Oxidant-antioxidant imbalance in the experimental interstitial lung disease induced in sheep by visna-maedi virus

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Oxidant-antioxidant imbalance in the experimental interstitial lung disease induced in sheep by visna-maedi virus. V. Cottin, I. Court-Fortune, J. Crevon, J-F. Mornex. ©ERS Journals Ltd 1996.
ABSTRACT: Infection of sheep by visna-maedi virus causes an interstitial pneumonitis similar to that associated with human immunodeficiency virus type-1 (HIV-1). Visna-maedi virus infection of alveolar macrophages leads to their activation.

In this study we determined whether an imbalance in oxidant-antioxidant activity may be involved in the pathogenesis of the disease.

We investigated the spontaneous and phorbol myristate acetate (PMA)-induced release of hydrogen peroxide (H_2O_2), and the activities of superoxide dismutase and glutathione peroxidase in alveolar macrophages from lambs experimentally-infected with visna-maedi virus, and in ovine alveolar macrophages infected *in vitro*.

Alveolar macrophages from lambs experimentally-infected *in vivo* exhibited normal spontaneous H_2O_2 release and had superoxide dismutase and glutathione peroxidase activities similar to those from control animals. In contrast, after *in vitro* stimulation with PMA the H_2O_2 production by macrophages from experimentally-infected lambs was significantly increased. Similarly, spontaneous and PMA-induced H_2O_2 production by *in vitro* infected macrophages was significantly increased as compared to controls.

In conclusion, the increased capacity of alveolar macrophages infected with the human immunodeficiency virus type-1-related visna-maedi virus to release hydrogen peroxide on stimulation suggests an oxidant-antioxidant imbalance, which may contribute to the pathogenesis of the observed chronic interstitial pneumonitis.
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Visna-maedi virus is a lentivirus related to human immunodeficiency virus type-1 (HIV-1). Spontaneous infection of sheep by visna-maedi virus is responsible for multivisceral degenerative and chronic inflammatory lesions, including a progressive interstitial pneumonitis (known as maedi) [1, 2], which shows similarities with HIV-1-associated interstitial pneumonitis [3]. Maedi is characterized by peribronchiolar and perivascular proliferative lymphoid follicles, smooth muscle hyperplasia, diffuse intraluminal and mural alveolitis, and fibrosis [4]. The process of alveolitis involves macrophages, lymphocytes and neutrophils [5]. Similar but milder pathological abnormalities can be reproduced by experimental intratracheal inoculation of newborn lambs with visna-maedi virus preparations [6, 7]. A lymphocytic alveolitis is also present in this experimentally-induced lung disease [8].

Visna-maedi virus infection of alveolar macrophages leads to their activation and induces a high level of expression of major histocompatibility complex (MHC) class II antigens, and the release of fibronectin, neutrophil chemotactic activity [5], and procoagulant activity [9]. Activated alveolar macrophages have been shown to release reactive oxygen species (ROS), including hydrogen

peroxide (H_2O_2), superoxide ($O_2^{·-}$), and nitric oxide (NO) [10, 11]. The toxicity of ROS are normally balanced by several intracellular and extracellular antioxidant defence systems. The major pulmonary antioxidant enzyme systems are superoxide dismutase, catalase, and the enzymes of the glutathione redox cycle, such as glutathione peroxidase and glutathione reductase [12]. An oxidant-antioxidant imbalance, through an increase in oxidant stress or a compromise in antioxidant resources, has been implicated in various lung conditions, including the adult respiratory distress syndrome, hyperoxic lung injury, cigarette-smoke effects, exposure to air pollutants (ozone), xenobiotic-induced lung injury, ischaemia-reperfusion lung injury, idiopathic pulmonary fibrosis, and HIV-1-associated lung disease [12, 13]. We hypothesized that an imbalance in oxidant-antioxidant activity may be involved in the pathogenesis of the interstitial lung disease induced in sheep by visna-maedi virus. We have investigated the release of H_2O_2 and the superoxide dismutase and glutathione peroxidase activities of alveolar macrophages from lambs experimentally-infected with visna-maedi virus, and of *in vitro* infected ovine alveolar macrophages.

Methods

Production of visna-maedi virus stock preparations

Virus stocks were prepared from field isolates of visna-maedi virus checked by reverse transcriptase activity, electron microscopy and/or positive immunofluorescence with a polyclonal sheep serum as described previously [8]. Each visna-maedi virus stock was made by mixing three different field isolates; one from blood monocytes and one from alveolar macrophages of animals with natural maedi and the third from a lung explant of an experimentally-infected animal. Each was obtained by productive viral replication at low passage (n=3) in ovine skin fibroblasts (IDO5 strain, kindly provided by J. Laplace, Rhône-Merieux, Lyon, France). The prototype visna-maedi strain K1514 (kindly provided by G. Chapuis, Rhône-Merieux) was prepared by productive viral replication in IDO5 fibroblasts. Culture supernatants were collected and stored frozen until use, the stock preparations titrated between $10^{5.3}$ and $10^{6.5}$ 50% tissue culture infective dose $(TCID_{50}) \cdot mL^{-1}$. The absence of endotoxin contamination of the virus preparations was checked by the Limulus amoebocyte lysate assay (QCL 1000; Bio-Whittaker, USA).

Inoculation of lambs

Lambs (Lacaune strain) were separated from the ewes at birth, before suckling, and inoculated transtracheally as reported previously [7]. Animals receiving virus (n=12) were inoculated with 2 mL of purified virus, whereas controls (n=5) received the same volume of saline. The different groups were stalled separately to prevent horizontal transmission of the virus and were maintained by artificial feeding. Care was taken to maintain and use the lambs in agreement with the French regulation on animal experimentation (authorization number 02623). All animals were slaughtered at 3 months by intravenous infusion of xylazine and exsanguination. Lungs from lambs and from adult sheep were excised immediately after death. For *in vitro* infection, lungs from a healthy adult sheep were obtained from the slaughterhouse.

Histological analysis

Fragments for tissue studies were taken from the dorsal part of the right posterior lobe of lungs. Histological examination was performed as described previously [4].

Preparation of alveolar cell suspensions

The alveolar cells from lambs and adult sheep were obtained by bronchoalveolar lavage (BAL) and analysed as described previously [5]. Cell viability, monitored at this step by counting the percentage of cells that excluded trypan blue, was regularly over 85%. Cell counts per microlitre of recovered BAL fluid were established with a haemocytometer. The cells were washed twice with phosphate buffered saline (Gibco BRL) and suspended at a concentration of 2×10^6 cells $\cdot mL^{-1}$ in Dulbecco-modified Eagle's minimum essential medium (DMEM) (Gibco BRL) containing 8% foetal calf serum (FCS).

Virus isolation

Cells obtained by BAL were co-cultured with IDO5 cells, as described previously [5], to detect the presence of virus. Virus infection was indicated by the appearance of syncytia (>8 nuclei·cell^{-1}) after 10–30 days of culture. The syncytia were confirmed by fixation and staining of the fibroblasts with May-Grünwald-Giemsa. Cultures with no syncytia after at least 60 days of co-culture and/or 10 passages were considered negative. In addition, supernatants of co-cultures were analysed for reverse transcriptase activity as described previously [14], except that 10 mM magnesium acetate was used instead of 1 mM manganese acetate.

Assay for hydrogen peroxide production

Production of H_2O_2 by alveolar macrophages was measured using the method described by PICK and KEISARI [15], based on the horseradish peroxidase-dependent oxidation of phenol red by H_2O_2, which results in the formation of a compound absorbing at 610 nm. Alveolar macrophages were isolated by differential adherence. The cells, suspended in DMEM containing 8% FCS, were seeded into 96-well tissue culture plates with flat bottoms (Microtest III™; Falcon 3072, Becton Dickinson) at 0.3×10^6 cells·well^{-1}. Incubation for 2 h at 37°C in 90% air/10% CO_2, allowed the adherence of macrophages to the plates. Nonadherent cells were then removed by rinsing with 100 µL Hanks's balanced salt solution (HBSS), (Gibco BRL) without phenol red, prewarmed to 37°C. Adherent cells were covered with phenol red assay solution, containing 140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red (Sigma, Nanterre, France), and 19 U·mL^{-1} of horseradish peroxidase (Sigma). Alveolar macrophages were stimulated with three different concentrations (0.01, 0.1, and 1 µg·mL^{-1}) of phorbol myristate acetate (PMA), and the plates were incubated for 1 h at 37°C.

At the completion of incubation, the supernatants were made alkaline by adding NaOH 1 N, 10 µL·well^{-1}. The samples were read at 610 nm (Titertek Multiskan, Flow, France) against a blank of phenol red assay solution to which 10 µL of NaOH 1 N had been added. Standard curves were made using the same preparation of phenol red solution, with 1–100 nM H_2O_2 solutions. The standards were treated in the same manner as the culture supernatants. The amount of H_2O_2 produced was calculated from the standard curve. In each experiment, three wells of adherent macrophages were used for determining the amount of cell protein. The monolayers were scraped into NaOH 1 N, and protein concentration determined according to the method of LOWRY *et al.* [16]. The results were expressed as nanomoles of H_2O_2 generated per milligram of cell protein.

Superoxide dismutase activity

The role of superoxide dismutase is to accelerate the dismutation of superoxide radicals $(O_2^{\cdot -})$ to hydrogen peroxide and molecular oxygen. The assay (Ransod superoxide dismutase™; Randox, Crumlim, UK) employed xanthine and xanthine oxidase to generate superoxide

radicals which reacted with 2-(4-iodophenyl)-3-(4-nitro-phenol)-5-phenyl tetrazolium chloride (INT) to form a red formazan dye. Superoxide dismutase activity was then measured by the degree of inhibition of this reaction. Briefly, alveolar cells suspended in DMEM containing 8% FCS were seeded into 96-well tissue culture plates as described above, and incubated for 24 h at 37°C in 90% air /10% CO_2. After removing nonadherent cells by rinsing with HBSS adherent cells were lysed with triton 0.1%. Assays were routinely performed at 37°C in 50 mmol^{-1} 3-cyclohexylamino-1-propanesulphonic acid, pH 10.2, containing 0.94 mmol^{-1} ethylenediamine tetra-acetic acid (EDTA), according to the recommendations of the manufacturer. The absorbance was measured at 505 nm (Multiskan) against a blank consisting of xanthine, INT, and phosphate buffer. A calibration curve was obtained using standards. The results were expressed as units of superoxide dismutase activity per milligram cell protein.

Glutathione peroxidase activity

Glutathione peroxidase activity catalyses the oxidation of glutathione by cumene hydroperoxide; in the presence of glutathione reductase and nicotinamide adenine dinucleotide phosphate reduced form (NADPH), the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to nicotinamide adenine dinucleotide phosphate (NADP+). Glutathione peroxidase was measured by the method described by PAGLIA and VALENTINE [17], in which the decrease in absorbance at 340 nm is measured. Alveolar macrophages were selected by 24 h adherence and lysed with triton 0.1%, as described above. Assays were performed according to the manufacturer's recommendations (Ransel glutathione peroxidase™; Randox).

In vitro *virus infection of macrophages*

Uninfected macrophages, obtained as described above from healthy adult sheep lungs, were seeded into 96-well tissue culture plates (Microtest III ™; Falcon 3072; Becton Dickinson), 0.3×10^6 cells·well^{-1} in 150 µL of antibiotic-supplemented DMEM. After 24 h adherence, cultures were inoculated with 50 µL of the K1514 stock (10^8 TCID$_{50}$·mL^{-1}) or with 50 µL of the heat-inactivated (56°C for 30 min) K1514 stock. Controls were mock-infected with medium alone, or with the supernatant of uninfected fibroblast cultures. The flasks were incubated overnight at 37°C in 95% air/5% CO_2. The cells were then stimulated with 0.01 µg·mL^{-1} of PMA, and the plates were incubated for 1 h in the same conditions before harvesting the cells for measurement of H_2O_2 production.

Statistical analysis

The results shown are the mean of duplicate determination for each sample. Results are expressed as arithmetic mean±SEM. The data were analysed by the nonparametric Mann-Whitney U-test using the Statwork™ package (DataMetrics, Philadelphia, PA, USA) run on a Macintosh™ computer. Differences were considered significant when p-values were below 0.05.

Results

Experimental infection and induction of lesions

Histological examination. Lungs from virus-inoculated lambs were normal in two out of 12 cases. Multifocal peribronchovascular lymphoid nodules were present in eight cases, and peribronchovascular and perialveolar lymphocytic infiltration in 10 cases out of 12; these were classified as minor lesions, according to criteria described previously [4]. Histological examination of lungs from the adult sheep and from three out of five control lambs showed a slight thickening of the alveolar septa by oedema and/or minor lymphocytic infiltration; lungs were histologically normal in the two remaining control lambs.

Lentivirus detection. No cytopathic effect was observed when fibroblasts were cultured alone. In contrast, positive viral isolation, as demonstrated by cytopathic effect in co-culture with BAL cells, was obtained in 11 out of 12 experimentally-infected lambs. Co-cultures from 10 out of 12 experimentally-infected lambs exhibited significant reverse transcriptase activity. Thus, viral infection was demonstrated in 10 out of 12 experimentally-infected lambs. A cytopathic effect in co-culture with BAL cells was observed in three out of five control lambs, and was not present in one control lamb; co-cultures were contaminated by fungi in the remaining control lamb. None of the five control lambs exhibited significant reverse transcriptase activity. Thus, the positive cytopathic effect in three control lambs was considered as a false positive.

BAL fluid cell counts. The total cell count was significantly higher in experimentally virus-inoculated lambs than in control lambs (table 1), demonstrating a process of alveolitis in infected animals, as described previously [8]. The alveolitis was characterized by a significant increase both in macrophage and lymphocyte counts (table 1). There was no increase in the number of neutrophils.

Hydrogen peroxide production by alveolar macrophages

The spontaneous H_2O_2 production by the bronchoalveolar macrophages was not increased in experimentally-infected lambs (46.2±16.3 nmol·mg^{-1} cell protein), as compared with values from control animals (84±44.9 nmol·mg^{-1} cell protein; p=0.22) (fig. 1) . In contrast, after

Table 1. – Bronchoalveolar lavage fluid cell counts in experimentally-infected lambs and controls

	Infected lambs (n=12)		Controls (n=5)	
	cells·µL^{-1}	%	cells·µL^{-1}	%
Total cells	668±50**		333±50	
Macrophages	464±36**	70	263±44	79
Lymphocytes	196±25**	29	59±11	18
Neutrophils	9±2	1.3	10±3	3.0

Values are presented as mean±SEM and as percentage of total cells. **: p≤0.01, significant differences between the two groups.
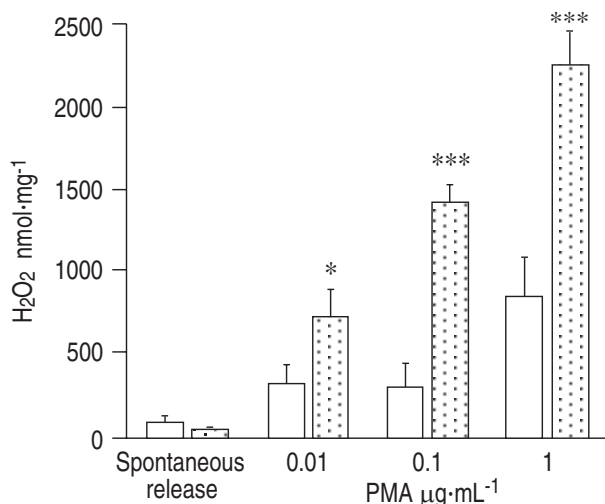
Fig. 1. – Spontaneous and phorbol myristate acetate (PMA)-induced release of hydrogen peroxide (H_2O_2) by alveolar macrophages from lambs experimentally-infected with virus (n=12) and control lambs (n=5). *: p<0.05; ***: p=0.001, significant difference between groups. : uninfected; : infected:

in vitro stimulation with 0.01, 0.1 or 1 $\mu g \cdot mL^{-1}$ of PMA the H_2O_2 production by macrophages was significantly increased in experimentally-infected lambs as compared with controls: 729±164 *vs* 326±116 $nmol \cdot mg^{-1}$ cell protein, (p<0.05); 1,412±119 *vs* 308±146 $nmol \cdot mg^{-1}$ (p=0.001); and 2,247±214 *vs* 850±238 $nmol \cdot mg^{-1}$ (p=0.001), respectively (fig. 1). The spontaneous and PMA-induced H_2O_2 production by macrophages was not increased in control lambs with a positive cytopathic effect in co-culture as compared with control lambs without viral cytopathic effect (not shown).

Results of H_2O_2 production after *in vitro* infection of alveolar macrophages from healthy adult sheep are shown on figure 2. Spontaneous and PMA-induced H_2O_2 production were increased in experimentally-infected macrophages (spontaneous: 1,209 $nmol \cdot mg^{-1}$ cell protein; PMA 0.01 $\mu g \cdot mL^{-1}$: 2,392 $nmol \cdot mg^{-1}$ cell protein) as compared

with control (spontaneous: 320 $nmol \cdot mg^{-1}$ cell protein; PMA 0.01 $\mu g \cdot mL^{-1}$: 1,002 $nmol \cdot mg^{-1}$ cell protein; p=0.03 for both spontaneous and PMA, infected *vs* uninfected) (fig. 2). H_2O_2 production was also progressively increased when infected macrophages were stimulated with 0.1 or 1 $\mu g \cdot mL^{-1}$ of PMA (data not shown). Spontaneous and PMA-induced H_2O_2 production was not altered by heat inactivation of the virus preparation (spontaneous: 1,416 $nmol \cdot mg^{-1}$ cell protein; PMA 0.01 $\mu g \cdot mL^{-1}$: 2,610 $nmol \cdot mg^{-1}$ cell protein) (fig. 2). H_2O_2 production was not increased when macrophages were treated with supernatants from uninfected fibroblast cultures (spontaneous: 304 $nmol \cdot mg^{-1}$ cell protein; PMA 0.01 $\mu g \cdot mL^{-1}$: 237 $nmol \cdot mg^{-1}$ cell protein) as compared with medium alone (fig. 2).

Superoxide dismutase and glutathione peroxidase activities

Glutathione peroxidase activity was not different in experimentally-infected lambs (0.09±0.01 $U \cdot mg^{-1}$ cell protein; n=12) and in control lambs (0.09±0.01 $U \cdot mg^{-1}$ cell protein; n=5) (p=0.38). Similarly, no significant difference was found in superoxide dismutase activity between infected (n=12) and uninfected (n=5) lambs (0.51±0.1 *versus* 0.43±0.1 $U \cdot mg^{-1}$ cell protein, respectively) (p=0.24).

Discussion

In this study, it was shown that alveolar macrophages from lambs experimentally-infected *in vivo* exhibit a normal spontaneous H_2O_2 release and superoxide dismutase and glutathione peroxidase activities as compared with macrophages from control animals. In contrast, after *in vitro* stimulation with PMA the H_2O_2 production by macrophages is significantly increased in experimentally-infected lambs as compared with controls, suggesting that the virus may have "primed" these cells to produce reactive oxygen species. Finally, the spontaneous and PMA-induced H_2O_2 production are increased in *in vitro* infected macrophages as compared with controls.

The effect of HIV-1 on the release of ROS by mononuclear phagocytes *in vivo* remains controversial [18–26]. However, *in vitro* experiments have demonstrated that HIV-1 infection of macrophages did not decrease their ability to generate H_2O_2 when treated with PMA or zymosan [27]. Similarly, an increase in lipid peroxidation has been reported [28, 29]. On the other hand, reduced concentrations of acid-soluble thiol (cysteine and reduced glutathione) have been reported in cell lysates of peripheral blood mononuclear cells [30], in venous plasma and in lung epithelial lining fluid [31] of symptom-free HIV-1-infected individuals, and it is likely that the glutathione deficiency contributes to the pathogenesis of HIV-1-related lesions [32].

The present study supports the concept of the activation of alveolar macrophages during visna-maedi virus infection. We have previously shown that visna-maedi virus infection of alveolar macrophages leads to the expression of MHC class II antigens on most of these cells [5]. Infected alveolar macrophages also release fibronectin, neutrophil chemotactic activity [5], procoagulant activity [9], and express tissue factor [9] and
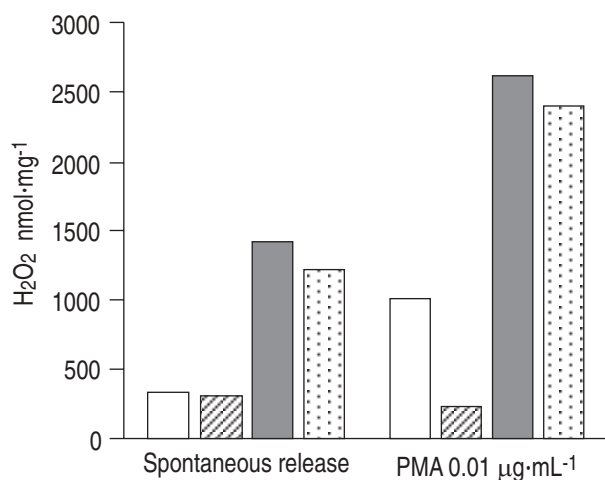

Fig. 2. Spontaneous and phorbol myristate acetate (PMA)-induced release of hydrogen peroxide (H_2O_2) by *in vitro* infected alveolar macrophages and controls. Results were obtained from duplicate experiments. : control (medium); : supernatant of uninfected fibroblast culture; : heat-inactivated virus; : active virus.

interleukin(IL)-8 messenger ribonuclear acid (mRNA) [33]. Together with these findings, the present demonstration of an increased oxidant release confirms that visna-maedi virus-infected alveolar macrophages are activated.

The molecular mechanisms by which the high level of ROS, particularly H_2O_2 release, contribute to the pathogenesis of lentivirus-associated pulmonary lesions remain poorly understood. ROS have been shown to increase the production of cytokines, such as IL-1 or IL-8 [34, 35]. Recently, *in vitro* studies have demonstrated that low concentrations of H_2O_2 activate intracellular transcription nuclear factor kappa-B (NF-κB) post-translationally, and that the activation of NF-κB by H_2O_2 is prevented by the antioxidant N-acetyl-L-cysteine [36]. Similarly, the transactivator, Tax, from human T-cell leukaemia virus type-1 (HTLV-1) enhances the production of ROS, which in turn activate NF-κB [37]. NF-κB activates the transcription of genes involved in immune responses, leading to the production of several cytokines [38]. In particular, NF-κB activates transcription of the IL-8 gene [39, 40]. The release of ROS and subsequent activation of NF-κB may explain, at least in part, the induction of IL-8 gene expression observed in alveolar macrophages from visna-maedi virus-infected animals [33]. The release of proinflammatory cytokines, such as IL-8, may in turn contribute to the alveolar recruitment of inflammatory cells and then to the pathogenesis of the disease. Moreover, ROS have the potential to increase HIV-1 gene expression [41, 42] *via* NF-κB-mediated transcription pathways [36], and they probably play a role in regulating HIV-1 replication *in vitro* [36, 43, 44]. The release of ROS during lentiviral infection may then lead to a self-amplifying loop of viral expression and replication. However, the role of ROS in regulating the visna-maedi replication *in vitro*, and to a greater extent *in vivo,* remains speculative.

Nevertheless, the high level of hydrogen peroxide release during visna-maedi infection, coupled with the non-stimulated level of antioxidant, are likely to contribute to an oxidant-antioxidant imbalance that can contribute to the pathogenesis of the chronic interstitial pneumonitis induced in sheep by the human immunodeficiency virus type-1 related visna-maedi virus.

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