Free Rad. Res. Comms., Vol. 18, No. 4, pp. 211-223
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TEMPORAL ASSOCIATION BETWEEN PULMONARY INFLAMMATION AND ANTIOXIDANT INDUCTION FOLLOWING HYPEROXIC EXPOSURE OF THE PRETERM GUINEA PIG

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(Received November 9, 1992)

The time course and nature of the pulmonary inflammatory and antioxidant responses, both during and after hyperoxic-induced acute lung injury were studied in the preterm guinea pig. Three-day preterm (65 days gestation) guinea pigs were randomly exposed to either 21% O_2 (control) or 95% O_2 (hyperoxia) for 72 hours. All pups were then maintained in ambient conditions for up to a further 11 days, during which time lung damage was monitored. In animals exposed to hyperoxia, evidence of acute lung injury and inflammation was characterized by a marked increase in microvascular permeability and elevated numbers of neutrophils in bronchoalveolar lavage fluid. Protein concentration, elastase-like activity and elastase-inhibitory capacity in lavage fluid were at a maximum at the end of the 72 hours hyperoxic exposure. Four days later, all values had returned to control levels. In contrast, increased numbers of neutrophils, macrophages and lymphocytes were recovered in the lavage fluid during this early recovery period. Coinciding with the influx of inflammatory cells, there was a significant increase in glutathione peroxidase, manganese superoxide dismutase and catalase activities in immature lung. Lung copper/zinc superoxide dismutase activity remained unchanged during both experimental periods. The strong temporal relationship between the influx of inflammatory cells to the lung and the induction of pulmonary antioxidant enzyme defences suggests that a common mechanism underlies both responses. These findings have led us to regard inflammation in the hyperoxic-injured immature lung as a beneficial event and not, as previously suggested, as part of the injurious process.

KEY WORDS: Pulmonary oedema, superoxide dismutase, glutathione peroxidase, catalase, interleukin 1, tumour necrosis factor, elastase activity.

ABBREVIATIONS

BAL	bronchoalveolar lavage
CAT	catalase
CLD	chronic lung disease
Cu/Zn-SOD	copper/zinc superoxide dismutase
EDTA	ethylenediaminetetra-acetate
EIC	elastase inhibitory capacity
ELA	elastase-like activity
GSH-Px	glutathione peroxidase
IL-1	interleukin 1
Mn-SOD	manganese superoxide dismutase
O_2	oxygen
PMSF	phenylmethylesulphonyl fluoride
PPE	porcine pancreatic elastase
RDS	respiratory distress syndrome
SLAPN	succinyl-1-trianyl-p-nitroanilide
TNF	tumour necrosis factor

INTRODUCTION

While the precise pathogenesis of respiratory disease of the preterm neonate remains unresolved, many reports emphasize the importance of inflammatory events.[1-3] The neutrophil is the predominant cell type found in tracheal aspirates obtained from neonatal infants during the first few days of life, whereas macrophages are more abundant during recovery from the respiratory distress syndrome (RDS). The continued presence of neutrophils in samples from infants with RDS has been suggested as a predictor of the subsequent development of chronic lung disease (CLD).[1-3]

Inflamatory cells contain potent proteolytic enzymes, have considerable free radical generating capacity and are widely assumed to contribute to lung injury in preterm neonates.[1-4] In contrast to several studies which supported such a view,[5,6] more recent investigations by a number of groups including our own[7-10] have no evidence to support such a role for inflammatory cells in acute oxidative-induced lung injury. Using a preterm guinea pig model we have established that free elastase is absent from bronchoalveolar fluid, even when there is a marked influx of neutrophils to the pulmonary parenchyma and airpsace's following the hyperoxic exposure.[9] In addition we found that even when circulating neutrophils were depleted by 90% by the use of a specific antiserum the extent of the oxidative lung injury was unaltered.[10]

As a result of these observations and as the immune system is usually considered to be a protective mechanism during periods of stress, we now hypothesize that the inflamatory response associated with hyperoxic exposure may be of benefit to the immature lung. In the present study we have addressed this possibility by investigating the movement of inflammatory cells into the lung, both during the development of, and recovery from, oxidative lung injury. At the same time we have determined the nature and time course of the protective pulmonary antioxidant enzyme response that occurs after the exposure of preterm guinea pigs to a sub-lethal oxidative stress.[11]

MATERIALS AND METHODS

Reagents

All reagents were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) unless otherwise stated.

Animals

Guinea pigs pups were delivered by caesarean section at day 65 of gestation (normal gestation is 68 days) under halothane anaesthesia (2–4%) maintained with nitrous oxide (0.4 l/min) and oxygen 1.5 l/min). After delivery, the oropharynx was cleared and the umbilical cord double clamped and cut, and the mother sacrificed by exsanguination. The pups were dried in a stream of warm air, weighed and placed with a lactating dam into 25 l perspex chambers containing ample food, water and hay. Cages were changed daily, the pups spending no more than two minutes out of the chamber. A full account of these procedures is given in Kelly *et al.*[9]

Experimental Protocols

Pups were randomly allocated to hyperoxic (95% O_2) or control (21% O_2) exposure for 72 h. As adult animals are more susceptible than pups to hyperoxia, the dams in the 95% O_2 cages were changed daily to avoid lethal oxygen toxicity. At the end of the 72 h period, all animals were transferred to large open cages and maintained in ambient conditions for up to 14 days. Subgroups (n = 8–10) of animals were removed at random for study at 72 h (3 days), 5 days, 7 days and 14 days after delivery.

Bronchoalveolar Lavage (BAL)

Bronchoalveolar lavage and determination of BAL total and differential leucocyte counts were performed as described previously.[11] BAL supernatant was divided into 0.5 ml aliquots and frozen at −70°C for subsequent analysis of total protein, elastase-like activity (ELA) and elastase inhibitory capacity (EIC). The lungs were then dissected from the thoracic cavity, washed in saline, blotted dry and frozen at −70°C for subsequent antioxidant analysis.

Protein Concentration

Total protein was measured in the unconcentrated lavage fluid using the bicinchoninic acid assay.[12] Absorbance was measured at 562 nm with a Dynatech MR 850 reader using bovine serum albumin standard. Results are expressed as mg of protein per ml of lavage recovered.

Elastase-like Activity (ELA)

The total ELA of BAL fluid was assessed using the synthetic substrate, succinyl-1-trianyl-p-nitroanilide (SLAPN).[13] ELA was determined in triplicate against a range of active-site-titrated standards of porcine pancreatic elastase (PPE; 0.72–2.16 ng/ml) as described elsewhere.[14] BAL fluid or standard (50 μl) were combined with 100 μl of buffer (0.2 M Tris-HCl, pH 8.0) and preincubated in individual wells of a 96-well microtitre plate at 37°C for 15 min. Prewarmed SALPN (50 μl; 2 mM in buffer) was then added to each well and the plate covered with aluminium foil and incubated at 37°C. the absorbance at 410 nm was determined at 2 h, 4 h, 20 h and 24 h with a Dynatech MR 850 plate reader. Linear regression analysis was performed on the standards, which were linear over the 24 h period, and a plot of change in absorbance per hour against PPE concentration was obtained. Similar rates were determined for BAL samples and the ELA calculated from the standard plot. The data are expressed as pmol of PPE equivalents/ml of BAL fluid. ELA was also characterized by the inhibition of activity following the addition of either 10 mM EDTA or 10 mM PMSF.

Elastase Inhibitory Capacity (EIC)

The capacity of BAL fluid to inhibit the degradation of the synthetic substrate SLAPN by exogenously administered PPE was determined as a measure of the lavage fluid's EIC. Briefly, 100 μl of BAL was serially diluted ten times with buffer (0.2 M Tris-HCl, pH 8.0) in a microtitre plate. Active-site-titrated PPE (50 μl; 11.5 ng/ml) was added

to each well and the plates preincubated for 15 min at 37°C. SLAPN (50 μl; 2 mM) was then added and the incubation continued for a further 30 min. Absorbance at 410 nm was recorded after 30 min and the remaining PPE activity, expressed as a percentage, was calculated from a standard containing only enzyme, buffer and substrate. This activity was then plotted against volume of BAL and the linear portion of the curve was extrapolated to the amount of lavage fluid required to inhibit completely the added PPE. This method of EIC determination is applicable even in the presence of alpha-2-macroglobulin.[15]

Tissue Preparation

Frozen tissue samples (200 mg) and antioxidant enzyme analysis (copper/zinc superoxide dismutase; manganese superoxide dismutase, catalase and glutathione peroxidase) were carried out as described previously.[11]

Data Analysis

The significance of differences between survival curves were assessed using the log rank test. Differences between experimental groups were analysed using a two-tailed Mann-Whitney-U test for non parametric data. A probability value of less than 0.05 was considered significant.

RESULTS

Survival. All preterm animals delivered at 65 days' gestation exhibited a variable degree of respiratory distress, with tachypnoea which persisted for up to 12 h after delivery. Those animals exposed to 95% O_2 developed further distress by 24 h, including cyanosis, tachypnoea, rib retraction and, in severe cases, pulmonary oedema. Overall survival rates at 72 h were 87% (n = 46) in the 21% O_2 and 68% (n = 59) in the 95% O_2 group, (log rank test $p < 0.01$). There were 2 deaths in each group amongst those animals that survived beyond 72 h.

Growth. Mean (SD) body weights at delivery were 78.9 (10.7)g and 85.6 (8.9)g in the 21% O_2 and 95% O_2 exposed groups, respectively. Both groups had lost 16–17% body weight by day 5 (21% O_2) or 7 (95% O_2). By day 14, body weight had increased to 147.6 (26.5)g in the 21% O_2 exposed group and to 145.3 (18.7)g in the 95% O_2 exposed group. There were no statistical differences between the body weights of the two groups at any of the time points studied.

BAL inflammatory cells. The total leucocyte count in BAL fluid from control animals remained low and relatively constant throughout the 14-day period (Figure 1a). In the 95% O_2 exposed group there was no significant difference in the total number of inflammatory cells recovered by lavage during the period of exposure. After return to ambient conditions, a significant increase was seen at the day 5 and day 7 time points (Figure 1a). The differential cell counts of control and hyperoxia-exposed pups are shown in Figure 1b–e. In control animals, the change in the cellular profile during the 14-day study period was similar to the normal maturational changes seen in term animals.[16] This included a gradual increase in the numbers of macrophages (Figure 1c) and a concomitant fall in the number of eosinophils (Figure 1b). Numbers of neutrophils (Figure 1d) and lymphocytes (Figure 1e) remained low throughout the

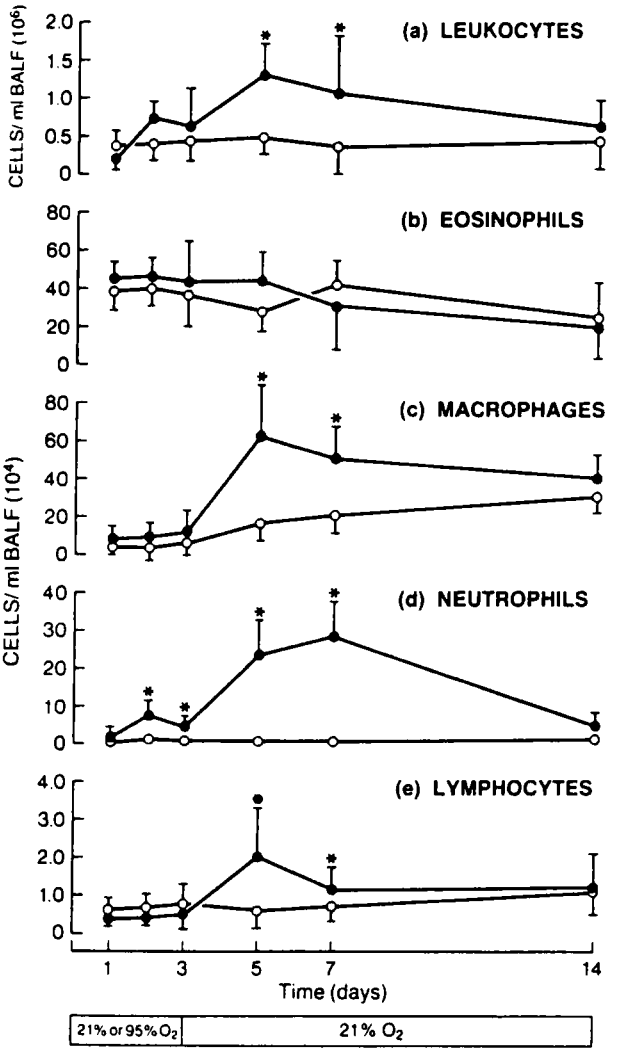

Figure 1 Inflammatory cell values in BAL from 3-day preterm guinea pig pups exposed to either 21% O_2 (○——○) or 95% O_2 (●——●) for 72 h and then maintained in ambient conditions. Each point represents the mean and standard deviation for 8–10 animals per group. *$p < 0.05$; 21% vs 95% O_2.

study period in the control animals. Those animals exposed to 95% O_2 showed a significant increase in the number of neutrophils recovered in the lavage fluid during the exposure period (Figure 1d). On cessation of the oxidative insult, or during the early recovery period, two important changes were observed in the BAL fluid. First, the number of neutrophils continued to increase for at least 4 days post-exposure (Figure 1d) and second, the numbers of macrophages and lymphocytes increased significantly (Figure 1c, 1e). Numbers of all inflammatory cell classes had returned to control levels by day 14.

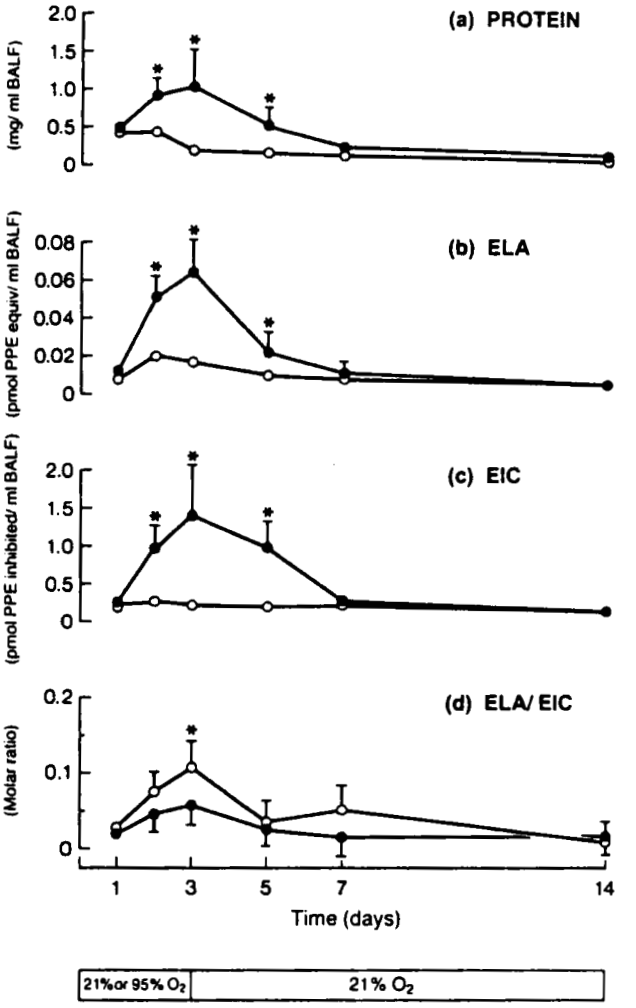

Figure 2 (a) Total protein concentration, (b) total elastase activity (ELA) and (c) total elastase inhibitory capacity (EIC) in BAL from 3-day preterm guinea pig pups exposed to either 21% O_2 (○——○) or 95% O_2 (●——●) for 72 h and then maintained in ambient conditions. Each point represents the mean and the bars the standard deviation for 8–10 animals per group. *$p<0.05$; 21% vs 95% O_2.

Changes in pulmonary microvascular permeability. BAL fluid protein was used as an index of lung permeability and hence of tissue injury. We have previously shown in this model that acute lung injury is followed by an influx of plasma proteins, primarily albumin, to the airways.[9] In the present study, the concentration of BAL fluid protein in the control group remained low throughout the study period (Figure 2a). In the hyperoxia-exposed group, total protein concentration in BAL fluid increased significantly during the period of oxygen-exposure but, in contrast to the inflammatory cell changes, levels returned to normal 4 days after the removal of the oxidative stress (Figure 2a).

BAL fluid ELA and EIC. ELA in the BAL supernatant followed a similar pattern to that of the total protein, with significantly elevated activities in the 95% O_2 exposed group at days 2, 3 and 5, but returned to control levels by day 7 (Figure 2b). At all time points considered, approximately 90% of the ELA was EDTA sesitive (metalloelastase) and therefore likely to be of macrophage origin.[2] The EIC of the BAL fluid also followed a similar pattern, with significantly elevated capacities at days 2, 3, and 5, falling to control levels by day 7 (Figure 2c). Comparisons of the ELA with the EIC indicated a substantial imbalance in favour of EIC at all time points considered (Figure 2d).

Lung antioxidant enzyme activities. These are shown in Figure 3a–d. With the exception of GSH-Px at 72 h, lung antioxidant activities were similar in control and hyperoxia-exposed pups during the period of exposure (Figure 3a–d). During the early recovery period, that is days 5 and 7, Mn-SOD, CAT and GSH-Px activities all increased significantly ($p < 0.05$) with respect to control animals. By day 14, these differences were no longer evident (Figure 3 a,c,d). Cu/Zn-SOD activities were similar in control and hyperoxic-treated pups throughout the 14-day period (Figure 3b).

DISCUSSION

The protocol for this study was designed so that the kinetics of both inflammatory cell infiltration to, and antioxidant induction in, the immature lung, during both acute hyperoxic stress and the subsequent recovery period might be investigated. We have previously reported that exposing 3-day preterm guinea pig pups to 95% O_2 is associated with acute inflammatory lung injury that is maximal and often lethal at 96 h.[9] In the present study the duration of the exposure to oxygen was reduced to 72 h and the animals were then returned to ambient conditions where recovery took place. During this phase, from 3 to 14 days of age, deaths were few although animals from the 95% O_2 group often displayed respiratory distress following the reduction in oxygen concentration. Generally, however, the pups had recovered within 36 h of this change in pO_2 and thereafter appeared clinically well and gained weight normally.

In the past few years it has become apparent that the cytokines IL-1 and TNF, which are released from inflammatory cells such as the macrophage, can induce antioxidant defences.[17–20] Antioxidant induction forms the basis of increased tolerance of oxidative stress and reduces the extent of lung injury after hyperoxic exposure.[11,21,22] Since we have recently shown that antioxidants are induced in the immature lung of the preterm guinea pig and since we were unable to identify a detrimental role for the pulmonary inflamatory response associated with hyperoxic exposure, we hypothesized that the antioxidant induction may be regulated by the inflammatory response.

With the exception of GSH-Px activity at 72 h, no major changes in antioxidant defences were apparent during the period of hyperoxic stress when the numbers of inflammatory cells were low in BAL fluid. During the early recovery period, antioxidant activities (with the exception of Cu/Zn-SOD) increased, coinciding with increased numbers of inflammatory cells in the lung. The marked influx of neutrophils, macrophages and lymphocytes to the lung after hyperoxic exposure was one of the most intriguing observations of the present study. It has previously been shown that BAL chemoattractant activity for neutrophils increases 10-fold after 66 h of hyperoxic

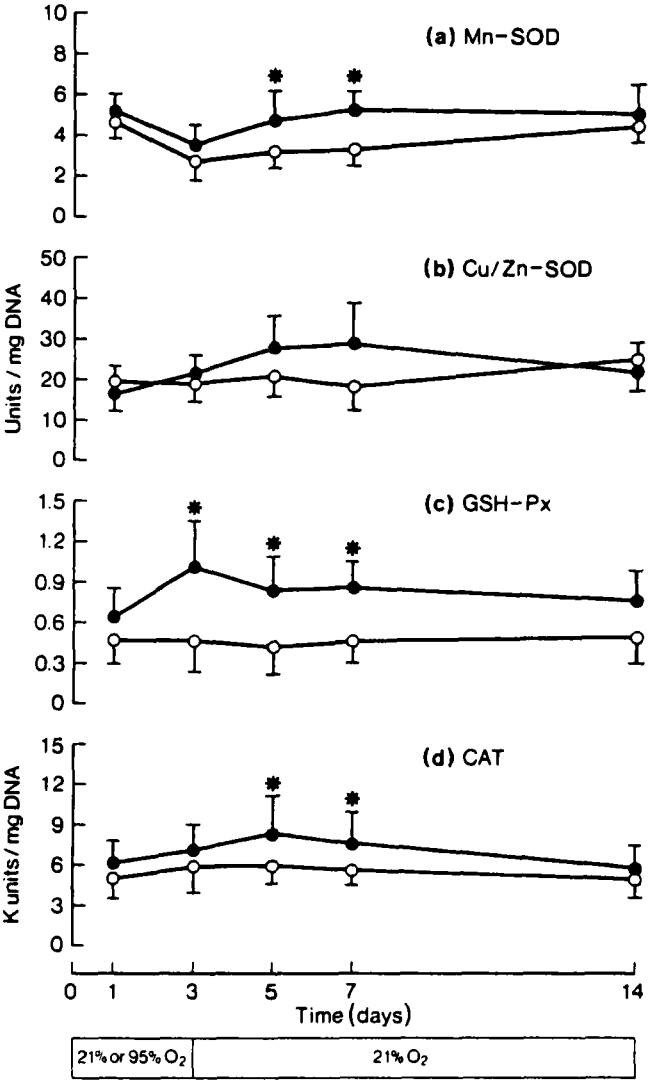

Figure 3 Lung antioxidant activities in 3-day pretrm guinea pig pups exposed to either 21% O_2 (○——○) or 95% O_2 (●——●) for 72 h and then maintained in ambient conditions. Each point represents the mean and the bars the standard deviation for 8–10 animals per group. *$p < 0.05$; 21% vs 95% O_2.

exposure in rats.[5] Due to the relatively short half-life of most known chemoattractants, it would appear that in the present study the cessation of exposure to oxygen did not immediately reduce the generation of chemoattractants, since neutrophils, macrophages and lymphocytes continued to accumulate in the alveoli for at least 4 days after the return to 21% oxygen.

The accumulation of macrophages after the cessation of oxygen treatment is a phenomenon noted in other animal models and in human infants with RDS.[1,23]

Although, in the preterm monkey, there was no increase in the number of macrophages during the acute phase of oxygen exposure, a marked increase occurred during the recovery stages.[23] These data have been taken to suggest that macrophages are not essential to the development of acute lung injury since their influx followed that of neutrophils. The precise role of the macrophage in this process has not been clarified but an immuno-modulator role has been proposed whereby the macrophage may down-regulate the inflammatory process and phagocytose neutrophils and debris.[24,25] Other studies have, alternatively, suggested that the macrophage may contribute directly to the lung injury through the release of pro-inflammatory mediators or through the generation of free radicals,[26] a role that has also been proposed for the neutrophil.[24] To our knowledge the present study is the first to assign an antioxidant-inducing role to the macrophage during hyperoxia-induced pulmonary inflammation.

As it is not presently possible directly to determine concentration of IL-1 and TNFα in guinea pig BAL fluid, we were unable to assess whether a direct relationship existed between macrophage influx and antioxidant enzyme induction. However, indirect evidence of macrophage activation was apparent from the increases in BAL fluid ELA, which were primarily of metalloelastase and hence of macrophage origin, during both the acute lung injury (day 2) and recovery (day 5) phases. We have previously shown that concentrations of protein and albumin in BAL fluid are elevated progressively after 24 h of exposure to 95% O_2, reflecting injury of pulmonary endothelial and epithelial cells.[27] In this study, concentrations of protein in BAL fluid were maximal at 72 h and fell progressively thereafter. The resolution of lung injury coincided with both the cessation of hyperoxic exposure and the time at which pulmonary antioxidant enzyme defences were increasing. The subsequent infiltration of neutrophils, which continued for at least a further 4 days did not result in further perturbation of the endothelial barrier. These results can be interpreted to suggest either that neutrophils attracted to the immature lung during hyperoxic exposure do not contribute to pulmonary injury or alternatively that neutrophils recruited during the recovery phase are not activated and hence do not participate in further lung injury.

In summary, we propose that the influx of inflammatory cells to the hyperoxic-injured lung reflects a protective response leading to antioxidant-induction and hence to the provision of protection against further oxidative stress. This mechanism is probably orchestrated through the release of the inflammatory cytokines TNF and IL-1 from macrophages attracted to the injured tissue. If correct, this will have important implications for the design of future treatments of neonatal lung disease which should therefore attempt to encourage this process.

Acknowledgements

This study was supported by grants from the Wellcome Trust and the Medical Research Council.

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Accepted by Professor B. Halliwell