was due to a hyperactive uterus. Marx *et al.*[12] showed that both halothane and enflurane 0,5 MAC (MAC = minimum alveolar anaesthetic concentration producing immobility in 50% of subjects exposed to a noxious stimulus) impaired spontaneous uterine activity in post-partum patients. Suppression of oxytocin response, however, was only evident at concentrations above MAC (halothane MAC = 0,76%; enflurane MAC = 1,68% in 100% oxygen).

Low concentrations of a volatile anaesthetic agent will ensure maternal anaesthesia during caesarean section. Convincing evidence that partial or complete reversal of intra-uterine fetal biochemical asphyxia could also result from this practice, would further justify regular use of the anaesthetic vapours in obstetric anaesthetic practice.

Unfortunately, this study provided only tentative evidence of enflurane's efficacy in improving placental supply to the fetus. Moreover, the intermittent analysis of the fetal scalp blood gas/acid-base components is only relevant at the moment the sample is taken and does not necessarily reflect continuous change.[13] However, the recent introduction of continuous recording fetal scalp [H+] and blood gas electrodes offer new and exciting prospects for continued investigation of our hypothesis.[8,13]

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The Effect of Paraquat on the Aerobic Metabolism of Rabbit Alveolar Macrophages and Lung Fibroblasts

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SUMMARY

In this study the effects of paraquat on the aerobic metabolism and viability of isolated rabbit alveolar macrophages and lung fibroblasts were investigated, and compared with the effects of other known metabolic inhibitors, i.e. sodium fluoride (NaF) and potassium cyanide (KCN).

The manometrically and polarographically determined endogenous oxygen consumption of lavaged alveolar macrophages compared very well (180,9 ± 35,8 and 169,3 ± 26,8 nmol per 10^6 viable cells per hour respectively). Exogenous glucose (10 mM) and autologous serum (1:3 v/v) added to the medium had no significant effect on the basal respiration rate. The mean cell protein content, determined by the micro-Kjeldahl and Lowry techniques, amounted to 242,6 ± 37,6 μg/10^6 macrophages.

Paraquat (2 mM), like NaF (20 mM) and KCN (5 mM), decreased the viability of the macrophages far less than it did the oxygen utilization of the viable cells, and resulted in an 80% inhibition of oxygen uptake. In contrast, paraquat (1 mM) induced a marked stimulation (230%) of the cyanide-insensitive respiration of alveolar macrophages.

The concentrations of paraquat (nmol/10^3 cells) which reduce macrophage metabolism to almost zero were virtually non-toxic to fibroblasts, as measured by their oxygen consumption.

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Previous *in vitro* investigations done in this laboratory showed that paraquat interferes with the aerobic metabolism of lung tissue and subcellular fractions.[1,2] The lung consists of many different cell types, and the pattern of oxygen or substrate utilization by the whole lung may mask critical differences in the oxygen/substrate requirements of specific cells.

After oral or parenteral administration, paraquat is apparently retained by the lung, which leads to non-specific pulmonary lesions,[3] and an increase in the number of alveolar macrophages as well as fibroblastic prolifera-

tion.[4] The fibrosis may not be simply the result of damage to alveolar cells, since this does not occur after exposure to paraquat aerosols.[4]

The lung lesion produced by paraquat involves both alveolar macrophages and fibroblasts. Styles[5] investigated the effect of the bipyridylium herbicides on the viability of cultured rat macrophages and fibroblasts and found irreversible damage to macrophages with concentrations of paraquat that did not reduce the viability of fibroblasts.

The purpose of this study was therefore to compare the effects of paraquat on the metabolism of alveolar macrophages and fibroblasts, and to relate these observations to the known effects of paraquat on whole lung tissue.

MATERIALS AND METHODS

The technique used for procuring alveolar macrophages was a modification of that described by Myrvik et al.[6] and Brain and Frank.[7]

New Zealand White rabbits weighing 2,0 - 2,5 kg were anaesthetized with a sublethal dose of thiopentone sodium through the marginal ear vein. The thoracic cavity was opened and the trachea was dissected free and fitted with an intratracheal cannula. A catheter was inserted in the pulmonary artery and the lungs were perfused in situ with 50 ml of ice-cold 154 mM saline. The left atrium was excised to minimize outflow obstruction and pulmonary oedema. The lungs, heart and trachea were dissected out, whereafter the heart was carefully removed, avoiding any injury to the lungs or bronchi.

Hereafter, the lungs were transferred to a stainless-steel bucket buried in ice and containing isotonic saline. Then 50 ml saline was instilled through the intratracheal cannula over a period of 1 minute, left in the lungs for another minute, and withdrawn from the lungs during the third minute. The first wash was discarded owing to contamination with red blood cells, leucocytes and mucus/surfactant material. The second and subsequent six tracheo-bronchial washings were performed as follows: the same 50 ml of saline which was instilled primarily into the lungs was withdrawn and infused for four cycles, and then stored on ice. The cells were pelleted by centrifuging in a precooled bench centrifuge (Model IHS) at 500 g, the supernatant was decanted and the pellets resuspended in a Krebs-Ringer phosphate (KRP) buffer — pH 7,4. Total cell counts were made with a Spencer Bright-Line haemocytometer on each washing, and differential cell counts of smears stained with May-Gruenwald-Giemsa stain were performed.

Lung fibroblasts were prepared from lungs of fetuses delivered by caesarean section 2 days before full term. The fetal lungs were minced with a sterile razor blade in a laminar flow bench (Afronix). The pieces of lung tissue were incubated at room temperature in a trypsinizing flask containing a filter-sterilized enzyme mixture (0,1% collagenase and 0,2% hyaluronidase; Sigma Chemical Co.) in sterile phosphate-buffered saline (PBS) — pH 7,4, with continuous stirring on a magnetic stirrer. The supernatant was decanted every 20 minutes and replaced with

fresh enzyme solutions. The enzyme activity was stopped by the addition of 1 mM ethyleneglycol tetra-acetic acid (EGTA), and after 1 hour the cells in the pooled supernatants were washed three times with sterile PBS. Total counts were done as for macrophages, and the cells were resuspended in a predetermined volume of culture medium. Cells were then seeded at equal concentrations in 100-mm plastic culture dishes (Falcon Plastics) containing 7,5 ml of medium.

The minimum essential medium (phosphate-buffered) was used, supplemented with 10% fetal calf serum and penicillin/streptomycin at final concentrations of 50 U/50 μg/ml. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air (Hotpack CO_2 incubator). After 12 hours non-adherent cells were removed and fresh medium was added. Thereafter the medium was replaced every 3 days. Within 5 - 10 days the culture dishes contained a complete monolayer of typical fibroblasts. After trypsinization (0,125 g in 25 ml PBS) the free cells were collected in PBS, washed and resuspended in KRP buffer for metabolic experiments.

The oxygen consumption of alveolar macrophages and fibroblasts was determined manometrically (Warburg) and polarographically (Oxygraph) as previously described.[1,2] The viability of cells before and after Oxygraph procedures was measured in nanomoles O_2 per 10^6 viable cells per hour. Protein determinations were done by both the micro-Kjeldahl procedure[9] and the Lowry technique.[10] Paraquat dichloride was obtained from Aldrich Laboratories, Wisconsin, USA.

Standard methods were used to compute the mean and standard deviations, and pairwise comparisons (P values) were made, using a two-sided t test.

RESULTS

The mean number of alveolar macrophages lavaged from the lungs of rabbits with our technique amounted to 1,15 × 10^{8}, with a mean viability of 95 ± 3%. Differential cell counts showed that about 97% of these cells were intact alveolar macrophages.

Several basal values for alveolar macrophages from normal control rabbits are summarized in Table I. The endogenous oxygen uptake as measured manometrically and polarographically compared well, and the addition of glucose (10 mM) or autologous serum (1:3 v/v) to the suspension medium did not alter the rate of oxygen

TABLE I. BASAL VALUES FOR ALVEOLAR MACROPHAGES FROM NORMAL CONTROL RABBITS*

Parameter and technique	Mean	±1 SD
Oxygen uptake (nmol/10^6 cells/h)		
Warburg (endogenous)	180,9	±35,8
Oxygraph (endogenous)	169,3	±26,8
Oxygraph (+ 10 mM glucose)	167,6	±12,2
Oxygraph (+ 1:3 v/v serum)	174,7	±32,1
Protein content (μg/10^6 cells)		
Micro-Kjeldahl	221,6	±29,3
Lowry	263,6	±46,0

* Each value represents the mean ± 1 SD of 20 determinations.

uptake significantly. Cell protein contents, determined with the micro-Kjeldahl and Lowry techniques, are well in agreement.

Fig. 1 demonstrates the effects of an uncoupler of oxidative phosphorylation (dinitrophenol (DNP) 0,05 mM), two metabolic inhibitors (sodium fluoride (NaF) 20 mM and potassium cyanide (KCN) 5 mM), anaerobiosis (excess sodium dithionite ($Na_2S_2O_4$)) and paraquat (2 mM) on the viability and oxygen consumption of alveolar macrophages. These results indicate that the viability of the cells was not dramatically affected by these substances, and even after being under totally anaerobic conditions for 40 minutes nearly 50% of the macrophages still had the ability to exclude eosin. There is no correlation between the percentage decrease in the viability and the percentage increase or decrease of the oxygen uptake of viable cells. Paraquat, NaF and KCN reduce the oxygen consumption to values well below 40% of the control values, while DNP increases the oxygen uptake to nearly 60% above the control values.
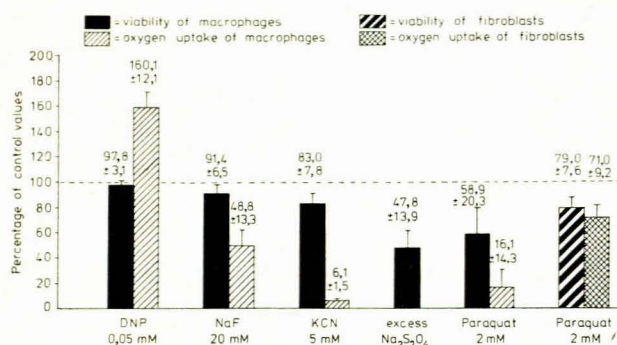

Fig. 1. Histograms to illustrate the effect of some metabolic inhibitors and paraquat on the viability and oxygen consumption of alveolar macrophages and lung fibroblasts.

With time and increasing paraquat concentrations the decrease in viability becomes more evident, and this tendency is slightly more marked in the presence of glucose as substrate (Table II). The oxygen uptake, on the other hand, is markedly depressed with time by paraquat in concentrations of 1 mM and higher. In contrast to the viability, paraquat in the absence of glucose causes a greater inhibition of oxygen uptake than when glucose is present.

The results recorded in Table III show the effect of paraquat on the cyanide-insensitive respiration of alveolar macrophages. The cyanide-insensitive respiration of control macrophages varied from 5,7% to 6,5% of the total oxygen utilization, and 1 mM and 10 mM paraquat induced a 230% and 90% increase in respiration respectively.

TABLE III. EFFECT OF 1 mM AND 10 mM PARAQUAT ON THE CYANIDE-INSENSITIVE (5 mM KCN) OXYGEN CONSUMPTION OF RABBIT ALVEOLAR MACROPHAGES*

Oxygen consumption (nmol/10^6 cells/h)

Control values	KCN 5 mM	Paraquat	
$179,4 \pm 19,1$	$10,4 \pm 3,1$	1 mM	$33,9 \pm 10,0$
$178,7 \pm 12,7$	$11,5 \pm 2,6$	10 mM	$21,8 \pm 6,1$

* Each value represents the mean ± 1 SD of determinations on 5 animals.

The fibroblasts harvested from culture had a mean viability of 85 ± 3% and a mean oxygen consumption of 381,7 ± 112,3 nmol O_2 per 10^6 viable cells per hour. When fibroblasts were exposed to paraquat concentrations of 1 nmol per 10^3 cells for 40 minutes — conditions which invariably led to almost total inhibition of the oxygen uptake of macrophages — the viable fibroblasts still had an oxygen uptake of 60 - 80% of the control values (Fig. 2).
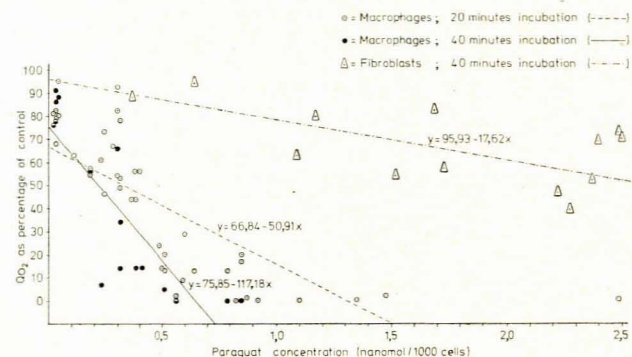

Fig. 2. A scatter diagram with regression lines to illustrate the effect of various concentrations of paraquat on the oxygen consumption (QO_2) of alveolar macrophages and cultured lung fibroblasts.

TABLE II. EFFECT OF PARAQUAT ON THE VIABILITY AND OXYGEN CONSUMPTION OF RABBIT ALVEOLAR MACROPHAGES*

Paraquat concentration (mM)	Glucose concentration (mM)	Viability (%)			Oxygen uptake (nmol/10^6 cells/h)	
		0 min	20 min	40 min	20 min	40 min
0 (control)	—	$94,8 \pm 1,1$	$93,0 \pm 1,6$	$88,4 \pm 1,8$	$197,8 \pm 31,4$	$173,5 \pm 26,7$
0 (control)	10	$95,2 \pm 0,8$	$86,4 \pm 5,2$	$84,2 \pm 6,3$	$186,3 \pm 16,6$	$148,9 \pm 20,8$
1,0	—	$94,0 \pm 1,9$	$80,2 \pm 8,6$	$65,0 \pm 19,4$	$112,4 \pm 11,1$	$56,6 \pm 36,3$
1,0	10	$94,4 \pm 1,8$	$77,0 \pm 9,5$	$56,2 \pm 23,9$	$147,9 \pm 36,2$	$86,9 \pm 48,6$
2,0	—	$95,4 \pm 1,1$	$77,2 \pm 16,2$	$56,2 \pm 32,1$	$39,9 \pm 32,2$	$6,5 \pm 10,6$
2,0	10	$95,0 \pm 0,7$	$69,4 \pm 12,1$	$50,2 \pm 22,4$	$37,4 \pm 41,0$	$12,8 \pm 15,0$

* Each value represents the mean ± 1 SD of determinations on 5 animals.

DISCUSSION

The control and baseline values used by research workers to express results of metabolic studies on lung tissue and cells differ from one laboratory to another.[11-14] These differences in oxygen consumption of macrophages may be due to species differences, type of macrophage (alveolar, peritoneal), or functional state (resting, activated, elicited), or may depend on whether the macrophages were harvested from pathogen-free animals or animals kept under routine conditions. Although most of these factors are well appreciated by research workers in this field, differences still exist between animals kept under similar environmental conditions. The majority of authors expressed their results in terms of cell protein content. Regarding the protein values of macrophages, the following are recorded in the literature: Powell and Green[11] — 104 μg/10^6 cells; York et al.[12] — 1 mg/10^6 cells; Stubbs et al.[13] — 70 μg/10^6 cells; Oren et al.[14] — 400 μg/10^6 cells.

To ascertain the validity of our findings, a large number of control experiments were performed to compare the results obtained with different techniques (Table I). The manometric and polarographic methods compared well when oxygen consumption was expressed in terms of viable cells, and the normal control values were well in accordance with results obtained by Ouchi et al.[15] and Gee et al.[16] on rabbit alveolar macrophages. As regards the methods for the estimation of cell protein, the Lowry method gave reproducible results only when the protein concentration in the reaction tube was less than 30 μg/ml or when the optical density was below 0,4 at 660 nm.

Our results regarding the effects of KCN, DNP and NaF on macrophage respiration are in agreement with those reported in the literature.[14,17,18] The effect of paraquat, KCN and NaF on the viability of alveolar macrophages indicated that enough energy must somehow be available for the exclusion of eosin. Although Styles[5] showed that paraquat and diquat reduced the viability of macrophages in tissue culture, our results indicated that the decrease in viability of cells, as measured by the dye exclusion technique, is a very insensitive parameter for measuring toxicity. On the other hand, a decrease in the oxygen uptake of cells seems to be a far more sensitive indicator of the relative toxicity of substances. When the effect of paraquat on the oxygen consumption of viable cells is compared with that of NaF and KCN, it appears as if paraquat may likewise inhibit certain enzymes. Furthermore, there exists no correlation between the percentage decrease in viability and the percentage decrease or stimulation of the oxygen uptake due to paraquat. This finding confirms earlier results that the dye exclusion test indicates damage to the cell membrane but not necessarily cell death.[5]

Paraquat in the presence of glucose has a lesser depressing effect on the oxygen uptake of alveolar macrophages than when glucose is absent. This could perhaps be due to an increase of the pentose phosphate pathway and the associated higher production of NADPH, which is oxidized by microsomes.[2] Under the prevailing experimental conditions, where paraquat reduces mitochondrial oxygen uptake to about 20% of its normal value,[2] the highly significant stimulation of the cyanide-insensitive (microsomal) respiration could well make a considerable contribution to the observed increase in oxygen uptake in the presence of glucose.[2]

Alveolar macrophages and lung fibroblasts differed in their response to paraquat. Styles[5] showed that paraquat reduces the viability of macrophages markedly more than that of cultured fibroblasts, and it is possible that this differential damaging effect may be related to differences in the membrane transport systems for paraquat. Considering that paraquat is a divalent cation that will not penetrate cell membranes readily, the higher pinocytotic activity of macrophages certainly favours an increased ingestion of paraquat. However, if this also holds true for the in vivo effect of paraquat after absorption from the blood into the lung, it may well explain the observed lung necrosis and subsequent fibrogenesis. Further research is necessary to verify these suggestions.

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