Impaired Pulmonary Clearance of Pneumococci in Neonatal Rats

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ABSTRACT. Lung infections are a common cause of morbidity and mortality in neonates. To evaluate neonatal lung defenses against pneumococci, we challenged rats with aerosols of encapsulated pneumococci in an airborne infection apparatus. Whereas adult rats cleared >95% of inhaled type 1 or type 25 pneumococci within 4 h, pneumococci proliferated in the lungs of newborn rats and reached 200–600% of the baseline value by 4 h and 1000–1700% by 24 h. As neonatal rats matured, their ability to clear inhaled pneumococci improved, but compared with adults some impairment in clearance was present until approximately 4 wk of age. Newborn rats had significantly fewer resident alveolar macrophages per g of lung tissue than did adults ($p < 0.001$). Although the number of resident macrophages increased with time, a significant deficit in alveolar macrophages persisted for the first 3 wk of life ($p < 0.01$). Aerosols of pneumococci caused an influx of granulocytes into the lungs of adult rats within 4 h, compared with 24 h for neonatal rats. Even at 24 h after pneumococcal challenge, newborn rats had significantly fewer granulocytes per g of lung tissue ($p < 0.05$) than did adults, although 7-day-old rats had reached an adult level by this time. Significant ($p < 0.05$) increases in granulocyte chemotactic activity were observed in lavage fluids of adult, but not newborn, rats after pneumococcal challenge. Thus, impaired clearance of pneumococcal aerosols by neonatal rats was associated with an age-dependent deficiency in numbers of resident alveolar macrophages and impaired generation of chemotactic activity and recruitment of granulocytes to the lung. (*Pediatr Res* 22: 736–742, 1987)

Abbreviations

PBS, phosphate-buffered saline
i.p., intraperitoneal
FMLP, N-formyl methionyl leucyl phenylalanine

Respiratory infections cause much morbidity and mortality in neonates, but only in recent years have detailed investigations of neonatal pulmonary host defenses been carried out. In rabbits, aerosols of *Staphylococcus aureus* (1) and group B streptococci (2) are poorly cleared in the first few weeks of life, particularly in the immediate postnatal period. In comparison with adult rabbits, neonatal rabbits have decreased numbers of resident

pulmonary alveolar macrophages, and decreased bactericidal activity of alveolar macrophages for staphylococci (3, 4) and group B streptococci (2). In the present studies, pulmonary clearance of pneumococci was studied by use of a sublethal, inhalation challenge in rats. Clearance of pneumococcal aerosols was profoundly depressed in newborn and developing rats. Neonatal rats had lower numbers of resident alveolar macrophages than did adults, and impaired ability to generate chemotactic activity and to recruit granulocytes to the lungs.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats with an established date of pregnancy were obtained from Harlan Sprague-Dawley, Inc., Indianapolis, IN. Rats were housed in plastic boxes under positive pressure ventilation with filtered air. Unweaned rats were kept with their mothers. Rats were given food and water *ad libitum*. During studies of bacterial clearance, unweaned rats were removed permanently from the colony along with their mothers. Developing rats of either sex were used as available for studies. Unless indicated otherwise, adult rats were at least 4 months old.

Bacteria. Pneumococcal serotypes 1 and 25 were obtained from the American Type Culture Collection, Rockville, MD. Encapsulation was maintained by periodic intraperitoneal passage in mice. Pneumococci were grown overnight under 5% CO₂ in brain heart infusion broth containing 7% heat-inactivated serum and were then inoculated into fresh media and incubated another 5–6 h to obtain log-phase growth. The organisms were washed in PBS (pH 7.4) and brought to a concentration of about 5×10^8 viable pneumococci per ml for nebulization. The number of viable pneumococci in slurries was determined by serial dilution and culture on agar plates containing 5% sheep blood.

Bacterial clearance. Clearance was carried out as previously reported (5). Briefly, log-phase pneumococci in PBS were aerosolized with a no. 40 nebulizer (The DeVilbiss Co., Somerset, PA) into an airborne infection apparatus (Tri-R Instruments, Rockville Center, NY) with a switchable in-line particle sizer (Andersen Samplers, Atlanta, GA). More than 85% of the particles were $<3.3 \mu\text{m}$ as determined by cultures of the aerosol collected on Andersen air sampler plates. Rats were placed in individual compartments in the chamber to prevent huddling. Nebulization was carried out for 30 min and was followed by a 10-min washout period before the animals were removed from the chamber. Rats were killed by an overdose of pentobarbital administered intraperitoneally immediately after removal (0 h) or at intervals thereafter. Lungs were removed aseptically, and the parenchyma was stripped from the large airways into 4 ml of PBS in glass vials and homogenized (Tissumizer, Tekmar Co., Cincinnati, OH) for 1 min. Aliquots (0.1 ml) were serially diluted in PBS and streaked on blood agar for culture and quantitation. The mean number (N) of viable pneumococci remaining in the lung at time, t, was divided by the number viable at 0 h (No),

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and the quotient multiplied by 100 to give the % remaining viable, or:

$$\% \text{ bacteria remaining viable} = \frac{N_t}{N_0} \times 100$$

Dry lung weights. Standards for dry lung weights of developing rats were established by weighing lyophilized lungs of eight to 12 rats per age group on a microbalance (Cahn Instruments, Cerritos, CA). The range of values obtained were as follows: 0–2 days of age (0.024–0.028 g); 3–5 days (0.035–0.036 g); 6–8 days (0.059–0.060 g); 13–15 days (0.093–0.096 g); 20–22 days (0.120–0.124 g); 4 wk (0.155–0.165 g); 5 wk (0.193–0.196 g); 6 wk (0.300–0.311 g) and > 4 months (0.330–0.390 g).

Histology. Lungs were fixed in 10% buffered formaldehyde, impregnated with paraffin, and the sections stained with hematoxylin and eosin for light microscopy.

Bronchoalveolar lavage. Rats were killed with an overdose of pentobarbital i.p. and bronchoalveolar lavage was carried out as described previously (6). In brief, the trachea was exposed, incised, and tubing was inserted and attached to a syringe holder (19-gauge Butterfly, Abbott Hospitals, Chicago, IL). A tie was placed to secure the catheter, and the trachea above the catheter was occluded with a hemostat. Cold sterile PBS was injected into the catheter and withdrawn for two cycles. After the procedure was repeated the two volumes were pooled. The volumes of PBS used for each cycle of lavage in the different age groups were as follows: 0- to 6-day-old-rats (0.20 ml); 7–13 days (0.35 ml); 14–20 days (0.75 ml); 21–27 days (1.0 ml); 4 wk (1.5 ml); 5 wk (3.0 ml); and ≥ 6 wk (5.0 ml). The volume of lavage fluid recovered ($\sim 75\%$ of the original volume in all age groups) was recorded and a leukocyte count was performed. Leukocytes were removed by centrifugation and stained with Wright's stain for the differential leukocyte count and with α -naphthol acetate esterase (Sigma Chemical Corp., St. Louis, MO) for detection of nonspecific esterase activity.

Complement receptors. Complement receptors were detected by use of erythrocyte complexes as previously described (7, 8). Briefly, sheep erythrocytes (E) were coated with IgM anti-Forssman antibody (EA_M) (Cordis Laboratories, Miami, FL), and the complexes were mixed with a dilution of fresh rat serum that yielded approximately 50% hemolysis in 30 min at 37°C to give EA_MC. EA_MC contained C3 almost entirely as iC3b (7). Leukocytes from bronchoalveolar lavage fluid ($>95\%$ macrophages by morphology and nonspecific esterase staining) were suspended in RPMI with 10% heat-inactivated fetal calf serum with gentamicin sulfate (Sigma Chemical Co., St. Louis, MO), penicillin G and streptomycin sulfate (GIBCO, Grand Island, NY) to a concentration of 5×10^6 cells/ml, and 0.2 ml of the suspension was added to wells on glass slides (Lab Tek, Miles Laboratories, Inc., Naperville, IL). After 2 h, nonadherent cells were removed by washing, and EA_MC (1×10^7 in 0.5 ml) were added. After 30 min at 37°C nonadherent EA_MC were removed by washing and 200 or more macrophages per well were examined for formation of rosettes. Macrophages with three or more complexes attached were considered positive for C-receptors.

Chemotaxis. Leukocyte-free lavage fluids were prepared from adult rats (one rat per lavage sample) and from rats aged <1 day (six rats per pool). The fluids were stored at -70°C and thawed only once, just before use.

Granulocytes were isolated from 140 ml of human peripheral venous blood (21 to 35-year-old, nonsmoking, male volunteers) by use of dextran (average mol wt 234,000) sedimentation of heparinized whole blood, separation of the granulocytes and mononuclear cells by the Hypaque-Ficoll technique, and hypotonic saline lysis of residual erythrocytes (9–11). The granulocyte suspensions were more than 93% pure and viability was $\geq 95\%$ as determined by trypan blue exclusion. For labeling with ^{51}Cr (12), granulocytes were suspended in Gey's balanced salt solution without bovine serum albumin to give a final concentration of

2.0×10^7 cells/ml. ^{51}Cr (as sodium chromate) was added to the cell suspension ($2 \mu\text{Ci}/10^6$ granulocytes) and incubated with agitation at 37°C for 1 h in an atmosphere of 95% air and 5% CO_2 . The incubation was stopped by adding cold Gey's medium. The ^{51}Cr -labeled leukocytes were collected by centrifugation at 5°C , washed three times with cold Gey's medium, and resuspended in Gey's medium with 2% (w/v) bovine serum albumin. Cell counts were determined with a Coulter counter (model ZBI) and the granulocyte concentration adjusted to 3×10^6 cells/ml.

Chemotaxis assays were done using blind well chambers. Two membrane filters (a $5\text{-}\mu\text{m}$ pore size top filter and a $3\text{-}\mu\text{m}$ pore size bottom filter) were used to separate the two compartments of the blind well chamber. ^{51}Cr -labeled granulocytes (0.2 ml at a concentration of 3×10^6 cells/ml) were placed in the upper compartment, and lavage buffer alone, chemotactic standard or lung lavage fluid (0.2 ml) in the lower compartment. For each assay, dose-response curves were generated using FMLP as the standard chemotactic agent at concentrations of 0 (random movement control), 1×10^{-9} , 5×10^{-9} , 1×10^{-8} , and 5×10^{-8} mol/liter. All chemotaxis assays were done in triplicate chambers. After 3 h of incubation at 37°C in an atmosphere saturated with water and containing 95% air and 5% carbon dioxide, the contents of the upper compartment were aspirated and the filters removed. The filter on the attractants side was dip rinsed 10 times in isotonic saline. Its radioactivity was determined with a γ -counter and expressed as corrected counts/min adjusting for the radioactivity of the labeled granulocytes (mean of 10,000 cpm/ 10^6 cells) as previously described (10, 12). Chemotaxis results were expressed as mean cpm for triplicate samples after subtraction of counts for random migration. The intraassay variation averaged $\pm 10.7\%$ from the mean, and the interassay variation was $\pm 21.9\%$.

Statistical analysis. Data were analyzed with the *t* test or by the Mann-Whitney U test, as appropriate.

RESULTS

Pneumococcal clearance. Bacterial slurries containing approximately 10^8 viable pneumococci per ml were nebulized for 30 min into an aerosol chamber in which developing and adult rats had been placed. At the end of the period of bacterial inhalation (0 h), rats were killed at intervals and the number of viable pneumococci remaining in their lungs was determined. Pulmonary pneumococcal clearance was strikingly different in adult and developing rats over a 24-h period. Studies with type 25 pneumococci (Fig. 1) showed that adult rats could clear the bulk of inhaled pneumococci within 90 min, whereas there was net bacterial growth at this time in the lungs of rats that were <1 day old. The ability of the latter to clear pneumococci was significantly lower at all times compared with adults ($p < 0.01$). Six-day-old rats did achieve significantly ($p < 0.02$) better clearance of type 25 pneumococci than did <1 -day-old rats, but the 6-day-old rats also had depressed clearance throughout the study period compared with adults ($p < 0.01$). Results with type 1 pneumococci were similar (Fig. 1). Whereas adult rats cleared the bulk of type 1 pneumococci within 24 h, there was striking proliferation of these organisms in <2 -day-old rats, and 7-day-old rats had an intermediate level of clearance. Differences in clearance of type 1 pneumococci among adult rats and developing rats were significant at all times ($p < 0.02$), and the 7- and <2 -day-old rats also differed significantly from each other at 4 and 24 h ($p < 0.01$).

Despite the marked depression in clearance of inhaled type 1 and type 25 pneumococci, neonatal rats never showed obvious respiratory distress after pneumococcal aerosols, and they remained active and nursed normally. When neonatal rats were killed at 24 h after inhalation of type 1 or type 25 pneumococci, none had gross evidence of pulmonary consolidation. To determine if newborn rats observed longer than 24 h would develop evidence of pneumonia, 12 rats aged <1 day were exposed to

aerosols of type 25 pneumococci and observed for a 2-wk period; all remained well. Six were killed at 1 wk after the aerosol exposure, and the other six were killed after 2 wk. None of these rats had detectable pulmonary consolidation grossly or microscopically and cultures of the lungs were sterile. These findings indicated that despite the marked impairment of early clearance in developing rats, host defenses operating after 24 h eliminated inhaled type 25 pneumococci.

To compare clearance as a function of developmental age in greater detail, rats ranging in age from <1 day to 4 wk were exposed to aerosols of type 25 pneumococci and clearance was measured 30 min after deposition of pneumococci. The results (Table 1) showed that clearance was lower in all developmental

groups compared with adults. The defect was age related, and some impairment was present in rats aged 2–4 wk.

Alveolar macrophages of developing rats. Because alveolar macrophages are believed to play a major role in clearance of several species of inhaled bacteria (13), bronchoalveolar lavage was carried out on normal neonatal and adult rats and the number of resident alveolar macrophages in the lavage fluids was quantified. Resident alveolar macrophages were identified by their characteristic morphology and by nonspecific esterase staining. These cells accounted for 94% or more of all the leukocytes present in the bronchoalveolar lavage fluids in all age groups. Macrophages were at least 92% viable, as assessed by trypan blue exclusion, and there was no difference with devel-

Fig. 1. Clearance of inhaled pneumococci by developing rats. Points represent means $\pm$ SE for seven to 15 rats in each age group at each time period. The mean number of viable pneumococci deposited in the lungs was not significantly different at different ages, and ranged from 4.7×10^5 – 7.1×10^5 /g of wet lung tissue for type 25 pneumococci and from 1.5×10^6 – 3.1×10^6 for type 1 pneumococci. Clearance of type 25 or type 1 pneumococci in developing rats was significantly less than in adults at all times ($p < 0.02$).

Table 1. Age-related differences in clearance of type 25 pneumococci in rats

Age	No. litters	No. rats	Zero time	No. rats	30 min after aerosol
			Pneumococci deposited ($\times 10^5$) per g lung*		Pneumococci % viable
<1 day	2	13	$5.82 \pm 1.28^{\dagger\ddagger}$	13	$111 \pm 10^{\dagger\ddagger}$
1 day	2	10	$5.10 \pm 1.20^{\ddagger}$	13	$115 \pm 23^{\ddagger}$
2 days	2	12	$5.70 \pm 1.50^{\ddagger}$	13	$73 \pm 10^{\ddagger}$
4 days	2	10	$3.75 \pm 1.00^{\ddagger}$	9	$62 \pm 16^{\ddagger}$
5–6 days	2	9	$2.29 \pm 0.95^{\ddagger}$	8	$76 \pm 10^{\ddagger}$
7–8 days	3	13	$2.67 \pm 0.74^{\ddagger}$	15	$54 \pm 4^{\ddagger}$
2–4 wk	3	14	$2.28 \pm 0.37^{\ddagger}$	15	$67 \pm 4^{\ddagger}$
Adult (≥ 4 mo)	2	8	3.09 ± 0.20	10	34 ± 4

* Wet lung wt.

$\dagger$ Mean $\pm$ SE/rat.

$\ddagger$ Not significantly different from adult ($p > 0.05$).

$\S$ Significantly different from adult ($p < 0.05$).

opmental age. Alveolar macrophages obtained from rats during their first few days of life frequently contained a multitude of pale cytoplasmic inclusions; these inclusions were not seen in rats older than 1 wk.

Quantitative counts of macrophages in lavage fluids revealed striking differences between adults and neonates (Table 2). Rats that were less than 1 day old had fewer than 1/10 the number of alveolar macrophages of adults, even when differences in lung weight were taken into account. The number of macrophages per g of lung tissue increased steadily with age and by 4 wk approached the number in adults.

Immune receptors of macrophages of developing rats were evaluated by determining the percent of alveolar or peritoneal macrophages that would form rosettes with EA_MC (Table 3). The percent of resident alveolar macrophages of adults that formed rosettes with EA_MC was low compared with peritoneal macrophages, as previously reported (9, 10). Resident alveolar macrophages of developing rats, however, formed even fewer EA_MC rosettes than their adult counterparts, whereas peritoneal macrophages from neonates and adults formed rosettes equally well.

Granulocyte recruitment. In adult mice, granulocytes have been shown to play an important role in the clearance of inhaled pneumococci within several hours after pneumococcal deposition in the lung (14–16). Levels of granulocytes in neonatal lungs after pneumococcal aerosols were, therefore, of interest. Preliminary information on granulocyte movement into lungs after type 25 pneumococcal aerosols was obtained by histologic examination of hematoxylin and eosin-stained sections of lungs. Seven unchallenged adults and seven newborn rats were examined to provide baseline information. These rats had no granulocytes in their alveoli and few in the interstitium. After pneumococcal challenge (average of 2.1×10^6 viable pneumococci per adult lung) seven adult rats that were studied at 1 h showed a few granulocytes in the pulmonary interstitium, and another seven, studied at 24 h, showed occasional alveolar as well as interstitial granulocytes. Newborn rats studied in parallel with the adults (also seven in each group) showed no detectable granulocytes in lung sections obtained before or at 1 h after pneumococcal challenge, and the number in sections obtained at 24 h appeared to be less than in adults. To obtain quantitative information on granulocyte influx, lavage was carried out on rats exposed to aerosols of type 25 pneumococci, and total and differential counts of leukocytes in lavage were carried out. Results (Fig. 2) showed that rats in all age groups had numbers of granulocytes immediately following pneumococcal challenge (0 h), but by 4 h adult rats had a significant increase in the number of granulocytes in their lavage. One-week-old and <1-day-old rats mounted a significant granulocyte response within 24 h after pneumococcal challenge, but the number of granulocytes in lavage fluids of <1-day-old rats was consistently lower than in fluids from adults or 1-wk-old rats. In all age groups, alveolar macrophages remained the predominant cell type, and adults had significantly more macrophages per g lung weight at all times compared with newborn rats or 1-wk-old rats. Adults

also had more lymphocytes, but the differences did not reach significance.

Chemotactic activity of lavage. To determine if differences in granulocyte recruitment in neonatal and adult rats were associated with differences in chemotactic activity in lavage fluids, adult and 1-day-old rats were exposed to aerosols of type 25 pneumococci and their leukocyte-free lavage fluids were tested for chemotactic activity. Lavage was carried out using a comparable ratio of lung weight to lavage volume for adults (0.035 g/ml) and <1-day-old rats (0.051 g/ml). Leukocyte-free fluids from the neonatal rats were pooled (six lavages/pool) to allow an adequate working volume for triplicate assays of chemotactic activity. Fluids from adults were tested without pooling. The assays were carried out in blind well chemotactic chambers with ⁵¹Cr labeled human granulocytes. The results (Fig. 3) showed that adults had increased chemotactic activity after pneumococcal aerosols. The activity peaked at 4 h and the activity (following 30 min of bacterial inhalation and a 10-min period to flush the aerosol chamber) at 0 and at 4 h was significantly higher than the activity observed in lavage fluids of unchallenged adults. In contrast, neonatal lavages showed no significant change in activity after pneumococcal aerosols compared with unchallenged neonates.

DISCUSSION

In the present studies the ability of neonatal rats to clear pneumococci from the lungs was evaluated. This model, which provided a diffuse but sublethal inhalational challenge, showed that neonatal rats had impaired net clearance of inhaled pneumococci. Impairment was most marked in the immediate postnatal period, but some impairment persisted for the first 4 wk after birth. These observations are consistent with results obtained by Sherman *et al.* (1, 2) who found that inhaled staphylococci are cleared more slowly in rabbits at 1 day after birth

Table 3. Percent of alveolar and peritoneal macrophages that formed rosettes with EA_MC

Age	No. of pools or individuals tested	% Macrophages rosetting with EA _M C	
		Alveolar	Peritoneal
Prenatal*	5	1.2 ± 1.0†	
0–2 days	3	3.1 ± 1.2†	81.5 ± 3.1‡
3–8 days	5	1.6 ± 0.3†	78.8 ± 6.7‡
2–3 wk	6	1.4 ± 0.7†	87.0 ± 3.5‡
4–6 wk	3	6.8 ± 1.1†	80.2 ± 4.6‡
Adult (≥4 mo)	6	11.6 ± 2.5	84.8 ± 2.8

* Macrophages in the amniotic fluid of unborn, term fetuses were tested.

† Significantly different from adult ($p < 0.05$).

‡ Not significantly different from adult ($p > 0.05$).

Table 2. Number of alveolar macrophages in bronchoalveolar lavage fluid of developing rats (mean ± SE)

Age	No. of pools or rats tested	Macrophages × 10 ⁵		
		Per rat	Per g lung	
			Wet wt	Dry wt
<1 day	12	0.20 ± 0.02	1.56 ± 0.18*	7.49 ± 0.84*
1 wk	7	0.81 ± 0.25	2.41 ± 0.74*	13.76 ± 4.24*
2 wk	7	2.47 ± 0.86	5.15 ± 1.79*	26.60 ± 9.30*
3 wk	14	6.30 ± 0.63	10.40 ± 1.07*	51.66 ± 5.20*
4 wk	5	10.96 ± 1.28	15.13 ± 1.29†	68.51 ± 6.74†
Adult (≥4 mo)	12	27.8 ± 3.74	15.71 ± 1.51	81.82 ± 7.01

* Significantly different from adult ($p < 0.05$).

† Not significantly different from adult ($p > 0.05$).

Fig. 2. Leukocytes in lung lavage fluids of rats following inhalation of type 25 pneumococci. Bars indicate the mean number ($\pm$ SE) of leukocytes per g dry lung tissue. Six to 14 rats were studied in each age group at each time period. Values that were significantly different from adults are indicated with an asterisk ($p < 0.05$) and values significantly different from rats of the same age at 0 h are indicated with a cross ($p < 0.05$). The number of viable pneumococci deposited per g of dry lung tissue for rats less than 1-day-old, 1-week-old, or adults (≥ 4 months old) were $5.5 \pm 2.3 \times 10^6$, $7.4 \pm 3.8 \times 10^6$, and $8.6 \pm 2.5 \times 10^6$, respectively. These differences in inocula were not significant ($p > 0.05$).

than at 7 or 14 days of age, and that inhaled group B streptococci actually proliferate in the lungs of 1-day-old rabbits.

Quantitation of leukocytes in bronchoalveolar lavage fluids in the present studies revealed that newborn rats, like neonatal rabbits and monkeys (3, 17), had markedly fewer resident alveolar macrophages per g of lung tissue than did adults. The number of resident alveolar macrophages in rats increased steadily after birth and reached adult levels at about 4 wk of life. Low numbers of resident alveolar macrophages certainly might have contributed to impaired clearance of pneumococci, considering the important role these cells have in clearance of several other bacterial species following aerosols (13). Alveolar macrophages may play a central role in killing inhaled pneumococci either by phagocytosis and intracellular killing, or, as discussed below, via antibacterial secretory products (18).

The efficacy of neonatal alveolar macrophages in phagocytes and killing of pneumococci *in vitro* has not been reported to our knowledge. Such studies would be difficult to carry out because neonatal rats have a very low total number of resident alveolar macrophages compared with adults or with neonates of larger species, such as rabbits. Studies of the phagocytosis of *Staphylococcus aureus*, *Escherichia coli*, and *Candida* *in vitro* by rabbit neonatal alveolar macrophages suggest that neonatal macrophages are about as effective in phagocytosis of these pathogens as are adult macrophages (4, 19). However, macrophage uptake

Fig. 3. Chemotactic activity of bronchoalveolar lavage fluids of neonatal and adult rats before and after challenge with aerosolized type 25 pneumococci. Each point represents the mean activity ($\pm$ SE) obtained with six to 14 individual adult or pooled neonatal lavage fluids. Activity is expressed as cpm of ^{51}Cr -labeled human granulocytes attracted by 0.20 ml lavage fluid after correction for random activity. Values after aerosol exposure that were significantly different from the unchallenged rats in the same age group are marked with an asterisk ($p < 0.05$).

of inhaled *S. aureus* *in vivo* appears to be less in neonatal rabbits than in adults (26), and lapine neonatal alveolar macrophages show less chemotactic and microbicidal activity than adult macrophages both *in vitro* (19) and *in vivo* (1, 2). Other studies indicate that postphagocytic oxidative metabolism and superoxide release is not as well developed in alveolar macrophages of neonates compared with adults either in rabbits (20) or in guinea pigs (21). Thus, to the extent that rat neonatal alveolar macrophages resemble those of other species, they may have less microbicidal activity than macrophages of adults.

Abnormalities of immune receptors of neonatal rat alveolar macrophages have been described (22). In the present study, we were able to confirm that neonatal rat alveolar macrophages have significantly fewer C3 receptors detectable by rosetting with $\text{EA}_{\text{M}}\text{C}$ than do adults. However, the relevance of this finding to impaired clearance of inhaled pneumococci in neonatal rats is uncertain. Even in adult rats only a minority of alveolar macrophages will bind $\text{EA}_{\text{M}}\text{C}$ or pneumococci opsonized with complement (7, 8), and depletion of circulating C3 in rats has little effect on early clearance of inhaled pneumococci (23).

The present studies extend previous observations on mechanisms of clearance of bacterial aerosols in the neonatal lung by showing that there is impairment in the development of the inflammatory response to inhaled pneumococci and impaired movement of granulocytes into the lungs of developing rats. These defects were most marked in rats aged less than 1-day, but 7-day-old rats also showed impairment. The degree of the defect in granulocyte recruitment correlated well with the degree of impairment in pneumococcal clearance. It has been documented in adult mice that movement of granulocytes into the lung is critical for eventual killing of pneumococci that escape destruction by resident alveolar macrophages or extracellular antibacterial factors (14–16). Although the present observations are the first that deal with the pulmonary granulocytic response to pneumococcal aerosols in neonates, analogous studies have been done on clearance of group B streptococci from the peritoneal

space in a murine neonatal model. In those studies, impaired peritoneal clearance was associated with delayed recruitment of granulocytes (24). Despite the host deficiencies demonstrated in the present studies, even newborn rats were able eventually to eliminate inhaled pneumococci without any mortality. The reason for this favorable outcome is not certain, but may have been due to the fact that rats in each of the age groups were able eventually to develop a degree of pulmonary inflammation.

The delay in recruitment of granulocytes into the lung after pneumococcal aerosols in the present studies was shown to be associated with poor generation, or conceivably inhibition, of chemotactic activity in the lungs of neonatal rats. Pneumococci themselves may provide a weak chemotactic stimulus to granulocytes, but they lack endotoxin and do not provide the same level of chemotactic stimulus as seen with gram-negative bacilli. In adult mice it has been demonstrated that C5 is an important source of chemotactic activity in the lung after pneumococcal challenge, and pulmonary granulocyte recruitment after pneumococcal challenge is significantly less in genetically C5-deficient mice than in their C5-sufficient counterparts (15). Chemotactic factors other than C5 also appear to be important in eliciting a granulocyte influx into the lungs, in that genetically C5-deficient mice are able to develop appreciable chemotactic activity in bronchoalveolar lavage fluids after pneumococcal challenge (15). Phagocytosis by alveolar macrophages is accompanied by release of chemotactic factors that are distinct from C5a (25–28), and these factors could be a source of chemotactic activity in the alveoli after bacterial aerosols. One would expect lower levels of macrophage-derived chemotactic factors in developing animals simply because of the low numbers of alveolar macrophages in the early stages of postnatal development. Increased levels of chemotactic inhibiting activity have been found in the cord blood and serum of newborn humans (29), but the role of chemotactic inhibitors in the lung, if any, has not been established.

Poor granulocyte recruitment to the lungs in neonates could be a consequence of low granulocyte reserves or functional defects of granulocytes which have been noted in developing animals and in man (30–38). Hyde *et al.* (39), using a rabbit model, have found that neonatal granulocytes are deficient in their ability to emigrate into the alveolar spaces of adult rabbits challenged with C5f. These investigators, however, suggest that unique features of the alveolar and vascular anatomy of the neonatal lung may help to compensate for functional deficiencies of neonatal granulocytes. Additional studies will be needed to determine whether intrinsic granulocyte deficiencies contributed to the impaired granulocyte response in neonatal rats after pneumococcal aerosols. The lower chemotactic activity of neonatal rat lavage in the present studies was separable from possible functional defects of neonatal rat granulocytes, because adult human granulocytes were used in the chemotactic assays.

Although phagocytosis and intracellular killing of inhaled pneumococci by granulocytes appears to play an important role in the eventual sterilization of the alveoli, the bulk of inhaled pneumococci (80–90% of the inoculum) are cleared within 45 to 60 min after deposition, and there is increasing evidence that much of this early killing may take place extracellularly (23, 40, 41). Several extracellular factors have been found in bronchoalveolar lavage fluid which could contribute to the extracellular killing of pneumococci, including lysozyme, peptides, transferrin, lactoferrin, and free fatty acids (18, 42). Lysozyme and transferrin are secreted by alveolar macrophages, and lipoprotein lipase, another alveolar macrophage secretory product, can release free fatty acids from triglycerides (18). Surfactant contains triglyceride and lipoprotein lipase along with antipneumococcal free fatty acids (18, 42, 43). Recently, we found that there is an age-related deficiency in the antipneumococcal activity of bronchoalveolar lining material of developing rats (44). In these studies the mean bactericidal titer of lining material from adult rats for pneumococci was 1:40, whereas the same amount of lining material from newborn rats had no antipneumococcal

activity. Bactericidal activity increased with developmental age and normal levels were reached at about 4 wk of age. Lysozyme levels in lavage fluids from newborn rats were markedly lower than in adults ($p < 0.01$) and free fatty acid levels were moderately lower ($p < 0.1$). Studies are currently in progress to determine whether these differences affect clearance of inhaled pneumococci *in vivo* and whether macrophages play a role in production of antipneumococcal factors within the alveoli.

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