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Linda Svensson, Lenore Johansson, Marlene Lundström, Anders Bucht

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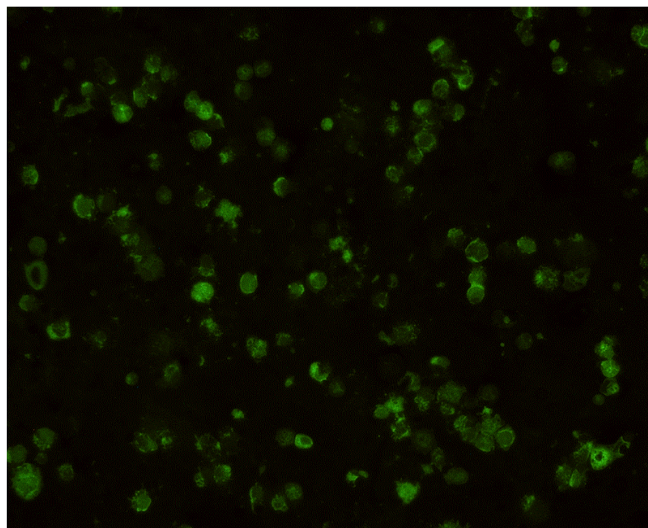

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Report title (In translation)

Method for analysis of intracellular production of free radicals in vivo

Abstract (not more than 200 words)

Inhalation of toxic substances may give rise to an acute inflammatory response leading to recruitment of neutrophils into the lung. Upon activation, these cells start to produce reactive oxygen metabolites that are finally released into the surrounding tissue. Because of their microbicidal properties, these oxygen radicals play an important role in the host defence to microbes, but since their action is non-specific they could also be harmful to the host.

Research on treatment of acute pulmonary inflammation is on-going at the Swedish Defence Research Agency, Division of NBC Defence. By using a method of intracellular staining with a fluorescent probe (dichlorodihydrofluoresceindiacetate) *in vitro*, the neutrophil production of reactive radicals can be detected. In order to improve the analysis of cell activation during inflammation, the aim of this study was to develop a method for intracellular staining of oxygen radicals *in vivo*.

Inflammation in peritoneum or the lung was triggered in mice through intraperitoneal injection or aerosol inhalation of the bacterial toxin lipopolysaccharide (LPS). To avoid dilution effects, the probe was administered directly into the target organ of inflammation either by intraperitoneal injection or by tracheal instillation. Cells from either locality was isolated and labelled with a specific antibody against neutrophils followed by analysis of oxidative activity by flow cytometry or fluorescence microscopy.

Keywords

Inflammation, free radicals, dichlorodihydrofluoresceindiacetate, flow cytometry

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Metod för analys av intracellulär produktion av fria radikaler in vivo

Sammanfattning (högst 200 ord)

Toxiska ämnen kan vid inhalation ge upphov till ett akut inflammatoriskt svar vilket leder till rekrytering av neutrofiler till lungan. Aktivering av dessa celler initierar en intracellulär produktion av reaktiva syreradikaler. Radikalerna är kraftigt oxiderande och fungerar som mikrobicider men kan efter frisättning även orsaka skador på den omgivande vävnaden.

Vid FOI, NBC-skydd bedrivs sedan tidigare forskning kring behandling av skador som uppstår vid akut lunginflammation. För att mäta oxidativ aktivitet i neutrofilerna har då cellerna färgats in med en fluorescerande prob (diklorodihydrofluoresceindiacetat) *in vitro*. Proben tas upp i cellen och börjar fluorescera när den oxideras av syreradikaler. Syftet med denna studie var att utveckla en metod för infärgning av aktiverade neutrofiler *in vivo*, för att på så sätt bättre återspegla den naturliga cellaktiveringen vid inflammationer.

Inflammationer framkallades i bukhåla och lunga genom att exponera försöksdjur för bakterietoxinet lipopolysackarid (LPS) i form av injektioner intraperitonealt respektive inhalationer av aerosol. För att undvika utspädningseffekter tillfördes proben direkt i målorganet för inflammationen. Detta utfördes genom intraperitoneal injektion eller trakeal instillation. Celler från de båda lokaliteterna isolerades slutligen genom buk- respektive lungsköljning och neutrofilerna färgades in med en specifik antikropp. Den oxidativa aktiviteten kvantifierades därefter med hjälp av flödescytometri eller fluorescensmikroskopi.

Nyckelord

Inflammation, fria radikaler, diklorodihydrofluoresceindiacetat, flödescytometri

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Introduction

Inhalation of toxic substances (e.g. bacterial endotoxin) can lead to acute lung inflammation. Macrophages in the lower respiratory tract, the so-called alveolar macrophages, initiate the inflammatory reaction through production of mediators (cytokines and chemokines) which diffuse into surrounding tissues and activate cells such as neutrophils and endothelial cells in blood vessels. On activation, these cells begin to express specific surface receptors that permit neutrophil adhesion to the blood vessel wall and subsequent migration out in the tissues. Binding to epithelial cells and stimulation with cytokines initiates synthesis of reactive oxygen intermediates in neutrophils (1). Production of free oxygen radicals intracellularly is through a cascade of events and requires a unique enzymatic complement that exists in all phagocytosing cells, such as neutrophils, monocytes and macrophages. Radicals function as microbicids and fulfil a prominent role in cellular defence to bacteria (2). The first enzyme involved in radical synthesis is NADPH oxidase which catalyses the formation of superoxide, which in turn facilitates production of several other reactive oxygen intermediates (figure 1). These oxygen radicals are a defence against foreign organisms that invade the body, but they can also damage the surrounding tissues. Many inflammatory diseases are linked to oxidative injury caused by an imbalance in the production and regulation of free oxygen radicals.

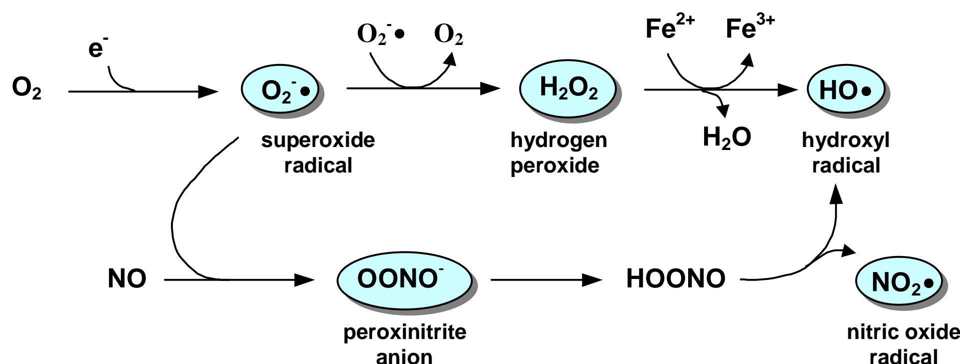

Figure 1. Chemical reactions leading to formation of some reactive oxygen species produced intracellularly in neutrophils. The cascade-like production is called ’respiratory burst’.

Through measurement of the oxidative potential of cells it is possible to assess their degree of activation. Quantitative analysis of oxygen radicals is hampered by their short half-lives. Cells are usually stained with probes that fluoresce when oxidised, which permits an indirect measurement of oxygen radical production. Such techniques have been effectively utilised in cell culture and other *in vitro* systems (3).

One probe that is commonly used in measurements of oxidative activity is dichlorodihydro-fluoresceindiacetate (H_2DCFDA), a hydrophobic molecule, which is directly taken up by cells by diffusion. Within the cytosol the probe is catalysed by intracellular esterases to a hydrophilic metabolite which is then trapped within the cell (figure 2). If reactive oxygen radicals are produced intracellularly, the probe is oxidised to a fluorescent form that can be detected using flow cytometry or fluorescence microscopy (4). Studies have demonstrated that the probe is oxidised by a variety of different radicals and not just by hydrogen peroxide as previously believed. H_2DCFDA is useful for studies of oxidative activity in cells as it is only taken up by living cells and although it reacts with many different oxygen radicals it does not yield unacceptable levels of background fluorescence (4, 5).

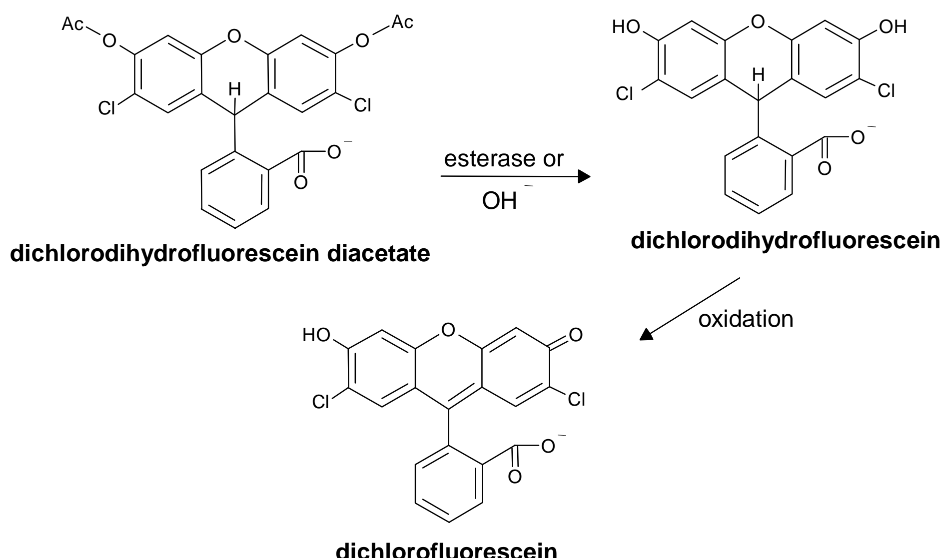

Figure 2. Dichlorodihydrofluoresceindiacetate (H_2DCFDA) enters living cells where it is catalysed by esterases to dichlorodihydrofluorescein, a hydrophilic molecule, which cannot leave the cell. The probe is finally oxidised by intracellular radicals to the fluorescent dichlorofluorescein form (1, 2).

Studies of free oxygen radicals have previously been conducted *in vivo* in perfused organs using H_2DCFDA and other fluorescent molecules such as manganese diaminobenzidine (Mn-DAB) and dihydrorhodamine 123 (DHR) (3, 6). However, the experimental set-up for microcirculation in organs is relatively complicated and does not really reflect the natural cellular environment (4). H_2DCFDA has previously been used for detection of free oxygen radicals in an *ex vivo* system in which lung lavage cells are incubated in test-tubes together with the probe and analysed by flow cytometry (7). To stain cells directly in experimental animals under as normal conditions as possible and to then study the activity of specific cell populations *ex vivo* using flow cytometry or fluorescence microscopy would give a better understanding of oxidative activity *in vivo*.

Purpose and Aims

The purpose of the study is to develop a method for staining of free oxygen radicals *in vivo*. In order to minimise the time of analysis of intracellular oxidative activity *ex vivo*, the probe is administered directly into animals. This yields a better picture of the natural cell activation status during an inflammatory response.

The aim is that the method should allow for analysis of tissue-specific oxidative activity and also for studies of the effects of antioxidant treatments.

Materials and Methods

Animals

Female C57BL/6J mice 10-14 weeks old were obtained from in-house breeding at FOI NBC-defence or were purchased from M & B (A/S, Ry, Denmark) and used in all experiments. The latter were allowed to acclimatise for one week prior to use. All experiments complied with

the Swedish Defence Research Agency regulations and were approved by the local ethical committee in Umeå (DNR A48-98 and A82-00).

Intraperitoneal administration of LPS and probe

Peritoneal inflammation was induced by intraperitoneal (i.p.) injection of 400 µl lipopolysaccharide (LPS) solution from *Escherichia coli* serotype 0128:B12 (Sigma, St. Louis, MO, USA, cat no. L-2755; batch no. 87H4112). The dose of LPS injected varied between 0.1-1 mg/ml. After 3.5 hours, H_2DCFDA (Molecular Probes, Eugene, OR, USA) was administered by injection of 500 µl probe i.p.. The probe was dissolved in 100 µl ethanol (99,6 %) and further diluted in phosphate buffered saline (PBS) to the required concentration. An appropriate dose was defined by titration between the range 0.1-0.8 mg/ml. Thirty minutes after probe injection the animals were killed by cervical dislocation and peritoneal cells were recovered through washing with 5 ml ice-cold Hanks' balanced salt solution (HBSS) pH 7.4. Control animals were first injected with physiological saline and then received a PBS solution with an equivalent ethanol content to that of the probe (about 2-8 %).

Analysis of oxidative activity by fluorescence microscopy

Peritoneal cells were isolated from LPS-treated animals, washed in PBS (285 g, 4°C, 10 min) and resuspended in 0.4 ml PBS. The number of living leukocytes was assessed by trypan blue exclusion and microscopical evaluation. In order to increase the proportion of neutrophils in the cell suspension, macrophages were removed by adhesion through incubation of the cells in polystyrene petri dishes for 30 min at 37°C. The supernatant was recovered and approximately 20 000-25 000 leukocytes were applied to microscope slides using a cytospin (190 g, 5 min). Slides were then analysed using a fluorescence microscope (Leica DMR, Wetzlar, Germany).

Aerosol exposure to LPS and intratracheal administration of probe

Mice were exposed to an aerosol of LPS (nebuliser concentration 1 mg/ml, dissolved in sterile water) for 15 min with an air flow of 7.4 l/min (8). The probe was introduced into the lungs through tracheal instillation of 100 µl solution (0.2-1 mg/ml) of which 0.5-2.5 % comprised ethanol. Instillation of mice was conducted under anaesthesia (15 mg Rapinovet™/kg intravenously; Schering-Plough Animal Health, Madison, NJ, USA) approximately 19 h after LPS exposure. After 30 min animals were killed by cervical dislocation and cells were recovered through broncheoalveolar lavage (BAL) with 4x1 ml ice-cold HBSS. Control animals received water aerosols and were instilled with PBS/ethanol.

Analysis of oxidative activity by flow cytometry

Lung and peritoneal cell suspensions were kept on ice and in the dark throughout the analysis. Cell suspensions were centrifuged (285 g, 4°C, 10 min) and resuspended in 0.4 ml PBS. 20 µl was removed for estimation of living cell numbers by trypan blue exclusion in a Bürker chamber and microscopical evaluation. For antibody staining 200 000 cells from each sample were transferred to FACS tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The cells were washed in 4 ml ice-cold PBS/bovine serum albumin (BSA) (0.1 %) before 100 µl PBS/BSA containing 4 µl Fc-block (diluted 1:5 in PBS/BSA) (Pharmingen, San Diego, CA, USA) and 4 µl normal rat serum were added. Cells were then incubated for 5 min before staining with 4 µl GR-1, PE-conjugated antibody diluted 1:5 in PBS/BSA (Pharmingen). Following incubation for 30 min in the dark, cells were washed with PBS. Cells were then

resuspended in 0.4 ml PBS and analysed by flow cytometry (FACSort; Becton-Dickinson, San Jose, CA, USA) (8).

Quantification of oxidative activity in neutrophils

Neutrophils were defined by positive GR-1 staining and were gated (positive signal in the fl-2 flow cytometer channel). The proportion of neutrophils in BAL fluid was used as a measure of the extent of inflammation in the lung. Those neutrophils which were activated and which produced free oxygen radicals were stained green by the probe and could be detected in the fl-1 channel. Through analysis of the GR-1 positive cells in a histogram, changes in the median fluorescence of the neutrophils could be investigated.

Statistical analysis

Results are presented as means and standard deviations. Statistical significance was assessed using analysis of variance (ANOVA) with the Bonferroni ’post-hoc’ test if more than two groups were compared. The null hypothesis was rejected if $p<0.05$ (double-sided). Groups containing less than 3 values were not statistically analysed.

Results

Development of a protocol for administration of probes

To enable method development the starting point was initiation of alveolar inflammation in mice through aerosol exposure to LPS. Staining of cells was accomplished *in vivo* via intravenous or intraperitoneal injection of the probe about 19 h after exposure. The oxidative activity of neutrophils from blood and BAL fluid was then investigated. Due to the dilution effects of the probe associated with these systemic routes of administration, detectable concentrations in the target organ of inflammation, the airways, were not obtained. Neither was there any measurable oxidative activity in blood neutrophils during experimentation. As *in vitro* staining with the same probe demonstrated that blood neutrophils had a very low oxidative activity following LPS aerosol exposure, this implies that the induced lung inflammation did not induce any significant cellular activation in the peripheral blood. However, in a previous study *in vitro* we have reported that following inhalation of LPS the oxidative activity in neutrophils in BAL fluid is strongly enhanced (7). Taken together, these results indicate that the probe must be introduced into the target organ of inflammation so that a sufficient quantity is taken up by activated neutrophils. In a first experiment this was achieved by injection of both LPS and the probe intraperitoneally 3.5 h apart. This protocol induced peritoneal inflammation and a significant staining of neutrophils (figure 3). Through exposure of mice to LPS aerosols and subsequent instillation of the probe into the lungs, the method was adapted to enable studies of alveolar neutrophils.

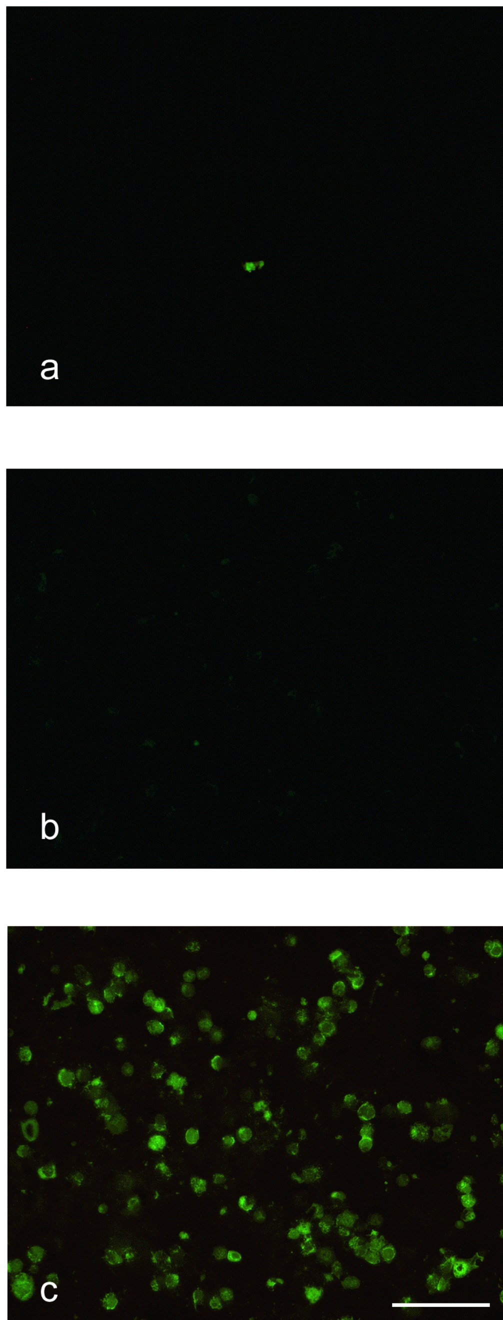

Figure 3. Oxidative activity of peritoneal neutrophils following i.p. stimulation with LPS (40 µg/mouse), depicted as green fluorescence after staining with the probe H_2DCFDA (200 µg/mouse) for 30 min *in vivo*. The oxidative activity was visualised by fluorescence microscopy. The figures represent peritoneal neutrophils treated with **(a)** saline and PBS, **(b)** saline and H_2DCFDA, or **(c)** LPS and H_2DCFDA. Bar = 50µm.

In order to study cellular oxidative activity by flow cytometry the cells were stained with a PE-conjugated antibody (GR-1) specific for neutrophils. The positively staining neutrophils were detected by flow cytometry analysis in fl-2. Cells in an activated state producing reactive oxygen species, including free radicals, were stained green when the probe was oxidised. This fluorescence was detected in the fl-1 flow cytometer channel and quantified through measurement of the median fluorescence of the neutrophil population (figure 4).

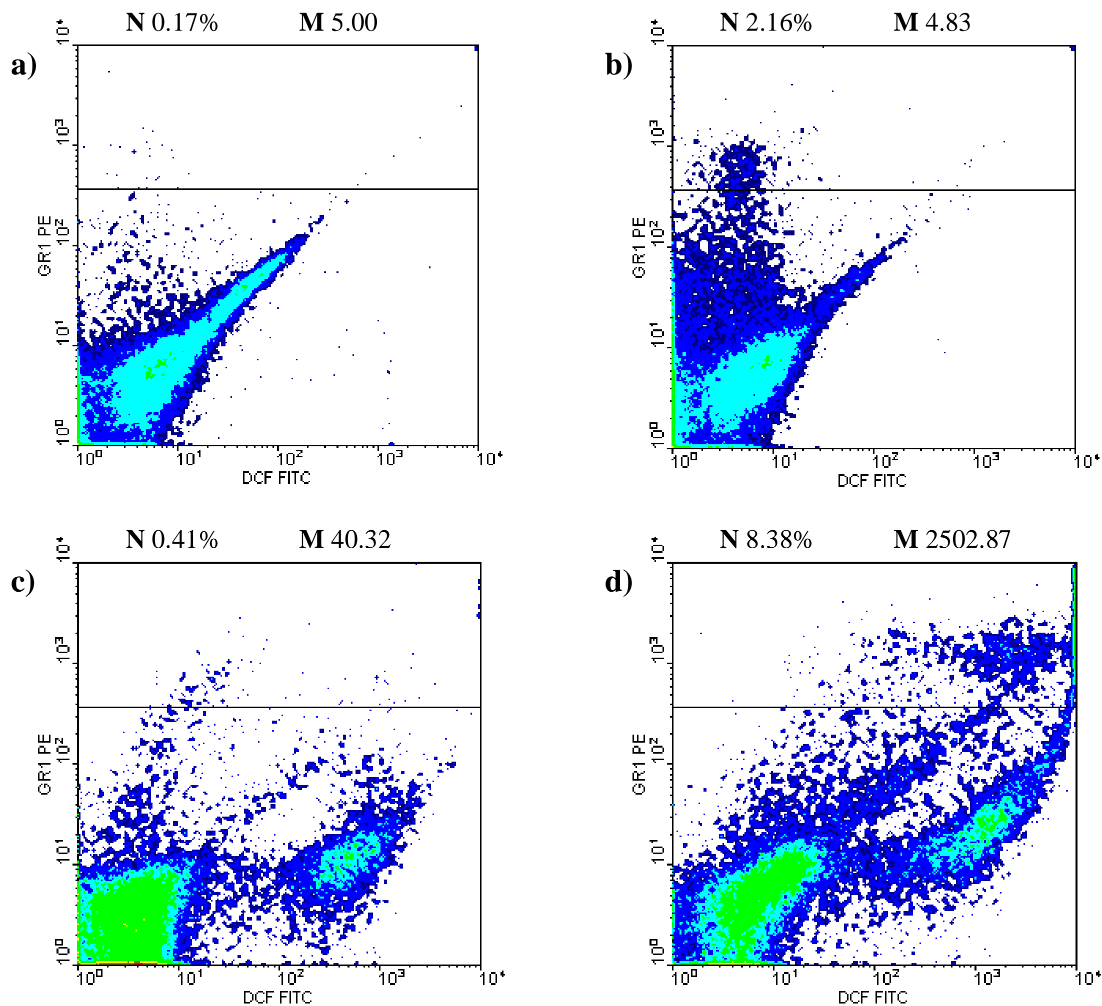

Figure 4. FACS analysis of the oxidative activity in peritoneal neutrophils (GR-1$^+$ cells) following stimulation by LPS (40 µg/mouse) and staining with H_2DCFDA (200 µg/mouse). The dot plots depict cells from **(a)** untreated mice, **(b)** control mice injected with saline and PBS, **(c)** mice injected with saline and H_2DCFDA, or **(d)** mice injected with LPS and H_2DCFDA. (Neutrophils were detected in the fl-2 channel and are expressed as % of the total number of leukocytes **(N)** in the sample. The cellular oxidative activity is expressed as median fluorescence **(M)** in the fl-1 channel).

Analysis of oxidative activity in peritoneal neutrophils

Analysis of peritoneal cells from untreated mice revealed few neutrophils in the peritoneum in the normal state (figure 4a). However, the saline injections in control mice caused some influx of neutrophils into this compartment (figure 4b), which indicates that the injection *per se* caused a limited inflammatory response. However, the probe that was used did not induce any further inflammation in the control mice (figure 4c). An increased influx of neutrophils was recorded following exposure to LPS (figure 4d). The cells activated by LPS showed increased oxidation of the probe and thus increased fluorescence. Enhanced oxidative activity following LPS exposure *in vivo* could also be detected in other peritoneal cells, which were most probably activated macrophages.

Peritoneal inflammation was induced by injection of 400 µl LPS solution that was titrated to a concentration of 0.1 mg/ml (40 µg LPS/mouse). Thereafter, 50 µl probe solution with a concentration of 0.4 mg H_2DCFDA/ml (200 µg/mouse) was injected. Intraperitoneal injection of LPS causes an influx of leukocytes into the peritoneum, of which about 10% are

neutrophils (figure 5a). The number of neutrophils obtained following peritoneal lavage decreased after staining with the probe (figure 5b), although the changes in cell number could not be confirmed statistically ($p>0.05$). Even injection with physiological saline induced minor inflammation. However, these neutrophils were not statistically significantly ($p<0.001$) activated to the same extent as those exposed to LPS (figure 5c).

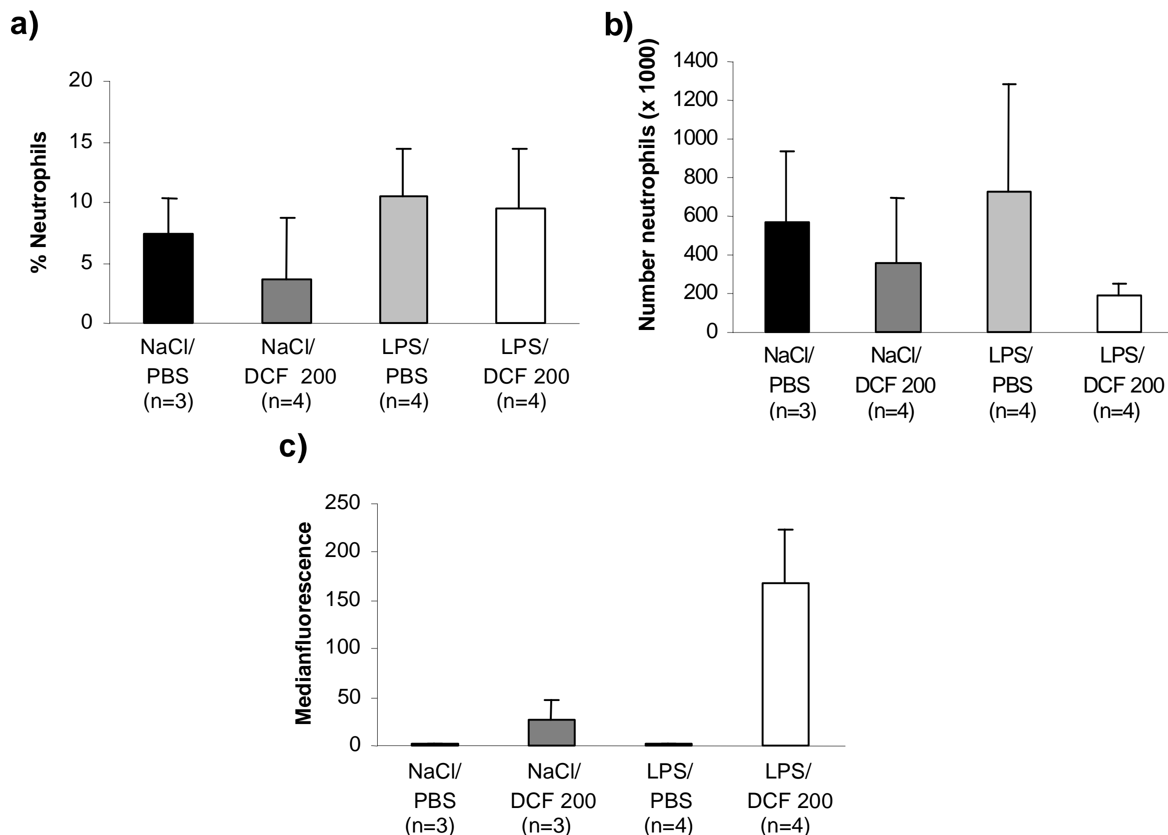

Figure 5. Peritoneal inflammation, 4 h after injection of 40 µg LPS (i.p.), represented as percentage neutrophils (GR-1^+ cells) of the total peritoneal leukocytes **(a)**, total number of neutrophils in the peritoneum **(b)**, and production of free radicals in neutrophils **(c)** (depicted as median fluorescence in the flow cytometer fl-1 channel after *in vivo s*taining with 200 µg/mouse of the probe H_2DCFDA). All parameters were analysed by flow cytometry. Control animals were injected with physiological saline (NaCl) instead of LPS with either PBS or probe. Significant increases in median fluorescence were recorded after LPS treatment and staining with the probe compared to control groups without LPS or probe, respectively ($p<0.001$).

Analysis of oxidative activity in broncheoalveolar neutrophils

Inhalation of LPS aerosols gave rise to an acute inflammatory reaction characterised by a dramatic influx of neutrophils into the lungs. The cells congregated in the airways and about 19 h after exposure there was a maximal inflammatory reaction (8). The proportion of neutrophils in the lung leukocyte population was 80 %, in contrast to the untreated animals in which the proportion was less than 5 % (figure 6a). The probe was instilled intratracheally in order to study activation of the neutrophils during airway inflammation. 100 µl of the probe H_2DCFDA was instilled into the lungs of anaesthetised mice (20 µg/mouse) at a time of maximal inflammatory reaction and 30 min later cells were recovered from the lungs by broncheoalveolar lavage. Neutrophils in BAL fluid were stained with the GR-1 antibody and analysed for conversion of the probe to its fluorescent form. The chosen dose of probe did not induce any inflammatory reaction in the lungs *per se*, but a reduction in the number of

neutrophils obtained in the BAL fluid was observed (figure 6b). The oxidative activity detected in the neutrophils was restricted to those cells exposed to LPS and stained with H_2DCFDA (figure 6c). The median fluorescence of these cells was more than ten times higher than that of the control group.

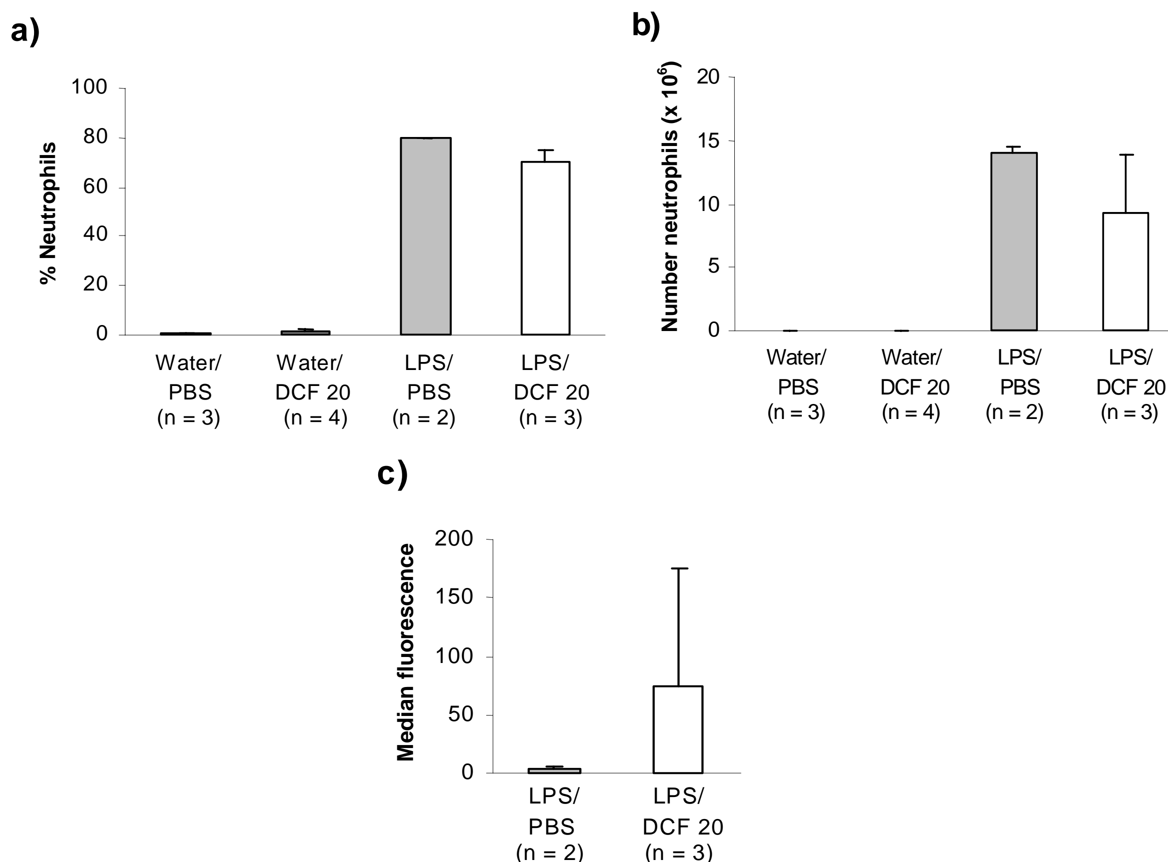

Figure 6. The influx of GR-1^+ neutrophils into the lungs, approximately 19 h after aerosol exposure to 1 mg/ml LPS, depicted as **(a)** proportion of neutrophils (% of total lung leukocytes), or as **(b)** total number of neutrophils in BAL fluid. Cellular oxidative activity is given as median fluorescence **(c)** in the flow cytometer fl-1 channel following tracheal instillation of the probe H_2DCFDA (20 µg/mouse). All parameters were analysed by flow cytometry. Control animals were exposed to endotoxin-free water instead of LPS, or PBS instead of the probe. LPS exposure resulted in a significant increase of both the proportion and number of neutrophils in the lungs ($p<0.001$).

Discussion

The results demonstrate that it is possible to stain neutrophils with a probe *in vivo* in order to study intracellular oxidative activity during inflammation. To obtain a clear neutrophil staining but at the same time avoid dilution effects the probe must be administered directly into the target organ of inflammation. This was achieved in the first experiment through intraperitoneal injection of both LPS and probe. The method was modified to enable study of alveolar neutrophils through LPS aerosol exposure and instillation of the probe directly into the lungs.

The optimal concentration of probe was determined by titration for both peritoneal and tracheal staining, although the results indicate that these concentrations are still somewhat

toxic. Cells treated with the probe tended to exhibit membrane changes and the number of neutrophils in BAL or peritoneal wash was reduced.

Using *in vitro* staining protocols it is impossible to completely recreate the normal environment for cells, as they are isolated and resuspended in artificial media on ice. The advantage of *in viv*o staining is that it occurs without significant alteration of the normal cellular conditions. Despite this, the *in vivo* method has certain limitations as at present the probe must be introduced directly into the target organ, since it is difficult to prevent its nonspecific uptake by tissues. Such studies are also currently limited to organs that are easily accessible to administration of the probe.

Analyses of the oxidative activity in specific neutrophils are most easily performed using flow cytometry (9). Study of activated neutrophils using fluorescence microscopy requires definite identification of the fluorescing cells. It would also be desirable to be able to study oxidative activity directly in tissue sections and to thus localise the precise areas of activity within the inflammatory focus. However, fluorescent microscopical studies of lung tissues are complicated by the high background fluorescence evident in the tissues. Combination of staining with a neutrophil-specific antibody and the probe should be a possible solution to such problems associated with this type of analysis.

There is a good potential to further improve the method. In addition to studies of tissue-specific activity, future applications would include studies of the effects of antioxidants as well as of the oxidative activity of other organs, such as the brain.

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