Long-Term Protection of Polyaspartic Acid in Experimental Gentamicin Nephrotoxicity

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Polyaspartic Acid (PAA) protects the kidney from experimental gentamicin nephrotoxicity despite large increases in renal cortical gentamicin content. In these experiments, prominent cytoplasmic vacuoles were noted in all animals that received PAA with or without gentamicin. The present study showed that there were no renal structural or functional consequences of PAA given alone or with gentamicin for up to 14 days, followed by a 16-week washout period. Creatinine clearance was similar to that of controls in animals that received gentamicin and in those that received PAA alone. Thus, complete functional protection was conferred by PAA and gentamicin, confirming previous reports from our laboratory. There was no protection by PAA from the nephrotoxic effects of mercuric chloride and cis-platinum.

Aminoglycoside antibiotics continue to be widely used to treat serious gram-negative infections, despite ototoxic and nephrotoxicity complicating 10 to 20% of all courses of treatment (6). Although there has been a proliferation of new antibiotics in recent years, there is often no substitute for the proven efficacy of an aminoglycoside in life-threatening infections. Attempts have been made with animal models to define manipulations which attenuate ototoxicity and/or nephrotoxicity while preserving antimicrobial activity. Moderate attenuation of nephrotoxicity is demonstrable in rats with concomitant antipseudomonal penicillin therapy or by substituting a once-daily dosage regimen for the three-times-a-day dosage regimen. While these latter maneuvers modify the severity of nephrotoxicity, they do not eliminate it, even in experimental animals. In contrast, polyaspartic acid (PAA) has been reported by several investigative groups to prevent all functional and histologic evidence of aminoglycoside nephrotoxicity (2, 4, 8, 10). Paradoxically, PAA nephroprotection is associated with increased renal parenchymal gentamicin accumulation (2, 4, 8). We have shown that animals given a PAA preparation with a mean molecular weight (MW) of 16,000 demonstrated no functional or histologic evidence of nephrotoxicity. Prominent vacuoles were observed in the cytoplasm of the proximal tubular cells of animals administered PAA with or without gentamicin, and their functional significance was not apparent in previous short-term experiments. In addition, it is unknown whether the PAA nephroprotection was specific for aminoglycosides or was also applicable to other nephrotoxins. The studies described below had four purposes: (i) to determine whether there were any long-term effects of the PAA-induced vacuoles during 7- and 14-day aminoglycoside treatments and a 16-week washout period; (ii) to assess the posttreatment consequences of the high renal cortex gentamicin concentrations in rats given gentamicin plus PAA; (iii) to assess the influence of PAA on mercuric chloride (HgCl2)- and cis-platinum (cisP)-induced renal injury; and (iv) to assess the influence of a PAA preparation with a lower MW on aminoglycoside nephrotoxicity.

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MATERIALS AND METHODS

Purified PAA polymer (approximately MW, 3,700) was a gift of Bristol-Myers-Squibb (Syracuse, N.Y.). PAA was administered at 330 mg/kg/day. The PAA was characterized at Bristol-Myers-Squibb by D. McGregor. It was found to be a mixture of D and L optical isomers and a mixture of alpha and beta peptide linkages. Gentamicin (Elkins-Sinn, Cherry Hill, N.J.) was administered at 40 mg/kg/day subcutaneously. All animals received a total of 12.5 mmol of sodium daily. All test compounds were given subcutaneously in two equally divided doses at different sites at 8 a.m. and 4 p.m. daily. Animals were acclimatized male Fischer 344 rats with an average initial weight of approximately 200 g (Charles River Breeding Laboratories, Wilmington, Mass.).

Seven-and fourteen-day nephrotoxicity studies. Rats were divided into four groups: gentamicin-NaCl, PAA-Na2O, gentamicin-PAA, and NaCl2O (controls). Within each group, animals were treated for 7 or 14 days (n = 4 in each). At 24 h prior to sacrifice, the rats were transferred to individual metabolic cages for determination of urine osmolality (Uosm), urine creatinine, and creatinine clearance (CLCR). At sacrifice, blood was obtained for determination of serum blood urea nitrogen and creatinine by using standard methods.

Washout study. Rats were divided into four treatment groups: gentamicin-NaCl, PAA-Na2O, PAA-gentamicin, and NaCl2O (controls). All rats were treated for 14 days. Within each group, animals were sacrificed in groups of four at 1 to 2, 4, 6, 8, 10, and 16 weeks following the 14-day treatment period. At 24 h prior to sacrifice, rats were transferred to individual metabolic cages to allow determination of Uosm, urine creatinine, and CLCR. At sacrifice, blood was obtained for determination of serum blood urea nitrogen and creatinine by standard methods. One kidney of each animal was processed for light microscopy. The second kidney was weighed, homogenized, and digested in 1 N NaOH. The digest was diluted, the pH was returned to 7.0, and then the material was assayed for gentamicin with a polarized fluorescence immunoassay (Abbott TDX, North Chicago, III.). The tissue assay has a lower limit of detection of 0.2 μg/g with a coefficient of variation of 5%. Recovery studies show greater than 90% yield using known standards.
and the tissue assay standard curve is linear up to 5,000 ng/g of kidney cortex.

Additionally, glomerular filtration rates determined by [14C]inulin clearance (CL\textsubscript{IN}) were measured in rats immediately following the 14-day treatment period and at 2, 6, and 16 weeks after PAA discontinuation by methods which are standard in our laboratory (3). Briefly, a loading dose of 0.25 µCi of [14C]inulin (New England Nuclear, Boston, Mass.) in 6 ml of 1% NaHCO\textsubscript{3} was given at a rate of 52 µl/min. After a 30-min equilibration period, urine was collected over four periods of 20 min each via a cannula directly sutured into the bladder. Blood was drawn in 0.35-ml samples at the midpoint of each clearance period and replaced with equal volumes of 1% NaHCO\textsubscript{3}. Serum and urine radioactivities were measured with a scintillation counter, and clearance values were calculated by using the standard formula and expressed as milliliters per minute per 100 g of body weight. The CL\textsubscript{IN} for each animal was taken as the mean of the four sequential clearance periods.

Renal histopathology. One half of one kidney from each animal was fixed in 10% buffered formalin and embedded in paraffin; sections were cut at 5 µm and stained with hematoxylin-eosin. For each kidney, a pathologist examined coded slides and estimated the percentages of necrotic and regenerating cortical tubular cells. A semiquantitative scoring system was established for the number of vacuoles present: 1+ if <10%, 2+ if 10 to 25%, 3+ if 25 to 40%, and 4+ if 25 to 40% of the cortical tubular cell contained vacuoles.

HgCl\textsubscript{2} and cisP nephrotoxicity models. Rats were divided into six groups (four rats in each): HgCl\textsubscript{2}-NaCl, HgCl\textsubscript{2}-PAA, cisP-NaCl, cisP-PAA, PAA-H\textsubscript{2}O\textsubscript{2}, and NaCl-H\textsubscript{2}O (control). HgCl\textsubscript{2} (Sigma Chemical Co., St. Louis, Mo.) was administered subcutaneously at 2 mg/kg/day for 2 days, cisP (Bristol-Myers-Squibb, Syracuse, N.Y.) was administered intraperitoneally at 6 mg/kg/day for 3 days, and PAA was administered at 320 mg/kg/day. All animals received equivalent millimolar concentrations of sodium. At 24 h prior to sacrifice, rats were transferred to individual metabolic cages for determination of urine creatinine and CL\textsubscript{CR}. At sacrifice, blood was obtained for determination of blood urea nitrogen and creatinine by using standard methods.

Statistical methods. Differences between groups were analyzed by analysis of variance with repeated measures and Student’s t test. Statistical significance was present if the null hypothesis was rejected \( P \leq 0.05 \).

RESULTS

Seven- and fourteen-day nephrotoxicity washout studies. Functional studies. After 7 days of treatment, renal function (CL\textsubscript{CR}) did not differ between groups. After 14 days of treatment, renal function, as assessed by CL\textsubscript{CR}, was markedly impaired in animals that received gentamicin alone compared with all other groups (\( P < 0.001 \)) (Fig. 1). CL\textsubscript{IN} measured at selected time points, was significantly reduced after 14 days of treatment with gentamicin alone (0.10 ± ml/min/100 g) versus all other groups (0.55 ± 0.16 for PAA-H\textsubscript{2}O, 0.75 ± 0.1 for gentamicin and gentamicin-PAA, and 0.69 ± 0.33 for controls) (\( P < 0.005 \)). During the washout period, CL\textsubscript{IN} remained within the normal range for rats given gentamicin-PAA and did not differ from the PAA-H\textsubscript{2}O and control groups. Similarly, \( U_{\text{cr}} \) was significantly reduced after 14 days of gentamicin alone (558 ± 78 mosm) but well maintained in all other groups (1,914 ± 306 for PAA-H\textsubscript{2}O, 1,722 ± 197 for gentamicin and gentamicin-PAA, and 2,475 ± 538 for controls) (\( P < 0.005 \)). During the washout period, CL\textsubscript{CR} normalized in gentamicin-treated
animals and did not differ from that of the other groups after week 2. CL_{CR} was normal in all groups after week 16 of the washout period. Animals treated with gentamicin alone recovered urinary concentrating ability after week 12 of the washout period. Interestingly, U_{osm} after week 16 of washout was lower in gentamicin-treated animals (2,270 ± 276 mosm) than in the control group (3,093 ± 146 mosm; P < 0.05). The PAA-gentamicin and PAA-alone groups maintained a normal U_{osm} through the washout period and did not differ from controls.

**Histopathology.** Tubular necrosis and regeneration were found only in rats given gentamicin alone; such changes were absent in all other groups immediately following the 14-day treatment period. During the washout period, tubular necrosis and regeneration resolved in the gentamicin-treated group by week 8. Extensive microcalcification of renal cortical tissue was present only in rats treated with gentamicin alone after week 12 of the washout period.

Administration of PAA, with or without gentamicin, led to cytoplasmic vacuole formation in renal tubular epithelial cells. The vacuoles associated with PAA alone were large and homogeneous, in contrast to those associated with PAA-gentamicin, which were smaller, contained electron-dense material, and were more heterogeneous. Resolution of all vacuoles occurred during the washout period; after 6 weeks, PAA-exposed kidneys (with and without gentamicin) were identical to untreated controls in appearance.

After 14 days of treatment, animals that received gentamicin-PAA had accumulated approximately 10 times more gentamicin in their renal cortical tissue (5,439 ± 528 μg/g of tissue) than had animals given gentamicin alone (603 ± 28 μg/g of tissue; P < 0.001), despite preservation of CL_{CR} in the former group (Fig. 2). During the washout period, the elimination half-life of gentamicin from renal parenchyma differed between the two groups. It took 11 days to eliminate half of the gentamicin content in the kidneys of rats treated with PAA-gentamicin, compared with 18.6 days in rats administered only gentamicin (Fig. 2).

**HgCl₂ and cisP study.** CL_{CR} was markedly reduced in rats that received either HgCl₂ alone (0.42 ± 0.24 ml/min/100 g), HgCl₂-PAA (0.06 ± 0.02), cisP alone (0.13 ± 0.004), or cisP-PAA (0.39 ± 0.18) compared with controls P < 0.25 (Fig. 3). Additionally, CL_{CR} did not differ significantly between animals that received HgCl₂ or cisP with or without PAA coadministration. Despite a trend toward better function in cisP versus HgCl₂-PAA, differences did not reach statistical significance, possibly because of the small number of animals.

**DISCUSSION**

Williams and Hottendorf first described PAA protection from experimentally induced amikacin nephrotoxicity (9). Subsequently, three investigative groups have verified this observation (2, 4, 8). Previously, we reported that PAA prevented functional and pathologic evidence of experimental gentamicin nephrotoxicity for dosing periods of up to 27 days (4). The current study was undertaken to evaluate the long-term effect of PAA on aminoglycoside-induced nephrotoxicity by using a purified PAA with a lower MW over 7- and 14-day treatment periods and a 16-week washout period. Additionally, the specificity of nephroprotection by PAA was evaluated by using standard mercuric chloride and cisP nephrotoxicity models.

Administration of PAA with or without gentamicin resulted in formation of cytoplasmic vacuoles in renal tubular epithelia. The pattern of vacuolation was identical to that of our previous study, despite the administration, in the earlier study (4), of a PAA with a mean MW of 16,000, as opposed to the PAA used in the present study, which had a
mean MW of 3,700. Coadministration of PAA and gentamicin resulted in a mixed population of small to medium-sized vacuoles, while PAA alone resulted in a more homogeneous population of large, clear vacuoles. The vacuoles had no functional consequences as measured by CLCR and CLIN, which remained in the normal range throughout the treatment and washout periods. No necrosis or regeneration of tubular epithelial cells could be directly correlated with the presence of these vacuoles. Histologically, the vacuoles resolved during the washout period; after 6 weeks, vacuoles were no longer present in PAA (with or without gentamicin)-exposed kidneys. No long-term structural changes could be attributed to previous PAA treatment. Maunsbach et al. reported the formation of cytoplasmic vacuoles in the proximal tubular epithelium of rats given a glucose, mannitol, sucrose, or dextran solution (7). The underlying mechanism of this osmotic nephrosis is unclear; however, and whether this represents the same physiologic event in PAA-exposed kidneys remains to be determined. On the basis of current data and our previous report, the presence of vacuoles, implying tubular cell PAA uptake, correlates with nephroprotection.

The paradoxical finding of markedly elevated renal parenchymal gentamicin accumulation with preservation of cell integrity and renal function was again documented in animals that received gentamicin and PAA. The mechanism that allows for this dissociation between nephrotoxicity and renal cortical aminoglycoside accumulation remains unclear. It is tempting to speculate that the polyanionic PAA acts as an intracellular electrostatic binding “sponge” for the polycationic aminoglycoside. Our results indicate that gentamicin elimination from the renal parenchyma was more rapid, with an elimination half-life of 11 days, in animals given both PAA and gentamicin, as opposed to an elimination half-life of 18.6 days in rats given gentamicin alone. It is not apparent whether this difference in half-life is related directly to the presence of PAA or is simply a reflection of enhanced drug excretion by proximal tubular cells less damaged by the aminoglycoside. Alternatively, cells injured by aminoglycosides might be able to accumulate less drug because of damage to drug transport pathways. There are no direct data available to exclude the alternative explanation.

In a series of recent reports by Kishore et al. and Beauchamp et al., coadministration of PAA and gentamicin to rats prevented aminoglycoside-induced inhibition of lysosomal lipase, reduced cell turnover as measured by diminished [3H]thymidine DNA incorporation, and blocked gentamicin-induced increases in kidney phospholipid content (1, 5). The diminished cell turnover rate may simply reflect the lack of necrosis found when PAA and gentamicin are coadministered. While prevention of aminoglycoside-induced phospholipidosis is conferred by PAA, the mechanisms of cellular protection remain unclear.

Little is known of the structure-activity relationships of PAA. All of the PAA preparations analyzed to date have proved to be a mixture of D and L optical isomers and alpha and beta peptide linkages. It is tempting to speculate that some degree of D optical isomer is desirable for avoidance, or retardation, of hydrolysis by endogenous peptidases. Similarly, the relationship between MW and nephroprotection is unknown. All PAA preparations represent a family of polymers, and the quoted MW is a mean value. In theory, it is the net total negative charge that is functionally important. Hence, the same degree of nephroprotection might be achievable by a lower-MW polymer administered in a larger dose.

In separate trials, PAA was coadministered with HgCl₂ and cisP. CLCR was significantly lower in animals that
received HgCl₂ with or without PAA than in controls and rats that received PAA alone. Likewise, animals that received cisP were not protected by the presence of PAA, although renal function tended to be better than with PAA-HgCl₂. Thus, nephroprotection was not apparent when PAA was coadministered with either HgCl₂ or cisP. It should be noted, however, that protection from cisP nephrotoxicity may have been missed, given the small number of animals studied.

In conclusion, PAA with a mean MW of 3,700 prevented functional and morphologic nephrotoxic injury in rats administered a predictably nephrotoxic dose of gentamicin. Neither the tubular epithelial cytoplasmic vacuoles associated with PAA administration nor the high renal gentamicin concentration seen with coadministration of gentamicin and PAA produced functional impairment over a 16-week washout period. The exact strategy for employing PAA as a nephroprotectant clinically is unknown; pharmacokinetic and pharmacodynamic studies are in progress to determine optimal dosing regimens.

REFERENCES