Effects of Dexamethasone on Macrophages in Fetal and Neonatal Rat Lung

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Summary. The effects of injecting dexamethasone to pregnant and newborn rats on the subsequent production of macrophages in the lung and on their phagocytic activity and lysosomal enzyme content were evaluated from late gestation to postnatal day 10 using an organ culture system to collect macrophages. Pieces of lung tissue cultured 6 days on cover glasses produced a halo of macrophages adherent to the glass around the explants. Thymidine labeling showed that the macrophages were derived from dividing precursors in the pulmonary interstitium. DNA synthesis was reduced after steroid treatment, and fewer cells were obtained on cover glasses, particularly just after birth when the macrophage number usually increases. Phagocytic function was also diminished in cells collected after dexamethasone injection, particularly when derived from neonatal animals. In contrast, intracellular levels of non-specific esterase and glucosaminidase were increased, probably indicative of lower phagolysosome formation and lower enzyme secretion. These results demonstrate that steroid administration to fetal or newborn animals subsequently reduces the number and phagocytic activity of macrophages in the lung. This could reduce the defense mechanisms of the neonate and increase susceptibility to infection.


Key words: Organ culture; macrophage migration; thymidine labeling; phagocytic index; non-specific esterase, N-acetyl-β-glucosaminidase determination.

INTRODUCTION

The alveolar macrophage (AM) plays an important role in pulmonary defense against inhaled bacteria and particulates. However, in the neonatal period, the number of AM and their principal cellular functions of phagocytosis and digestion are poorly developed. These factors have been implicated in the increased risk of newborns to infection, and any conditions that lead to a depletion in the number and/or function of these immature AM in the perinatal period may further compromise the newborn. In recent years, steroid treatment of the fetus to prevent respiratory distress syndrome and of the newborn to limit bronchopulmonary dysplasia have resulted in the exposure of developing lungs to corticosteroids. Although these agents are associated with accelerated lung maturation or limiting alveolitis, their effects on the pulmonary macrophagic system in the perinatal period may not be beneficial. Some in vitro studies indicate that steroids can alter phagocytosis and decrease other macrophagic functions in general but the effects of directly administered glucocorticoids in vivo on lung macrophages recovered from fetal and newborn animals are not known. It is postulated that steroid injection may reduce the number and function of AM in the perinatal period.

The usual method of obtaining AM is by bronchoalveolar lavage. This has been used to obtain cells from larger animals, but is not feasible for small animals or for fetal studies. In the present experiments, we have used an organ culture system to collect macrophages that migrate from the lungs of rats of different ages. The effects of steroid administration to pregnant rats and to newborns on the number of macrophages produced subsequent to steroid treatment.
quenty from the lungs and on various cellular functions are compared between steroid-injected and non-treated groups at various pre- and postnatal times.

MATERIALS AND METHODS

Groups of four timed pregnant Sprague-Dawley rats were sacrificed at days 18, 20, and 22 of gestation (full term: day 22). Two days before death, each rat received 2 mg/kg of dexamethasone intraperitoneally. This treatment schedule was chosen based on a previous study on accelerated lung development following steroid injection. Control pregnant rats received a saline injection and were killed at the same time. Litters of newborn rats and rats 8 days old were also injected directly with the same dose of steroid and killed at days 2 and 10, respectively. Age-matched controls were also studied.

Organ Culture

From each fetus or neonate, the lungs were removed aseptically and tissue from eight to ten fetuses per litter or six newborns per litter was pooled. The lung samples were carefully sliced into approximately 1 mm³ pieces. These explants were placed on multiple sterile cover glasses, nine per glass, in 35 mm culture dishes containing 1 mL Waymouth's medium with 20% fetal calf serum and 1% antifungal agent. The liquid level was just below the top of the explants so that they did not float. After 24 h, when they had attached, more medium could be added to each dish. In some cases, two dishes of extra explants of normal lung were incubated with dexamethasone (1 µg/mL) added directly to the culture medium. All tissues were incubated for 6 days at 37°C in 95% oxygen and 5% carbon dioxide with the media being changed after 3 days.

Migrating macrophages formed a halo on the cover glass around each explant and were readily visible after 6 days of culture. (After longer culture periods, many fibroblasts were found, particularly where the explants attached to the glass.) After the 6 day period, the explants were removed and some tissue pieces were prepared for microscopy. The explants were fixed in buffered glutaraldehyde, post-fixed in osmic acid before dehydration, and embedded in Spurr plastic. The cells on the cover glasses, which were identified as AM by their morphology, ability to phagocytize particles, and their enzymatic content, as detailed below, were compared between culture and steroid-treated groups.

Macrophage Quantitation

For each determination, the cells on at least 12 separate cover glasses (three per litter) were scraped off, resuspended in 1 mL of media, and quantitated on a Coulter counter. The explants removed from each cover glass were pooled, freeze-dried, and weighed. The AM numbers at each time were expressed as cells/mg dry lung weight.

Proliferation Studies

On two additional cover glasses per litter, after 6 days the media was changed to fresh media containing 0.1 µCi/mL of tritiated thymidine, for 4 h. Then the dishes were rinsed and the explants removed. Cover glasses were prepared for autoradiography by dipping in Kodak NTB2 emulsion in a dark room; after 2 weeks they were developed and the cells stained. Tissue explants were fixed and embedded in plastic, then 0.5 µm-thick sections were prepared for autoradiography as above. The percentage of labeled cells in the AM preparations and the percentage of labeled non-epithelial cells in tissue explants were calculated after counting 800 cells each time.

Phagocytic Index

After 6 days of culture, three cover-glass preparations from each group were incubated for an additional 4 h in media containing a 1 mg/mL carbon particle suspension. Cover glasses were then rinsed and stained for light microscopy. The percentage of AM containing carbon were determined after counting 2,000 cells for each day studied.

Enzyme Determinations

Non-specific Esterase

From three cultures per time, cells on cover glasses were air-dried and stained for non-specific esterase. Nuclei were counterstained briefly with hematoxylin. The percentage of esterase positive cells (red-brown granules) was counted by light microscopy on 200 cells each time.

N-Acetyl-β-Glucosaminidase

Additional cover glasses were treated with 0.5 mL of 0.2% Triton-X-100 to lyse the cells; then 100 µL of lysate was incubated at 37°C with 2.5 mM 4-methylumbelliferonyl-N-acetyl-β-D-glucosaminide in 100 µL citrate buffer at pH 5. After 1 h, the reaction was terminated by the addition of glycine buffer at pH 10.3. Enzyme activity was determined by fluorimetry and expressed as picomoles/hour/cell for each time studied.

Statistics

For each endpoint, a mean value per litter was calculated for steroid-exposed and control groups. Using values obtained from each of four litters, overall mean ± S.E. was calculated, and each experimental group was compared to an age-matched control group at each pre-natal and postnatal time by the paired Student's t-test.
RESULTS

Explant Morphology

When the lung tissue is cut sharply and cultured for a few days, fibroblasts tend to form a linear array at the edges and lay down a collagen matrix analogous to a wound healing reaction. After 3 days of culture, macrophages were observed migrating across this peripheral boundary in sections of the lung explants (Fig. 1). By 6 days, a distinct halo of cells, firmly adherent to the glass, was visible by phase microscopy around each explant. The lung tissue could be removed by forceps and the halo of cells stained for light microscopy (Fig. 2). These cells were large mononuclear cells with pseudopodia, typical of macrophages. They were also phagocytic and contained lysosomal enzymes (see below).

Macrophage Number

The number of cells attached to cover glasses was counted for the various experimental groups (Fig. 3). The number in each steroid-treated group was compared independently to the AM number of controls at the same time point. The number of macrophages derived from normal fetal rat lung was highest in the perinatal period.

When fetal rats were exposed to dexamethasone transplacentally, fewer macrophages were produced from tissue taken at days 20 and 22 of gestation compared to time-matched controls. Neonatal rats injected directly with steroid also showed a significant reduction in AM per dry tissue weight. Postnatally on day 2, when control numbers increased, the number of macrophages from
steroid-injected animals was greatly reduced. In some cases, dexamethasone was added directly to organ cultures of normal rat lung. In these experiments also, lower macrophage numbers were found, especially in the perinatal period (Fig. 3). There was no obvious size difference between macrophages from steroid-treated and control groups.

Cell Proliferation

To determine whether the differences in the numbers of macrophages obtained on cover glasses were due to reduced division among free cells or in the explants, DNA synthesis was examined by autoradiography at two representative times. The free cells on cover glasses from fetal and newborn rats showed a very low level of cell division, with less than 1% of cells labeled (Table 1). No significant differences from day to day, or between control and steroid-exposed, macrophages were observed. When sections of the explants were examined, the cell types present were almost totally epithelial and interstitial, including fibroblasts and macrophages. Labeled cell counts were made on the interstitial population, and the percentage of cells labeled by the thymidine pulse was 3–4% for controls (Table 1). At both times examined, exposure to steroid resulted in a significant reduction in the percentage of labeled interstitial nuclei, and few labeled cells resembling macrophages were seen in these explants.

Phagocytic Index

Carbon particles were added to macrophage preparations to quantitate phagocytosis (Fig. 4). About 50% of cells from days 18 and 20 lungs contained carbon (Fig. 5). The phagocytic activity increased steadily from day 22 gestation onward in macrophages derived from normal lung tissue until 80% of cells were positive. Less than 50% of macrophages from rats exposed to dexamethasone at day 22 and postnatally were capable of phagocytosis, and the differences from controls were significant at these times.

Table 1—Percentages ± SE of Thymidine-Labeled Nuclei of Free Macrophages (Cover Glass) and Interstitial Cells (Sections)

<table>
<thead>
<tr>
<th>Age</th>
<th>Free macrophages</th>
<th>Interstitial cells in explants</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Steroid</td>
</tr>
<tr>
<td>Gestational</td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 22</td>
<td>0.8 ± 0.3</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Postnatal</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.2</td>
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*P < 0.05, steroid group less than age matched control.

Fig. 4. Macrophages from lung explant of postnatal day 2 rat after adding a carbon suspension. Most cells show phagocytized particles. ×850.

Fig. 5. Percentages of macrophages that phagocytized carbon in control and steroid exposed cells from rats of different ages. *P < 0.05.

Enzyme Activity

Non-specific Esterase

Macrophages from normal lung showed a steady increase in the percentage of positive cells with age, as shown by histochemistry. Tissue from steroid-exposed rats produced fewer macrophages, but significantly more of these cells were positive for esterase (Fig. 6).
Glucosaminidase Activity

As an index of lysosomal enzyme content, glucosaminidase was measured biochemically. Enzyme activity per cell of normal macrophages was relatively constant throughout the period of study. Steroid exposure in vivo, however, increased the level of the enzyme found in cells from postnatal day two rats (Fig. 7). When dexamethasone was added directly to cultures of normal lung, an increased level of glucosaminidase was also found in cells obtained from newborn rats.

DISCUSSION

Maintenance of a population of alveolar macrophages in adult lung appears to occur by local proliferation of alveolar or precursor macrophages within the pulmonary interstitium.\(^{14,15}\) In situations of increased demand, this mechanism is supplemented by migration of blood monocytes.\(^{16}\) In fetal lung, very few free macrophages are present in the developing air sacs,\(^{2}\) and there is evidence for a pulmonary origin of these cells, since a replicating population of macrophage-like cells has been shown to reside in the stroma, before monocytes appear in the circulation.\(^{17,18}\) In the present study, we have used this observation for developing an organ culture system to collect pulmonary macrophages. The explants were essentially free of circulating leukocytes, and from thymidine uptake, labeled macrophages were seen in the tissue rather than in the free cell population. This indicates that the cells collected on cover glasses arose by division of precursor macrophages in the pulmonary interstitium. The migration of macrophages from the explanted tissue onto cover glasses is somewhat similar to the in vivo situation where macrophages migrate across the epithelium to become free AM. Thus, the explant system described here allows collection of pulmonary macrophages from tissue if bronchoalveolar lavage is not feasible.

The free cells were identified as macrophages by morphology (large mononuclear cells with inclusions and pseudopodia), phagocytic activity, and enzymatic contents. The large increase in macrophage numbers recovered from lung tissue after birth is consistent with the observation made in vivo that AM are few at birth, then increase rapidly in number.\(^{2}\) This increase may be due to hormonal changes or to the presence of pulmonary surfactant in the alveoli.\(^{19}\) In our study, lung explants, when cultured from rats exposed to dexamethasone in vivo, produced fewer macrophages than did controls from day 20 onward. The difference was especially marked at postnatal day 2. The lower macrophage number could be due to reduced migration induced by steroid\(^{20}\) or to lower cell division by precursor cells, since we observed a significant reduction in thymidine uptake by interstitial macrophages in the explants. It is known that dexamethasone decreases epithelial cell division in the lung,\(^{2}\) so that the decrease in macrophage replication may be due to a non-specific effect of steroid. Although interstitial cell counts include both AM and fibroblasts, upon microscopic examination it was evident that fewer clearly identifiable macrophages were labeled in explants from steroid-injected animals. Other studies reported a decreased rate of in vitro macrophage cell division, when exposed to glucocorticoids.\(^{21}\) Whether as a result of reduced mobility and/or lower cell production, the results indicate that steroid exposure of the fetus or newborn reduces the number of macrophages produced by the lung.

Although there are likely to be maturational changes that occur in the normal cell population over the 6 day culture period, comparisons of normal and treated groups are still valid. The present study indicates that macro-
phages derived from steroid-exposed lung do not function as efficiently as those originating from normal lung. Phagocytosis was decreased by dexamethasone treatment, which may be due to the ability of steroids to stabilize cell membranes and thus prevent closure of phagosomes. In contrast to the reduction in phagocytosis, lysosomal enzyme activity per cell was increased following steroid injection, as shown by histochemical and biochemical determinations. The higher enzyme levels may not represent a rise in enzyme production, but, rather, an increase in retention. Enzyme production may continue unchanged, but membrane stabilization could lead to decreased phagolysosomal formation and reduced exocytosis of enzymes. Thus, dexamethasone has the potential to both decrease AM production in the lung and render these cells less effective as phagocytes. This combination of fewer cells and reduced function is most apparent in the perinatal period when the cell number is normally low and the phagocytes are immature. Since neonatal susceptibility to infection has been attributed to inadequate macrophage number and function, steroid exposure is likely to further compromise the newborn.

As further evidence that these changes were due to the direct effects of steroid, the addition of dexamethasone to cultures of normal lung also induced a large reduction in migrating macrophages and an increase in lysosomal activity, predominantly soon after birth. Since glucocorticoid receptor sites on macrophages have been shown previously, it is likely that the effects seen here are a result of direct action of dexamethasone on pulmonary macrophages and their precursors. Although the dose of glucocorticoid used in this study is much higher than that used clinically, the effects of dexamethasone on the macrophages are likely to be dose dependent. In this study, the drug was administered to pregnant rats and newborns at the same dose per kilogram body weight. Since the transplacental distribution of dexamethasone is less than one to one, fetal rats were exposed to a lower concentration, which could account for the smaller effects of steroid seen in the fetal period.

In clinical studies it appears that the dose of steroid administered during pregnancy is sufficient to accelerate lung maturation without increasing the susceptibility of newborn to infection. However, it appears that the neonate is more vulnerable to infection if glucocorticoids are administered in the early postnatal period. Direct administration of glucocorticoids to respirator-dependent neonates with bronchopulmonary dysplasia (BPD) has been implicated as a cause of increased pulmonary infection in these infants, though some improvement in lung function has been demonstrated. Immediately after birth, AM numbers normally increase, and our results suggest that a steroid-induced reduction in macrophage number and phagocytic activity could contribute to a possible higher infection rate in this period. On the other hand, recent evidence indicates that glucocorticoid administration may be beneficial in BPD due to its action in reducing free radical release by AM in association with reduced alveolitis. Thus, the combined evidence from these various studies suggests that the reduction in AM number and phagocytic activity may increase the risk of infection in neonates exposed to steroid, whereas the stabilization of phagosomes and reduced secretion by AM may have beneficial effects in circumstances of lung injury and alveolitis.

REFERENCES