

Pulmonary platelet trapping induced by bleomycin: correlation with fibrosis and involvement of the $\beta 2$ integrins

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Received for publication 17 March 1994

Accepted for publication 6 June 1994

Summary. Platelet trapping was explored during the course of bleomycin induced pulmonary fibrosis by the injection of indium-111 labelled platelets and by light and electron microscopy (EM) of the alveolar capillaries. An i.v. injection of bleomycin markedly increased the localization of labelled platelets in the lung (but not in other organs) for about 3 weeks. On day 7 after bleomycin injection, a significant increase in the number of platelets in contact with the alveolar endothelium was seen with EM. Platelet trapping was strongly correlated ($P < 0.005$) with collagen deposition when examined in mouse strains genetically susceptible (CBA, C57BL/10, BL10 A.2R), or resistant (Balb/c, BL10.D2, BL10.A), to bleomycin induced fibrosis. In addition, several treatments known to decrease bleomycin induced collagen deposition and synthesis, namely administration of antibodies against CD11a, CD11b, TNF- α and IL-1ra, also decreased platelet trapping. As evaluated by EM, anti CD11a mAb significantly decreased the number of platelets in contact with the alveolar endothelium. This study indicates that bleomycin induced pulmonary fibrosis is strongly correlated with platelet trapping and that platelets probably interact, via their CD11a, with the CD54 born by the alveolar endothelium.

Keywords: bleomycin, fibrosis, collagen, platelet, $\beta 2$ integrins, TNF- α , IL-1ra

Ross *et al.* (1974) observed that platelets contain a large amount of fibrogenic factors. This finding raised the possibility that they might play a role in fibrogenesis as observed during atherosclerosis, wound healing or pathological fibrosis (Ross *et al.* 1974). This hypothesis however received little support from subsequent investigations of the influence of thrombocytopenia on fibrogenesis. Thus, bleomycin induced pulmonary fibrosis in rat was not greatly influenced by thrombocytopenia (Evans *et al.* 1990), and we have made a similar

observation in the mouse. Since the fibrogenic factors present in platelets, such as TGF- β or PDGF, are also present in other cells, notably macrophages, platelets are no longer regarded as a major contributor to fibrogenesis (Kovacs 1991).

Recently, we became interested in a possible role for platelets in bleomycin or silica induced pulmonary fibrosis in the mouse because the administration of anti CD11a (or LFA1) or CD11b (or CR3) mAbs, which markedly decrease the lung collagen content, appeared also to decrease pulmonary platelet trapping, as seen on histological sections (Piguet *et al.* 1993b). Since the CD11/CD18 complex, also called the $\beta 2$ or 'leucocytic integrin' is expressed not only on leucocytes but also on

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platelets (McCaffery & Berridge 1986), we reasoned that the anti $\beta 2$ mAbs might decrease pulmonary fibrosis by their influence on platelet trapping.

In this work, we explored quantitatively platelet pulmonary trapping in correlation with various aspects of bleomycin induced pulmonary fibrosis.

Methods

Mice

CBA/Ca, Balb/c and C57BL/10 (B10) and H-2 recombinant mice were purchased from OLAC Ltd (Blackthorn, UK). Experiments were performed with 3–5-months-old male mice.

Bleomycin administration

Bleomycin (Lundbeck, AVS, Copenhagen, Denmark) was dissolved in saline and injected i.v. (50 U/kg).

Antibodies

Anti CD11a (LFA1); H35.89.9, a rat IgG2b (Pierres *et al.* 1982). Anti CD11b (CR3); 5C6, a rat IgG2b (Rosen & Gordon, 1987). Anti CD18; M18/2, a rat IgG2a (Sanchez-Madrid *et al.* 1983). Anti CD54 (ICAM-1); Y-N 1/1, a rat IgG2b (Prieto *et al.* 1989). Anti mouse TNF- α was derived from rabbits immunized with mouse recombinant TNF- α , as described before (Piguet *et al.* 1989). IgG fractions were prepared by protein A sepharose chromatography. For platelet studies, 200 μ g were injected i.v. 12 hours before the injection of platelets. For the treatment of fibrosis, 1000 μ g were injected every 5 days: this schedule ensured a detectable anti TNF activity within the serum of the treated mice (Piguet *et al.* 1989).

IL-1 ra

The human IL-1ra was a gift from Synergen, Boulder, Co, USA. IL-1 ra or its solvent was administered by a continuous abdominal infusion during 7 days, using an Alzet osmotic minipump (2001, Palo Alto, Ca, USA).

Microscopy

Mice were bled by opening the abdominal aorta under light ether anaesthesia. The thorax was opened and the lungs were fixed by an intratracheal instillation of glutaraldehyde (2% in 0.1M cacodylate buffer, pH 7.4) under a hydrostatic pressure of 20 cm. Material from the left lung was embedded in Epon and processed for light or electron microscopy (EM), two blocks from each

mouse being examined. About 20 pictures of the alveolar septa for each block were taken at random at a magnification of $\times 6000$. In addition, alveolar septa was examined by EM at a magnification of $\times 3600$, and the individual platelet and red blood cells within the alveolar capillaries were counted to evaluate platelet retention, 30–50 alveolar red blood cells being counted for each grid. Thin sections were examined with a Philips 400 electron microscope at 60 kV.

Distribution of ^{111}In -labelled platelets

Blood was collected, in citrate/glucose solution (ACD), from the retro-orbital plexus of isologous mice and platelets were isolated and labelled with ^{111}In as described elsewhere (ICSH Panel 1988). About 10^8 platelets, corresponding to $50\text{--}150 \times 10^3$ c.p.m., were resuspended in 0.2 ml saline and injected i.v. Mice were sacrificed, the lungs were flushed by the injection of saline into the right ventricle and excised for counting. As observed in preliminary studies, the c.p.m. per lung increased for about 3 hours after injection and subsequently decreased. Unless stated otherwise, mice were sacrificed 3 hours after the injection of labelled platelets.

Lung hydroxyproline content

This was determined according to established procedures (Thrall *et al.* 1979). In brief, the lungs were submitted to acid hydrolysis and the hydrolysates were neutralized and extracted with phenol–chloroform–isoamylalcohol to clarify the aqueous phase. Hydroxyproline concentration was determined colorimetrically (Huszar *et al.* 1980).

Northern blots of collagen $\alpha 1(I)$ mRNA

RNA was extracted from a whole lung, as described before (Piguet *et al.* 1989). Briefly, the lung was washed by the injection of saline in the right ventricle, excised, and frozen in liquid nitrogen. It was subsequently thawed and homogenized in guanidine/thiocyanate, and the total lung RNA was isolated by guanidine/caesium chloride centrifugation. RNAs, denatured with glyoxal, were separated on a 1.2% agarose gel (10 μ g/lane) and transferred onto nylon membranes. Collagen type I mRNA was detected with a 1.6 kb cDNA fragment, inserted in a pBR322 plasmid which was labelled by random priming (Genovese *et al.* 1984). This probe hybridizes with two rat $\alpha 1(I)$ mRNAs of 4.7 and 5.7 kb (Genovese *et al.* 1984). After hybridization at 43°C in 50% formamide, membranes were washed 2×20 min in

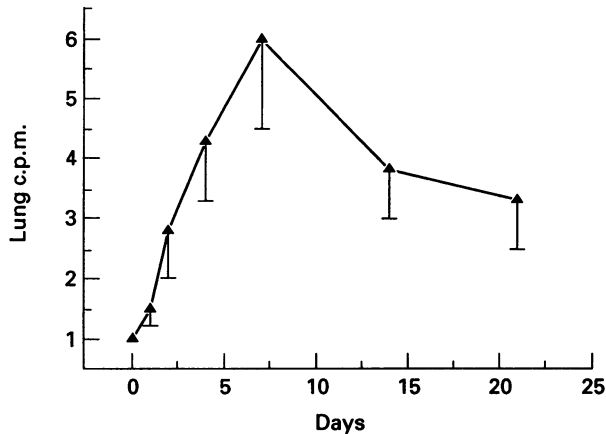


Figure 1. Bleomycin-induced platelet pulmonary localization. Localization of labelled platelets, in c.p.m. $\times 10^{-2}$, as a function of time after an injection of bleomycin (CBA mice).

$3 \times$ ssc at 43°C and 2×20 min in $0.2 \times$ ssc/0.2% SDS at 65°C .

Statistical evaluation

Groups of values were compared using a non-parametric Mann-Whitney *U*-test and correlations were evaluated using Spearman's rank correlation test.

Results

Bleomycin induced platelet trapping as a function of time

Platelet trapping was evaluated at various times after a single i.v. injection of bleomycin. An injection of

bleomycin induced a lasting increase of the pulmonary localization of ^{111}In -labelled platelets, as seen in Figure 1. Bleomycin did not induce significant changes in the c.p.m. recovered from blood, liver, kidney or spleen (not shown).

Morphological aspect of bleomycin induced platelet trapping

An i.v. injection of bleomycin induced moderate changes of the alveolar septa, detectable by light microscopy. An increase in the cellularity was observed in small areas, either below the pleura or around pulmonary veins (Figure 2). In the alveoli, the mean (s.d.) number of nucleated cells per microscopic field increased from 15 (5) in controls to 22 (8) in bleomycin injected mice. Platelet aggregates were occasionally observed in the medium sized blood vessels, as described before (Piguet *et al.* 1993b).

As evidenced by EM, the alveolar septa, notably the endothelium and the interstice (Figure 3B and C), were enlarged in bleomycin injected mice. Platelets were observed in the alveolar capillaries, and some of these were in contact with the endothelium (Figure 3A and C). To evaluate a possible preferential retention of platelets in the alveolar capillaries, independent of blood stasis, we determined the ratio between platelets and red blood cells by EM: this was significantly elevated by the injection of bleomycin (Table 1), thus indicating a more selective adherence of platelets when compared with red blood cells. Areas of thickening of the endothelium, suggesting the recent engulfment of platelets, were also observed (Figure 3A).

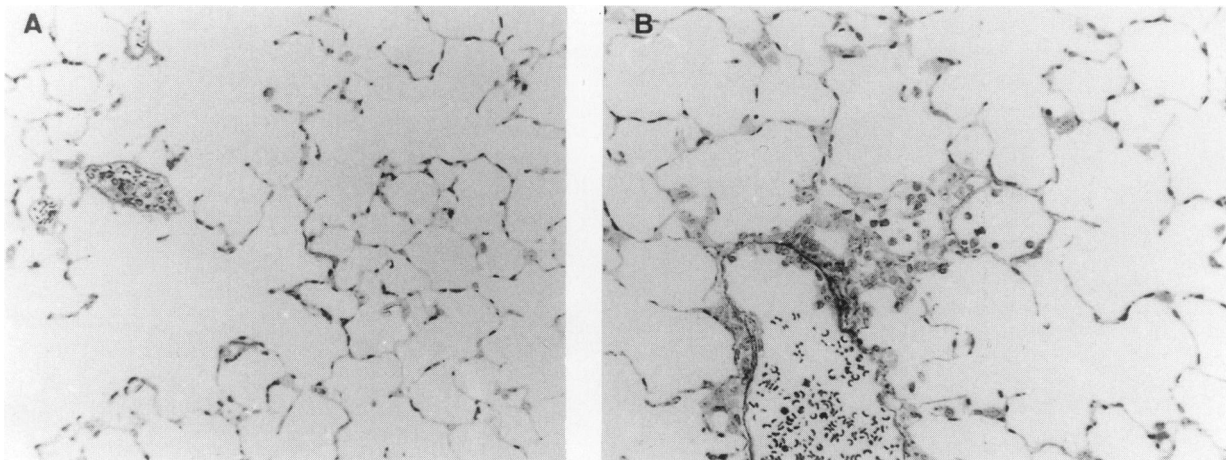


Figure 2. Lung as seen 15 days after an injection of bleomycin. Alveoli from A, normal mice or B, bleomycin injected mice which show a limited cellular reaction near a medium sized blood vessel. Toluidine blue, $\times 140$.

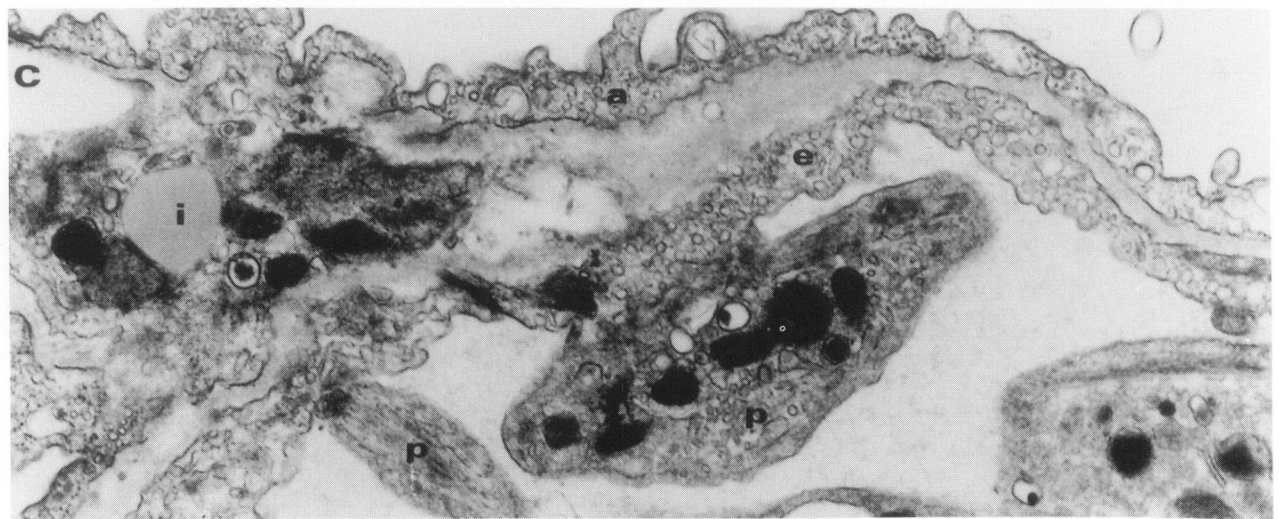
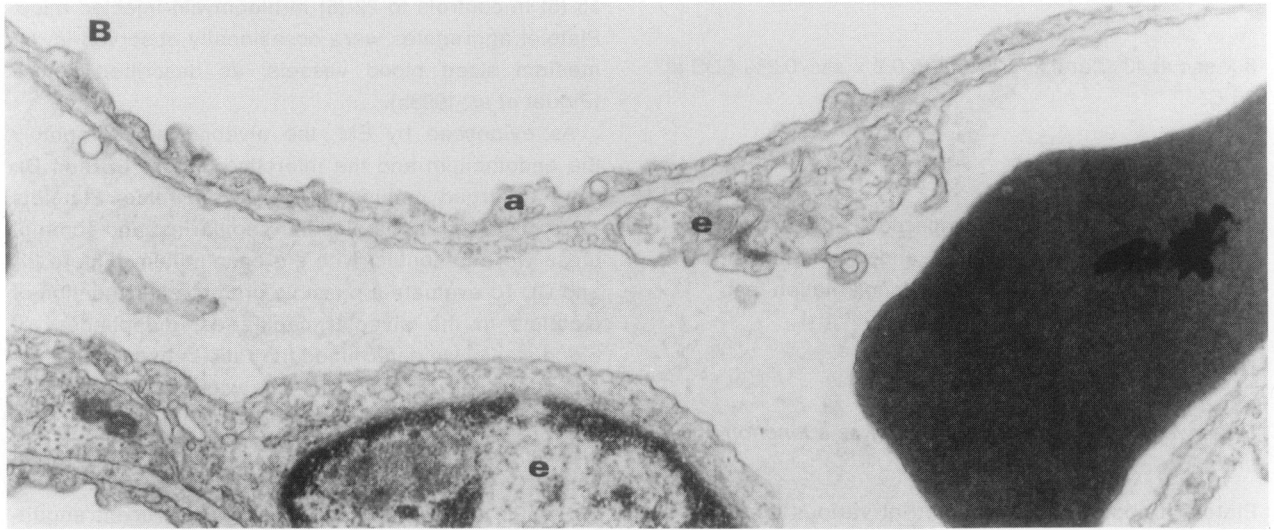
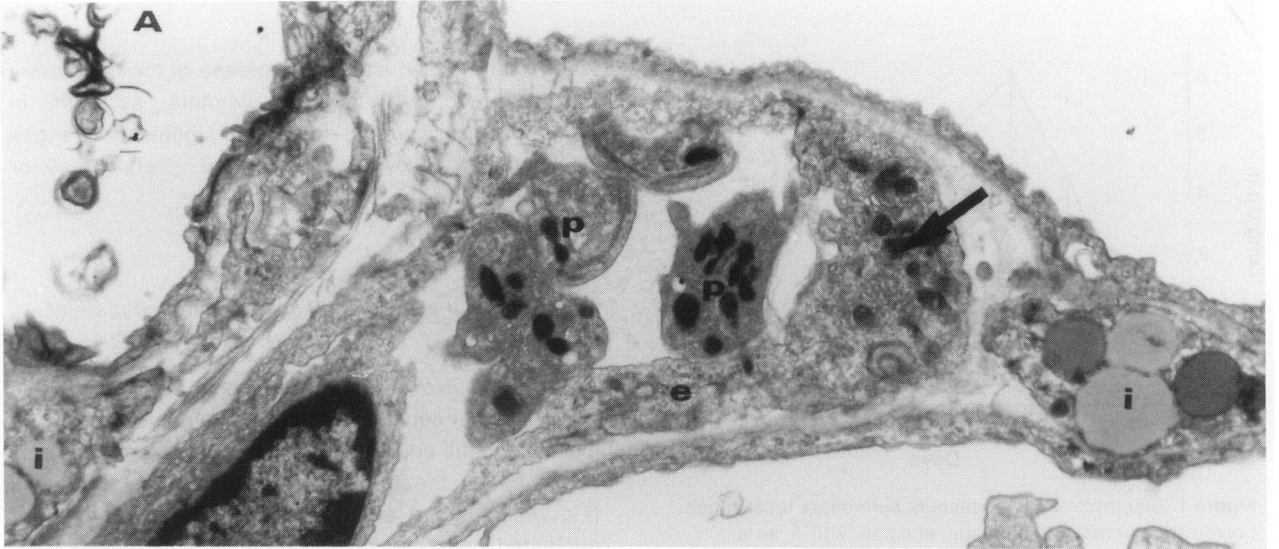


Figure 3

Table 1. Platelet retention in the alveolar capillaries

Mice and treatment (number of mice)	Platelet/r.b.c.
Normal (5)	0.04 (0.01)
Bleomycin (4)	0.12 (0.04) $P < 0.01$
Bleomycin, nx igG (6)	0.12 (0.06)
Bleomycin, CD11a (5)	0.05 (0.01) $P < 0.01$

Platelets and red blood cells were counted within the alveolar capillaries by EM and the results are the mean (s.d.) of the ratio obtained in individual mice, sacrificed on day 5 after an injection of bleomycin.

Correlation between bleomycin induced fibrosis and platelet trapping in susceptible or resistant mouse strains

The pulmonary fibrosis elicited by intratracheal instillation of bleomycin has been reported to vary widely in different mouse strains (Rossi *et al.* 1987). Pulmonary fibrosis elicited by an i.v. injection of bleomycin showed a similar pattern of resistance or susceptibility to that elicited by intratracheal instillation (Table 2). Platelet pulmonary trapping and fibrosis were evident in susceptible (CBA, B10, B10.A 2R), but not in resistant (Balb/c, B10.D2, B10.A), strains. Thus, platelet trapping and fibrosis were strongly correlated (Table 2).

The resistant character of Balb/c mice was conferred by their major histocompatibility complex (MHC) (H-2d), since B10 mice with the H-2d MHC (B10.D2) are also

Table 2. Correlation between platelet trapping and fibrosis

Strain	MHC KAED	Platelet/lung bleo/ctrl	Lung H-proline bleo/ctrl
CBA	kkkk	2.3 (0.3)	1.8 (0.4)
Balb/c	dddd	0.9 (0.3)	1.2 (0.2)
B10	bbbb	3.3 (1.1)	1.9 (0.3)
B10.D2	dddd	1.1 (0.3)	0.9 (0.2)
B10.BR	kkkk	1.4 (0.2)	1.4 (0.2)
B10A	kkkd	1.2 (0.2)	1.2 (0.2)
B10.A (2R)	kkkb	2.8 (1.0)	2.1 (0.4)

Results are the mean (\pm s.d.) of 5 mice for the platelet distribution and more than 7 mice for the lung hydroxyproline, expressed as a ratio with normal mice. For platelet trapping, mice were sacrificed 5 days after bleomycin injection, while lung hydroxyproline was measured 15 days after bleomycin injection. Platelet trapping and the lung hydroxyproline content were correlated at a $P < 0.005$ by Spearman rank correlation.

Table 3. Bleomycin-induced platelet trapping: prevention by anti CD11a, CD11b, CD18 and CD54 antibodies

Treatment		Platelet/lung %	H-proline (μ g/right lung)
None	none	14 (5)	60 (4)
Bleomycin	nx IgG	100 (38)	135 (18)
Bleomycin	anti CD11a	28 (14) $P < 0.004$	68 (4) $P < 0.01$
Bleomycin	anti CD11b	56 (4) $P < 0.001$	n.d.*
Bleomycin	anti CD18	72 (26) $P < 0.05$	n.d.*
Bleomycin	anti CD54	30 (10) $P < 0.01$	112 (12)

Platelet trapping was measured on day 5 and is expressed as a percentage of the c.p.m. per lung obtained with bleomycin treated mice and the lung hydroxyproline was measured on day 15 after injection of bleomycin. Mean (\pm s.d.) from groups of 6 or more CBA mice.

* This treatment markedly decreased the collagen deposition elicited by an intratracheal instillation of bleomycin (Piguet *et al.* 1993b and unpublished observations).

resistant. Furthermore, the study of MHC recombinant strains indicates that the region conferring resistance is located in the E-D region of the MHC (i.e. present in B10.A but absent in B10.A 2R mice), a region which also contains the TNF locus (Spriggs *et al.* 1992).

Effect of anti CD11, CD18 and CD54 mAbs on bleomycin induced fibrosis and platelet trapping

The collagen content in lungs from mice treated with an intratracheal instillation of bleomycin is profoundly decreased by the injection of anti CD11a, CD11b (Piguet *et al.* 1993b), or of anti CD18 or CD54 mAbs (not shown). Results were similar after an i.v. injection of bleomycin and the fate of labelled platelets indeed showed that anti CD11a, CD11b, CD18 or CD54 mAbs decrease the pulmonary platelet localization (Table 3).

Modulation of platelet trapping by TNF- α and IL-1 antagonists

As seen in Table 4, the injection of anti TNF- α antibodies decreased both bleomycin induced platelet trapping and fibrosis.

Administration of IL-1ra has been reported to decrease markedly the collagen deposition elicited by an intratracheal instillation of bleomycin (Piguet *et al.* 1993a). This treatment also decreased the platelet trapping induced by bleomycin (Table 4).

Figure 3. Platelet-lung endothelium interaction induced by bleomycin. Alveolar capillaries from B, a normal mouse or A and C, from a mouse injected with bleomycin 5 days before. A, An alveolar sinus contains several platelets, and most of these are in contact with the endothelium. An area of the endothelium is markedly enlarged, perhaps by the recent engulfment of platelets (arrow). p, Platelet; i, lipid laden interstitial cells; a, alveolar epithelium; e, endothelium. A, $\times 4000$. B and C, $\times 7000$.

Treatment		Platelet/lung %	H-proline ($\mu\text{g}/\text{right lung}$)
None	none	22 (5)	74 (6)
Bleomycin	nx IgG	100 (38)	140 (13)
Bleomycin	anti TNF- α IgG	62 (36) $P < 0.004$	110 (8) $P < 0.02$
Bleomycin	solvent	100 (13)	n.d.
Bleomycin	IL-1ra	51 (21) $P < 0.02$	n.d.*

Table 4. Bleomycin-induced platelet trapping: prevention by TNF- α and IL-1 antagonists

Platelet trapping was measured 5 days, and the lung hydroxyproline 15 days after an injection of bleomycin. Anti TNF- α antibodies or control IgG (1.5 mg) were injected i.p. IL-1 ra (1 $\mu\text{g}/\text{h}$) or its solvent, was administered by an osmotic minipump implanted i.p. one day after bleomycin injection. Mean (\pm s.d.) from groups of 6 mice.

* This treatment markedly decreased the collagen deposition elicited by an intratracheal instillation of bleomycin (Pignet *et al.* 1993a).

Effect of anti CD11a antibody on collagen $\alpha 1(I)$ mRNA level

The collagen $\alpha 1(I)$ mRNA level was substantially elevated by injection of bleomycin (Figure 4) and this increase was markedly reduced when mice were treated with anti CD11a mAb (Figure 4).

Discussion

The present results demonstrate that bleomycin increases the binding of platelets to the alveolar endothelium and that this event is closely correlated with collagen synthesis and deposition. Furthermore, the binding of platelets to the endothelium appears to be mediated by the platelet $\beta 2$ integrins.

A close correlation between platelet trapping and collagen deposition was indeed observed in several

conditions: in genetically susceptible or resistant mouse strains and in mice receiving treatments, such as administration of anti TNF- α antibody or antibodies to the $\beta 2$ integrins, which decrease the lung collagen content. Because of the abundance of platelets and their rich content of fibrogenic factors, such as TGF- β and PDGF (Assoian & Sporn 1986), these correlations argue in favour of a causal relation between platelet trapping and collagen deposition. This interpretation is also consistent with the marked decrease of collagen I mRNA level, and most likely of collagen synthesis, induced by treatment with anti CD11a mAb (Figure 4). This conclusion seems however to contradict that derived from the study of thrombocytopenic animals, since the injection of an anti platelet antibody did not significantly decrease the bleomycin induced collagen deposition (Evans *et al.* 1990), and our own unpublished observations. This discrepancy might be due to the fact

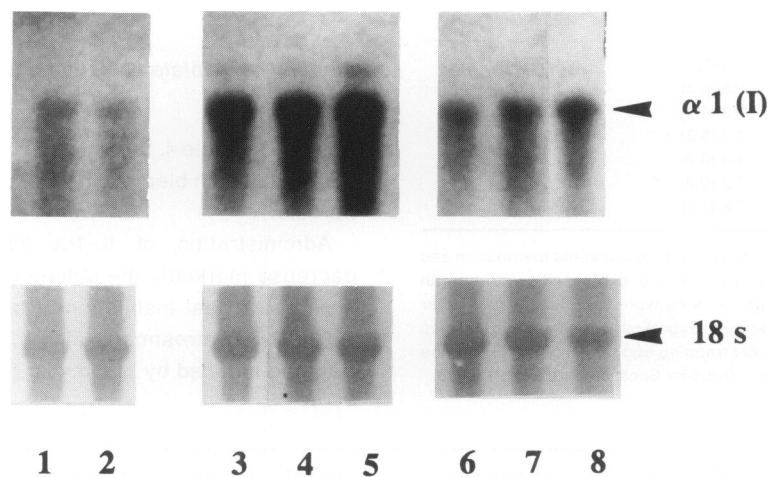


Figure 4. Collagen $\alpha 1(I)$ mRNA levels. RNAs were isolated from the lungs of normal mice (lanes 1 and 2) or mice injected 7 days before with bleomycin (lanes 3–8) and treated with normal (3–5) or anti CD11a (6–8) IgG. Position of the 4.7-kb collagen I mRNA and that of the 18s RNA, stained with methylene blue, are indicated by an arrow.

that the number of platelets really localized in the lung is influenced more by the availability of the ligands involved (the $\beta 2$ integrin and CD54 as discussed below) than by the number of platelets passing through the lung.

Platelets are known to bind, by their $\beta 1$ and $\beta 3$ integrins, to various proteins of the extra-cellular matrix (ECM), which may become accessible after the severe endothelial damage of an acute inflammation (reviewed by Kunicki & Newman 1992). Bleomycin induced fibrosis is most commonly elicited in rodents by intratracheal instillation, a protocol which induces a patchy fibrosis associated with alveolar haemorrhages. To avoid haemorrhages, which would complicate the study of platelet-endothelium interactions, bleomycin was administered by the i.v. route which induces a more diffuse but less severe fibrosing alveolitis. In this condition, platelets were found either as aggregates within the medium sized blood vessels, as described before (Piguet *et al.* 1933b), or as a more scattered binding to the alveolar endothelium, as observed by EM (Figure 3). The occurrence of platelet aggregates was not consistently increased by an i.v. injection of bleomycin and we suspect that these might represent fixation artefacts, a possibility which has already been discussed in more detail elsewhere (Rosenblum 1986). In the alveolar capillaries, semi-quantitative evaluation by EM indicated that bleomycin significantly increases the number of platelets in the alveolar capillaries. These platelets had frequently a zone of contact with the endothelial cell membrane, but not with the basement membrane (Figure 3).

Several arguments indicate that platelet endothelium interaction is mediated by platelet $\beta 2$ integrins and endothelium CD54. Mouse platelets bear CD11a (McCaffery & Berridge 1986; Piguet *et al.* 1993c) and pulmonary platelet trapping was significantly decreased by anti CD11a, and to a lesser extent by anti CD11b and CD18 mAbs, a difference which corresponds to the relative abundance, as detected by FACS analysis, of these molecules on platelets (Grau *et al.* 1993). Platelets localize at sites of acute inflammation and this can be abrogated by anti CD11a and anti CD54 antibodies (Piguet *et al.* 1993c). It is most likely that the $\beta 2$ integrin of platelets interacts with its ligand CD54, whose expression on endothelial cells is markedly increased by the inflammatory cytokines TNF- α and IL-1 (reviewed by Gamble *et al.* 1992). The expression of the TNF- α mRNA level in the lung is increased by bleomycin in susceptible, but not in resistant, mice (Piguet *et al.* 1989; Phan & Kunkel 1992) and the bleomycin resistant phenotype appears to be closely linked to the TNF

region of the MHC (Table 2); it is therefore likely that in susceptible mice the induction of TNF- α increases the endothelial expression of CD54, thus leading to the trapping of platelets and leucocytes.

Platelet interactions with the endothelium have been extensively investigated in the past and evidence has been presented for a fusion of platelets with the endothelium (Wojcik *et al.* 1969), although the quantitative significance of this observation is controversial (Weksler 1987). A platelet-endothelium interaction leading to fusion is compatible with the morphological aspect of platelet-endothelium interaction seen in the present study (see Figure 3A) and, furthermore, the profound influence of anti CD11a mAb on both collagen synthesis and platelet trapping indeed suggests that platelets might transfer their rich content of fibrogenic factors to the endothelial cells and the interstitium.

Acknowledgements

This work is supported by grant no 31-28855.91 from the Swiss National Science Foundation. We are grateful to R. Thompson, Synergen Boulder, Co for the gift of IL-1ra, and to Mr and Mrs Anne RoCHAT, for the preparation of the Northern blots, and to G. Leyvraz, P. Henchoz, J. Stalder and Le Minh Tri for the preparations for light and electron microscopy.

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