Lipopolysaccharide Alters Aggrecan Metabolism in the Growth Plate (44029)

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Abstract. **In this study, we examined the influence of lipopolysaccharide (LPS) on aggrecan metabolism and structure in the growth plate. Two experimental approaches were used: (i) *in vivo* administration of LPS to 10-day-old chicks; and (ii) *in vitro* addition of LPS to explant culture of normal chick growth plate. Twelve-day-old male broiler chicks were killed 48 hr after intravenous injection of LPS (3 mg/kg) or saline (control), and growth plate from the femur or tibia was cultured or frozen. Tissue for explant culture was (i) cultured for 5 days with daily medium change (glycosaminoglycan release into the medium estimates proteoglycan breakdown rates), or (b) incubated with $^{35}SO_4$ to determine the rate of proteoglycan synthesis. Proteoglycan structure was determined by associative (0.5 *M* sodium acetate) and dissociative (4 *M* guanidine HCl) Sepharose CL2B chromatography. Explant culture of growth plate from LPS-injected chicks (*in vivo*) showed a decrease ($P < 0.05$) in the rate of proteoglycan synthesis. There were a greater proportion of small monomers and a reduced ability to aggregate in growth plate from LPS-injected chicks. *In vitro* addition of LPS (100 μg/ml) to explant culture medium reduced proteoglycan synthesis ($P < 0.02$), and the rate of release was increased ($P < 0.001$). In addition, the total and newly synthesized proteoglycans released into the medium from LPS-treated explant culture had a reduced aggregation and a majority of monomers that were smaller than control. These results demonstrate that LPS disrupts the normal metabolism and structure of growth plate aggrecan, and we hypothesize that this may adversely influence longitudinal growth.** [P.S.E.B.M. 1996, Vol 212]

The growth plate is immature skeletal tissue responsible for virtually all longitudinal growth of the skeleton. Long bone growth is accomplished through cell proliferation and maturation, matrix production, and mineral and endochondral ossification. When adult length is achieved, chondrocyte proliferation ceases and the growth plate closes (1). The growth plate consists of an extracellular matrix composed primarily of cartilage collagens (2) and proteoglycans (3). The predominant proteoglycan is aggrecan, which is a large molecule (M_r 1–4 × 10^6) with a protein core of M_r 225–250,000 (4). Aggrecan contains many proteoglycan monomers, with chondroitin sulfate and keratin sulfate chains and *N*- and *O*-linked oligosaccharides attached to a core protein. The core protein binds to hyaluronate and is stabilized by the interaction of link protein. In cartilage, aggrecan contributes to the compressive properties and may have a similar function in the growth plate.

Lipopolysaccharide (LPS), also known as endotoxin, is an essential component of gram-negative bacteria and is considered to be the major factor responsible for the toxic effects of an infection (5). It has been shown that LPS can activate the immune response by triggering the release of cytokines. Cytokines result in a protease imbalance, which may be responsible for the ensuing joint destruction (6, 7). Chondrocytes exposed to LPS have shown a deleterious effect on proteoglycans in articular cartilage (8–12). An increased degradation of proteoglycans and other matrix compo-

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nents is believed to be responsible for the subsequent tissue damage associated with inflammatory joint disease (13). This same mechanism may occur in the growth plate, and contribute to the growth retardation observed in children with a history of neonatal joint infection (14) or those exposed to bacterial infections (15, 16). However, the mechanism by which chondrocytes regulate aggrecan metabolism in the growth plate during pathological stress or infection has never been addressed. In this study, we investigated whether LPS affects the metabolic events and ultrastructural organization of aggrecan in the growth plate *in vivo* and *in vitro*.

Materials and Methods

Two approaches were used to examine the effect of LPS on proteoglycan metabolism and structure in the growth plate: (i) *in vivo* LPS was injected into 10-day-old chicks; and (ii) *in vitro* LPS was added to the medium of normal chick growth plate explant culture. Newborn male broiler chicks (Avian Services, Frenchtown, NJ) were maintained on a semipurified diet (Research Diets Inc., New Brunswick, NJ) and tap water *ad libitum*, and housed in stainless steel, heated (35°C) brooder cages with eight birds per cage.

Protocol for *In Vivo* Studies. Twenty-two chicks were grown to 10 days of age and monitored for food intake and growth rate (weight) twice weekly. Half of the chicks received an intravenous injection of LPS into the wing vein at a dose of 3 mg/kg body wt (17). This dosage and the route of administration were chosen because they produced the desired symptoms (i.e., lethargy, elevated temperature, anorexia) without any deaths. The LPS solution was prepared by suspending 3 mg of the lyophilized powder (serotype 055:B5; Sigma Chemical Co., St. Louis, MO) in 1 ml of saline. A control group received an injection of sterile saline into the wing vein at a volume equivalent to the experimental group. The chicks were sacrificed 48 hr postinjection by asphyxiation with CO_2. Blood samples were obtained by cardiac puncture, and growth plate was removed.

The control chicks were pair-fed with the chicks in the LPS-injected group because food intake was reduced in chicks who received the LPS injection. The LPS-injected group was allowed to eat *ad libitum*, and the food was weighed to determine the amount to give to the control chicks on the following day. This ensured that both groups of animals received the same energy intake during the 48-hr period.

The presence of endotoxin in the plasma was determined using the E-Toxate Limulus Amebocyte Lysate test (Sigma Chemical) at 24–48 hr after the LPS injection. Blood samples were centrifuged at 4°C and 3000 RPM for 10 min to obtain the plasma. A solution of lysate was added and all samples were incubated for one hour at 37°C. The formation of a hard gel is indicative of endotoxin in the sample.

Tissue preparation. The joints of 12-day-old chicks were removed, and superficial tissue was excised. The epiphyseal growth plate of the proximal and distal tibia and distal femur was dissected away from the metaphyseal and epiphyseal bone. The perichondrium was removed, and the growth plate was sectioned sagitally with a scalpel and cultured or frozen. Growth plate tissue used to determine tissue composition and proteoglycan structure and size was frozen at −25°C for later analysis. Tissue for histology was immediately frozen on dry ice and made into cryostat chunks in optimal cutting temperature (O.C.T.) compound (Miles Scientific, Naperville, IL) and stored at −80°C until further sectioning, and then stained with hematoxylin and eosin.

Tissue composition. The composition of the growth plate tissue was determined by measuring the water (percentage by weight) and estimating proteoglycan and collagen content of the tissue. After determination of water content, tissue was digested with papain for 16 hr at 60°C in 0.1 *M* sodium acetate, pH 5.6, containing 0.05 EDTA and 0.01 cysteine HCl, and the papain was inactivated by heating at 95°C for 45 min.

Total sulfated-glycosaminoglycan (S-GAG) content was analyzed using the 1,9-dimethylmethylene blue (DMB) assay, and measured with a spectrophotometer at 540 and 595 nm (18). Chondroitin-6-sulfate from shark cartilage was used as the standard. Collagen content was estimated by measuring the hydroxyproline (OH-P) content in the tissue. Samples were hydrolyzed overnight with 6 *M* HCl, and then diluted in 1:10 dilution of the stock buffer (0.26 *M* citric acid monohydrate, 12% (v/v) glacial acetic acid, 1.5 *M* sodium acetate and 0.85 *M* sodium hydroxide, pH 6.0). A solution of chloramine-T in *n*-propanol and stock buffer was then added, and samples were left at room temperature for 20 min. Then *p*-dimethyl-aminobenzaldehyde in *n*-propanol and perchloric acid was added and incubated at 60°C for 15 min. Absorbance was measured with a spectrophotometer at 540 nm.

Values for S-GAG and OH-P were normalized to DNA content of the growth plate. DNA was determined fluorometrically with the Hoechst dye 33258 by a modification of the method of LaBarca (19). A 50-μl sample of papain-digested material was added to 1.94 ml of phosphate saline buffer (0.05 *M* $NaPO_4$, 2 *M* NaCl, 2 m*M* EDTA, pH 7.4) and 10 μl of 20% bisbensimidazole. After 1 hr of incubation, the fluorescence was analyzed on a MicroFLUOR Reader (Dynatech Laboratories, Alexandria, VA) using an excitation wavelength of 365 nm and an emission maximum of 458 nm. Highly polymerized DNA (100–500 ng/ml) was used as standard.

Growth plate explant culture. Growth plate explant tissue was cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal calf serum and supplemented with 1% nonessential amino acids, penicillin (1 IU/ml), and streptomycin (1 μg/ml) at 37°C with 5% CO_2. Cell viability using trypan blue exclusion was similar in both control and LPS-treated groups.

To determine rates of proteoglycan synthesis after 24 hr in culture, growth plate tissue was incubated with $Na^{35}SO_4$ (20 μCi/ml; Dupont NEN Products, North Billerica, MA) for 4 hr. The tissue was frozen to stop proteoglycan synthesis, digested with papain, and eluted on PD-10 columns (Pharmacia LKB Biotechnology, Uppsala, Sweden) to separate the incorporated radiolabel from the free isotope. Radioactivity was determined by liquid scintillation counting. Proteoglycan synthesis was expressed as counts per minute (cpm) per microgram S-GAG content.

To study the proteoglycan release from explant tissue, growth plate tissue was maintained in culture for 6 days. The medium for Day 1 was discarded, thereafter it was collected and frozen daily for the next 5 days. The amount of S-GAG in explant tissue and that released into the medium was determined.

Protocol for *In Vitro* Studies. Rates of proteoglycan synthesis and release from the matrix were determined. Growth plate tissue from 18 normal 12-day-old chicks was cultured in the absence and presence of LPS. Lipopolysaccharide was added to explant culture of experimental groups at a concentration of 20 or 100 μg/ml (8, 9, 12) and incubated for 48 hr before determining the rate of proteoglycan synthesis. For proteoglycan breakdown, culture medium from the first 24 hr was discarded. Thereafter, media containing LPS was replaced and collected every 24 hr for five additional days.

Pulse-chase experiments were performed to determine the structure and size of newly synthesized proteoglycan released into the medium. The growth plate was incubated with or without LPS at a concentration of 20 μg/ml for 16 hr before the pulse-chase. The explant tissues were then incubated with 20 μCi/ml $Na^{35}SO_4$ for 4 hr, after which time it was replaced each day with unlabeled medium containing LPS. The medium was collected 48 hr after the pulse and run through a Sepharose CL-2B column (120 × 0.6 cm, Pharmacia) to separate the proteoglycans by size and the ability to aggregate.

Gel filtration chromatography. Proteoglycans were extracted from the growth plate in a 4 *M* guanidine-HCl, 0.05 *M* sodium acetate solution with protease inhibitors for 48 hr at 4°C. The diluted extraction was applied to a Sepharose CL-2B column (120 × 0.6 cm) equilibrated in a dissociative buffer (2 *M* guanidine HCl, 0.5 *M* sodium acetate, pH 5.8, at 4°C) to determine the size distribution of proteoglycan monomers.

To determine the ability of the proteoglycans to aggregate, the extraction solution was dialyzed against 0.5 *M* sodium acetate for 24 hr, with high molecular weight hyaluronate (2% w/w per S-GAG) added to maximize aggregation. The sample was then applied to a Sepharose CL-2B column equilibrated under associative conditions (0.5 *M* sodium acetate, pH 5.6) (20). Fractions were analyzed for total S-GAG and ^{35}S-sulfate content. Aggregation was determined by the elution in the void volume of the column.

Data analysis. One-way analysis of variance (ANOVA) was used to analyze the rates of synthesis and release that involved more than two LPS concentrations (0, 20, and 100 μg/ml). Post hoc analysis was carried out on significant ANOVA results by using the Fisher PLSD test performed on the Macintosh StatView statistical package, version 4.5. Comparisons of synthesis and breakdown between LPS and control groups were analyzed by unpaired Student's *t* test. The rate of proteoglycan release from explant tissue over a 5-day period was analyzed by comparing the slopes between groups using linear regression analysis. Values are expressed as mean ± SD, unless otherwise noted.

Results

Effect of LPS *In Vivo*. At 10 days of age, prior to treatment, the chicks weighed an average of 215 ± 33 g. LPS-injected chicks tested positive for plasma endotoxin, demonstrated symptoms of lethargy, and showed a 15% reduction in food intake. LPS-injected chicks increased body weight by 20% over 48 hr from 200 ± 27 to 253 ± 28 g. This was similar to pair-fed control chicks who showed a 20% increase from 229 ± 34 to 286 ± 40 g.

The water (percentage by weight), proteoglycan (μg S-GAG/μg DNA), and hydroxyproline (μg/μg DNA) content of the growth plate were 80% ± 3%, 283% ± 69%, 11% ± 3%, respectively, in LPS-treated animals, and 79% ± 5%, 293% ± 45% and 10% ± 3%, respectively, in the saline-treated animals. Values were not significantly different between the two groups of animals. Histological examination was done in two or three of LPS-treated or saline-treated tissues and showed that the hypertrophic zone of the LPS-treated tissue was 31% ± 2% of the growth plate and greater than the 24% ± 1% observed in the saline-treated tissue ($P < 0.05$).

Rate of proteoglycan synthesis. The rate of $Na^{35}SO_4$ incorporation into S-GAG of LPS and saline-treated chicks is shown in Figure 1. There was a 48% decrease ($P < 0.05$) in the rate of proteoglycan synthesis in the growth plate of the LPS-injected group,

428 ± 44 cpm/μg S-GAG, compared with the control, 817 ± 155 cpm/μg S-GAG.

Rate of proteoglycan release from explant tissue. Proteoglycan turnover is expressed as the percentage of total proteoglycan released from the tissue explant each day. One Day 1, there was a 3% release of proteoglycan from growth plate in both the LPS-treated and control groups. The rate of release was approximately maintained so that at Day 5 there had been a 16% and 18% release in the LPS-treated and control groups, respectively. There was a trend ($P < 0.06$) towards a small increase in the release of S-GAG in the LPS-treated group compared with the control group (Fig. 2).

Proteoglycan structure. Analysis of aggregating proteoglycans in the growth plate show a reduction in the LPS-injected group, with 39% of total proteoglycans aggregating compared with 68% for the control group (Fig. 3a).

Monomer size was examined under dissociative conditions, and was polydisperse in the control group. However, the larger-sized monomers present in the control group were absent from the elution profile of the LPS-injected group (Fig. 3b).

Effect of LPS on Normal Growth Plate Explant *In Vitro.* *Rate of proteoglycan synthesis.* Proteoglycan synthesis rates were determined in explant tissue cultured for 48 hr in the presence or absence of LPS at a concentration of 20 or 100 μg/ml. The rate of synthesis in the LPS-treated group (at 100 μg/ml) was 29% lower and was significantly lower than the control group ($P < 0.02$; Fig. 4).

Rate of proteoglycan release. Daily proteoglycan release from the matrix was determined in tissue explants that were cultured continually in the presence of LPS. There was a significant increase in the rate of proteoglycan released into the medium in the 20 and 100 μg/ml LPS groups compared with control (Fig. 5).

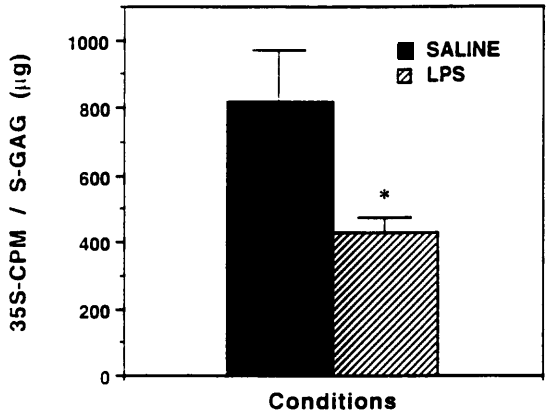

Figure 1. The rate of ^{35}S-sulfate incorporation in the growth plate of LPS- or saline-injected chicks. Mean ± SEM; $n = 8$ for each group. *$P < 0.05$, differs from control.

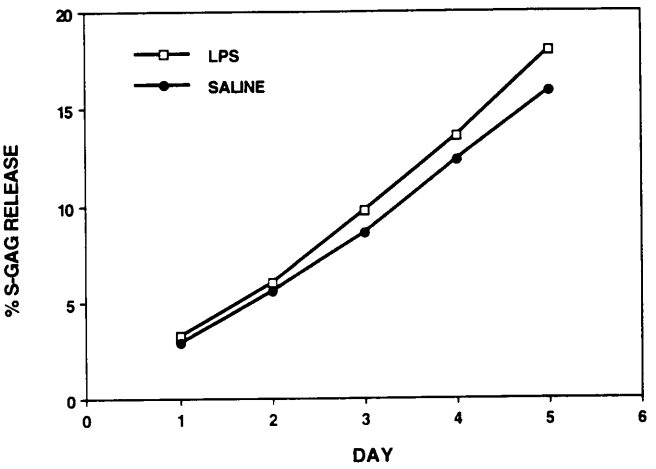

Figure 2. Release of proteoglycans from the growth plate of LPS- or saline-injected chicks. $n = 8$ for each group.

By Day 3, both of the LPS-treated cultures had a significantly greater ($P < 0.0001$) amount of proteoglycan released into the medium, at 8% ± 2% and 12% ± 3%, compared with 5% ± 1% in the control group. The difference in the rate of release became greater on Day 4 and 5 (Fig. 5).

Pulse-chase. Under conditions to examine the proteoglycan's ability to aggregate, the total proteoglycan population released into the media showed that 9% and 34% were able to aggregate in the LPS-treated and control groups, respectively (Fig. 6a). Eight percent and 27% of the newly synthesized proteoglycans released into the medium in the LPS and control groups, respectively, were able to aggregate (Fig. 6b).

The elution profiles to determine size distribution showed a broad polydisperse peak, indicating a wide distribution of monomer size for both the total proteoglycan population and the newly synthesized proteoglycans in control tissue. However, in the LPS-treated group the peak in the total proteoglycan pool and newly synthesized proteoglycans eluted later than the control group, indicating that the majority of the proteoglycan monomers were small (Fig. 6c and d).

Discussion

The data presented in this article provide *in vivo* and *in vitro* evidence that LPS is a potent stimulator of aggrecan breakdown in the growth plate. The growth plate of LPS-injected animals and explant cultured with LPS showed a reduction in proteoglycan synthesis, aggregation, and monomer size. In addition, there was an increased rate of release of proteoglycans from growth plate cultured with LPS. Our study complements and augments findings of studies in articular cartilage exposed to LPS. In those studies, cartilage explant cultured with LPS depressed proteoglycan synthesis and accelerated proteoglycan catabolism (8,

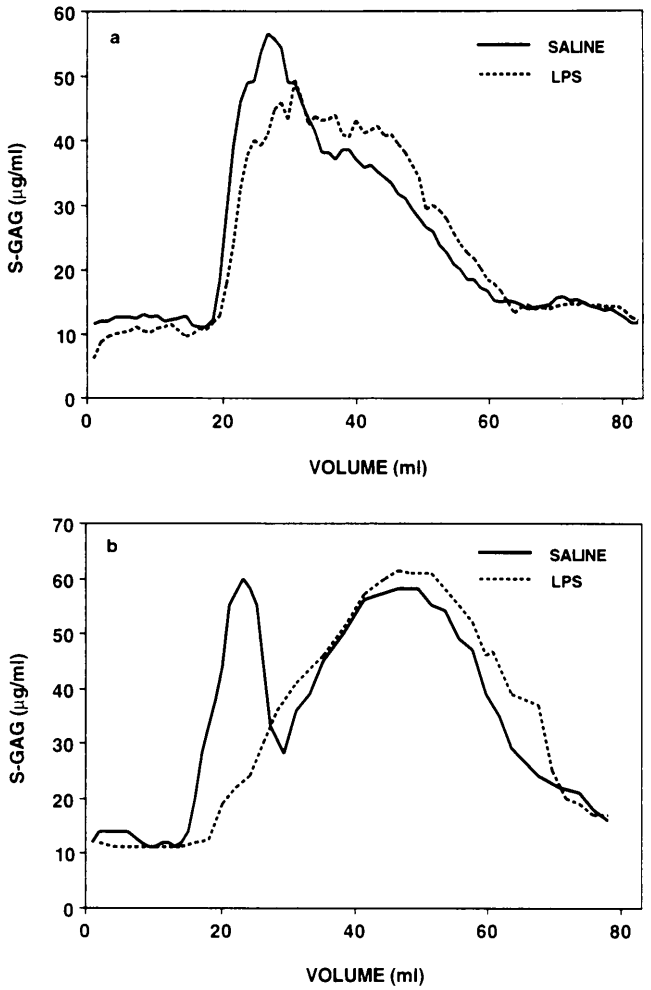

Figure 3. Ten-day-old chicks were injected with either LPS (3 mg/kg) or saline and growth plate was excised 48 hr after injection. Proteoglycans were extracted from the growth plate in a 4 *M* guanidine-HCl solution applied to a Sepharose CL-2B column. The column was equilibrated with (a) 0.5 *M* sodium acetate to determine the ability of proteoglycans to aggregate or (b) with 2 *M* guanidine HCl to determine size distribution.

10–12), and did not appear to affect general protein synthesis (8). To our knowledge, this is the first evidence to show that LPS alters proteoglycan metabolism in an *in vivo* model and that it occurs in growth plate tissue.

In the presence of LPS, we found that there is an accelerated loss and breakdown of aggrecan from growth plate explants similar to the findings with articular cartilage explants. Autocrine production of soluble mediators such as interleukin-1 (IL-1) or tumor necrosis factor (TNF) by chondrocytes is one mechanism by which LPS-mediated cartilage degradation is thought to occur (8). The detrimental effects of IL-1 on proteoglycan metabolism are well established (20–22) and appear to use the same chondrocytic pathways as LPS (i.e., decreased aggrecan synthesis and accelerated breakdown, and increased prostaglandin E_2 synthesis) (8). In addition, the increased prostaglandin E_2 synthesized by chondrocytes cultured with LPS is an

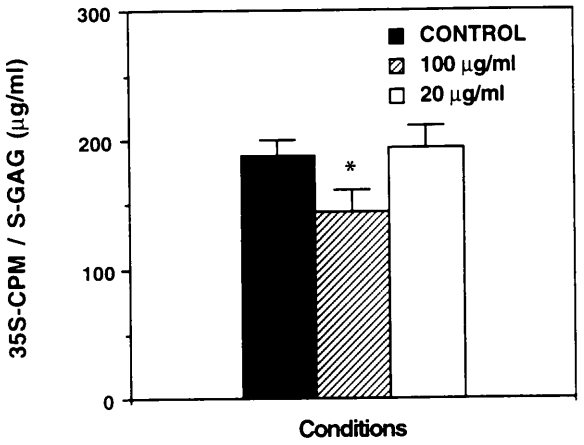

Figure 4. The rate of [^{35}S]-sulfate in growth plate explants exposed to varying concentrations of LPS in culture. Mean ± SEM; $n = 9$ for each group. *$P < 0.02$, differing from control.

important mediator of the acute inflammatory response (8, 10, 12, 23). The increase in aggrecan release from growth plate tissue exposed to LPS may also be controlled by an increased production of metalloproteases which degrade proteoglycans (24, 25). The LPS required in our and other's (8, 9, 12) explant culture systems are higher than those used in cell culture systems and probably reflects differences between systems. It is likely that the effect of LPS on growth plate chondrocytes occurs through a host of secondary mediators (23). However, additional effects of high concentrations of LPS are also possible.

Aggrecan are normally found as large aggregates in the growth plate, but during pathological conditions the structure and size of the aggrecan are altered. For example, Buckwalter and Rosenberg (26) demonstrated with electron microscopy that aggregate size decreases substantially with age. In addition, during osteoarthritis proteoglycan aggregates in articular cartilage are smaller and monomer size is reduced and

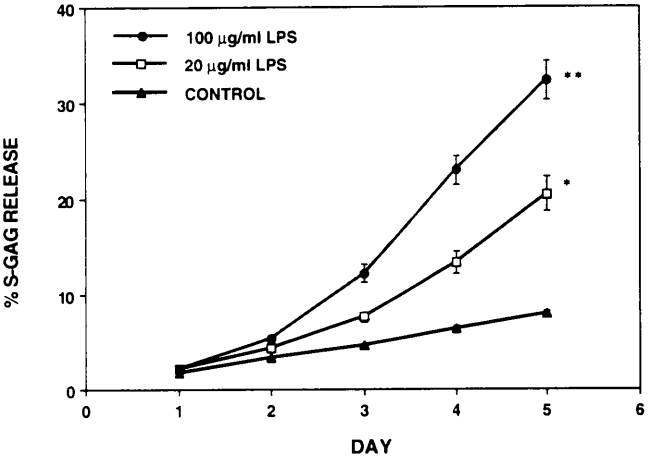

Figure 5. Release of proteoglycans from growth plate explants cultured with LPS at 20 or 100 μg/ml over a 5-day period. Mean ± SEM; $n = 9$ for each group. *$P < 0.05$, differing from control; **$P < 0.025$, differing from control.

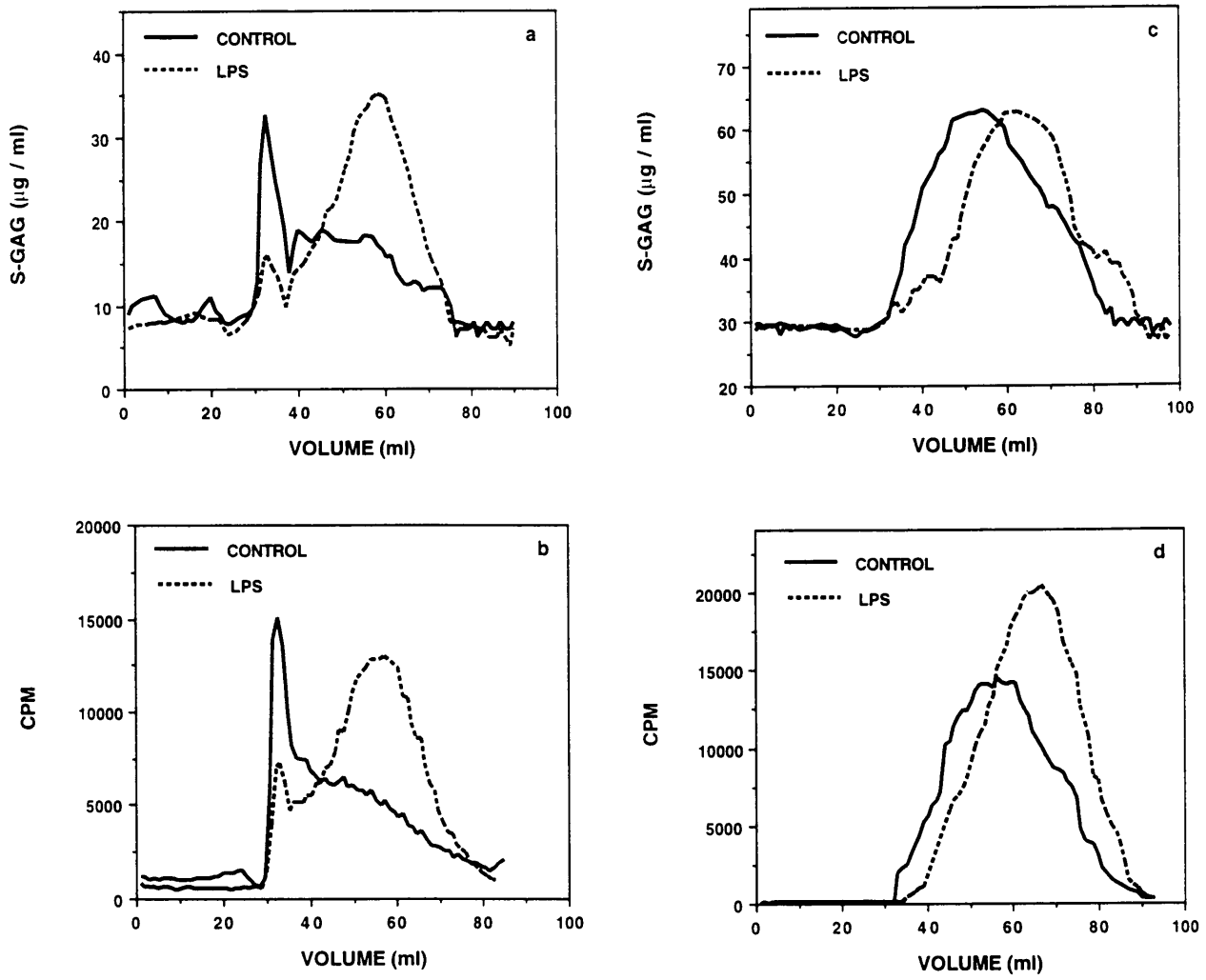

Figure 6. Sepharose CL2B chromatography of proteoglycans released into the media from growth plate explant incubated for 16 hr with or without 20 μg/ml LPS before a 4-hr incubation with $Na^{35}SO_4$, and then replaced, daily, with cold media containing LPS for 48 hr. Aggregating proteoglycans of the total population (a) and the newly synthesized proteoglycans (b); and monomer size for the total population (c) and newly synthesized proteoglycan (d).

lack hyaluronate binding region (HABR) (27). Our results suggest that the presence of LPS increases variability in monomer length and decreases the number of intact aggregates by approximately 20%. It is likely that the reduced ability to aggregate is due to either an absence of link protein, or an increased breakdown of link protein and HABR. These components may be present, but in a nonfunctional form (20). In addition, the smaller subunits of the protein core in the LPS-treated tissue are likely due to elevated enzyme activity (28). If proteoglycan structure plays a role in regulating mineralization of the growth plate, as suggested by others (3), this would contribute to endotoxin-mediated growth abnormalities.

Growth plate heterogeneity in cell morphology and expression is a unique characteristic of this tissue. The differential regulation of aggrecan metabolism and structure in the reserve, proliferative and hypertrophic zones has recently been described (29, 30). Most mRNA for aggrecan core protein is found in the proliferative zone of the growth plate and less in the hypertrophic zones (30, 31). In addition, the various types of collagen demonstrate a heterogenous distribution through the depth of the growth plate (2). Hence, it is likely that there are differential effects of LPS on chondrocyte regulation of the major matrix constituents in discrete zones of the growth plate, but should be confirmed in future studies.

Proteoglycan synthesis is reduced in the presence of LPS and release is accelerated. This could be expected ultimately to affect proteoglycan content within the growth plate. The extent of proteoglycan loss from the growth plate would ultimately depend on the continued imbalance of proteoglycan turnover degradation, and the length of exposure to LPS. In preliminary experiments, we found that the daily rate of proteoglycan synthesis in growth plate explants cultured with LPS remained reduced, over the course of 4 days, compared with control (32). In addition, others (8, 9) have showed that chondrocytes cultured with LPS

show a reduced rate of proteoglycan synthesis up to 50 days in culture. This lends support to the idea that the proteoglycan content of the matrix would eventually be reduced if continually exposed to LPS, even if degradation was not accelerated.

In summary, the evidence that LPS alters metabolism and structure of aggrecan in growth plate tissue supports the hypothesis that systemic bacterial infections will contribute to growth abnormalities. In addition, because we controlled for energy intake, a reduced food intake in children chronically ill with bacterial infections (33) is not likely to be the only factor responsible for their subsequent growth retardation. Understanding how LPS modulates the components in the extracellular matrix in future studies will contribute to our knowledge of longitudinal bone growth and how it is impaired, and could potentially be prevented, in pathological states.

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