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Chemokine involvement in tetracycline-induced pleuritis

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ABSTRACT: Sclerosants such as tetracycline (TCN) have often been used in the control of malignant pleural effusions. Although the resultant inflammatory response is probably important in the ensuing pleural fibrosis, the signals responsible for the cellular influx into the pleural space following TCN instillation are not well understood. This study, therefore, sought to determine whether the chemokines interleukin-8 (IL-8), growth-related protein (Gro), and monocyte chemotactic protein-1 (MCP-1) were locally elaborated within the first 72 h following intrapleural TCN administration.

TCN induced an exudative effusion with high lactate dehydrogenase activity. Although there was no significant change in the pleural fluid total leukocyte content, the median polymorphonuclear neutrophil concentration decreased from 1.067×10^6 to 2.03×10^5 cells·mL^{-1} between 24 and 72 h, whereas the median macrophage concentration increased from 1.44×10^5 to 5.98×10^5 cells·mL^{-1} over the same period. Furthermore, IL-8, Gro and MCP-1 concentrations decreased between 24 and 72 h. Immunocytochemistry indicated expression of IL-8 by pleural mesothelial cells 24 h, but not 72 h, following TCN administration.

The data suggest that local elaboration of interleukin-8 and growth-related protein, in part of mesothelial origin, may influence neutrophil recruitment in tetracycline-induced pleuritis.

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Each year, in the USA, ~1.5 million individuals develop a pleural effusion [1]. Malignant pleural effusions can occur as a result of tumour growth, and are often associated with various neoplasms, including carcinomas of the lung, breast or intestinal tract. Treatment of patients with malignant effusions can involve pleural fluid aspiration for symptomatic relief. In some cases, more aggressive palliative measures are necessary to manage the pleural effusion.

Pleurodesis, the production of adhesions between the visceral and parietal pleura, is often effective in the relief of malignant pleural effusions. Chemical pleurodesis can be achieved by the injection of an irritant, such as tetracycline (TCN) or one of its derivatives doxycycline or minocycline, into the pleural space [2–4]. Intrapleural TCN administration produces intense inflammation with initial neutrophil influx, followed by an increase in pleural macrophage and lymphocyte numbers over the subsequent 48 h. This is accompanied by mesothelial denudement, and the rapid deposition of pleural fibrin, which serves as a scaffolding for fibroblasts to span the pleural compartment. The ensuing fibrosis results in the fusion of the visceral and parietal pleura and the obliteration of the pleural space. While the anatomical and temporal features of the fibrotic response are well appreciated, the mechanisms involved in the inflammatory reaction to TCN are incompletely understood.

Interleukin-8 (IL-8), a neutrophil chemoattractant of the α-chemokine family, has been implicated in lung inflammation and injury [5] and pleuritis in both humans and rabbits. IL-8 can be secreted by numerous cell types including mesothelial cells, which cover the surfaces of the lung, mediastinum, thoracic wall and diaphragm. However, its role in TCN-induced pleuritis has not been fully elucidated. The purpose of this study was to determine whether α- and β-chemokines are involved in the TCN-associated inflammatory response, and to determine the temporal pattern of their expression. In particular, this study sought to determine whether IL-8 could contribute to the early influx of neutrophils in TCN-induced pleuritis in rabbits.

Materials and methods

Study animals

All animal work was approved by the Institutional Animal Care and Use Committees at the University of Texas Health Center and Vanderbilt University. Three groups (six animals per group) of female New Zealand White rabbits, 4–5 months old and weighing 2 kg, were used.

Study design

Three groups of rabbits were used with each animal acting as its own control. In the first two groups, a baseline blood sample was drawn before TCN administration and

compared to samples drawn prior to sacrificing the animal at either 24 or 72 h post-TCN administration. Since baseline pleural fluid samples could not be taken prior to TCN administration, the cellular and selected chemokine content of pleural fluid collected after 24 or 72 h, immediately *post mortem*, were compared. The third group, which received talc as a sclerosant, were only examined for selected chemokine content of pleural fluid collected after 24 or 72 h.

Induction of pleuritis

The animals were anaesthetized with ketamine (250 mg) and xylazine (30 mg) *i.m.* A small incision was then made over the lateral aspect of the 3rd or 4th left rib using a No. 10 scalpel. TCN (Sigma, St Louis, MO, USA) was freshly prepared as an injectable solution (TCN 20 $mg \cdot mL^{-1}$; ascorbic acid 2.5 $mg \cdot mL^{-1}$; lidocaine 1 $mg \cdot mL^{-1}$), and filter sterilized. TCN solution (3 mL) was then injected into the pleural space using a 5.08-cm 18-G animal feeding needle. Animals were sacrificed at 24 and 72 h after intrapleural TCN administration by intravenous Nembutal (35 $mg \cdot kg^{-1}$). A further group of animals received talc (200 $mg \cdot kg^{-1}$) *i.p.* as previously described [6].

Fluid sampling and tissue collection

A baseline peripheral blood sample, from each animal, was collected (into 15% ethylenediaminetetraacetic acid) 1 h prior to treatment (Pre-Tx). Peripheral blood samples were then collected just prior to euthanasia at either 24 or 72 h after administration of TCN. The animals were then sacrificed and pleural fluid was collected in 0.9% sodium citrate *post mortem*. The pleural fluids were centrifuged at $500 \times g$ for 10 min and then stored at -70°C until use. Pleural tissue was also removed by *en bloc* resection of the thorax and chest wall *post mortem* for histological examination and immunocytochemistry.

Assays

Total white cell counts, haemoglobin concentration and haematocrit were determined, for each blood sample, using a Coulter-S cell counter (Coulter Electronics, Inc., Hialeah, FL, USA). Pleural fluids were analysed for cellular content by the clinical laboratory at the University of Texas Health Center at Tyler, TX, USA. Total white blood cell (WBC) counts were determined using haemocytometry and differential cell counts were assessed using cytospin preparations stained using the Diff-Quick procedure (Baxter Scientific, McGaw Park, IL, USA). Total protein and lactate dehydrogenase (LDH) assays were performed as previously described [7]. In addition, the blood and pleural fluids were examined for IL-8, growth-related protein (Gro) and monocyte chemotactic protein-1 (MCP-1) using specific enzyme-linked immunosorbent assays (ELISAs) as previously described [8, 9].

The lungs and hemithoraces were removed *en bloc* and formalin fixed for immunohistochemistry. Samples of tissue were embedded in paraffin and 5 μm thick sections prepared. The tissue sections were stained with haematoxylin and eosin for morphological assessments and with trichrome stain in order to detect collagen deposition. Immunochemical analyses were performed as previously described [10]. Negative controls included substitution of isotypic control immunoglobulin G for the primary antibody. The tissue sections were stained for IL-8 using a polyclonal antiserum [11].

Statistical analysis

The data are expressed as mean±SD unless otherwise indicated. The differences between the groups were compared using one-way analysis of variance or the Mann-Whitney rank-sum test and an all pairwise multiple comparison procedure (Dunn′s method or Tukey test) to test the statistical significance of the differences in median or mean values, respectively.

Results

Two groups of six New Zealand White rabbits were treated *i.p.* with TCN (20 $mg \cdot mL^{-1}$, 3 mL) under sterile surgical conditions. One group was sacrificed after 24 h, the other group after 72 h. The early interval was chosen in order to evaluate the expression of IL-8 and other selected chemokines during an initial acute inflammatory phase (24 h) during which pleural loculation and fibrosis had not yet occurred. In addition, the animals were studied at a later phase (72 h) characterized by florid loculation, pleural fibrosis and collagen deposition within transpleural adhesions. The animals developed unilateral pleural effusions (up to 10 mL) after 24 h, and the effusion persisted for 72 h.

Intrapleural instillation of TCN caused a significant fall in the median haematocrit from the Pre-TX value after 24 h ($p<0.05$, Dunn′s method) (fig. 1a). However, after 72 h, the median value had returned to its Pre-TX value. There was also a significant drop in the mean haemoglobin concentration from its Pre-TX value 24 h after TCN administration ($p<0.05$, Tukey test). However, the haemoglobin concentration after 72 h had also returned to its Pre-TX value. There were significant increases in the numbers of both total peripheral WBCs (fig. 2a) and total peripheral polymorphonuclear neutrophils (PMNs; fig. 2b) following intrapleural instillation of TCN. After 24 h, the mean WBC concentration increased from the Pre-TX value ($p<0.05$, Tukey test), but had returned to normal after 72 h. Furthermore, there was a significant increase in the mean peripheral blood PMN concentration from that during the first 24 h ($p<0.05$, Tukey test). As with WBCs, after 72 h, the PMN concentration was not significantly different from the Pre-TX value.

Since the leukocytosis was initially neutrophilic and resolved into a more monocytic predominance after 72 h, the plasma concentrations of IL-8, Gro and the β-chemokine MCP-1 were measured prior to, and 24 and 72 h after, TCN administration using specific ELISAs. No significant changes ($p>0.05$) in the plasma chemokine concentrations were detected following intrapleural administration of TCN (fig. 3).

To characterize the local inflammatory response, the pleural fluid cellular and selected solute concentrations were examined. Cellular damage, as indicated by the high LDH activity, occurred within the first 24 h following TCN

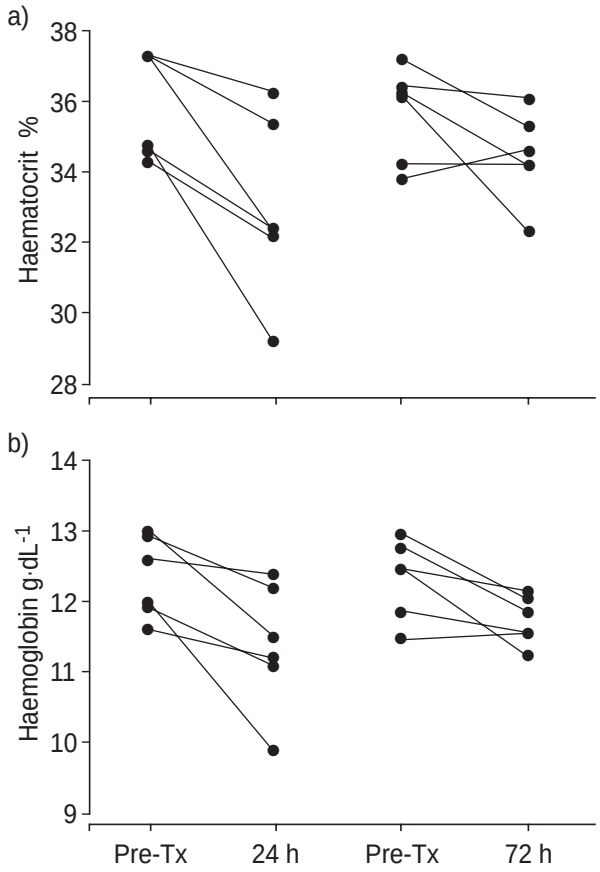

Fig. 1. – Blood haematocrit and haemoglobin concentration. a) Intrapleural instillation of tetracycline caused a significant drop in the median haematocrit from the pretreatment (pre-TX) value after 24 h ($p<0.05$, Dunn′s method). The median value after 72 h was not significantly different from that pre-TX. b) There was also a significant drop in the mean haemoglobin concentration from the pre-TX value ($p<0.05$, Tukey test). The haemoglobin concentration after 72 h was not significantly different from that pre-TX.

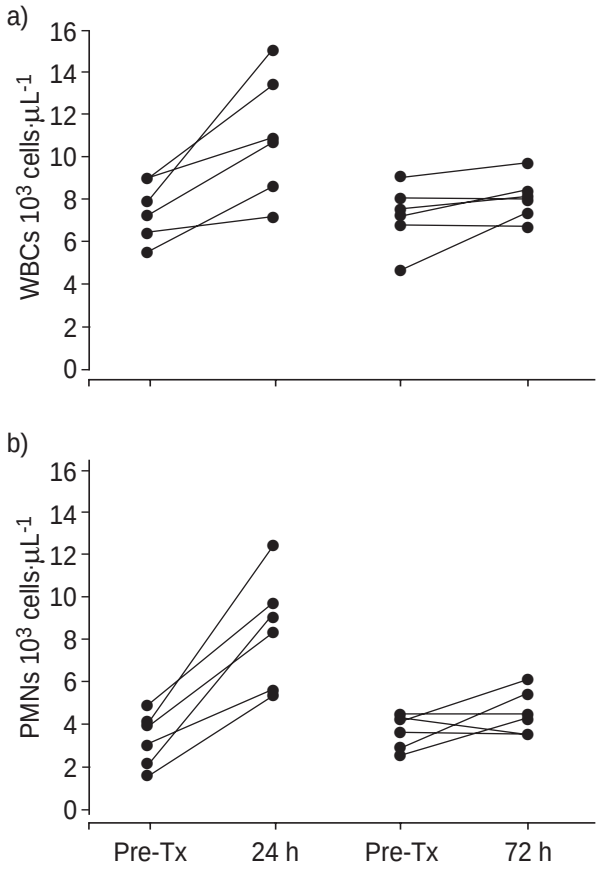

Fig. 2. – Peripheral blood total white blood cell (WBC) and polymorphonuclear neutrophil (PMN) concentration. a) Intrapleural instillation of tetracycline caused a significant drop in mean WBC from the pretreatment (pre-TX) value ($p<0.05$, Tukey test). The value at 72 h was not significantly different from that pre-TX. b) There was a significant drop in the mean peripheral blood PMN concentration after 24 h ($p<0.05$, Tukey test). After 72 h the PMN concentration was not significantly different from that pre-TX.

injection (fig. 4). However, the median LDH activity of the pleural fluid decreased significantly between 24 and 72 h following TCN administration ($p=0.002$, Mann-Whitney). This was not the case with respect to the total protein concentration of the effusion, and there was no significant change in the total protein concentration over the same period ($p=0.41$) (fig. 4). There was an initial leukocytosis, primarily neutrophilic in nature, which had lessened by 72 h (fig. 5). The Mann-Whitney rank-sum test indicated that the median concentration of PMNs in the pleural fluid 24 h after TCN administration was significantly higher ($p=0.009$) than that after 72 h. However, there was no significant difference in the concentration of total WBCs in the pleural fluid after 24 or 72 h ($p=0.485$). Although there was a small increase in the mean pleural macrophage concentration between 24 and 72 h after TCN administration, the increase was not statistically significant (fig. 5).

Next the effusions were examined for specific chemokines. The effusions from a group of nine rabbits which had received talc as the sclerosing agent were also examined. In cases in which TCN had been used as the sclerosant, the concentrations of the α-chemokines IL-8 and Gro and the β-chemokine MCP-1 were all higher after 24 than after 72 h (fig. 6a). The fluids contained 25–50 times more MCP-1 than IL-8 or Gro, with Gro concentrations being higher than those of IL-8. There was a significant decrease in the median Gro concentration from 400 pg·mL^{-1} at 24 h to <200 pg·mL^{-1} after 72 h ($p=0.004$, Mann-Whitney). The mean IL-8 concentration also fell significantly from 175±17 pg·mL^{-1} after 24 h to 121±47 pg·mL^{-1} after 72 h ($p=0.025$). Furthermore, the median concentration of the macrophage chemoattractant MCP-1 significantly decreased from 9,400 pg·mL^{-1} to 3,050 pg·mL^{-1} over the same time period ($p=0.004$, Mann-Whitney). In the group in which talc was used as the sclerosant, the concentration of each chemokine was higher than that in the group in which TCN was used. Unlike in the TCN-treated rabbits, there was no change in Gro concentration, and the median IL-8 concentration increased significantly from 600 pg·mL^{-1} at 24 h to 6,330 pg·mL^{-1} at 72 h ($p=0.028$). The MCP-1 concentrations in talc-treated rabbits, however, paralleled those seen in the TCN group, and significantly decreased from a mean of 30,400 pg·mL^{-1} to 4,400 pg·mL^{-1} between 24 and 72 h post-sclerosant administration ($p=0.0002$).

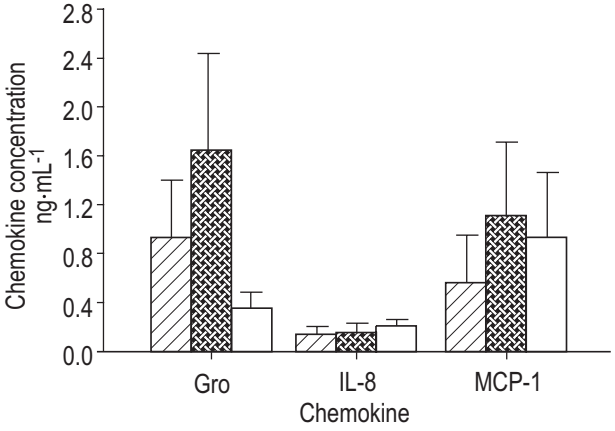

Fig. 3. – Peripheral blood chemokine concentrations following tetracycline (TCN) administration. Chemokine concentrations in the blood were measured prior to (▨), and 24 (▩) and 72 h after (□), TCN administration using specific enzyme-linked immunosorbent assays. There were no significant changes in any of the blood chemokine concentrations following TCN administration ($p>0.05$). Data are presented as meansd. Pre-TX: pretreatment; Gro: growth-related protein; IL-8: interleukin-8; MCP-1: monocyte chemotactic protein-1.

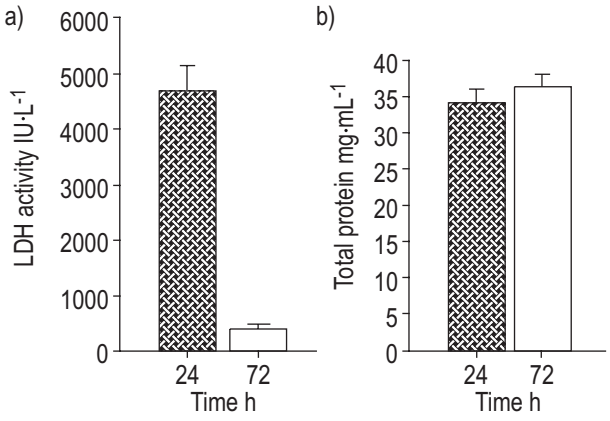

Fig. 4. – Lactate dehydrogenase activity (a) (LDH) and total protein concentration (b) in pleural fluid following tetracycline administration. After 72 h, the median LDH activity of the pleural fluid decreased significantly from that at 24 h ($p=0.002$, Mann-Whitney). However, there was no significant change in the total protein concentration over the same period ($p=0.41$). Data are presented as mean±SD.

Histopathological analysis confirmed that the neutrophilic pleocytosis noted at day 1 in pleural fluids was accompanied by acute inflammation associated with infiltration of the pleural and subpleural areas by neutrophils (fig. 7a). By day 3, more macrophages were noted in the pleural infiltrate (fig. 7b). At day 3, fibrinous transpleural adhesions had formed and were noted to contain detectable collagen fibrils (fig. 7c). The mesothelial surface of the intact rabbit parietal pleura stained positively (red) for IL-8 (fig. 7d). At day 1, the mesothelial surface retained IL-8 reactivity (fig. 7e), whereas little staining was detectable by day 3 (fig. 7f). These data demonstrate that the mesothelial lining of the pleural compartment constitutively expresses IL-8. IL-8 expression by the mesothelium is retained early after TCN-induced pleuritis but falls when pleural remodelling and fibrosis are apparent.

Immunohistochemistry showed that the IL-8 was derived, at least in part, from production by the pleural mesothelial cell layer (fig. 7). The staining was consistent with the pleural fluid IL-8 content, and was evident after 24 h, but no IL-8 expression was detected after 72 h. Neither Gro nor MCP-1 could be demonstrated in rabbit pleural tissue using polyclonal antibodies directed against the rabbit antigens.

Discussion

TCN and its derivatives have proven to be effective sclerosing agents in the treatment of pleural effusions [2, 3], particularly those that occur in patients with malignancy [1]. These compounds are relatively inexpensive, and when used to accomplish pleurodesis are associated with low morbidity rates [12]. Systemic and topical administration of TCN has been shown to suppress neutrophil chemotaxis [13]. Conversely, when injected directly into the pleural space, TCN causes an initial neutrophil influx into the pleural space, which is followed by an increase in macrophage number during the subsequent 48 h [14]. The inflammatory cell response is probably an important component in the progression to subsequent pleural fibrosis. While chemokines and IL-8 are implicated in the inflammatory response, their expression and temporal distribution in TCN-induced pleuritis has not, to the authors' knowledge, been previously characterized.

In the present study, the elaboration of chemokines within the first 72 h following sclerosant administration was investigated. The 24 and 72 h intervals were chosen for study in order to evaluate the expression of selected chemokines during an initial acute inflammatory phase (24 h) during which pleural loculation and fibrosis had not yet occurred and a later phase (72 h) characterized by florid loculation, pleural fibrosis and collagen deposition within transpleural adhesions. Previously, it had been found that IL-8, a neutrophil chemotactic member of the α-chemokine family, is expressed in the pleural space of patients

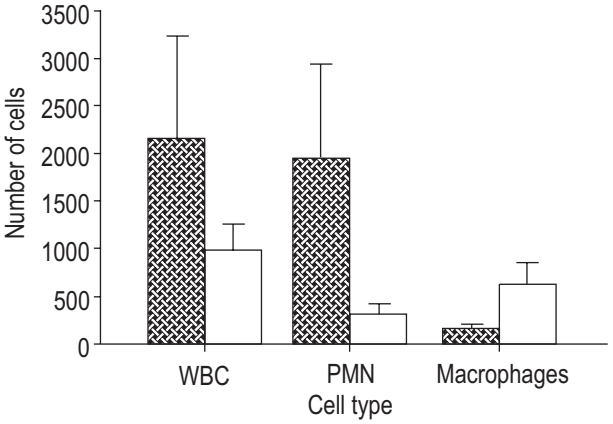

Fig. 5. – Changes in cellular content of pleural fluid 24 (▩) and 72 h (□) following tetracycline (TCN) administration. The Mann-Whitney rank-sum test indicated that the median concentration of polymorphonuclear neutrophil (PMNs) in the pleural fluid 24 h after TCN administration was significantly higher ($p=0009$) than that after 72 h. However, there was no significant difference in the median concentration of total white blood cells (WBCs) in the pleural fluid after 24 or 72 h ($p=0.485$). Nor was there a significant difference between the mean pleural macrophage concentration at 24 and 72 h after TCN administration. Data are presented as mean±SEM.

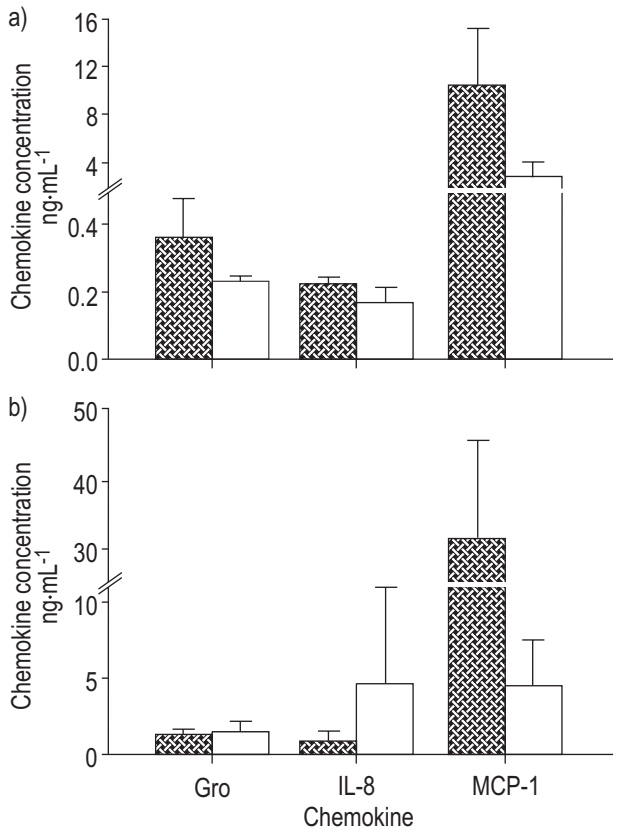

Fig. 6. – Changes in pleural fluid chemokine content following sclerosant administration. Chemokine concentrations in the pleural fluid were measured 24 (▩) and 72 h (□) after: a) tetracycline; and b) talc administration, using specific enzyme-linked immunosorbent assay. Following TCN administration, there was a significant decrease in the median growth-related protein (Gro) concentration after 72 h (p=0.004, Mann-Whitney). The mean interleukin-8 (IL-8) concentration also fell significantly between 24 and 72 h (p=0.025). Furthermore, the median concentration of the macrophage chemoattractant monocyte chemotactic protein-1 (MCP-1) significantly decreased over the same time period (p=0.004, Mann-Whitney) and a decrease in the mean MCP-1 concentration (p=0.0002, Mann-Whitney). There were no significant changes, between 24 and 72 h, in Gro concentration (p=0.84).

with exudative pleural effusions [15]. Since TCN itself induces an exudative effusion [16], this study sought to determine whether IL-8 and Gro, another neutrophil activator of the α-chemokine family, were locally expressed during the course of TCN-induced fibrosing pleuritis. Also, a group of rabbits were treated with a separate sclerosing agent, talc, so that the chemokine expression in the two models could be compared.

It was found that the pleural fluid mean IL-8 concentration fell significantly between 24 and 72 h following TCN administration. The production of IL-8 appears, at least in part, to derive from the mesothelial cells. No IL-8 immunoreactivity was identified in these cells after 72 h, which was consistent with the fall in IL-8 concentration noted in the pleural fluid. Given that IL-8 immunoreactivity was identified in the native mesothelial lining, it may be that local release into the pleural exudate that forms after injury establishes a chemotactic gradient that attracts neutrophils into the pleural space. Other chemoattractants, such as thrombin, may likewise differentially attract neutrophils to the injured pleura. While the control of intrapleural inflammatory cell traffic is probably multifactorial, the present data suggest that local elaboration of IL-8 contributes to the pleural fluid neutrophilia observed at day 1. The decrease in pleural fluid IL-8 concentration at day 3 in the TCN-treated animals correlates with a fall in pleural fluid neutrophil numbers, further suggesting that IL-8 is involved in the local regulation of inflammatory cell traffic in this form of pleuritis. This scenario is similar to the pattern of events that occur in acute lung injury associated with adult respiratory distress syndrome (ARDS) [5]. Irrespective of the precipitating cause of ARDS, IL-8 is found in elevated concentrations in the airspaces, and is largely responsible for the neutrophil chemotactic activity. The prolonged production of IL-8 seen in the talc-treated rabbits may be due to the particulate nature of the sclerosant, resulting in a slower clearance of the irritant from the pleural space. The influx of neutrophils and subsequent alveolitis in animal models of acute lung injury can be blocked by either α-chemokine receptor inhibitors [17] or antibodies to IL-8 [18, 19]. IL-8, therefore, appears to regulate inflammatory cell traffic during evolving injury in both pleural and alveolar components and the authors infer that its expression may likewise contribute to the progression of pleural injury during pleuritis induced by sclerosing materials.

VILLARD *et al.* [20] found that, in pneumonia and ARDS, the IL-8 concentration in the airspaces correlated with the concentration of Gro, another neutrophil chemotactic member of the α-chemokine family. The present study, therefore, sought to determine whether Gro was also expressed following TCN challenge. Local expression of Gro, as well as MCP-1, was found to be altered during the course of TCN-induced pleuritis. There was a significant decrease in the median Gro concentration from 400 pg·mL^{-1} at 24 h to <200 pg·mL^{-1} after 72 h. In the talc-treated animals, there was considerably more Gro in the pleural fluid, with a mean concentration of 1,300 pg·mL^{-1} being detected on both days. This pattern of Gro expression is consistent with that described in a study examining the elaboration of cytokines in dermal inflammation induced by sulphur mustard [21]. In that study, TSURUTA *et al.* [21] found that mononuclear cell expression of messenger ribonucleic acid (mRNA) encoding Gro was at a maximum 2 days following dermal injury. *In situ* hybridization techniques using probes that recognize all three forms of Gro (α, β and γ) showed that Gro mRNA in mononuclear cells in the lesion was only slightly decreased by 6 days following injury. In the present study, using antibodies raised against rabbit Gro, it has been shown that the accumulation of this specific cytokine in pleural effusions decreases more rapidly, and is significantly decreased after 72 h. Previous *in vitro* studies of Gro production in rabbit alveolar macrophages [22] showed that maximal accumulation occurred between 4 and 22 h after stimulation. This study also used *in situ* hybridization techniques to show the production of Gro by macrophages and neutrophils in a rabbit model of *Escherichia coli* pneumonia. These observations suggest that some of the Gro in the pleural fluid may, at least in part, be derived from the neutrophils that entered the pleural space. Since Gro is also a neutrophil chemotaxin, it could also stimulate pleural neutrophilia and amplify

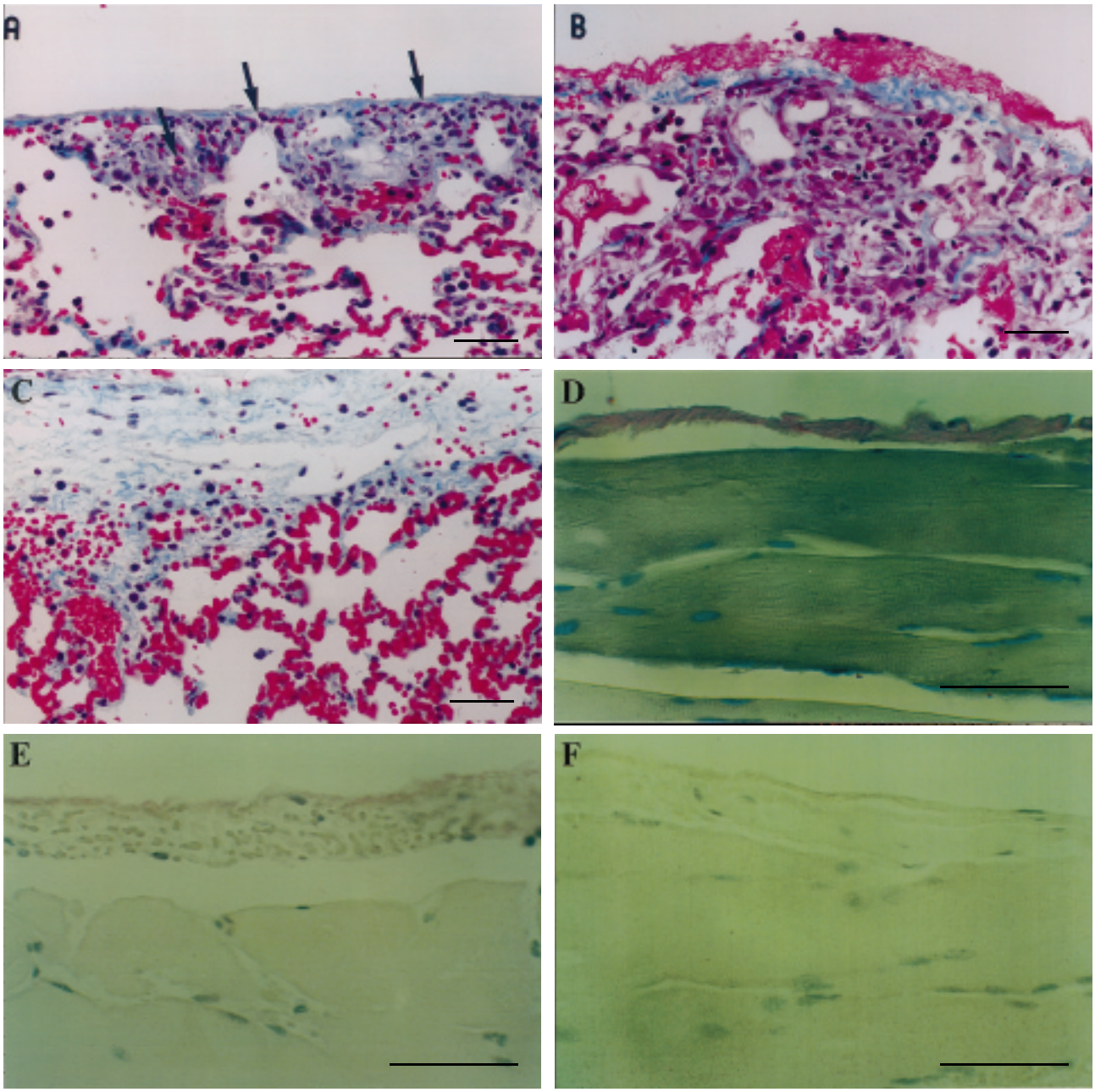

Fig. 7. – A) Pleural and subpleural acute inflammation 24 h after induction of tetracycline (TCN) pleuritis. Arrows indicate polymorphonuclear neutrophils within the inflammatory foci. (haematoxylin and eosin stain) B) Persistent pleural and subpleural inflammation 72 h after administration of intrapleural TCN. C) Formation of collagen within intrapleural adhesions 72 h after intrapleural TCN administration. Collagen fibrils are stained blue. (Trichrome stain). D) Immunohistochemical identification of interleukin-8 (IL-8) (red staining) in the mesothelial lining of the intact (uninjured) rabbit pleura, using polyclonal antiserum. E, F) Immunohistochemical identification of IL-8 in rabbit pleural tissue 24 and 72 h after intrapleural TCN administration, respectively (Internal scale bar=25 μm).

the neutrophil influx early in the course of both TCN- and talc-induced pleuritis.

Since there were changes in the macrophage population, the pleural fluid was also examined for the β-chemokine macrophage chemoattractant MCP-1. The median concentration of MCP-1 significantly decreased from 9,400 $pg \cdot mL^{-1}$ at 24 h to 3,050 $pg \cdot mL^{-1}$ after 72 h, inversely correlating with the increase in pleural macrophages at this same time interval. Similar changes were noted in the talc-treated animals. Although the concentrations of this chemokine were again higher than in the TCN-treated animals, there was a significant decrease from the 24 to the 72 h value. These observations suggest that other factors may be responsible for the increase in the number of macrophages in the pleural space. In a recent study, BIRDSALL *et al.* [23] examined the attraction of monocytes into the canine myocardium following reperfusion and determined that monocyte migration was induced by a series of factors. The initial attraction resulted from complement component C5a, transforming growth factor-β1 (TGF-β1) and MCP-1 acting sequentially. However, after 3 h, the chemotactic activity was mainly due to MCP-1 and TGF-β1

acting together. Therefore, it is probable that 24 h after TCN administration, the monocytes in the pleural space have been recruited by a series of attractants including MCP-1.

In conclusion the expression of at least two neutrophil and one macrophage chemoattractants following the instillation of either tetracycline or talc into the pleural space have been confirmed. This is associated with leukocyte influx into the area. The initial influx of neutrophils is, at least in part, attributable to interleukin-8 and is replaced by an increased macrophage presence after 72 h. However, the role of monocyte chemotactic protein-1 is less certain, and locally elaborated chemoattractants, may affect the pleural fluid macrophage predominance observed later on in the course of tetracycline- and talc-induced injury.

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