRAIL) pathway [89]. NDV-induced apoptosis is caspase dependent and is suppressed by caspase inhibitors. Compared with NDV, the primary mechanism of apoptosis induction in host cells by influenza, measles, and rabies viruses is through the death domain receptors, whereas herpes simplex virus and reovirus cause apoptotic death by inducing the release of mitochondrial pro-apoptotic proteins (nucleases). Human tumor cells resistant to NDV-induced apoptosis became susceptible to apoptosis induction by the recombinant viruses, including the P-antigen editing NDV mutant rBC-Edit strain [391]. A mechanism of NDV resistance emerged in mouse cells; mutant mouse cells gain resistance to NDV infection by altering cell surface receptors or by up-regulating IFN production [11]. In mouse spleen cells, the NDV hemagglutinin-neuramidase (HN) protein is the major IFN inducer [188], whereas NDV structural proteins other than HN also serve as inducers of IFNs [443].

Autocrine opiate-to-receptor circuit in glioblastoma? There is a conspicuous lack of studies on the mechanisms of resistance expressed by some human cancer cells (including those of glioblastoma from patients who relapsed during therapy) to MTH-68/H or OV001/HUJ [108, 432]. Some NDV strains are known to activate in mammalian cells (first shown in mouse spleen cells) the pro-opiomelanocortin gene [82, 444]. However, the viral structural, or otherwise encoded, protein that enters the cell nucleus and activates the opiate gene (the receptor, the ligand, or both) has not been identified. It has been claimed that the NDV-H/MTH-68/H virus ameliorated pain in cancer patients treated with this virus and therefore it was assumed that this NDV strain activates the endomorphin system in the brain of patients receiving NDV oncolytic therapy [63]. Since glioblastoma cells are known to use endogenous opiates (as ligands) and their receptors for autocrine growth circuits [39, 70, 117, 445], the possibility should be entertained (and investigated) if NDV (MTH-68/H) could activate such a growth cascade in glioblastoma cells of patients with glioblastoma who relapse and die after NDV therapy. Reference is made to two reports describing such relapsing patients [108, 432].

In general, it should be taken into account when malignant gliomas are treated with oncolytic viruses that IFNs may interact with opiate receptors expressed in glioma cells. In particular, NDV is a major inducer of type I IFNs. Preparations of IFN-α induce endorphin-like activity. Virally stimulated lymphocytes produce IFN-α and "low-molecular-weight-related proteins", which bind opiate receptors and thus induce the analgesia reaction [380]. Thus IFN induction by an oncolytic virus (NDV) could possibly stimulate the growth of malignant glioma cells through a paracrine circuitry.

The opioid-cytokine connection [296] exerts influence on host immune reactions [44, 341] and it may be immunosuppressive [29, 86]. If so, and the nonpathogenic oncolytic virus is tolerated by the host, viral oncolysis may proceed unabated. However, the immunosuppression should not embrace the tumor. Opioids may induce FasL-mediated apoptosis [460]. If so, and if this involves tumor cell death, then the induction of opioids is advantageous to the host. However, immune T cells should be spared apoptotic deaths. In endotoxin shock,

reactive leukocytes release opioids [149]. If tumor-reactive leukocytes (see below) also release opioids, certain neuroendocrine tumor cells may thus gain growth factors. The non-negligible effects of NDV infection in the mammalian host, i.e. the activation of the opioid-cytokine network, may also influence the outcome of viral oncolysis. It may occur that an NDV strain exerts direct oncolysis in infected tumor cells, whereas by inducing the opioid-cytokine network it stimulates the growth of those subclones of tumor cells that operate an autocrine-paracrine circuit of opioid receptor expression and further capture IFN-induced opioids as ligands for mitosis induction. This complicated virus-tumor interrelationship so far remains uninvestigated.

NDV oncolysis. Genetic maps of avirulent (for example D26) [326] and freshly isolated virulent (for example Herts'33 genotype IV) [19, 64] NDV strains are available to identify the genetic machinery leading to oncolytic activity. Attenuated NDV strains remain (or become) oncolytic either by viral replication or by apoptosis induction in tumor cells. Thus the loss of virulence genes does not deprive the virus of its oncolytic efficacy.

Inactivated NDV (and influenza A virus) particles can cause apoptotic death in normal or tumor cells exposed to such viral inocula [458]. Live NDV is apoptotic to chicken cells and it induces agglutination and lysis of chicken lymphocytes [219, 220]. If the live NDV inoculum induces apoptotic death of the tumor cells before the maturation of the new viral progeny, there will be no continuation of the oncolytic process due to the lack of mature virus particles. Repeated viral inocula may generate virus-neutralizing antibodies, which may antagonize the continuation of viral oncolysis. However, if apoptotic tumor cells are engulfed by macrophages and DCs, tumor antigens (or tumor antigens combined with viral peptide antigens) may be processed for presentation to CD4 lymphocytes. If the presenting DCs are mature and activate co-stimulatory molecules and if the reacting CD4 cells secrete IFN-γ, cell-mediated (immune CD8 T cell-mediated) Th1-type immune reactions will be generated and the viral therapy will be converted into an endogenous tumor vaccine therapy. If the presenting DCs are immature and if the reacting CD4 cells secrete IL-4 (and IL-10), an antibody-mediated Th2-type immune reaction will be generated. In both settings, $CD4^+CD25^+FoxP3^+$ T cells may arise and gain support by chemokines (stroma-derived factor-1 and its receptors CXCR4/7) and transforming growth factor (TGF)-β). These tumor-protective T_{REG} cells antagonize the anti-tumor immune T cell clones. The Th1 and Th2 types of immune reactions may overlap. The antibody-mediated reactions may further antagonize immune T cells, neutralize the oncolytic virus, or, in contrast, co-operate with Fc receptor--expressor macrophages and NK cells in an antibody--directed cellular cytotoxic reaction (ADCC) directed against virus-infected tumor cells [198]. It is not known what anticellular immune reactions are induced by tumor cells lysed by the fully mature new viral progeny bursting out of the cells. The anti-NDV host immune reaction contributes to the lysis of virally infected tumor (mouse lymphoma) cells [84]. Direct *in vitro* cytolysis of Burkitt's lymphoma cells by NDV [417], if it occurred *in vivo*, would predictably induce anti-lymphoma cell host immune reactions.

F proteins. NDV strains possess fusogenic viral envelope proteins [229, 254, 467] and induce small syncytia of infected tumor cells. For viral oncolysis in general, fusogenic oncolytic viruses (such as the VSV) are preferred so that the virus may spread directly from cell to cell without an extracellular phase, thus escaping neutralization by host antibodies. Some genetically engineered oncolytic herpes viruses were rendered fusogenic by the insertion of the fusogenic protein gene of the gibbon ape leukemia virus [347]. However, tumor cell populations are heterogenous. Tumor cell syncytia consisting of multinucleated tumor cells may unite hitherto separately functioning oncogenic cascades [429]. If the oncolytic virus fails to eventually lyse tumor cell syncytia, the patient may succumb to a tumor of artificially increased malignancy (growing now in syncytia). As for NDV and VSV, it has been repeatedly shown that virally induced tumor cell syncytia were eventually lysed by these oncolytic viruses [239, 364, 368, 369]. The NDV fusion protein motif (112)R/K-R-Q-K/R-R(116) at the terminus of the F2 protein with phenylalanine at residue 117 at the N terminus characterizes virulent NDV strains. The motif (112)G/E-K/R-Q-G/E-R(116) with leucine at residue 117 is the faculty of weakly virulent (mesogenic) NDV strains (faculties most recently extensively discussed) [64, 65, 302] (D – aspartate, E – glutamic acid, glutamate, G – glycine, K – lysine, R – arginine, Q – quatamine, glutamine).

The NDV envelope glycoprotein HN. These and other NDV structural proteins induce IFN production in chicken fibroblasts, mouse spleen cells, and human cells [29, 102, 409, 477]. Expression of the class I major histocompatibility complex (MHC) is stimulated by NDV and IFN in normal cells, but stimulation fails in embryonal carcinoma cells due to the lack of cooperation between IFN-responsive factor-1 and nuclear factor (NF)-κB [409]. However, in mice and hamsters NDV may be immunosuppressive (inhibiting hemolytic antibody production against red blood cells) [86]. Some NDV strains are immunosuppressive by agglutinating and lysing chicken lymphocytes [220]. No such effect on healthy human lymphocytes was observed. Some NDV strains attack RBC membranes and are hemolytic [400]. No hemolytic anemia was reported in patients receiving NDV therapy.

Recent academic clinical trials with NDV

Emory University, Atlanta, GA. The first advanced academic phase II clinical trial of NDV therapy of human tumors is that for stage III (gross regional lymph node metastases) malignant melanoma conducted at Emory University in Atlanta, GA [28, 49, 50]. After sur-

gical removal of clinically evident lymph node metastases regional to the primary melanoma, patients received protracted vaccinations (for years, sometimes for two decades) with 73-T NDV viral oncolysates (VOs). The virus was a field isolate of NDV provided by Lederle Laboratories, American Cyanamid Company (Pearl River, NY). The virus was serially passaged in Ehrlich's ascites carcinoma by W. Cassel; the virus lysed these mouse tumor cells *in vitro*. At Emory in 1965, W. A. Cassel and R. E. Garrett declared NDV to be an "antineoplastic agent" [46] (cited and commented on in [50, 165, 358]).

The clinical trial was not prospectively randomized. It enlisted patients with regional (and other) lymph node metastases after surgical removal of gross disease (in the pre-CT-scan era). Contemporary local and worldwide control patients with stage III disease treated surgically only experience a high relapse rate, exceeding 70%, and in some series up to 80%; practically all these relapses eventually terminate in fatal hematogenous metastases. The relapse rate in 10 years (and above) of recipients of postoperative VO therapy remained below 20%. Over 60% of the VO-treated patients were alive and tumor free over twenty years after the beginning of VO therapy [49, 50].

St. Joseph's Hospital, Tampa, FL. In the mid-1990s and thereafter, the authors of this article used Cassel's 73-T NDV strain and his technology (with his permission) to vaccinate patients with very deep stage I (primary melanoma, excised), stage II (satellite metastases excised, or not excised), stage III (regional clinically evident lymph node metastases, excised), and stage IV (hematogenous metastases) disease. However, changes were introduced into the original Emory protocol, which was written before the discovery that IFN and IL-2 have efficacy in the treatment of melanoma. The vaccination period was reduced to one year; the vaccines were given either with 5 million units of IFN-α2b or with 5 million units of IL-2 or with 250 micrograms of granulocyte-macrophage colony stimulating factor (GM-CSF, sargramostim, Leukine, Berlex, Richmond, CA) subcutaneously on Monday, Wednesday, and Friday of the week when VO was administered on Wednesday, at 3- to 4--week intervals. It was expense reductions (Health Maintenance Organization denials of reimbursements, especially for GM-CSF) that dictated the reduced dosages and short administration periods of the biologicals. In a small group of patients (n=8), low-dose cyclophosphamide *per os* was combined with the VO in order to reduce suppressor CD4 T_{REG} cell generation (inasmuch as a study indicated that macrophages generate and protect these suppressor cells in NDV-infected mice) [75].

By 1999, in the fifth year, there were 3 stage I (deep primary melanoma), 14 stage II (satellitosis), 23 stage III (regional lymph node metastases), and 5 stage IV (distant hematogenous metastases) patients with melanoma enrolled receiving either autologous (preferred, when available) or allogeneic VO vaccine. Of the stage III patients, 14 remained tumor free and 9 patients with early relapses who were also accepted in the protocol continued with advancing disease. Patients with kidney cancers surgically removed and with adverse prognostic factors were also enrolled in a similar protocol (because of a German clinical trial utilizing an NDV-treated tumor cell vaccine with IFN-α and IL-2 was on-going) [207, 352, 477]. Relapsed patients with melanoma received a biochemotherapy regimen alternating with autologous immune lymphocyte (lymphokine-activated natural killer cell and immune T cell) infusions [370–372]. Patients in early relapses after primary surgery were also accepted; all patients complying with the protocol admission criteria were accepted. There were no randomized untreated control patients.

The Cancer Research Laboratory was owned and operated by St. Joseph's Hospital and the program was financed by the Hospital. Its scientific and medical directors were the authors of this article, who were also affiliated with the Department of Clinical Microbiology and Immunology at the University of South Florida College of Medicine, Tampa, FL. The protocols submitted by the scientific and medical directors were approved by the Hospital's supervisory Board of Clinical Research. The Joint Commission on Accreditation of Healthcare Organizations certified the Laboratory and its personnel and inspected the premises biannually (the laboratory and clinics) where the work was conducted. The Joint Chiefs of the American College of Surgeons, the Commission on Cancer, and the Association of Community Cancer Centers visited annually and approved the program. The program was initiated in the early 1990s and was discontinued in 2006. In 2006, the Cancer Institute of St. Joseph's Hospital (the Hospital) was affiliated with the H. L. Moffitt Comprehensive Cancer Center, and investment in clinical cancer research (not reimbursed by insurance companies) was discontinued in the Hospital (in the expectation to be continued at the Moffitt Cancer Center).

By early 2006 there were 84 viral oncolysate-vaccinated patients, including those patients who were in rapidly progressing early relapse at the initiation of treatment. These advancing patients received less than four vaccinations and were switched to biochemotherapy and immune lymphocyte therapy. The vaccinated patient population ultimately defied stratification and statistical analysis. For example, patients who were denied reimbursement for GM-CSF were switched to receive IFN-α2b or IL2 and patients who refused to continue on pre-vaccination cyclophosphamide (due to nausea) were switched to another arm of the master protocol. One of three female patients with deep stage I disease relapsed after vaccination and died with brain metastasis (for which she declined therapy) and another patient with newly discovered liver lesions underwent surgical resections; however, the liver lesions were benign adenomas (not melanoma metastases). A third

patient developed distant lymph node metastases after allogeneic viral oncolysate vaccine therapy; she was among those patients who entered rapidly durable (in the fifth year) complete remission after biochemotherapy, supporting the tenet that pre-vaccination increases the susceptibility of tumors to subsequent biochemotherapy [372].

Only 54 patients with adverse prognoses completed the entire protocol. Of these patients, 27 (50%) remained alive and relapse free for 5 to over 10 years. For various reasons, 30 patients failed to complete the entire protocol (including those who were enrolled in the stages of early postoperative relapses and were transferred to other treatment protocols). Of these patients, 24 (80%) succumbed to progressive disease, 2 patients (6.6%) are alive, and 4 patients (13%) were "lost to follow-up" (some of these patients must have died of relapsed disease under the care of private practitioners outside of the reach of St. Joseph's Hospital, where the initial treatment was administered).

The principal investigators (the authors of this article) conclude that Cassel's 73-T VO performed satisfactorily as a preventive vaccine in controlling postsurgical micrometastases, but failed as a therapeutic vaccine against rapidly advancing early relapses or established metastatic disease. The 50% relapse-free survival rate is 15–30% better than what patients treated only surgically for disease with the most adverse prognosis experience. However, prospectively randomized clinical trials do not accept comparisons of patients on the protocol with historical control patients; even the so-called matched historical controls would be unacceptable due to the biased selection of these control patients. By the rigid criteria of prospectively randomized clinical trials, the Tampa clinical trial does not appear to be acceptable as a proof of efficacy for the viral oncolysate therapy of human cancer (melanoma). However, the investigators involved in the laboratory work and clinical patient care are highly impressed by the tumor-free survival of several patients originally presenting with extremely adverse prognostic factors. Independent testimonial is provided by Mary Pritchard, RN, protocol nurse and a St. Joseph's Hospital employee, who maintains contact with patients surviving tumor-free over five years despite the most adverse prognostic factors. It appears to be an acceptable conclusion that viral oncolysate therapy provided a "favorable trend" in preventing melanoma relapses emanating from micrometastases. Even higher relapse-free survival rates are recorded in the Emory University clinical trial (see above) [49, 50]. If quantitative improvements of tumor-specific immune faculties were documented (autologous melanoma cell-directed immune T cells, increasing numbers of NK cells practicing ADCC on autologous tumor cells) and were shown to have correlated with the remission maintenance by viral oncolysate therapy, a more convincing case favoring the efficacy of viral oncolysate therapy could have been concluded. The efficacy of viral oncolysate therapy could possibly be improved by better vaccination schedules (more frequent and longer duration, increased live viral dosages, and co-administration of Th-1 type immune modulators) in a newly designed protocol.

One of us (J. C. Horvath) prepared a DC vaccine for a patient with kidney carcinoma metastatic to the lungs using autologous DCs and NDV-infected irradiated tumor cells. The patient experienced a documented partial remission and prolonged stabilization of disease, which was lost to brain metstases later (presentation by J. C. Horvath and J. G. Sinkovics at the 50th Annual Jubilee Conference of the Hungarian Microbiological Society) [168]. A reference cited [61] indicates that DCs preferentially phagocytose and process virally infected apoptotic tumor cells and induced rejection-strength anti-tumor immunity in hosts vaccinated with DC vaccines prepared with herpesvirally infected apoptotic tumor cells (see below).

Krebsforschungszentrum, Heidelberg, Germany. The third advanced academic NDV VO clinical trial is the most extensive as to the tumor categories involved and it is the most compliant as to the rules of academic medicine since it unites molecular immunovirology (work carried out in the laboratory) with supervised clinical trials. This work is being conducted at the Krebsforschungszentrum of Germany in Heidelberg by V. Schirrmacher and his clinical associates. The "extremely avirulent" non-lytic Ulster NDV strain was used to alter the surface antigenicity of various X-irradiated human tumor cells [332–334]. The hemagglutinin-neuraminidase-0 precursor of the hemagglutinin-neuraminidase glycoprotein of the Ulster NDV shows an unusual C-terminal extension, a reduced number of basic amino acids at the F0 cleavage site, and a leucine residue instead of a phenylalanine residue at the N terminus of the F1 cleavage fragment [254] (see above). Ulster-virus infected cells induce local antibody production [318].

Basic science work revealed how healthy cells stop NDV replication by type I IFN production and how malignantly transformed cells fail to produce antiviral enzymes: sRNA-activated protein kinase (PKR) and the IFN-inducible Mx proteins. The human genes *mxA* and *mxB* encode these proteins (MxAB). The human MxA protein in the cytoplasm inhibits the replication of a number of RNA viruses (measles virus, NDV, and VSV). Healthy peripheral blood mononuclear cells stopped the replication cycle of NDV after the production of its positive-strand RNA, whereas in tumor cells, viral replication and translation of viral genomes continued uninhibited [102].

Patients with surgically removed (microscopic or detectable residual disease left behind) breast, colon, and ovarian carcinomas, malignant melanoma, glioblastoma, and other tumors were vaccinated with autologous tumor cells with cell surface antigenicity modified by the co-expression of NDV Ulster and tumor antigens: "Aktiv spezifische Immuntherapie mit autologen virusmodifizierten Tumorzellen" [333]. These patients generated immune T cells, NO-synthesizing macropha-

ges attacking their tumor cells, and stored tumor-reactive memory cells in their bone marrow [332]. Clinically, postoperative relapses were delayed or entirely canceled and some residual tumors actually showed regression [332–334, 441]. Most remarkable is the remission inductions and significant delays in relapses of glioblastoma [387]. The US NCI failed to recognize the value of immunovirological research and immunotherapy for glioblastoma multiforme, as expressed in an editorial released from the US NCI (cited in [373]) and published in the same issue of the Journal of Clinical Oncology in which the article by Steiner et al. appeared [387]. The spokesman with unparalleled simplism stated that the alkylator temozolomide remains the standard treatment for that condition (cited in [373]). Nevertheless, the Heidelberg clinical trials stand out as the most convincing ones for the value of viral oncolysate therapy as applied to various human malignant tumors.

Wellstat Biologics. In the fourth advanced, FDA--approved but privately sponsored (Wellstat Biologics, Gaithersburg, MD) NDV clinical trial, the PV701 naturally attenuated NDV strain [240] is administered intravenously in large repeated doses to tumor-bearing patients; most of the recipients are patients with metastatic cancers. Adverse reactions to the first administration of the virus are avoided by "desensitization", consisting of a very slow infusion rate. The tumor-site--specific events consist of temporary enlargement of the lesions due to edema and inflammation. The latest report counts 4 "major responses" in 19 patients receiving, after desensitization, large repeated doses of the virus intravenously at a slow infusion rate [170, 239, 241]. In comparison with the reported extraordinary response rates to the MTH-68/H virus (20 complete and 9 partial remissions in 29 patients with metastatic cancers) [96], the PV701 response rates appear much more realistic. Deciphering results from anecdotal reports [235], it appears that the response rates to MTH-68/H viral therapy were claimed to be between 15% (after intravenous administration) and 21% (after intranasal administration). The intravenously administered PV701 virus induced 3%, 6%, and 22% response rates. Responses were stabilization of disease, minor and partial tumor regressions, and occasionally claimed complete remissions [239, 241, 293]; most of the complete remissions were lost to relapses. As to the durable complete remissions, the question is if they were induced with viral therapy alone and how long they lasted.

Apoptosis induction by the MTH-68/H virus in rat pheochromocytoma cells [96, 399] may not be representative of the mode of oncolysis by all NDV strains in human tumor cells. PV701 viral persistence in mesothelioma cells was observed [293]. If the virus replicates in human tumors, then the host cell is preserved until after the new fully matured viral progeny lyses it. Or is there a viral persistence state (mediated by IL-10, the promoter of viral persistence) in which a low level of viral replication in surviving tumor cells occurs and tumor cells and viruses coexist in a tolerant host? Furthermore, not in the artificial *in vitro* environment [74, 95], but *in vivo* in the host, antiviral antibodies with complement or with macrophages or NK cells in the ADCC reaction may lyse the virally infected tumor cells by the release of perforins from the NK cells. The CD8 immune T-cell attack may also result in cytolysis with perforins. Nuclear DNA laddering due to caspase and endonuclease cascades activated in an extrinsic apoptotic pathway may be induced by lymphocytes expressing FasL and related ligands, and not by the virus. There must be more than one mode of tumor cell death under NDV attack.

The PV701 virus [240] induces an anaphylactic reaction when rapidly infused intravenously. In a new protocol, this reaction was significantly diminished by lowering the rate of infusion [170, 241]. After safe dose-seeking phase I trials of PV701 virus [240], phase II trials will reveal the most sensitive tumor entities and if the clinical responses, not only "stabilization of disease", but actual remissions, are truly durable.

Jerusalem, Israel. For the treatment of patients with glioblastoma [108], the Hadassah and Hebrew Universities engaged in phase I/II clinical trials with the intravenously infused HUJ strain attenuated NDV. It is not clear if the previously used attenuated NDV strain OV001 and the currently used HUJ NDV are derivatives of each other or are different attenuated NDV strains. The OV001 virus induced a remission of a glioblastoma in a patient whose case history was presented at the 40th American Association Clinical Oncology meeting in New Orleans in 2004 [107]; however. this patient later relapsed and died [181]. In a summary report, of 14 enrolled and 11 treated patients, one complete remission was observed, but it is not clear if this case was the one reported earlier in New Orleans. No details are as yet available as to what other treatments were given and how long this remission lasted [108]. On behalf of the same authors [108], C. Irving (Theravir Management, Jerusalem, Israel) gave a verbal presentation of this project at the 4th International Conference on Oncolytic Viruses as Cancer Therapeutics in 2007 (funded by the Rochester Mayo Clinic College of Medicine), printed in the abstract book [181]. Patients with advanced glioblastoma received HUJ virus therapy intravenously, and the one patient achieving a temporary complete remission (see above) was again mentioned. The lentogenic NDV strain HUJ is oncolytic in mouse and human lung cancer cells, but IFN-β exerted an antiviral effect and protected the cancer cells from oncolysis [455].

Influenza virus

Complete dissolutions of mouse ascitic carcinomas. Influenza viral oncolysis for murine ascitic carcinomas was often complete and not less, but probably more efficient than that achieved with NDV [1, 48], since some NDV strains were rather oncostatic than oncolytic in

this system [349, 358]. Even inactivated influenza virus could elicit an apoptotic response in the exposed malignant (Daudi lymphoma) cells [442]. Augmented immunogenicity of cell membranes of influenza virus-infected tumor cells was documented [37, 234, 442].

Clinical trials with influenza virus

The melanoma and sarcoma viral oncolysates. Influenza VO used in the early and mid-1970s at M.D. Anderson Hospital [352] were prepared from autologous (preferred, when available) or allogeneic tumor (sarcoma or melanoma) cells. Tumor cells of established tumor cell lines [366] were targeted by patients' lymphocytes before and after active immunization with viral oncolysates in the quantitative chamber/slide assay. The results were expressed in graphs comparing the growth curves of control and lymphocyte-exposed tumor cells [353, 357, 363, 370, 375]. Tumor cells of established cell lines [366] were infected *in vitro* with the PR8 influenza A virus of allantoic fluid derivation by a Hospital Surveillance Committee-approved procedure [352] and the cultured and infected tumor cells were lysed. The lysates were rendered free of live tumor cells, quantitated by protein content and PR8 virus titers, checked for viral (especially cytomegalovirus, herpes simplex virus) and bacterial (including mycoplasma) contaminants, submitted to limulus test (performed by Dr. Dieter Gröschel, chief of the Laboratory of Medical Microbiology at M. D. Anderson) for freedom from endotoxins, aliquoted, and stored deep frozen. At one point, the Surveillance Committee requested ultraviolet (UV) light treatment of the VO fluids; by titrations, substantial amounts of live PR8 virus remained in the UV-treated VO preparations [166].

Bacille Calmette-Guerin. In the era when Bacille Calmette-Guerin (BCG) was promoted and supported by the USA NIH/NCI extensively for the treatment of leukemic and solid tumor-bearing patients, but viral therapy of cancer was not in the VO protocol, BCG was scarified over the intra- or subcutaneous injection sites of VO administration [379].

Sarcoma VO. For patients with metastatic sarcomas, VOs were used in combination with chemotherapy [352]. Control patients received chemotherapy only; patients enrolled in the protocol received either chemotherapy and BCG or chemotherapy, BCG, and VO. Clinical evaluation (in the pre-computerized tomogram era) of these patients was the result of team work; the principal investigator (J. G. Sinkovics) was just one member of the team. The third group of 19 patients receiving chemotherapy, BCG, and VO surpassed in remission induction and duration that of the first and second groups of patients. During the same observation period for all three groups of patients, progression of disease occurred in 32% of these (the third group) patients. In contrast, a 72% progression rate in 49 patients of the chemotherapy group and a 53% progression rate in 19 patients of the chemotherapy and BCG group occurred by the conclusion of the trial [167, 355, 356, 370–372, 379]. However, the patient population could not be stratified according to an even distribution of metastatic sarcoma subtypes and extent of the disease (the "tumor load") at the beginning of the treatment within the three groups of the patients (in which, however, every patient harbored hematogenous metastases of a sarcoma, most commonly in the lungs) and the protocol was not "prospectively randomized". Therefore, in the era of evidence-based medicine, this early clinical trial is judged retrospectively as representing a most promising "favorable trend" toward an effective treatment modality of the future in which the co-administration of VO is to be favored. Attempted statistical calculations for significance indicate that the two immunotherapy-chemotherapy-treated groups of patients surpassed the 28% response rate of the group of patients treated with chemotherapy only; however, most of the patients in this latter group were contemporary historical controls receiving the same chemotherapy at about the same time, but without being registered in the protocol. The important comparison is between the 47% response rate of the chemotherapy- and BCG-treated patients versus the 68% response rate of the chemotherapy-, BCG-, and VO-treated patients. This significant difference was valid for the duration of the clinical trial. In the next two years, in the off-therapy period, most of these patients relapsed with advancing disease; however, the patients receiving VO sustained the longest remissions. This clinical trial also indicates that chemotherapy and vaccination may not necessarily be antagonistic, but may even be additive or even synergistic [372]. Clinical results like these suggest that new prospectively randomized trials should be designed with VO vaccinations continuing after the conclusion of the chemotherapy.

Immunization of patients with *in vitro* prepared VO (made preferably from autologous tumor cells by chief technicians Jimmy Romero and Jerry Cabiness) was observed to mobilize large numbers of tumor-reactive NK cells in addition to lesser numbers of small compact lymphocytes (immune T cells) and, occasionally, macrophages. Slides of chamber/slide culture vessels, in which virally infected and non-infected cultured tumor cells and blood buffy coat cells or ficoll/hypaque-purified lymphocytes (prepared by Dr. Cameron Tebbi) of immunized patients were interacting, were inspected by research associates Drs. H. David Kay and Harikishan Thota, chief technicians Jerry Cabiness and Jimmy Romero, and the principal investigator (J. G. Sinkovics) before and after immunization of the patients with sarcoma viral oncolysates. The types and numbers of the lymphoid cells reacting with the tumor cells were visually observed and counted. In postvaccination samples, an impressive outpour of large granular lymphoid cells (NK cells) which surrounded and lysed tumor (sarcoma) cells was observed. These pictures of historical value are among the first showing human NK cells attacking autologous and allogeneic tumor cells (Figs. 1, 2, 3)

[353, 357, 361, 367, 371]. Quantitative measurements of the effect of patients' lymphocytes on tumor cell growth were expressed in graphs (growth curves of lymphocyte--targeted tumor cells as summarized in contemporary and recent review articles [353, 361, 371]). Increased numbers of cytotoxic lymphocytes have been repeatedly documented in VO vaccinated patients (Figs. 2, 3) [361, 371]. The cytotoxicity of the patients' small compact lymphocytes (later recognized to be immune T cells) was often reduced when tested against antibody-pretreated autologous tumor cells, whereas antibody pretreatment of autologous, or allogeneic, tumor cells often increased the cytotoxicity of the patients' large granular lymphocytes (later recognized to be natural killer, NK, cells) [357, 358, 361, 371]. The discovery of Fc receptors on NK (but not on immune T) cells and that of the ADCC reaction offered the most plausible explanation for these phenomena [198]. The cytokines that mobilized the NK cells were not as yet discovered in the early 1970s.

The observations made in human patients in the early 1970s can readily be documented in mice in the present era in which recently discovered chemokines and cytokines reveal the mechanism of the phenomenon. In athymic nude mice carrying human hepatocellular carcinoma xenografts, adenoviral rAd and ganciclovir therapy eradicated the tumors. The rAd adenoviral vector expressing herpesviral thymidine kinase (TK) and monocyte chemoattractant protein-1 induced in responding mice IL-12 and IL-18 production and mobilized large numbers of NK cells attacking tumor cells [414] (see below).

Malignant melanoma VO. Early favorable trends [376], as evaluated by clinical associates Drs. Carl Plager, Nicholas Papadopoulos (medical oncology), Marion J. McMurtrey, Marvin M. Romsdahl (surgical oncology), and the principal investigator (J. G. Sinkovics), were slowly diminished in time in patients treated, in addition to contemporary standard therapy (chemotherapy and BCG), with autologous (preferred) or allogeneic VO, i.e. relapses were delayed in the patients receiving additional VO therapy. Curves showing 8–12% fewer relapses of patients treated with VO in addition to standard therapy (chemotherapy and BCG) at 48 months relative to standard therapy without VO were read in the late 1970s as "negative" [377]. That was the era in which complete and partial remissions were counted only as a tumor response, and minor responses and stabilization of disease were dismissed as negative results. When an immunotherapy regimen in the pre-CT scan era induced a tumor response manifesting as edema within the tumor, thus with no visible shrinkage of the tumor, the results were dismissed as negative. Retrospectively, there is a strong hint that those survival curves really showed some advantage of the VO vaccinations (Fig. 4) [377]. In two groups of patients with postoperative stage III disease (gross regional lymph node metastases surgically removed), the 34 patients who received chemotherapy and BCG continued to succumb to relapses up to the fifth year; 17 patients (50%) died in the first two years and of the remaining 17 patients, 7 died between the third and the fifth year, leaving 10 patients (30%) alive and tumor free at the end of the fifth year. In the group of 30 patients receiving chemotherapy, BCG, and VO, 17 relapses occurred within the first two postoperative years, which is to say that against disease already in early relapse (not detectable in the pre-CT scan era), when the patient was enrolled in the protocol, the treatment failed to control the rapidly advancing tumors. However, from the third year on, not one single melanoma relapse occurred in this group of patients, which is to say that between the third and fifth year these patients remained relapse free. In this group, 11 (36%) patients remained alive and tumor free at the end of the fifth year. Of the 2 remaining patients, 1 died of prostate and 1 died of colon cancer, but with freedom from melanoma relapse, even at autopsy. Thus all 13 patients receiving VO in addition to standard therapy remained melanoma relapse free

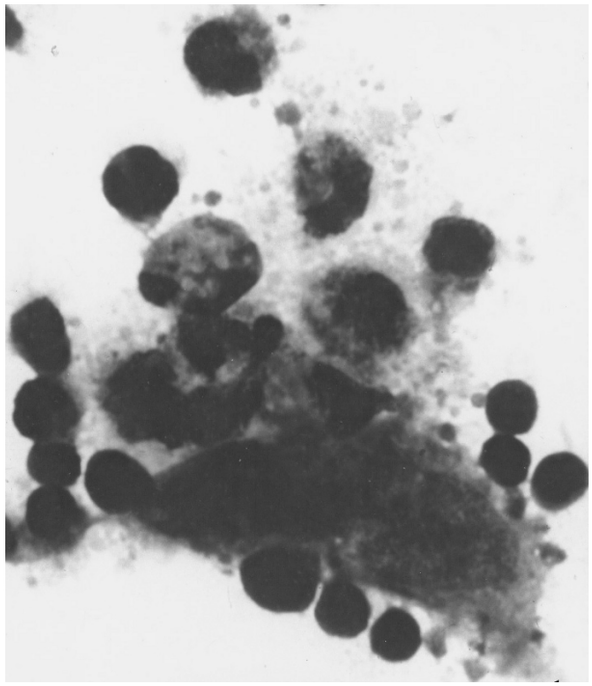

Fig. 1. Outpour of natural killer (NK) cells after immunization of patient with sarcoma viral oncolysate (VO)**.** An allogeneic human sarcoma cell (from cell line #3743) [366] is attacked by small compact (immune T cells) and large granular (NK cells) lymphocytes of a male patient (L. N., MDAH#90641) with metastatic liposarcoma in remission who was immunized with allogeneic sarcoma viral oncolysates (VO) in the mid-1970s at M. D. Anderson Hospital in Houston, TX. Lymphocyte preparations of this patient killed several allogeneic sarcoma cell lines; his serum intensified the cytotoxicity of his large granular (NK cells) lymphocytes. In contrast, the patient's lymphocyte preparations stimulated the growth of the control squamous cell carcinoma cell line #2043. A much diminished format without any details of this figure was published in reference [371]. Professor D. Spandidos, editor, International Journal of Oncology, permitted reprinting this picture in a larger format showing details. Wright-stained slides viewed with objective ×54, ocular ×10 in a Zeiss microscope.

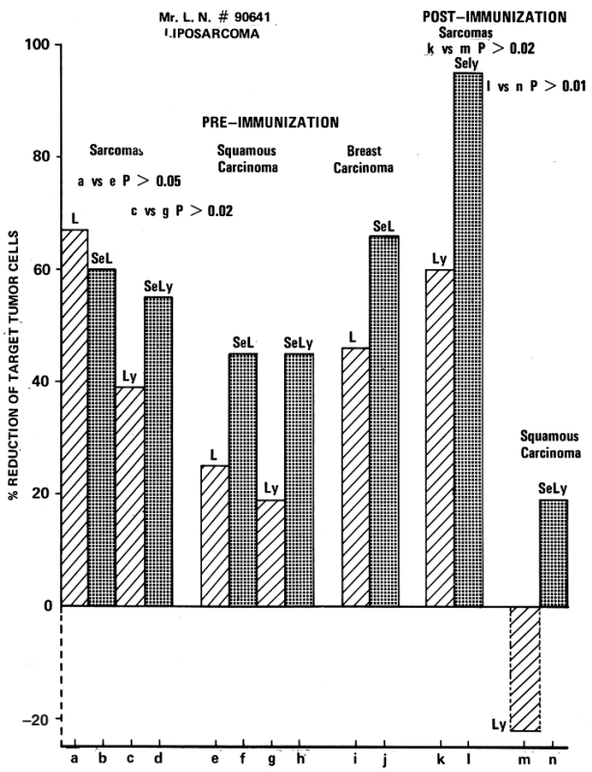

Fig. 2. Serial evaluation of lymphocyte-mediated cytotoxicity. Serial evaluation with statistical analysis of quantitated cell- and antibody-mediated immune responses to cultured human sarcoma cells (and to control non-sarcoma cells) of this patient (L. N. MDAH#90641) before and after serial immunizations with sarcoma viral oncolysate (VO) vaccine. Anti-sarcoma immune reaction significantly increased after immunization with the VO vaccine, but stimulation rather than suppression of growth occurred in control carcinoma cell cultures exposed to the immune lymphocyte preparations. The growth stimulatory effect was due to cytokines and hematopoietic growth factors released from the immune lymphocytes, but they had not yet been discovered in the early 1970s. This figure was published in Sinkovics J. G. et al. (1975): Prospectives for immunotherapy for sarcomas. Cancer Chemotherapy. Year Book Medical Publishers, Chicago, IL, 417–443. (Permission for reproduction has been applied for).

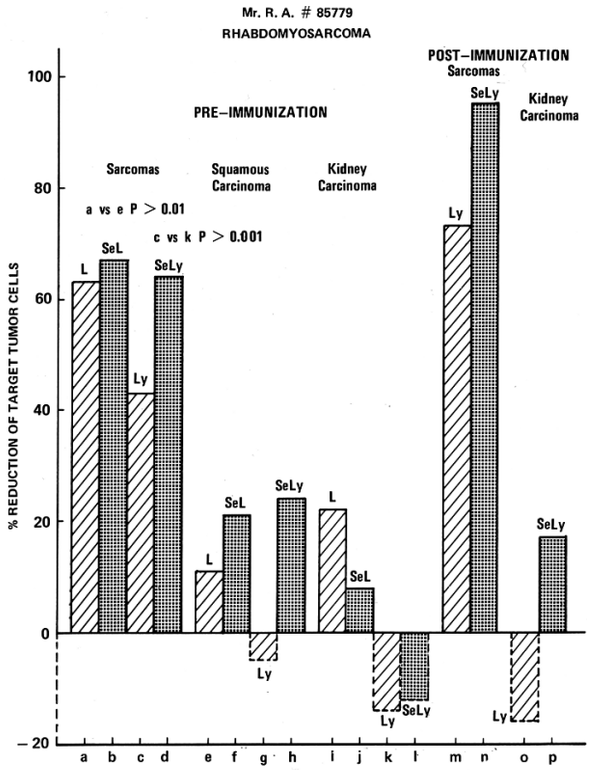

Fig. 3. Serial evaluation of lymphocyte-mediated cytotoxicity. Serial evaluation with statistical analysis of quantitated cytolytic immune lymphocyte reactions to sarcoma and carcinoma (squamous cell carcinoma; kidney carcinoma) cells of a young male patient (R. A. MDAH#85779) with rhabdomyosarcoma who received viral oncolyste (VO) vaccination (with chemotherapy and BCG) at M. D. Anderson Hospital in the early 1970s [372]. Prevaccination samples showed cytolytic reactions of his lymphocytes to sarcoma, but not to carcinoma cells. After vaccination with VO, the lymphocyte-mediated cytolytic rections significantly increased toward sarcoma target cells, but not to control carcinoma cells. Previously shown in reference [367]. (Permission for reproduction has been applied for).

(43% vs 30%) [369, 375–377]. If the two patients who died with cancers other than melanoma, but free of melanoma relapse, are accepted as melanoma-free survivors, then the 13% difference favoring those patients who received VO vaccinations becomes of borderline significance.

Other patients with active stage IV disease (hematogenous metastases) formed three groups. In Group I, 19 patients received chemotherapy and died in 5.1 months; in Group II, 24 patients received chemotherapy and BCG and died in 6.2 months; and in Group III, 11 patients received chemotherapy, BCG, and VO and died in 12.8 months (mean, from beginning of treatment, in all three groups of patients) [378]. The fact that the patients were not well stratified (as to an even distribution of metastatic sites and tumor burden) in each of the three groups and not prospectively randomized at enrollment places these clinical trials in the category of a "favorable trend." These clinical pilot studies with viral oncolysates provide a platform from which prospectively randomized and better designed and financially supported (more intensive immunizations with certain cytokine reinforcement) clinical trials should be launched.

Antibodies, immune T cells, and NK cells. There is extensive laboratory documentation of immune reactions to tumors cells prior to and after VO administration [353, 363, 375]. Lymphocyte-mediated cytotoxicity was quantitatively measured against autologous and allogeneic tumor cells *in vitro*, with or without antibody (patients' sera) pretreatment of the tumor cells. There was a tendency of correlation between the strength of the immune reactions as measured *in vitro* and the patients' clinical responses and length of survival [355]. However, disturbing events emerged. Non-immunized patients

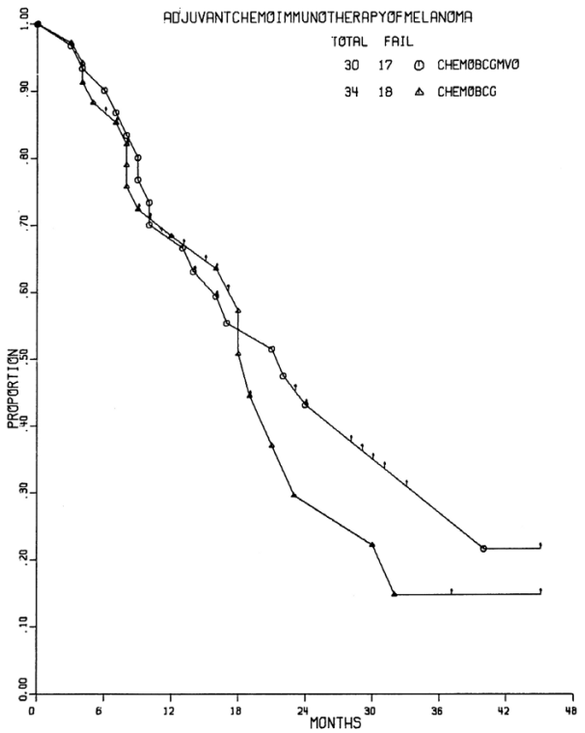

Fig. 4. Patient survival in graficon. The graph drawn before the finalization of the clinical trial with melanoma viral oncolysate (VO) as presented at the 12th International Cancer Congress in Buenos Aires, Argentina, in 1978. The graph shows relapse-free survival of patients with stage III (gross regional lymph node metastases of melanoma) disease in a 13% better range for the recipients of the standard treatment plus VO vaccine than the relapse-free survival curve of the patients who received the standard treatment (surgery, chemotherapy, and BCG scarifications) without VO [377]. Six months after its presentation, the difference between the two groups of patients remained valid (for comments on signifance, see the text).

also showed such immune reactions. Some patients showing immune reactivity to their tumor cells *in vitro* succumbed to relapses *in vivo.* These events remained unexplained until after the discovery of T_{REG} cells and tests for their detection. Healthy controls (first the senior author of this article) circulated large lymphocytes with granular cytoplasm that killed various human tumor cells *in vitro* [371]. In the early 1970s, these reactions were first mistakenly referred to (by representatives of the granting agency) as "non-specific *in vitro* artifacts." The doctrine specifying that no immune reaction may exist without specific pre-immunization guided the beliefs of the granting agencies, so much so that efforts were initiated to eliminate these "non-specific reactions" [371]. Only after healthy male donors' lymphocytes were found to kill cancer cells (breast and ovarian) of female patients and patients with various tumors also possessed the large granular lymphocytes that attacked and killed autologous and allogeneic tumor cells was the existence of natural killer (NK) cells accepted [371].

These authors are strongly impressed that patients receiving VO vaccinations mobilize NK cells. Exposure to viral particles attached to cell membranes, as in the VO preparations, may be exquisitely stimulatory to NK cell responses. Figure 4 in reference [372] shows the appearance of the PR8 influenza A virus-infected tumor cells' surface membranes in confluence with the lipid bilayers of the viral membranes as the tumor cell is undergoing the lytic process. Such co-presentation of tumor cell surface antigens with those of the viral membranes appears to "xenogenize" the tumor antigens, thus breaking the host's tolerance toward them. This mechanism of tumor antigen presentation may be the "forte" (the strong point) of VOs when it comes to the induction of immune reactions to an autologous tumor.

Gynecologic tumors. The Gynecologic Oncology Department of M. D. Anderson Hospital referred to the PR8 VO as "virus-modified autologous or homologous tumor extracts" and used them for the treatment of ovarian adenocarcinomas and squamous cell carcinomas of the uterine cervix [104–106]. Antitumor immune reactions were documented in skin tests, and by the generation of tumor cell-specific cytotoxic T cell clones [179, 180]. These immune reactions correlated with partial remission inductions and delayed relapses in immunized patients [106].

Reovirus oncolysis

Respiratory enteric orphans. Human reoviruses belong to the genus *Orthoreovirus*. Human reoviruses are not orphans anymore; in Malaysia, fruit bats spread newly discovered reovirus strains that cause pneumonia [57]. Members of the family *Reoviridae* are nonenveloped and contain 10 (large, L1-3, medium, M1-3, and small, S1-4) segments of a double-stranded (ds) RNA genome; the segments encode the viral structural and nonstructural proteins. The infectious viral population consists of mature virions and infectious subviral particles. The cellular receptors are sialic acids and Ig-like junction adhesion molecules. Reoviral particles enter the cell by receptor-mediated endocytosis, undergo acid-mediated proteolytic cleavage in the endosomal and lysosomal vesicles, exit through the vacuolar membranes, and release their ten capped viral mRNAs. The ten viral mRNAs serve as templates for negative-strand RNA synthesis. The viral mRNAs are translated into viral structural proteins. The viral dsRNAs are RNase resistant. Subviral particles are assembled and united with preformed complexes of outer capsid proteins. The cell bursts as it releases mature virions.

Early recognitions. It is seldom recognized that Lindenmann and Klein published a chapter on "Tumor immunity following reovirus oncolysis" in 1967. They cite reports on reoviral oncolysis of murine ascitic tumors first recognized in 1960. The monograph shows the electron microscopic picture (Fig. 22 on page 60) of a macrophage with phagocytosed debris of a reovirus-infected tumor cell. However, only antibody-response to mediate postoncolytic anti-tumor cell immunity was rec-

ognized. Today it would be recognized that the macrophage was presenting viral and tumor antigens to CD4 lymphocytes. Lindenmann and Klein explained the immunogenicity of viral oncolysates with the adjuvant effect of the virus on the tumor antigens [234].

IFN-deficient human tumor cells succumb to reovirus. The up-regulated and phosphorylated cellular protein kinase RNA-activated (PKR) mediates intracellular IFN action. It is up-regulated by transcription factor E2F-1 or by the melanoma-associated gene-7 product protein. Activated PKR phosphorylates the α subunit of eIF2α and eIF2-GDP entraps eIF2B; when eIF2B is so sequestered, protein synthesis comes to a standstill. IFNs inhibit viral replication by signaling through IFN receptors, thus inducing those cellular genes which establish the intracellular antiviral state. In the antiviral state, the association of mRNA with polysomes is inhibited, thus translation cannot take place. Various viral proteins target PKR to disable it or dephosphorylate eIF2α or function as IFN decoy receptors. The reoviral sigma3 protein binds dsRNA and inhibits PKR [395]. Influenza viral NS1 protein blocks PKR activation. Herpes simplex viral proteins US11 and ICP34.5 dephosphorylate eIF2α. Vaccinia viral protein E3L binds dsRNA and blocks PKR. Vaccinia and myxoma viral proteins B18R and M-T7 are decoy receptors and capture IFN-α/β, and IFN-γ, respectively. In malignant cells transformed by one of the *ras* oncogenes, the Ras oncoproteins block PKR phosphorylation; thus Ras-transformed tumor cells are naturally IFN deficient. Reovirus readily replicates in and lyses Ras-transformed human tumor cells [221].

*Mechanisms other than ras-mutation***.** Various mutations other than those of the *ras* sequences are also operational in the reovirus-tumor cell relationship. Oncogenes v-*erb*B or c-*myc* render tumor cells susceptible to reoviral infection. Some tumor cells, including those of malignant gliomas, undergo apoptotic death upon reoviral infection [58, 138, 449] without producing new viral progeny. Some other tumor cells, human fibrosarcoma (HT1080) cells, retain Ras mutation, become latently infected, stay alive resisting lysis, but fail to grow as tumors in xenografts; these cells remain susceptible to apoptosis inducers and infection with E1B-defective adenovirus. Those reovirus-resistant cells (HT1080/HTR1) that achieved freedom from viral persistence regained their tumorigenicity in xenografts [204, 205]. Tumor cells in the human host undergoing reoviral treatment (Reolysin, Oncolytics Biotech, Calgary, Alberta, Canada) [393] may escape oncolysis, tolerate persistent low level viral presence, or acquire freedom from the virus and display resistance to reinfection. In Burkitt's lymphoma cell line Raji, persistently reovirus-infected tumor cells and tumor cells purging all viral particles ("cured tumor cells") survive and resist re-infection with the virus *in vitro*. Reovirus-infected tumor cells, upon their re-inoculation, are not able to grow into xenograft tumors, but cured tumor cells grow into xenograft tumors and succumb to oncolysis upon re-infection with reovirus [5]. Thus tumor-bearing hosts should benefit from reoviral oncolysis: 1) tumor cells may die due to apoptosis or lysis, 2) they may become persistently virally infected and lose their tumorigenicity, and 3) tumor cells eliminating the virus regain susceptibility *in vivo* to re-infection with the virus.

Therapeutic trials with reovirus

Preclinical and clinical. Oncolytics Biotech (Calgary, Alberta, Canada) released an unauthored early report on phase I clinical trials in the Expert Reviews of Anticancer Therapy in April 2003 and elsewhere (references cited [358]). In contrast to what may happen in the tumor-bearing human host, in tissue cultures, and *in vivo* in xenografted human tumors, reovirus is highly oncolytic. The Dearing serotype 3 reovirus readily replicates and kills human tumor cells (adenocarcinomas of the breast, colon, ovary, and pancreas and squamous cell carcinomas of the head and neck) in culture and in xenografts *in vivo* [160, 177, 204, 274, 275].

In twelve patients with relapsed malignant gliomas treated with intratumorally inoculated reovirus, neither harmful effects nor complete or partial remissions occurred; however, unexpectedly prolonged survivals are being observed. The abstracted Forsyth et al. report is cited in a review [373]. In a phase I clinical trial, 33 patients with advanced cancers tolerated intravenous reoviral therapy well and without shedding infectious virus. Viral particles were visualized within tumors, tumor necrosis was observed (by computed tomography, CT), and prostate-specific antigen (PSA) and carcinoembryonic antigen (CEA) levels dropped in patients with metastatic prostate or colon cancers. Intralesional (direct intratumoral) injections of reovirus, now referred to by its registered trade name, Reolysin, induced an only 11% response rate in patients (reviewed in references [204, 393]). Even patients with advanced cancers were able to produce reovirus-neutralizing antibodies in response to intravenous viral inocula. In these patients, both CD4 and CD8 lymphocyte and $CD56^+$ NK cell counts increased. The aggregate immune response was a combined Th1- and Th2--type reaction [448]. It is not clear how much these immune reactions were directed against the tumor; did tumor cell deaths occur due to viral oncolysis, to host immune reactions to virally infected tumor cells, or both? Were reovirus therapy combined with a *ras* oncogene/oncoprotein inhibitor (farnesylthiosalicylic acid) [478], would the tumor cells resume IFN production and eliminate the reovirus?

Vesicular stomatitis virus

The VSV rhabdovirus is extremely sensitive to inhibition by IFN-α (as is NDV, while herpes simplex virus is more sensitive to IFN-β than to IFN-α). VSV enters cells through a phosphatidylserine receptor. The VSV genome is a nonsegmented single-stranded (ss) linear

negative RNA strand serving as template for a leader RNA and for five monocistronic capped and polyadenylated mRNAs to encode five (N, P, M, G, L) viral structural proteins. The L (RNA-dependent RNA polymerase) and P proteins copy from the negative RNA strand the five subgenomic mRNAs; L protein does the capping. The mRNAs are translated by cytoplasmic and by endoplasmic reticular ribosomes. The viral leader RNA and the viral M protein inhibit the transcription of cellular RNA polymerases, thus shutting down cellular RNA synthesis. The leader RNA translocates from the cytoplasm to the nucleus to inhibit DNA-dependent RNA transcription. The negative viral RNA strand serves as a template for the RNA genomic strands of the new viral progeny. The glycosysated G protein is expressed on the plasma membrane and mediates the assembly of mature virions (packing the genomic RNA into the hollow nucleocapsid N, binding the matrix protein M to the internal domain of the envelope glycoprotein G and to the cell membrane). N and P proteins assemble in disc-like oligomers containing ten N and five P protein molecules forming one turn of the ribonucleoprotein helix. Mature virions leave the cell by budding from the cell membrane.

Lysis of VSV-induced onco-syncytia. As early as the mid-1960s, the VSV Indiana strain was found to lyse a mouse lymphomatous tumor *in vivo* [364, 374]. By then it was well known that VSV induced plaques of killed cells in normal mouse embryo cell monolayers grown under agar and that IFN-α protected the cells and inhibited plaque formation. However, in mouse sarcoma cells carrying a mouse leukemia virus, VSV induced multinucleated giant cells and syncytia [364, 374]. These were the times when the IFN-deficient environment of tumors was attributed to "anti-interferons" of tumor cell derivation; it was discovered much later that *ras*-mutated tumor cells do not produce IFNs. Within the syncytia, VSV replicated even in the presence of exogenously added crude (NDV-infected mouse brain-derived) IFN-α [364]. The syncytia of the malignantly transformed cells harboring a murine leukemia/sarcoma virus and superinfected by VSV eventually disintegrated [364, 374]. These observations remained unrecognized in their time, but the phenomenon of polykaryocyte formation by VSV in mouse cells was confirmed [272, 407]. The unrecognized original observations are now reproduced in a more recent publication [361, 372]. Quite similarly, VSV induced syncytia formation of Rous sarcoma virus (RSV)-infected cells and these syncytia also disintegrated [54], thus VSV-induced events of "oncolysis" occurring *in vitro*. Later, VSV-lysed Ehrlich carcinoma cells were shown to release immunogenic antigens. The viral G protein was identified as the one responsible for cell fusions. A VSV melanoma oncolysate induced antitumor antibodies in patients without obvious clinical benefits (citations in [358]). More recently, VSV is finally recognized as a potent oncolytic agent for orthotopically xenografted human tumors (colorectal and hepatocellular carcinomas, head and neck squamous cell carcinomas) and possibly for conjunctival adenocarcinomas (reviewed in [118, 232, 358]). Furthermore, immune T cells (targeting ovalbumin antigens in ovarian carcinoma cells) infected with VSV carry the virus into the target tumor and thus launch an additive to synergitic antitumor attack in combining viral oncolysis with T-cell immune reactions. VSV-infected immune T cells may be used for adoptive immunotherapy of malignant tumors [301].

Human tumor cell oncolysis with VSV

The Canadian team. In Calgary it was established that VSV is not a human pathogen and that it is exquisitely sensitive to IFN-α; thus it would replicate in IFN--deficient human tumor cells (in which *ras*-mutations disabled PKR), while healthy host cells would defend themselves. Some M protein mutants of VSV (strains AV1 and 2) fail to suppress IFN production in healthy cells; healthy cells infected with AV1 and 2 VSV mutants overproduce IFNs. The AV2 VSV readily kills human ovarian carcinoma xenografts in the peritoneal cavities of mice [232, 243]. The virally induced inflammatory reaction compromises blood flow to the tumor [41]. The matrix protein mutant deltaM51 strain of VSV displayed high affinity to cultured human high-grade malignant glioma cells and suppressed the growth of these tumors xenografted in nude mice [243]. Xenografted and VSV-infected tumors released tumor antigens that induced immunity in the hosts and were protective against challenge with live, not virally infected, tumor cells (reviewed in reference [358]). VSV Delta M51-injected pediatric rhabdoid tumor xenografts frequently completely regressed [454]. These tumor xenografts are also susceptible to myxoviral therapy (see below).

The Miami team. At the Department of Microbiology and the Sylvester Cancer Center, VSV-mediated oncolysis in cultured and xenografted human tumors, including that of glioblastoma, was documented [22, 299]. Thereafter, tumor cells expressing herpesviral thymidine kinase and infected with VSV were shown to be increasingly susceptible to ganciclovir, inducing cell death. To genetically engineered VSV-transfected tumor cells with the IL-14 gene insertion, the murine hosts mounted Th2-type antibody-mediated immune reactions. While immune T cells cytotoxic to tumor cells were not generated, granulocytes infiltrated the VSV--infected tumors [22]. The genetically engineered VSV strain transduced with the fusion gene of the *E. coli* enzyme cytosine deaminase/uracil phosphoribotransferase enabled infected host cells to convert the innocuous 5-fluorocytosine into the cytotoxic 5-fluorouracil. Human multiple myeloma, murine lymphoma, breast cancer, and melanoma cells infected with the rVSV acquired sensitivity to 5-FU. The tumor-bearing host treated with rVSV produced IFN-γ and generated IFN--γ-secreting T cells cytotoxic to the tumor cells [299].

The British and Mayo Clinic team. Mouse melanoma cell syncytia induced with VSV were shown to be highly immunogenic "syncytiosomes" in the xenografted tumor-bearing hosts. It is DCs loaded with tumor antigens of syncytiosome-derivation that induce the immune response [27, 92, 233].

International efforts. Human colon cancer, hepatocellular carcinoma, and head and neck squamous carcinoma xenografts are susceptible to VSV therapy [85, 173, 346]. A replicating IL-12 gene-expressor VSV strain (rVSV-IL12) surpassed in oncolytic potency against orthotopic floor-of-the-mouth carcinomas the rVSV-F strain in immunocompetent mice; the rVSV--IL12 agent is in human clinical trials [346]. Tumor cells with an active Ras/Raf1/MEK/ERK signaling pathway and defective IFN-α-upregulated responsive factor MxA are the most susceptible to VSV cytolysis. However, the mitogen-activated protein kinase (MEK) inhibitor U0125 restored IFN responsiveness of the tumor cells and suppressed viral replication in the treated tumor cells [276]. The expression of fusogenic viral proteins transfected into tumor cells by adenovirus vectors or herpes simplex virus amplicon vectors (measles virus-H/F, respiratory syncytial virus-F, and VSV-G) increased the efficacy of FOLFOX chemotherapy (a combination of 5-fluorouracil, leucovorin, and oxaliplatin) in xenografted colon and pancreas carcinoma cells; RSV-F was the most effective [161, 163, 164]. Human T-cell leukemia virus-I-infected T cells were super-infected and then lysed *ex vivo* with VSV; naïve $CD4^+$ healthy T cells resisted VSV infection. however, activated healthy $CD4^+$ T cells were permissive, but fourfold less than HTLV-I^+ cells, to VSV infection [51].

Vaccinia virus

Ancient interactions. Poxviruses are prominent among those ancient viruses that coexisted with and infected living cells probably even before the time of the Cambrian explosion. Large dsDNA viruses infect the dinoflagellate (*Heterocapsa*), the green alga, chlorella (*Paramecium bursaria*), and the hydra (*Hydra viridis*). Preserved from the flora of the primordial Earth, the *Sulfolobus* archaebacterial virus is related to the chlorella virus. The huge mimiviruses (*mi*micking *mi*crobes) are extant poxviruses. According to an announcement from the Université de la Méditerranée, Marseille, France, a 18.3-kb circular dsDNA virus, a "virophage", parasitizes the mimivirus [479]. *Chordopoxviridae* infect vertebrates and *Entomopoxviridae* exist in insects [297]. The poxviral genome is large, exceeding 191,600 bp, and encodes well over 200 proteins. Some of these proteins antagonize host IFN-α, -β, and γ, IL-1β, TNF-α, and several chemokines [227]. The vaccinia viral NS1 protein binds the host cell's dsRNA engaged in RNA--silencing-based antiviral response [24]. Ancient poxviruses served as vectors for the horizontal transfer of retroposons from reptiles to mammals [297]. In the laboratory, attenuated poxviruses are enlisted for oncolysis and as vectors for gene therapy [103].

Accidents with favorable outcome. Levaditi's laboratory discovered that the vaccinia and mouse ectromelia viruses were oncolytic (reviewed in [348]). Cassel and Garrett successfully treated murine ascites carcinomas with vaccinia virus [47]. Salmon et Baix (*sic*) [322] inoculated vaccinia virus into a large breast carcinoma metastatic to axillary lymph nodes of a female patient ("cancer au sein" and "ènorme tumeur des ganglions de l'aisselle") by scarification and by direct intratumoral injection ("la vaccine est injectèe en abondance dans le nodule cancèreux"); however, nothing other than the localized pustules of vaccination occurred and the tumor was infected with the virus ("la vaccine reste localisèe au point d'inoculation et ne se gènèralise pas à la totalitè de la tumeur" and "la masse nèoplasique … contaminèe par le virus vaccinal"), but there is no straightforward statement in the brief text about the regression of the virally infected tumor [322]. Yet this report is often quoted by title as if it were an early example of successful viral therapy of a malignant human tumor. Much later, the staff at M. D. Anderson Hospital witnessed remission of chronic lymphocytic leukemia in a patient who was inadvertently vaccinated with vaccinia virus [78]. Another patient with CLL became so ill after vaccinia virus vaccination that he had to receive vaccinia virus immune serum to recover; however, his CLL remitted and remained in remission over three years [147]. A patient with multiple myeloma received vaccinia virus intravenously and responded with a partial remission [197]. In two patients with metastatic pulmonary or renal adenocarcinomas, the Japanese attenuated vaccinia virus AS strain given intravenously induced partial tumor regressions [13, 197]. Paradoxically, in healthy people, malignant tumors (basal cell carcinomas) arose in the scar of prior vaccinia vaccination [128, 249], but could be cured by wide excision. Vaccinia viral oncogenesis by whatever mechanism remains speculative and unproven.

Oncolysis. Melanoma skin tumors were treated with repeated direct inoculation of vaccinia virus [93, 175]. Powerful oncolysis could be observed in large extensive melanoma satellitosis by direct intratumoral injections of genetically engineered vaccinia virus encoding the GM-CSF protein. In seven patients, responses included no response, mixed response (with regression of some of the un-inoculated tumors), partial response (rendering the patient a complete responder by surgically removing residual tumors), and one complete response of scalp metastases [251]. Further topical administrations of vaccinia virus into the cancerous urinary bladder (Dryvax, Wyeth-Ayerst, Philadelphia, PA) and prostate resulted in partial responses and recruitment of reactive lymphocytes [126]. Those vaccinia viral strains (Western Reserve) that replicate to full maturation in melanoma cells kill the tumor cells, but also infect DCs and incapacitate them, prohibiting them from functioning immunologically. However, vaccinia virus strains (Ankara MVA) that induce apoptotic death of melanoma cells and fail to yield mature viral progeny

spare DCs and allow engulfment of dying melanoma cells by DCs, which express the MelanA antigen and generate CD8 immune T cells cytotoxic to melanoma cells [136]. In the replication cycle of the vaccinia virus there is an early phase, when the first viral progeny is released from the host cell before the cell is lysed. These viral populations consist of the cell-associated enveloped virus, which remains tethered to the cell, and the extracellular enveloped virus (EEV), which spreads rapidly, transgressing the matrix and escaping virus-neutralizing antibodies inasmuch as the viral surface is covered by the cell membrane. In mouse tumor models, the EEV exerted rapid enhanced oncolysis [208, 209]. However, the EEV particles are not stable and lose their cell membrane coating.

The JX-594 thymidine kinase gene-depleted vaccinia virus is genetically engineered to express the hGM-CSF gene. It is being tested in rabbits and rats harboring lung and liver cancers. The intravenously injected virus invaded the tumors and induced lymphocytic infiltrates [203]. The recombinant VACV GLV--1h68 agent (with inserted protein genes) serves as a therapeutic (oncolytic and immune stimulatory) and tumor diagnostic agent, thus erasing human breast cancer xenografts. Expression cassettes for galactosidase/glucuronidase, luciferase, and green fluorescent protein were inserted into the thymidine kinase and hemagglutinin loci of the viral genome. For diagnostic purposes, the tumor-infiltrating virus emits green fluorescence [471]. The virus vvDD-Cd expresses the yeast cytosine deaminase gene; it infects ovarian carcinoma cells and converts the prodrug 5-fluorocytosine into a cytotoxic agent [52]. The JX-795 IFN-β-expressing B18R gene-deleted vaccinia viral construct (TK^-/ /$B18R^-$/IFN-β^+) selectively infects tumor cells and vascular endothelial cells of the tumor bed in a mouse model; healthy cells were not infected by the JX-795 oncolytic vaccinia virus. Treated mice rendered tumor--free rejected tumor cell challenges [208].

The Wistar Institute's vaccinia viral oncolysate

The clinical trial in the USA (and in France). The Wistar Institute developed and patented a human melanoma vaccinia viral oncolysate (VMO), an allogeneic virus-augmented polyvalent melanoma cell lysate for "tumor-specific immunotherapy" of patients with melanoma. Patients with stages I and II melanoma seldom circulated antibodies reacting with their melanoma cells, but after VMO therapy, the staphylococcus protein A and the C3-mixed hemadsorption assays showed both IgM and IgG melanoma cell-reactive immunoglobulins [435].

In France, Western blot assay identified the M(r) 31,000-glycoprotein antigen to which the antibodies responded. Anti-ganglioside IgG antibody production correlated with freedom from relapse. VMO immunization increased lymphocyte-mediated responses to melanoma cells *in vitro*. Of 32 patients surgically rendered tumor free and immunized with VMO, 19 relapsed, 13 early and 6 late. Tumor-free survival at 40 months for the group was 35%, overall survival (some patients alive, but in relapse) at 30 months was 60%; there was no valid control group for comparison [31, 81].

In the USA, the phase III prospectively randomized double-blind multi-institutional postoperative adjuvant clinical trial for patients with stage II disease (positive metastatic lymph nodes, now called stage III) had two arms: VMO versus live vaccinia virus (VV); there was not a "no treatment" third arm. There was no statistically significant disease-free survival benefit for those patients who received VMO therapy (38 mo) in comparison with VV-treated patients (37 mo) ($P=0.99$). Overall survival favored the VMO-treated patients, especially in certain selected subgroups. At the 4th year, VMO-treated male patients experienced a 17–37% overall survival advantage over VV-treated male patients ($P=0.9$, $P=0.13$). Later analysis revealed no survival advantage of VMO-immunized patients over the VV-treated controls. However, subsets of VMO--treated male patients experienced 7–30% survival advantage over their VV-treated counterparts. At the 50^{th} month, 217 patients were immunized with VMO and 113 patients received VV. Disease-free/overall survivals for the VMO group at 2, 3, and 5 years were 48%/70%, 44%/60%, and 42%/49%. Disease-free/overall survivals for the VV group at 2, 3, and 5 years were 51%/65%, 45%/56%, and 40%/48%. However, the subgroup of VMO-treated male patients continued to show 19–27% survival advantage over their VV-treated controls. The great disadvantage of this trial is that the VV group was not a valid control group to the VMO group because it is assumed that it exerted weak effects of viral therapy in comparison to surgically treated historical control patients. Nevertheless, this clinical trial inadvertently showed a favorable trend for MVO immunotherapy over direct VV oncolysis. In comparison with surgically treated control groups of several other prominent clinical trials (testing IFN-α or IFN-γ against surgically treated controls), the VMO and the VV groups together and the VV group in itself performed better than the surgically treated control groups [201, 435–438]. Thus, in the VV group, live vaccinia virus exerted some oncolytic effect, but less than the VMO.

The clinical trial in Australia. Patients in relapse with lymph node metastases of melanoma were treated with allogeneic vaccinia melanoma cell viral lysates (VMCL) postoperatively. In addition, one group of patients received both VMCL and low-dose cyclophosphamide. At the beginning of the trial, melanoma relapses were delayed in both groups of VMCL-treated patients in comparison with the patients treated only surgically. By the end of the trial there were 353 surgically and VMCL-treated and 347 only surgically treated patients. At the 8th year, the median overall survival was 88 months for the control patients and 151 months for the VMCL-treated patients. At 5 and 10 years, overall survivals of control patients were 55 and 41% versus

VMCL-treated patient survivals of 61 and 53%. Median relapse-free survival was 43 months for control and 83 months for VMCL-treated patients. Relapse-free survivals for control and VMCL-treated patients at 5 years were 47 and 51%. However, the benefits the VMCL--treated patients seemingly received were not statistically significant [155, 156]. A "favorable trend" emerged toward the continuation of viral oncolysate therapy for melanoma with the aim to eradicate micrometastases after the surgical removal of all gross disease. Most elaborate clinical trials [50, 201, 376–378, 435–438] in which academic standards had been strictly adhered to support this effort.

Myxomavirus

An oncogenic virus becomes oncolytic. It has been known since the 1970s that Shope fibroma virus--induced tumors were permissive to co-infections with other viruses [284]. For example, VSV readily infected and inhibited the growth of these tumors [62, 397]. "Plaque production" in cultured cells by the fibroma--myxoma group of viruses [453] could have suggested that under certain circumstances some oncogenic viruses could practice cell- or onco-lysis; the term "plaques" is used correctly to indicate clear areas in a culture produced by the destruction of cells by a virus. Vaccinia and leporipox (myxoma) viruses encode serpins (serine protease inhibitors) and protect their host cells from apoptosis induction by FasL or tumor necrosis factor (TNF)-α. In the infected cell, the natural antagonist of TNF-α, nuclear factor (NF)-κB, is active. These viruses encode decoy receptors for IFN-γ and TNF-α. The virally encoded cell surface proteins B18R (by vaccinia virus) and M135R (by myxomavirus) are non-signaling decoy receptors for IFN-α/β [23, 24]. Poxviruses encode v-GAAP (Golgi anti-apoptotic protein). The human c-GAAP exhibits as much as a 72% amino-acid homology with some poxviral v-GAAP. The oncogenic rabbit myxomaviruses possess IFN-R and TNF-R (receptor) homologues and anti-apoptotic gene-product proteins (M11L) inhibiting pro-apoptotic host proteins of mitochondrial origin: BAK (Bcl-2 antagonist killer) and BAX (Bcl-2-associated X protein). Mouse cells protect themselves against infection with rabbit myxoma virus by IFN-α production, whereas human cells produce TNF-α for their protection against this virus [384, 439].

Oncolysis of human cancer cells. Myxomavirus kills human tumor cells by lysis as the fully mature virions of the new viral progeny burst out of the lysed cells. Imatinib mesylate, the inhibitor of the *scr*/*bcr*/*abl* oncogenic pathways, prevents the egress of poxvirus (vaccinia) particles from infected cells. For the permissiveness of human tumor cells to oncolytic myxomavirus infection, Akt cell survival pathway activation by the ankyrin protein M-T5 host range factor of myxoma virus is necessary [439]. For important clarification: the c-*akt* oncogene was found transduced from its host cell in a murine lymphoma retrovirus as v-*akt*. The phosphorylated serine-threonine oncoprotein kinase Akt (formerly protein kinase B, PKB) with phosphatidyl inositol kinase 3 (PI3K) is active in malignantly transformed cells. Tumor cells with active Akt are susceptible to myxomavirus infection and lysis, whereas cells with inactive Akt (most resting normal cells, certain breast carcinoma and other tumor cells) resist myxomavirus infection and lysis. Type I human tumor cells exhibit high levels of endogenous Akt activation and are highly permissive to myxomaviral replication and oncolysis. Type II human tumor cells operate low levels of endogenous Akt phosphorylation. Type III human tumor cells (the MDA--MB435 breast cancer cell line) are completely devoid of endogenous Akt activation and resist myxomaviral infection and lysis.

The 289-kDa serine/threonine kinase, the mammalian target of rapamycin (mTOR), regulates the Akt pathway. Rapamycin binds FKBP12 (FK506-binding 12--kDa protein, FK506 – tacrolimus). The rapamycin-FKBP12 complex associates with mTOR. Akt/PKB is activated by the mTOR-Rictor/Raptor complex. Rapamycin (sirolimus, Rapamune, Wyeth, Philadelphia, PA), the antibiotic produced by *Streptomyces hygroscopicus* (from Rapa Nui of the Easter Islands), inactivates the system. Low-permissive human type II tumor cells are rendered permissive to myxomaviral oncolysis by the mTOR inhibitor rapamycin [384, 439]. There is a suppressive effect by this antibiotic on the tumor-bearing host's antiviral immune reactions. However, another paradoxical effect is induced. If the phosphorylated and constitutively activated Akt promotes intracellular myxomavirus replication, the mTOR and Akt inhibitor rapamycin would be expected to protect the tumor cell from viral cytotoxicity and lysis, while it would reduce its malignancy by suppressing the cell survival Akt/PI3K pathway. Two factors intervene in favor of AKT activation in myxomavirus-infected tumor cells. The myxomaviral protein M-T5 activates the cellular ubiquitin ligase cullin-1, which secures the completion of the cell cycle and prevents premature cell death (thus allowing full maturation of the new viral progeny). In type II tumor cells with low levels of endogenous Akt activation (see above), the myxomaviral protein M-T5 endeavors to activate Akt/PKB. Rapamycin inhibits the mTOR pathway downstream of the Akt locus; paradoxically, it can induce upstream receptor kinase signaling and thus activate Akt. The sum of these interactions is that in the myxovirally infected type II tumor cells, Akt activation prevails and the virus completes its lytic infectious cycle [385].

Human malignant gliomas are subjected to viral therapy in xenografts and in patients involving naturally oncolytic viruses (NDV, reo-, vaccinia, and VS viruses) and genetically engineered viruses (adeno-, herpes-, and recombinant polio-rhinoviruses, reviewed in [373]). For orthotopically xenografted human malignant glioma cells, directly injected myxomaviral inocula were apparently "curative"; myxomavirus was apathogenic to

resting brain cells, but established persistent or lytic infection of the glioma cells. In contrast to vaccinia virus, rabbit myxomavirus is non-pathogenic in the human host, but displays extraordinary tropism to human malignant cells, including those of glioblastoma multiforme and medulloblastoma [244, 245]. The rabbit myxomavirus is highly oncolytic in human brain tumors (medulloblastoma, glioblastoma) and it synergizes with rapamycin [23]. The chemo- and radiotherapy-resistant pediatric rhabdoid tumors grow in xenografts; the xenografts of these tumors are susceptible to intartumoral injections of myxomavirus or attenuated deltaM51 VSV [454].

Parvovirus

A most peculiar behavior. "Peculiar" refers to the unusual affinity of these viruses to dividing cells, whereas resting cells are seldom, if ever, infected by parvoviruses. The subfamily *Parvoviridae* includes the genera *Parvovirus* (minute virus of mice, MVM H1), *Erythrovirus* (the pathogenic human B19 virus), and *Dependovirus* (human adeno-associated viruses, AAV). These small (20–25 nm) icosahedral viruses are not enveloped and possess a linear ssDNA genome of 5 kb in length operating the open reading frame that encodes the nonstructural proteins NS-1 and NS-2. The MVM NS-1 protein induces cell death through the alteration of the cytoskeleton and activation of endogenous casein kinase II (CKII). The NS-1 adaptor molecule directs CKII-α to link to tropomyosin, thus altering the kinase's phosphorylation pathways. These viruses enter host cells through heparan sulfate proteoglycan receptors, replicate only in those host cells that undergo the S phase of the cell cycle (divide), and some of them (adeno-associated virus, AAV) integrate their DNA genome in the genome of their host cells. The AAV2 genome consists of 4679 nucleotides and integrates into the long arm of human chromosome 19 (the well-known "latent infection"). In productive infection, the viral DNA replicates very rapidly by the "single-strand displacement mechanism" after the Rep78/68 proteins have recognized the terminal resolution site of the viral DNA strand; the protein links covalently to the viral DNA. Dependoviruses require helper adenoviruses for their replication. Parvoviral replication is up-regulated in *ras*-transformed cells.

The AAV group of parvoviruses did not evolve in the simian-hominoid autonomous primate parvoviral pathway; the AAVs (and parvovirus B19) are linked to the parvoviruses of birds [242] and show close nucleotide sequence analogy (identity) [468]. The two features of tumor cells that attract parvoviruses are mitosis and hypoxia. The environment ruled by hypoxia-inducible transcription factor-α and hypoxia-responsive element favors parvovirus replication [338]. The oncotropic oncoselective minute viruses of mice (MVMi and prototype MVMp) replicate in human tumor cells *in vitro* without displaying any pathogenicity in the human host. MVMs replicate with the help of the P4 promoter, cyclic AMP responsive element (CRE), parvovirus initiation factor, and CRE-binding protein (CREB); active CRE regulates parvoviral oncoselectivity [286, 294]. If MVMp replicates in CD11c myeloid DCs [310], it may inhibit the myeloid DCs (mDCs), which are well known to act as inhibitors of immune T cells. In tumor-bearing hosts, mDCs are recognized to be powerful antagonists of immune T cells; thus MVMp may act in favor of the tumor-bearing host.

Toll-like receptors (TLRs) recognize evolutionarily conserved molecules from pathogens (pathogen-associated molecular patterns). TLR9 recognize non-self DNA, such as CpG (cytosine-guanine islands) in ODN (oligonucleotides with nonmethylated deoxycytidyl--deoxyguanosine dinucleotide). TLRs remain the initiators of innate and adaptive immune reactions. CpG motifs incorporated into the ssDNA genome of parvovirus H-1PV trigger antitumor immune reactions in the parvovirus-infected tumor-bearing host. In draining lymph nodes of lung metastases of hepatocellular carcinoma (in a rat model), CD80/CD89 DC activation and IFN-γ-secreting immune T-cell generation occurred and contained the number and growth of the metastases [308, 309].

The chicken anemia circovirus (CAV, formerly considered to be a parvovirus) invades the gonads and infects the embryos [99]. MVM may be present in murine ascites [368]. In theory, these parvoviruses may have contributed to the extraordinary efficacy of myxo- and paramyxo- (and other) viruses in the eradication of murine ascitic carcinomas [368]. Oncolytic NDV stock viruses are grown in embryonated eggs and the Cassel 73-T oncolytic NDV strain was developed by passages through Ehrlich carcinoma cells. These unusually effective oncolytic viruses should be checked for the coexistence of parvoviruses in the stock virus preparations [368].

Preclinical and clinical trials. In vitro observations and preclinical trials involving tumor xenografts established the oncolytic potency of parvoviruses. The Sloan-Kettering Cancer Institute tested as early as in the mid-1960s the possible oncolytic efficacy of the H1 parvovirus in patients (cited as reference 517 in the review chapter [358]). In patients with metastatic osteosarcoma, the H1 parvovirus induced viremia and rapid virus-neutralizing antibody production without a tumor response. After parvoviral oncolysis was observed in murine tumors (some induced by oncogenic viruses such as SV40), further human clinical trials were initiated. In patients with various tumors metastasizing to the subcutaneous tissues, direct intratumoral injections of the H1 parvovirus induced tumor regressions in 4 out of 7 patients with adenocarcinomas. It was not addressed if distant, not directly inoculated tumors responded or not (reviewed in [358]). In animal models, H1-infected and lysed tumors or lysates of infected tumor cells induced, through macrophages and DCs, tumor cell-reactive immune T cells (reviewed in [123, 358, 396]). Mouse

melanoma cells with the MCP-3 (known also as chemokine ligand CCL7) gene inserted by parvovirus MVMp induced cytotoxic T-lymphocyte and NK-cell reactions resulting in the suppression of tumor growth [446]. The autonomous H1 parvovirus lyses human melanoma cells. Parvoviral melanoma cell lysates induced immature monocyte-derived DCs to mature and generated immune T cells; the immune T cells reacted with the melanoma cells and released cytokines to amplify the immune reaction [256].

In human hepatoma xenografts, the H1 parvovirus infected and killed the tumor cells via "necrosis" [344]. Human melanoma cells infected with AAV2 showed signs of differentiation; they achieved diploid chromosomal modes, failed to form colonies in soft agar, and displayed density-arrested growth [21]. Parvovirus H1 shows tropism to human glioma cells, whereas resting normal brain cells escape infection. Glioma/glioblastoma cells replicate H1 virus up to cytotoxic death of the cells and release of fully mature viral progeny [154]. In Bcl2--overexpressed and TRAIL-resistant human glioma cells, the H1 virus rendered lysosomal membranes permeable to cathepsins and reduced the level of cathepsin-inhibitory cystatins. Lysosomal cathepsins accumulating in the cytosol induced non-apoptotic tumor cell death [77]. In animal models, H1 virus-infected irradiated tumor cell vaccines delivered live virus which was able to infect viable tumor cells, while the irradiated tumor cells immunized the host [308]. In a comparison of nine viruses for oncotropism to and oncolysis for human glioblastoma cells *in vitro*, VSV, Sindbis virus, and MVMi and MVMp were the most active and are listed as candidates for clinical trials [451]. The tendency of parvoviral therapy is shifting from the induction of direct viral oncolysis to gene therapy with parvoviral vectors [103]. The wnt signaling pathway-responsive MVM and H1 virus hybrids do not grow well in HeLa cells with inactive *wnt* signaling ("wingless" in drosophila, *int* in mice, reviewed in [360]), but replicate excessively up to cytolysis in *wnt* signaling lung and colon cancer cells [60]. Tumor cells use CKII for protection against parvovirus (MVM)--induced cytoxicity; the parvoviral NS1 protein binds with CKII and tropomyosin and negates their cytoprotective effect [278]. Adeno-associated parvo- and circoviruses are the smallest of the naked ssDNA viruses, yet they are capable of exerting oncolyitc effects (see below).

The resident viral flora (endogenous retroviruses, potentially oncogenic dna viruses, passenger viruses) in human tumors and hosts

Retroviruses. There are numerous endogenous and exogenous passenger retroviruses in some of the human tumors that are targeted for viral therapy (teratocarcinomas, melanoma, certain adenocarcinomas of breast, ovary, and prostate, certain sarcomas) (reviewed in [360]). Endogenous retroviruses are activated by stress signals [56] and by the impediment of innate and adaptive immune faculties in immunosuppressed individuals [470]; however, the ancient microRNA/small inhibitory RNA defense reactions remain active [142]. Oncolytic viral therapy is being extended for the treatment of these tumors. There will be interactions between endogenous retroviral genomes and those of the exogenous oncolytic viruses. Viruses (a genetically engineered influenza virus expressing truncated NS1 protein) can activate immune T cells to attack and kill non-virally infected human prostate cancer cells [87], opening up new avenues to unknown mechanisms and virus-to-virus interactions within the context of the viral therapy of human tumors. An infectious exogenous retrovirus (xenotropic murine leukemia virus-related virus, XMRV) resides in stromal fibroblasts of a subset of human prostate adenocarcinomas; the DNA provirus is inserted next to cellular transactivation factors. The virus is suppressed by IFN-β and an RNase enzyme [80, 98]. When prostate cancer cells are transfected by various poxviruses inserting transgenes (see below), does the presence of the XMRV in the stroma or in the cancer cells influence in any way the outcome of virally--induced gene therapy? An activated human endogenous retroviral type E genome residing in chromosome 6q in a patient with metastatic kidney carcinoma encoded a protein to which allogeneic immune $CD8^+$ T cells reacted. The allogeneic lymphocytes derived from an allograft the non-myeloablated patient received in the form of adoptive immunotherapy [406]. Attention should be paid to the viral flora (consisting of endogenous retroviruseses and passenger exogenous viruses) which the tumors (melanoma, breast, ovarian, and prostate adenocarcinomas and others) or the tumor--bearing host may harbor.

Potentially oncogenic DNA viruses. Are viral oncogene-driven human tumors more or less susceptible to viral oncolysis? Is there a distinction between virally induced (HPV, SV40, polyomavirus) and virally promoted (EBV, KSHV/HHV-8, SV40, polyomavirus) and endogenous retrovirus-expressor human tumors when it comes to the induction of host immune reactions, and to susceptibility to oncolysis by an exogenously introduced other (oncolytic) virus? Is HPV-induced squamous cell carcinoma of the uterine cervix unusually susceptible to oncolysis by rhabdoviruses (attenuated rabies virus, vesicular stomatitis virus)? It has not been investigated whether the endogenous viral flora influences the outcome of viral oncolysis. Some pediatric brain tumors express the SV40 genome [15, 340, 428] and Merkel cell carcinomas express a polyomaviral genome [101]. Would the presence of these oncoviral genomes render these tumors more or less susceptible to viral oncolysis?

The Torque teno circovirus. The Torque teno transfusion-transmitted circoviruses are widely spread worldwide [76, 140, 404, 423]. These agents emerge as the inducers of anti-DNA auto-antibodies and as the causative agents of "idiopathic" inflammatory myositis [35, 121, 122]. The TTVs negatively interact with the NF-κB pathway [473]. The TTV ORF-2 and HTLV-

-encoded HRES-1/p28 are in "antigenic mimicry" [20, 122].

The tumor's response to oncolytic viral therapy may depend on the interaction between the resident viral flora of the host and the oncolytic virus and on the host's immune response to the complex situation. Here the host receives contradictory signals of tolerance induction versus "danger signals" for the induction of a rejection reaction. Will the host choose to tolerate the tumor or the oncolytic virus, or will it reject the virally infected tumor?

Bacteriophages inhibit tumor metastases

Expertise in phage therapy of bacterial infections gives birth to a new concept. Each gp24 head corner protein of some bacteriophages (coliphage T4) incorporates the Lys-Gly-Asp amino-acid sequences of one KGD motif. This is the very same sequence the integrins of certain eukaryotic cells capture in a ligand-to-receptor reaction. The αIIb-β3-intergrins are considered to be the only integrins known to bind KGD sequences. Endothelial cells, monocytes, and many tumor cells operate β-integrins for interactions with the stroma in their microenvironment. In cell communities it is the KGD^+ natural ligand CD40 (CD154) with which αIIb-β3-integrins reacts. The CD40 ligand interacts with B and T lymphocytes in processes of graft acceptance versus rejection, in atheromatous plaques, and in autoimmune diseases. The CD40 ligand-to-receptor interactions are important in B- and T-cell activation; interruption of these interactions results in strong immunosuppression. One activity of KGD^+ phages is competition with the natural CD40 ligand in various biological processes of multicellular organisms, including those of mammalian species. According to Górski and Weber-Dabrowska, the omnipresent phages in the mammalian hosts (including humans) practice immunosurveillance. Phages exercise control over the endogenous bacterial flora and the hosts' reactions to it and to exogenous foreign (infectious) or endogenously arising invaders (autoimmune lymphoid cells, malignantly transformed stem cell clones) [131].

Based on this basic information in phage biology, A. Górski postulated that KGD^+ phages should interact with the β-integrins that tumor cells express and that such interaction may result in the suppression of the tumor cells' locomotion and other biological activities [130]. The phage-tumor cell relationship may be more intimate than just an attachment of the phage particle to the cell surface. The cell's integrins may serve as portals of entry for the virus (as they do in the case of some hantaviruses). Furthermore, tumor cells suppressed in their biological activities and/or excluded from their micro-environment (anoikis: homelessness, detachment-induced cell death) may eventually undergo programmed cell death (apoptosis) [130, 131]. The experimental testing of this concept showed that phages interact with mammalian cells as powerful biological response modifiers [66, 67]. Purified phages are available in high concentration; in tumor-bearing hosts, such phage preparations not only inhibited the locomotion of tumor cells, thus acting antagonistically to metastasis formation. Phage therapy actually resulted in the deceleration of growth of established tumors, in decrease of tumor sizes, and in the induction of leukocyte-mediated anti-tumor immune reactions of the host (see below).

Not oncolysis, but growth inhibition with anti-tumor immunogenicity. The coliphage T4 and its "higher affinity" mutant HAP1 virus induce growth inhibition of large subcutaneous melanoma tumors in intraperitoneally injected mice. These experiments showed that phage lysates of *E. coli* actually stimulated tumor growth. It was the purified phage preparation that exerted the inhibitory effect on the tumors [66, 67]. The mechanism of the interaction was documented to be due to the binding of the KGD ligand of the phage particle to the β3-integrins of the tumor cells. The tumor-binding ability of the phage preparations, as observed *in vitro*, was abrogated by synthetic peptides mimicking the ligand and competing with the phage for the receptor or by anti-β3-integrin antibodies [66, 67].

Murine DCs phagocytose T4 phage particles. Such mature DCs were loaded with colon carcinoma MC38/D antigens in the form of tumor cell lysates. Vaccination of CD57BL mice with such DC preparations conferred immunity against MC38/D tumors [287]. It remains to be shown 1) if the immune reactions so induced were tumor-specific and 2) by what mechanisms (CD8 immune T cells, NK cell acting in an ADCC reaction, or both). Furthermore, 3) if the uptake of phage particles by DCs contributed to the presentation of tumor antigens in an immunogenic, instead of tolerogenic, manner to CD4 T cells.

Phages selected by high-affinity binding to B16-F10 mouse melanoma tumors or cell lines ("panning") from the filamentous bacteriophage display peptide library Ph.D-12™ (sounds as if some phage particles earned a doctoral degree!) could induce complete regression of established B16 melanoma tumors in mice. These phages express a 12-amino-acid peptide on their protein III which recognizes a tumor surface protein. These phages induced tumor regression that was comparable to the response induced by HLA-A2 antibody-expressing phages. The antibodies encoded by these phages were the HLA-A2 Fab/scFV fragments directed against B16-HLA-A2-positive melanoma cells. These B16 melanoma cells were stably transfected with, and thus expressed, the targeted human HLA-A2 gene. The phage-displayed HLA-A2 immunoglobulin fragments lack the Fc region and therefore cannot induce an ADCC reaction by NK cells or macrophages. Tumors infected with these phage particles induced a heavy infiltrate by polymorphonuclear neutrophilic leukocytes. Under these circumstances, some tumors completely regressed [91]. It appears that the host mobilized an ancient innate immune reaction to the antibody-frag-

ment-carrier phage-infected tumor cells [91]. The reviewing authors point out that the phages did not lyse the tumor cells. It had to be the antibody fragments FAb/svFV within the phage particles that must have "opsonized" the tumor cells for phagocytic leukocytes. Is it possible that the TLRs recognizing phage proteins and/or nucleic acids activate innate immune responses resulting in the secretion of inflammatory cytokines/ /chemokines? In the mouse, the chemokines CXCL1 and CXCL2 recruit neutrophilic granulocytes to bacterial targets [69]. If the target is a tumor cell, the leukocytes will attack and kill that target [91].

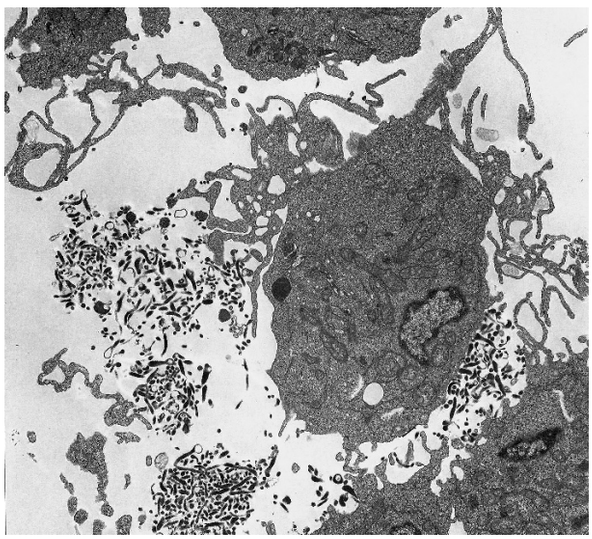

Fig. 5. Mycoplasma-infected cell line. A salivary species of mycoplasma infected the human angiosarcoma cells from cell line #2177, which was set in culture on March 23, 1971, from patient Dr. J. C. (MDAH#46448) and discontinued on September 9, 1974. Transmission Philips electron microscopy. Original magnification ×8500.

Tumor immunologists recognize the ability of tumor cells to express the Fas ligand for the elimination of Fas receptor-positive immune T cells of the host. However, $FasL^+$-expressor tumor cells attract, and are attacked by, polymorphonuclear leukocytes [370]. Mobilization of oncolytic polymorphonuclear leukocytes also occurred when mycoplasma-infected tumor cells were exposed to buffy coat preparations of patients or healthy donors [365] (Figs. 5 and 6AB). An uncommon but powerful leukocyte-mediated innate anti-tumor reaction may be induced by FasL-expressor or mycoplasma-infected or phage-carrier tumor cells, especially when the phages express a globulin fragment.

What happens in the colon when colon cancer arises? Stem cells at the bottom of the crypts of Lieberkühn regenerate the entire colonic mucosa at approximately five-day intervals. Singly mutated stem cells generate polyps and multiply mutated stem cells generate adenocarcinomas. Malignantly transformed stem cells masquerade as self, and even if the host mobilizes immune reactions to the protein products of the oncogenes or to the improperly expressed carcinoembryonic substances, the malignant cells have means to evade the attack. Therapeutic attacks on the replicating derivatives of the malignantly transformed stem cell (the cancer cell) are futile unless the entire underlying resting malignant stem cell population is eradicated. Potentially curative therapy must be directed against the malignantly transformed stem-cell population. The environment certainly is not sterile. There, phage lysates of bacteria may promote tumor growth [66, 67], probably by providing paracrine growth factors. However, oral administration of phage lysates of bacteria was not observed to pro-

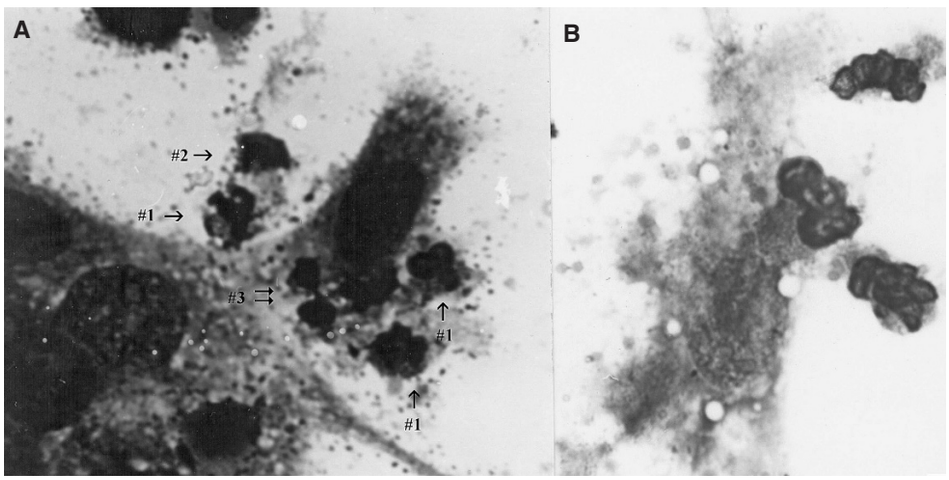

Fig. 6. Patient's buffy coat cells reacting (**A**). Polymorphonuclear leukocytes of the buffy coat of the patient react to the mycoplasma-infected autologous tumor cells: three large cells resembling leukocytes (arrows #1), two small compact lymphocytes resembling immune T cells (arrows #3), and one large lymphocyte resembling an NK cell (arrow #2) surround a tumor cell. Technician's buffy coat cells reacting (**B**). Exclusively polymorphonuclear leukocytes react to mycoplasma-infected tumor cells from the buffy coat of the technician who handled the culture. Figures A and B: Wright-stained slides viewed in Zeiss microscope with objective ×54 and ocular ×10.

mote tumor growth (such an event has never been reported). What remains to be documented is that a large population of phage particles attaching to or actually entering the tumor cells may inhibit tumor growth. If phages could attach to or enter the resting malignant stem cells and thus "xenogenize" them, a most powerful innate and adaptive host immune reaction could be mobilized and incipient colon cancers could be eliminated [66, 67, 91, 130, 287]. Indeed, the T4 phage substrain HAP1 phage (see above) binds tumor cells preferentially over healthy cells. Intraperitoneal administration of the purified HAP1 phage proved to be inhibitory to murine lung cancers and B16 melanoma lung metastases [66, 67]. Due to its natural history, its intimate association since its inception with phages and phage lysates of bacteria, the established colon cancer is the tumor most educated in the means of evading "phage immunosurveillance" [131].

In the case of colon cancers bathing in the phage lysates of Gram-negative bacterial flora, how many polyps or malignantly transformed stem cells in the crypts of Lieberkühn are eliminated by colonic phages directly or indirectly at their inception, before one tumor cell colony escapes and emerges as a clinically manifest neoplasm? Genetically engineered phage populations given in enemas to patients with polyposis of the colon could answer the first question. What type of immune reactions, if any, are induced by incipient colon cancers and how would attachment of phages to the transformed tissues change these host reactions? Leukocyte- and NK cell-mediated innate, antibody- and T lymphocyte-mediated adaptive, or sequentially both types of immune reaction are expected to be induced. The second question would be whether intracellular phages could promote cytoplasmic β-catenin degradation and thus switch off an oncogenic pathway. Could, under natural circumstances, the abundant phage flora of the colonic contents inhibit or correct the first malignant transformation in polyps or induce the complete regression of an incipient adenocarcinoma by eliminating its malignantly transformed stem cells? The colon is the battlefield where incipient tumor cells and oncosuppressor phages vie. These matters of interest will be elaborated further in the Discussion.

GENETICALLY ENGINEERED ONCOLYTIC VIRUSES

Adenoviruses

The host cell surrenders. Masterpieces of nature, the adenoviral capsids drew admiration [211] and commanded the most precise structural measurements [266, 267]. The fiber protein of the adenovirus particle binds the fiber receptor of the cell surface, the coxsackie-adenovirus receptor (CAR), and the viral penton base attaches to the integrin receptor of the cell membrane. In the acidified endosome, the internalized virus lyses the endosomal membrane and escapes to the cytosol. Attached to microtubules through dynein, the microtubule-associated molecular motor, the nucleocapsid travels through the cytoplasm until it reaches the nucleus. There it docks on the nuclear pore complex filament protein. Histones and importins disassemble the capsid and release its dsDNA into the nucleus. The cellular enzyme, RNA polymerase II, transcribes the viral early gene E1A. Back in the cytoplasm, mRNA of E1A is translated into the E1A protein, which binds the cellular Rb tumor suppressor protein and disables it (inhibits Rb-E2f complex formation). The cell enters the S phase of the cell cycle uninhibited. The adenoviral E1B protein interacts with another tumor suppressor, the cellular p53 protein. The tetramer p53 protein binds to DNA and activates gene transcriptions leading to the arrest of the cell cycle at G_1/S, because stimulation of the transcription of $p21^{Cip1}$, the G_1 cyclin-dependent kinase (CDK) inhibitor, a cell cycle inhibitor, occurs. Another cause of apoptotic death is the transcription stimulation of the pro-apoptotic proteins Bax and Fas of mutated and transformed cells. The *a*taxia *t*elangiectasia *m*utated (Atm) gene-product protein (when encoded by the healthy, non-mutated gene) stabilizes p53; this effect is lost when the *atm* gene is mutated. Attachment of the cellular MDM (mouse double minute) protein to p53 condemns p53 to ubiquitination (degradation in the proteasome), an event frequently activated in malignantly transformed cells. The adenoviral E1B protein binds and incapacitates p53; E1B-bound p53 is converted from a gene-activator transcription factor to a gene repressor. In healthy cells infected by adenoviruses, the "axis of evil" of seven viral genes (other than E1A and E1B), among them ADP (adenovirus death protein) and RID (receptor internalization and degradation), protects the infected cell from apoptotic death by TNF, immune T cells, or Fas ligand (FasL). FasL, captured by its receptor Fas of the death domain, induces extrinsic apoptotic death of the cell. FasL cannot act when the Fas receptor expression is down-regulated. If p53 acts first, these cells die apoptotic deaths before the full maturation of the new viral progeny. However, when adenovirus particles enter a tumor cell, they do not encounter functional Rb or p53 proteins. The basic mechanisms of malignant transformation have already eliminated these proteins. In tumor cells, adenoviral replication continues uninhibited and large numbers of viral particles burst out of the cell, causing its lysis [187] (see above).

These authors submitted hypotheses and drawings for the genetic engineering and recombination of adeno-, parvo-, and retroviruses for viral oncolysis in the early 1990s [368].

The birth of ONYX and its siblings. Understanding the difference between the interactions of virus and host cell in healthy cells and in malignantly transformed cells led to the idea that E1A/E1B gene-deleted adenovirus particles could not incapacitate Rb and p53 in healthy

cells, which would prohibit the generation of new mature viral progenies, whereas tumor cells without functional Rb and p53 proteins would succumb to cytolysis upon the egress of fully mature new viral progenies [6, 151, 202]. The efficacy of ONYX could be enhanced. For anaplastic carcinoma cells of the thyroid (ACT), lovastatin (the coenzyme A reductase inhibitor) in itself is an apoptosis-inducer. The oncolytic activity of the dl1520 ONYX-015 (see below) against ACT xenografts is greatly intensified in combination with lovastatin [231]. Etanercept, the recombinant dimer of the extracellular domain of the TNF-α receptor (linked to the Fc portion of human IgG1), performs extracellular binding and neutralization of the ligand, TNF-α. Co-administration of subcutaneous etanercept and intravenous ONYX-015 to patients with metastatic solid tumors resulted in significantly higher titers of the oncolytic virus [269]. The etanercept gene could be transduced into the cells of the synovial membrane in rheumatoid arthritis by an adeno-associated viral vector created by Targeted Genetics (Seattle, WA) for the treatment of that condition. The neutralization of TNF-α sometimes promotes the flare-up of very severe (fatal mycobacterial) infections.

Furthermore, ONYX-015dl1520 (E1B-deleted), dl922/947 (E1A-deleted), and E1A/E1B double-deleted oncolytic adenovirus strains were constructed for use in clinical trials. It is worth mentioning that the E6 protein of the human papilloma virus types 16 and 18 also binds p53, sending it to ubiquitination. Next, by binding IFN regulatory factor-3, E6 suppresses IFN production of the cell. The papillomavirus E7 protein binds both the cellular Rb and the cell cycle-inhibitory p21 proteins. Hypophosphorylated Rb, by binding to E2F1,2,3, inhibits the cell cycle; the Cip, Kip, Ink proteins inhibit CDK and thus the cell cycle. The papillomaviral E7 protein binds the CDK inhibitory protein $p21^{Cip1}$ and liberates the progression of the cell cycle from G_1 through S. Thus the papillomavirally induced squamous cell carcinomas of the uterine cervix could not defend themselves from a replication-competent oncolytic virus, whereas healthy cells of the host could eliminate the virus. Human uterine cervical carcinoma xenografts are readily lysed by the E1B 19-kDA and E1B 55-kDa gene-deleted adenovirus Ad-DeltaE1B19/55 and its derivatives [202]. Here, an oncolytic virus (which, on occasion, could be oncogenic in hamsters) eliminates tumor cells induced by a highly oncogenic virus. With its modified RGD fiber protein, the E1A E1B double-deleted AxdAdB3 is also re-targeted from the CAR entry site to adenocarcinoma cells of the biliary tract, which overexpress integrins-α/β as viral entry receptors [433].

In China, ONYX-like genetically engineered adenoviruses performed well in clinical trials of human cancers, especially in head and neck squamous cell carcinomas (especially in combination with chemotherapy). Virotherapy of human cancers with these agents has now been licensed in China [464]. Non-ONYX-like adenoviral constructs are also in use. The telomerase reverse transcriptase promoter (overexpressed in tumor cells) drives the growth of conditionally replicating adenovirus KH901, which inserts the GM-CSF (sargramostim) gene into the tumor cells and kills human cancer xenografts better than 5-FU and cisplatin while mobilizing anti-tumor DCs [343]. The p53-expressing conditionally replicative CNHK500-p53 adenovirus kills hepatocarcinoma cells [464] and is being enlisted into clinical trials.

Genetically engineered new oncolytic adenoviruses. The large number of genetically engineered adenoviruses for human cancer therapy has been repeatedly reviewed [6, 151, 358]. The herpesviral TK gene therapy principle and transgenic adenoviral insertions of GM--CSF, monocyte chemotactic protein-1 genes in colonic, or hepatocellular adenocarcinomas and other human tumor cells have been applied in preclinical trials since 2000. Monocyte chemoattractant protein-1 potentiated the oncolytic efficacy of the HSV TK gene expressing adenoviral construct to hepatocellular carcinoma cells by recruiting NK cells [413, 414] (see above). It has also been documented that athymic mice carrying human tumor xenografts, after adenoviral lysis of these human tumor implants, develop NK cell-mediated rejection--strength immunity against re-implantation of the same, but not virally infected, xenografts. New ideas are implemented in the genetic designs of newer oncolytic adenoviruses (Table 1) [114, 133, 300, 303, 306, 307, 330, 383, 456]. There are replication-competent adenoviral agents and replication-incompetent vectors for gene therapy in advanced pre-clinical investigational stages. Conditionally replicative adenoviruses (CRAds) are tissue (tumor) specific; for example, tyrosinase promoters drive adenoviruses in melanoma cells. The adenoviral E1A promoter can be replaced by the human tyrosinase enhancer/promoter construct (hTyr2E/P); mutation of the viral E1A gene (delta 24) attenuates the virus in healthy cells, but the expression of hTyr2E/P renders the virus highly cytotoxic to melanoma cells [270]. Of the many new CRAds, the CNHK500-p53 viral construct replicates in hypoxic and telomerase-overexpressing hepatocellular carcinoma cells and inserts the wild--type p53 gene into the p53-deprived tumor cells [472]. The CRAd-S.RGD and CRAd-SF5/3 viruses enter and replicate selectively in survivin-overexpressing mesothelioma cells and glioma cells [475]. In a telomerase-specific conditionally replicating adenovirus, hTR/hTERT promoters control the expression of the viral E1A gene [34]. The virus reached titers lethal to tumor cells, whereas normal cells resisted much lower viral titers [114].

De-targeting and re-targeting a given adenovirus by deleting its natural cell binding structures aimed at CAR and replacing it with the insertion of peptide ligands specific to chosen receptors preferably expressed by tumor cells has been accomplished [157, 433]. The new ligands are expressed in the loops of the adenoviral short fiber knob. Further modifications of the retargeted adenovirus particles are the internal ligand incorpo-

Table 1. New ideas implemented in oncolytic adenoviral designs

Idea	Design	Results	References
Deliver the prodrug ZD2767P activator enzyme CP gene into tumors	Construct gene carrier AdV.hTERT-CPG2 vector	Colon carcinoma SW620 xenografts were eradicated by the prodrug	330, 383
Combine TMZ with oncolytic adenovirus for melanoma therapy	Tyrosinase promoter to drive Ad replication in melanoma cells	TMZ increased Ad5/3 2xTyr replication in melanoma cells	303
Neutralize MDM-2 to preserve p53	Combine MDM-antagonist Nutlin with Adp53	Apoptotic death rate of tumor cells increased; large viral progenies burst tumor cells	133
Deliver HSV-TK into tumor cells; treat host with GCV	Ad5/3Delta24-TK-GFP (re-targeted)	Adenovirus alone cytotoxic to ovarian carcinoma cells; GCV ineffective	306, 307
hTRT proximal promoter to drive Ad5 (Telomelysin)	Promoter drives E1A/E1B linked with IRES	High rate of Ad5 replication in tumor cells; normal cells exempt	114
Ad E1A neutralizes Rb; normal Rb protects against Ad replication	Mutated E1A unable to block Rb. AxdAdB-3 grows in tumor cells, not in normal cells	In esophageal cancer cells AxdAdB-3 neutralized NF-κB promoting apoptosis	456
HIF-1 promotes hypoxic metabolism in tumor cells; induces neoangiogenesis	HIF-1 promoter drives E1A of HYPR-Ad-IL-4; hypoxia--dependent IL-4 expression induces leukocyte infiltration	Human tumor xenografts supported high viral titers; underwent necrosis and fibrosis. No effect in normoxic healthy cells	300

CPG – carboxypeptidase gene, ZD2767P – phenol aniline mustards, hTERT – human telomerase reverse transcriptase, MDM – mouse double minute, HSV – herpes simplex virus, TK – tyrosine kinase, TMZ – temozolomide, GCV – ganciclovir, IRES – internal ribosome entry site, HIF – hypoxia inducible factor, HYPR-Ad-IL4 – attacks and lyses hypoxic tumor cells; IL-4 exerts anti-neoangiogenic effect by down-regulating vascular endothel growth factor receptor (VEGF-R) expression in infected tumor cells; in immunocompromized hosts, eosinophil leukocytes and macrophages invade infected tumors (an innate immune reaction); in immunocompetent hosts, immune T cells react to infected tumors.

ration into the extended short-shafted fiber and the insertion of tandem copies of the peptide ligands. Fiber--modified p53 gene-bearing replication-selective adenoviruses (AdFLAGp53, Ad.hTC.GFP/E1a.RGD) entered xenografted human leiomyosarcoma cells in which the viruses replicated, inserted the wild p53 gene, and induced the upregulation of the cell cycle inhibitor $p21^{Cip1/Waf1}$ pathway [146]. Bypassing the CAR entry--site, mosaic fiber adenovirus serotype 5, containing reovirus sigma-1 and adenovirus serotype 3 knob, readily infected ovarian carcinoma cells [416]. The adenoviral protein E1A is cytotoxic to $HER2^+$ ovarian carcinoma cells, especially to the clear cell type, which seldom mutates the p53 gene. Even trastuzumab non-responsive and platinol-resistant ovarian cancer cells responded to E1A intraperitoneal therapy (see below) [183]. Ad5IL-12-infected prostate cancer LNCaP cells cannot conceal MHC class I expression from immune T cells and NK cells and remain vulnerable to cell-mediated immunity of the host [116]. The survivin-driven fiber--modified Ad5CRAD-S-pk7 adenoviral construct is highly cytolytic to human glioblastoma xenografts [418–420] (see below).

Ad-Delta24 carries a 24-bp deletion in the E1A region preventing its binding the Rb protein [112, 187]. The adenovirus Ad5/3-Delta24 and its derivatives are E1A-deleted/mutated and RGD-4C peptide motif--inserted adenoviruses anchoring on cell surface integrins overexpressed on brain tumor (glioblastoma, medulloblastoma) and other tumor cells which down--regulate CAR expression [394]. Normal cells with intact Rb pathway resist Ad-Delta24 infection. Ad-Delta24 viruses are driven to replicate by the human E2F-1 promoter in Rb pathway-defective tumor cells; the E2F-1 promoter is selectively activated/de-repressed in tumor cells, but is quiescent in resting healthy cells. In human brain tumor cells (glioblastoma, medulloblastoma) the E2F-1 promoter and in human melanoma cells the tyrosinase enhancer/promoter (hTyrE/P) drive the replication of the Ad-Delta24 viruses [415]. Ad5CMV--p53 and Ad5Delta24 doubly infected glioma cells were killed more efficiently than with either one of the viruses applied singly [255]. In killing glioblastoma cells, the topoisomerase antagonist irinotecan (CPT-11), or temozolomide, and an Ad-Delta24 derivative acted additively/synergistically [127]. The Ad5/3Delta24hCG adenovirus is re-targeted to the adenovirus serotype 3 receptor, which is overexpressed in human tumor cells, including those of prostate cancer. Hormone therapy--refractory human prostate cancer cells are readily infected *in vitro* and in xenografts and are killed by this virus [305]. Prostate cancer cells overexpressing Bcl-2

and $BCL\text{-}_{xL}$ resisted adenoviral constructs carrying the melanoma differentiation associated gene-7 and the IL--24 gene (Ad*mda*-7/IL-24). This adenoviral construct ("the cancer terminator virus") (see below), when driven by progression elevated gene-3 (Ad.PEG-E1A--*mda*-7/IL-24), surpassed tumor-cell resistance to the extent that it destroyed not only the directly injected tumor xenograft, but also its distant metastases [323]. Hypoxic tumors (glioblastoma) are radioresistant. The heterodimer Ku proteins (Ku70/Ku80) protect against DNA strand breaks and facilitate DNA repair; the absence of Ku proteins renders tumor cells radiosensitive. The DNA-dependent protein kinase DNA-PK consists of large catalytic subunits and the DNA-targeting components Ku70/Ku80. Replication-defective adenoviral expression of the dominant negative construct of Ku70, DNKu70, controlled by a CMV promoter, rendered glioblastoma and colon carcinoma cells highly radiosensitive [152]. Human brain tumor stem cells grown in xenografts died autophagic deaths upon exposure to Ad-Delta-RGD targeted to the p16INK4/Rb pathway [184, 190, 191]. While many genetically engineered adenoviruses transfect tumor cells with the wild p53 gene [6, 10, 151, 472], the ICOVIR-5 constructs aim at the restoration of the Rb pathway. In the ICOVIR-5 construct, the viral gene for Rb-binding viral protein E1A is deleted, the E1A promoter is substituted for E2F-responsive elements, and an RGD-4C peptide motif is inserted in the fiber structures. In the tumor cell, the ectopic adenoviral E2F1-responsive elements interact with the endogenous E2F1 protein (an oncogene), the E2F transcriptional activity is enhanced, and the Rb/E2F1 repressor complexes are restored. ICOVIR-5 exerts additive/synergistic effect in combination with the mTOR inhibitor, rapamycin-derivative, RAD001, or temozolomide [8, 9, 45]. To the conquest of glioblastoma, virotherapy will mightily contribute, perhaps with the help of adoptive therapy with immune T and lymphokine-activated killer (LAK) cells in an environment with down-regulated T_{REG} cell compartment in the background [373].

Adenoviruses possess virus-associated I RNAs to facilitate the translation of virally generated mRNAs; deletion of the adenoviral virus-associated gene I (VAI) renders the adenoviral genome non-replicative. In EBV-associated tumors, EBV-encoded small RNA molecules abound. VAI-depleted adenoviruses selectively replicate in EBV-infected tumor (Burkitt's lymphoma, nasopharyngeal lymphoepithelioma) cells and thus exert oncolysis [440].

Harvest of 2007–2008. Adenovirus AxdAdB-3 has a nonfunctional E1A and a deleted E1B gene. Hormone--resistant prostate cancer cells defective in the p53 and Rb/E2F/p16 pathways are highly susceptible to oncolytic infection with this virus [327]. Adenoviruses AdTIMP-1/2 express tissue inhibitors of metalloproteinases. These viruses suppress the recruitment of osteoclasts by prostate cancer cells and thus inhibit the induction of osteolytic metastases [72]. Of conditionally replicating adenoviruses, CRAd-CXCR4.F5/3 is driven by the chemokine CXCR4 promoter that is overexpressed in squamous carcinoma cells of the head and neck. The modified fiber F5/3 of the virus endows it with exclusive affinity to these tumor cells [476]. The TRAIL is expressed by adenovirus Ad5/Ad35.IR-E1A/TRAIL; this virus is oncolytic in glioblastoma xenografts [450]. The Ad5-Delta-24-RGD (arginine-glycine-aspartate) adenovirus targeted to the mutated p16INK4/Rb pathway of tumor cells attacks human glioblastoma stem cells and induces in them lethal autophagy; thus it has eradicated human glioblastoma xenografts [190, 193]. Melanoma-associated tyrosinase antigens are overexpressed in mouse glioblastoma cells. The conditionally replicative adenovirus (CRAd) construct Ad24TYR infected these glioblastoma cells and significantly prolonged the life of treated mice [418–420]. The conditionally replicative adenoviral vector CRAd-survivin5/3 with its E1a under the control of the survivin promoter was highly cytopathic to gliobastoma cells [418–420]. The Division of Neurosurgery at the University of Chicago, Chicago, IL, published reviews on adenoviral therapy of glioblastoma in the spring 2008 issues of Expert Opinion on Drug Delivery and in Stem Cells. The replication-defective adenovirus expressing the cDNA of TNF and containing the radioinducible promoter element Egr (Ad.Egr-TNF) given in combination with ionizing radiation and temozolomide completely eradicated glioblastoma xenografts [457]. Due to its modified capsid fiber and penton base, adenovirus Ad5/3 enters ovarian carcinoma cells and kills human ovarian carcinoma xenografts in combination with chemotherapy [285]. Viral oncolysis in ovarian carcinoma cells is intensified when the virus expresses TNF under the effect of a MUC-1 promoter or when it is combined with chemotherapy (gemcitabine; epirubicin) [264, 285, 307]. Adenoviral E1A stabilizes wild-type p53 and induces Bax and caspase-9 upregulation in cisplatin--resistant clear cell carcinomas of the ovary; xenografts of this tumor stagnate and allow prolonged survival of tumor-bearing hosts [183]. Adenovirus Ad5.R1-scTCR is endowed with recombinant fiber consisting of a trimer of tumor antigen-specific T-cell receptors that replaced proteins in their native fiber knob responsible for binding the CAR. These viruses lost their ability to infect normal cells through binding to CAR, but rather enter tumor cells which express the target antigen MAGE-A1 presented by HLA-A1. Thus these viruses kill exclusively melanoma cells (or other tumor cells which express MAGE-A1) [337]. The adenoviral construct Ad5/ /3.2xTyr replicates preferably in melanoma cells under the effect of the cells' tyrosinase promoters; temozolomide further promoted cytolytic replication of this adenovirus in melanoma cells [303]. Melanoma cell-directed replication-defective adenoviruses are not cytotoxic to DCs, allow DC maturation and antigenic expressions and thus, in addition to oncolysis, induce anti-tumor host immune reactions [331]. The ΔNP73α-expressor adenovirus induces DCs to present the N-terminal trun-

cated p73α oncoprotein to T cells and thus generate a T cell-mediated anti-tumor immune reaction [172]. The well-established technique of adenovirus-mediated delivery of HSV thymidine kinase (see below) was applied to the treatment of human hepatocellular carcinoma in China. Patients with large tumors confined to the liver received liver transplants (LT) followed by Adv-TK virotherapy. The recurrence-free survival and the overall survival in the LT/Adv-TK-treated patients at three years were 43% and 69%. In contrast, for patients receiving LT only these values were 9% and 20%, respectively. Those patients who showed nonvascular invasion of their hepatocellular carcinoma experienced 100% overall and 83% recurrence-free survival after LT-Adv-TK therapy [225].

Virally induced autophagy in infected host cells seldom serves the survival of the cell; it reduces herpesviral replication in autophagic cells. In contrast, poliovirus replicates best in autophagosomes. The oncolytic adenovirus Delta-24-RGD genetically interacts with the infected tumor cell. It forces the cell to up-regulate its autophagic complexes, Atg12-5. The egress of infectious virions from the cell through ruptured membranes is greatly facilitated. The cell passively surrenders to lysis; when autophagy is induced by the oncolytic adenovirus, the cell actively contributes to the release of larger yields of infectious virions [193].

Two fiber-mutant adenoviral vectors were constructed. One (adRGD-FKN) expressed fractalkine (CX3CL1), the membrane-attached chemokine, the other (AdRGD-IL-12) expressed the cytokine IL-12. Ovarian carcinoma cells infected with these adenoviral vectors attracted perforin-postive $CD3^+$ immune T cells into the tumor [119]. An improved technology yields "high-capacity" adenoviral vectors for the delivery of herpesviral type-1 TK gene in glioma cells. Transfection of glioma cells with TK gene occurred even in hosts immune to the adenovirus. Anti-herpesviral drugs (ganciclovir) induced tumor regressions [206]. The fiber knob is the target of adenovirus-neutralizing antibodies. Changing the fiber knob allows the escape of the capsid--modified virus from neutralization; thus capsid-modified adenoviruses are able to deliver transgenes [328]. The "highly expressed in cancer gene 1" is targeted by RNA interference. Adenoviral constructs (Ad-siRNA--Hec1 and Ad-siRNA-Hec1.F5/3) carry out these reactions. Quantitative PCR assays determined that in human ovarian carcinoma cells, these adenoviral vectors neutralized (knocked down) the Hec (human endometrial carcinoma) gene mRNA and thus induced apoptotic deaths of the tumor cells [224, 277]. Introgen at M. D. Anderson Cancer Center, Houston, TX, continues the re-insertion by adenoviral vectors (INGN 201) of the healthy p53 tumor suppressor gene into patients with Li--Fraumeni syndrome or with p53 deficiency due to somatic mutations of the gene or ubiquitination of its gene-product protein [10]. Adenoviral vectors continue to deliver IFN and IL genes to patients with cytokine deficiencies. Adenoviral IFN-β delivery into the ascites of patients with ovarian carcinoma is in phase I of clinical trial [390].

The "cancer terminator virus" driven by the progression-elevated gene-3 and expressing mda-7/IL-24 (Ad.PEG-E1A-mda-7) (see above) killed human melanoma cells by apoptosis induction. In *in vivo* xenografts, not only the injected tumors, but also their distant metastases became infected and were dissolved [323, 324]. The MDA7/IL-24 proteins upregulate the dsRNA-activated protein kinase (PKR). This is the kinase that mediates the antiviral effects of IFN (see above). Its functions include that of tumor suppression by apoptosis induction. When guanine triphosphate Ras oncoprotein dephosphorylates PKR, it deprives the cell of a tumor suppressor, but it also creates an IFN-free environment that oncolytic viruses (HSV R3616, reovirus, NDV, VSV) take advantage of [431]. Reactivation of PKR by MDA7/IL-24 may restore its tumor suppressor efficacy, but also enables the tumor cell to get rid of the oncolytic virus by restoring its IFN activity. An Ad-mda7 adenoviral vector is being tested for the treatment of ovarian carcinoma at M. D. Anderson Cancer Center, Houston, TX [129]. In this system, the FasL-to-Fas pathway activated by IL-24 results in the apoptotic death of the cancer cells. Adenoviral vectors (adCN205-IL-24) armed with the melanoma differentiation-associated gene-7 (mda-7)/ /IL-24 will be available in China for the treatment of hepatocellular carcinoma [246].

Adeno-associated- (AA-), circo-, and parvoviruses

Apoptin. The CAV belongs to the genus *Gyrovirus* in the family *Circoviridae*. The virus particle is nonenveloped and icosahedraI; its genome is a circular ssDNA. Even though it is a circovirus, it acts as a parvovirus. It induces apoptotic death of thymocytes and precursor T cells and hemocytoblasts and erythroblasts, i.e. it attacks immature replicating and differentiating stem cells in its natural host. In macrophages it forms "botryoid" cytoplasmic inclusions. This virus immunosuppresses its host. However, its genome encodes the protein apoptin. Apoptin translocates into the nucleus guided by its nuclear localization signals and kills p53--defective or Bcl-2 up-regulated human tumor cells; in healthy cells (normal diploid fibroblasts), apoptin remains in the cytoplasm and exerts no cytopathic effects [68, 316]. The viral apoptin gene was cloned and expressed in the pVAX-CAV-VP3 vector. The vector induced apoptosis in RSV-transformed chicken cells; *in vivo* it caused regression of RSV-induced tumors [268]. Apoptin expressed in the recombinant adenovirus AdAptVP3 increased the susceptibility of human osteosarcoma, non-small-cell lung carcinoma, and prostate carcinoma cells *in vitro* to either etoposide or paclitaxel [281]. The reviewing authors ask if oncolytic NDV preparation obtained from embryonated chicken eggs could possibly harbor CAV [368].

Vectors. MVM and H1 parvoviruses infect human tumor cells (see above). In expressing the IL-2 or the IFN-γ inducible IP10 protein or the monocyte chemotactic protein-3, these parvoviruses increased their oncolytic potency [60]. The *wnt* pathway is mutated in colon cancer cells. MVM was re-targeted by inserting binding sites for β-catenin transcription factor (Tcf). Hybrid MVM/H1 line viruses containing Tcf promoters replicated best in *wnt*-mutated colon cancer cells and practically not at all in healthy cells with inactive *wnt* signaling pathways (including the *wnt* mutation negative HeLa cells) [248]. The recombinant hH1 parvovirus expresses the apoptin gene and when administered in combination with chemotherapeutic agents (paclitaxel, etoposide), it exerts increased cytotoxicity to human tumor cells *in vitro* [281]. The eukaryotic nucleic expression plasmid pVVP3IL-18HN carries the apoptin gene, the NDV HN gene, and the IL-18 gene. The expression plasmid suppresses the growth of human HEp-2 hepatocellular and laryngeal carcinoma cell line Hep-2 (G. F. Guan et al., Jilin University, Changchun, China, 2005, in Chinese, PMID 16270869 for translation). The cytopathic effect of the wild fowlpox virus and the recombinant fowlpox virus expressing the apoptin gene (vFV--Apoptin) was compared in human HepG2 and murine hepatoma cell lines *in vitro* and *in vivo*; the vFV-Apoptin agent killed tumor cells and exerted therapeutic effect [228].

An AAV vector expressing angiostatin potentiated the efficacy of transcatheter chemoembolization of liver tumors (in rats) by suppressing neoangiogensis [192]. Highly expressed in dividing cancer cells, but not in resting cells, the Hec1 proteins regulate spindle checkpoints for cell division; Hec1 (see above) depletion results in cancer cell death. Kinetochore-associated proteins (Kntc2) regulate microtubule interactions in transformed cells. Recombinant rAAV containing mRNA--specific small interfering and short hairpin RNA (siRNA/shRNA) depleted Hec1 and Kntc2 proteins in human tumor cells causing human tumor cell deaths *in vitro* [224]. Transgenes carried by MVM and H-1 and inserted into the genome of non-immunogenic tumor cells (mouse melanoma B78) are those of IL-2 and human monocyte chemotactic protein-3. Tumor cell lysis by transgene-loaded replicating viruses resulted in the generation of IFN-γ-secreting immune T cells (CD4/CD8 lymphocytes) and NK cells; thus non-immunogenic tumors were rendered immunogenic [446] (see above).

Herpesviruses

The herpesviral genome genetically re-engineers the host cell. The herpesviral genome consist of unique long (126-kb) and unique short (26-kb) sequences of linear dsDNA, UL and US, with over 84 ORFs. Heparan and chondroitin sulfate proteoglycans are the viral entry receptors. The viral nucleocapsid and the tegument proteins enter the nucleus. Immediate early mRNAs are released and translated in the cytoplasm. The immediate early α proteins enter the nucleus and activate gene transcriptions. The early β proteins direct DNA replication. Late γ proteins are translated from late mRNAs into viral structural proteins. The naked viral nucleocapsids exit from the nucleus by utilizing their glycoproteins gB and gH/gL to fuse with the outer nuclear membrane [100]. The nucleocapsids are tegumented and enveloped in the Golgi endosome. Cytoplasmic vesicles with mature virions within ascend to the cell surface and release the virus particles. For latency, the viral DNA assumes a circular shape in the nucleus and further transcriptional cascades are blocked, but may be reactivated years later. During latency, the viral Lat protein protects neurocytes from cytotoxicity and from apoptosis. Viral thymidine kinase is encoded by viral gene UL23; the enzyme phosphorylates thymidine (and other nucleosides). The gene UL39 encodes "infected cell protein" ICP6, the ribonucleotide reductase (RR). HSV ORF 16 encodes an anti-apoptotic Bcl-2 homolog; protein US3 blocks apoptosis by forming a complex with BAD (Bcl-2 associated domain). Viral ICP4 and ICP27 maintain Bcl-2 mRNA and protein. HSV glycoprotein D [474] binds TNF-β receptor. Puromycin inhibits the translation of some ICP species. ICP6 encodes a viral ribonuclease reductase and the host cell in mitosis provides its own ribonucleotide reductase to complement the function of the viral gene γ34.5; thus cyclic cells promote viral replication. Resting malignant cell may escape productive viral infection. However, HSV strains of defective ICP6 may also replicate in quiescent cells, including resting tumor cells. Such HSV strains are considered for brain tumor therapy to encompass also the resting tumor cell population [2, 3]. However, normal brain cells are resting; if the oncolytic virus prefers to infect mitotic cells, the tumor is targeted, but the normal brain cells are spared. If the oncolytic virus does not distinguish between resting and dividing cells, may it target the resting healthy cells as well? HSV protein US11 blocks PKR activation; ICP34.5 dephosphorylates eIF2α and reverses PKR activation (if it occurred). In healthy cells, HSV infection switches off IFN production. In tumor cells transformed by the point-mutated Ras oncogene, PKR remains inactive and no IFN is produced. The α0 gene-product protein ICP0 does not bind DNA, but interacts with other proteins, promoting their degradation, while acting as an ubiquitin ligase [141]. Delta α0 herpesviral mutants are depleted of ICP0. Herpesviral gene α47 encodes protein ICP47 (infected cell protein), which inhibits recognition of viral antigens within MHC-I by CD8 T cells [463]; CD4 T cells react instead. Viral gene UL41/γ(1)34.5 product proteins suppresses MHC-II expression. UL41 is the virion's host shutoff protein, functioning as an endoribonuclease [402]. The two copies of the viral gene γ(1)34.5 and the protein ICP34.5 are responsible for the viral neurovirulence. The Syn locus' UL1 gene encodes cell fusion proteins. In syncytia of infected cells, the virus spreads from cell to cell without an extracellular phase, thus escaping

neutralization by antibodies. Mature viral particles capture antibodies by the antibody's heavy chain and neutralize IgG molecules. HSV can infect T lymphocytes, which are killed by immunoreactive T cells, committing "fratricide" [304]. Herpesvirally infected cells express HLA-E, which inactivates the CD94/NKG2A NK cell receptor; infected host cells thus escape immune attacks of the host and secure the full maturation of the new viral progeny. Human DCs productively infected with HSV-2 induce $CD8^+$ T cells that suppress the clonal expansion of viral antigen-specific $CD4^+$ cells; even $FoxP3^-$ and $CD25^-$ $CD8^+$ T cells could exercise this suppressive activity [273]. Some herpesviral genomes encode the tolerogen and immunosuppressive cytokine IL-10, modulating host immunity toward the Th2-type setting. Nevertheless, oncolytic herpesviruses encounter innate immune reactions consisting of the activation of TLRs and the production of complement, chemokines, cytokines (IFNs and ILs) by macrophages and microglia, and cellular reactions of neutrophils, and NK cells [434]. Adaptive immune reactions (antibodies and immune T cells) could further curtail the oncolytic herpesviruses.

Herpesviruses serve as gene vectors because the large herpesviral genome can accommodate many transgenes. These viruses promiscuously infect a large variety of cells. The viral genome is not inserted into the genome of the host cells. Herpesviral replication in healthy cells can be curtailed because antiviral drugs can halt unwanted viral replications.

New genetically engineered oncolytic herpesviruses. HSV TK and RR (ribonucleotide reductase) deletion mutants were the first-generation oncolytic herpesviruses produced at Massachusetts General Hospital in Boston. The γ(1)34.5 and the UL39 gene (encoding ICP6/RR) were deleted from the second-generation G207 herpesvirus. This virus performed very well against *in vitro* cultured and xenografted human tumors. In the first phase I clinical trials in patients with malignant gliomas, initial partial tumor regressions eventually converted into progressive disease. Co-administration of corticosteroids reduced antiviral antibody production. Postmortem examinations of the brain showed no encephalitis. In a patient, complete regression of a glioblastoma was observed at autopsy; the cause of death was "cerebral infarction". Further clinical trials for intraperitoneal administration in patients with ovarian carcinoma and with co-administration of transgene (IL-2, GM-CSF, B7, TK)-expressing plasmid vector HSV particles (without viral genes) were initiated in 2002 and continue at the Center of Cell and Gene Therapy, Baylor College of Medicine, Houston, TX (see below); G207/G47delta (Δ) herpesviruses armed with inserted thrombospondin or platelet factor-4 genes actually kill proliferating vascular endothelial cells and reduce tumor (glioma, malignant peripheral nerve sheath tumor) microvessel density [2, 237, 410, 411]. The G207 virus with its α47 gene also deleted (G47Δ) is in investigational use for the treatment of malignant nerve sheath tumors (schwannomas) and malignant gliomas [189]. Based on three clinical trials for the treatment of malignant gliomas (cited in [410, 411]), G207 of MediGene AG (Deutsch-amerikanisches Biotechnologieunternehmen, Martinsried, Germany) and γ34.5 mutant HSV1716 are expected to be licensed. Insertion of the hCMV PKR-evasion genes TRS1 and IRS1 (terminal resolution site, inverted repeats site) into HSVΔγ(1)34.5 vectors significantly increased the replication of these viruses within murine malignant glioma cells while showing no pathogenicity to healthy brain cells [339]. Malignant human peripheral nerve sheath tumors are driven by mutated Ras oncogene and by overexpressed epidermal growth factor receptor signaling; xenografts of these tumors are susceptible to combined treatment with G207 and erlotinib [247]. Of twelve patients receiving through the hepatic artery replication-competent and attenuated NV1020 HSV-1 for the treatment of colorectal cancer metastatic to the liver, two experienced minor response, seven remained stable, and three progressed [199].

At the Baylor College of Medicine (Houston, TX) HSV-2 was converted into a fusogenic oncolytic virus (FusOn-H2) by deleting the serine/threonine protein kinase domain from its ICP10 protein. This virus targets Ras-transformed tumors and eradicated human ovarian carcinoma cells xenografted intraperitoneally [109–111]. FusOn-H2 killed syngeneic mouse neuroblastoma cells; not only directly injected tumors, but un-injected distant tumors were also rejected. Mice rejecting tumors resisted challenge with virulent tumor cells and yielded splenocytes protecting the mice against tumor cell implants [222]. The HSV-1-derived fusogenic Synco-2D oncolytic herpesvirus kills human kidney carcinoma cells in nude mice by direct or by intravenous inoculations [109–111].

Derivative of the avirulent HSV-1F with deleted γ(1)34.5 gene and rendered fusogenic (syncytium-forming) by recombination with the gBsyn3 gene, the OncSyn oncolytic herpesvirus (Louisiana State University, Baton Rouge, LA) kills human breast cancer xenografts without any pathogenicity to the tumor-bearing host [182]. The anti-apoptotic US3 locus-deficient L1BR1 herpesvirus is replication competent in pancreatic carcinoma cells; in combination with 5-FU and cisplatin, the L1BR1 herpesvirus kills tumor cells by inducing their apoptotic death [196]. A HSV-1 G47Δ amplicon vector expressing the measles virus fusogenic glycoproteins acted synergistically with the FOLFOX regimen (5-fluorouracil, leucovorin, oxaliplatin) or with irinotecan and cetuximab against xenografted colon carcinoma cells; not only the directly injected tumors, but not-injected distant tumors were also infiltrated with immune T cells, NK cells, and macrophages and regressed [161–164]. The importance of fusogenic protein expression by oncolytic viruses other than herpesviruses was shown by an adenovirus construct expressing respiratory syncytial virus fusion protein in colon carcinoma cells [162]. An HSV vector expressing GM-CSF (OncoVEX, BioVex, Cambridge, MA) was

injected into cutaneous metastases of various tumors (carcinomas and melanoma) of patients. Inflammatory responses and stabilizations of disease occurred (but no tumor regressions were reported) [171]. Wild-type HSV and its mutated oncolytic derivative, the G207 virus, (γ34.5$^-$, ICP6$^-$, LacZ$^+$) induce neovascularity due to reduction of intratumoral thrombospondins; thus initially regressing tumors resume growth. The G207 virus, with further deletion of its α47 gene and promoter gene of US11 gene, is deprived of its neovascularity inducing effect [2] (see above). Table 2 lists some ideas leading to the construction of new oncolytic herpesviruses [145, 174, 195, 216]. Dying tumor cells engulfed by DCs form DC vaccines, inasmuch as mature DCs express tumor antigens not in a tolerogenic, but in an immunogenic manner. A HSV construct was deprived of all its immediate early genes except for ICP0; tumor cells infected with HSVd106 became apoptotic and were highly preferred for engulfment by DCs. Such DC vaccines generated more effective anti-tumor immunity than DC vaccines prepared with UV-light-inactivated tumor cells. The DC/HSVd106 vaccines upregulated chaperone heat shock protein HSP70 in the DCs and induced IFN-γ--secreting immune T cells [61] (see above).

Harvest of 2007–2008. The replication-competent attenuated HSV NV1023 is subjected to a 15-kb deletion of its genome (deleting the UL56 gene, one copy of the diploid genes ICP0 and ICP4, and the neurovirulence gene $\gamma_1$34.5). The virus retains its perineural invasive ability. It attacks and lyses perineurally spreading human tumor (pancreatic, squamous cell, and adenocystic carcinoma) cells [124]. The NV1023 herpesvirus kills human salivary gland mucoepidermoid carcinoma cells *in vitro* and *in vivo* in xenografts [313]. The NV1042 herpesvirus is a variant of the NV1023 virus by expressing IL-12. It eradicates xenografts of an aggressive human prostate cancer in transgenic mice [425]. By degrading collagens within the tumor mass (human sarcoma cell line HSTS26T), overexpression of MMP-1/8 promoted the spread of oncolytic herpesvirus MGH2 within the tumor [257]. The HSV mutant G207 is devoid of its ribonucleotide reductase and $\gamma_1$34.5 genes. The Japanese derivative of this virus, G47delta, replicates in glioblastoma cells and induces better anti-tumor-cell than anti-viral host immune reactions [410, 411]. The R7041 herpesvirus has been deprived of its anti-apoptosis gene Us3; thus it fails to prevent normal host cell apoptosis and viral replication is thus reduced in apoptotic normal host cells. In tumor cells with strong anti-apoptotic forces in effect, the oncolytic viral progenies reach full maturity and exert oncolysis. In Us3$^-$ herpesvirus-infected tumor cells, the PI3K-Akt pathway is active and is susceptible to inhibition by LY294002 [236, 237]. Herpesvirus R3616 lacks both copies of its $\gamma_1$34.5 neurovirulence genes [426]. Human tumor cells frequently overexpress the mitogen-activated protein kinase and extracellular signal-regulated kinase kinase pathways, which suppress protein kinase R, and create an IFN-free environment. The oncolytic R3616 herpesvirus readily replicates in such human tumor cells

Table 2. New ideas implemented in oncolytic herpesviral designs

Problem	Idea and design	Results	References
Gamma(1)34.5- and RR-depleted HSV are avirulent and of reduced oncolytic efficacy	Tumor-specific promoter of neural RNA-binding protein of glioma cells (RBP) will be selective	HSV-1 KeM34.5 driven by RBP replicates in high titer in glioma cells	195
ICP34.5-deleted HSV should deliver TNF-α for better efficacy	CMV promoter highly augments, US11 moderately increases TNF-α expression	US11-driven TNF-α expression increased oncolytic without toxicity	145
Without expression of viral entry receptor nectin-1, HSV cannot infect anaplastic thyroid carcinoma cells	HSV NV1023 must express gp D and entry mediators for nectin-1	Nectin-1 gene transfections facilitated viral entry for cytotoxic infection	174
β-catenin gene mutations increase malignancy of colon carcinoma and hepatoblastoma cells	TE promoter drives the Wnt/β-catenin pathway. TE-driven oncolytic HSV was constructed: bM24-TE vector	Oncolytic bM24 vector selectively replicates to high titer in β-catenin gene-mutated cancer cells, killing them	216

HSV – herpes simplex virus CMV – cytomegalovirus, ICP – infected cell protein; ICP22 upregulates the US11 viral gene; ICP34.5 neurovirulence factor inhibits cellular autophagic response to CMV infection; ICP34.5-deleted HSV (HSV-1716) is oncolytic. ICP34.5-deleted oncolytic herpesvirus expresses TNF-α driven by US11 promoter (Biovex Ltd., Abingdon, Oxon, OX14 4RX, UK), TNF – tumor necrosis factor TE – T cell factor responsive element, US11/Us – unique short sequences, herpesviral (HSV, CMV) immunoevasive genes and gene product proteins inhibiting expression of major histocompatibilty complex (MHC) class I molecules in infected cells, protecting infected cells against apoptosis, counteracting the the dsRNA-responsive eIF2α kinase, protein kinase R, and inhibiting 2'-5' oligoadenylate synthetase in infected cells, thus creating an interferon-free intracellular environment, eIF2α – eukaryotic initiation factor α subunit.

and in their metastases [426]. Innate immunity, especially microglia activation in the brain, eliminates oncolytic viruses injected directly into brain tumors. Oncolysis by the fusogenic herpesvirus FusOn-H2 is augmented by the co-administration of cyclophosphamide [223]. Cyclophosphamide and depletion of $CD163^+$ and $CD68^+$ monocytes-macrophages allow for increased intratumoral titers of the oncolytic herpesviruses hrR3 with its UL39 locus inactivated and rHSVQ with both copies of its $\gamma_1$34.5 gene deleted [115]. In decreasing reactive inflammatory neoangiogenesis in gliomas infected with the oncolytic herpesvirus hrR3, viral titers increased and resulted in augmented oncolysis. The cyclic peptide arginine-glycine-aspartic acid (cRGD) antagonizes integrins αv/β5 ($\alpha_v\beta_5$); in the absence of these integrins, sprouting new endothelial cells die in apoptosis [218, 314]. T-cell survival is secured by the 4-1BB receptor uniting with its ligand 4-1BBL. Replication-defective herpesviral amplicons transfect mouse lung cancer cells with 4-1BBL. Such tumor cells generated specific immune T-cell clones cytolytic to the tumor cells and long-lived memory cells of these T-cell clones [459]. Claims like this are printed in the literature: "HSV-1 is the most potential oncolytic virus", but admittedly bio-safety and risk management issues remain to be resolved [253].

Influenza virus

Deleted NS1 gene. The N-terminal of the influenza viral NS1 protein binds RNA and the C terminal inhibits polyadenylation of cellular mRNAs. NS1 inhibits cellular PKR activation, thus suppressing IFN-α production and allowing the viral progeny to mature. NS1-deleted influenza A virus is inhibited by IFN-α, whereas Ras--mutated tumor cells with disabled PKR allow viral replication leading to cytotoxicity (oncolysis) [265]. Prostate cancer cells were not good hosts for *del*NS1 influenza viral oncolysis, but weak oncolytic activity could be augmented by IL-12. Breast and ovarian carcinoma cells were lysed by replicating NS1Δ viral progenies. These infected tumor cells generated $CD8^+$ immune T cells (among them, immune T cells reacting to Her2/*neu*) and NK cells which were cytolytic to infected and not-infected tumor cells [87]. This observation parallels the findings made in patients who were immunized with the PR8 H1N1 viral sarcoma-melanoma oncolysates [358]. A genetically engineered A/PR8/34 virus with truncated NS1 and a Her2/*neu* epitope inserted in its neuramidase protein infected DCs. Infected DCs generated $CD8^+$ memory cells in the exposed peripheral blood lymphocytes of patients with ovarian cancer [87]. The NS1 mutant/deleted influenza viruses stimulate macro-phages for the release of cytokines (IFN-α/β, IL-6, TNF-α) and the chemokine CCL3 (monocyte inflammatory protein-1α) [386]. Oncolytic live influenza viruses in the human community pose some chances for recombinations with wild influenza viruses. Surveillance committees appropriately advise utmost caution in the design of clinical trials with oncolytic influenza viruses (see below).

Influenza virus, live attenuated intranasal vaccine. These authors (unpublished) proposed the clinical use of the live attenuated and cold adapted (viral growth best at 25 C) and licensed Wyeth flu vaccine (Physicians' Desk Reference, 2007, pp. 1901–1904) for the treatment of squamous cell carcinomas of the oral and nasal cavities and the conjunctiva. However, the project is "on hold"; one contradictory factor is the high antigenicity of the vaccine excluding its repeated use. However, if antiviral immunity were turned against virally infected (and non-infected) tumor cells, the vaccine could still be effective as an oncolytic viral agent.

Measles virus

A most immunosuppressive wild-type virus becomes oncolytic. It is most remarkable that the wild-type measles virus, a cause of fatal infections, could be genetically engineered to the degree that it limits its pathogenic effects to malignantly transformed human cells. Wild-type measles virus infects DCs, interferes with the generation of antiviral immune T cells, and probably induces Th2-type immunity and the clonal expansion of suppressor T cells (T_{REG}) [113, 194, 388]. The antiviral antibodies enhanced the growth of measles virus-infected hamster glioma cells (cited in [369]). Burkitt's lymphoma cells express either the CD46 or the CD150/SLAM receptor (signaling lymphocyte activation molecule) through which the wild measles virus enters the lymphoma cells. Either by inducing apoptosis or by bursting the lymphoma cells by the egress of fully mature new viral progenies, the measles virus eventually kills the lymphoma cells. It is worth recalling (see above) that natural measles can lead to clinical remission of African Burkitt's lymphoma and Hodgkin's disease [36, 134, 329, 408], but relapses ensue later. The wild-type measles virus inhibits the host's IFN response by its P gene expression; the attenuated measles virus induces an IFN response. However, some of the genetically engineered oncolytic measles virus strains (MV--eGPF), being attenuated, induce IFN reactions in infected tumor (myeloma, ovarian carcinoma) cells. The wild-type P gene-armed oncolytic measles virus (MV--eGFP-Pwt) reduces the IFN response in the cells (lymphoma, myeloma) it infects [148]. The wild-type measles virus could be attenuated by passages through human kidney, amnion cells, or fibroblast cultures and in embryonated chicken eggs (Edmonston-Enders, Moraten, and Zagreb attenuated measles virus strains).

Intentional use of attenuated measles virus for the treatment of a malignant disease was reported from China in 1981 [465]. Patients with acute myelogenous leukemia received chemotherapy and experienced a 31% remission rate; when BCG was added to chemotherapy, the remission rate rose to 45% (confirming the French data concerning BCG co-therapy for

acute lymphoid leukemia, first reported from Villejuif by G. Mathé, cited in [367]). When chemotherapy and live attenuated measles virus were co-administered, a 46% response rate was achieved; patients with acute myelogenous leukemia receiving chemotherapy, measles virus, and BCG experienced a 79% response rate [465]. This report confirms the results of a protocol of chemotherapy, BCG, and influenza A viral oncolysate for the treatment of patients with metastatic sarcomas reported from M. D. Anderson Hospital, Houston, TX, in 1977 [355, 356, 358, 370, 372]. Therefore, chemotherapy and live oncolytic viral therapy are not antagonistic; rather, these modalities of treatment may be additive or even synergistic. Direct injections of the Edmonston attenuated measles virus into xenografts of human lymphoma cells, myeloma cells, and ovarian carcinoma cells induced regression of these tumors; passive transfer of antiviral immune sera did not abolish measles viral oncolysis [150, 176].

Preclinical trials. The entry of measles virions into cancer cells through receptors other than CD46 or SLAM (see above) can be accomplished by re-targeting the virus to enter through the epidermal growth factor or insulin growth factor (EGF, IGF) receptors that are abundantly overexpressed by certain human cancer cells. The re-targeted measles virus [318–320, 335] enters tumor cells even through the mutated EGFRvIII, which is overexpressed by human malignant glioma/glioblastoma cells [7, 290]. Since healthy cells never express the mutated EGFRvIII, the retargeted measles virus is restrained from entry into normal cells, including brain cells. The cytopathic effect of the retargeted measles viruses to human tumor cell xenografts surpasses that of the standard attenuated measles virus vaccines. The retargeted measles virus fuses the infected glioma cell membranes and the tumor cell syncytia are surrounded by host inflammatory cells, with NK cells and macrophages dominating in the infiltrates [290]. The retargeted measles virus MV-GFP-H(AA)--scEGFR is incapacitated by ablating mutations to enter cells through CD46 and SLAM receptors; in its hemagglutinin protein it displays a single-chain antibody directing it to EGFRs [320]. Without causing any neurotoxicity, the re-targeted measles virus eradicated orthotopic human glioblastoma xenografts. Interactions of measles virus with its natural receptors are ablated and a single-chain antibody specific to the α folate receptor is inserted. The α folate receptor is overexpressed in ovarian carcinoma cells. The re-targeted MV-αFR almost exclusively infects and lyses ovarian carcinoma cells [150]. The MV-NIS measles virus expresses the sodium iodide symporter molecule, which is an iodide ion channel; by transporting I^{131} into infected tumor cells, the oncolytic effect of the virus is potentiated. When re-targeted to the myeloma cell surface antigen CD38, this virus supposedly selectively infects myeloma cells. The biotechnology company Houston Pharma (Houston, TX) is applying for an FDA license for the virotherapy of human tumors (glioblastoma, multiple myeloma, ovarian carcinoma) with genetically engineered measles viruses [320].

A genetically engineered MV enters lymphoma cells through cell-surface antigen CD20 and carries the gene for the prodrug convertase enzyme purine nucleoside phophorylase; the enzyme converts fludarabine to a highly diffusibe cytotoxic substance. This MV was cytotoxic to the Burkitt's lymphoma Raji xenografts and to the Granta 519 mantle cell lymphoma xenografts when co-administered with the prodrug F-araAMP. Mantle lymphoma cells were susceptible to this MV *in vitro* [421]. A concise review lists those oncolytic viruses (coxsackie A21, measles, vaccinia, VSV), which are well--tested candidate agents for the virotherapy of multiple myeloma [392].

Polio-rhinovirus recombinants

Poliovirus-rhinovirus oncolytic recombinants (PVS--RIPO). Poliovirus enters motor nerve cells through the nectin subfamily immunoglobulin superfamily (IGSF) cell-surface receptor CD115. The polioviral genome carries an untranslated region containing non-initiating AUG codons. Translation initiation occurs through the internal ribosomal entry site (IRES) at the 5' end and in a cap-independent manner. IRES dictates first the formation of a large polyprotein that is a substrate of virally encoded proteases; the proteases liberate from it four structural and seven nonstructural proteins. Using the viral RNA as a template, the viral enzyme RNA--dependent RNA polymerase synthesizes negative template RNA strands from which positive viral genomic RNA strands are formed for encapsidation. A chimeric poliovirion was constructed by replacing its IRES element with that of the rhinovirus type 2. The chimeric virus replicated in HeLa cells, but lost its neuropathogenicity. Even in CD115 transgenic mice or in cynomolgus monkeys, the chimeric virus, termed PVS-RIPO, failed to infect healthy nerve cells, including those of the anterior horn motor cells of the spinal cord; even neuroblastoma cells resisted infection with PVS-RIPO [252].

Serial passages of PVS-RIPO in these cells did not produce neurovirulent revertant subclones of the virus. The PVS-RIPO virus retained extraordinary pathogenicity to human glioblastoma cells [252, 279]. The Sabin attenuated poliovirus vaccine strains all exhibit point-mutated IRES elements; neurovirulence resides in the native polioviral IRES element, which is removed and replaced by HRV2 non-neurovirulent IRES in the PVS-RIPO virus. Herpes simplex viral neurovirulence could also be attenuated by placing its neurovirulence gene γ(1)34.5 under the control of HRV2 IRES; while the neurovirulence gene-deleted herpesvirus did not grow well in glioblastoma cells, the HRV2 IRES-attenuated herpesvirus replicated up to cytotoxicity in human glioblastoma cells [43].

Preclinical trials. In malignantly transformed cells, cell adhesion molecules of the IGSF (immunoblobulin

superfamily) are overexpressed, and it is CD115 which dominates in neuroectodermal malignant tumor cells. The cluster of differentiation in CD115 expression in normal human brain cells is low, but in glioblastioma cells it is exceedingly high. The high level of tropism of PVS-RIPO to CD115 indicates that the virus would infect and lyse human glioblastoma cells. Human glioblastoma xenografts are eradicated by intratumorally or intravenously injected PVS-RIPO virus. In rodent models of human glioblastoma multiforme (hGBM) cell leptomeningitis in hCD115-expressing transgenic mice/rats, the intrathecally introduced live and UV light-inactivated (control) PSV-RIPO viruses reduced and even eradicated tumor growth with very significant tumor-free survival in the live virus-inoculated groups [279]. For human use, the PVS-RIPO virus is being licensed by the NIH/NCI Rapid Access to Inventional Development Program. The Duke University team [252] and these authors reviewed recent data concerning the viral [94] and immune lymphocyte adoptive therapy [373] for malignant glioma.

Naturally oncolytic viruses genetically engineered or otherwise modified

Alphaviruses. The attenuated Venezuelan equine encephalitis virus expressing either the tyrosinase of the melanocyte differentiation antigen or human prostate--specific membrane antigen (PSMA) induced B- and T--cell-mediated immune reactions in syngeneic mice harboring B16 melanoma. Th1-type cytokines, cytotoxic immune T cells, and IgG antibodies were produced. These immune reactions resulted in tumor growth cessation and prolongation of life. These experimental vaccines are planned to be further developed for human use [83].

Sindbis virus is not known to cause any human disease. The AR339 strain of Sindbis virus is replication competent and cytotoxic in human cervical carcinoma (HeLa and C33A) and several ovarian carcinoma cell lines both *in vitro* and *in vivo* (in xenografts). Healthy human keratinocytes resisted infection, even though these cells also express the viral entry receptor laminin. The replication-competent green fluorescent protein--expressing Sindbis virus allowed detection of tumor--infecting virus at distant tumor sites not directly infected [422]. The avirulent A7(74) strain of Semliki forest virus yielded a replication competent vector marked with enhanced green fluorescent protein, VA7-EGFP (A. I. Virtanen Institute, Kuopio, Finland). This vector kills xenografted human melanoma cells, but enters the brain of tumor-bearing mice and tumor cells growing in nodules acquire resistance to it (cited in [424], which is a highly recommended review on cancer virotherapy).

Coxsackie virus. The CVA21 strain of coxsackie virus entered human multiple myeloma cells *in vitro* through the intercellular adhesion molecule-1 and decay-accelerating factor (ICAM, DAF) receptors and produced lytic infection of the malignant cells, while normal peripheral blood leukocytes resisted infection [17].

NDV. Semliki Forst virus replicons transferred expression of NDV HN and F proteins into transfected cells; both proteins were expressed and the transfected cells formed syncytia [469]. Possession of the bispecific fusion protein αHN-IL-2 by the NDV particle directs the viral particle to the IL-2 receptor, but viral infection of the targeted cells occurred only in the presence of the viral F protein [33]. Malignant cells overexpressing the IL-2R (for example, Burkitt's or other lymphoma cells) could be targeted by the recombinant NDV. Expressing the β-glucuronidase gene, the virus enables tumor cells to cleave cytotoxic drugs from prodrugs [139]. Melanoma cells transfected with the MHC gene and infected with NDV proved to be highly immunogenic vaccines [298]. The IL-2-expressing NDV strain exerts enhanced oncolysis in mouse tumors (ALVAC Cancer Vaccine Research, 5 Upon Rd, United Kingdom BH 7AA) [427]. The Hertfordshire attenuated NDV H(Ph/02) strain, referred to as MTH-68/H virus in Hungary (see above), is a most potent IFN-α-inducer. Its systemic application was toxic and failed, while its intratumoral application succeeded in rejecting susceptible mouse tumors. It could induce host cell-mediated immune reactions against virus-resistant tumors [12].

Picornavirus. Seneca Valley Virus strain NTX-010 replicates exclusively in human tumor cells with neuroendocrine features (small-cell undifferentiated carcinoma of the lung, large-cell lung cancer undergoing neuroendocrine differentiation, Rb). In phase I/II clinical trials, the virus was not pathogenic in patients, but replicated in the targeted tumors [143]. Antiviral antibodies did not neutralize viral oncolysis. Neotropix, Malvern, CA, is applying for license to have a Food and Drug Administration approved oncolytic virus for the therapy of small-cell undifferenciated and related carcinomas. The native picornavirus SVV-001 displays high affinity to human neuroectodermal tumors (small-cell undifferentiated lung cancer, neuroendocrine Rb), while it is not known to be pathogenic in human (and in many animal) hosts. It is in phase I/II human clinical trials [312].

Respiratory syncytial virus. The fusion protein of RSV was expressed within tumor cells by a replication-defective adenovirus (Ad.RSV-F). Intratumoral administration of Ad-RSV-F into murine colonic carcinoma cells induced tumor cell syncytia formations. Co-administration of adenoviral vectors expressing cytokines (IL-12, IL-18, IL-21, GM-CSF) significantly enhanced tumor cell-directed cytotoxicity of Ad-RSV-F; the host generated immune reactions directed against tumors not virally injected and located distantly from the virally injected tumor [162]. Murine colonic tumor cell syncytia fused by measles virus, RSV, or VSV fusion proteins expressed by a replicating adenoviral vector became exquisitely susceptible to the FOLFOX (leucovorin, 5-FU, oxaliplatin) chemotherapy protocol [161–164] (see above).

Poxviruses. These large viruses readily accept transgenes and yield readily as vectors for gene therapy [24]. Patients with CLL inadvertently vaccinated with vaccinia virus experienced remissions (see above). The highly attenuated modified Ankara strain of vaccinia virus (MVA) (see above) expresses TRICOM: costimulatory molecules B7-1, intercellular adhesion molecule-1, and leukocyte function-associated antigen-3 [250]. Patients with CLL vaccinated with MVA-TRICOM expressed the three antigens on the surface of CLL cells and generated immune T cells reacting with infected and non-infected CLL cells [288]. Vaccinia and fowlpox viruses expressing the cancer/testis antigen NY-ESO induced in patients specific antibodies and immunoreactive CD4 and CD8 T cells which lysed NY-ESO$^+$ melanoma cells. One of 8 patients with metastatic melanoma experienced a complete response lasting over 32 months; other patients with melanoma and other tumors experienced stabilization of disease and minor/partial responses, but three patients with sarcomas failed with progressing disease [186]. Vaccinia, canary pox, and fowlpox viruses expressing PSA or CEA are in clinical trials to treat patients with hormone therapy-refractory metastatic prostate cancer expressing PSA or with adenocarcinomas expressing CEA [250]. The patients with prostatic carcinomas who progressed during vaccine therapy were treated with docetaxel; these patients experienced progression-free survival of 6 months versus the 3.7 months progression-free survival of those patients who were treated with docetaxel without pre-vaccination. The vaccine might have increased the tumors' susceptibility to chemotherapy [14]. Vaccinia virus-primed and PSA-expressing fowlpox virus-vaccinated patients with hormone-refractory metastatic prostate cancer also received GM-CSF; anti--PSA immune reactions were generated (more PSA--reactive T cells than antibodies), but the clinical response was mainly stabilization of the disease [14]. Patients with various metastatic cancers received priming with vaccinia virus and immunizations with CEA/TRICOM-expressing fowlpox virus plus GM-CSF. Of 58 patients treated for metastatic disease, one with small-cell lung cancer experienced a complete remission. Twenty-three patients (40%) stabilized their advancing disease; in 14 patients, the stabilization of disease exceeded 6 months. Clinical responses correlated with immune reactions to CEA [250]. The following genetically engineered vaccinia viruses are currently in clinical trials [430]: JX-594 thymidine kinase-negative but hGM-CSF gene-expressing vaccinia virus [203], ALVAC canary pox virus expressing melanoma antigen gene gp100m [382], and ALVAC canarypox virus co--expressing CEA and B7.1 genes. This last-named agent induces leukocyte infiltrates at the vaccination sites, reduces the blood level of CEA, and stabilizes the course of the disease. The more prior chemotherapy the patient received, the less CEA-specific immune T cells he generates [38, 430].

Sendai virus. The UV light-inactivated hemagglutinating virus of Japan (as it is referred to in the cited literature and in the cited article) induces DCs and immune CD4$^+$ and CD8$^+$ T cells to infiltrate tumors. In addition, Sendai viral envelope HVJ induces IL-6 release from DCs. IL-6 suppresses the proliferation of Foxp3$^+$CD4$^+$CD25$^+$ T_{REG} cells. By removing T_{REG} cells, anti-tumor immune reactions act unopposed [217]. The recombinant virus (rSeV/DCs) infects, and induces maturation of, immature DCs, thus accelerating DC-mediated anti-tumor immune reaction in mice via the "immunostimulatory virotherapy" reaction [462].

VSV. The VSV negative-strand RNA genome is quite pliable; chimeric VSV/hCMV particles could be produced in hamster cells [132]. The genomes of mouse mammary tumor virus, avian and murine leukemia, and sarcoma viruses could replace the VSV genome and be encapsidated in the VSV envelope [53]. The VSV genome accepts and expresses the IL-15 transgene, thus promoting the recruitment and replication of NK cells and immune T cells in the microenvironment of the tissues (tumors) in which it is injected [389]. Not only the measles virus, but also VSV accepts and expresses the human sodium iodide symporter gene; I^{131} selectively accumulates in VSV-NIS-infected tumor cells [125]. With mutated or deleted matrix protein gene, VSV remained oncolytic in human glioma xenografts [243]. The oncogenic cascade RAS/Raf1/MEK/ERK and the loss of IFN-α-responsive factor render tumor cells exquisitely susceptible to VSV lysis [18, 282, 451]. The D2F2/E2 mouse tumor is transfected with the HER2/*neu* gene and expresses the oncoprotein. A Sindbis virus glycoprotein (gp) contains an Fc antibody targeting HER2/*neu* oncoprotein. The recombinant replicating rrVSV expresses the Sindbis virus gp and a mouse GM-CSF. The HER2/*neu*$^+$ mouse tumors implanted intraperitoneally kill the mice. Treatment of the tumor-bearing host with rrVSV reduces or eliminates tumor growth; mice rendered tumor-free resist challenge with tumor cells of the same line. Tumor rejection is immune T cell dependent [30, 120]. Human healthy hepatocytes exclude VSV infection by type I IFN production, but hepatocellular carcinoma cells do not respond to VSV infection with IFN production; even dsRNA fails to induce IFN-β in hepatocellular carcinoma cells, rendering them highly susceptible to VSV oncolysis. Intratumoral VSV replication was reduced after the third day by IgM antibody production [85]. In a mouse model, recombinant VSV lysed hepatic metastases of colon carcinoma [172]. Both myxomavirus and VSV (DeltaM51 strain) kill human pediatric rhabdoid tumor xenografts [454].

Retro- and lentiviruses

Murine leukemia viruses enter the genomes of dividing cells; resting host cell genomic sequences are resistant to retroviral genomic insertions. Replication-competent retroviruses serve as vectors of suicide gene delivery into solid tumors, including glioblastoma [403]. Are there any possible recombinations with endogenous

retroviral sequences harbored by the host cells? Nondividing DCs accept infection by lentiviral vectors. DCs transduced by the melanoma antigen NY-ESO-1 (ESO – embryonic stem cells/ovaries) gene-encoding lentivirus expressed the antigen and generated NY--ESO-1-specific immune T cells, both in murine and human systems [289]. Human DCs cultured from peripheral blood mononuclear cells could be transduced by lentiviral vector to express the Melan A (MART-1 – melanoma antigen recognized by T cells) antigen. These DCs cocultured with autologous T cells induced the expansion of T-cell clones recognizing HLA-A2-restricted Melan-A epitope expression. The responding T cells secreted IFN-γ [238]. Retroviral vectors tranduce dividing melanoma and glioblastoma cells; only a minority of these tumor cells expressed the transgenes for costimulatory factor B7-2 and GM-CSF. Of over 100 glioblastoma cell specimens transduced, only 5, and of over 30 melanoma cell specimens transduced, only 3 expressed the transgenes. Immunized patients did not show clear-cut specific anti-tumor responses [292]. There is yet hope that retro-lentiviral vectors will deliver and insert useful genes for cancer immunotherapy [40]. The well-known anti-angiogenic effect of IFN-α [359] was taken advantage of in incorporating its gene into a lentiviral vector (LV-IFN). Human ovarian carcinoma xenografts growing as hemorrhagic ascitic tumors in SCID (severe combined immunodeficient) mice were inhibited by LV--IFN treatment. In the ascitic tumors, as IFN-α levels rose, microvessel density was reduced and ischemic necrosis of the tumors set in [178].

First, children with severe combined immunodeficiency were cured with gene therapy. However, retroviral integration into the genome of recipients may activate and/or induce recombination of leukemogenic proto-oncogenes. The recent past and immediate future of gene therapy of human "blood disorders" is discussed by A. W. Nienhuis of St. Jude Children's Research Hospital, Memphis, TN, in a very recent article [271].

CONCLUDING DISCUSSION

Would viral oncolysis be more effective in an immunosuppressed host, and should it involve both the innate and adaptive immune faculties? Of the adaptive immune faculties, the Th1-type immune environment appears to favor the host, as immune T cells attack virally infected tumor cells and low levels of virus-neutralizing antibodies fail to eliminate the oncolytic virus. As to the innate faculties, granulocytes, DCs, NK, and LAK cells should certainly be spared and macrophages may be activated in the ADCC reaction. The ideal situation would be the exquisite suppression of virus-neutralizing antibodies sparing from neutralization of a non-pathogenic oncolytic virus, without any outer immunological compromise.

There appears to be a discrepancy between the actual cure with oncolytic viruses of murine cancers, especially mouse ascites tumors, and metastatic human cancers. In the murine host, the success rate is high; in the human host, oncolytic viruses induce remissions, or at least stabilize tumor growth, but the actual cure of metastatic human cancers with viral therapy alone, and exclusive of other therapeutic interventions, still remains to be clearly and convincingly documented.

Furthermore, the mechanisms of tumor cell killing by some oncolytic viruses is studied exclusively in artificially created laboratory tumor cell lines, which are unnatural laboratory products [74], and not in native tumor cells obtained from the patients. The spectacular results ("apoptosis inductions") obtained in the artificial systems would very likely fail to be validated in the real situation, i.e. in the native tumor cell in its natural host, the patient. In addition, human tumor xenografts, in their new environment, are also very vulnerable to viral therapy, to which tumor cells may react quite differently, that is resist it, in their natural host [359]. When the high success rates in artificial systems lead to controlled clinical trials, medical oncologists may arrive at conclusions much less dramatic than what was expected in the laboratory. The congenial design of the ONYX adenovirus (after onyx, the microscopic chalcedonic silica cryptocrystals) was not translated into dramatic cancer cures in actual clinical trials in patients. In China, the ONYX-like licensed adenovirus is used with combination chemotherapy to treat nasopharyngeal carcinoma [464]. Finally, will it be ethical to replace standard cancer therapy with first-line oncolytic viral therapy, or will only patients with established advancing tumors, failing standard therapy, be allowed to be treated with oncolytic viruses?

Viral oncolysates prepared with naturally oncolytic viruses (influenza A virus, NDV, or vaccinia virus) have repeatedly shown a favorable trend in clinical trials. Indeed, there was a very favorable trend in the Emory University melanoma viral oncolysate trial [50] toward the elimination of subclinical metastases left behind after surgical removal of gross tumors (melanomas). In these cases, results are not expressed by the exact tumor size measurements before and after treatment, but by statistical comparisons of patients treated only surgically and patients who received viral oncolysate immunotherapy postoperatively. For the validity of such a comparison, nearly identical patients in the compared cadres are needed. In patients with stage III melanoma, active "tumor-specific" immunization with vaccinia viral oncolysates yielded slightly better results than direct oncolysis induced by vaccinia virus injected into the patients [155, 201, 370, 436–438].

The hope for the success of virotherapy with naturally oncolytic viruses for human cancers rests on the fact that both immunization with viral oncolysates prepared *ex vivo* and viral oncolysis accomplished *in vivo* induce specific postoncolytic anti-tumor immunity [213, 234, 358, 372]. It is essential to pick out not just the IFN--deprived viral oncolysis-sensitive tumor cells for biochemical studies, but rather the resistant ones. The cur-

rent Canadian studies of tumor cells resistant to reovirus are the most educational [5], and tumor cells exposed to viral oncolysis should always be submitted to such studies. It is just as important to dissect the immune responses of the host during and after viral oncolysis. Which is more effective: tumor cell apoptosis without viral replication or full maturation of the viral progeny that accomplishes continuous and complete oncolysis (or both simultaneously)? Is it cell-mediated (Th1-type), antibody-mediated (Th2-type), or their overlapping modalities (oversimplified in this sentence) of immune reactions aimed at the virus and to the virally infected tumor cells that secure the long-term success of oncolyis? Knocking down the oncogenic signal cascades in the infected tumor cells by genetically engineered viruses is the essential platform on which the host may build a rejection-strength immune reaction.

The discovery that bacteriophages may be oncosuppressive opens up a new field of investigation [130]. These agents are not pathogenic. Genetically engineered mass-purified and immunologically manipulated bacteriophages and the tumor-bearing host together may achieve the rendering of a tumor-hostile and oncolytic environment. An astute and most helpful reviewer of this manuscript brought up the question if the authors could specify the antigens that are to be targeted by phages in colon cancer cells. The senior author proposes that these antigens should be in the MAGE (melanoma-associated antigens) class. Filamentous phage virions delivered HLA-2-restricted MAGE peptides into tumor cells to elicit potent and highly specific CTL responses. Further, MAGE-expressing phage particles used as vaccines elicited anti-tumor immune reactions of the host. MAGE expression is not the exclusive property of melanoma cells; among other tumors, colon carcinoma cells naturally express MAGE. CEA-, MUC1-, and MAGE-expressor allogeneic colon carcinoma cells used as vaccines presented these antigens in an immunogenic manner and elicited anti-tumor CTL reactions [212, 325, 342]. It probably will be first in the colon where phages will prove themselves to be oncosuppresssive.

The hope for the success of virotherapy with genetically engineered viruses for human cancers rests in the brilliance and congeniality of the designs of these altered viral agents. Could naturally oncolytic and genetically engineered oncolytic viruses be used in combinations simultaneously (if they do not interfere with one another) or sequentially? Viral therapy of human cancer is now in the front lines of the "war on and conquest of cancer".

Acknowledgment: The Authors are grateful to Sister Mary Celeste Sullivan, President of St. Joseph's Hospital, Tampa, FL, for the establishment and support of the Clinical Cancer Research Laboratory within the Cancer Institute of the Hospital in 1992. Gratitude is expressed to Dr. Marianna Szabó-Szabari, Dr. Andrea Horák, and Dr. Elada Neville for meritorious work in the Laboratory and to the board-certified oncology nurses Mary Pritchard, RN, ONC, and Cynthia Crumpton, RN, ONC, for assistance in the clinical trials and to the several certified technicians and medical laboratory technologists for assistance in preparing viral oncolysate and immune lymphocyte preparations for the immunotherapy of patients at the Cancer Institute, St. Joseph's Hospital, Tampa, FL (1993–2006).

Gratitude for assistance in the Senior Author's work at the University of Texas M. D. Anderson Hospital throughout the 1960s and 1970s to the clinical investigators Dr. Carl Plager and Dr. Nicholas Papadopoulos at the Clinical Melanoma--Sarcoma Service and to the laboratory investigators Drs. H. David Kay, Francisco Gonzalez, Philip DiSaia, and Harikishan Thota at the Section of Clinical Tumor Virology and Immunology, Department of Medicine (1962–1979), has been expressed in the text, in publications cited, and remains vividly preserved in the mind and heart of the Senior Author.

The authors thank Dr. Steven Specter (sspecter@health.usf.edu), Associate Dean, University of South Florida College of Medicine, Tampa, FL, for encouraging the authors to write and publish this article. The authors express appreciation to Dr. Adorjan Aszalos (aszalosa@mail.nih.gov) of the National Cancer Institute/National Institutes of Health, (formerly Department Head at the Food & Drug Administration) Bethesda, Maryland, and to Dr. Robert W. Simpson, (oldvirushunter@yahoo.com/bobglen@patmedia.net), distinguished professor of virology, Waksman Institute, Rutgers, State University of New Jersey, New Brunswick & Piscateway, NJ, for reading and favorably commenting on various segments of the drafts of the manuscript.

The senior author dedicates his contributions to this article to the memory of "öregmama" Ágnes Godavszky, "nagymama" Erzsébet Beliczky, and "édesanya" Mária Rajnocha.

The authors declare no financial association with or dependence on (no grants, no salaries, no consultation fees) any drug companies or entrepreneurs promoting or practicing viral therapy of human cancer.

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