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Oxidative Metabolism of Neonatal and Adult Rabbit Lung Macrophages Stimulated with Opsonized Group B Streptococci†

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We compared the oxidative metabolism of alveolar macrophages (AM) from adult and neonatal (1- and 7-day-old) rabbits before and after their in vitro exposure to type Ia group B streptococci (GBS) opsonized with immune rabbit serum. Nonstimulated AM from 1-day-old, 7-day-old, or adult rabbits consumed O_2 at a rate of 17 to 20 nmol/10^6 AM per 10 min under basal conditions and released minimal amounts of superoxide (O_2^-) into the medium. Approximately 80% of this basal respiration was of mitochondrial origin, based on its inhibition by NaCN. Exposure to GBS opsonized with immune rabbit serum stimulated O_2 consumption approximately half as effectively in the neonatal AM as in the adult AM. Little O_2^- was released into the medium unless the cells were pretreated with dihydrocytochalasin B. Under such conditions, 1-day-old, 7-day-old, and adult AM released 3.6, 5.3, and 13.9 nmol of O_2^-/10^6 AM per 10 min, respectively. The uptake of opsonized GBS by 1-day-old AM was not affected by 1 mM NaCN, whereas phagocytosis by adult AM was substantially reduced under these conditions. Overall, our findings suggest that neonatal AM have less-well-developed postphagocytic oxidative metabolic responses and release less superoxide after exposure to opsonized GBS than do adult AM. They also demonstrate that the energy for phagocytosis is derived principally from mitochondrial metabolism in adult AM but not in neonatal AM. We conclude that metabolic differences between neonatal and adult AM may contribute to neonatal pulmonary susceptibility to GBS infections and account, in part, for the ability of GBS to succeed as neonatal pulmonary pathogens.

During the past decade, group B streptococci (GBS) emerged as a leading cause of bacterial infections in human newborns within the United States (2, 5). Infections caused by type Ia serotypes of *Streptococcus agalactiae* have usually been associated with pneumonia and bacteremia occurring within 24 h after birth (5, 9, 28).

Alveolar macrophages (AM), key components of lung defenses against bacterial challenge (11, 12), are likely to utilize both oxygen-dependent and oxygen-independent mechanisms of microbicidal activity (8, 15). Sherman et al. (23) exposed neonatal rabbits to aerosols of type Ia GBS and found that AM from animals less than 1 day old were unable to restrict the intracellular proliferation of ingested GBS in vivo. This finding contrasted with the behavior of AM from older neonates and may have accounted for the greater susceptibility of rabbits under 1 day old to aerosol-induced streptococcal pneumonia. After birth, the oxygen-deficient alveolus of the fetus becomes the relatively hyperoxygenated alveolus of the newborn, an event which may profoundly influence oxygen-dependent phagocytic killing in the AM. In this communication, we report studies dealing with differences in the metabolism of neonatal and adult AM incubated in vitro with opsonized type Ia GBS.

MATERIALS AND METHODS

Animals. Commercially bred New Zealand white rabbits were observed closely to determine the time of parturition. Pups 6 to 24 h old were designated "1 day old." The maximal litter size was restricted to eight pups by culling any runts or ill-appearing newborns, typically 1 to 3 per litter, on day 1. Neonatal litters appeared healthy and well nourished. Male and female adult rabbits, 1 to 2 years old and weighing 3.5 to 4.5 kg, served as controls.

AM. Cells were obtained by in situ bronchoalveolar lavage as described previously (24). A portion of the initial lavage effluent from each adult animal was serially diluted, inoculated onto blood agar plates, and incubated at 37°C, and any resultant colonies were counted to ascertain the degree of respiratory colonization. A portion of pooled lavage fluid from individual neonatal litters was processed in the same manner. Organisms isolated on these plates were identified with the API system (Analylab Products, Plainview, N.Y.). AM were maintained in balanced salts-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (26) at a concentration of 5×10^6 cells per ml until tested.

Bacteria. *S. agalactiae* type Ia strain Flem isolated from a prematurely born human infant with fatal early onset pneumonia and bacteremia was incubated for 16 h at 37°C with shaking in 50 ml of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.). The bacteria were washed twice by centrifugation in sterile normal saline, suspended to an optical density corresponding to 10^{10} CFU/ml, heat killed (60°C, 30 min), washed two more times with saline, and adjusted to 10^{10} CFU equivalents per ml. Such GBS particles neither consumed O_2 nor produced superoxide (O_2^-).

Antibody. Adult rabbits were injected intravenously for 6 weeks with Formalin-killed type Ia strain Flem as described by Anthony (1). The resultant antiserum showed markedly positive (>1:64) capillary precipitin reactions against hot hydrochloric acid extracts of the immunogen (27). Immune serum was stored at −70°C in 3-ml aliquots.

Opsonization. GBS (10^{10} CFU equivalents) were mixed with 1 ml of immune serum for 30 min at 37°C, washed twice with sterile normal saline by centrifugation in an Eppendorf

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Model 3200 Microfuge (Brinkmann Instruments, Inc., Westbury, N.Y.), and resuspended in 1 ml of normal saline. This suspension was vigorously agitated to disperse the opsonized particles before they were added to the reaction mixture. GBS opsonized with immune serum were designated GBS/IRS.

Oxygen consumption. A Gilson KM-I dual recording oxygraph with duplicate 1.2-ml heated chambers and Clark-Yellow Spring Instrument electrodes was used to measure O_2 consumption. Each chamber contained (in 1.2 ml of balanced salts-HEPES buffer at 37°C) 10^6 AM, 1 mM sodium cyanide or no sodium cyanide, and 5.0×10^8 GBS/IRS. The reaction mixture was stirred continuously with a Teflon-coated magnetic bar. After chamber equilibration, basal O_2 consumption was measured for 10 min. After GBS/IRS were added, O_2 consumption was recorded for an additional 10 min.

Superoxide production. The rates and kinetics of O_2^- release by AM were measured at 37°C by the ferricytochrome *c*-superoxide dismutase spectrophotometric method as described previously (24). In some assays, cells were preincubated with dihydrocytochalasin B (2.5 μg/ml) for 10 min before the stimulus (GBS/IRS) was added. The final reaction mixtures contained (in 1 ml of balanced salts-HEPES buffer) 0.5×10^6 AM, 2.5 μg of dihydrocytochalasin B, 50 μM ferricytochrome *c*, 30 μg of superoxide dismutase or no superoxide dismutase, and 2.5×10^8 GBS/IRS. Average basal and stimulated rates of O_2^- production were calculated over 10-min intervals, and peak (maximal) rates of O_2^- production were calculated over 3-min intervals.

Phagocytosis. Phagocytosis was measured by a differential fluorescence-quenching procedure modified from the method of Hed (13) and Hed and Stendahl (14). Heat-killed *S. agalactiae* type Ia strain Flem was conjugated with fluorescein isothiocyanate (FITC) (isomer I; Sigma Chemical Co., St. Louis, Mo.) as previously described (7). The fluorescent organisms were extensively washed, opsonized with immune rabbit serum, and used in place of nonfluorescent GBS in some measurements of O_2 consumption. After these measurements (10 min of basal O_2 consumption + 10 min of stimulated O_2 consumption) were made, 1 mM (final concentration) *N*-methylmaleimide was added to stop further ingestion of bacteria. A portion of the stimulated O_2 consumption reaction mixture (20 μl) was then mixed with an equal volume of 0.25% methylene blue in phosphate-buffered saline (pH 7.0) for ca. 1 min, and a portion of this mixture was examined by fluorescence microscopy. Extracellular or surface-adherent GBS adsorbed methylene blue, resulting in the quenching of their fluorescence. Such quenched cocci were readily distinguishable from brightly fluorescent intracellular organisms (Fig. 1). Under light microscopy, extracellular or surface-adherent quenched organisms stained blue, whereas intracellular organisms remained unstained and were not visible. At least 30 macrophages were examined within 10 min from each sample under oil immersion (970×), and the number of nonquenched cocci per macrophage was determined. We calculated both the percentage of macrophages containing one or more fluorescent GBS (percent phagocytosis) and the mean number of fluorescent cocci per phagocytic AM.

RESULTS

Cell yield. The mean number of alveolar cells recovered per rabbit was influenced by the postnatal age of the animal, as follows: 1 day old, 0.88×10^6 ($n = 107$); 7 days old, 4.3×10^6 ($n = 77$); and adult, 39×10^6 ($n = 20$). Because the cell yield from individual neonatal rabbits was insufficient for multiple testing, the cells from a single litter were pooled. Thus, the designation $n = 4$ means that four experiments were performed with pooled cells from a single (and different) litter of rabbits on each occasion. For example, each litter had seven to eight pups, so four neonatal rabbit experiments required AM obtained from 28 to 32 animals. Adult cells were never pooled, so $n = 4$ for adults means that AM from four separate adults were tested. Cytocentrifuge preparations of neonatal or adult lavage fluids were stained with Giemsa stain and contained the following mean numbers of AM per age: 1 day old, 97.6%; 7 days old, 96.7%; and adult, 95.5%. Neonatal lavage fluids always had fewer than 1% heterophils (granulocytes), and adult lavage fluids always had fewer than 3% heterophils. Cell viability was ascertained by trypan blue exclusion and exceeded 95% at all ages.

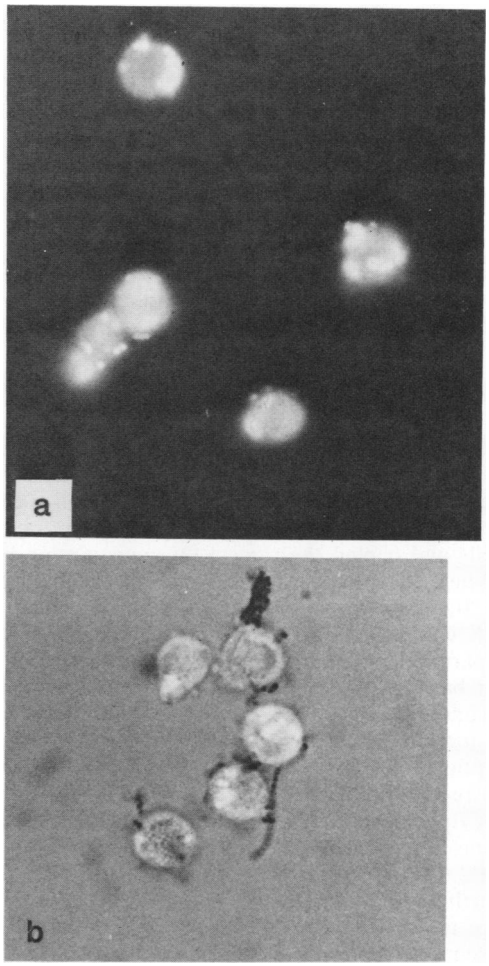

FIG. 1. Analysis of phagocytosis by fluorescence quenching. (a) Five neonatal AM incubated with FITC-labeled GBS/IRS as described in the text. Methylene blue (final concentration, 0.125%) was added to quench the numerous extracellular streptococci, which are not visible. Ingested GBS protected from the methylene blue by the cytoplasm of the macrophage retained their intense fluorescence. (b) Five adult AM incubated with FITC-labeled GBS/IRS in the presence of 1 mM NaCN. Methylene blue was added, and under epi-illumination, blue-stained extracellular streptococci and ingested fluorescent streptococci were visible. The effects of cyanide on the ingestion of GBS/IRS are described in the text.

TABLE 1. Effect of GBS/IRS on the oxidative metabolism of neonatal and adult AM

AM donor age	O_2 consumption			O_2^- release		
	Basal[a]	Stimulated (GBS/IRS)	Δ O_2 comsumption	Basal[b]	Stimulated (GBS/IRS)	After dihydrocytochalasin B-GBS/IRS treatment
1 Day old	18.5 ± 0.5	40.7 ± 3.5	22.2 ± 2.9[c]	0.04 ± 0.04	0.33 ± 0.20	3.58 ± 0.98[d]
7 Days old	19.1 ± 0.5	41.5 ± 3.4	23.4 ± 3.8[c]	0.14 ± 0.09	0.46 ± 0.21	5.30 ± 0.72[d]
Adult	19.6 ± 0.5	65.0 ± 2.0	45.4 ± 2.0	0.08 ± 0.04	0.25 ± 0.11	13.93 ± 3.20

[a] Nanomoles of O_2 consumed per 10^6 AM per 10 min; mean ± standard error of the mean of five neonatal and five adult rabbit experiments.
[b] Nanomoles of O_2^- released per 10^6 AM per 10 min; mean ± standard error of the mean of 6 to 8 neonatal and 10 adult rabbit experiments.
[c] $P < 0.001$ versus adult; two-tailed unpaired t test.
[d] $P < 0.02$ versus adult; two-tailed unpaired t test.

Oxidative metabolism. We measured the effect of GBS/IRS on the rates of O_2 consumption and O_2^- generation by neonatal and adult AM (Table 1). In the absence of stimulation (basal conditions), neonatal and adult AM showed equivalent rates of O_2 consumption. The addition of GBS/IRS approximately doubled the rate of O_2 consumption by neonatal cells and tripled the rate of O_2 consumption by adult cells. When the basal rate of O_2 consumption was subtracted, the stimulated O_2 consumption of adult AM to GBS/IRS was approximately double that of their neonatal counterparts.

Under basal conditions, O_2^- release was barely detectable. The addition of GBS/IRS stimulated O_2^- release slightly, but the increment was small relative to the change in O_2 consumption. GBS/IRS-induced O_2^- release was accentuated by preincubating the AM for 10 min with 2.5 μg of dihydrocytochalasin B per ml. Under such conditions, adult AM released almost four times as much O_2^- as 1-day-old AM and 2.6 times as much O_2^- as 7-day-old AM.

To further characterize the effect of GBS/IRS on AM oxidative metabolism, we performed a series of studies in the presence of 1 mM NaCN. This compound, a classical inhibitor of mitochondrial metabolism, does not block the O_2^--generating NADPH oxidase responsible for the "respiratory burst" of phagocytes. Because phagocytosis is an energy-dependent process that might be impaired by mitochondrial inhibition, we modified our procedure to permit bacterial uptake to be assessed along with the measurement of O_2 consumption. This was done by covalently binding FITC to heat-killed GBS and using the principle of fluorescence quenching (see Fig. 1) to discriminate intracellular from extracellular or surface-adherent GBS. The results of these studies are shown in Table 2. Basal O_2 consumption was inhibited by 75 to 80% in the presence of 1 mM NaCN, suggesting that it was largely of mitochondrial origin. In the absence of NaCN, the addition of FITC-labeled GBS/IRS stimulated O_2 consumption by 130% in adult AM and 79.0% in 1-day-old AM, suggesting that FITC-labeled GBS/IRS were somewhat less effective stimuli than their nonfluorescent counterparts (Table 1). In contrast, in the presence of NaCN, the neonatal AM manifested twice the stimulated O_2 consumption (designated Δ O_2) as the adult AM.

The greater responsiveness of 1-day-old cells under these conditions probably reflects the dissimilar effects of NaCN on the uptake of GBS by adult and neonatal AM. Although adult cells showed a substantial decrease both in the percentage of phagocytic cells (91.7%, control; 42.0% when treated with NaCN) and in the mean number of GBS per phagocytic AM (23.5%, control; 11.1% when treated with NaCN; $P < 0.005$, two-tailed paired t test), the ingestion of GBS/IRS by neonatal cells was essentially unimpaired by the presence of NaCN. Thus, neonatal AM were more uniformly and heavily phagocytic than adult AM in the presence of NaCN.

Representative oxygraph tracings showing the metabolism of neonatal and adult AM exposed to GBS/IRS with or without NaCN during these studies of phagocytosis are shown in Fig. 2.

Other studies. The temporal features of O_2^- release by dihydrocytochalasin B-treated AM exposed to GBS/IRS were independent of postnatal age. Both neonatal (1- or 7-day-old) and adult AM began to release O_2^- ca. 60 s after the addition of GBS/IRS. The peak rate of release was maintained for ca. 4 min, and then release rapidly decreased. Peak O_2^- release rates (nmol of O_2^- per 10^6 AM per min) were as follows: 1 day old, 0.53 ± 0.14; 7 days old, 1.04 ±

TABLE 2. Effect of cyanide on interactions between neonatal and adult AM and FITC-conjugated GBS/IRS

AM donor age	Condition	Phagocytosis			O_2 consumption			Δ O_2 consumption/ phagocytic index ratio
		% Phagocytic AM	GBS per phagocytic AM	Phagocytic index[a]	Basal[b]	Stimulated (GBS/IRS)	Δ O_2 consumption	
Adult	Standard	91.7 ± 1.7	23.5 ± 2.9	21.5	18.0 ± 1.3	41.4 ± 2.1	23.4 ± 2.9	1.09
	Cyanide (1 mM)	42.0 ± 5.7	11.1 ± 2.5	4.66	3.4 ± 0.9	9.7 ± 0.8	6.3 ± 0.4	1.35
1 Day old	Standard	82.2 ± 4.6	19.0 ± 1.4	15.6	17.9 ± 0.6	31.9 ± 1.7[c]	14.1 ± 1.2[c]	0.90
	Cyanide (1 mM)	76.2 ± 7.4[d]	20.2 ± 2.1[c]	15.4	4.4 ± 1.0	16.6 ± 1.5[d]	12.2 ± 0.8[d]	0.79

[a] Phagocytic index = (mean percentage of phagocytic AM/100) × (mean GBS per phagocytic AM); e.g., (91.7/100) × 23.5 = 21.5.
[b] Nanomoles of O_2 consumed per 10^6 AM per 10 min; mean ± standard error of the mean of four neonatal and five adult rabbit experiments.
[c] $P < 0.05$ versus adult; two-tailed unpaired t test.
[d] $P < 0.01$ versus adult; two-tailed unpaired t test.

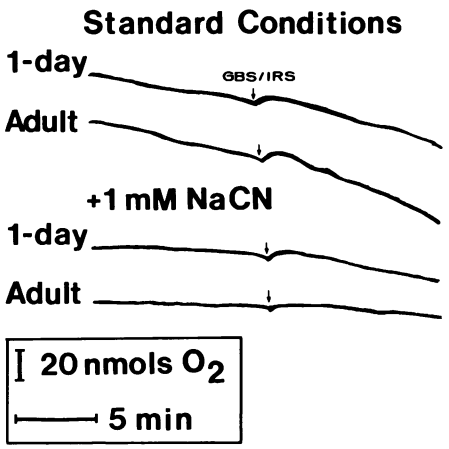

FIG. 2. O_2 consumption by neonatal and adult AM. These polarographic measurements were made by using 10^6 neonatal (1-day-old) or adult AM that had been exposed to a 500:1 multiplicity of GBS/IRS added at the arrows. The portion of the tracing to the left of the arrows represents basal O_2 consumption, and the portion to the right of the arrows represents stimulated O_2 consumption. The effects of 1 mM NaCN on O_2 consumption and the ingestion of GBS/IRS are described in the text. Oxygen and time scales are shown within the box.

0.17; and adult (mean ± standard error of the mean, $n = 8$ to 10), 3.30 ± 0.67. The differences between peak O_2^- release rates for 1- or 7-day-old AM and adult AM were statistically significant ($P < 0.01$).

Bacteriological analysis of lung lavage fluid revealed that the diminished levels of O_2^- release seen in neonatal AM were not associated with excessive colonization of the lung with *Bordetella bronchiseptica*. This organism was recovered from pooled lavage fluids of one of eight 1-day-old litters (mean, 2.0×10^4 CFU/ml) and from four of eight 7-day-old litters (mean, 3.2×10^4 CFU/ml). Neither neonatal nor adult animals had substantial pulmonary colonization with other bacteria (<200 CFU/ml), and most 1-day-old lung lavage fluids were sterile.

DISCUSSION

In leukocyte physiology, the term respiratory burst denotes a pronounced cyanide-insensitive increase in O_2 consumption subsequent to the activation of a primarily or secondarily membrane-associated NAD(P)H oxidase complex (4). This response, which may be triggered by a wide variety of soluble or particulate stimuli, provides reactive oxygen intermediates such as O_2^-, H_2O_2 and $OH^\cdot$. Such reactive intermediates may exert antimicrobial activity directly or by acting in concert with other cellular components (3).

Although some workers earlier experienced difficulty in detecting the production of O_2^- or H_2O_2 by rabbit AM (6, 8, 10), their release is readily demonstrable when appropriate stimuli and detecting systems are used (20, 21, 24). However, the role of oxidative metabolism in the microbicidal mechanisms of AM remains uncertain (17), and alternative host defense mechanisms that operate independently of reactive oxygen intermediates have been proposed (18, 19, 22).

It was striking to note the substantial contribution of mitochondrial metabolism to basal O_2 consumption by neonatal and adult AM. Zeligs et al. showed by electron microscopy that the cellular content of mitochondria in 1-day-old AM was approximately half that in 7-day-old AM (29). We found equivalent mitochondrial (i.e., cyanide-sensitive) respiration in neonatal and adult AM under basal conditions. Simon et al. reported that rabbit AM contain substantially more cytochrome oxidase, a key mitochondrial marker enzyme, and less pyruvate kinase, an important glycolytic enzyme, than peritoneal macrophages (25) and that normal AM show higher O_2 utilization and lower aerobic and anaerobic glycolysis than peritoneal macrophages (25). Anaerobic cultivation of AM in vitro for 96 h caused their pyruvate kinase content to rise fivefold and their cytochrome oxidase content to halve. The reverse situation occurs in newborns, when birth causes a fivefold increase in alveolar oxygen tension and probably induces the appearance of mitochondria and the enhanced oxidative phosphorylation which we observed in the developing AM. The substantial inhibition of phagocytosis consequent to cyanide exposure that we observed in adult AM did not have a counterpart when we tested neonatal (1-day-old) AM, suggesting that the energy requirements for phagocytosis by the neonatal cells arose from nonmitochondrial (presumably glycolytic) metabolism. Hoffman and Autor reached generally similar conclusions in a comparative study of phagocytosis by neonatal and adult rat AM (16).

Our studies suggest that O_2^- production by 1-day-old AM is somewhat diminished relative to that by adult AM. Our studies with dihydrocytochalasin B and GBS/IRS indicated that 1-day-old and, to a lesser extent, 7-day-old AM released significantly less O_2^- than similarly treated adult AM. The overall stimulation of O_2 consumption by adult AM exposed to GBS/IRS was approximately twice that by neonatal AM exposed to GBS/IRS.

Our data on O_2 consumption and O_2^- production should not be juxtaposed to infer stoichiometry, as they were not obtained under identical experimental conditions. Among the important differences were the presence (O_2 consumption) or absence (O_2^- release) of effective stirring and the presence or absence of cyanide or dihydrocytochalasin.

Curiously, we also found that cyanide-insensitive O_2 consumption by GBS/IRS-exposed 1-day-old rabbit AM exceeded that by GBS/IRS-exposed adult AM. However, the significance of this finding is uncertain, given the substantially smaller degree of particle uptake by cyanide-treated adult AM. For example, when the respiratory burst of cyanide-treated neonatal AM was normalized for phagocytosis, their respiratory burst per particle ingested was only 58.5% of the normalized values obtained for cyanide-treated adult AM. This estimate is in excellent agreement with our previously reported observation that AM from 1-day-old rabbits produced O_2^- at 52.0 and 40.7% of adult AM rates after exposure to the potent soluble respiratory burst stimuli cytochalasins D and E, respectively (24). Our findings with GBS/IRS emphasize the importance of performing simultaneous measurements of phagocytic uptake and metabolic responses in studies of leukocyte metabolism.

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