RESEARCH ARTICLE
Complement activation by recombinant adenoviruses

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Recombinant adenoviruses are currently the most important vector system in gene therapy. Adenoviruses frequently cause upper respiratory tract infections in humans and anti-adenoviral antibodies are found in 35–70% of the population. Therefore in the majority of potential patients receiving adenoviral gene therapy, the contact of virus particles and blood will lead to the formation of antigen–antibody complexes. These complexes have the ability to induce inflammatory reactions via an activation of the complement system. We have determined the level of C3a (the most reactive complement component) generated in isolated citrate plasma of healthy individuals after challenge with recombinant and wild-type adenoviruses in amounts corresponding to virus blood levels to be expected in patients during adenoviral gene therapy. All plasma samples containing anti-adenoviral antibodies showed a substantial, dose-dependent gener-ation of C3a. A virus plasma level of about 7.5×10^9 particles/ml (which was calculated to be the highest blood level reached during clinical trials in the past) induced an average release of about 3000 ng/ml C3a (baseline levels <140 ng/ml). Analyzing the nature of anti-adenoviral antibodies showed, that not only antibodies with neutralizing properties (anti-Ad5), but also non-neutralizing anti-adenoviral antibodies are capable of complement activation. This study suggests that complement activation can be ignored in local low-dose applications of recombinant adenoviruses, but warrants attention after systemic application of large viral quantities. In clinical protocols aiming at systemic virus application, measures for monitoring and controlling the complement system should be included on a regular basis. Gene Therapy (2001) **8,** 1794–1800.

Introduction

Recombinant adenoviruses are highly efficient *in vivo* gene transfer vehicles and offer the basis for a broad spectrum of therapeutic strategies especially in cancer therapy. Current concerns regarding their application in humans are derived from the appearance of severe life-threatening inflammatory reactions which were seen in one patient during a clinical trial for the correction of an inborn liver defect. This patient received a dose of 3×10^{11} Ad5 particles/kg via hepatic artery infusion.[1,2] Some hours after virus infusion he developed a complex of clinical symptoms including hyperpyrexia, clotting disorders, thrombocytopenia and jaundice. Three days after virus application he died due to the onset of severe lung perfusion disturbances (adult respiratory distress syndrome, ARDS) and multiple organ failure (MOF). Four of five patients who received a comparable high virus dose also developed hyperthermia of about 40°C, but recovered without major problems.[2] Viral capsid proteins have been attributed to be responsible for this inflamma-tory reaction, but the precise pathomechanism remains unclear. In this article, we would like to provide evidence that a strong complement activation might have been involved in the observed reactions. It has been known for a long time that adenoviruses are able to induce an antibody-dependent activation of the complement system in human plasma, but the level of activation which is reached during clinical adenoviral gene therapy (especially in intravascular infusions) might have been underestimated in the past. We will show that challenge of isolated human plasma with serotype 5 adenoviruses in amounts corresponding to blood levels reached in the above-mentioned trial, generates a level of complement activation, which holds the potency to induce serious inflammatory reactions.

The complement system
Complement activation is a physiological defense mechanism which induces rapid destruction and phagocytosis of inoculated microbes and foreign bodies. Complement activation can occur by antigen–antibody complexes (classical pathway) and directly after plasma contact with a number of microbial components or foreign surface (alternative pathway).[3,4] The majority of adults carry antibodies against adenoviral hexon group antigens[5,6] due to previous adenoviral infections. The direct inoculation of

viral particles into the circulation in such individuals will usually induce the formation of antigen–antibody complexes and subsequently complement activation. Factors influencing the number of antigen–antibody complexes will depend on the route of administration (systemic or local), the virus dose, the amount and type of pre-existing antibodies and the activity of regulatory proteins. During the early phase of complement activation, the anaphylatoxins C3a and C5a are generated. These mediators induce a broad variety of inflammatory reactions, such as IL-1 and TNF release,[7–9] activation of neutrophilic granulocytes and macrophages,[10] contraction of pulmonary vasculature,[11] changes in vascular permeability,[12] and platelet aggregation.[13] Systemic activation of the complement system, as known from patients suffering from sepsis, severe burns or injuries, could induce autodestructive inflammatory reactions, such as adult respiratory distress syndrome (ARDS) or multiple organ failure (MOF).[14–18] Both types of clinical symptom complexes have been observed in the patient who died during the OTCD trial, which suggests an involvement of the complement system. In this article, we will discuss prophylactic measures which might help to control or avoid excess activation of the complement system after adenoviral gene therapy in humans.

Results

Anti-adenoviral antibodies in human plasma
Citrate blood was taken from 18 healthy volunteers by brachial vein puncture and anti-adenoviral antibody titers were determined by ELISA, complement fixation test (CBR) and neutralizing assay. Sixteen of 18 citrate plasma samples contained anti-adenoviral antibodies titers ranging between 11 and 708 U/ml (IBL-ELISA). The presence of anti-adenoviral antibodies was also determined by CBR (titers 1/10–1/1/80). In 12 of 16 samples, neutralizing antibodies were detected (1/256–1/1024), while four samples did not contain antibodies with neutralising properties (<1/4). Two samples did not contain any detectable anti-adenovirus antibodies (negative in all three test systems).

Dose-dependency studies
Plasma samples were challenged with virus particles (Ad5) in four different concentration (5×10^7–5×10^{10} particles/ml plasma) at 37°C. Reaction was stopped after 90 min by addition of 1/10 vol 0.1 M EDTA and C3a-desArg levels were determined by ELISA (Figure 1). To evaluate possible inhibitory effects of cellular components of the blood, citrate blood samples were challenged with equivalent amounts of virus particles (40% cellular volume was considered). After challenging plasma (blood) samples with 5×10^7 and 5×10^8 particles/ml, a moderate generation of C3a-desArg (180–430 ng/ml and 460–700 ng/ml, baseline <140 ng/ml) was determined, while a substantial further increase in complement activation was measured after incubation with 5×10^9 and 5×10^{10} particles (2300–4900 ng/ml and 8800–24000 ng/ml) (Figure 1). The presence of cellular components of the blood reduced the level of complement activation by about 20% (data not shown). Comparing C3a-desArg levels of plasma samples containing neutralizing antibodies and those without neutralizing antibodies revealed

higher levels of activation in the first group (about 20 900 ng/ml *versus* 9300 ng/ml). The data show that antibody-dependent complement activation does also occur in the absence of neutralizing antibodies.

To judge the complement-activating potency of Ad5 particles, control experiments were performed with heat aggregated human IgG (HAAG, 1–12 mg/ml) one of the most potent activators of the complement system (Figure 1). A linear dose dependency of complement activation was seen after challenge with 1 and 2 mg/ml HAAG, while incubation in higher amounts (5 and 12 mg/ml) led to saturation of C3a release.

Complement activation by different adenoviral serotypes
To investigate whether complement activation is a specific problem of serotype 5 adenoviruses which might be solved by switching the adenoviral serotype, we challenged six plasma samples with Ad serotype 1, 3, 4, 5 and 9 particles. Complement activation was also found after challenge with serotype 1, 3 and 9 while only low values were seen after stimulation with Ad4 (subgenus E) (Figure 2). The level of C3a-desArg release (after stimulation with the same viral serotype) varied between plasma samples from different volunteers on average by a factor of 2 to 3.
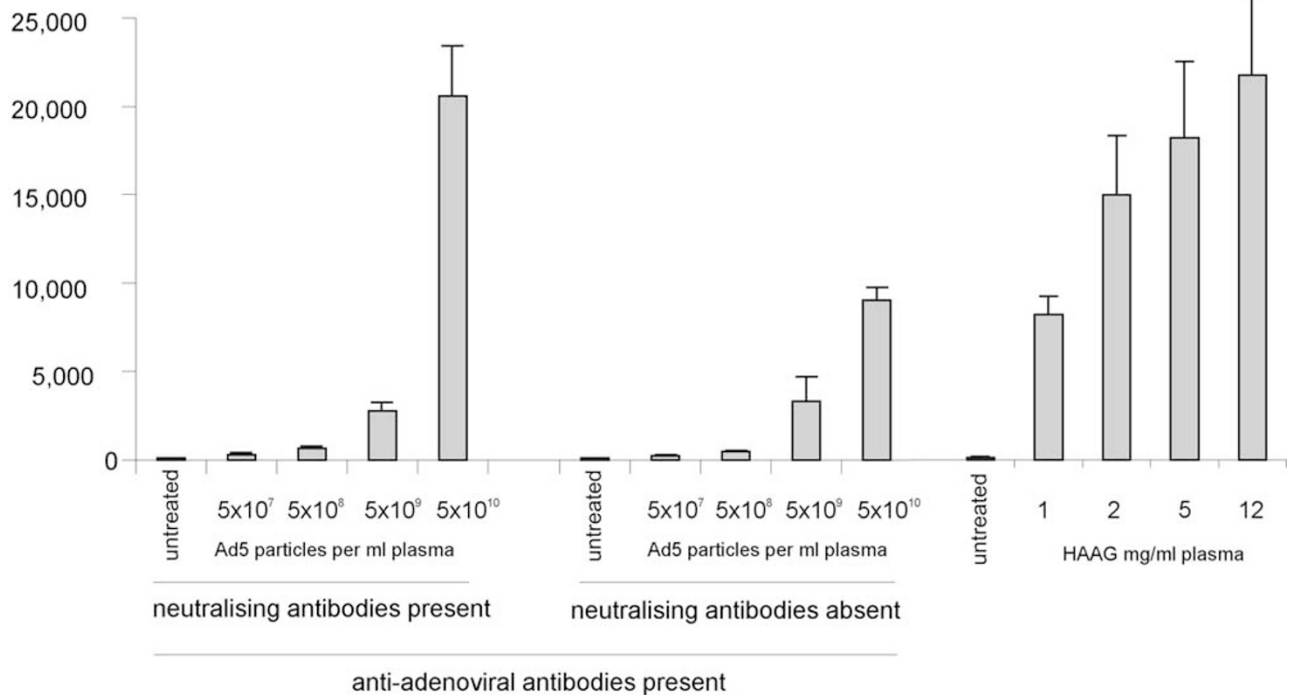
Antibody dependency of complement activation
In samples with no detectable anti-adenovirus antibodies, no complement activation was noted after challenge with Ad5 particles. This finding suggests a strict antibody-dependence of complement activation in human plasma. To further verify the role of pre-existing antibodies for complement activation in this system, immunoglobulin was removed from citrate plasma samples by gel filtration on protein-G sepharose. The filtration procedure itself induced some complement activation, but after removal of the immunoglobulins, the addition of virus particles no longer induced C3a release (Figure 3). After reconstitution of Ig-depleted plasma with polyvalent immunoglobulin (containing an average amount of neutralizing anti-Ad antibodies at a titer of 1/256) to a concentration of 2.5 mg Ig/ml (about 20% of physiological Ig serum concentration), the previous responsiveness to adenovirus challenge was demonstrated. Complement activation via the classical pathway by adenovirus was confirmed in an additional experimental system. Plasma samples were incubated with adenoviruses in the presence of EGTA. The calcium-binding capacity of EGTA blocks the antibody-dependent classical pathway of complement activation, whereas the alternative pathway remains unaffected, thus allowing differentiation of both pathways. EGTA blocked the virus-induced complement activation completely (Figure 3). These findings reveal that the alternative, ie antibody-independent pathway of complement activation after challenge with adenoviruses, is not relevant in human plasma.

Control experiments
Complement activation after challenge with recombinant adenoviruses does not depend on the nature of the encoded transgene as no difference was observed after challenge with Ad-p53, Ad-hAAT and Ad-hLDL (data not shown).

To exclude a potential role of host cell proteins present as contaminants in the virus preparation, Hela, A549 and

C3a-desArg ng/ml plasma

Figure 1 Dose-dependency of complement activation in human plasma after stimulation with recombinant serotype 5 adenoviruses in the presence and absence of neutralizing antibodies. Citrate plasma samples of healthy volunteers (n = 4) were incubated with viral particles in four different concentrations (5×10^7–5×10^{10} particles Ad-lacZ/ml plasma). The reaction was stopped after 90 min at 37°C by addition of EDTA and C3a-desArg levels were determined subsequently. All samples included in this comparison contained anti-adenoviral antibodies (ELISA; 11–702 U/ml), while half of the samples contained neutralizing antibodies (titer 1/256–1/1024) and the second half did not contain neutralising antibodies (titer <1/4). Control experiments were performed with heat-aggregated IgG (HAAG). Average levels of plasma C3a-desArg (n = 3) generated after stimulation with 1/5 vol 1, 2, 5 and 12 mg/ml HAAG. After challenge with 5 and 12 mg/ml HAAG C3a-desArg generation starts to saturate.

293 cell lysates at different concentrations were used as additional control. The protein content in 10 µl virus suspension (4×10^{12} particle/ml) was determined as 0.06 µg. A comparable dose of cell lysate had no influence on complement activation (data not shown).

Discussion

The data presented suggest that complement activation could be expected in the majority of potential patients receiving adenoviral gene therapy. Since complement activation is a physiological defense mechanism, controlled local activation does not carry any health risk for a patient. Potential hazardous reactions could occur if complement-activating components enter the circulation and induce the formation of large numbers of circulating antigen–antibody complexes. Therefore the level of induced complement activation will depend on the applied dose and the route of application (local, systemic). After local low-dose adenovirus applications, complement activation can be most likely ignored. However, direct intravascular high dose applications (>5 × 10^{10} particles/kg) might carry an increased risk for the development of adverse inflammatory reactions.

In a previous study in rabbits, we have shown that local application via the portal vein cannot prevent a rapid spread of virus into the circulation.[19] In clinical trials for the treatment of malignant tumors[20] or the correction of monogenetic diseases,[1] doses of up to 3 × 10^{11}

particles per kg body weight have been applied by intravascular application via the hepatic artery. Immediately after virus infusion, the blood or blood plasma, respectively, will contain about about 7.5×10^9 viral particles per ml. Higher concentration could be expected at the site of inoculation. A particle concentration of 7.5×10^9 Ad5 particles per/ml induces an average level of complement activation (in anti-Ad antibody carriers) of about 3000 ± 1000 ng C3a-desArg/ml in isolated human plasma. An *in vivo* level of 3000 ng/ml C3a would bear a high risk for serious inflammation of the vascular bed with subsequent organ damage. Clinical experience regarding serious effects of complement activation is derived from patients suffering from burns or severe injuries. The mobilization of tissue factors from damaged tissue has the potency to induce systemic complement activation. Clinical symptoms of complement-mediated inflammation normally arise in the first days after the initial trauma. Characteristic manifestations are edema and gas exchange disturbances of the lungs (adult respiratory distress syndrome, ARDS) and failure of liver and kidney function (multiple organ failure, MOF). Patients often die not as a direct consequence of the initial accident, but from the subsequent inflammatory immune response. In clinical studies, the plasma values for the onset of these syndromes has been found in the range of 1000 ng C3a-desArg/ml.[15,21,22]

The *in vitro* data presented here, only took into account blood-derived inhibitory factors. After *in vivo* application,

C3a-desArg ng/ml plasma
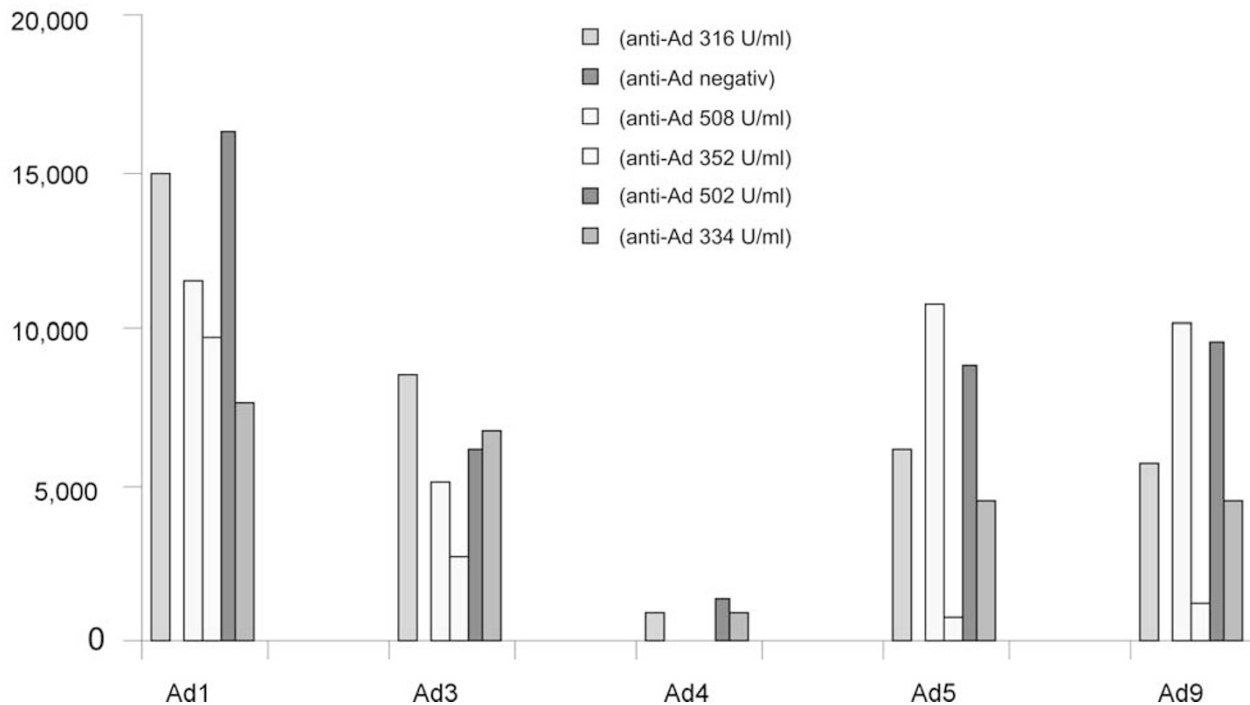
Figure 2 Crossreactivity between different adenoviral serotypes. Citrate plasma samples of six probands (anti-adenovirus antibodies in U/ml; IBL-ELISA) were challenged separately with 5×10^{10} particles/ml wt Ad1, 3, 4, 5 and 9. Ad1 induced the strongest and Ad4 (subgenus E) the weakest C3a release compared with each other serotype.

additional regulatory mechanisms involving vascular endothelial cells and the reticulo-endothelial system (liver, spleen) will influence complement activation. However, the level of C3a-desArg released after *in vitro* challenge strongly suggests complement activation to be relevant for *in vivo* applications, particularly in high-dose intravascular applications in humans.

The studies show with serotype 1, 3, 4 and 9, that there is a broad cross-reactivity between different serotypes with regard to complement activation and, therefore, serotype switching might be of only limited value.

There is only a weak correlation between the level of complement activation and the number of pre-existing antibodies. It is interesting that it is the presence rather than the level of anti-adenovirus antibodies that appears to be important for triggering the complement system.

Prophylactic measures
Apart from monitoring complement activation on a regular basis during the trial, the data indicate that a number of additional measures might be appropriate. Before virus application, the level of all anti-adenoviral antibodies should be determined. Measuring only the titer of neutralizing antibodies provides no information about the total amount of relevant, ie complement-activating antibodies. The individual differences in complement reactivity against adenoviruses (up to a factor of 5) will make it difficult to define a dose threshold which would reliably exclude uncontrolled complement activation. It might be more appropriate to define an individual threshold performing *in vitro* complement studies with patients' plasma before gene therapy, in a kind of bedside

test to predict the amount of *in vivo* activation to be expected. Patients showing a strong *in vitro* reactivity could benefit from appropriate pharmacological measures. Prophylactic steroid treatment will have no influence on the extent of complement activation, but steroids are known to reduce subsequent inflammatory reactions, like cytokine release and macrophage activation.[23,24] C1-esterase inhibitors have been proven for many years to be beneficial for the treatment of hereditary angioedema. They are currently tested as a protective measure against complement-mediated inflammatory tissue damage[25–27] and would help to avoid or reduce adenovirus-induced complement activation.

Recombinant adenoviruses hold an important potency especially for tumor therapy. Current applications are restricted to patients in an advanced and otherwise untreatable state of malignant growth. Measures which could help to control vector-related side-effects, might allow earlier intervention and improve the therapeutic benefits of adenoviral vector technology.

Materials and methods

Adenoviral procedures
Adenovirus wild-type specimens of serotype 1, 3, 4, 5 and 9 were provided by the Institute of Virology of the Medizinische Hochschule Hannover, Germany. Wild-type viruses were grown on Hela and A549 cells. Recombinant serotype 5-derived adenoviruses encoding the human p53 gene were kindly provided by Wei-Wei Zhang (Anderson Cancer Center, Houston, TX, USA), and

C3a-desArg ng/ml plasma
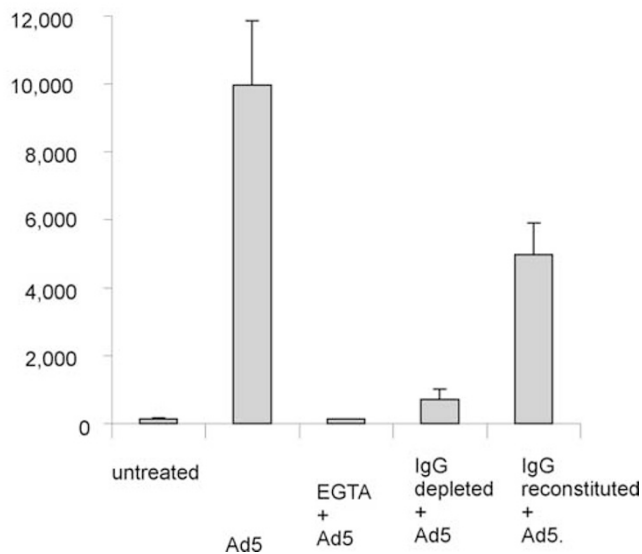
Figure 3 Evaluation of antibody independent complement activation by serotype 5 adenoviruses. To distinguish antibody-dependent and antibody-independent activation pathways, plasma samples were incubated with adenovirus particles under varying conditions: (a) untreated (control), (b) adenovirus only, (c) pretreatment with EGTA (MgCl₂) (blocks the antibody-dependent activation), (d) depletion of immunoglobulin (Ig) by G-sepharose pretreatment and (e) reconstitution of previously depleted plasma with purified polyvalent human Ig. C3a-desArg release was fully blocked by EGTA pretreament. The increased background levels in Ig-depleted plasma are the result of the G-sepharose treatment. In Ig-depleted plasma, only minimal response was measured after adenovirus challenge, while reconstitution of Ig (20% of physiological levels) reinstalled the previous sensitivity to adenovirus exposure. The outcome illustrates the strict antibody dependence of adenovirus-mediated complement activation in human plasma.

recombinant adenoviruses encoding the human alpha-1-antitrypsin (hAAT) gene and the gene for bacterial β-galactosidase (*LacZ*) were a kind gift of Mark Kay (Stanford University, USA). A recombinant virus encoding the gene for the human LDL receptor was generated according to the procedure of McGrory *et al*.[28] Recombinant viruses were propagated on 293 cells and lysed by three freeze–thaw cycles. Purification was performed by two rounds of cesium chloride density gradient centrifugation and cesium chloride was removed by gel filtration on sephadex G-25 columns (Pharmacia, Uppsala, Sweden). Equilibration was performed with a buffer containing 3 mM KCl, 1 mM MgCl₂, PBS. Sterile filtration was carried out using a 0.45 μM filter (Schleicher and Schuell FP 030/2, Dassel, Germany). Virus suspensions were supplemented with 10% glycerol and stored in aliquots at −78°C. Titration was carried out in endpoint dilution assays on 293 cells in 96-well plates as described elsewhere.[19] In addition, particles were counted spectrophotometrically as described by Mittereder *et al*.[29] Biological titers were presented as infectious particles (i.p.)/ml and physical titers as particles/ml. Particle concentration of adenoviral stock solutions ranged between 2–5 × 10¹² particles/ml. Biological titers were on average one order of magnitude below physical titers.

Anti-adenoviral antibodies

Citrate plasma was obtained from 18 healthy volunteers (A–L) and anti-adenoviral antibodies were determined in a commercial ELISA (anti-adenovirus; IBL, Hamburg, Germany). Viral target antigens employed in the IBL-ELISA are derived from serotype 5 adenoviruses (subgenus C) only. In addition anti-adenoviral antibodies were also determined in a complement fixation test (CFT) for adenovirus antibodies (Virion-Serion, Würzburg, Germany) and by neutralizing assay. The CFT employs a mixture of adenovirus antigens derived from subtype 2, 3, 4, 7 (subgenera B, C, E).

The titers of neutralizing antibodies were determined by the ability of proband sera to prevent infection and subsequent replication of 293 cells by recombinant viruses. 30 μl previously heat-inactivated (30 min at 56°C) serum samples were serially diluted in three independent tests and incubated with 30 μl virus suspension, containing 5×10^2 i.p. Ad-hLDL (diluted in medium without FCS) for 60 min at 37°C in 96-well plates. 50 μl of the serum-treated virus samples were transfered to 293 cells, seeded in 96-well plates at a density of 3×10^3 cells/well (in 150 μl medium, 10% FCS) on the previous day. The neutralizing antibody titer was calculated from the reciprocal cytopathic effect observed 7 days after infection.

Complement studies

Citrate blood was obtained from healthy volunteers by cubital vein puncture in 4.5 ml vials containing 0.129 M sodium citrate (vacutainer system 367705; Becton Dickinson, NJ, USA). In one part of the sample citrate, plasma was immediately separated by centrifugation (10 min, 800 g) and aliquots of 350 μl plasma were incubated with 10 μl virus suspension/storage buffer containing 5×10^7, 5×10^8, 5×10^9, and 5×10^{10} particles/ml plasma for 90 min at 37°C. For complement studies in full blood 590 μl citrate blood were incubated with the same amounts of virus particles as applied in the studies with plasma. The reactions were stopped by addition of 1/10 vol 0.1 M EDTA. Plasma was separated from blood samples (after challenge with Ads) by centrifugation and stored at −78°C for further analysis. For discrimination of the classical activation pathway from the alternative pathway, citrate plasma was pretreated with EGTA (1/10 vol 0.1 M EGTA) before incubation with adenoviruses. In a parallel experiment, EGTA was supplemented with 3 mM MgCl₂. Comparable results were observed as with EGTA alone. The reaction was stopped after 90 min at 37°C by addition of EDTA (1/10 vol. 0.1 M EDTA) and samples were immediately stored at −78°C for further analysis.

For studying complement activation in the absence of immunoglobulin, depletion was performed by gel filtration of the plasma samples twice using protein-G sepharose columns (High Trap Protein G columns; Pharmacia, Freiburg, Germany). Reconstitution experiments with purified Ig showed in controls that the reactivity of the complement system was not impaired in the depleted plasma. Human gamma-globulin from pooled plasma (25 mg Ig/ml; Dianova, Hamburg, Germany) of a known anti-adenovirus antibody titer was used and incubations with four different concentrations of viral particles were performed as described above. C3a-desArg levels were determined as previously described in an ELISA system

using a monoclonal antibody to a neoantigenic epitope of C3a-desArg.[30,31]

Control experiments

Heat-aggregated IgG (HAAG) was generated according to standard techniques. In brief, 15 mg/ml human IgG in PBS (Dianova) were heated for 30 min at 63°C. Particle aggregates were removed by microcentrifugation (5 min, 12 000 g) and the remaining protein concentration was determined photometrically. Heat-aggregated IgG was diluted in PBS to a final concentration of 1, 2, 5 and 12 mg and 50 μl of these dilutions were incubated with 200 μl citrate plasma for 90 min at 37°C. Reactions were stopped by addition of 1/10 vol 0.1 M EDTA. A549, Hela and 293 cell lysates were generated by harvesting and pelleting 5×10^7 cells of each line. Cells were resuspended in 1 ml complete medium (10% FCS), three freeze–thaw cycles were performed. Protein content in the supernatant was measured spectrophotometrically using the Bradford assay. Citrate plasma was incubated with a mixture of complete medium and cell lysates in four different concentrations (0.045, 0.45, 4.5 and 45 μg/ml plasma). The reaction was stopped after 90 min at 37°C by addition of EDTA as described above. The protein content at the highest virus dose employed (5×10^{10} particles) was determined as 0.06 μg. To exclude a direct interaction between the surface proteins of recombinant viruses and the C3a-desArg ELISA-system recombinant viruses (5×10^7–5×10^{10} particles) were diluted in 5% bovine serum albumin or in heat-inactivated plasma, respectively. No activation was noticed after 90 min incubation. In addition injection buffer (containing 10% glycerol) was incubated with plasma in the absence of viruses. Again no activation was found.

Acknowledgements

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