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ORIGINAL ARTICLE

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The immunocytochemical localization of tumour necrosis factor and leukotriene in the rat heart and lung during endotoxin shock

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Abstract After the intravenous administration of lipopolysaccharide at a dose of 3.0 mg/100 g to rats, immunoreactive sites for tumour necrosis factor (TNF) and peptide leukotrienes (LTs) were examined in the heart and lung. Immunoreaction for TNF is preferentially localized on the apical endothelial cell surface of the vessels and in lysosomes of inflammatory and interstitial cells. Lysosomes of cardiac muscle cells which undergo degeneration are also reactive. Peptide LTs in inflammatory cells give almost the same reactions as those for TNF. However, the production of peptide LTs occurs uniquely in cardiac muscle cells in the media of the pulmonary vein, although lysosomes of intracardiac muscle cells which undergo degeneration do not show immunoreactivity. These results suggest that the degeneration of cardiac muscle cells may be induced not only by endogenous TNF but also by peptide LTs which are produced in muscle cells of the venous media and are transported to the myocardium via the coronary circulation.

Key words Endotoxin shock · Immunocytochemistry
Tumour necrosis factor · Peptide leukotriene · Heart

Introduction

Endotoxin shock has some clinical resemblance to septic shock induced by gram-negative rods and produces often lethal disturbance of the cardiovascular (Natanson et al. 1989) and respiratory systems (Pauwels et al. 1990; Kips et al. 1992). Endotoxaemia followed by endotoxin shock is associated with the generation of inflammatory cell-derived mediators such as leukotrienes (LTs) and tumour necrosis factor (TNF). Our previous studies revealed that LTs and TNF, produced by treatment with lipopolysaccharide (LPS), induce a variety of morphological damage in the liver (Nagano et al. 1992) and kidney (Kita et al. 1993).

The existence of endogenous mediators in endotoxin-induced cardiovascular and respiratory dysfunction has been reported by several workers (Brigham and Meyrick 1986; Parker and Parrillo 1990), but the process in which these mediators induce morphological injuries in these organs has not been elucidated. TNF has been implicated as one of the endogenous mediators which induces endotoxin shock similar to that caused by sepsis or infusion of purified LPS. TNF, secreted after exposure to endotoxin (Giroir et al. 1992), impairs various aspects of cardiovascular and respiratory performance (Warren et al. 1989; Arbustini et al. 1991; Pagani et al. 1991). LTs, other endogenous mediators which are produced by inflammatory cells during endotoxin shock (Rouzer et al. 1980; Samuelsson 1983), also have profound effects on cardiovascular function, including coronary flow rates and myocardial contractions (Sprague et al. 1989). The question of what kind of cells in the cardiovascular and respiratory system are able to synthesize these two chemical mediators during endotoxin shock is unresolved. In the present immunocytochemical study, we investigated localization sites of TNF and LTs in the rat heart and lung. The aim of this study was to elucidate TNF- and LT- producing cells involved in the cardiorespiratory shock syndrome.

Materials and methods

Ten male Wister rats (Seiwa Experimental Animal Co., Ooita, Japan) weighing 300–350 g were used for the experiments. Under light ether anaesthesia, rats received intravenous injection of LPS (*Escherichia coli* 0127: B8; Difco, Detroit, Mich., USA) at a dose of 3.0 mg/100 g body weight. Control rats received sterile saline only. Both experimental and control animals (five rats each) were sacrificed under ether anaesthesia at 5 h after injection.

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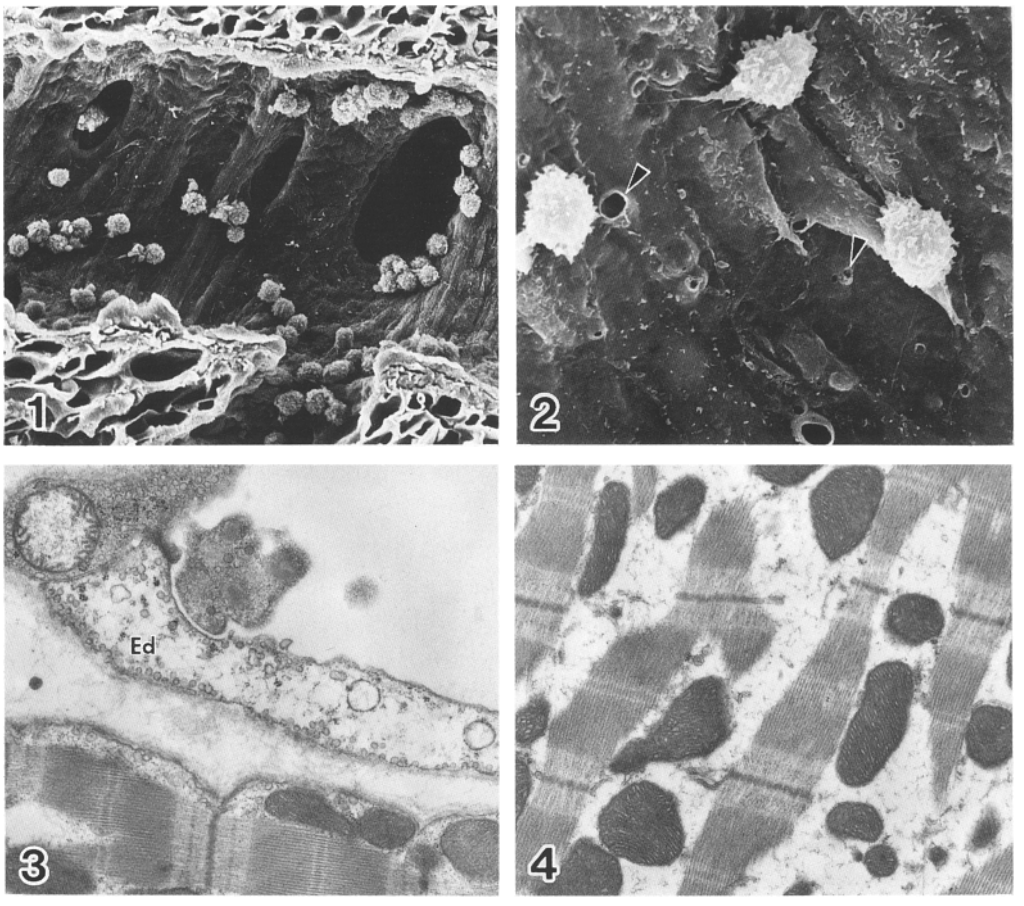


Fig. 1 Scanning electron micrograph showing the pulmonary vein at 5 h after injection of lipopolysaccharide (LPS). A considerable number of blood cells are attached to the endothelial cell surface. × 700

Fig. 2 Higher magnified scanning electron micrograph showing the pulmonary vein at 5 h after injection of LPS. Abundant pores (*arrows*) are seen in the endothelial lining. × 3,000

Fig. 3 Electron micrograph showing endothelial cells (*Ed*) of the myocardial capillary at 5 h after injection of LPS. The endothelial cells appear to be oedematous having swollen mitochondria and dilated rough endoplasmic reticulum. Stained with uranyl acetate and led citrate, × 16,000

Fig. 4 Electron micrograph showing a cardiac muscle cell at 5 h after injection of LPS. The sarcoplasm shows oedematous changes. The arrangement of myofibrils become irregular. Mitochondria appear to be morphologically intact. Stained with uranyl acetate and lead citrate, × 14,000

For scanning electron microscopy (SEM), rats were first infused through the inferior vena cava with a solution of 0.1M phosphate buffer followed by Karnovsky solution at 4° C. The heart and the lung were isolated, cut into approximately 2-mm-thick slices, and postfixed in 2% osmium tetroxide in 0.1M phosphate buffer at 4° C for 1 h. Specimens were dehydrated in ascending alcohol series, dried by the *t*-butyl alcohol freeze-drying method, coated with platinum-palladium, and examined in a Hitachi S–700 type field emission SEM.

For transmission electron microscopy (TEM), the heart and the lung were perfused in the same way, cut into approximately 2-mm-thick slices, and postfixed in 2% osmium tetroxide in the buffer at 4° C for 1 h. Specimens were dehydrated in ascending alcohol series, and embedded in Quetol 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a JEM 1200 EX electron microscope.

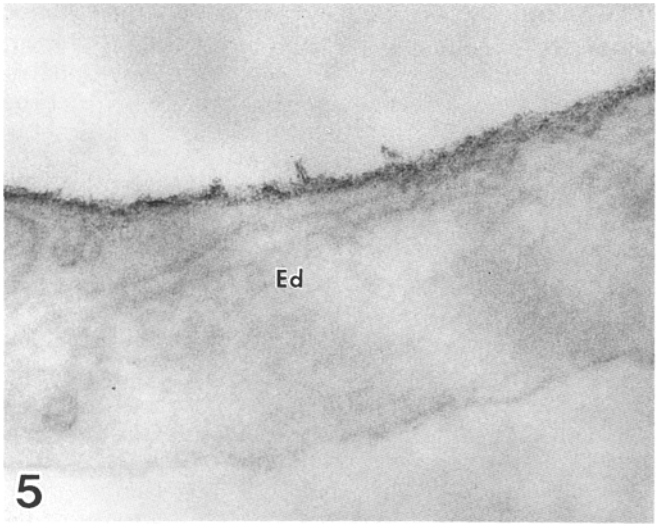
To detect immunoreactions of TNF, hamster anti-murine TNF (α and β) monoclonal antibody (Genzyme Corporation, Boston) was used at a working dilution of 1:100 in 0.1M phosphate buffer containing 0.1% bovine serum. The activity of the antibody was tested by neutralizing the bioactivity of mouse TNF in an L929-cell cytotoxicity assay (Sheehan et al. 1989). This antibody is able to neutralize 2,500–5,000 units of mouse TNF bioactivity in the L929 assay, and reacted both to the 17-kDa (TNFα) and 24.7 kDa (TNFβ) exclusive of mouse and rat. It does not exhibit any detectable reactivities to interferon-α , -β or interleukin (IL)-1.

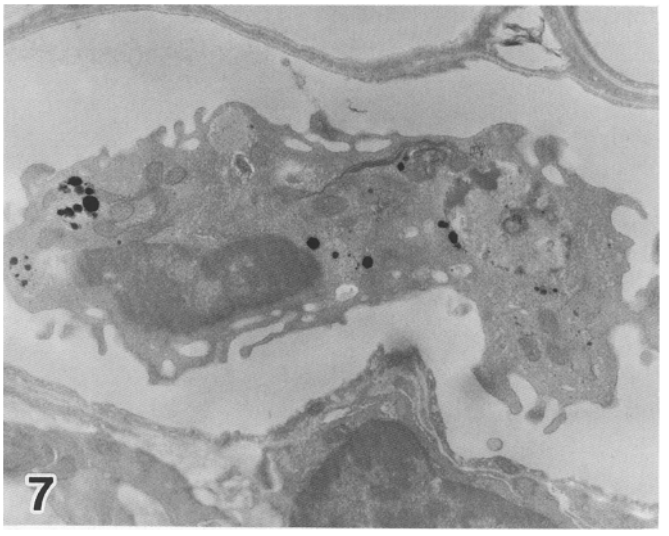
For immunoreactions of LTs, polyclonal rabbit anti-bovine LTC_4 antibody (Advanced Magnetics, catalogue no. 1052) was used at a working dilution of 1:200 in 0.1M phosphate buffer containing 0.1% bovine serum. The cross-reactivity of this antibody at 50% B/Bo value was 100% with LTD_4, 64.0% with LTC_4, 7.3% with LTE_4 and less than 0.1% with other eicosanoids.

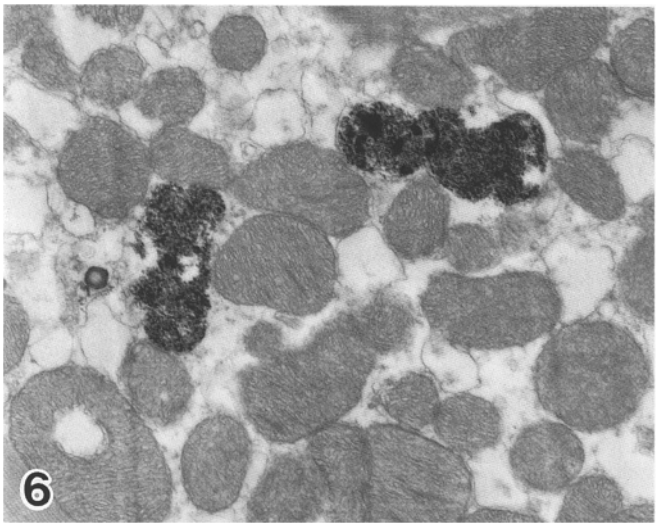
The animals were perfused with 0.1M phosphate buffer and then with a mixture of 2.5% paraformaldehyde and 0.5% glutaraldehyde in 0.1M phosphate buffer. The lung and the heart were cut into approximately 2-mm-thick slices and fixed in the same fixative for 1 h. Approximately 30- to 40-μ m-thick sections were made on a microslicer (Dosaka EM, Osaka) and inserted into a sample-mesh-pack (Shiraimatsu, Osaka).

The streptavidin-biotin technique according to the method by Shi et al. (1988) was utilized. The endogenous peroxidase activities were blocked by incubation in periodic acid solution (Histofine; Nichirei, Tokyo, code no. 292971) for 45 s. After treatment with normal goat serum at room temperature, sections were incubated overnight at 4° C with dilutions of the primary antibody described above. Sections were treated for 1 h at 37° C with the biotinylated secondary antibody (goat anti-mouse and anti-rabbit immunoglobulins: Histofine, code no. 292835) diluted 1:200 with 0.05M TRIS buffer, and then for 1 h at 37° C with streptavidin-peroxidase complex (Histofine SAB-PO kits) diluted to 1:600 with 0.05M TRIS buffer. After incubation, they were treated with a solution of 3,3'-diaminobenzidine tetrahydrochloride (in 0.05M TRIS buffer containing 0.05% hydrogen peroxide at pH 7.6) for 10 min at room temperature. Sections were thoroughly washed in 0.05M TRIS buffer, post-fixed in 1% osmium tetroxide in the buffer for 1 h at 4° C, dehydrated in ascending alcohol series, embedded in Quetol 812, and examined in a JEM 1200 EX electron microscope.

For each test, two negative control preparations were used for each section; at the stage when the test preparations were treated with the primary antibodies the controls were incubated in TRIS buffer or a suitable dilution of the appropriate non-immune serum respectively.







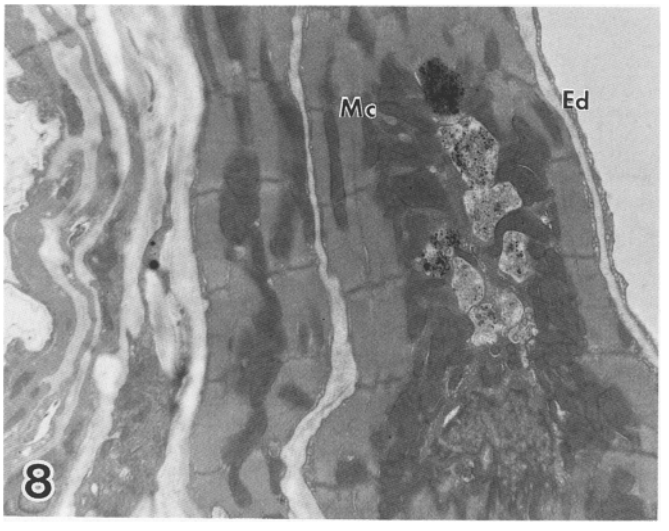


Fig. 5 Electron micrograph showing immunoreactions of tumour necrosis factor (TNF) on the apical plasma membrane of an endothelial cell (*Ed*) of the myocardial capillary at 5 h after injection of LPS. Unstained, × 110,000

Fig. 6 Electron micrograph showing immunoreactions of TNF in lysosomes of a degenerating muscle cell of the myocardium at 5 h after injection of LPS. Unstained, × 23,000

Fig. 7 Electron micrograph showing immunoreactions of peptide leukotrienes (LTs) in lysosomes of a macrophages in the pulmonary capillary at 5 h after injection of LPS. Unstained, × 13,000

Fig. 8 Electron micrograph showing immunoreactions of peptide LTs in lysosomes of a cardiac muscle cell (*Mc*) in the media of the pulmonary vein at 5 h after injection of LPS. *Ed* endothelial cell of the vein. Unstained, × 9,000

Results

At 5 h after injection of LPS, a considerable number of blood cells are attached to the endothelial lining of the pulmonary vessels when compared with control specimens (Figs. 1, 2). Pores of various sizes are seen in the endothelial lining. By TEM, we find an abundance of macrophages and leucocytes in the heart and pulmonary capillaries. The endothelial cells occasionally show cytoplasmic oedema in association with swelling of mitochondria and dilatation of rough endoplasmic reticulum, as shown in Fig. 3. Aggregates of platelets and fibrin in these capillaries are occasionally observed. Some muscle cells of the myocardium also show oedematous changes in the sarcoplasm and disturbance of myofibrillar arrangement, although the mitochondria appear to be morphologically intact (Fig. 4).

Immunoreaction for TNF was observed only in LPS-treated samples. In the pulmonary and heart blood vessels, the apical plasma membrane of the endothelial cells shows intense immunoreactions, although the basal one does not (Fig. 5). Immunoreactivity is also seen in lysosomes of inflammatory cells such as leucocytes, monocytes and macrophages, interstitial cells such as fibroblasts and alveolar septal cells, and degenerating muscle cells of the myocardium (Fig. 6).

Immunoreactions of LTs in LPS-treated samples are localized preferentially in specific granules of neu-

trophils, eosinophils and in lysosomes of pulmonary macrophages (Fig. 7). In addition to these cells, cardiac muscle cells in the media of the pulmonary vein are immunoreactive to LTs (Fig. 8), although those which undergo degeneration in the myocardium do not show immunoreactivity.

Discussion

As in the LPS-treated liver and kidney previously reported (Nagano et al. 1992; Kita et al. 1993), free cells such as macrophages and leucocytes adhere to the endothelial lining of the heart and pulmonary vessels and immunoreactivity for TNF is localized preferentially on the apical plasma membrane of the endothelial cells. This may be a morphological manifestation of a combination of TNF released from these inflammatory cells with specific receptor sites of the endothelial cell membrane, as described by Tsujimoto et al. (1985). Several workers have suggested effects of cytokines such as TNF and IL-1 as chemotatic factors for adhesion in the process of contact between endothelial and blood cells (Nawroth et al. 1986; Pohlman et al. 1986; Rampart and Williams 1988; Walsh et al. 1991). The present finding adds further weight to this assumption.

Coronary oedema and thrombosis induce the myocardial degeneration by ischaemia (Barroso-Aranda et al. 1991). However, other factors for the induction of myocardial degenerations also exist, such as alterations in rates of myocardial metabolism (Parker and Parrillo 1990) and inhibition of oxygen transport in septic shock (Edwards 1991).

In the present study, positive immunoreactivity for TNF is localized in lysosomes of degenerating cardiac muscle cells. This means that the TNF synthesized in such cells is stored in lysosomes as suggested by Giroir et al. (1992) and Nagano et al. (1992). It seems likely that this endogenous TNF in lysosomes is one of the direct toxic agents causing the induction of myocardial degeneration, without the occurrence of ischaemia.

The myocardial dysfunction in endotoxaemia is most frequently induced by mediators liberated under the influence of endotoxin (Bruni et al. 1978; Natanson et al. 1986). Among them, the peptide LT family are known as the slow-reacting substance of anaphylaxis (Samuelsson 1983). This substance has been considered to originate from the smooth muscle cells of the porcine pulmonary artery (Piper and Galton 1984) and rat and mouse myocardial cells (Karmazyn and Moffat 1984; Ikeda et al. 1987). The present study also reveals immunoreactions of LTs in those cardiac muscle cells constituting the media of the pulmonary vein (Almeida et al. 1975). This may imply that LTs synthesized in these cells are promptly released into the coronary circulation via the left ventricle, since the deleterious effects of LTs on the coronary circulation have been pointed out by Sprague et al. (1989) and Karmazyn and Moffat (1990). Our TEM observations indicate that the heart capillaries of LPS-treated samples show marked oedematous change in endothelial cells and occasional thrombosis by focal platelet aggregations (Fig. 3). Therefore, it is reasonable to assume that such changes in the microvascular beds may be due to LTs via effects on the media of the pulmonary vein, although we do not rule out the effects of other mediators such as the direct influence of LTs derived from inflammatory cells (Goldstein 1990). Further investigations as to toxic effects of LTs on the myocardium are necessary, and such studies are now in progress in our laboratory.

In conclusion, an intravenously administrated shock-inducing dose of LPS (3.0 mg/100 g) enhances of the production of TNF in the myocardium and LTs in the media of the pulmonary vein. This induces rat myocardial degeneration.

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References

Almeida OP, Bohm GM, Carvalho MP, Carvalho AP (1975) The cardiac muscle in the pulmonary vein of the rat: a morphological and electrophysiological study. J Morphol 145:409–434

Arbustini E, Grasso M, Diegoli M, Bramerio M, Foglieni AS, Albertario M, Martinelli L, Gavazzi A, Goggi C, Campana C, Vigano M (1991) Expression of tumor necrosis factor in human acute cardiac rejection. Am J Pathol 139:709–715

Barroso-Aranda J, Schmid-Schönbein GW, Zweifach BW, Mathison JC (1991) Polymorphonuclear neutrophil contribution to induced tolerance to bacterial lipopolysaccharide. Circ Res 69:1196–1206

Brigham KL, Meyrick B (1986) Endotoxin and lung injury. Am Rev Respir Dis 133:913–927

Bruni FD, Komwatana P, Soulsby ME, Hess ML (1978) Endotoxin and myocardial failure: role of the myofibril and venous return. Am J Physiol 235:H150–H156

Edwards JD (1991) Oxygen transport in cardiogenic and septic shock. Crit Care Med 19:658–663

Giroir BP, Johnson JH, Brown T, Allen GL, Beutler B (1992) The tissue distribution of tumor necrosis factor biosynthesis during endotoxemia. J Clin Invest 90:693–698

Goldstein RE (1990) Involvement of leucocytes and leukotrienes in ischaemic dysfunction of the coronary microcirculation. Eur Heart J 11:16–26

Ikeda U, Toyo-oka T, Arisaka H, Hosoda S (1987) Stimulated synthesis of prostaglandin E_2 or leukotriene C_4 from myocardial cells is not a cause but a result of their injury under hypoxia. J Mol Cell Cardiol 19:523–526

Karmazyn M, Moffat MP (1984) Calcium-ionophore stimulated release of leukotriene C_4-like immunoreactive material from cardiac tissue. J Mol Cell Cardiol 16:1071–1073

Karmazyn M, Moffat MP (1990) Positive inotropic effects of low concentrations of leukotrienes C_4 and D_4 in rat heart. Am J Physiol 259:H1239–H1246

Kips JC, Tavernier J, Pauwels RA (1992) Tumor necrosis factor causes bronchial hyperresponsiveness in rats. Am Rev Respir Dis 145:332–336

Kita T, Tanaka N, Nagano T (1993) The immunocytochemical localization of tumour necrosis factor and leukotriene in the rat kidney after treatment with lipopolysaccharide. Int J Exp Pathol 74:471–479

Nagano, T, Kita T, Tanaka N (1992) The immunocytochemical localization of tumour necrosis factor and leukotriene in the rat liver after treatment with lipopolysaccharide. Int J Exp Pathol 73:675–683

Natanson C, Fink MP, Ballantyne HK, Mac Vitte TJ, Conklin JJ, Parrillo JE (1986) Gram-negative bacteremia produces both severe systolic and diastolic cardiac dysfunction in a canine model that simulates human septic shock. J Clin Invest 78:259–270

Natanson C, Danner RL, Elin RJ, Hosseini JM, Peart KW, Banks SM, MacVittie TJ, Walker RI, Parrillo JE (1989) Role of endotoxemia in cardiovascular dysfunction and mortality. J Clin Invest 83:243–251

Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D (1986) Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J Exp Med 163:1363–1375

Pagani FD, Baker LS, Knox MA, Cheng H, Fink MP, Visner MS (1991) Load-insensitive assessment of myocardial performance after tumor necrosis factor in dogs. Surgery 111:683–693

Parker MM, Parrillo JE (1990) Myocardial function in septic shock. J Crit Care 5:47–61

Pauwels RA, Kips JC, Peleman RA, Van Der Straeten M (1990) The effect of endotoxin inhalation on airway responsiveness and cellular influx in rats. Am Rev Respir Dis 141:540–545

Piper PJ, Galton SA (1984) Generation of leukotriene B_4 and leukotriene E_4 from porcine pulmonary artery. Prostaglandins 28:905–914

Pohlman TH, Stanness KA, Beatty PG, Ochs HD, Harlan JM (1986) An endothelial cell surface factor(s) induced in vitro by lipopolysaccharide, interleukin 1, and tumor necrosis factor-α increases neutrophil adherence by a CDw18-dependent mechanism. J Immunol 136:4548–4553

Rampart M, Williams TJ (1988) Evidence that neutrophil accumulation induced by interleukin-1 requires both local protein biosynthesis and neutrophil CD18 antigen expression in vivo. Br J Pharmacol 94:1143–1148

Rouzer CA, Scott WA, Cohn ZA, Blackburn P, Manning JM (1980) Mouse peritoneal macrophages release leukotriene C in response to a phagocytic stimulus. Proc Natl Acad Sci USA 77:4928–4932

Samuelsson B (1983) Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science 220:568–575

Sheehan KCF, Ruddle NH, Schreiber RD (1989) Generation and characterization of hamster monoclonal antibodies that neutralize murine tumor necrosis factors. J Immunol 142:3884–3893

Shi Z, Itzkowitz SH, Kim YS (1988) A comparison of three immunoperoxidase techniques for antigen detection in colorectal carcinoma tissues. J Histochem Cytochem 36:317–322

Sprague RS, Stephenson AH, Dahms TE, Lonigro AJ (1989) Proposed role for leukotrienes in the pathophysiology of multiple systems organ failure. Crit Care Clin 5:315–329

Tsujimoto M, Yip YK, Vilcek J (1985) Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. Proc Natl Acad Sci USA 82:7626–7630

Walsh LJ, Trinchieri G, Waldorf HA, Whitaker D, Murphy GF (1991) Human dermal mast cells contain and release tumor necrosis factorα , which induces endothelial leukocyte adhesion molecule 1. Proc Natl Acad Sci USA 88:4220–4224

Warren JS, Yabroff KR, Remick DG, Kunkel SL, Chensue SW, Kunkel RG, Johnson KJ, Ward PA (1989) Tumor necrosis factor participates in the pathogenesis of acute immune complex alveolitis in the rat. J Clin Invest 84:1873–1882