Blood clearance rates of adenovirus type 5 in mice

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Persistence of adenovirus type 5 in blood has implications for the pathogenicity of the virus infection and for the use of this virus in oncolysis and gene therapy. In this study, the kinetics of adenovirus clearance from blood in mice has been evaluated. After a single inoculation of concentrated virus into the vena cava, virus half-life was less than 2 min. Depletion of Kupffer cells (KC) resulted in increased viraemia. After tail-vein injection, virus and latex beads co-localized within KC. An important factor in clearance by KC is the negative charge of particles. Deletion of the hexon hypervariable region 1 acidic stretch decreased the negative charge of the virion but it did not increase blood persistence. Coating with PEG ('PEGylation') reduced the clearance rate but also reduced infectivity.

Adenovirus type 5 (Ad5) infects the respiratory epithelium and can reach the bloodstream through lymphatics (Horwitz, 1996). Persistence in the circulation can affect virus pathogenesis and the outcome of therapies that use recombinant adenoviruses (Hitt et al., 1997; Kirn & McCormick, 1996). Interventions that slow clearance would favour tissue- or tumour-specific targeting approaches based on systemic delivery. We studied the kinetics of blood clearance of Ad5 in mice to obtain a better understanding of the limitations of adenovirus as a therapeutic agent.

Different viruses are cleared from the blood by Kupffer cells (KC) (Kim et al., 1982). Blockage of macrophages has indicated the interaction of adenovirus and KC (Lieber et al., 1997; Wolff et al., 1997; Worgall et al., 1997). When KC are not blocked, 90% of the adenovirus genome is cleared from the liver in 24 h (Worgall et al., 1997). To demonstrate the direct interaction of adenovirus with KC, we injected Ad-CMV-LacZ virus (Alemany et al., 1996) labelled with red fluorophore (Cy3, Amersham; Leopold et al., 1998) into the tail vein of BALB/c mice (10^{11} virions per mouse). Ten minutes later, we injected fluorescein-labelled latex beads (2 µm diameter; Sigma) as a KC tracer. After a further 10 min, livers were resected and embedded for cryosection. The co-localization of virus and latex beads within KC was readily observed as patchy aggregates all over the sinusoids (Fig. 1C). In most instances, co-localization was evident as yellow fluorescent regions containing several latex beads. Some red fluorescence was also observed with no beads, probably due to incomplete marking of all KC. In order to confirm further the role of KC in virus uptake, KC were depleted with GdCl₃ (three tail-vein injections of 200 µl of a 2 mg/ml solution at 54, 30 and 6 h before injection of adenovirus and latex beads). The distribution of adenovirus in the liver changed from the patchy pattern to a regular, diffuse pattern enriched towards the perivenous region of the liver (Fig. 1D). Whether this periporal to perivenous enrichment reflects differences in the levels of coxsackievirus/adenovirus receptor (CAR) in parenchymal or endothelial cells or the effect of other factors requires further investigation. The patchy distribution of adenovirus capsids taken up by KC reflects the main deposition site of the inoculum. More sensitive techniques to detect viral capsid proteins would probably reveal the distribution of smaller amounts of virus in other cell types. An attempt has recently been made to quantify the distribution of an adenovirus vector among different liver cell types after intravenous injection in rats (Davern et al., 1999). Although hepatocytes, stellate cells, KC and endothelial cells are transduced, the measurements of gene expression probably do not reflect the amount of virus adsorption. Indirect evidence that each liver cell type interacts with adenovirus comes from studies of liver toxicity (Lieber et al., 1997).

Viraemia varies among different viruses (Kirn et al., 1982). One of the most important determinants of blood clearance is size. Rapid clearance (> 99% of an intravenous inoculum within 1 h) occurs with large viruses or small viruses opsonized with antibodies or complement. The net charge of the viral particle also affects the clearance kinetics (Jahrling & Eddy, 1977). To measure blood clearance, we flanked the GFP-expression cassette from pTracer-SV40 (Invitrogen) with Ad5 sequences (left, bp 27040–28045; right, bp 30863–31948) and inserted it into E3 by homologous recombination (Chartier et al., 1996). GFP transduction provides a faster and more
Fig. 1. Distribution of adenovirus capsid after intravenous injection in BALB/c mice. Fluorescent microscopy of liver sections after injection of $10^{11}$ particles of red-labelled virus (Ad-CMV-LacZ) and fluorescein-labelled latex beads to mark KC. Nuclei were counterstained with Hoech 33342. Red, green and blue fluorescence were captured independently and superimposed. (A) Control of latex beads alone. (B) Control of virus alone. (C) Virus and latex beads: co-localized virus and KC are encircled. (D) Virus and latex beads: distribution of virus after depletion of KC with GdCl$_3$. CV, Centrilobular vein. Magnification, $\times$100.

sensitive measurement of functional virus particles (transducing units, t.u.) than a plaque assay (Mittereder et al., 1996). The recombinant virus was generated by transfection of the viral genome into 293 cells, purified by CsCl and dialysed against PBS. The titre was determined by serial dilution in DMEM/5% FCS and infection of A549 cells. No differences in titre were observed when virus was serially diluted in plasma or whole blood and incubated for 1 h prior to the addition of medium, indicating no direct inactivation of virus by blood components (data not shown). Direct inactivation was also ruled out by using quantitative transduction assays of A549 cells with a luciferase-expression Ad5 vector. Serial dilution of purified virus in whole heparinized blood followed by 1 h incubation did not affect the transduction levels at all m.o.i. tested (e.g. at m.o.i. = 40, $5.1638 \pm 10725$ luciferase units per 25 000 cells in 24 h in PBS compared to $67452 \pm 18101$ in blood).

These results agree with the lack of haemagglutination of murine erythrocytes by Ad5, a property also observed with monkey and human erythrocytes (Hierholzer, 1973). To study blood clearance, a single dose of $10^{10}$ t.u. in 200 µl PBS was injected into the vena cava under anaesthesia (100 mg/kg ketamine/xylazine). A small aliquot of blood (20 µl) was collected at different time-points from the tail vein and virus in plasma was measured by serial dilution and infection of A549 cells. Fig. 2(A) shows a representative fluorescence microscopy field of the same dilution of blood samples collected 1–60 min after virus injection and Fig. 2(B) shows the virus titre (measured as t.u.). Considering a blood volume of 2 ml (7.3% of body weight), the estimated virus concentration in blood at $t_0$ was $5 \times 10^6$ t.u./µl. At 1 min post-injection, we found only 10% of the estimated injected dose. Comparing levels after this first minute is more meaningful, because these are actual titres.
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A total of $10^{10}$ t.u. virus containing a GFP-expression cassette was injected via the vena cava into BALB/c mice in a surgical procedure. At the indicated times post-injection, blood was drawn from the tail into heparinized tubes. Clotting of the tail section was prevented with heparin and bleeding between intervals was suppressed with pressure. The titre at each time-point was assessed by serial dilution of the plasma followed by infection of A459 cells. Transducing units were measured 16 h post-infection. (A) Fluorescence microscopy of wells corresponding to the 1000-fold dilution. The three rows show Adwt, Adwt after GdCl₃ treatment (Adwt Gd) and AdhN at different times (in min). (B) Quantification of the virus titre (as t.u./µl blood) with Adwt (■), Adwt after GdCl₃ treatment (○), AdhN (▲) and PEGylated Adwt (▼). The $t₀$ value was calculated as the total injected dose distributed in 2 ml (7-3% body weight) ($n =$ 3 mice).

From 1 to 5 min, we found a 7-fold reduction in virus titre from $6.5 \times 10^5$ t.u./µl to $10^5$ t.u./µl, indicating that the half-life of adenovirus in blood is less than 2 min. The virus level after 1 h was around 0.01% of the level found after the first minute. This clearance curve of Ad5 can be compared to the clearance rate of other non-blood-borne viruses that are efficiently phagocytosed by KC in mice, such as frog virus 3, vesicular stomatitis virus and Newcastle disease virus (Brunner et al., 1960; Steffan, 1997). For these viruses, viraemia decreases according to an exponential function of time, the slope of which indicates the clearance efficiency. A clearance rate constant, $K$, can be defined as the negative slope of the line obtained by plotting the log of the virus concentration against time. A clearance rate proportional to the amount of circulating virus would appear as linear in log–linear graphs such as Fig. 2(B). Benacerraf has argued that the $K_{max}$ of particulate material by KC is 0.35 (Brunner et al., 1960). If we fit the concentration of wild-type Ad5 (Adwt) from 1 to 30 min to a line, we get the value $K = 0.08$. However, the clearance of Adwt would fit better with a non-linear decrease in the log–linear plot. This would indicate that the rate of clearance continually decreases with time, suggesting that the clearance mechanisms are more efficient when there is more virus in circulation (from 1 to 5 min, $K = 0.2$).

KC are responsible for the clearance of most foreign particles in the blood (Steffan, 1997). To assess their role in
adenovirus clearance from blood, we depleted KC by GdCl₃ treatment. The persistence of adenovirus in circulation increased markedly after KC depletion (Fig. 2). From 1 to 5 min, only a 2.5-fold reduction in titre was observed, indicating a value of $K = 0.009$, or 2.2-fold slower than in the presence of KC. Between 5 and 10 min, the reduction was only by 2-fold. In the next 10 min, it dropped by 2.8-fold, with a slight 2-fold decrease between 20 and 60 min. If we consider the first 30 min after injection, the value of $K$ is 0.04, 2-fold slower than in the presence of KC. Even with this slower clearance, the levels after 1 h were still much lower (around 10%) than the levels after the first minute. This could reflect the fact that GdCl₃ depletes mainly the large periportal KC (Lieber et al., 1997). However, other mechanisms of clearance besides KC are likely to be involved. The liver, spleen and bone marrow vasculature has pores up to 100 nm in diameter, allowing adenovirus extravasation. In hepatocytes, the presence of zv integrins and high CAR levels can mediate specific virus uptake (Fechner et al., 1999) and explain the better gene transduction after KC depletion (Wolff et al., 1997). Adenoviruses unable to bind CAR and integrins will be useful to study the role of these receptors in clearance. In spleen, low CAR levels preclude this specific uptake and explain the poor transduction of this organ; however, uptake by spleen macrophages (not depleted with GdCl₃) could be important. Depletion of these macrophages with clodronate will help to understand their role in the adenovirus clearance presented here.

The mechanism of adenovirus uptake by KC or other macrophages is not known. Human alveolar macrophages are not transduced efficiently with adenovirus vectors due to low CAR levels, although transduction is blocked by soluble fibre (Kaner et al., 1999). Monocytes and their derived macrophages also express adenovirus transgenes very inefficiently (Huang et al., 1996). Attachment and expression in this case are not blocked by soluble fibre. Adenovirus entry into alveolar macrophages by a non-CAR pathway could lead to virus degradation rather than transgene expression. From the point of view of clearance, these scavenger pathways are most important. KC clear bacteria, parasites, colloids, erythrocytes and any particulate material in circulation (such as latex beads) (Steffan, 1997). Size and net charge are major determinants of clearance, and it has been noted that the charge of the adenovirus capsid may play a decisive role in tropism (Mei & Wadell, 1995). Interestingly, the neutral mouse adenovirus is adapted to circulate in blood and has a tropism for endothelial cells. To study the effect of Ad5 charge on blood clearance, we deleted 13 residues (EDEDDNEDDEVDE) of the hexon hypervariable region 1 (HVR1) (Crawford-Miksza & Schnurr, 1996). HVR1 contains the largest number of charged residues that differ among serotypes. We used the oligonucleotide 5′ctgctcttgaaataaacctACAGCTGAGCAGCAAAAAACTC 3′ (capital letters indicate residues after the deletion point) for site-directed mutagenesis (Transformer; Clontech) in a plasmid containing a portion of the hexon gene (Xmal fragment, bp 18918–20373 of Ad5). The mutated Xmal fragment was inserted into the plasmid containing the GFP cassette in E3 described above. Virus AdhN was generated by transfection of 293 cells and purified as above. The deletion was confirmed by DNA sequencing. Zeta potential charge analysis (PALS analyser) of a solution containing 10¹³ virus particles/ml PBS (pH 7.4) of Adwt gave a value of $-9.76 \pm 0.98$ mV compared with $-6.28 \pm 0.62$ mV for AdhN, indicating a substantial charge modification (three independent measurements). When AdhN was injected into the vena cava of three BALB/c mice, we did not observe any significant delay in virus clearance (Fig. 2). A more laborious analysis is warranted to rule out the possibility that charge does not affect clearance, because most other negative residues are scattered throughout the hexon and fibre sequences. An alternative approach would be to compare the clearance of different adenovirus serotypes.

Coating with PEG (‘PEGylation’) is commonly used to avoid protein clearance by KC and other macrophages. PEGylated ‘stealth’ liposomes and nanoparticles show increased blood persistence (Allemann et al., 1995; Gabizon et al., 1994). Adenovirus has been PEGylated to avoid neutralization by antibodies (O’Riordan et al., 1999). Due to the potential use of PEGylated adenoviruses as gene-delivery vehicles, we studied their blood clearance rate. Tresyl-monomethoxypolyethylene glycol (TMPEG) from PolyMASC Pharmaceuticals was obtained from Fluka. Adwt (2 × 10¹¹ virus particles/ml in PBS) was diluted 2-fold with 130 mM sodium phosphate pH 7.5% sucrose, and 0.1 ml was added to 4 ml TMPEG (i.e. 4·1%, w/v). After 30 min incubation at room temperature, virus was injected into the vena cava of mice. Infectivity of the PEGylated virus was measured by serial dilution and infection of A549 cells. In agreement with previous reports (Chillon et al., 1998), we found a 300- to 1000-fold reduction of infectivity with 4·1% TMPEG and a proportionally smaller reduction when using less TMPEG (not shown). The clearance rate during the first 30 min gave $K = 0.02$, 4-fold slower than the non-PEGylated virus (Fig. 2B).

In summary, adenovirus clearance from blood results in a virus half-life of less than 2 min. Similar to other non-blood-borne viruses, adenovirus is efficiently phagocytosed by KC and the blood clearance kinetics suggest a high capacity, non-specific uptake. Adenovirus clearance is not affected by partial charge neutralization via deletion of the hexon acidic stretch. More extensive charge depletion, or other modifications of the viral capsid such as elimination of CAR binding and integrin binding, need to be evaluated as ways of increasing blood persistence. Finally, it must also be noted that, whereas murine models are most commonly used in preclinical gene therapy research, clearance studies in other animal species are needed to allow generalization from these results.

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References


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