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Oxidant-antioxidant balance in alveolar macrophages from newborn rats

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Oxidant-antioxidant balance in alveolar macrophages from newborn rats. C. Delacourt, M-P. d'Ortho, I. Macquin-Mavier, S. Pezet, B. Housset, C. Lafuma, A. Harf. ©ERS Journals Ltd 1996.

ABSTRACT: An oxidant-antioxidant imbalance in neonatal alveolar macrophages (AMs) may contribute to the increased susceptibility to lung injury described in the neonatal period.

We therefore evaluated oxygen radical production by rat AMs at various postnatal ages, and measured in parallel cellular antioxidant enzyme activities. AMs were obtained by bronchoalveolar lavage from rats aged <24 h, 21 days and 5 weeks, and results were compared to those obtained with adult rat AMs.

Intracellular production of oxygen radical species, estimated fluorometrically using 2',5'-dichlorofluorescein diacetate as the substrate, was significantly reduced in neonates as compared with adults, both in the presence and in the absence of cell stimulation with phorbol myristate acetate (PMA) or opsonized zymosan. A similar pattern was observed for the extracellular release of oxygen radical species, evaluated by lucigenin-enhanced chemiluminescence (CL) or peroxidase-catalysed CL oxidation of luminol: peak CL values measured after cell stimulation with PMA or opsonized zymosan remained significantly lower for AMs from newborn rats than for AMs from adults. By contrast, high values for antioxidant enzyme activities (superoxide dismutase and glutathione peroxidase) in AMs were demonstrated in newborns as compared to adults.

We conclude that high antioxidant activity in rat AMs after birth may be at least partly responsible for the low production of oxygen metabolites observed during the same period.

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Alveolar macrophages (AMs) represent the main cellular component of the defence system that maintains the integrity of the lower respiratory tract [1]. Macrophages modulate a variety of complex host functions, including immunoregulatory, phagocytic and secretory processes. Among the numerous secretory products of AMs are the reactive oxygen metabolites [2]. After *in vitro* stimulation of AMs, *via* either phagocytosis or specific or nonspecific stimuli, oxygen is reduced by the enzyme nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase to the superoxide anion (O_2^-), which quickly dismutates to hydrogen peroxide (H_2O_2) [1]. H_2O_2 can then either generate hydroxyl radical (OH·) in the presence of transition metals or be converted to hypochlorous acid (HOCl) by peroxidases [3]. These intermediate compounds are highly reactive and can cause cell injury and death by altering a variety of cell structures and functions. However, cells also contain antioxidant systems, whose effect is to eliminate O_2 radicals and hydroperoxides with a potential for oxidizing crucial cellular structures [4]. Superoxide dismutase (SOD) catalyses the dismutation of O_2^- to H_2O_2; the latter compound is further detoxified to H_2O by reactions involving glutathione peroxidase (GPX) and catalase.

Whether neonatal AMs are capable of mounting an adequate protective response to lung injuries has been questioned, since the neonatal lung is particularly exposed to impaired development and long-term sequelae after injuries, such as hyperoxia [5–7] or infections [8, 9]. Decreased oxidant production by neonatal macrophages has been found in some studies [10–12], but not in others [13]. Similarly, studies of AM intracellular antioxidants are contradictory, showing either low SOD activity [14] or high SOD activity [15] immediately after birth. Simultaneous estimation of both oxidant production and antioxidant enzyme activities in neonatal alveolar macrophages has not been performed. We therefore undertook this study to evaluate the oxidant-antioxidant balance in neonatal alveolar macrophages and its changes with postnatal age.

Methods

Reagents

Dulbecco's modified Eagle Medium (DMEM) was obtained from Gibco Laboratories (Paisley, UK). 2',5'-dichlorofluorescein diacetate (DCFH-DA) was supplied

by Molecular Probes Inc. (Eugene, OR, USA). All other reagents were supplied by Sigma (Saint Quentin Fallavier, France), unless otherwise stated.

Animals

Pregnant Sprague Dawley rats (Charles River, Saint Aubin-les-Elbeuf, France) were obtained at 17 days of gestation. They were housed in individual plastic cages until spontaneous delivery (term day=22 days of gestation). Newborn rats were then kept with their mother and tested during the first 24 h of life, and on Day 21 after birth, when major postnatal changes in lung structures are accomplished [16, 17]. Adult rats of either sex weighing 300–400 g were also tested.

Harvesting of alveolar macrophages

Alveolar macrophages (AMs) were obtained by *in situ* bronchoalveolar lavage (BAL) using a technique described previously [18]. Briefly, prior to BAL, rats were anaesthetized with 5 mg·100 g^{-1} body weight (BW) of intraperitonally-injected sodium pentobarbital and then killed by exsanguination. The thorax was opened wide to expose the lungs and trachea. A small length of tubing was inserted into the trachea and ligated. BAL was carried out using 8–10 separate aliquots of warmed saline (37°C). Volumes ranged from 0.2 mL per aliquot for newborns (6 g) to 2.5 mL per aliquot for adults. Lavage fluids from several animals were pooled to obtain a sufficient number of cells (from two rats at 21 days to 12 rats in the first 24 h of life). BAL cellularity was determined using a haemocytometer. The lavage fluid was centrifuged at 300×g for 7 min and the cell pellet was then resuspended at 2×10^6 cells·mL^{-1} in DMEM supplemented with 0.5% bovine serum albumin (BSA), 100 IU·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin, 0.25 μg·mL^{-1} fungizone and 2 mM glutamine. Cell smears were stained with standard May-Grünwald-Giemsa for each pool; differential cell counts consistently showed >98% AMs. Cell viability was estimated by exclusion of trypan blue. Cell lysates were obtained using 0.1% Triton X-100 and were stored at -80°C before use in enzymatic assays.

AM stimulation

Phorbol myristate acetate (PMA) and opsonized zymosan were used as stimulants. PMA was dissolved as a 10^{-3} M stock solution in dimethyl sulphoxide (DMSO) and stored at -80°C. Before use, stock solution was further diluted in DMEM to a final concentration of 10^{-7} M. Zymosan was suspended in Hank's balanced salt solution (HBSS) to a concentration of 50 mg·mL^{-1}. One volume of zymosan was incubated with four volumes of normal rat serum for 30 min at 37°C. Opsonized zymosan was centrifuged, resuspended in HBSS to a concentration of 20 mg·mL^{-1} and stored at -80°C. The final concentration to be used was 40 μg·10^{-5} cells.

Oxygen species analysis

Intracellular production of oxygen radical species. Oxygen radical production by AMs was estimated fluorometrically using DCFH-DA as a substrate, according to a previously described method [19, 20] in the presence or absence of stimulants. The cells (3×10^5) were incubated first with 5 μM DCFH-DA for 15 min, then with PMA or zymosan for 60 min, at 37°C. Generation of 2',7'-dichlorofluorescin (DCF) was estimated as the maximal fluorescence over 2 min at 37°C with the excitation and emission wavelengths at 488 and 520 nm, respectively (spectrofluorophotometer RF-5000; Shimadzu Co., Kyoto, Japan). To positively identify the intracellular fluorescent reaction product, we checked emission spectra for reagent DCF (Sigma) and for cellularly oxidized DCFH from AMs with 488 nm excitation. Serial dilutions of reagent DCF were monitored on the spectro-fluorometer to obtain a standard curve of fluorescence per nanomole DCF. Oxygen radical species produced in AMs were expressed as picomoles DCF per 3×10^5 cells.

As cellular DCFH oxidation could be influenced by the presence of intracellular antioxidant enzymes, the measured fluorescence would not strictly measure the production of intracellular oxidant production, but would rather represent the intracellular balance between oxidants and antioxidants.

Extracellular production of oxygen radical species: chemiluminescence (CL). Chemiluminescence was monitored in a LKB-Wallac 1251 luminometer (Wallac Co., Turku, Finland) connected to a microcomputer. Two different luminescent probes were used: 1) O_2^- production was evaluated using lucigenin enhanced CL [21]. Lucigenin (5 mg) was dissolved in HBSS to obtain a 10^{-3} M solution. Further dilutions were in DMEM and the final concentration was 10^{-5} M; 2) H_2O_2 release was evaluated using peroxidase-catalysed CL oxidation of luminol [22, 23]. Luminol (5 mg) was suspended in DMSO to obtain a 10^{-2} M solution and further diluted in DMEM to a final concentration of 10^{-4} M. Horseradish peroxidase (HRP) was added at a concentration of 0.2 U·10^{-5} cells to optimize the reaction between luminol and H_2O_2.

All procedures were performed in the dark. Each vial contained 10^5 AMs (final volume 300 μL) and was placed in a luminometer at 37°C with continuous stirring. The luminescent probe was added first, and two measurements 1 min apart were obtained in the absence of stimulation. Either PMA (10^{-7} M) or zymosan (40 μg·10^{-5} cells) was then added, and measurements were performed every minute for 60 min. Controls were performed using AMs without PMA or zymosan. CL peak after stimulation was determined as the maximal increase above control value. Results were expressed as mV·10^{-5} cells.

To validate the chemical specificity of chemiluminescent probes under these experimental conditions, separate experiments were performed. Firstly, we compared *in vitro* the relative sensitivity of lucigenin and luminol to H_2O_2, by adding incremental doses of H_2O_2 (10 nM–100 mM) to the reaction mixture. Secondly, using adult rat AMs stimulated with opsonised zymosan, we tested the inhibitory effect of SOD (500 U) and catalase (1,000 U) on lucigenin and luminol-enhanced CL, respectively. Furthermore, the dependence of luminol-induced light emission under the presence of peroxidase was also tested.

Antioxidant enzyme activities

Peroxidases are the key enzymes in the redox cycle responsible for the reduction of hydroperoxides [4]. Experimental and clinical data suggest that the glutathione redox cycle is the most important antioxidant peroxidase system in mammalian species [24]. GPX activity was measured as described by PAGLIA and VALENTINE [25]. The assay mixture contained 1 mM glutathione (reduced), 150 μM β-NADPH, 1.5 U glutathione reductase, 200 μM t-butyl hydroperoxide and sample, in a final volume of 1.65 mL 50 mM Tris buffer, pH 7.6. Reaction rates were measured at 340 nm. One unit of GPX activity was measured as the reduction of 1 nmol hydroperoxide per min.

We also evaluated SOD which reacts specifically with O_2^-. SOD activity was determined using the SOD-525 kit (Bioxytech SA, Bonneuil sur Marne, France) whose characteristics have been described previously [26]. Briefly, the assay is based on the SOD-mediated increase in the rate of auto-oxidation of 5,6,6α,11β-tetra-hydro-3,9,10-trihydroxybenzo(*c*)fluorene (BXT-01050) in aqueous alkaline solution. This auto-oxidation yields a chromophore with a maximal absorbance wavelength of 525 nm, which is perfectly stable during the time of measurement. The optimized assay of SOD activity is performed at pH 8.8, 37°C, in 50 mM air-saturated 2-amino-2-methyl-1,3-propanediol buffer containing 3 mM boric acid and 0.1 mM diethylenetriamine pentaacetic acid. With such conditions, optimal assay sensitivity is achieved without affecting activities of known SODs such as Cu/Zn-, Mn-, or Fe-SOD. As the cyanide inhibition of Cu/Zn-SOD will lead to erratic results, it precludes the discrimination between Cu/Zn-SOD and MnSOD activities if both enzymes are present in the sample. The addition of another reagent, 1,4,6-trimethyl-2-vinylpyridinium trifluoromethanesulphonate, directly eliminates interference due to sample mercaptans, such as glutathione. The precision of this method was determined by performing 30 series of measurements under the same experimental conditions within a single day. The standard errors obtained on mean values for three distinct concentrations of SOD were all lower than 5%. The reproducibility was measured by performing the same experiment 3 days later, which again gave standard errors lower than 5% in the two experimental series. Results are expressed as U·10^{-6} AMs.

Data analysis and statistics

At least three pools of AMs were tested in each experiment. Results are expressed as mean ±SEM. Analysis of variance and Fisher's protected least-squares difference test was used for statistical analysis of differences in oxidant or antioxidant production according to the age. A p-value of <0.05 was considered significant.

Results

Intracellular production of oxygen radical species

The fluorescent product was identified as DCF based on the fact that emission spectra of both reagent DCF and AMs preincubated with DCFH-DA were identical with 488 nm excitation, with a maximal emission wavelength at 520 nm.

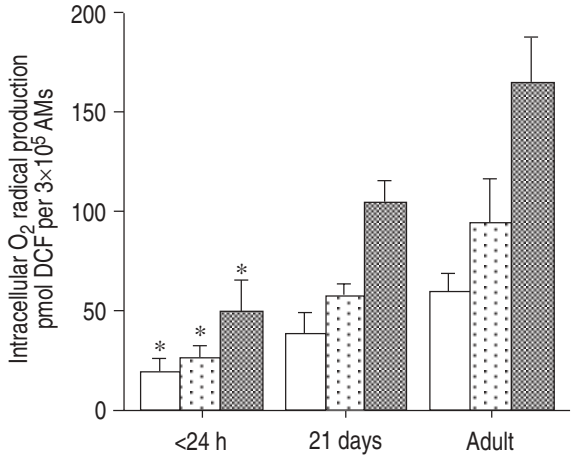

Fig. 1. – Intracellular oxygen radical production by alveolar macrophages (AMs) estimated by fluorometric analysis. AMs (3×10^5) were treated with 5 μM DCFH-DA and incubated without stimulant (▭) or in the presence of 10^{-7} M PMA (⁘) or opsonized zymosan (▩). Values are presented as mean±SEM. Tests were performed at three different ages: <24 h of life (n=3); 21 days of life (n=3); and adults (n=4). DCFH-DA: 2'5'-dichlorofluorescein diacetate; PMA: phiorbol myristate acetate. *: p<0.05, as compared with adult values.

Oxygen radical production patterns by AMs are shown in figure 1. AMs from neonatal rats (<24 h of life) produced significantly less oxygen radical species than adult AMs, both in the absence of stimulation (20±6 *versus* 59±9 pmol DCF per 3×10^5 AMs, respectively) and in the presence of 10^{-7} M PMA (26±6 *versus* 93±22 pmol DCF per 3×10^5 AMs, respectively) or opsonized zymosan (49±16 *versus* 162±24 pmol DCF per 3×10^5 AMs, respectively). AMs from 21 day old rats produced intermediate amounts of oxygen radical species, but the difference with adult AM production was not significant.

Chemiluminescence assays

Validation experiments. The sensitivity for H_2O_2 of peroxidase-catalysed CL oxidation of luminol was determined. Under our reaction conditions (10^{-4} M luminol, 0.2 U HRP, 300 μL), the lowest concentration of H_2O_2 that could be detected was 10 μM. A linear increase in light emission was observed when H_2O_2 concentrations were increased from 10 to 100 μM (fig. 2a). Catalase (1,000 U) completely inhibited the HRP-luminol-enhanced CL induced by 100 μM H_2O_2. When no HRP was added to the reaction mixture, 100 μM H_2O_2, did not induce any increase in chemiluminescence above the background level. From this dose-response curve, it could be estimated that higher CL peaks obtained in the present experimental conditions with adult rat stimulated macrophages always corresponded to H_2O_2 concentrations lower than 40 μM. We therefore verified that this H_2O_2 concentration range (10–100 μM) did not produce light when combined with lucigenin in our reaction conditions (10^{-5} M lucigenin, 300 μL). It was found that lucigenin produced light only when combined with 10 mM H_2O_2.

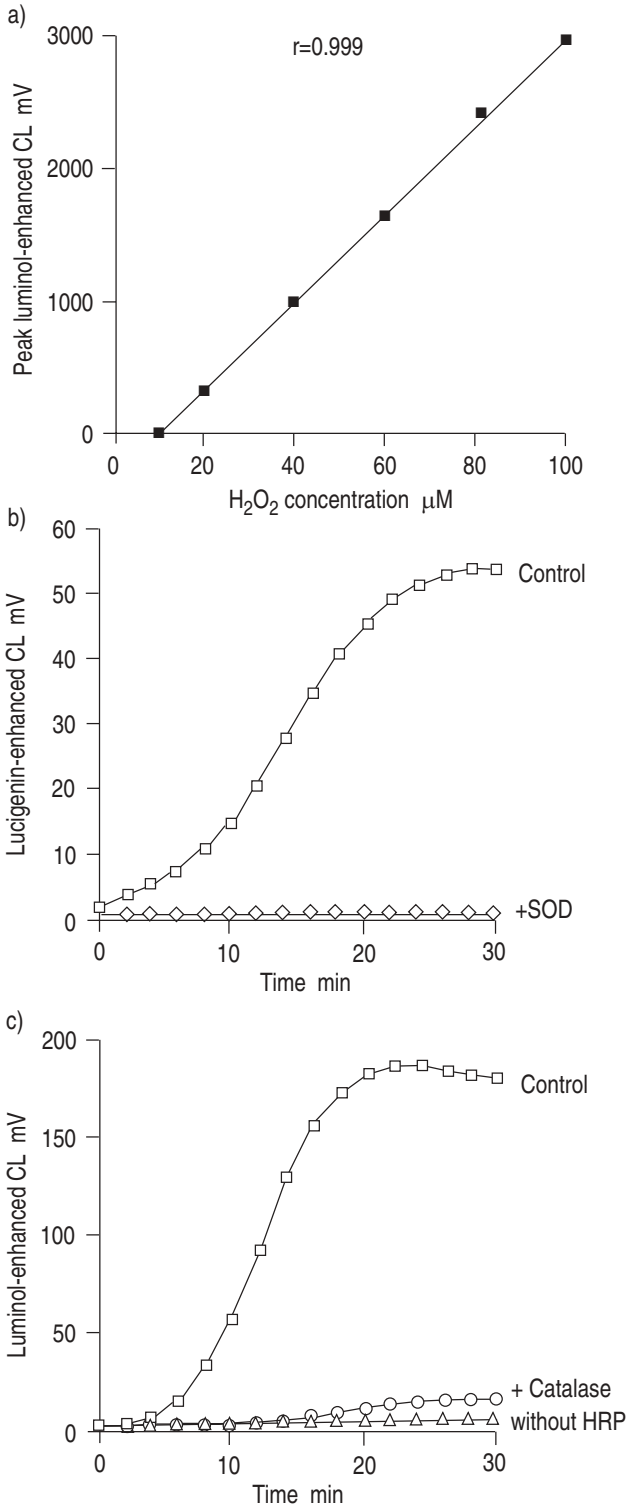

Fig. 2. – Validation of the chemical specificity of chemiluminescent probes under the present experimental conditions. a) Determination of the sensitivity for hydrogen peroxide (H_2O_2) of peroxidase-catalysed chemiluminescence (CL) oxidation of luminol under the present reaction conditions (10^{-4} M luminol, 0.2 U HRP, vol.=300 μL). Lucigenin (10^{-5} M) produced light only when combined with 10 mM H_2O_2. b and c) Chemiluminescence generated from adult rat AMs (10^5 per 300 μL^{-1}) stimulated by opsonized zymosan: b) complete inhibition of lucigenin-enhanced CL by SOD (500 U); and c) of HRP-luminol-enhanced CL by catalase (1,000 U) or by the absence of added peroxidase (bottom). HRP: horseradish peroxidase; AMs: alveolar macrophages; SOD: superoxide dismutase.

Thus, under the present experimental conditions, lucigenin-enhanced CL was not related to H_2O_2 production.

Finally, in experiments with adult rat AMs stimulated with opsonized zymosan, it was demonstrated that lucigenin-enhanced CL was totally inhibited by SOD (500 U) and that HRP-luminol-enhanced CL was inhibited by catalase (1,000 U) or by the absence of added peroxidase (fig. 2b and c).

Lucigenin-enhanced CL: O_2^- generation. CL peak values obtained after stimulation increased gradually with age (PMA: $p<0.005$; zymosan: $p=0.0001$) (fig. 3). Mean CL peak was significantly lower for neonatal AMs than for adult AMs, both after 10^{-7} M PMA stimulation (1.4±0.1 *versus* 8.2±2.2 $mV \cdot 10^{-5}$ AMs, respectively), and after zymosan stimulation (4.8±2.0 *versus* 29.5±7.6 $mV \cdot 10^{-5}$ AMs, respectively). Since CL peak values for 21 day old rat AMs remained significantly lower than adult values, AMs from 5 week old rats were also tested. At this age, CL peaks after PMA or zymosan stimulation were 5.4±0.4 and l2.0±4.6 $mV \cdot 10^{-5}$ AMs, respectively. Although these values remained lower than adult values, the difference did not reach statistical significance.

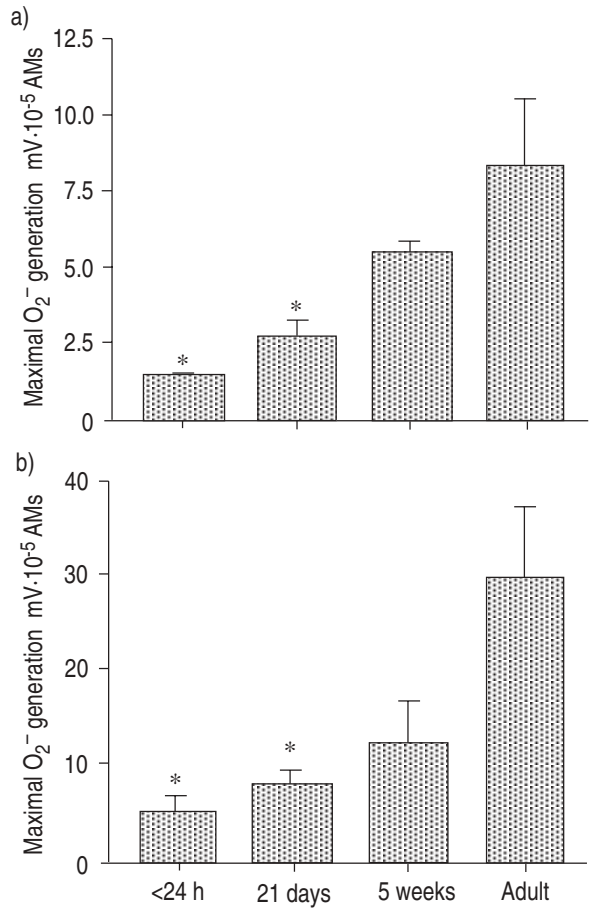

Fig. 3. – Maximal superoxide anion generation from AMs using luminometer analysis. Lucigenin (10^{-5} M) was used as enhancer for the chemiluminescence (CL) analysis. Tests were performed at four different ages: <24 h of life (n=4); 21 days (n=5); 5 weeks (n=3); and adults (n=4). CL peaks were obtained after stimulation of AMs with either: a) PMA; or b) opsonized zymosan. The results were expressed as mean±SEM. AMs: alveolar macrophages; PMA: phorbol myristate acetate. *: $p<0.05$, as compared with adult values.

Luminol-enhanced CL: H_2O_2 analysis. The maximum capacity of AMs for releasing H_2O_2 was evaluated after adding peroxidase to the reaction mixture. After stimulation, production of H_2O_2 by AMs increased gradually with age (PMA: p=0.0002; zymosan: p=0.0001). CL peak values obtained after stimulation were significantly lower for neonatal AMs than for adult AMs (fig. 4). both with 10^{-7} M PMA (1.7±0.3 *versus* 29.0±7.1 mV·10^{-5} AMs, respectively) and with zymosan (22±14 *versus* 269±61 mV·10^{-5} AMs, respectively). CL peaks measured after PMA or zymosan stimulation in 21 day old rat AMs were significantly increased as compared with neonatal values (12.1±2.1 and 84±13 mV·10^{-5} AMs, respectively) but remained significantly lower than adult values. At 5 weeks of age, CL peaks after PMA or zymosan stimulation were 14.2±1.4 and 168±91 mV·10^{-5} AMs, respectively.

Antioxidant enzyme activities in alveolar macrophages

Gradual decreases in activity as postnatal age increased were observed for SOD (p=0.0004) and GPX (p=0.01) (fig. 5). SOD activity was 1.33±0.22 U·10^{-6} AMs in neonates and fell to 0.99±0.06 U·10^{-6} AMs at 21 days of age and to 0.12±0.05 U·10^{-6} AMs in adults. Similarly, GPX activities were 98.2±21.4, 40.6±4.0 and 28.1±5.8 U·10^{-6} AMs in cells from neonates, 21 day old rats, and adults, respectively.

By plotting PMA-induced CL responses both for O_2^- and H_2O_2 with the levels of SOD and GPX activities, respectively, we demonstrated an inverse relationship between CL response and antioxidant activity (fig. 6).

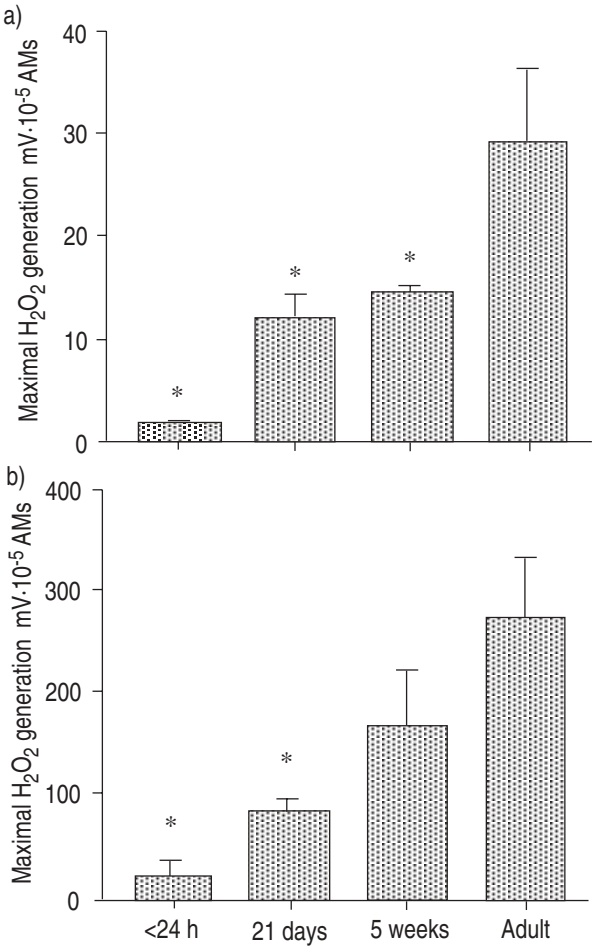

Fig. 4. – Maximal hydrogen peroxide generation from AMs using luminometer analysis. Luminol (10^{-4} M) was used as enhancer and horseradish peroxidase (0.2 U·10^{-5} cells) to optimize the reaction for the chemiluminescence (CL) analysis. Tests were performed at four different ages: <24 h of life (n=4); 21 days (n=5); 5 weeks (n=3) and adults (n=4). CL peaks were obtained after stimulation of AMs with either: a) PMA; or b) opsonized zymosan. The results were expressed as mean±SEM. For definitions see legend to figure 3. *: p<0.05, as compared with adult values.

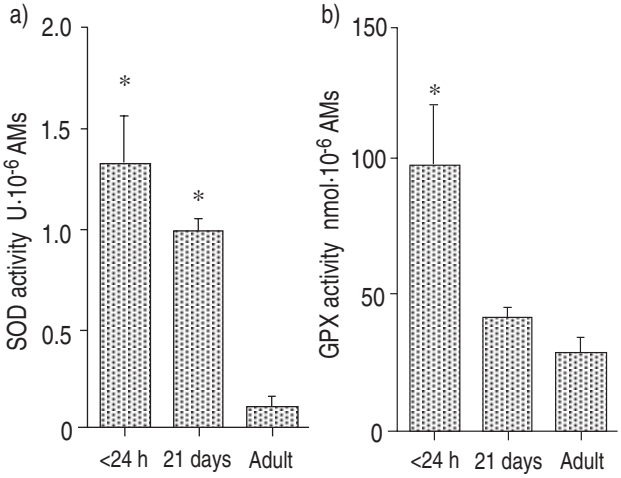

Fig. 5. – Activities of antioxidant enzymes in rat alveolar macrophages (AMs) according to the postnatal age. The activities of: a) superoxide dismutase (SOD); and b) glutathion peroxidase (GPX) were estimated as described in the Methods section. Tests were performed at three different ages: <24 h of life (n=4); 21 days (n=3); and adults (n=4). Values are expressed as mean±SEM. *: p<0.05, as compared with adult values.

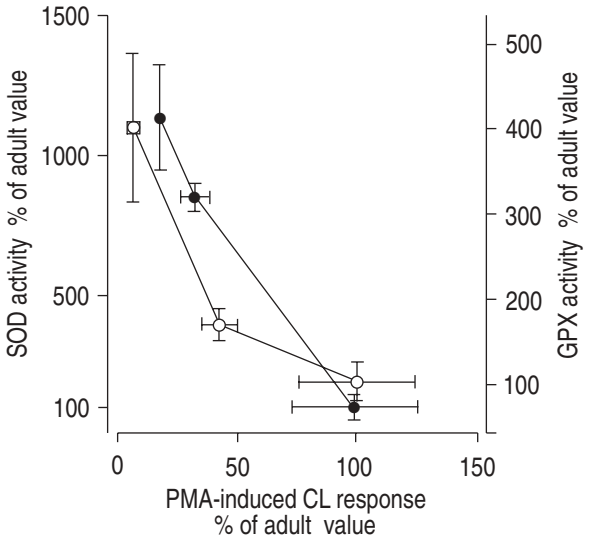

Fig. 6. – Relationship between maximal chemiluminescence (CL) response from AMs and intracellular antioxidant enzyme activities. Production of superoxide anions (lucigenin enhanced CL response) by AMs to PMA stimulation was plotted against superoxide dismutase (SOD) activity (—●—) and production of hydrogen peroxide (luminol enhanced CL response with horseradish peroxidase) was plotted against glutathion peroxidase (GPX) activity (—○—). The three points on each curve represent the mean±SEM values measured at three different postnatal ages: <24 h; 21 days; and adults. Values are expressed as percentage of adult value (adult value = 100%). For definitions see legend to figure 5.

Discussion

A decreased ability of AMs to generate O_2^- was reported during the neonatal period, and an inadequate production of oxygen radicals may contribute to the increased risk of pulmonary infection in neonates [11, 12]. However, the mechanisms underlying this decreased O_2^- production remain to be established. Because spontaneous increases in antioxidant activity have been demonstrated in neonatal lung homogenates [27], we hypothesized that the low oxidant-generating capacity of neonatal AMs may be related to high levels of cellular antioxidant activity. Our estimations of oxidant production levels and antioxidant enzyme activities in rat AMs at various postnatal ages showed inversion of the oxidant-antioxidant balance in these cells with postnatal maturation. Increases in production of oxygen radical species and decreases in SOD and GPX activities occurred as postnatal age increased.

Among endogenous free radicals, some are generated and act intracellularly, whereas others are produced within the cell and are subsequently released into the surrounding area [28]. In our study, oxygen metabolite production in AMs was monitored both by chemiluminescence (release of free radicals) and by intracellular oxidation of DCFH (intracellular free radicals). We found a defective response to exposure to PMA or to opsonized zymosan particles in neonatal AMs with the chemiluminescence method, in keeping with results of earlier studies [10–12]. In addition, this study demonstrated that intracellular production of oxygen metabolites was also decreased during the neonatal period. This simultaneous depression both of extracellular and intracellular O_2 radical production in neonatal AMs argues for a common developmental control mechanism. Furthermore, the deficiency in O_2 radical production persisted in AMs from 21 day old rats and was perhaps also present in cells from 5 week old rats, since these were characterized by decreased light emission.

This defect in the O_2 radical production response has generally been ascribed to AM immaturity during early postnatal development [11, 12, 29], but our previous report of high gelatinase activity from neonatal rat AMs [18] suggests that cell immaturity, if present, may not affect all AM functions. Alternatively, the low capacity of neonatal AMs for producing oxygen metabolites may be due to the presence of inhibitory factor(s) in the surface lining material of airways, such as pulmonary surfactant [10]. However, this finding may be species-dependent, since pretreatment of rat AM with rat surfactant has been reported to increase their luminol-dependent CL response [30]. Thus, surfactant may not account for the defective AM response observed in young rats. From the present data, we propose that upregulation of intracellular antioxidant activity is, at least in part, responsible for the observed defective response in O_2 radical production.

Changes in AM antioxidant enzyme activities with postnatal age have been the focus of few investigations, and these have yielded conflicting results. STEVENS and AUTOR [15] reported high basal levels of both mitochondrial SOD and catalase in neonatal rat AMs. On the contrary, NERURKAL *et al.* [14] found low levels of SOD activity in rabbit AMs immediately after birth. To our knowledge, no data on GPX activity are available. Our results argue for a tight connection between the antioxidant enzyme activity and the presence of critical concentrations of oxygen metabolites in AMs (fig. 5).

These results are, at first sight, at variance with the report by SPEER *et al.* [13], who found similar oxygen metabolite production in neonatal and adult monocyte-derived macrophages in a study involving testing of macrophages after a long incubation period (7 days). However, as demonstrated by NERURKAR *et al.* [14], such a delay is associated with an overall loss in antioxidant enzyme activities in cells. We suggest that the key role of antioxidants is, paradoxically, further supported by the results of SPEER *et al.* [13].

Developmentally, a number of studies performed in several mammalian species have demonstrated that a rise in antioxidant enzyme activity was an important event in the preparation of the foetal lung for birth [27, 31–33]. At birth, lung cells are exposed to much higher levels of oxygen tension (PO_2) than those experienced in the comparatively hypoxic *in utero* environment. In particular, in lung homogenates of late gestation foetal rats, a rapid increase in activity was demonstrated for the three pulmonary antioxidant enzymes, SOD (up to 150%), catalase (up to 350%) and GPX (up to 250%) [27]. The present study shows that a similar increase occurs in the components of the primary antioxidant defence system in AMs. Both SOD and GPX were elevated in AMs immediately after birth, and decreased to adult levels during the first weeks of life. In the absence of such an increase, exposure of alveolar cells to threefold to fourfold increase in alveolar O_2 tensions would be expected to result in a marked increase in production of intracellular O_2 radical [34, 35]. On the contrary, we found that the spontaneous intracellular production of oxygen radical species was considerably lower in neonatal AMs than in adult AMs. Furthermore, this lower O_2 radical production rate in neonatal AMs persisted after AM stimulation, demonstrating a strikingly different pattern for intracellular oxidant-antioxidant balance between neonates and adults.

The effect of this antioxidant shield may be to protect newborns from cytotoxic injury on initiation of air breathing. However, there is no direct evidence indicating whether or not the intracellular oxidant-antioxidant imbalance in AMs contributes to pathological changes in neonatal lungs. Intracellular antioxidants produced by phagocytic cells may help to scavenge oxygen radical species released outside the cells, thus decreasing free radical-mediated tissue injury [36]. On the other hand, intracellular antioxidants may regulate the amount of oxygen metabolites released outside the cells in some situations, such as during respiratory bursts. Indeed, increasing the intracellular antioxidant defences of phagocytes with N-acetylcysteine has been shown to reduce the chemiluminescence response of phagocytes to opsonized zymosan [37]. The present data, demonstrating a similar pattern of AM response both for intracellular and extracellular oxygen metabolites support this hypothesis. In case of prematurity, appropriate antioxidant protection should not have been developed, thus rendering the premature lung prone to O_2 radical-induced toxicity [38].

The deficiency in O_2^- and H_2O_2 production demonstrated in AMs immediately after birth in this study persisted during the first weeks of life, suggesting that AMs acquire their full oxidant-releasing capacity only when major pulmonary structural changes are completed, *i.e.* when lung tissue is more resistant to oxidant injury. Within 3 weeks, the saccular lung present at birth undergoes dramatic changes, including remodelling of lung structure, thinning of the interstitial region, and production of new alveolar walls with supporting extracellular matrix. During this period, the production of oxygen species by rat AMs remains significantly lower than during adulthood. After the third week, when production of AM oxygen species nears adult values, the rat lung exhibits an essentially mature structure, although it continues to undergo maturational changes, including an increase in capillary volume [39]. Although this developmental pattern for AM oxidant production may be an important factor in normal lung development, it may also contribute to the physiological impairment of lung antibacterial defences characteristic of the neonatal period.

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