Effect of uremia on HDL composition, vascular inflammation, and atherosclerosis in wild-type mice

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Bang CA, Bro S, Bartels ED, Pedersen TX, Nielsen LB. Effect of uremia on HDL composition, vascular inflammation, and atherosclerosis in wild-type mice. Am J Physiol Renal Physiol 293: F1325–F1331, 2007. First published August 8, 2007; doi:10.1152/ajprenal.00039.2007.—Wild-type mice normally do not develop atherosclerosis, unless fed cholic acid. Uremia is proinflammatory and increases atherosclerosis 6- to 10-fold in apolipoprotein E-deficient mice. This study examined the effect of uremia on lipoproteins, vascular inflammation, and atherosclerosis in wild-type C57BL/6J mice. Uremia was induced by 5⁄6 nephrectomy (NX) and increased plasma urea and creatinine concentrations 2.5- to 4.5-fold; control mice were sham operated. After NX, mice were fed a Western-type diet or the same diet with 0.5% cholic acid. Cholic acid-fed NX mice did not thrive and were killed. In NX mice fed the Western-type diet (n = 7), the total plasma cholesterol concentration was similar to that in sham mice (n = 11), but on gel filtration the LDL/HDL cholesterol ratio was increased. HDL from NX mice contained more serum amyloid A and triglycerides and less cholesterol than HDL from sham mice. Plasma concentrations of sICAM-1 and sVCAM-1 and aortic mRNA expression of ICAM-1 and VCAM-1 did not differ between NX and sham mice. Twenty-six weeks after NX, the average oil red O-stained area of the aortic root was similar in NX and sham mice fed the Western-type diet, while it was increased in cholic acid-fed sham mice. The results suggest that moderate uremia neither induces aortic inflammation nor atherosclerosis in C57BL/6J mice despite increased LDL/HDL cholesterol ratio and altered HDL composition.

inflammation; serum amyloid A

CHRONIC RENAL FAILURE INCREASES the risk of cardiovascular disease (11, 12). Although often encountered in uremic patients, hypercholesterolemia, hypertension, and smoking cannot explain the increased risk (9). However, less firmly established risk factors such as increased plasma concentrations of triglyceride-rich apolipoprotein B-containing lipoproteins (1, 18) (with normal total plasma cholesterol concentrations) and inflammation (18) may play a role. In addition, the maturation of HDL seemingly is a defect implying that uremia could impair the otherwise cardioprotective effect of HDL (31). Moderate uremia (induced by 5⁄6 nephrectomy) leads to markedly increased atherosclerosis in hypercholesterolemic apolipoprotein E (apoE)-deficient mice (3–7, 15, 20). This effect cannot be ascribed to changes in the total plasma cholesterol level (3). Nevertheless, in apoE-deficient mice uremia confers increases in the expression of inflammatory genes in the arterial wall (5, 6), suggesting that inflammation could play a role. Interestingly, systemic inflammation has been suggested to convert HDL from a protective to a harmful lipoprotein with proatherogenic effects (30). This effect has been suggested to be related to the increase in serum amyloid A (SAA) plasma concentrations that occurs during inflammation (22, 30). SAA is an amphipathic protein that can incorporate in HDL (10, 14). SAA-enriched HDL is believed to have proatherogenic effects, and SAA has been detected in atherosclerotic lesions where it colocalizes with apoA-I (25). It is unknown, however, whether SAA-enriched HDL may be formed during uremia and how it may affect development of atherosclerosis. The apoE-deficient mouse model may not be suited to address this issue, since their plasma lipoprotein composition is dominated by a vast accumulation of atherogenic apoB48-containing lipoproteins in plasma (34).

Mice normally have very low plasma levels of apoB-containing VLDL and LDL, with the major portion of the plasma cholesterol being in HDL. Nevertheless, the inbred C57BL/6J mouse strain is susceptible to development of fatty-streak-like atherosclerotic lesions when fed a plasma cholesterol-raising cholic acid-containing diet (24, 27), whereas lesion formation occurs but is minimal when the mice are fed a Western-type diet enriched with cholesterol and fat (29). Interestingly, dietary cholic acid raises plasma SAA concentrations (26), although the importance of this effect in relation to atherosclerosis is unclear (17, 28). Two previous studies have examined atherosclerosis in 5⁄6 nephrectomized C57BL/6J mice with moderate uremia. One study showed that uremic mice indeed can develop oil-red-O-stained lesions when fed a cholic acid-free, cholesterol- and fat-enriched diet (29). A more recent study noted that three of eight uremic wild-type mice had aortic lesions 12 wk after 5⁄6 nephrectomy when fed a low-cholesterol, low-fat diet (7).

In this study, we sought to examine to what extent moderate uremia might affect plasma lipoproteins, vascular inflammation, and development of atherosclerosis in C57BL/6J mice. For this purpose, we examined plasma lipids and lipoprotein profiles, SAA, hepatic expression of genes involved in synthesis of apoB- and apoA-I containing lipoproteins, and aortic expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) mRNA, as well as nitrotyrosine staining and lesion formation in aortas of uremic and control mice fed a cholesterol- and fat-enriched diet and in mice fed a cholic acid-containing diet.

MATERIALS AND METHODS

Animals. C57BL/6J wild-type mice (Taconic M&B Laboratory Animals and Services for Research, Ry, Denmark) were housed in a

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temperature-controlled facility (21–23°C) with a 12:12-h light-dark cycle. The mice were allowed free access to food and water. The experiments were performed according to the principles stated in the Danish law on animal experiments and were approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark. Before induction of uremia, the mice were fed a standard mouse chow (Altromin 1314, Altromin, Lage, Germany). After induction of uremia or sham operation, mice were switched to a high-cholesterol/high-fat diet (C13002, Research Diets, New Brunswick, NJ) with or without sodium chloride (0.5% wt/wt) produced by mixing Purina mouse chow 5015 (75 g); casein, 80 mesh (75 g); dextrose (25 g); maltodextrin (42 g); sucrose (16.25 g); cellulose BW200 (12.5 g); cocoa butter (75 g); mineral mix S10001 (8.75 g); vitamin mix V10001 (2.5 g); choline bitartrate (1.25 g); and cholesterol USP (12.5 g).

Moderate uremia was induced by a two-step surgical procedure (3). Briefly, the upper and lower poles of the right kidney were resected in 10wk-old mice. Two weeks later, the left kidney was resected. Control mice underwent sham operations at both 10 and 12 wk of age. Anesthesia was achieved with a mixture (ratio 3:0.2:4) of fentanyl (0.050 mg/kg), midazolam (5.0 mg/ml), and droperidol (2.5 mg/ml, 20 µg/g body wt sc), and buprenorphine 0.3 mg/ml (0.1 µg/g body wt sc twice daily for 3 days) was used as an analgesic after the surgical procedures. In mice on the C57BL/6J background, 3% NX does not affect blood pressure and as such does not inflict insufficient adrenal function (3). NX and sham-operated mice were allocated to a cholestero- and fat-enriched diet either with or without cholic acid. The NX mice fed the cholic acid-containing diet did not thrive and were killed before the end of the study. The cause of the poor compliance to the cholic acid-containing diet is unknown. At the time of death, several of the mice had grossly enlarged livers, gallstones, and elevated plasma liver enzyme concentrations.

**Plasma biochemistry.** Blood from the retroorbital venous plexus was collected in heparinized microtubes (Capjet, Terumo Medical, Elkton, MD) and centrifuged at 2,000 g for 10 min at 4°C. Plasma was stored at −80°C. Plasma sodium, potassium, chloride, creatinine, total calcium, phosphate, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, albumin, and bilirubin concentrations were measured with a Modular P Hitachi Automatic Analyzer (Roche, Mannheim, Germany). Plasma SAA, C-reactive protein (CRP), total triglycerides, total cholesterol, HDL cholesterol, calcium, phosphate, alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase, albumin, and bilirubin concentrations were measured with Modular P (plasma) or manually (gel filtration and ultracentrifugation of plasma liver enzyme concentrations).

**Molecular analyses.** RNA was isolated from liver and aorta (TRIzol; Invitrogen). Total RNA was isolated from liver and aorta with TRIzol (Invitrogen). A total of 500 ng of RNA was used for reverse transcription with a Tissuelyzer (Qiagen Retsch, Hann, Germany). Total RNA was isolated with TRIzol (Invitrogen). A total of 500 ng of RNA was used for reverse transcription with a Tissuelyzer (Qiagen Retsch, Hann, Germany). Total RNA was isolated with TRIzol (Invitrogen). A total of 500 ng of RNA was used for reverse transcription with a Tissuelyzer (Qiagen Retsch, Hann, Germany). After homogenization of mouse liver biop- sies with a TissueLyser (Qiagen Retsch, Hann, Germany), total RNA was isolated with TRIzol (Invitrogen). Aortic RNA was made as previously described (5). RNA concentrations were assessed by a BioPhotometer (Eppendorf). For semiquantitative analyses of plasma ap-A-I, we loaded 0.16 µl plasma/lane. Analyses of plasma dilutions with loadings of 0.0625, 0.125, and 0.25 µl showed proportionality between band intensities and amounts of loaded apo-A-I.

**mRNA quantification.** After homogenization of mouse liver biopsies with a TissueLyser (Qiagen Retsch, Hann, Germany), total RNA was isolated with TRIzol (Invitrogen). Aortic RNA was made as previously described (5). RNA concentrations were assessed by a BioPhotometer (Eppendorf). For semiquantitative analyses of plasma apo-A-I, we loaded 0.16 µl plasma/lane. Analyses of plasma dilutions with loadings of 0.0625, 0.125, and 0.25 µl showed proportionality between band intensities and amounts of loaded apo-A-I.

**Cell culture.** boyfriend (Vienna, Austria) was used to normalize hepatic gene expression. In aortas, the expression of β-actin, GAPDH, and HPRT was similar in NX and sham-operated mice, and the average expression of the three genes was used to normalize aortic gene expression. Importantly, the overall results and conclusions were similar whether the results were analyzed with or without normalization with housekeeping gene expression.

**Evaluation of aortic pathology and quantification of atherosclerosis.** To study aortic atherosclerosis, mice were anesthetized and the thoracic aorta was removed, freed of adventitial tissue, opened longitudinally, and the intimal surface was photographed with a digital camera. The heart with 1–2 mm of the aortic root was placed in OCT compound (Tissue-Tek, Sakura Fine tec, Varlose, Denmark) and frozen on dry ice. Before sectioning, hearts were fixed in phosphate-buffered parafomaldehyde (4% wt/
Effect of NX and a cholic acid-containing diet on plasma markers of kidney and liver function. Plasma urea was increased 2.5- to 4.5-fold by NX and was unaffected by the cholic acid-containing diet (Fig. 1). Also, the plasma potassium, creatinine, and calcium concentrations were increased in NX mice.

Table 1. Effect of NX and a cholic acid-containing diet on plasma biochemical parameters related to liver function

<table>
<thead>
<tr>
<th></th>
<th>NX (n = 7)</th>
<th>Cholic Acid (n = 7)</th>
<th>Sham (n = 11)</th>
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<tbody>
<tr>
<td>Creatinine, μmol/l</td>
<td>28±3.5‡</td>
<td>12±0.7</td>
<td>10±0.5</td>
</tr>
<tr>
<td>Total calcium, mmol/l</td>
<td>2.59±0.06†</td>
<td>2.45±0.05†</td>
<td>2.26±0.03</td>
</tr>
<tr>
<td>Phosphatase, mmol/l</td>
<td>2.18±0.31</td>
<td>1.73±0.08*</td>
<td>2.13±0.10</td>
</tr>
<tr>
<td>Ca × phosphate, mmol/l²</td>
<td>5.73±0.98</td>
<td>4.24±0.20</td>
<td>4.83±0.26</td>
</tr>
<tr>
<td>Sodium, mmol/l</td>
<td>159±3.2</td>
<td>160±5.1</td>
<td>155±2.2</td>
</tr>
<tr>
<td>Potassium, mmol/l</td>
<td>6.98±0.33½</td>
<td>5.86±0.51</td>
<td>5.18±0.15</td>
</tr>
<tr>
<td>Chloride, mmol/l</td>
<td>109±2.1</td>
<td>107±4.9</td>
<td>108±1.3</td>
</tr>
<tr>
<td>Body weight, % baseline</td>
<td>120±5.8⁵</td>
<td>115±4.6</td>
<td>160±6</td>
</tr>
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</table>

Values are means ± SE 26 wk after NX or sham operation. *P < 0.05, †P < 0.01, and ‡P < 0.001 compared with sham mice.

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Fig. 2. Effect of NX and a cholic acid-containing diet on plasma cholesterol and lipoprotein distribution in C57BL/6J mice. A: total plasma cholesterol in NX (●, n = 7), sham (○, n = 11), and cholic acid-fed mice (▲, n = 7). Values are means ± SE. *P < 0.05 and †P < 0.001 compared with sham mice. B: pooled plasma (200 μl) from 7–11 mice at 26 wk after NX was fractionated by gel filtration chromatography and cholesterol was measured in collected fractions. Solid line, NX mice (●, n = 7); long dashed line, sham mice (○, n = 11); short dashed line, cholic acid-fed mice (▲, n = 7).
the NX compared with the sham group, whereas the cholic acid-containing diet increased plasma calcium and decreased plasma phosphate (Table 1). NX did not affect plasma sodium and chloride concentrations (Table 1). Both NX and the cholic acid-fed mice gained less weight than the sham-operated controls (Table 1). Plasma alkaline phosphatase and lactate dehydrogenase were both increased 1.8-fold, whereas plasmaalanine aminotransferase and albumin were decreased by 60 and 10%, respectively, in the NX group compared with the sham group (Table 2). The cholic acid-containing diet increased plasma alkaline phosphatase, lactate dehydrogenase, and alanine aminotransferase 2.3-, 3.8-, and 5.3-fold, respectively, compared with the sham group, but did not affect plasma bilirubin or albumin (Table 2).

Table 3. Lipid composition of HDL from NX and sham mice

<table>
<thead>
<tr>
<th></th>
<th>NX</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid, mmol/l</td>
<td>0.62±0.16 (22.1±4.7)</td>
<td>0.67±0.14 (24.7±3.2)</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>0.29±0.04* (11.8±1.8*)</td>
<td>0.15±0.03 (6.5±0.6)</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>0.84±0.06 (15.0±0.8*)</td>
<td>0.95±0.03 (18.1±1.0)</td>
</tr>
<tr>
<td>Protein, mg/l</td>
<td>1.10±0.07 (51.1±2.2)</td>
<td>1.03±0.07 (50.7±2.7)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Three pools of HDL each from 3–8 mice were isolated from plasma from NX or sham mice with sequential ultracentrifugation. Data are plasma HDL lipid and protein concentrations assuming that the recovery of HDL during purification by ultracentrifugation was 100%. Numbers in parentheses are percentages of total HDL mass. Phospholipid, triglyceride, and cholesterol masses were calculated using molecular masses of 773.2, 885.0, and 386.6 g/mol, respectively. *P ≤ 0.05 vs. sham.
plasma apoA-I concentration and hepatic apoA-I mRNA expression did not differ in a statistically significant manner between NX and sham mice (data not shown).

The mean plasma SAA concentration increased markedly in the NX compared with the sham-operated mice (Fig. 4A). The cholic acid-containing diet also increased plasma SAA but to a lesser extent than NX (Fig. 4A). On gel filtration and Western blotting analyses of plasma from NX mice, SAA and apoA-I were associated with HDL-sized lipoproteins (Fig. 4B). It was striking that the magnitude of the increase in plasma SAA concentrations above the average value in sham-operated mice varied greatly between NX mice (0- to 53-fold). The cause of this variation is unknown. The mice were kept in a clean facility with frequent testing excluding infections, none of the mice had signs of wound infection after surgery and the rise in SAA was not associated with the extent of uremia (judged from plasma urea concentrations). To examine whether an increase in plasma SAA was associated with a decrease in plasma apoA-I, we compared the plasma apoA-I concentration in individual NX mice with the plasma SAA concentration. NX mice with high-plasma SAA had low apoA-I concentrations (Fig. 4C). However, the hepatic apoA-I mRNA expression was not decreased in parallel with the increase in SAA (Fig. 4C).

**Effect of NX on aortic expression of adhesion molecules.** Vascular inflammation is a hallmark of the accelerated atherosclerosis occurring in hypercholesterolemic, uremic, apoE-deficient mice (5, 6). To examine whether moderate uremia

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**Fig. 5. Effect of NX on aortic expression of ICAM-1 and VCAM-1 mRNA** and plasma sICAM-1 and sVCAM-1 in C57BL/6J mice. A: aortic expression of ICAM-1 and VCAM-1 mRNA [normalized with hypoxanthine phospho ribosyl transferase (HPRT), β-actin, and GAPDH as described in MATERIALS AND METHODS] 12 wk after NX. The aortas were harvested from NX and sham-operated control mice used in a previous study (5). Filled bars, NX mice (n = 18); open bars, sham mice (n = 11). B: plasma sICAM-1 and sVCAM-1 concentrations 26 wk after NX. Filled bars, NX mice (n = 7); hatched bars, cholic acid-fed mice (n = 6); open bars, sham mice (n = 11). Values are means ± SE. There were no statistically significant differences between NX and sham-operated mice for any of the variables depicted.

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**Fig. 6. Effect of NX and a cholic acid-containing diet on aortic atherosclerosis and nitrotyrosine staining in C57BL/6J mice.** A: area of oil red O-stained lesions 26 wk after NX. Filled bars, NX mice (n = 7); hatched bars, cholic acid-fed mice (n = 6); open bars, sham mice (n = 11). Values are means ± SE. Brackets and *p* values show results of 2-group comparisons. B–D: immunohistochemical nitrotyrosine staining (brown) and negative control staining (insets) of aortas from a C57BL/6J NX mouse (B), a C57BL/6J sham-operated control mouse (C), and an apoE-deficient NX mouse with atherosclerosis (D; the aorta was harvested 36 wk after NX and also used in another study) (4). Note the uniform staining of nitrotyrosine in the media of NX and sham C57BL/6J mice as opposed to the strong nitrotyrosine staining of the foam cell intimal lesion in the apoE-deficient NX mouse.
may have a similar effect in wild-type mice without overt hypercholesterolemia, we measured markers of aortic inflammation and oxidative stress. The aortic expression of VCAM-1 and ICAM-1 mRNA did not differ between in NX and sham-operated control mice when measured 12 wk after NX using aortic RNA from a previous study (Fig. 5A) (5). Also, the plasma concentrations of sICAM-1 and sVCAM-1 were similar in NX and sham-operated controls (Fig. 5B).

**Effect of NX and a cholic acid-containing diet on aortic atherosclerosis and aortic inflammation.** Twenty-six weeks after NX, there were no macroscopically visible atherosclerotic plaques in the thoracic aorta in either NX, cholic acid-fed, or sham-operated control mice (data not shown). On analyses of cross sections of the aorta proximally to the aortic valves, the oil red O-stained area was similar in NX and sham-operated control mice, whereas it was increased ~2-fold by the cholic acid-containing diet (Fig. 6A).

NX apoE-deficient mice display increased accumulation of nitrotyrosine-stained material in the aorta, suggesting that oxidative stress may play an integrated role in uremic atherosclerosis (3). To examine aortic oxidative stress in wild-type NX mice, aortic sections were stained with an anti-nitrotyrosine antibody. Nitrotyrosine-stained material in NX and sham-operated control mice was uniformly distributed in the aortic media (Fig. 6, B and C). In contrast, in atherosclerotic aortas from uremic apoE-deficient mice harvested 26 wk after NX (4), nitrotyrosine was predominantly in intimal atherosclerotic lesions rather than in the media (Fig. 6D). There was no difference in the nitrotyrosine-staining intensity between aortas from NX and sham-operated control mice as judged from blinded semiquantitative assessments of four to five sections per mouse by two independent persons (data not shown).

As in our previous studies of apoE-deficient mice (3–5), we did not see any calcifications in the aortic root of NX, cholic acid-fed, or sham mice on von Kossa staining of four to five sections from each mouse (data not shown).

**DISCUSSION**

The present study suggests that moderate uremia increases VLDL/LDL cholesterol in wild-type mice. Human individuals with uremia also have increased VLDL due to impaired metabolism in plasma, involving decreased lipoprotein lipase activity and lipoprotein clearance in the liver (13, 31). Although we did not assess plasma lipoprotein turnover, the present results in mice may similarly reflect impaired clearance of apoB-containing lipoproteins since the gene expression of MTP in the liver was slightly decreased and that of apoB unchanged in the moderately uremic compared with the control mice.

Although NX did not affect the plasma HDL cholesterol concentration, the composition of the HDL particles was changed. NX conferred a marked elevation of HDL-associated SAA in wild-type mice. SAA is mainly produced in the liver. Thus the data suggest that moderate uremia causes an inflammatory response in the mouse liver. The effect of NX on plasma SAA levels varied greatly between mice, and the NX mice with the highest SAA concentrations also had low plasma apoA-I concentrations. Despite that the hepatic apoA-I gene expression is decreased in uremic rats (32), the hepatic apoA-I mRNA expression was not reduced in the NX mice. This suggests that chronic inflammation in mice with moderate uremia confers a decrease in plasma apoA-I, which is not necessarily due to lowering of the hepatic apoA-I gene expression. HDL from uremic mice also contained more triglycerides and less cholesterol per microgram protein than control HDL. These changes in HDL lipid composition are similar to those described after induction of acute inflammation with an injection of croton oil in rabbits or with LPS in baboons (8). Nevertheless, in mice LPS-induced acute inflammation does not change the lipid composition of HDL (despite increasing plasma SAA), suggesting that, at least in mice, chronic inflammation associated with uremia affects HDL differently than acute inflammation perhaps due to reduced hepatic lipase activity (16) and/or plasma LCAT activity (33). Interestingly, stable isotope turnover studies support the notion that triglyceride-enrichment of HDL increases its catabolic rate (19).

NX dramatically accelerates the development of atherosclerosis in the aortas of apoE-deficient mice, which have elevated plasma VLDL/LDL cholesterol (3). In apoE-deficient mice, NX also increases aortic expression of the genes encoding ICAM-1 and VCAM-1 as well as plasma concentrations of sICAM-1 and sVCAM-1 (4, 6). The increase in ICAM-1 expression (and perhaps also VCAM-1) precedes lesion formation in NX apoE-deficient mice, suggesting that vascular inflammation may be an important precipitating factor in uremic atherosclerosis. In the present study, neither the aortic expression of VCAM-1 and ICAM-1 mRNA nor the plasma concentrations of sICAM-1 and sVCAM-1 were increased in NX wild-type mice. This supports the idea that vascular inflammation in uremic mice to a large extent is dependent on concomitant hypercholesterolemia. Similarly, when aortic oxidative stress was examined with nitrotyrosine staining, we saw no difference between NX and sham-operated control mice. Of note, in NX apoE-deficient mouse aortas nitrotyrosine staining was predominantly within the intimal lesions. These results further suggest that the proatherogenic effect of uremia in mouse arteries relies on hypercholesterolemia. Accordingly, there was no difference in atherosclerotic lesion areas in the aortic arch between wild-type NX and sham-operated control mice, and the average lesion area in NX mice was less than in the cholic-acid fed mice. Thus the small increase in VLDL/LDL cholesterol and the proinflammatory changes in HDL composition were insufficient to induce atherosclerosis in NX C57BL/6J mice. Notably, this result does not exclude that formation of proinflammatory HDL contributes to accelerated lesion formation in the setting of more markedly elevated VLDL/LDL cholesterol concentrations such as in apoE-deficient mice and possibly humans.

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