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THE EFFECTS OF IRON AND DESFERRIOXAMINE ON THE LUNG INJURY INDUCED BY INTRAVENOUS BLEOMYCIN AND HYPEROXIA

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The development of acute lung injury in rats following the intravenous injection of bleomycin was assessed by measuring the total pulmonary extravascular albumin space. Intraveous bleomycin alone produced no evidence of lung injury, yet when combined with a simultaneous exposure to hyperoxia or simultaneous tracheal instillation of ferric iron or ascorbate a severe lung injury evolved. Neither ferric iron or ascorbate alone produced lung injury when assessed in this manner, and ferrous iron, ferritin and haemoglobin did not potentiate bleomycin induced lung injury.

A continuous subcutaneous infusion of desferrioxamine enhanced hyperoxia induced lung injury, and had no modulating effect on the lung injury produced by combined intravenous bleomycin and hyperoxia. These results indicate that ferric iron can potentiate bleomycin induced lung injury, and that the metal chelator desferrioxamine can have adverse effects on the development of acute lung injury.

KEY WORDS: Bleomycin, iron, desferrioxamine, lung, pneumonitis.

INTRODUCTION

Bleomycin is an important chemotherapeutic drug derived from *Streptomyces verticillus*.[1] Its major adverse effect is the unpredictable development of lung injury in the form of a pneumonitis which is frequently fatal.[2-4] This feature has led to the extensive use of bleomycin in animal models of pulmonary fibrosis where the drug is usually instilled directly into the lungs via the trachea. The subsequent influx of inflammatory cells, accumulation of collagen (see 5 & 6 for reviews), and changes in the extravascular albumin space[7] are well documented. However, the exact mechanism by which bleomycin induces lung injury is less clear.[8] *In vitro* studies suggest that bleomycin acts by forming an active species after complexing with iron and oxygen.[9] This activated form of bleomycin is highly unstable and degrades spontaneously,[10] but will cause severe damage to any available DNA. The availability of iron and oxygen within the lung may therefore be an important factor in the development of pulmonary toxicity. We have previously shown that *in vivo* hyperoxia markedly potentiates lung injury following intravenous bleomycin.[11] We have here attempted to alter the amount of iron available within the lungs of rats at the time of an intravenous injection of bleomycin by instilling directly into their lungs iron in its ferrous or ferric form, and also when complexed to haemoglobin or ferritin. We have also examined

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the effects of the iron chelator desferrioxamine administered by continuous subcutaneous infusion on the lung injury induced by combined intravenous bleomycin and hyperoxia. Pulmonary injury was assessed by measuring the total pulmonary extravascular albumin space at 72 hours.[11]

MATERIALS AND METHODS

Laboratory Animals

Experiments were performed on male Lewis rats 175–225 g. in weight. They were allowed food and water *ad. libitum*. Before every procedure, including intravenous injections, the animals were anaesthetised with intramuscular fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml (Hypnorm) at a dose of 0.05 ml/100 g body weight.

Experimental protocol

Iron instillation experiments. Each experiment comprised a group of 12 animals, 6 receiving an intravenous injection of 5 mg bleomycin (Lundbeck, U.K.) dissolved in 0.5 ml sterile saline, and 6 receiving saline only. Immediately following the injection of saline or bleomycin, each group received one of the following by peroral tracheal instillation; the total volume instilled was always 0.3 ml, sterile water being used as the carrier.

Group 1, no tracheal instillation control; Group 2, normal saline instillation control; Group 3, ferrous ammonium sulphate; Group 4, ferric chloride; Group 5, rat haemoglobin; Group 6, equine ferritin; Group 7, ascorbic acid. The rat haemoglobin or equine ferritin were dialysed before use against water containing chelex. All chemicals were obtained from Sigma U.K., and the quantity of each substance was calculated to obtain 800 n.mol of iron to be administered to each animal. 170 μ.mol of ascorbic acid was administered as the free acid.

Iron chelation experiments. Animals received an intravenous injection of 5 mg bleomycin or received saline as controls, and were then exposed to an atmosphere of 90% oxygen for 48 hours. This has previously been shown to induce severe lung injury in the bleomycin group and very minor injury in the saline control group.[11] Half of the animals in addition received desferrioxamine administered at a dose of 100 mg/day by continuous subcutaneous infusion via 'Alzet osmotic mini pumps' (Alza, Palo Alto, USA.). This commenced 24 hours prior to receiving bleomycin or saline intravenously, and was supplemented with an intramuscular injection of 75 mg at the time of mini-pump insertion and at the time of bleomycin (or saline) injection. The remaining half were sham operated and received saline via mini pumps in the same manner. A further control group of animals received saline intravenously, desferrioxamine as before, and remained in room air.

Assessment of lung injury by measurement of total pulmonary extravascular albumin space

The total pulmonary extravascular albumin space (TPEAS), derived from the work of Wangensteen *et al.*,[7] was measured as follows:[11] All animals were killed 72 hours

following receiving intravenous bleomycin or saline. Twenty four hours before this they received an intravenous injection of 2 micro curies of ^{125}I human albumin (Amersham International). Animals were exsanguinated by aspiration of blood from the aorta after receiving 500 units of mucous heparin intravenously. The lungs were then flushed free of blood by perfusing 12 ml of phosphate buffered saline through the pulmonary vasculature by ligating a canular in the right ventricle, excised and placed in a counting vial. Duplicate plasma samples from each animal were also counted in a gamma counter. The total pulmonary extravascular albumin space was calculated as the ratio of the radioactivity in the whole lungs to that in 1 ml of plasma. Statistical analysis was performed with the Mann-Whitney U test.

RESULTS

The extent of lung damage as assessed by the total pulmonary extravascular albumin space following the tracheal instillation of iron containing substances simultaneous with intravenous saline or bleomycin is shown in Figure 1. None of the iron containing solutions or ascorbic acid produced a change in TPEAS when administered with intravenous saline. Bleomycin in combination with instilled saline, or alone, resulted in no change in TPEAS when compared to intravenous saline alone. However, ferric chloride or ascorbic acid in combination with intravenous bleomycin produced a significant ($p < 0.002$) increase in TPEAS. In contrast, ferrous ammonium sulphate and dialysed haemoglobin or ferritin produced no significant changes.

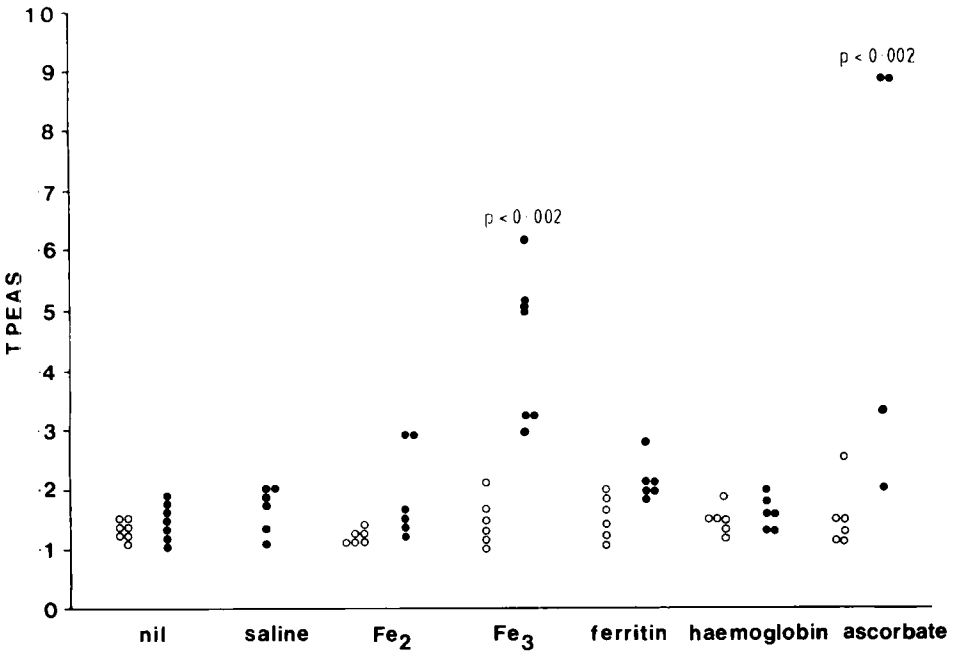

FIGURE 1 The effects of the combination of intravenous saline (open circles) or bleomycin (closed circles) with the simultaneous tracheal instillation of iron containing compounds on the development of acute lung injury as assessed by the total pulmonary extravascular albumin space at 72 hours.

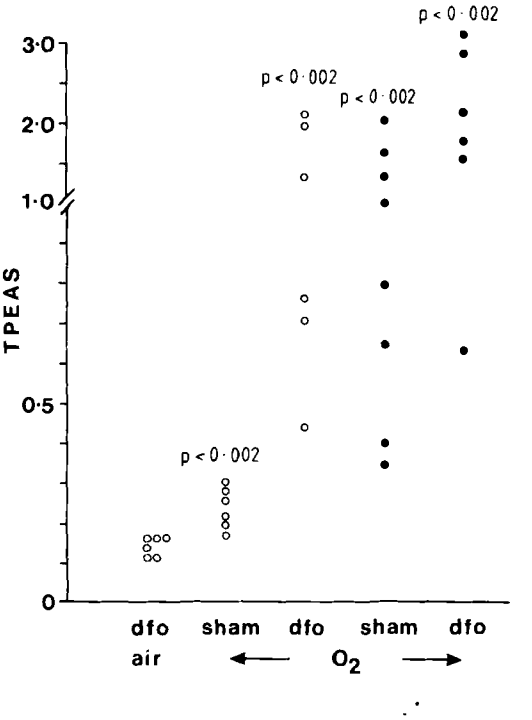

FIGURE 2 The effect of a continuous subcutaneous infusion of desferrioxamine (dfo) or sham operation on the acute lung injury induced by an atmosphere of 90% oxygen for 48 hours in combination with intravenous saline (open circles) or intravenous bleomycin (closed circles). Lung injury is assessed by measurement of the total pulmonary extravascular albumin space at 72 hours.

The effects of continuous subcutaneous desferrioxamine on hyperoxia induced and combined hyperoxia plus bleomycin induced lung injury is shown in Figure 2. TPEAS values for animals receiving saline and desferrioxamine are not different from animals receiving saline alone (the latter group shown in Figure 1). Hyperoxia plus sham operation results in a small but significant lung injury ($p < 0.002$). A marked and significant ($p < 0.002$) increase in TPEAS is shown for animals exposed to hyperoxia in combination with desferrioxamine alone, bleomycin alone, or bleomycin and desferrioxamine together when compared to the group exposed to hyperoxia and sham operated. Eight animals died before the end of the experiment in the hyperoxia plus desferrioxamine group, and 3 in the bleomycin plus desferrioxamine and hyperoxia group.

DISCUSSION

This study has shown that the instillation of ferric iron into the lungs is able to potentiate lung injury. This potentiation is also seen with ascorbic acid, but not with dialysed solutions of ferritin and haemoglobin or ferrous iron.

The role of iron in bleomycin induced lung injury has not been studied previously *in vivo*. There have, however, been *in vitro* studies of the mechanism whereby bleo-

mycin exerts its cytotoxicity. Bleomycin is known to avidly bind ferrous iron to become activated,[9] but in the absence of DNA spontaneously degrades to an inactive form.[10] When complexed with ferrous iron in the presence of DNA, bleomycin is able to release free radicals in the form of superoxide and hydroxyl radicals[12] and subsequently damage DNA. The importance of these radicals is not clear, as radical scavengers are not able to protect DNA in these experiments, but the iron chelator desferrioxamine is.[13]

The potentiation of lung injury in these experiments by ferric iron may not only result from the enhanced formation of active species of bleomycin, iron is also able to directly increase lipid peroxidation[14] and potentiate the production of oxidant species from netrophils.[15] These later two mechanisms are thought to be important in the potentiation of cobra venom factor pulmonary injury in rats seen with simultaneous intravenous injections of ferric but not ferrous iron.[16] The reason why ferrous iron does not have a similar enhancing effect in both of these models is not clear. It is possible that the ferrous iron in our experiment had bound bleomycin and become inactivated before reaching a cellular location, the ferrous-bleomycin complex being a highly unstable species.

Our original experiments suggested that ferritin and haemoglobin might similarly be able to enhance lung injury following intravenous bleomycin. This effect was not seen when the solutions were dialysed before use, and was probably due to contaminating iron in the preparations used. The potentiation seen with ascorbic acid may result from increased extraction of iron from carrier proteins in the lung, ascorbic acid as well as being a reducing agent is known to aid the release of iron from carrier proteins. The lung has a plentiful supply of reducing agents[17] which may have a detrimental effect in the presence of excess iron. Iron is known to be diverted to parenchymal locations in chronic illness and malignancy,[18] in rheumatoid arthritis iron accumulates in the synovium and the taking of oral iron compounds[19] or the adminstration of iron-dextran intravenously[20] can enhance joint inflammation. If this process of iron redistribution to parenchymal locations were to operate in the lung, it may partly explain the variable pulmonary toxicity of bleomycin.

In vitro studies demonstrate that the activation of bleomycin is absolutely dependant on the formation of a complex with iron. We have demonstrated in this paper that the instillation of ferric iron into the lungs enhances the pulmonary toxicity of bleomycin. It might therefore be expected that iron chelators would diminish pulmonary toxicity. There have been three previous studies which have examined the effects of desferrioxamine on the pulmonary fibrosis produced by intratracheal bleomycin, but the results have been conflicting.[21-23] In only one of the studies was the desferrioxamine administered continuously, and there have been no studies examining the effects of desferrioxamine on the acute lung injury produced by bleomycin. Any given agent may have quite different effects on the development of acute lung injury and the evolution of fibrosis. In this study desferrioxamine was administered as a continuous infusion which is the most effective means of delivery. Desferrioxamine provided no protection against the pulmonary injury induced by the combination of bleomycin and hyperoxia. A marked potentiation of the small degree of lung injury seen in the saline controls exposed to hyperoxia was seen. The most probable explanation for the failure of desferrioxamine to inhibit lung injury in the combined bleomycin and hyperoxia group, would be the failure of desferrioxamine to influence intracellular levels of iron as it does not cross membranes into cells. It is most probable that bleomycin exerts its action on intracellular DNA. The mechanism by which desfer-

rioxamine enhances oxygen toxicity is unclear. It may have a direct toxic effect on pulmonary cells which is only seen when they are also injured by other means, e.g. hyperoxia, perhaps by chelating other essential metals required for repair and regeneration.

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