Glucocorticoid regulation of surfactant components in immature lambs

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ANTENATAL GLUCOCORTICOID therapy is routinely used to reduce the incidence of respiratory distress syndrome in premature infants. Efficacy of this treatment requires at least 24 h of exposure to glucocorticoid before delivery; however, there is limited information regarding the duration of benefit from treatment and the need for retreatment (20). Because many women with preterm labor do not progress to delivery, clinicians are faced with the decision of when and how often to repeat treatment with antenatal glucocorticoids. Current clinical practice varies from a single course of glucocorticoid treatment to multiple treatments at a 7- to 10-day interval even in the absence of continuing premature labor. Although a single course of glucocorticoid treatment is considered safe, a number of concerns that include growth retardation, adrenal suppression, and susceptibility to infection have been raised regarding repetitive treatments. The issue of the time course of response to glucocorticoid therapy was identified by the National Institutes of Health Consensus Conference as one of the areas requiring further investigation (20).

The beneficial effects of glucocorticoids on lung maturation are mediated through effects on lung structure, capillary-alveolar permeability, and the surfactant system. In studies with cultured fetal lung tissue, glucocorticoids increase production of both lipid and protein components of surfactant (1). Glucocorticoid induction of surfactant proteins SP-A, SP-B, and SP-C involves regulation at the level of gene transcription, and these responses are reversible on removal of glucocorticoid. The increased transcription rates for SP-B and SP-C in human fetal lung explants, for example, are decreased within 4 h of removal of the hormone (3). These observations predict that increases in surfactant proteins in vivo after glucocorticoid treatment would be reversible events as hormone is cleared. Culture systems have not been useful in addressing the possible reversibility of other non-surfactant-related changes in the developing lung.

We previously studied effects of in vivo glucocorticoid treatment of fetal sheep using both maternal and direct fetal administration of betamethasone. Because of the relatively long gestation, this species is appropriate for addressing the timing and duration of glucocorticoid effects in the fetal lung. We found that as little as 15 h of exposure to betamethasone improves physiological parameters of lung function in prematurely delivered lambs (13). However, treatment for 48 h before delivery did not increase saturated phosphatidylcholine (Sat PC) in lung tissue or lavage fluid or lavage levels of SP-A (26). In the current study, we hypothesized that longer exposure of fetal lambs to glucocorticoid would increase fetal lung content of surfactant lipid and proteins and that the response would be reversible in the absence of repetitive dosing. To address this question, we administered betamethasone to pregnant sheep using short-term (48 h) and longer term (3 wk) clinically relevant, repetitive dosing regimens and assessed pulmonary function, Sat PC, SP-A, and SP-B after premature delivery of lambs. Preliminary results from these studies have been previously reported in abstracts (10, 21).

METHODS

Protocols for these experiments were approved by the animal use committees at both Harbor-University of California, Los Angeles Medical Center and The Western Australia Department of Agriculture. A description of the animals and treatment procedures have been reported elsewhere (12).

Treatment protocol A. Time-dated ewes with single fetuses were identified by ultrasound at 60 days gestation and were...
treated with 150 mg of medroxyprogesterone (Depo-Provera, Upjohn, Kalamazoo, MI) at 101 days gestation to reduce the occurrence of preterm labor and abortion after glucocorticoid treatment (9); progesterone is not known to influence lung development or the response to glucocorticoid treatment. At 104 days gestation, the ewes were randomized to receive intramuscular saline injection or intramuscular betamethasone (0.5 mg/kg) (Celestone Chronodose, Schering, Australia). The animals were allowed to freely feed in paddocks between subsequent injections of saline or betamethasone at 7-day intervals. There were four treatment groups of 11 animals each that received betamethasone at 104 days gestation only (1 dose); at 104 and 111 days gestation (2 doses); at 104, 111, and 118 days gestation (3 doses); or at 104, 111, 118, and 124 days gestation (4 doses). Saline was injected on those weeks that betamethasone was not administered. A control group of animals received four weekly injections of saline. The animals remained undisturbed except for the injections, and all fetuses were delivered by cesarean section at 125 days gestation.

Treatment protocol B. Time-dated pregnant ewes carrying a single fetus were randomized into one of four maternal treatment groups: intramuscular betamethasone (0.5 mg/kg) at 123 and 124 days gestation (IM-48/24 h); intramuscular betamethasone (0.5 mg/kg) at 123 and 124 days gestation (IM-48/48 h); intra-ami-nioglycosyl injection of betamethasone (0.5 mg/kg) at 123 days gestation (IA-48 h); and saline injection either intramuscularly (IM, n = 7) or intra-amniotically (IA, n = 5). Each treated group contained 9 or 10 animals. Results for the two groups of control animals (IM vs. IA) were comparable, and the data were pooled. All fetuses in this protocol were also delivered at 125 days gestation.

Term animals. For comparison with the premature animals of the study groups, lung tissue and lavage fluid were collected from eight near-term, untreated newborn lambs of 144–145 days gestation.

Postnatal evaluation. Delivery and assessment of premature lambs were identical for both treatment protocols A and B and have been previously described in detail (12, 17). In brief, at 125 days gestation the ewes were sedated and the fetuses were delivered by cesarean section after sedation with ketamine and placement of an endotracheal tube. Newborn lambs were mechanically ventilated with a fractional inspired O2 of 1.0, a rate of 40 breaths/min, and standardized ventilator settings. Tidal volumes were measured with a pneumotachometer, and compliance was calculated by dividing total lung volume by ventilatory pressure and normalized for body weight. A ventilatory efficiency index, which integrates ventilation with respiratory support and is directly proportional to ventilatory pressure and Pco2, was calculated as previously described (22).

Physiological outcomes for animals of protocol A are described in a separate study (12).

After 40 min of ventilation, the lambs were killed with pentobarbital sodium, the chests were opened, and a pressure-volume study was performed. We previously found that a 40-min postnatal study period was sufficient for respiratory mechanics to stabilize (17). The lungs were then removed from the thorax, and the left lungs were lavaged with five aliquots of cold normal saline that were pooled and constituted the lavage sample. Pieces of the right lower lobe of each lung were frozen for assay of surfactant components.

Assays. Lung tissue and lavage fluid were assayed for Sat PC as described by Mason et al. (19), and data are expressed as micromoles per kilogram body weight. Under the conditions of our assay, we found only saturated fatty acids, predominantly palmitate, on gas chromatography of the oxidation product. The content of SP-A and SP-B in lung tissue and lavage was assayed by a modification of the immunoassay described by Beers et al. (5). Alliquots of tissue sonicate or lavage fluid were serially diluted in phosphate-buffered saline (pH 7.4) and applied to a nitrocellulose membrane in a multiwell dot-blot apparatus under negative pressure. Each assay contained an equal number of samples from each of the treatment groups to minimize the impact of interassay variability. Typically, six dilutions of each sample were applied along with twofold serial dilutions of pooled tracheal aspirate fluid from term human infants, which served as an internal standard. After being blocked with a 5% milk protein solution and being washed, the blot was exposed to polyclonal antibodies raised in rabbits against purified human SP-A (1:10,000 dilution) or human SP-B (1:5,000 dilution) as previously described (4, 30). Specificity of the antibodies in the assay was indicated by greatly reduced signal after each antibody was preabsorbed with the respective purified surfactant protein. Secondary antibody conjugated with horseradish peroxidase was used along with enhanced chemiluminescence reagents according to the manufacturer’s instructions (DuPont NEN, Boston, MA), and the blots were exposed to X-ray film for varying times to provide signals for each sample within the linear range of film responsiveness. The X-ray films were analyzed with a scanning densitometer (Hoeffer, San Francisco, CA), and arbitrary densitometric units were plotted against the log of protein concentration, which was assayed by the method of Bradford (8).

The concentration of surfactant protein for each sample was calculated from the linear portion of the dose-response curve. Data for preterm animals were normalized to values obtained for the near-term animals, and results are expressed as a percentage of the level at term. The intra- and interassay coefficients of variation were 15.3 and 28.1% for SP-A and 6.0 and 25.9% for SP-B, respectively.

Data analysis. All values are given as means ± SE for each treatment group. Comparisons between groups were done by analysis of variance using Fisher’s exact test, and significance was accepted at P < 0.05. Correlations between different parameters were evaluated by Peacock’s linear regression or by exponential power regression, and correlation coefficients were determined.

RESULTS

Description of animals. Two different glucocorticoid treatment protocols were used in these studies. In the repetitive treatment protocol (protocol A), pregnant ewes received one to four weekly doses of betamethasone beginning at 104 days gestation with delivery on 125 days of gestation (term ∼147 days) as described in METHODS. Thus this protocol provided 3 wk of glucocorticoid exposure for the fetus with zero to three weekly retreatments. Lambs from each of the five treatment groups were delivered on each study day in a random order, and deliveries were continued until 11 live-born lambs had been studied for each group. There were no fetal losses for the saline-injected controls or the one-dose group and one to four abortions within the other three treatment groups. The effect of repetitive glucocorticoid treatment on maternal and newborn weights, cord blood pH and gas values, and lung weight, protein, and DNA content have been reported (12). Exposure to two to four doses of betamethasone decreased both body and lung weight (Table 1).

In the 48-h treatment protocol (protocol B), pregnant sheep received betamethasone by an intramuscular or
Values for $V_{40}$ were also increased by two or more doses of betamethasone, with a maximal fourfold response after four doses. Similar to the increase in $V_{40}$, the ventilatory efficiency index increased was greater than threefold with four doses compared with the control value. There were no differences in PO2 values for the five groups.

In animals of treatment protocol B, dynamic compliance increased for all three treatment groups, and there were significant increases in $V_{40}$ and ventilatory efficiency index for two of the groups (Table 2). The magnitude of the changes approximated those observed in animals receiving two betamethasone doses in protocol A and were <50% of the maximal increase observed after four doses. Highest values for all three parameters were noted in animals injected twice with betamethasone (IM-48/24 h), but these responses were not different from those observed in the other treatment groups. Values for PO2 were variable and not different among the four groups.

Sat PC content. Figure 1 summarizes results for Sat PC content in lung tissue and lavage fluid and the percentage of secreted lipid [lavage Sat PC/(lavage Sat PC + tissue Sat PC)] for fetuses of protocol A. There were modest but significant increases in Sat PC content of lung tissue after two, three, and four doses of betamethasone, with a maximal increase of 80% vs. control after three doses (Fig. 1A). Sat PC content in lavage fluid was also increased after two, three, and four doses, with a maximal increase of >11-fold in fetuses receiving three treatments (Fig. 1B). The percentage of total lung Sat PC recovered in lavage fluid, representing secreted phospholipid, was 0.4% in control animals compared with ~2.5% (P < 0.05) after three or four doses of betamethasone (Fig. 1C). Secretion tended to increase in animals after one and two doses (3- to 4-fold), but these changes were not statistically significant.

Figure 2 shows similar data for Sat PC content and secretion in fetuses of protocol B. None of the 48-h treatment regimens significantly affected Sat PC content or distribution, although pulmonary function improved in these animals (Table 1).

### Table 1. Body and lung weights in premature lambs

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Body Wt, kg</th>
<th>Wet Lung Wt, g</th>
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<tbody>
<tr>
<td>A Control</td>
<td>2.82 ± 0.11</td>
<td>108.4 ± 4.5</td>
</tr>
<tr>
<td>Betamethasone (0.5 mg/kg)</td>
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<tr>
<td>1 Dose</td>
<td>2.40 ± 0.07*</td>
<td>103.3 ± 5.3†</td>
</tr>
<tr>
<td>2 Doses</td>
<td>2.28 ± 0.08*</td>
<td>88.9 ± 2.3*</td>
</tr>
<tr>
<td>3 Doses</td>
<td>2.09 ± 0.09*</td>
<td>86.2 ± 3.6</td>
</tr>
<tr>
<td>4 Doses</td>
<td>2.02 ± 0.13*</td>
<td>76.4 ± 5.8*</td>
</tr>
<tr>
<td>B Control</td>
<td>2.73 ± 0.07</td>
<td>112.0 ± 4.5</td>
</tr>
<tr>
<td>Betamethasone (0.5 mg/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IM-48 h</td>
<td>2.68 ± 0.10</td>
<td>91.0 ± 5.3</td>
</tr>
<tr>
<td>IM-48/24 h</td>
<td>2.55 ± 0.08</td>
<td>88.1 ± 6.7</td>
</tr>
<tr>
<td>IA-48 h</td>
<td>2.71 ± 0.23</td>
<td>106.4 ± 10.7</td>
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</tbody>
</table>

Values are means ± SE. Protocol A ewes received intramuscular saline (control) or intramuscular betamethasone at 104 (1 dose), 104 and 111 (2 doses), 104, 111, and 118 (3 doses), or 104, 111, 118, and 124 days (4 doses) gestation. Protocol B ewes received intra-amniotic or intramuscular saline (control), intramuscular betamethasone at 123 (IM-48 h) or 123 and 124 days gestation (IM-48/24 h), or intra-amniotic betamethasone at 123 days gestation (IA-48 h). All fetuses in both protocols were delivered at 125 days gestation. *P < 0.05 vs. control; †P < 0.05 vs. 4 doses.

Table 2. Physiological parameters of pulmonary function in premature lambs

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Dynamic Compliance, ml·kg⁻¹·cmH₂O⁻¹</th>
<th>$V_{40}$, ml/kg</th>
<th>VEI, ml·kg⁻¹·cmH₂O⁻¹</th>
<th>PO2, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>0.176 ± 0.008</td>
<td>12.7 ± 0.6</td>
<td>0.028 ± 0.002</td>
<td>109 ± 29</td>
</tr>
<tr>
<td>Betamethasone (0.5 mg/kg)</td>
<td></td>
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</tr>
<tr>
<td>1 Dose</td>
<td>0.225 ± 0.017 (128)</td>
<td>19.4 ± 2.5 (153)</td>
<td>0.035 ± 0.004 (120)</td>
<td>82 ± 19 (75)</td>
</tr>
<tr>
<td>2 Doses</td>
<td>0.312 ± 0.034* (177)</td>
<td>36.1 ± 6.4* (284)</td>
<td>0.049 ± 0.006* (174)</td>
<td>95 ± 35 (87)</td>
</tr>
<tr>
<td>3 Doses</td>
<td>0.353 ± 0.021* (201)</td>
<td>44.7 ± 3.8* (352)</td>
<td>0.056 ± 0.005* (199)</td>
<td>139 ± 35 (128)</td>
</tr>
<tr>
<td>4 Doses</td>
<td>0.439 ± 0.038* (249)</td>
<td>54.9 ± 6.6* (432)</td>
<td>0.088 ± 0.038* (315)</td>
<td>101 ± 26 (93)</td>
</tr>
<tr>
<td>B Control</td>
<td>0.212 ± 0.021</td>
<td>17.5 ± 2.5</td>
<td>0.035 ± 0.004</td>
<td>75 ± 16</td>
</tr>
<tr>
<td>Betamethasone (0.5 mg/kg)</td>
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<tr>
<td>IM-48 h</td>
<td>0.306 ± 0.044* (141)</td>
<td>29.9 ± 7.6* (201)</td>
<td>0.052 ± 0.010 (137)</td>
<td>90 ± 21 (120)</td>
</tr>
<tr>
<td>IM-48/24 h</td>
<td>0.351 ± 0.019* (166)</td>
<td>39.6 ± 4.1* (226)</td>
<td>0.060 ± 0.004* (171)</td>
<td>45 ± 8 (60)</td>
</tr>
<tr>
<td>IA-48 h</td>
<td>0.290 ± 0.030* (144)</td>
<td>35.1 ± 4.9 (171)</td>
<td>0.048 ± 0.006* (149)</td>
<td>143 ± 36 (191)</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses are % of control value. Protocol A and B groups are defined in Table 1. Protocol A data are from Ref. 12. $V_{40}$, lung volume at 40 cmH2O pressure; VEI, ventilatory efficiency index. *P < 0.05 vs. control.
Protein content of lavage fluid. Lungs of all lambs were lavaged with saline by a standardized protocol, and total protein concentration was assayed. In animals of protocol A (Fig. 3A), protein concentration in lavage after three and four doses of betamethasone was significantly decreased (0.27 mg/ml) compared with that in control premature lambs (0.46 mg/ml) but was somewhat higher than that found for near-term animals (0.17 mg/ml). In animals of protocol B (Fig. 3B), lavage fluid protein concentration was significantly decreased vs. control in each of the three treatment groups: control, intramuscular (IM) or intra-amniotic (IA) saline injection; IM-48 h, intramuscular betamethasone (0.5 mg/kg) at 123 days gestation; IM-48/24 h, intramuscular betamethasone (0.5 mg/kg) at 123 and 124 days gestation; IA-48 h, intra-amniotic betamethasone (0.5 mg/kg) at 123 days gestation. None of the treated values was significantly different from control.
groups (0.24–0.31 mg/ml). The reduced protein content of lavage fluid observed after both repetitive and short-term glucocorticoid treatment likely reflects decreased capillary-alveolar permeability for plasma proteins, as previously observed in experiments using injections of iodinated albumin (13, 29).

Content of SP-A and SP-B. In animals of treatment protocol A, two or more doses of betamethasone significantly increased levels of SP-A in both tissue (Fig. 4A) and lavage (Fig. 4B). The maximal increase in tissue SP-A was approximately twofold compared with that in controls and was equivalent to ~11% of the concentration in lungs of near-term lambs. SP-A concentration in lavage increased ~10-fold, reaching ~17% of the value for term animals. Data for SP-A in lavage were also calculated per milligram total lavage protein. With this denominator, SP-A concentration increased 9-, 22-, and 14-fold after two, three, and four doses of betamethasone, respectively, with the highest value representing 13% of the SP-A concentration at term.

Tissue content of SP-B was increased two- to threefold with two and three doses of betamethasone (Fig. 4A). Lavage fluid SP-B was also increased after multiple doses of betamethasone, with a maximal increase of ~15-fold after three doses (Fig. 4B). This level of SP-B in lavage was equivalent to ~18% of the term value, whereas the maximal level in lung tissue represented ~3% of SP-B content at term. Expressed per milligram protein, SP-B concentration in lavage increased 12-, 31-, and 15-fold after two, three, and four doses, respectively.

Figure 5 shows surfactant protein data for animals of protocol B. Values for tissue SP-A were somewhat higher than control after intramuscular betamethasone treatment, but the differences were not statistically significant (Fig. 5A). There was no effect of intramuscular betamethasone on SP-A concentration in lavage fluid, whereas intra-amniotic treatment reduced SP-A concentration compared with that in IM-48 h animals (P < 0.05). Results were comparable when data were expressed per microgram protein (data not shown). Tissue levels of SP-B were increased after short-term intramuscular betamethasone treatment (Fig. 5A). The magnitude of the increase (2.8-fold) was comparable to that observed with repetitive betamethasone treatment. In contrast to the results for lung tissue, the concentration of SP-B in lavage fluid was not altered by short-term glucocorticoid treatment (Fig. 5B).

Correlations between measurements. Regression analysis was performed to examine the data for correlations between biochemical and/or physiological parameters of lung maturity in the animals of protocol A.
Values for the three physiological parameters of lung function were strongly correlated with each other, with r values ranging from 0.79 to 0.93. The strongest correlation between a surfactant component and a physiological measure of lung function occurred for lavage Sat PC content and V_{40} values (Fig. 7). This

Table 3. Selected correlations between biochemical and physiological parameters of lung maturity in premature lambs

<table>
<thead>
<tr>
<th>Parameter No.</th>
<th>1</th>
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<tbody>
<tr>
<td>1) Tissue SP-A</td>
<td>0.43</td>
<td>0.56</td>
<td>0.39</td>
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<td>0.38</td>
<td>0.40</td>
<td>0.28</td>
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<td>2) Lavage SP-A</td>
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<td>0.87</td>
<td>0.65</td>
<td>0.68</td>
<td>0.66</td>
<td>0.62</td>
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<td>3) Tissue SP-B</td>
<td>0.36</td>
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<td></td>
<td>0.44</td>
<td>0.60</td>
<td>0.50</td>
<td>0.45</td>
<td>0.40</td>
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<tr>
<td>4) Lavage SP-B</td>
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<td></td>
<td>0.74</td>
<td>0.45</td>
<td>0.50</td>
<td>0.30</td>
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<tr>
<td>5) Tissue Sat PC</td>
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<td></td>
<td></td>
<td>0.65</td>
<td>0.72</td>
<td>0.57</td>
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<tr>
<td>6) Lavage Sat PC</td>
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<td></td>
<td>0.89</td>
<td>0.93</td>
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<tr>
<td>7) Compliance</td>
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<td>0.79</td>
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<tr>
<td>8) V_{40}</td>
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<td>9) VEI</td>
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</table>

Values are linear correlation coefficients (r) for data on all animals (n = 55) of protocol A. SP-A and SP-B, surfactant proteins A and B, respectively; Sat PC, saturated phosphatidylcholine.
GLUCOCORTICOIDS AND SURFACTANT COMPONENTS

Fig. 7. Correlation between Sat PC content of lung lavage and lung volume measured at 40 cmH₂O pressure (V₄₀) for animals of protocol A. Data are best described by an exponential regression line with the equation as shown.

relationship was best described by an exponential regression, which had an r value of 0.87 (r² = 0.75). The strongest correlations for surfactant proteins and a measure of lung function occurred with compliance values, with r values of 0.65 and 0.60 for SP-A and SP-B, respectively. Similar to the finding for Sat PC, somewhat higher r values were observed between both SP-A and SP-B and the physiological parameters by exponential curve fit (data not shown). As expected, strong correlations also existed between surfactant components and physiological parameters when mean values for the five treatment groups were compared (data of Figs. 1 and 4 and Table 2). The r values for all comparisons were higher than those observed for individual data (Table 3), and the strongest correlations occurred with V₄₀ vs. lavage values for SP-A (r = 0.95), SP-B (r = 0.80), and Sat PC (r = 0.81).

DISCUSSION

In this study, we investigated pulmonary responses of the immature sheep fetus to clinically relevant short-term and long-term glucocorticoid treatment regimens. Prolonged glucocorticoid treatment enhanced all measures of lung maturity (content of surfactant components, protein content of lavage fluid, and indexes of pulmonary function), and all of the responses were influenced by duration and doses of treatment. By contrast, short (48 h) exposure improved indexes of lung function without demonstrable effects on surfactant components of lavage fluid. These results along with previous findings in this animal model using short treatment intervals (13) suggest that glucocorticoids improve lung maturity by at least two distinct mechanisms: 1) a relatively rapid change (within 15 h) in lung structure that is associated with improved compliance, increased lung volume, and decreased capillary protein leak, and 2) a slower, coordinated increase in production and secretion of surfactant components. Each of these responses represents acceleration of normal developmental events in the fetal lung, and together they provide a physiological basis for improved lung function and outcomes of premature infants after prenatal exposure to glucocorticoid.

None of the 48-h treatment regimens of protocol B significantly increased tissue content of Sat PC or distribution to air spaces, confirming previous results using both maternal and fetal treatment for intervals of 48 h or less (13, 26). In other experiments, we found an ~40% increase in lung Sat PC content in lambs delivered 7 days after betamethasone injection (27). In animals of protocol A, two or more doses of glucocorticoid over 3 wk increased tissue Sat PC content by 80% compared with that in controls. Considered together, these data indicate that a significant increase in lung phospholipid accumulation requires 1–3 wk of treatment. However, levels of both tissue and total lung Sat PC are relatively insensitive indicators of surfactant PC within lung type II cells, and thus it is possible that physiologically significant changes occur in a shorter time. The maximal induced level of tissue Sat PC represents 44% of the value for term lambs compared with 25% of the term level for control premature animals (16).

The content of Sat PC in lavage fluid increased >10-fold after three doses of betamethasone compared with 2- to 6-fold increases observed previously after a 7-day exposure (14, 27). The magnitude of the increase in lavage content with repetitive doses, compared with tissue and total lung levels, indicates that glucocorticoids increase both accumulation of PC in air spaces and production rate. This could reflect increased rate of secretion and/or decreased loss of Sat PC from alveoli. Because all of the lambs were ventilated for 40 min after delivery, our data do not indicate whether alveolar accumulation occurred before and/or after initiation of air ventilation. In an earlier study, however, Platzker et al. (25) found that glucocorticoid treatment of sheep fetuses increased surface-active material in fetal lung fluid before delivery. The relatively slow time course for accumulation of Sat PC in lavage fluid after betamethasone treatment may reflect the time required for induction of lipogenesis and formation of lamellar bodies, as well as the relatively slow rate of secretion that occurs in utero compared with postnatally (15).

This study provides the first demonstration that glucocorticoids increase the content of surfactant proteins in the in vivo fetal sheep model. In a previous study, no significant changes were found for SP-A content in lavage after 48 h of glucocorticoid treatment (26). In this study, we found a significant increase in tissue content of SP-B and a trend toward increased SP-A after the 48-h treatment regimens; lavage levels were not affected, suggesting that intracellular accumulation of surfactant proteins precedes secretion. The induction of SP-B within 48 h is consistent with the recent report that betamethasone treatment of fetal lambs for 24 h increases SP-B mRNA content (27) and with findings in explant culture of human fetal lung in which transcription rate and mRNA content are maximally induced within 2 and 12 h, respectively. The increase in tissue immunoreactive SP-B after 48 h of
treatment (2.8-fold) is comparable to the response observed after repetitive dosing (maximal 2.9-fold), and both values are similar to the 2.2-fold stimulation of SP-B mRNA after 24 h (27).

The glucocorticoid-induced increases for both SP-A and SP-B were considerably greater for lavage than for tissue, similar to results found for Sat PC, indicating that there is accumulation of newly synthesized surfactant proteins in air spaces. Additionally, the magnitude of the increase in lavage suggests that loss of alveolar surfactant proteins via reuptake, degradation, and efflux of lung fluid is relatively slow compared with the rate of secretion in the treated animals.

Glucocorticoid regulation of SP-A in cultured fetal lung of human and rabbit is biphasic with continued exposure to hormone (7, 11, 23). For example, SP-A content is increased in cultured human lung with exposure to relatively low concentrations of dexamethasone (≤10 nM) for time periods less than 48 h, but content is decreased with longer exposure at lower concentrations and at all time points after treatment with 100 nM dexamethasone (11). The physiological significance of these observations for responsiveness in vivo has been uncertain. The findings in the current study that SP-A is markedly increased after prolonged, repetitive exposure to glucocorticoid suggests that bi-phasic regulation does not occur in vivo in this species.

In the animals of protocol A, there was a close correlation between content of SP-A and SP-B in lavage fluid, and levels of both proteins correlated with content of Sat PC. A similar correlation was apparent when we examined only the animals that received three and four doses of betamethasone, which provided the maximal responses. Among these two treatment groups, there was considerable variability in the level of surfactant proteins, with increases of only ~2-fold in some animals compared with the control population. This finding is consistent with a sub-population of animals showing a relatively limited response to glucocorticoid treatment. The strong correlation between surfactant components suggests that these animals are equally poor responders with regard to all three surfactant components. The basis for this variability in responsiveness of the fetal lung is not known; however, these results implicate either an early event in glucocorticoid mechanism of action (e.g., glucocorticoid receptor content) or levels of a coregulator common to induction of both Sat PC and surfactant proteins (possibly prolactin or thyroid hormone). The biological variability in glucocorticoid responsiveness may contribute to the failure to observe statistically significant increases in SP-A and Sat PC after 48 h of treatment. The close correlation in levels of the three surfactant components indicates that induction of surfactant proteins and lipids is coordinated in vivo, consistent with production of surfactant that has a composition comparable to the surfactant of mature fuses and is functional (29).

A major goal of this study was to examine the effect of repetitive dosing during relatively long-term glucocorticoid treatment. Although mean values for both physiological and biochemical variables in lavage fluid were higher than those in controls after just one dose of betamethasone 3 wk before delivery, none of these increases reached statistical significance. In general, the maximal response occurred with three doses of betamethasone, indicating that all glucocorticoid effects were maintained for at least 1 wk after the last treatment, confirming earlier physiological results (14, 27).

The finding that repetitive dosing was required for maximal response suggests two nonexclusive possibilities. First, glucocorticoid induction of surfactant may be a partially reversible process in vivo, similar to findings for induction of surfactant proteins in cultured lung (3). Second, glucocorticoid effects may be irreversible but responsiveness is gestational age dependent. In this situation, one treatment at 104 days gestation would permanently increase the level of maturity, but the response would be less than that occurring with treatment at 111 or 118 days gestation. This possibility is supported by the observation of apparent reduced efficacy of antenatal glucocorticoids in very low birth weight human infants (20). Recent studies of plasma levels of betamethasone after treatment in sheep indicate that betamethasone is cleared from the fetal circulation with a half-time of ~6 h (6). Although tissue levels of betamethasone have not been determined, they likely follow a similar albeit somewhat slower time course. In studies of cord blood, endogenous cortisol concentrations, which reflect betamethasone downregulation of adrenocorticotropic hormone production by the pituitary, return to normal levels within ~7 days in both sheep (14) and premature infants (2). Thus it is likely that fetal sheep receiving one or two doses of betamethasone completely clear the steroid before delivery and assessment at 125 days gestation.

The treatment regimens of protocol B compared intramuscular vs. intra-amniotic administration of betamethasone as well as one vs. two intramuscular injections over 48 h. None of the data favored intra-amniotic treatment. Although there was improvement in compliance and ventilatory efficiency index in the IA-48 h group, the increase in V40 was not significant, and tissue SP-B content significantly increased with intramuscular but not with intra-amniotic administration. In general, two intramuscular doses of betamethasone were somewhat more effective than a single dose 48 h before delivery, consistent with the time course for clearance of betamethasone and supportive of current clinical practice.

We did not find an effect of betamethasone treatment on the P0.2 value after 40 min of ventilation despite improvements in the other respiratory outcomes. Oxygenation is the most variable assessment of lung function because of the unknown status of shunts. It is also possible that glucocorticoid effects on alveolar and capillary development in the sheep are not coordinated, resulting in regional variability in ventilation-perfusion matching.

In animals of protocol A, the values for three of the physiological parameters of lung function were strongly correlated with each other, suggesting that either compliance and maximal lung volume are coordinate.
regulated by glucocorticoid or that both reflect the same alteration in lung structure and/or surfactant content. In view of the correlation between $V_{40}$ and compliance, it is not surprising that both correlate strongly with the ventilatory efficiency index, which essentially measures required ventilatory pressures and efficiency of gas exchange. There was also a strong correlation between Sat PC content of lavage and $V_{40}$ values, consistent with a role for increased alveolar surface activity in improved lung volume. The relationship between Sat PC and $V_{40}$ was best described by a nonlinear regression (Fig. 7), which may indicate that the highest levels of lavage Sat PC achieved with treatment were sufficient to maximally improve total lung volume. This is supported by the finding that the mean value for $V_{40}$ after four doses of betamethasone (54 ml/kg) approached the level in term animals (80 ml/kg) (16). The same relationship may exist for the surfactant proteins because their relationships with $V_{40}$ were also best described by nonlinear equations. This interpretation is supported by the observations that levels of surfactant are higher at delivery of the term fetuses than in the adult, suggesting that surfactant levels at birth are greater than required for optimal lung function.

The fetal lamb has been a useful model for in vivo studies of hormonal modulation of lung development and provided the initial observations leading to clinical trials of antenatal glucocorticoid therapy (18). Concentrations of surfactant components (Figs. 1 and 4) and activity (25) in the fetal lamb increase manyfold between day 125 and term, similar to the increase in lecithin-to-sphingomyelin ratio and content of SP-A and SP-B in amniotic fluid during the final 20% of human gestation (28). The improved lung function in preterm sheep after 48 h of glucocorticoid treatment (Table 2) is consistent with the better respiratory outcome of human infants who deliver after this treatment interval (20). It should be noted, however, that lung alveolarization occurs earlier in the sheep than in the human and that this developmental difference could influence the response to glucocorticoids. Studies are currently under way to examine effects of betamethasone on lung morphology in the premature lamb.

Our findings provide experimental support for repetitive treatment courses of antenatal glucocorticoids for prevention of respiratory distress syndrome in premature infants. However, possible benefits from repetitive dosing must be weighed against increased risks for adverse effects. We found that the repetitive dosing regimen in fetal sheep resulted in a progressive decrease in birth weight and alterations in plasma hormone levels (12). Although repetitive dosing has not been studied in human infants in a controlled fashion, it is well documented that postnatal glucocorticoid treatment of premature infants is associated with failure to gain weight and decreased head circumference, as well as increased risk for infection and adrenal insufficiency (24). In view of these known complications of prolonged glucocorticoid exposure, retreatment should perhaps be reserved for cases in which there is active labor, recurring more than 1 wk after the last betamethasone administration, with high risk of delivering an infant at $\leq 32$ wk gestation.

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