Systemic circulation of poly(L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy

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Effective gene therapy for diseases of the circulation requires vectors capable of systemic delivery. The molecular weight of poly(L-lysine) (PLL) has a significant effect on the circulation of PLL/DNA complexes in mice, with PLL<sub>211</sub>/DNA complexes displaying up to 20 times greater levels in the blood after 30 minutes compared with PLL<sub>20</sub>/DNA. It is shown that PLL<sub>20</sub>/DNA complexes fix mouse complement C3 in vitro, independent of immunoglobulin binding; are less soluble in the blood in vivo; bind erythrocytes; are rapidly removed by the liver, where they associate predominantly with Kupffer cells; and result in a rapid increase in hepatic leukocytes expressing high levels of complement receptor 3 (CR3). The circulation properties of these complexes are also dependent on the type of DNA used, with circular plasmid DNA complexes exhibiting increased circulation compared with linear DNA. PLL<sub>211</sub>/DNA complexes bind erythrocytes and associate with Kupffer cells but, in contrast, do not fix mouse complement in vitro and are unaffected by the type of DNA used. In rats, both types of complexes produce hematuria and are rapidly removed from the circulation. Correlation of in vivo and in vitro results suggests that the solubility of complexes in physiological saline and species-matched complement fixation and erythrocyte lysis may correlate with systemic circulation. Analysis using human blood in vitro shows no hemolysis, but both types of complexes fix complement and bind IgG, suggesting that PLL/DNA complexes may be rapidly cleared from the human circulation. (Blood. 2001;97:2221-2229)

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Preparation of plasmid DNA and 32P- 35S-labeled DNA

A 6.4-kb plasmid encoding lac Z under transcriptional regulation of the cytomegalovirus immediate early promoter was used throughout these studies. Plasmid DNA was prepared in bulk by growth in *Escherichia coli* and purified using Qiagen columns (Qiagen, Crawley, United Kingdom). Linear plasmid was obtained by HindIII linearization followed by phenol/ chloroform extraction. Purity of the DNA was checked by ethidium bromide visualization of agarose gels. 32P- or 35S-labeled expression vector was prepared as follows: linearized plasmid was radiolabeled by template buffer at pH 7.5 to give a final DNA concentration of 10 \( \mu \text{g/mL} \). PLL 20 kd (pLL 20) or 211 kd (pLL 211) HBr DNA concentration of 20 \( \mu \text{g/mL} \) were obtained in-house and kept according to Home Female Balb/c mice (approximate weight, 20 g) and Wistar rats (approximate weight, 200 g) were obtained in-house and kept according to Home Office guidelines. 32P-labeled DNA complexes were administered by intravenous tail vein injection (0.1 mL in mice; 1.0 mL in rats; both corresponding to 0.6 \( \mu \text{g DNA/mL blood} \)). Blood was sampled from the end of the tail, smeared on glass slides (Superfrost +; BDH, Lutterworth, United Kingdom), air-dried, and fixed in methanol for 10 minutes. Animals were killed after 30 minutes for pharmacokinetic and liver immunohistochemistry studies and after 1 or 10 minutes for blood cell-binding studies. For body distribution studies, organs and carcasses were dissolved in 10 M NaOH at 75°C for 1 hour and assayed for radioactivity (values have been corrected for the levels of blood within the organs). For immunohistochemistry analysis, organs were removed, snap frozen in liquid nitrogen, and stored at −80°C. Mouse liver was sectioned on a cryostat (12-\( \mu \text{m} \) sections) and placed onto coated glass slides (Superfrost +; BDH). The sections were fixed in acetone for 10 minutes and air-dried. Blood cell-binding experiments were performed by centrifuging blood (without anticlotting agents) at 5000 rpm at 4°C immediately upon sampling. Radioactivity within blood samples and organs/carcass was measured in a liquid scintillation analyser (1900TR; Packard), and within blood smears and liver sections it was measured using quantitative analysis with a PhosphorImager (Molecular Dynamics).

Formation of pLL/DNA complexes

All complexes were formed in water at a charge ratio of 2.8:1 and a final DNA concentration of 20 \( \mu \text{g/mL} \); this was followed by dilution in HEPES buffer at pH 7.5 to give a final DNA concentration of 10 \( \mu \text{g/mL} \) and a final buffer concentration of 25 mM. PLL 20 kd (pLL 20) or 211 kd (pLL 211) HBr salt (35.84 \( \mu \text{g} \) of a 2.5 \( \mu \text{g} \)/\( \mu \text{L} \) stock solution in water) was added in a single addition to a solution of DNA (20 \( \mu \text{g} \) in 1.0 mL water), mixed, and left to stand for 1 hour at room temperature. HEPES buffer (1.0 mL, 50 mM, pH 7.5) was added to the samples and mixed by inversion before experimental analysis. Studies using radiolabeled DNA were performed using a spike of 32P-labeled DNA (0.6 ng/\( \mu \text{g} \) unlabeled DNA) in the DNA solution, and the complexes formed as above.

Immobilization of pLL/DNA complexes on magnetic streptavidin beads

Biotin-pLL was formed by reacting pLL (pLL 20 or pLL 211; 10 \( \mu \text{g/mL} \) in 1 M HEPES buffer, pH 8.5) with biotinamidocaproic acid 3-sulfo-N-hydroxy-succinimide ester (1.34 \( \mu \text{g} \)) at room temperature for 3 hours. The resultant mixture was centrifuged to remove particulates, and the supernatant was dialyzed against 20 mM NaCl for 48 hours. PLL concentration was determined using the TNBS method. The number of biotin moieties per protein was calculated using the ethidium bromide exclusion assay. PLL/DNA complexes were then formed using the method described above. Magnetic streptavidin beads (20 \( \mu \text{L} \), washed in phosphate-buffered solution (PBS) before use; Pierce, Chester, United Kingdom) were added to biotin-pLL/DNA complexes (0.4 \( \mu \text{L} \) and left at room temperature for 30 minutes. After magnetic isolation of the beads, the supernatant was removed, and the beads were washed twice in 25 mM HEPES buffer before experimental analysis.

Identification of proteins bound to pLL/DNA complexes

Radiolabeled biotin-pLL/DNA complexes bound to magnetic streptavidin beads were incubated in normal or heat-inactivated serum (0.4 \( \mu \text{L} \); heat-inactivated at 56°C for 30 minutes, with 10 mM EDTA added before incubation with complexes) for 5 minutes. Magnetic beads were isolated and washed in isotonic buffer (3 washes). Laemmeli buffer (0.1 \( \mu \text{L} \) was added to the immobilized complexes and boiled for 3 minutes. The resultant mixture was centrifuged to remove particulates, and the supernatant was removed, and the beads were washed twice in 25 mM HEPES buffer before experimental analysis.

Animal models for analysis of pharmacokinetics

Female Balb/c mice (approximate weight, 20 g) and Wistar rats (approximate weight, 200 g) were obtained in-house and kept according to Home Office guidelines. pLL/DNA complexes were administered by intravenous tail vein injection (0.1 mL in mice; 1.0 mL in rats; both corresponding to 0.6 \( \mu \text{g DNA/mL blood} \)). Blood was sampled from the end of the tail, smeared on glass slides (Superfrost +; BDH, Lutterworth, United Kingdom), air-dried, and fixed in methanol for 10 minutes. Animals were killed after 30 minutes for pharmacokinetic and liver immunohistochemistry studies and after 1 or 10 minutes for blood cell-binding studies. For body distribution studies, organs and carcasses were dissolved in 10 M NaOH at 75°C for 1 hour and assayed for radioactivity (values have been corrected for the levels of blood within the organs). For immunohistochemistry analysis, organs were removed, snap frozen in liquid nitrogen, and stored at −80°C. Mouse liver was sectioned on a cryostat (12-\( \mu \text{m} \) sections) and placed onto coated glass slides (Superfrost +; BDH). The sections were fixed in acetone for 10 minutes and air-dried. Blood cell-binding experiments were performed by centrifuging blood (without anticlotting agents) at 5000 rpm at 4°C immediately upon sampling. Radioactivity within blood samples and organs/carcass was measured in a liquid scintillation analyser (1900TR; Packard), and within blood smears and liver sections it was measured using quantitative analysis with a PhosphorImager (Molecular Dynamics).

Immunohistochemistry of mouse liver sections

Slides were exposed to either rat antimouse F4/80 antibody (Serotec, Oxford, United Kingdom; 1:50 dilution in 1% bovine serum albumin [BSA]/PBS) or rat antimouse CD11b antibody (Research Diagnostics, Flanders, NJ; 1:50 dilution in 1% BSA/PBS) for 2 hours and washed by immersion in 1% BSA/PBS solution (total, 4 washes). Sheep antirat immunoglobulin antibody (DAKO, Ely, United Kingdom; 1:50 dilution in 1% BSA/PBS) was added for 2 hours and washed by immersion in 1% BSA/PBS solution (4 washes). Rat APAAP antibody (DAKO; 1:50 dilution in 1% BSA/PBS) was added for 1 hour, and the slides were washed by immersion in 1% BSA/PBS solution (2 washes). Slides were placed in Tris-buffered saline (2.5 minutes; pH 7.5; 50 mM Tris base), followed by Tris buffer (2.5 minutes; pH 8.2, 50 mM Tris base) and then immersed in alkaline phosphate substrate (15 minutes; made in the following order: naphthol-phosphate AS-MX [10 mg]; NN-dimethyldiformamide [1.0 mL]; Tris buffer [49.0 mL]; levamisole [50 \( \mu \text{L} \) of 1 M stock]; Fast Red-TR [50 mg]; filtered before use). The slides were then exposed to Mayer’s hematoxylin (filtered) for 3 minutes, washed in tap water, and mounted.

Visualization of 35S-labeled pLL/DNA complexes in liver sections and blood smears

Slides were coated with liquid photographic emulsion (NTB-2; Anachem, Luton, United Kingdom) and stored in the dark at 4°C for various lengths of time (between 1 and 7 days). Slides were developed in D-19 developer (diluted 1:1 with deionized water), fixed in sodium thiosulphate (30% wt/vol), and washed gently in tap water to remove excess emulsion.

Erythrocyte lysis assay

Blood was taken from a healthy female human volunteer or a female Wistar rat and divided into EDTA-containing and empty blood tubes. The serum from the coagulated blood sample was mixed with blood cells from the EDTA-treated sample (cells washed × 2 in PBS to remove EDTA) to form an experimental blood substitute containing active serum. Blood (0.5 mL) was mixed with pLL/DNA complexes (30 \( \mu \text{L} \), corresponding to 0.6 \( \mu \text{g DNA/mL blood} \); formed in water and dialyzed in HEPES buffer as described above), incubated at 37°C for 1 hour, and centrifuged at 5000 rpm for 1 minute to pellet the blood cells, and then 100 \( \mu \text{L} \) supernatant was transferred to a flat-bottomed 96-well plate. Hemoglobin released into the supernatant was measured by absorbance at 550 nm in a microplate autoreader (Bio-Tek Instruments, Winooski, VT).
pLL/DNA complexes were formed with pLL20 or pLL211 and either DNAct, DNAcp, or DNAlp were administered intravenously to female Balb/c mice (0.1 mL of 10 μg/mL DNA). The mice were killed after 1 and 10 minutes, and the blood was sampled and immediately spun at 5000 rpm at 4°C to pellet the blood cells. Pellet and supernatant were assayed for 32P-labels, and the results show the percentage of radioactivity present in the blood associating with the cell pellet. Numbers in brackets indicate the percentage of the original 32P dose remaining in the blood at the time of sampling. Results show the mean ± SD of 3 independent experiments. Similar results were obtained using pLL/DNAct complexes (data not shown).

For abbreviations, see Table 1.

For example, the use of circular plasmid DNA (DNAcp) led to 9-fold greater levels of DNA remaining in the blood after 30 minutes (18.5% ± 2.0%) compared with that obtained for calf thymus DNA (DNAct) (2.1% ± 0.5%). This difference probably resulted from the physical properties of pLL/DNA complexes formed using linear DNA. For instance, when circular plasmid DNA was linearized (DNAlp), a 3-fold decrease in circulation was seen (7.0% ± 0.5%). Proteins present on DNAct were not responsible for the rapid clearance of pLL20/DNAct complexes because complexes formed with DNAct purified by phenol/chloroform extraction exhibited similar blood clearance profiles (data not shown). Administration of either DNAct, DNAcp, or DNAlp alone showed a similar level of 32P-labeled DNA remaining in the blood after 30 minutes. Additionally, complexes formed at ± charge ratios of 1:1 and 3:1 showed similar distribution profiles (data not shown).

Body distribution of pLL/DNA complexes

Table 1 shows the body distribution of 32P-labeled DNA in female Balb/c mice 30 minutes after intravenous injection of pLL/DNA complexes formed with pLL20 or pLL211 and either DNAct, DNAcp, or DNAlp. The values have been corrected for the levels of blood within the organs. Free DNA was rapidly removed from the blood (Figure 1, Table 2) on administration to mice, and, after 30 minutes, it was found primarily within the liver (84.9% ± 2.0%), with small levels within the carcass, the kidneys, and the intestine, and very low levels within the lungs and the spleen. In contrast, most complexes formed using either pLL20 or pLL211 exhibited significantly altered body distributions, with longer circulating vectors (all pLL211/DNA complexes, pLL20/DNAcp, or DNAlp) and, to a lesser extent,
The results in Table 2 show that pLL20/DNA ct and pLL211/DNA ct might have contributed to their differential removal from the blood. The degree of binding of pLL/DNA complexes to blood cells in vivo (150 mM NaCl) to pLL20/DNA cp complexes resulted in significant increases in the diameter of the complexes over time, with sharp increases observed after only 2 minutes. This increase in size was probably due to hydrophobic aggregation of the complexes as a result of increased solvent polarity. In contrast, pLL211/DNA cp complexes displayed significantly smaller increases in diameter on the addition of 150 mM NaCl; pLL20/DNA cp (1.5 nm; pLL211/DNA cp (2.2 nm); pLL20/DNA ct (54.1 ± 1.5 nm; pLL211/DNA ct (61.9 ± 7.2 nm)). Figure 2 shows that the addition of physiological levels of NaCl (150 mM) to pLL20/DNA ct complexes resulted in significant increases in the diameter of the complexes over time, with sharp increases observed after only 2 minutes. This increase in size was probably due to hydrophobic aggregation of the complexes as a result of increased solvent polarity. In contrast, pLL211/DNA ct complexes displayed significantly smaller increases in diameter on the addition of 150 mM NaCl, suggesting that these complexes may be more stable under physiological conditions. Similar results were obtained using pLL/DNA ct complexes.

Association of pLL/DNA complexes with mouse blood cells in vivo

Recent investigations have shown that polycation-DNA complexes bind to erythrocytes in vitro; therefore, we examined whether the degree of binding of pLL/DNA complexes to blood cells in vivo might have contributed to their differential removal from the blood. The results in Table 2 show that pLL20/DNA ct and pLL211/DNA ct complexes bind to blood cells in vivo, whereas free DNA does not. Autoradiography of blood smears taken from mice 1 minute after the administration of pLL/DNA complexes show that the pLL20/DNA ct complexes (Figure 3A) produced a punctate distribution of radioactivity, suggesting that the complexes might have aggregated in the blood. In contrast, neither pLL211/DNA ct complexes (Figure 3B) nor DNA (Figure 3C) displayed punctuate distribution of radioactivity, indicating that pLL211/DNA ct complexes might have been relatively soluble within the blood. Closer examination of the smears confirms that pLL20/DNA ct complexes associated with erythrocytes in vivo (Figure 3D), with no evidence of white blood cell association (data not shown). Radioactive quantification of the blood smears by PhosphorImager (Molecular Dynamics) analysis showed all smears to contain comparable amounts of radioactivity (data not shown). Similar results were obtained using pLL/DNA ct complexes.

Intrahepatic distribution of pLL/DNA complexes in mice in vivo

The liver is the main organ of accumulation of pLL/DNA complexes injected intravenously in mice; therefore, we investigated the intrahepatic distribution of complexes to identify any cells that might have been responsible for their capture. Figure 4 shows the distribution of pLL/DNA ct complexes in mouse liver using autoradiography after 1-day (Figure 4A) or 7-day (Figure 4B) exposure of the slides to the photographic emulsion. pLL20/DNA ct complexes (Figure 4Ai) showed punctate distribution of radioactivity within the liver after 1-day exposure, associating predominantly with Kupffer cells, whereas pLL211/DNA ct complexes (Figure 4Aii) did not give a detectable signal. This is unlikely to be due to less radioactivity on slides prepared from livers of mice treated with pLL211/DNA ct complexes, because PhosphorImager (Molecular Dynamics) analysis indicates that the levels of radioactivity are approximately 70% of liver slides from mice treated with pLL20/DNA ct complexes. The punctate appearance of radioactivity in the liver sections was most likely due to the presence of aggregates leading to “hot spots.” Seven-day exposure of liver sections from mice administered pLL20/DNA ct (Figure 4Bi) or pLL211/DNA ct (Figure 4Bii) complexes resulted in punctate
distribution of the radioactivity and, in both cases, associated predominantly with Kupffer cells. Livers from mice treated with pLL20/DNA ct complexes displayed "hot spots" with some high-intensity foci, thought to correspond with those detected after 1 day. The type of DNA used in the pLL/DNA complexes had no effect on their intrahepatic distribution, with both pLL20/DNA cp and pLL 211/DNA cp complexes displaying the same radiographic signal intensities as their respective pLL/DNA ct ones (data not shown). DNA alone (Figure 4Biii) was washed from the tissue sections during the autoradiographic development process, though 85% of DNA was found in the liver after 30 minutes (Table 1).

Activation of complement by pLL/DNA complexes in mouse serum in vitro

Complement activation by pLL/DNA complexes might have been a contributory factor for their rapid removal from the circulation of mice; therefore, we investigated the binding of complement protein C3 to pLL/DNA complexes incubated in mouse serum in vitro. Figure 5A shows, by dot blotting, that pLL20/DNA ct complexes bound C3, whereas pLL 211/DNA ct complexes did not. Binding of C3 to the pLL20/DNA ct complexes could be inhibited by heat treatment of the serum (Figure 5B), indicating that C3 binding was due to activation of complement and not nonspecific binding. Association of C3 with pLL20/DNA ct complexes might have been due to its interaction with the polycation, because pLL20 alone also binds C3. Similar results were obtained using pLL/DNA cp complexes (data not shown).

Figure 4. pLL20/DNA and pLL211/DNA complexes associate with Kupffer cells in mice. pLL20/DNAct or pLL211/DNAct complexes were administered intravenously to female Balb/c mice (0.1 mL of 10 μg/mL DNA). Liver sections were stained with hematoxylin (blue/purple nuclei) and the macrophage marker F4/80 (pink/red cytoplasm), and the radioactivity was viewed by exposure of the slides to liquid photographic emulsion for (A) 1 day or (B) 7 days. (i) pLL20/DNA ct; (ii) pLL 211/DNA ct; (iii) DNA alone (note: DNA is washed from the slides during the development process). Similar results were obtained using pLL/DNA cp complexes (data not shown).

Intrahepatic distribution of complement receptor 3 positive cells after administration of pLL/DNA complexes in mice

The complement receptor 3 (CR3) receptor is expressed on leukocytes and is involved in phagocytosis and cell motility. In normal liver, CR3 is differentially expressed on at least 2 subpopulations of liver cells (Figure 6Aii). The first expresses low levels of CR3 and is normally present throughout the parenchyma; this has been identified as tissue resident macrophages.24 The second cell population expresses high levels of CR3 (hereafter termed CR3h cells) and is present in low numbers within the parenchyma. PLL20/DNA ct (Figure 6Ai) and pLL 211/DNA ct (Figure 6A ii) complexes both associate predominantly with cells expressing low levels of CR3 (ie, Kupffer cells), though there is some association with CR3h cells, and both types of complexes led to higher levels of CR3h cells compared with the control. Quantitative cell analysis in liver sections (Figure 6B) showed that the administration of pLL20/DNA ct complexes led to the highest number of CR3h cells. This suggests that the pLL component of these complexes stimulated the immune system because the levels of pLL20/DNA ct and free DNA within the liver were comparable (Table 1), yet the

Figure 5. pLL 20/DNA ct complexes, but not pLL 211/DNA, fix mouse complement C3 independently of immunoglobulins. Biotin-pLL20/DNA ct or biotin-pLL 211/DNA ct complexes were immobilized on streptavidin magnetic beads and incubated in mouse serum at 37°C for 5 minutes. Beads were isolated, washed in isotonic buffer, and boiled in Laemelli buffer to isolate the bound proteins. Dot blots were prepared on nitrocellulose membrane, and proteins were identified by antibody blotting and radiography. (A) C3 binding to pLL/DNA ct complexes in normal mouse serum. (B) C3 binding to pLL20/DNA ct complexes in normal or heat-inactivated serum. (C) Total mouse immunoglobulins binding to pLL/DNA ct complexes in normal serum. Bead control represents serum-treated beads. Similar results were obtained using pLL/DNA cp complexes (data not shown).
number of CR3h cells was 50% lower when DNA alone was administered. Lower levels of CR3h cells were seen in liver sections of mice administered pLL 20/DNA cp and pLL 211/DNA complexes, possibly due to the reduced amounts of these complexes within the liver compared to pLL 20/DNA ct complexes. Mice administered either pLL 211/DNA ct or pLL 211/DNA cp complexes showed no increase in CR3h cells compared to DNA alone (Figure 6B).

pLL/DNA complex behavior in female Wistar rats

To assess whether the effect of pLL molecular weight on the circulation of pLL/DNA complexes was reproducible between species, we administered pLL 20/DNA ct or pLL 211/DNA ct complexes intravenously to female Wistar rats. Figure 7A shows that both pLL 20/DNA ct and pLL 211/DNA ct complexes were rapidly cleared from the bloodstream within minutes, and in both cases blood clearance was faster than it was in mice (data not shown). Additionally, erythrocyte toxicity was inferred for both complexes by the presence of hemoglobin in the urine at the time of killing. Rats treated with buffer or DNA alone showed no signs of hematuria. Figure 7B shows that rat complement C3 did not bind to either pLL 20/DNA ct or pLL 211/DNA ct complex.

Effect of pLL 20/DNA ct and pLL 211/DNA ct complexes in human blood in vitro

To predict the behavior of pLL/DNA complexes in humans, we investigated the interactions with human blood of pLL 20/DNA ct or pLL 211/DNA ct complexes. Figure 8A shows that neither complex caused hemolysis in human blood, whereas it was apparent in rat blood. Figure 8Bi shows that human complement C3 bound to both pLL 20/DNA ct and pLL 211/DNA ct complexes and that this binding was specific because it could be blocked by heat-inactivation of the serum (Figure 8Bii). Additionally, Figure 8C shows that IgG associated with both types of pLL/DNA complex (IgA, IgD, IgE, and IgM do not bind; data not shown), suggesting that complement activation may be through the classical pathway. Similar results were obtained using pLL/DNA cp complexes.

Discussion

Understanding the factors regulating differential circulation of polyelectrolyte DNA complexes in the bloodstream may provide insights into clearance mechanisms and possible means to evade them. Complexes formed using high- and low-molecular-weight pLL show quite different circulation properties in mice, and one clear morphologic difference between them is the appearance of a punctate distribution of radioactivity in the blood using pLL 20/DNA complexes not apparent using pLL 211/DNA. Although the precise reasons for this distribution are unclear, the observation of physiological salt-induced aggregation of pLL 20/DNA complexes in vitro suggests that these complexes may have relatively poor solubility in blood. We have also shown that pLL 20/DNA complexes bind erythrocytes in vivo, and it is possible that this, along with poor solubility, results in the punctate radioactivity distribution observed in the blood smears.

We have also shown specific binding of complement protein C3 to pLL 20/DNA, but not pLL 211/DNA, complexes in mouse serum. The influence of molecular weight of polycations on complement...
activation by pLL and pLL/DNA complexes has been reported previously. Siegal et al. have shown, in acute-phase human serum in vitro, that low-molecular-weight pLL (4 and 23 kd) activates complement significantly more than high-molecular-weight pLL (70 kd). In contrast, in normal serum, complement is activated independently of the molecular weight of pLL used. Additionally, they showed that in both acute-phase and normal human serum, complement activation is mediated by the acute-phase C-reactive protein (CRP). However, we have found that CRP does not associate with pLL/DNA complexes incubated in normal human serum. (data not shown), suggesting that either CRP is not involved in complement activation or that, more likely, CRP does not associate with pLL/DNA complexes (after complement activation). The phenomenon may reflect this phenomenon. The same effect may be observed in human serum. In vitro, that low-molecular-weight pLL (1, 4, 6, and 25 kd) activates complement significantly more than high-molecular-weight pLL (70 kd). In contrast, in normal serum, complement is activated by low-molecular-weight pLL (4 and 23 kd). The apparent contradiction between these 2 studies may reflect the different methods used to determine complement activation, with Siegal et al. using a direct measure of complement protein depletion and Plank et al. using an indirect sensitized sheep erythrocyte hemolysis assay. Despite the discrepancies between these 2 studies, which were both performed with human and mouse serum, they do show that complement is readily activated by pLL and that molecular weight can influence the level of activation achieved. Additionally, we show that complement activation may be through different complement pathways in mouse and human serum. Although the exact mechanisms of complement activation require further investigation, the binding of IgG (but not other immunoglobulins) to pLL/DNA complexes in human (but not mouse serum) suggests the presence of antibody-mediated immunity in normal human serum.

After intravenous injection to mice, pLL20/DNA complexes accumulate within the liver where they associate with macrophages (Kupffer cells). Kupffer cell-associated complement receptor 3 (CR3) is the major pathway for clearance of C3-opsonized particles from the mouse bloodstream,25 and it is possible that C3-opsonized pLL20/DNA complexes may follow this route. Administration of pLL20/DNA complexes also led to a rapid increase in the number of cells within the liver expressing high levels of CR3 (hereafter termed CR3h cells), with fewer CR3h cells being induced by DNA alone or by pLL211/DNA complexes. This is perhaps a reflection of the differential accumulation of polyelectrolyte complexes within the liver (Table 1). However, it is also possible that pLL211/DNA complexes may exhibit reduced interaction with the immune system, exemplified by their inability to mediate complement activation in mouse serum in vitro, resulting in lower numbers of CR3h cells. CR3h cells may be leukocytes infiltrating from the circulation—eg, monocytes24 or natural killer cells26—whose expression of CR3 is associated with phagocytosis and cell motility.27 Alternatively, CR3h cells may be stimulated Kupffer cells.28 Precise identification of CR3h cells has not been determined; however, the infiltration of CR3-expressing cells and the up-regulation of CR3 on phagocytic cells are associated with immune activation,29 especially by complement-mediated events.30-32 Hence, it is feasible that the increase in CR3h cells within the liver follows the interaction of pLL20/DNA complexes with components of the immune system. Although there is no direct evidence that complement activation by pLL20/DNA complexes leads directly to Kupffer cell capture and increased CR3h cells, it is clear that the immune system becomes provoked by these complexes in some way. pLL211/DNA complexes are eventually captured by Kupffer cells, albeit more slowly than pLL20/DNA complexes, but the mechanisms involved in their removal are unclear.

The properties of polyelectrolyte complexes formed using pLL20 are influenced by the DNA used. For example, pLL20/DNAcp complexes display extended circulation times in mice compared to pLL211/DNAcp or pLL20/DNAat complexes. Using circular dichroism, it has been shown that polyelectrolyte complexes formed using circular or linear plasmid DNA exhibit different topologies, with linear DNA forming less tightly organized structures than circular DNA.33 The greater circulation time of complexes containing the circular DNA may reflect this phenomenon. The same effect is not observed using pLL211, probably because the larger polycation enhances the stability of both types of DNA complex.

The body distribution of pLL/DNA complexes in mice appears to correlate with the circulation half-life of the vector, with pLL211/DNA and pLL20/DNAcp complexes exhibiting increased carcass accumulation and decreased liver accumulation compared to DNA alone. Decreased liver accumulation of these complexes...
may reflect reduced Kupffer cell capture, whereas increased accumulation within the carcass is most likely due to phagocytic capture of the complexes or trapping within capillary beds. All pLL/DNA complexes display decreased accumulation within the spleen, whereas DNA alone exhibited very low levels (0.4% ± 0.1%). Thus it appears that mechanisms within the spleen may be involved in pLL recognition and capture.

PLL20/DNAc complexes showed high levels of accumulation within the liver and low levels within the carcass, both similar to the levels obtained using free DNA. It is unlikely that the radiolabeled DNA probe within PLL20/DNAc complexes becomes dissociated from the PLL because more than 99% of the probe was retrieved intact after incubation in blood or serum (data not shown). In addition, PLL20/DNAc complexes bound blood cells in vivo whereas DNA alone did not (see Table 2), and liver-captured radioactivity of PLL20/DNAc (and also PLL211/DNAc complexes) was not eluted during the processing of liver sections, as is the case for free DNA (Figure 3).

Destabilization of PLL20/DNAc complexes has been described as a possible mechanism for the removal of polycationic complexes from the circulation.

Therefore, we hypothesized that incubation of PLL20/DNAc complexes in mouse blood might result in increased degradation of the DNA contained within the complexes compared with PLL211/DNAc complexes. Incubation of PLL/DNA complexes formed using either PLL20 or PLL211, and either DNAc or DNAa, in 100% serum or blood (mouse, rat, or human) resulted in negligible DNA degradation of the radiolabeled probe in all of the samples, whereas free DNA was substantially degraded (data not shown). This finding suggests that both types of PLL/DNA complexes protect the DNA from degradation by serum nucleases. Therefore, it seems unlikely that destabilization of PLL/DNA complexes, leading to DNA degradation or capture, is an important factor mediating their rapid removal from the blood.

Intravenous administration of pLL/DNAc complexes to Wistar rats resulted in rapid blood clearance, with signs of hematuria. The hematuria may be mediated by direct erythrocyte lysis, demonstrable by both types of complex in rat blood in vitro, and this may also contribute to their rapid clearance in vivo. High doses of DNA vectors have been used in mice to increase transgene expression, and it is possible that a high dose in rats would lead to considerable hematuria. Neither PLL20/DNAc, nor PLL211/DNAa complexes displayed significant levels within the spleen, whereas DNA alone exhibited very low levels (0.4% ± 0.1%). Thus it appears that mechanisms within the spleen may be involved in pLL recognition and capture.

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References


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