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Safety of Glucocorticoids in Cancer Patients Treated with Oncolytic Adenoviruses

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Abstract: Oncolytic adenoviruses are an emerging treatment option for advanced and refractory cancer. Such patients are often treated with corticosteroids to ameliorate tumor associated symptoms. Thus, it is important to evaluate whether safety is affected by immunosuppression possibly induced by corticosteroids. Concurrent low-dose cyclophosphamide, appealing for its immunomodulatory effects, could also impact safety. In a retrospective case-control study, we evaluated the effect of systemic corticosteroid use in cancer patients receiving oncolytic virotherapy. Four treatment groups were identified: (1) oncolytic adenovirus with oral glucocorticoids, (2) virus alone, (3) virus with glucocorticoids and cyclophosphamide and (4) virus with cyclophosphamide. Adverse events, neutralizing antibody titers, viral DNA in circulation and tumor responses were evaluated. The most common adverse effects were grade 1−2 fatigue, nausea, fever and abdominal pain. Common asymptomatic findings included self-limiting grade 1−3 hyponatremia and aspartate aminotransferase increase. Safety was good and no significant differences were observed between the groups. All patients had an increase in neutralizing antibody titers post-treatment, and no trends for differences between groups were observed. There were fewer post-treatment virus genomes circulating in patients receiving glucocorticoids when compared to their control groups. Overall, glucocorticoid use in cancer patients receiving oncolytic adenovirus, with or without low-dose cyclophosphamide, seems safe.

Keywords: Oncolytic adenoviruses; glucocorticoids; cyclophosphamide; safety; retrospective study

Introduction

Increasing numbers of cancer patients are treated with oncolytic virotherapy. The first product has been approved, and several phase 3 trials are ongoing.[1,2] Candidate patients for experimental therapies often have extensive disease causing tumor associated symptoms such as nausea, anorexia and fatigue. Glucocorticoids are regularly used to treat these symptoms, and it has been estimated that 50−70% of cancer patients use glucocorticoids in the last stage of their disease.[3,4] For example, daily oral dexamethasone in terminally ill patients can increase the feeling of well-being and reduce fatigue.[5]

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Cortisol and its synthetic analogues induce a variety of biochemical and physical effects and are well-known for their antiallergic and anti-inflammatory activities. The anti-inflammatory response is not dependent on the type of danger stimulus, and nearly all types of inflammation related symptoms can be attenuated by glucocorticoid intake.

Production of acute inflammatory cytokines by immune cells can cause systemic adverse effects in patients undergoing adenovirus (Ad) based gene therapy. For example, IL-6 has been suggested to predict Ad mediated toxicity.[6,7] Moreover, production of anti-Ad neutralizing antibodies may influence therapeutic outcome,[8] or safety, through antibody mediated vector uptake by immune cells and subsequent release of interleukins.[7] Therefore, the attenuation of these responses might improve the therapeutic outcome and reduce the side effects of oncolytic Ad therapies.[9,10] In contrast, Ad epitopes presented by infected cancer cells could help in tumor eradication and the inflammatory danger signal induced by productive oncolysis might be important for antitumor immunity.[11,12]

It has been proposed that dexamethasone increases adenoviral transduction and transgene expression *in vitro*.[13−15] Also, in several preclinical animal studies with Ads, glucocorticoids are associated with prolonged viral replication and enhanced transgene expression.[14,16] However, glucocorticoids may also have antiviral activity and dexamethasone has been shown to attenuate virus replication *in vitro* and *in vivo*.[13]

Although the utility of glucocorticoids for modulating the efficacy and/or safety of oncolytic viruses is well established in preclinical models, this has not been formally studied in humans. However, a patient series from 1956 reported the use of various wild type Ads as anticancer agents in 40 patients suffering from advanced cervical carcinoma. Fifteen of the study patients also received cortisone combined with viral treatment. Interestingly, it was observed that responses were more prominent in these patients and 4 out of the 7 most robust responses were seen in this group.[17]

CD4+CD25+FoxP+ regulatory T cells (Tregs) are immune cells contributing to tumor immunotolerance and progression.[18] They downregulate innate immune responses by means of inhibiting natural killer cell (NK) proliferation and activity.[19] In addition, they can inhibit dendritic- and CD8+ T-cell mediated tumor eradication by suppressing their function and augmenting their tolerance of malignant cells.[20] A selective reduction of the Treg population can be achieved

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by using low dose cyclophosphamide, an alkylating chemotherapeutic agent, in an oral daily (metronomic) manner or by low dose intravenous injection.[21−23] Previous preclinical and clinical work has shown that the relevant effector cells are not compromised and that Treg reduction can enhance antitumor immune responses.[21–23]

We report the safety of oral glucocorticoids, with or without low-dose cyclophosphamide, in 14 cancer patients treated with oncolytic Ads. Immunological and safety parameters were compared with matched control patients treated with virus alone or with virus and cyclophosphamide.

Materials and Methods

Patient Selection. Twenty-eight patients were selected in a retrospective manner from a Finnish Medicine Agency approved Advanced Therapy Access Program (ATAP). Inclusion criteria for oncolytic adenovirus treatment in ATAP include (I) solid tumors refractory to conventional treatments, (II) progressing disease, (III) written informed consent and (IIII) no major organ function deficiencies. The use of glucocorticoids and/or cyclophosphamide within 24 h prior to or post treatment was verified from patient records, and 14 patients were selected for the case groups in which 7 patients received virus + glucocorticoids and 7 received virus + glucocorticoids + cyclophosphamide (50 mg/daily or single 1000 mg intravenously) during virus treatment. Patients using topical or inhalable corticosteroids were not included. The controls were selected from patients not treated with glucocorticoids, by matching for as many as possible of these criteria (in order of importance): virus, virus capsid, virus arming, virus dose, round of treatment, cyclophosphamide route, WHO performance score, tumor, age and sex (Table 1).

Treatment. Twenty-six patients were treated for the first time with oncolytic adenoviruses. Patient Y36 and his control O24 had their second treatment. Patients received a single viral dose of 2×10^{10} to 9×10^{11} vp/mL administered intratumorally (intracavitary in case of pleural or peritoneal dissemination) and intravenously (Table 1) with ultrasound guidance as reported.[11,24−26] Post-treatment, patients were monitored for 24 h in the hospital and thereafter as outpatients. Treatments were performed with written informed consent according to Good Clinical Practice and the Helsinki Declaration of World Medical Association.

Viruses. The oncoytic viruses used in this study have been described earlier. All viruses feature a 24 bp deletion in the E1A for transcomplementation of a mutated Rb-pathway. Ad5/3-Cox2L-D24 is a serotype 3 receptor targeted adenovirus where E1A is under a cyclo-oxygenase 2L (Cox2L) promoter.[27,28] Ad5-D24-RGD binds to $\alpha_v\beta$ integrins.[29] Ad5/3-D24-GMCSF is targeted to Ad3 receptor and codes for granulocyte-macrophage stimulating factor (GM-CSF) under the native E3 promoter to enhance immunoresponse toward cancer cells.[24] Ad5-RGD-D24-GMCSF[30] and Ad5-D24-GMCSF[11] are similar to Ad5/3-D24-GMCSF but are retargeted to bind $\alpha_v\beta$ integrins and CAR, respectively. ICOVIR-7 has the RGD modification of Ad5 knob and binds to $\alpha_v\beta$ integrins. The expression of E1A is controlled by E2F-1 promoter binding site together with E2F binding hairpins.

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Table 1. Matching Criteria

patient ID	virus	dose (vp[a])	viral capsid[b]	viral arming[c]	glucocorticoid and dose	cyclophosphamide route and dose
			Group 1, Cases with Oral Glucocorticoids			
P123	Ad5/3-Cox2L-D24	3×10^{11}	Ad5/3		prednisolone 10 mg ×1	
N112	Ad5/3-Cox2L-D24	4×10^{11}	Ad5/3		dexamethasone 3−4.5 mg ×2	
S100	Ad5-D24-GM-CSF	2×10^{11}	Ad5	GM-CSF	prednisolone 5 mg ×1	
R39	ICOVIR-7	2×10^{10}	Ad5 + RGD		dexamethasone 3 mg ×3	
N56	ICOVIR-7	3×10^{11}	Ad5 + RGD		dexamethasone 3 mg +1.5 mg	
R85	ICOVIR-7	3×10^{11}	Ad5 + RGD		dexamethasone 6 mg ×2	
O92	ICOVIR-7	4×10^{11}	Ad5 + RGD		prednisolone 10 mg ×1	
			Group 2, Controls without Oral Glucocorticoids			
G11	Ad5/3-Cox2L-D24	2×10^{10}	Ad5/3			
P27	Ad5/3-Cox2L-D24	3×10^{11}	Ad5/3			
S23	Ad5-D24-RGD	2×10^{10}	Ad5 + RGD			
C31	Ad5-D24-RGD	7×10^{10}	Ad5 + RGD			
C65	Ad5-D24-RGD	4×10^{11}	Ad5 + RGD			
C104	ICOVIR-7	4×10^{11}	Ad5 + RGD			
C93	ICOVIR-7	4×10^{11}	Ad5 + RGD			
			Group 3, Cases with Oral Glucocorticoids and Cyclophosphamide			
G159	Ad5/3-D24-GMCSF	3×10^{10}	Ad5/3	GM-CSF	dexamethasone 4.5 mg ×1	1000 mg iv
P74	Ad5/3-D24-GMCSF	1×10^{11}	Ad5/3	GM-CSF	dexamethasone 6 mg ×2	metronomic 50 mg ×1 po
Y36	Ad5/3-D24-GMCSF	2×10^{11}	Ad5/3	GM-CSF	dexamethasone 4.5 mg ×2	metronomic 50 mg ×1 po
I116	Ad5/3-D24-GMCSF	4×10^{11}	Ad5/3	GM-CSF	prednisolone 7.5 mg ×1	metronomic 50 mg ×1 po
R42	Ad5-D24-RGD	1×10^{11}	Ad5 + RGD		prednisolone 20 mg ×1	metronomic 50 mg ×1 po
C143	Ad5-RGD-D24-GMCSF	9×10^{11}	Ad5 + RGD	GM-CSF	prednisolone 10 mg ×1	metronomic 50 mg ×1 po
C151	Ad5-RGD-D24-GMCSF	9×10^{11}	Ad5 + RGD	GM-CSF	prednisolone 30 mg ×1	metronomic 50 mg ×1 po
			Group 4, Controls with Cyclophosphamide but without Oral Glucocorticoids			
V136	Ad5/3-D24-GMCSF	3×10^{10}	Ad5/3	GM-CSF		metronomic 50 mg ×1 po
M158	Ad5-RGD-D24-GMCSF	1.5×10^{11}	Ad5 + RGD	GM-CSF		1000 mg iv
Y62	Ad5/3-D24-GMCSF	1×10^{11}	Ad5/3	GM-CSF		metronomic 50 mg ×1 po
O24	Ad5-D24-RGD	2×10^{11}	Ad5 + RGD			metronomic 50 mg ×1 po
O130	Ad5-RGD-D24-GMCSF	9×10^{11}	Ad5 + RGD	GM-CSF		metronomic 50 mg ×1 po
M126	Ad5-RGD-D24-GMCSF	9×10^{11}	Ad5 + RGD	GM-CSF		metronomic 50 mg ×1 po
R55	ICOVIR-7	3×10^{11}	Ad5 + RGD			metronomic 50 mg ×1 po

[a] vp, viral particles. [b] Ad5/3, Ad5 capsid with Ad3 knob; Ad5 + RGD, Ad5 capsid with RGD motif inserted in the HI-loop of capsid. [c] GM-CSF, granulocyte-macrophage colony stimulating factor. All patients received oncolytic adenovirus.

An insulator and Kozak sequence preceding E1A region ensures optimized transcription.[25,31]

Adverse Events. Adverse events were recorded according to CTCAE v3.0.[32] If pre-existing symptoms did not become worse after treatment, they were not scored. However, if the pre-existing symptom became more severe, e.g. pretreatment grade 1 changed to grade 2 after treatment, it was scored as grade 2.

Evaluation of Efficacy. RECIST criteria were used to evaluate antitumor efficacy. These criteria are as follows: partial response (PR), >30% reduction in the sum of tumor diameters; stable disease (SD), no response/progression; progressive disease (PD), >20% increase. For tumor markers, the same percentages were used and minor response (MR) was used to indicate 12−29% reduction.

Neutralizing Antibodies. 1×10^4 of 293 cells/well were seeded on 96-well plates and cultured overnight. Patient sera samples were incubated at 56 °C for 90 min to inactivate complement, and 4-fold dilution series (1:1 to 1:16384) were prepared in serum-free DMEM.[26,33] Nonreplicating luciferase

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expressing adenoviruses with identical capsids to respective oncolytic viruses were added to serum dilutions to obtain a final concentration of 100 vp/cell and incubated at room temperature for 30 min. The virus−serum mixture was then added on cells in triplicate and incubated +37 °C for 1 h, and DMEM with 10% FCS was added. 24 h postinfection, cells were lysed and luciferase activity was measured (Luciferase Assay System, Promega, Madison, WI, and TopCount luminometer, PerkinElmer, Waltham, MA). To evaluate the effect of neutralizing antibodies, luciferase readings were plotted relative to gene transfer achieved with respective luciferase expressing nonreplicating virus alone. The neutralizing antibody titer was determined as the lowest degree of dilution that blocked gene transfer more than 80%.

Quantitative Real-Time PCR (qPCR). Total DNA was extracted by adding 3 μg of carrier DNA (polydeoxyadenylic acid; Roche, Mannheim, Germany) to 400 μL of serum and using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Extracted DNA was eluted in 60 μL of nuclease-free water, and DNA concentration was measured by spectrophotometry. PCR amplification was carried out as previously reported.[26]

For determination of viral loads, a regression standard curve was generated using DNA extracted from serial dilutions of adenoviruses in normal human serum (1×10^8 to 10 vp/mL). The limit of detection and limit of quantification for the assay were 500 vp/mL of serum. For chart-making purposes, the value <500 vp/mL was set as 500 vp/mL. Positive samples were confirmed by real-time PCR using LightCycler480 SYBR Green I Master mix (Roche, Mannheim, Germany) and primers specific for RGD sequences (forward primer 5′-ACAAACGCTGTTGGATTTATGC-3′ and reverse primer 5′-GATGGGCAGAAACAGTCTCC-3′) or for other sequences as previously reported.[24–26]

Statistical Analysis. SPSS version 15.0 (SPSS Inc., IL) for Windows was used for statistical analysis. Levene's test was used to test the equality of variances. Nonparametric Spearman's rho and Mann−Whitney tests were used to evaluate correlations between glucocorticoid uptake and viral genomes in the serum at different time points or throughout the time period. A two-tailed *t* test was used to compare means between groups at indicated time points or at all time points. A *p*-value <0.05 was deemed statistically significant.

Results

Patients. Twenty-eight patients consisting of 14 cases and 14 controls were selected for the study in a retrospective manner. The most relevant matching criteria are listed in Table 1. Group 1 received glucocorticoids in combination with oncolytic adenovirus treatment. Group 2 is the control group for group 1, where patients received oncolytic adenovirus alone. Group 3 patients received glucocorticoids and cyclophosphamide during their oncolytic adenovirus treatment. Group 4 is the control group for group 3, where patients received cyclophosphamide together with oncolytic adenoviruses but no glucocorticoids. There were no notable differences between groups in WHO performance score, age, sex or previous treatments (Table 2).

Adverse Events. Adverse events were collected for 30 days after treatment and scored according to Common Terminology Criteria for Adverse Events (CTCAE) version 3. (Tables 3, 4). In all groups the most common findings were grade 1 or 2 fatigue and fever. In groups 3 and 4 grade 1−2 nausea was also a common finding. The majority of patients received virus injection into the abdominal region, which may be associated with abdominal pain reported commonly. One reversible incident of grade 4 hyperglycemia occurred in a diabetic patient (V136) in group four (virus and cyclophosphamide). The patient's blood glucose returned to normal level after insulin treatment. One grade 4 pulmonary embolism was observed 21 days after the virus treatment in an appendix carcinoma patient (C151) treated with glucocorticoids and cyclophosphamide (group 3). The patient was hospitalized for 4 days, antithrombotic medication was started and she recuperated without sequelae. It is unclear if the treatments played a role in this event, as thrombotic events are quite common in late stage cancer patients: Up to 15% of hospitalized colon cancer patients have symptomatic venous thromboembolism, and asymptomatic thrombosis is found in up to 40% of colon cancer patients undergoing surgery.[34,35]

The most common asymptomatic side effects were hyponatremia and AST increase. In groups 1 and 2, AST increases were typically grade 2 or 3 and all grade 3 elevations were observed in patients whose values were abnormal already at baseline.

In groups 3 and 4, AST increases were typically grade 1 or 2 and only one grade 3 AST increase was observed in a control patient. One grade 3 hyperbilirubinemia was observed in a melanoma patient on day 28 after treatment, while her ALT value remained normal, which suggested extrahepatic obstruction possibly due to tumor progression, as the patient had a normal bilirubin at baseline.

Neutralizing Antibody Response. Neutralizing antibodies against the adenovirus capsid were measured at baseline and at different time points after treatment (Figure 1). There were no significant differences between groups in the rate of antibody increase or final titer. All patients, except Y36 who had a maximum titer at baseline, had an increase in neutralizing antibodies against the virus that was used for the treatment. In group 1 (glucocorticoids + virus), more patients ($n = 5/7$) reached the maximum measured antibody titer (Figure 1A) than in group 2 (virus only controls, $n = 3/7$) (Figure 1B), and thus glucocorticoids did not seem to reduce formation of neutralizing antibodies. The same

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Table 2. Patient Characteristics

	group 1: cases[a]	group 2: controls[b]	group 3: cases[c]	group 4: controls[d]
no. of patients	7	7	7	7
women	5	5	3	3
men	2	2	4	4
age, median (range)	45 (37−69)	54 (43−70)	47 (30−69)	62 (51−78)
cortisole dose,[e] mg/day, median (range)	135 (5−360)		120 (30−360)	
WHO, median (range)	1 (0−4)	2 (1−3)	3 (0−3)	2 (1−2)
tumor type (*n*)				
appendix carcinoma			1	
breast cancer	2		1	1
bladder cancer				1
cholangiocarcinoma			1	1
colorectal carcinoma		4	1	
esophageal carcinoma			1	
gastric carcinoma		1		
melanoma			1	
mesothelioma				2
nasopharyngeal carcinoma	1			
neuroendocrine tumor	1			
ovarian cancer	1			2
prostate carcinoma	1	1	1	
renal carcinoma				1
sarcoma	1	1		
previous treatments				
surgery (*n*)	6	6	5	6
chemotherapy (*n*)	7	7	7	7
median no. of chemotherapeutics	4	4	3	2
radiation (*n*)	6	4	2	2

[a] Group 1, patients treated with oral glucocorticoids. [b] Group 2, controls without oral glucocorticoids. [c] Group 3, cases treated with oral glucocorticoids and cyclophosphamide. [d] Group 4, controls treated with cyclophosphamide but without oral glucocorticoids. [e] Doses of prednisolon and dexamethasone have been converted to cortisole equivalents; *n*, number of patients.

seemed true for low dose cyclophosphamide as 45% (5/11) of patients who received cyclophosphamide (groups 3 and 4) reached the maximum titer (Figure 1C,D) while in patients without cyclophosphamide (groups 1 and 2) this number was 57% (8/14).

Efficacy of Treatment. Eleven patients were evaluable for efficacy by computed tomography, and 5 patients could be evaluated with tumor markers (Table 5). The inclusion criteria of this case-control safety study did not require tumors to fulfill RECIST criteria or to be positive for tumor markers, and thus many patients could not be evaluated for efficacy. Radiological evaluation was performed by an experienced radiologist by comparing scans before and ca. 2 months after treatment. Overall, four patients had stable disease (SD) response and thus the radiological clinical benefit rate was 36%. All patients had progressing tumors before treatment, and thus disease stabilization might indicate antitumor activity.

Five patients were evaluable for tumor marker responses, and all of them had decrease or stabilization in at least one marker after treatment. Thus, overall, there was evidence of possible biological activity of the treatment in 9/16 (56%) evaluable patients. This nonrandomized case-control study was not planned to analyze efficacy, and thus formal conclusions cannot be drawn. Heterogeneity of the population and treatments also limits conclusions on efficacy in this safety study. Also, tumor marker measurements alone are not an established practice to evaluate efficacy. Nevertheless, no major differences were observed between cases and controls. In group one, 1/4 patients had evidence of antitumor activity, while in the controls, 2/4 did. In group three, 3/3 patients had stable disease or better, while the numbers were 3/5 in group four.

If all glucocorticoid treated patients (=cases) are grouped together, and both markers and radiology are included, 4/7 (57%) patients had SD or better, while in controls the numbers are 5/9 (55%). Cyclophosphamide treated patients in groups 3 and 4 seemed to have a trend for higher rate of clinical benefit compared to groups 1 and 2, 6/8 (75%) versus 3/8 (38%), but it should be kept in mind that this is not a randomized comparison.

Viral Particles in Post-Treatment Serum Samples. It has been proposed that prolonged shedding of virus into blood following treatment with oncolytic virus suggests virus replication in the tumor,[36,37] and therefore this was assessed with quantitative real-time PCR (qPCR). For 23 patients,

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Table 3. Side Effects in Patients Receiving Oncolytic Adenovirus Treatment with (Cases, $n = 7$) or without (Controls, $n = 7$) Oral Glucocorticoids[a]

	cases				controls			
	grade 1	grade 2	grade 3	grade 4	grade 1	grade 2	grade 3	grade 4
				General				
fatigue	3	2	0	0	2	5	0	0
fever	4	0	0	0	4	1	0	0
sweating	1	0	0	0	0	0	0	0
chills	0	0	0	0	4	0	0	0
				Dermatological				
pruritus	1	0	0	0	0	0	0	0
				Gastrointestinal				
vomiting	1	2	0	0	0	0	0	0
nausea	1	0	0	0	0	0	0	0
anorexia	0	0	0	0	2	0	0	0
constipation	0	4	0	0	1	0	0	0
other (heart burn, dehydration)	0	0	0	0	1	1	0	0
				Hematological				
hemoglobin decrease	2	1	0	0	1	1	0	0
thrombocytopenia	2	0	0	0	1	0	0	0
leukocytopenia	1	0	0	0	0	1	0	0
				Lymphatics				
abdominal edema	3	0	0	0	1	0	0	0
limb edema	0	1	0	0	0	0	0	0
				Metabolic or Laboratory				
hypokalemia	2	0	0	0	1	0	0	0
hyponatremia	3	0	0	0	4	0	1	0
ALT increase	0	0	2	0	2	0	0	0
AST increase	0	0	2	0	0	2	1	0
hyperbilirubinemia	0	1	1	0	1	1	0	0
INR increased	0	0	0	0	1	0	0	0
				Neurological				
limb numbness	0	0	0	0	1	0	0	0
diplopia	0	0	0	0	1	0	0	0
dizziness	0	0	0	0	0	1	0	0
				Pain				
back	2	1	0	0	1	0	0	0
abdominal	0	0	0	0	4	0	0	0
injection site	0	0	0	0	1	1	0	0
head	1	0	0	0	0	0	0	0
other	0	2	0	0	4	1	0	0
				Pulmonary				
cough	0	1	0	0	1	0	0	0
dyspnea	0	3	0	0	0	2	0	0

[a] The adverse effects are reported according to CTCAE v3.0 up to 30 days after virus treatment.

pretreatment samples were available and no viral particles were detected at this time point. For most patients (25/28) at least one sample was taken between days 1 and 6 after treatment.

In analysis of groups 1 and 2, control patients who did not receive glucocorticoids (group 2) had a trend for higher amounts of virus present in the serum (Figure 2A). The highest viral load observed was 5 259 766 vp/mL measured from patient P27 on day 4 after treatment. Also, patient C65 had 1 066 835 viral copies in circulation on day 8. The highest viral load in group 1 (glucocorticoid cases), 209 683 vp/mL, was measured from patient N112 on day 7. All analyzable patients in the control group were positive for viral particles in the circulation after treatment. Interestingly, two patients from the glucocorticoid cohort (group 1) did not show presence of circulating virus at any time point. The

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Table 4. Side Effects in Patients Receiving Cyclophosphamide and Oncolytic Adenovirus Treatment with (Cases, $n = 7$) or without (Controls, $n = 7$) Oral Glucocorticoids[a]

	cases				controls			
	grade 1	grade 2	grade 3	grade 4	grade 1	grade 2	grade 3	grade 4
General								
fatigue	1	4	0	0	2	5	0	0
fever	0	4	0	0	1	1	0	0
sweating	1	0	0	0	1	0	0	0
chills	0	2	0	0	1	0	0	0
weight loss	0	0	0	0	0	1	0	0
Cardiac								
hypotension	0	0	0	0	1	0	0	0
sinus tachycardia	0	0	0	0	1	0	0	0
Gastrointestinal								
vomiting	0	1	0	0	1	0	0	0
nausea	1	2	0	0	2	1	0	0
anorexia	0	0	0	0	2	0	0	0
diarrhea	0	0	0	0	3	0	0	0
constipation	0	0	0	0	1	0	0	0
dry mouth	1	0	0	0	1	0	0	0
Hematological								
hemoglobin decrease	0	1	0	0	0	1	1	0
leukocytopenia	0	0	0	0	1	1	0	0
Hemorrhage/Bleeding								
upper gastrointestinal NOS	0	1	0	0	0	0	0	0
Lymphatics								
abdominal edema	0	1	0	0	1	1	0	0
Metabolic or Laboratory								
hypokalemia	2	0	0	0	3	0	0	0
hyponatremia	2	0	2	0	3	1	0	0
ALT increase	1	0	0	0	1	0	0	0
AST increase	1	3	0	0	0	1	1	0
hyperbilirubinemia	0	1	1	0	1	0	0	0
hyperglycemia	0	0	0	0	0	0	0	1[b]
Neurological								
dizziness	0	0	0	0	1	0	0	0
Pain								
back	0	0	0	0	1	0	0	0
abdominal	3	1	0	0	1	2	0	0
injection site	2	0	0	0	0	1	0	0
other	0	0	0	0	5	2	0	0
Pulmonary/Upper Respiratory								
cough	0	1	0	0	1	0	0	0
dyspnea	0	1	0	0	0	1	0	0
voice hoarseness	1	0	0	0	0	0	0	0
Syndromes								
flu-like syndrome	0	0	0	0	1	0	0	0
Vascular/Coagulation								
INR increased	2	0	0	0	0	0	0	0
pulmonary embolism	0	0	0	1	0	0	0	0

[a] The adverse effects are reported according to CTCAE v3.0 up to 30 days after virus treatment. [b] Patient had diabetes.

mean vp/mL value of control group for days 1−6 ($n = 7$) was 493 904 vp/mL compared to 7269 vp/mL in the case group ($n = 6$). For days 7−14 the respective amounts were 229 077 vp/mL ($n = 4$) and 42 297 ($n = 5$). The mean amount of serum viral particles from day 15 onward remained ca. 800 vp/mL in the control group and less than

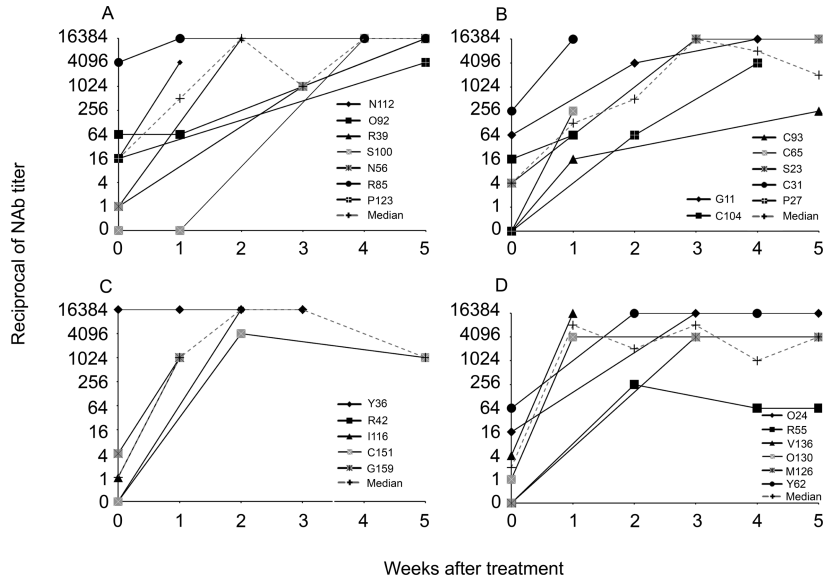

Figure 1. Neutralizing antibody (NAb) titer of patients treated with or without corticosteroids. (A) Titer in patients receiving corticosteroids in parallel with oncolytic adenovirus treatment. (B) NAb titer in the control group of (A), i.e. patients receiving oncolytic adenovirus only. (C) NAb titer in patients receiving simultaneously glucocorticoids, cyclophosphamide and oncolytic adenovirus. (D) NAb titer in control group of (C), i.e. in patients receiving cyclophosphamide together with oncolytic adenovirus.

500 vp/mL for cases. However, patient P123 (group 1, cases) had the most extended presence of virus and had samples positive up to day 52. Differences between groups were not statistically significant due to high variation and small patient number.

The qPCR readouts were generally lower in patients who received cyclophosphamide (Figure 2B). The mean titer for the group receiving cyclophosphamide and glucocorticoids was 4821 vp/mL for days 1−6 while it was 3273 vp/mL in the cyclophosphamide-only control group. On days 7−14 and 15−30 the results were in parallel with groups 1 and 2 as more circulating virus was detected in the control group, with mean values of 4618 vp/mL vs 1299 vp/mL and <500 vp/mL vs 0 vp/mL, respectively. In contrast to group 1, all cases were positive for virus on at least one time point while one control patient, M158, did not have any viral particles present in the blood after treatment. Again, the number of samples was too small to result in statistical significance.

Discussion

Corticosteroids could affect the efficacy of oncolytic adenoviruses in two opposing ways. They might act on the immune system for suppression of humoral and cellular immunity against the virus enabling more effective virus replication and cell killing.[10] In contrast, in vitro they have also been reported to directly reduce viral replication which might inhibit oncolysis and/or virus induced antitumor immunity resulting in attenuated antitumor efficacy.[13] Since this is the first study to investigate these issues in humans, emphasis was placed on safety, while virological and immunological aspects were secondary end points.

This is a first-in-man evaluation of the safety of glucocorticoids in patients treated with oncolytic adenoviruses (the 1956 study utilized wild type viruses[17]). The heterogenicity of the patient population and the retrospective nature of the study set limits on any final conclusions. However, we consider it important to report the observed trend for good safety in all groups, since glucocorticoids are widely used in patients with different cancer diagnosis and stages. Also, it may be challenging to test the safety of glucocorticoids in a randomized setting, as no efficacy benefit is expected. In these patients, glucocorticoids are not used to enhance efficacy of the treatment but to alleviate tumor mediated symptoms. In general, treatments were well tolerated with only one serious nonhematological adverse event, which was probably more related to the tumor than to treatments. Glucocorticoid use did not seem to increase the frequency of adverse events. Similar data were obtained in a clinical study where five mesothelioma patients were treated intrapleurally with a replication deficient adenovirus in combination with intravenous methylprednisolone 1 day before and 2 days after virus.[38] Gene transfer was not abrogated by the use of corticosteroids and was similar to a nonrandomized historical control group. Also, methylprednisolone was reported to reduce the febrile reaction and decrease the incidence of hypotension associated with vector administration. In parallel to our findings, glucocorticoid treatment did not prevent the development of neutralizing antibodies.

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Table 5. Tumor Responses

	response (radiology,[a] tumor markers[b])
Group 1, Cases with Oral Glucocorticoid Treatment	
P123	
N112	
S100	PD
R39	mMR
N56	PD
R85	
O92	PD
Group 2, Controls without Oral Glucocorticoid Treatment	
G11	mMR
P27	
S23	PD
C31	mSD
C65	
C104	
C93	PD
Group 3, Cases with Oral Glucocorticoid and Cyclophosphamide Treatment	
G159	
P74	
Y36	
I116	
R42	mPR
C143	SD
C151	SD
Group 4, Controls with Cyclophosphamide but without Oral Glucocorticoids	
V136	SD
M158	
Y62	
O24	mMR
O130	SD
M126	PD
R55	PD

[a] PR, partial response = more than 30% decrease in sum of tumor diameters; PD, progressive disease = more than 20% increase in sum of tumor diameters; SD, stable disease = no response or progression; blank, data not available. [b] The same percentages were used for tumor markers measured from blood (mPR, mSD, mPD), and mMR was additionally used to indicate a minor response = 12−29% decrease.

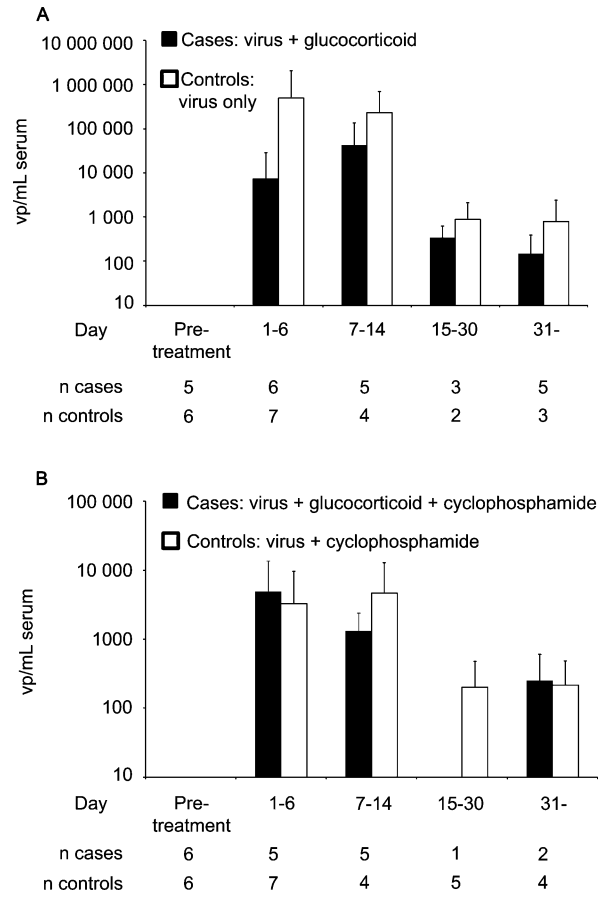

Figure 2. Detection of viral particles (vp) from patient serum samples. Quantitative PCR was used to measure viral particles/mL of serum at different time points. (A) Number of viral particles in patients receiving only oncolytic adenovirus or oncolytic adenovirus with oral glucocorticoids. (B) Number of viral particles in patients receiving oncolytic adenovirus and cyclophosphamide with or without glucocorticoids.

There is emerging data suggesting that oncolytic replication can break immunological tolerance for induction of antitumor immune responses.[11,39] Also, antiviral T-cell responses can help in tumor eradication,[12,40] and this phenomenon can be amplified by utilization of immunostimulatory transgenes such as GM-CSF.[11,41] Within the lymphocyte population, T-cells are affected most by corticosteroids.[42,43] Therefore, if cytotoxic T-cell responses are attenuated, it might reduce the overall antitumor activity of oncolytic adenoviruses. This was reported in a syngeneic murine neuroblastoma model where a high dose (5 mg/kg) dexamethasone reduced the overall antitumor activity of an oncolytic herpes simplex virus 1 by inhibiting the cytotoxic T-cell response against tumor cells. Thus, prolonged dexamethasone administration reduced the efficacy of treatment demonstrating the importance of cytotoxic T-cell response in oncolytic virus mediated tumor control. However, dexamethasone administration did not have any effect on the oncolytic potency of the virus.[44]

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Another aspect of glucocorticoid use that might negatively impact the immunogenic potential of oncolytic virotherapy is the antipyretic effect since previous data suggests that elevated temperature might help in development of antitumor immune responses.[45] In our study we did not detect reduced febrile reactions in patients receiving glucocorticoids and, in contrast, more patients in the case group receiving cyclophosphamide had fever than patients in their control group. This could be explained by the rather low glucocorticoid doses used here which were not designed to reduce the febrile reaction.

Patients in groups 3 and 4 were treated with concomitant cyclophosphamide. Although cyclophosphamide can cause immunosuppressive effects when used at high doses, the goal here was the opposite. Low dose cyclophosphamide, especially with metronomic dosing, can reduce suppressive cells, resulting in enhanced immunity.[21–23] In this regard, it was not surprising that neither the febrile reaction nor induction of antibodies was thwarted.

While glucocorticoids dampen the T-cell response in general, they also modulate the response from Th1 cellular immunity toward Th2 humoral immunity by altering macrophage response.[46] Cortisone restrains B cell proliferation but promotes generation of antibody secreting plasma cells, and antibody production is compromised only at very large doses.[47] Thus it was not surprising that differences in the neutralizing antibody response were not detected in our patients. Even if glucocorticoids might reduce virus replication, which might slow production of neutralizing antibodies, this would perhaps be counteracted by the concurrent switching from Th1 toward Th2 signals.

In the context of the overall effect that glucocorticoids might have on antitumor immunity, it is interesting to speculate that perhaps Th2 stimulation could also increase the formation of antibodies against the tumor, which might balance possible unwanted effects of glucocorticoids on T-cells. These topics are interesting for further studies. Optimally, samples should be obtained from a randomized trial to avoid confounding due to imbalance in patient populations.

This study was designed to evaluate safety and is underpowered to detect differences in efficacy. Thus, no definitive conclusions on efficacy can be drawn. Nevertheless, the preliminary data reported here suggests that glucocorticoid treatment does not prevent antitumor activity as possible clinical benefits were seen in both cases and controls. Corticosteroid treated patients had a trend for lower amounts of virus detected in the circulation. One explanation could be that glucocorticoids enhance the integrity of microcirculation by limiting the permeability of capillary endothelium[48] thus reducing viral spread from tumors. An obvious drawback of this phenomenon might be that the vascular infection of metastatic tumor sites could be compromised. Alternatively, the antiviral effects of glucocorticoids might have directly caused a reduction in virus replication.[13] Although much remains to be studied, one of our key findings was that glucocorticoid use did not seem to enhance viral replication which could have had significant consequences regarding patient safety.

These findings, if confirmed in larger patient series, could have important implications regarding the use of oncolytic viruses. For example, our data supports the safety of glucocorticoid use if patient symptoms or laboratory findings require it. An example of this situation would be virus replication in liver metastases and consequent inflammatory swelling of the tumor which might in turn constrict bile ducts. Further, it seems safe to administer oncolytic adenoviruses to patients who use glucocorticoids for other reasons, such as tumor associated loss of appetite or rheumatoid arthritis. Finally, as it has been reported that glucocorticoids can be useful for reducing virus related immediate effects such as cytokine release, hypotension, and induction of antibodies, our preliminary safety results suggest that these aspects could be studied formally in prospective trials. However, the possible negative impact on antitumor immunity is important to study further as it is a possible drawback for strategies aiming at immunogenicity.

In conclusion, using oral glucocorticoids in patients receiving oncolytic adenovirus, with or without low dose cyclophosphamide, does not seem to affect safety. Further prospective studies with larger patient populations are needed to confirm these findings and to evaluate the impact of these therapies on efficacy.

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