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Pulmonary Catabolism of Interleukin 6 Evaluated by Lung Perfusion of Normal and Smoker Rats

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Abstract

Cytokines such as interleukin 6 are involved in the pulmonary inflammation arising as a result of smoking. By use of isolated and perfused lung preparations we have evaluated the role of the lungs in the catabolism of human recombinant interleukin 6 both in normal rats and in rats subjected to an acute cigarette smoking episode.

When interleukin 6 was incorporated into the lung perfusion medium, neither control nor smoke-exposed rat lungs cleared the cytokine and only $0{\cdot}1 \pm 0{\cdot}2\%$ of the total dose was recovered in the bronchoalveolar lavage fluid. When, on the other hand, the same amount of interleukin 6 was instilled into the bronchoalveolar tree, concentrations of the cytokine in the perfusate increased progressively so that after 3 h up to $70{\cdot}1 \pm 9{\cdot}8\%$ and $40{\cdot}9 \pm 22{\cdot}5\%$ of the administered dose, as measured by immunoenzymatic test, had been transferred from the bronchial lumen to the perfusion medium of either control or smoker rat lungs, respectively, indicating significantly ($P \leqslant 0{\cdot}05$) different behaviour of the cytokine in the two experimental groups. Total recoveries of the administered interleukin 6 evaluated in smoke-exposed rat lungs were $55{\cdot}3 \pm 23{\cdot}2\%$, significantly lower than those for control rat lungs ($83{\cdot}9 \pm 11\%$). Determination of biological activity gave values always lower than those measured by immunoenzymatic test, indicating loss of biological activity during the transalveolar transit.

It appears that the transfer of interleukin 6, especially in smokers, is almost exclusively unidirectional, from the alveolar space to the plasmatic pool with degradation during the transalveolar passage.

The combustion of tobacco, generating an aerosol containing more than 10^9 particles mL^{-1} of cigarette smoke (McCusker 1992) in a gaseous medium consisting of more than 4000 chemical constituents such as polynuclear aromatic hydrocarbons and tobacco-specific nitrosamines (Roberts 1988), has been associated with inflammatory processes (Robbins et al 1990). The development of pulmonary inflammation is known to involve cellular events controlled by a variety of cytokines. These mediate interactions between macrophages, lymphocytes and granulocytes and are important in the regulation of airway inflammation (Kelley 1990). Interleukin 6 (IL-6), released after acute inflammation (Ohzato et al 1993), is a glycoprotein product of T-cells, fibroblasts, monocytes and other cells (Van Snick 1990) which can induce in the liver the production of the acute-phase proteins including C-reactive protein, serum amyloid-A, α_1-antichymotrypsin, and fibrinogen and can down regulate albumin synthesis (Gauldie et al 1987). IL-6 is often produced in association with IL-1 and both can synergize in regulating target-cell function (Kelley 1990). IL-6 increases transvascular fluid and serum protein flux in lesions and endothelial permeability in-vitro, moreover (Maruo et al 1992), and appears to have a significant role in mediating the various parameters of wasting (Strassmann et al 1993).

The disappearance of IL-6 from plasma is generally very rapid (Castell et al 1988; Mulé et al 1990) and the liver or the skin, or both, seem to be major organs responsible for the clearance (Castell et al 1990; Nesbitt & Fuller 1992), although the sequence of events is controversial. Very little is known about the role of the lungs in the catabolism of IL-6. We have already reported that interferon (IFN) α and tumour necrosis factor (TNF) α underwent little catabolism while circulating through perfused rabbit lungs (Bocci et al 1984; Pessina et al 1987) but that when both were instilled into the bronchi they underwent partial inactivation during transalveolar absorption (Bocci et al 1984; Pessina et al 1995b).

The aim of this work was to evaluate the effective role of the lungs in the catabolism of IL-6 by using isolated and perfused rat lung preparations. Because smoke constituents alter vascular permeability (Alving et al 1992) we also evaluated the metabolic behaviour of IL-6 either administered in the perfusion medium or instilled into the bronchi of normal and smoker rats.

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Materials and Methods

Materials

Recombinant, human, glycosylated IL-6 (Sigosix) was a kind gift from Ares-Serono Group-proteins (Geneva, Switzerland); the endotoxin content was < 2 EU (mg protein)$^{-1}$ and the specific activity was $1{\cdot}5 \times 10^7$ int units (mg protein)$^{-1}$.

ELISA tests for human recombinant IL-6 were performed using a commercial enzyme-linked immunosorbent assay (IL-6-EASIA, Medgenix Diagnostics, Belgium), which showed no cross-reaction with other animal species.

The Lymulus amoebocyte lysate gel test was used to prove all media were negative for endotoxin contamination.

Animals

The experiments were approved by the local ethics committee. Outbred Wistar male rats (Charles River), 300 g, were used throughout the experiments. Before perfusion, smoker rats were lodged in a smoke apparatus (Pessina et al 1993a) for 1 h,

during which time the animals breathed air containing the smoke of three cigarettes without filter. Immediately after the smoking session, the rats were anaesthetized with an intramuscular injection of Nembutal (Serva, Heidelberg) and prepared for laparotomy after withdrawing 1 mL of blood from the femoral vein for the determination of carboxyhaemoglobin.

Control animals (air-sham-exposed rats) were subjected to the same procedure except that the chamber was insufflated with air only.

Isolation and perfusion of rat lungs

After anaesthesia, a tracheotomy was performed and a polythene cannula was introduced into the trachea about 2·5 cm above the bronchial bifurcation, the animal was laparatomized, and 3 mL of heparinized (100 int. units mL^{-1}) saline was injected into the vena cava. A midline sternotomy was performed, the thorax was then opened and saline-filled catheters were advanced through incisions at the base of the right and left ventricles until the tips were positioned in the pulmonary artery and left atrium, respectively. The catheters were sutured in place, the lungs were removed and placed in the perfusion system previously described (Bocci et al 1984). The lungs were ventilated with a humidified gas mixture of 95% air and 5% CO_2 by use of a Harvard rodent respirator, the settings of which were: tidal volume, 3 mL; respiratory rate, 40 min^{-1}; and positive end expiratory pressure of 2 cm H_2O. The lungs were perfused using a roller pump (Watson-Marlow, England) with 150 mL sterile RPMI 1640 medium containing 6% human serum albumin (for therapeutic use) at a constant flow of 0·05 mL g^{-1} body weight min^{-1}. Mean pulmonary artery inflow pressure was measured by a pressure transducer (C. F. Palmer, England) via thin catheters connected to the cannula close to the lungs and maintained at 12 ± 1 cm H_2O. The pulmonary venous outflow drained into a heated water bag reservoir (37·5°C) and was recirculated after being aerated with a mixture of air and CO_2 (95% : 5%) to achieve and maintain a pH of 7·4 and an oxygen tension of approximately 100 torr.

Administration of IL-6

After an appropriate period of equilibration, in the first series of experiments, 100 μL containing 15 μg of human recombinant IL-6 were added to the perfusate; in the second series of experiments the same amounts of IL-6 were instilled directly into the left and right bronchi (7·5 μg each). Direct instillation into the bronchi was preferred to aerosol administration because of the impossibility of ascertaining the exact amount administered in an aerosol. Samples of perfusate were taken at predetermined intervals, were centrifuged (2000 *g* for 10 min at 4°C) and were stored at −80°C until IL-6 determination.

At the end of perfusion, the lungs were removed, weighed and a bronchoalveolar lavage was performed with heparinized sterile Hanks solution. Five washes of 5 mL each were performed in rapid succession and the bronchoalveolar lavage fluids were pooled. Recovery was about 90%. Samples were centrifuged and supernatant was stored at −80°C. Sediment was resuspended in a small volume of Hanks solution and cells were counted in a Bürker chamber.

IL-6 determination

A commercial enzyme-linked immunosorbent assay was used for determination of IL-6. All samples were diluted at least 1 : 2 with the appropriate diluent. Automatic 3-cycle washing was routinely performed. The reliability and precision of the assay was assessed by adding cytokine standards to negative samples, in the absence or presence of human albumin. Yields ranged between 90 and 108%. Results were expressed as pg mL^{-1}. To compare IL-6 antigen present in our samples with biological activity, B9 cell proliferation assay (van Oers et al 1988) was also performed, using the IL-6 dependent murine plasmocytoma cell line B9 kindly provided by Dr L. Aarden, Amsterdam, The Netherlands (Aarden et al 1987).

Statistical analysis

Results were expressed either as ng mL^{-1} or as percentage of the administered dose and reported as mean ± s.d. Statistical evaluation of experimental data was performed using two-tailed Student's *t*-test with $P \leqslant 0.05$ as the minimum level of significance.

Results

Carboxyhaemoglobin levels were assessed as a reliable indicator of the biological effects of cigarette smoking. Normally in non-smoker rats the average basal carboxyhaemoglobin level was -1 ± 1.1%; after the smoking period this increased to 11.6 ± 1.3%.

Fig. 1 shows the averaged results, evaluated by ELISA test, of several experiments during which human recombinant IL-6 was introduced into the perfusate of the lungs. With lungs of either control (middle) or smoker (bottom) rats inserted in the system, IL-6 concentrations remained practically constant in the perfusion medium, indicating the absence of IL-6 degradation. Only 0.12 ± 0.15% of the administered dose was recovered in the bronchoalveolar lavage fluid of both control and smoker lungs, without any difference between the two groups. When IL-6 was added to the perfusion medium and recirculated under the same conditions as above, without the lungs inserted in the system (Fig.1,top), kinetic curves remained constant for the 3 h of perfusion and recovery was complete, indicating that the molecule was not absorbed by the perfusion apparatus. Evaluation of the biological activity of the same samples gave kinetic curves practically overlapping the previous experimental data. The results were markedly different when IL-6 was instilled directly into the bronchial alveolar tree and its appearance in the perfusate was checked during the perfusion period. As shown in Fig. 2 (top), IL-6 concentration, measured by ELISA test, increased rapidly and progressively in the perfusate of lungs both of control and smoker rats until 70.1 ± 9.8% and 40.9 ± 22.5%, respectively, of the administered dose was transferred from the bronchi to the circulating compartment at the end of perfusion. About 14% of the administered dose was, moreover, recovered in the bronchoalveolar lavage fluid of both control and smoke-exposed rat lungs although, in the smoker group, total IL-6 recoveries at the end of perfusion were always significantly lower than those measured in controls (Table 1). When IL-6 biological activity was evaluated (Fig.2,bottom), only 49 ± 21% and 16.9 ± 13.3%, respectively, of the instilled dose was transferred from the bronchial lumen into the perfusate of lungs of either control or smoker rats after 3 h. In such a case the amount of IL-6 recovered in the bronchoalveolar lavage

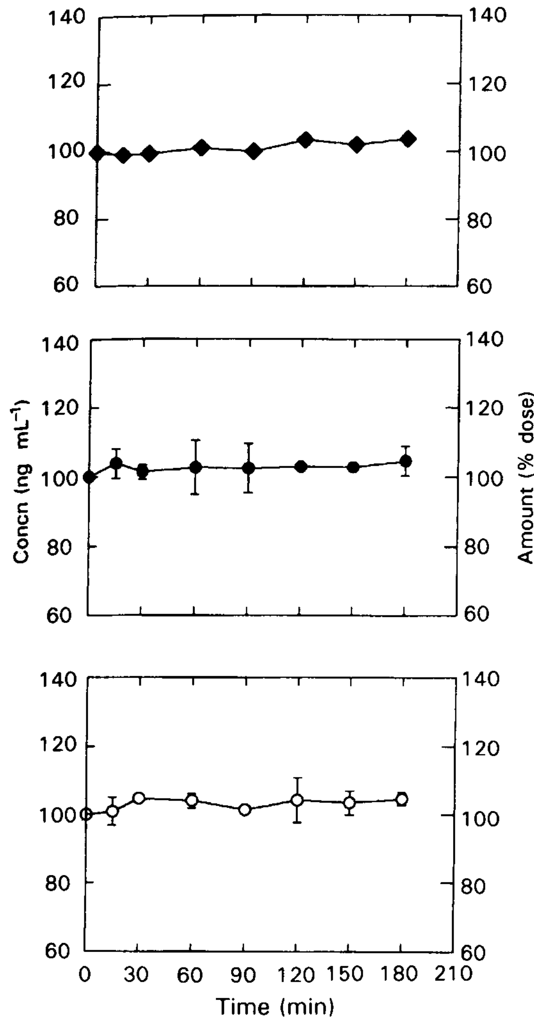

FIG. 1. Kinetics of perfusate levels observed after direct incorporation of IL-6 in the medium perfusing isolated rat lungs: ◆ no lungs inserted in the system; ● control rats; ○ smoker rats. (n = 3).

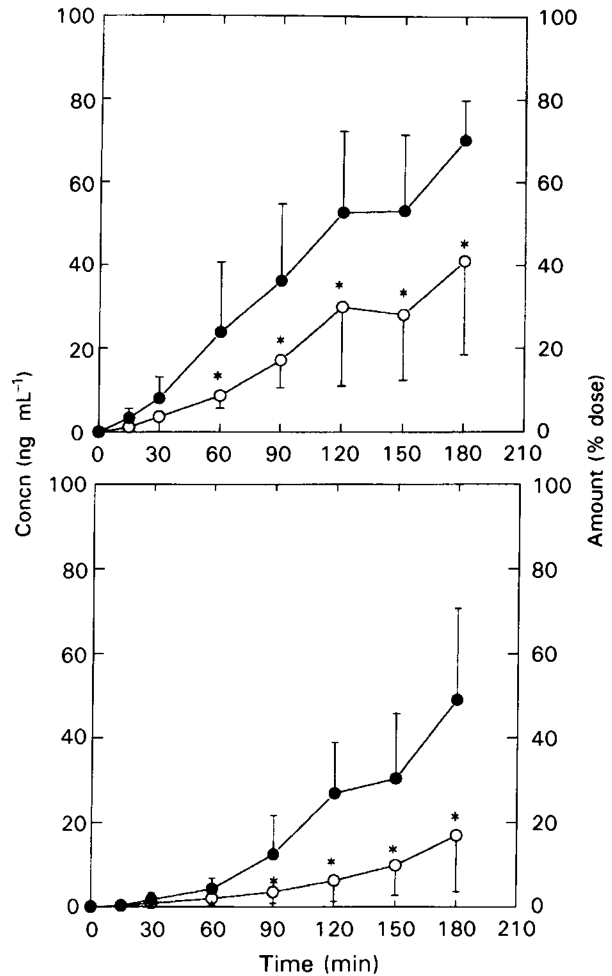

FIG. 2. Kinetics of appearance of IL-6, measured both by immunoassay (top) and by biological activity assay (bottom), in medium perfusing isolated rat lungs after direct instillation into the bronchi: ● control rats; ○ smoker rats (n = 6). $*P \leqslant 0.05$ compared with control values.

fluid was between 6 and 9% only. Total recoveries were, moreover, significantly lower for the smoker group (23·8 ± 18·3%) than for controls (58·8 ± 24%) and both values were also significantly lower than those obtained by immunoassay (Table 1).

Discussion

Although the half-life of intravenously administered human recombinant IL-6 in rats is about 3 min (Castell et al 1988), our results clearly indicate that the lungs are not responsible for such rapid clearance. In fact, after administration of IL-6 in the medium perfusing the lungs of both control and smoker rats its concentration in the perfusate remained constant during the whole perfusion period. This agrees well with the study of Castell et al (1990) which indicated that the lung distribution of IL-6 in the rat, after intravenous administration, was negligible. It is interesting to note that lungs, despite having one of the largest endothelial surfaces of the body, consisting of about

Table 1. Recovery of IL-6 (%) after 180 min of lung perfusion of control and smoker rats after administration into the bronchoalveolar tree.

	Activity tested	Perfusate samples	Bronchoalveolar lavage fluid	Total
Controls	Biological activity	49 ± 21[a]	9·7 ± 4·1	58·8 ± 24[a]
	Immunoassay	70·1 ± 9·8	13·9 ± 1·8	83·9 ± 11
Smokers	Biological activity	16·9 ± 13·3[a,b]	6·8 ± 5·4	23·8 ± 18·3[a,b]
	Immunoassay	40·9 ± 22·5[a]	14·5 ± 0·7	55·3 ± 23·2[a]

[a]$P \leqslant 0.05$ compared with control values (n = 6). [b]$P \leqslant 0.05$ compared with immunoassay values (n = 6).

3×10^{11} capillary segments (Weibel 1963), neither show any catabolic role, nor contribute to the IL-6 flux from the blood into the alveoli, because only traces of the drug were recovered in the bronchoalveolar lavage fluid. Accordingly, Schmekel et al (1992) have found that concentrations of both urea and albumin in the bronchoalveolar lavage fluid were practically identical in smokers and non-smokers, thus discounting the possibility of increased transuding of albumin from the pulmonary capillaries in smokers. They also demonstrated that the alveolar epithelium is much less permeable to small water-soluble molecules than the capillary endothelium and, as the alveolar epithelium represents the major barrier to solute flux (Staub 1974), its increased permeability might be expected to cause increased transfer of solutes from the alveoli into the plasma. TNF-α, generated after cigarette smoking (Pessina et al 1993b,c) increases pulmonary vascular permeability (Hocking et al 1990) and represents a factor in the pathogenesis of the oedema. Nitric oxide, a component of the smoke, is, moreover, likely to have a multifaceted role in the inflammatory reaction, ranging from the enhancement of vasodilation to the formation of oedema (Staub 1974); it also mediates both cigarette-smoke-induced vasodilatory responses in the lungs (Alving et al 1992) and TNF-α cytotoxicity in endothelial cells (Estrada et al 1992). Finally, Lannan et al (1994) demonstrated that cigarette smoke and its condensates causes injury to type II alveolar epithelial cells. With this in mind we considered it of practical relevance to explore the fate of IL-6 directly administered into the bronchial lumen. The two IL-6 determination methods used here are appropriate in order to correlate IL-6 biological activity with the amount of IL-6 antigen really present in our samples. An interesting aspect of this study was to demonstrate that total IL-6 recoveries were significantly lower in the smoker group than in the control group (Table 1). Recoveries obtained after IL-6 biological activity measurements in samples of both smoker and control rats were, moreover, always lower than those measured by immunoassay, indicating that during transalveolar passage IL-6 undergoes partial proteolysis with good recovery of the antigen but with a considerable loss of biological activity. It is, in fact, well known that removal of the three carboxy-terminal amino acids dramatically affects the biological activity of the molecule (Krüttgen et al 1990). Our data agree well with our previous results obtained after bronchial instillation in rats of either rat recombinant IFN-γ (Pessina et al 1995a), or human recombinant TNF-α (Pessina et al 1995b). Clinical results are also in line with these data because IFN-α is hardly detectable in the plasma of patients after aerosol administration (Kinnula et al 1989; Maasilta et al 1991). The aerosol delivery of IFN-γ has, moreover, been investigated also in man as a means of activating alveolar macrophages and hence enable organ-specific cytokine therapy (Jaffe et al 1991). In such cases the IFN-γ serum concentrations were undetectable, because the small amount emerging in the circulation underwent rapid catabolism (Bocci 1987). After smoking, neutrophil kinetics are modified and more neutrophils are retained into the lungs (MacNee & Selby 1990). It is, indeed, known (Weitz et al 1987; Nortier et al 1991; Van Kessel et al 1991) that cigarette smoking leads to the production of several secretory products and proteinases from neutrophils; these might be responsible for the increased cleavage of IL-6 observed after instillation of the molecule into the bronchial alveolar tree of the smoker lungs. Cigarette smoking also reduces bioactive IL-6 secretion by alveolar macrophages, probably by the cosecretion of an IL-6 inhibitor (Soliman & Twigg 1992). It is unlikely that protein breakdown occurs in the alveolar fluid, owing to an excess of proteinases insufficiently blocked by inhibitors partly inactivated by oxidation, because we obtained in the bronchoalveolar lavage fluid identical recoveries of administered IL-6 after the 3-h perfusion period. Other sites of breakdown might possibly be the several cell types of the bronchial mucosa (Reid & Jones 1979), the interstitial fluid especially in the case of oedema and the endothelial cells during the cellular transit.

In conclusion, smoking, although having no effect on the plasmatic pulmonary catabolism of IL-6, appears to reduce the potential biological effects of the molecule after bronchial instillation by enhanced local catabolism and inactivation during the transalveolar transit. If this interpretation is correct, it clarifies why, after a smoking session, we have not detected either TNF-α or IFN-γ in the bronchoalveolar lavage fluid (Pessina et al 1993c).

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