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Dexamethasone modulation of tumour necrosis factor-α (cachectin) release by activated normal human alveolar macrophages

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ABSTRACT: Recurrent infections of the lower respiratory tract are a frequent and serious side-effect of chronic corticosteroid treatment. Since alveolar macrophages (AM) are currently thought to play a central role in the protection of the lower respiratory tract against infectious agents, it is likely that a steroid-induced deficiency of AM is involved in this process. In this respect, when activated, AM are major producers of tumour necrosis factor-α (TNF or cachectin), a versatile cytokine with several biological properties including antiviral and anti-infectious activities. A deficit of TNF production induced by corticosteroids may be one mechanism of the sensitivity to infections. Thus normal human AM obtained by bronchoalveolar lavage were pretreated with dexamethasone (DXM) before activation with lipopolysaccharides (LPS) and the amounts of TNF released in culture were quantified. Pretreatment with DXM resulted in a marked decrease of TNF release in a dose-dependent fashion. In contrast, when AM were activated with LPS before DXM treatment, TNF release by AM was suppressed in a more limited fashion. Thus DXM suppression of LPS-activated AM ability to release TNF may play a role in the susceptibility to infections of patients chronically treated with corticosteroids.

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Cachectin, or tumour necrosis factor-α (TNF), a cytokine produced by mononuclear phagocytes, can stimulate numerous biological activities [1–4] including: cytotoxicity for certain tumour cell lines, induction of cachexia and endotoxic shock, antiviral [5–7] and anti-infectious activities [8–12]. In this respect, the human lower respiratory tract is physiologically sterile and this property is currently thought to be related to the presence of alveolar macrophages (AM) [13, 14]. Thus, the ability of AM to release *in situ* large amounts of TNF may participate in the protection of the lower respiratory tract against infections [15, 16].

Impairment of lung defences against infections is a common and serious side-effect of chronic corticosteroid treatment [17] and, since TNF is a cytokine likely to play a role in the protection of the lower respiratory tract, lipopolysaccharide(LPS)-activated normal AM were evaluated with or without pretreatment with corticosteroids (dexamethasone (DXM)), demonstrating that pretreatment with DXM suppressed TNF release, while, in contrast, when DXM treatment was applied after LPS activation, TNF release was reduced in a limited fashion.

Materials and methods

Isolation of normal alveolar macrophages

Fourteen normal subjects (9 men, 5 women, mean age 37±5 yrs), all nonsmokers, with no current or past history of lung disorders and with normal chest X-ray and lung function test, underwent bronchoalveolar lavage (BAL), as described previously [18]. Briefly, after local anaesthesia, successive aliquots of 50 ml of sterile saline solution were infused and subsequently recovered by gentle aspiration. For each subject, three different lobes were lavaged with a total maximum of 250 ml of saline solution instilled. The recovered cells were separated from the epithelial lining fluid by centrifugation and resuspended for total cell count and lavage cell differential (by cytocentrifugation preparation): AM: 92±3%, lymphocytes: 6±2%, neutrophils 1±1%, and eosinophils <1%. AM were purified by adherence to plastic dish (1 h, 37°C, 10% CO_2) in 24-well Falcon plates (Becton Dickinson Labware, Lincoln Park, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO, USA). In order to have a relatively fixed number of AM in

each well for every subject ($1\pm0.3 \times 10^6 AM \cdot ml^{-1}$), the number of lavage cells plated depended on the initial number of AM counted by cell differential. After adherence, non-adherent cells were eliminated by several washes. AM purity was >97% and viability >93% (assessed by trypan blue exclusion).

Activation of alveolar macrophages

Purified AM were activated by addition of lipopolysaccharides (LPS, *Escherichia coli* 0127; B8, Sigma) and cultured (24 h, DMEM, 10% CO_2). As an initial step, different concentrations of LPS were tested and subsequently, if not otherwise stated, an optimal standard concentration of 10 $\mu g \cdot ml^{-1}$ was used. At this concentration, LPS had no significant toxic effect on AM and did not interact with TNF quantification.

Tumour necrosis factor-α quantification

TNF present in AM supernatants was detected and quantified by its cytotoxic activity on mouse L-929 cells (CCL1; American Tissue Culture Collection, Rockville, MD, USA) using the assay described by AGGARWAL and KOHR [19]. Recombinant TNF was used as a standard, and anti-TNF antibody was added in positive samples for specificity. Both TNF and anti-TNF antibody were generous gifts from Boehringer Ingelheim (Vienna, Austria). Media alone was the negative control and guanidium hydrochloride (3 M, Sigma) was used for maximum lysis. After incubation of L-929 cells (18h, 37°C, 10% CO_2) in different conditions, the cells were washed with cold phosphate buffered saline (PBS), and cell viability was assessed by crystal violet (Sigma) incorporation (30 min, 24°C) by viable L-929 cells [19], and quantified at 540 nm after cell lysis in distilled water as shown in figure 1A. Successive dilutions (1 to 1 in DMEM) of each sample of supernatant were tested in duplicate and the amount of TNF present was determined in regard to TNF standard curves (fig. 1B). The results are expressed as number of TNF units released by 10^6 AM. Several controls were used to assure that the cytotoxicity observed was due to the specific presence of TNF: 1) since interleukin-1 (IL-1), another cytokine produced (but in small amounts) by AM, can have, in some experimental conditions, a cytotoxic activity on L-929 cells [20], IL-1-α and IL-1-β were tested for cytotoxicity on the cells used. In our hands, neither IL-1-α nor IL-1-β had cytotoxic activity in the test, consistent with the fact that some subclones of L-929 cells are not sensitive to IL-1 cytotoxicity; 2) to further confirm the specific detection of TNF by this test, each positive sample was retested in parallel, straight and after incubation with anti-TNF antibody (1 h, 24°C) demonstrating that only 4±2% of the cytotoxicity measured was related to the presence of mediator(s) other than TNF-α.

Dexamethasone treatment of alveolar macrophages

Dexamethasone (DXM, Sigma) if not otherwise stated, was added to AM for 1 h, at 37°C, in 10% CO_2; the cells were then washed three times to remove DXM present in the supernatants before activation by addition of LPS, and culture (24 h, DMEM, 10% CO_2). On the other hand, when DXM was applied to AM after LPS activation, LPS (10 $\mu g \cdot ml^{-1}$) was added for 1 h, at 37°C, in 10% CO_2, before treatment with DXM applied as described above. Several dilutions of DXM (10^{-5} to 10^{-10}M) were tested. However, DXM at 10^{-5}M concentration had some effects on cell viability. At the concentrations used (10^{-6} to 10^{-9}M), DXM had no specific effect on TNF assay.

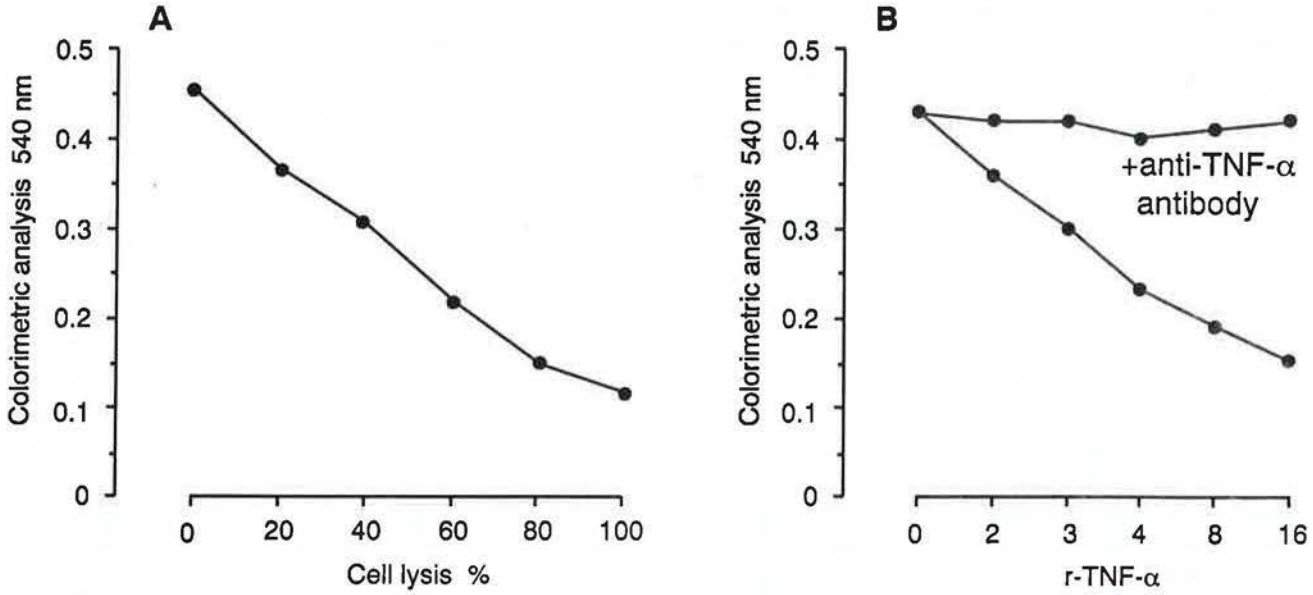

Fig. 1. – Quantification of tumour necrosis factor-α (TNF-α) by its cytotoxic activity on mouse L-929 cells. A) Colorimetric assessment by crystal violet incorporation of L-929 cell lysis as described in Materials and Methods. B) Example of standard curve of cytotoxicity of increasing concentrations of recombinant TNF (r-TNF-α $u \cdot ml^{-1}$) tested straight or after incubation with anti-TNF-α antibody.

Statistical analysis

All data are presented as mean±standard error of the mean. All statistical comparisons were made using a two-tailed Student's t-test.

preliminary observation, AM were subsequently activated with 10 μg·ml^{-1} of LPS. LPS-activated AM release of TNF was confirmed for all subjects (fig. 2B): 839±72 TNF units *vs* 2±1 TNF units released by inactivated AM, $p<0.01$.

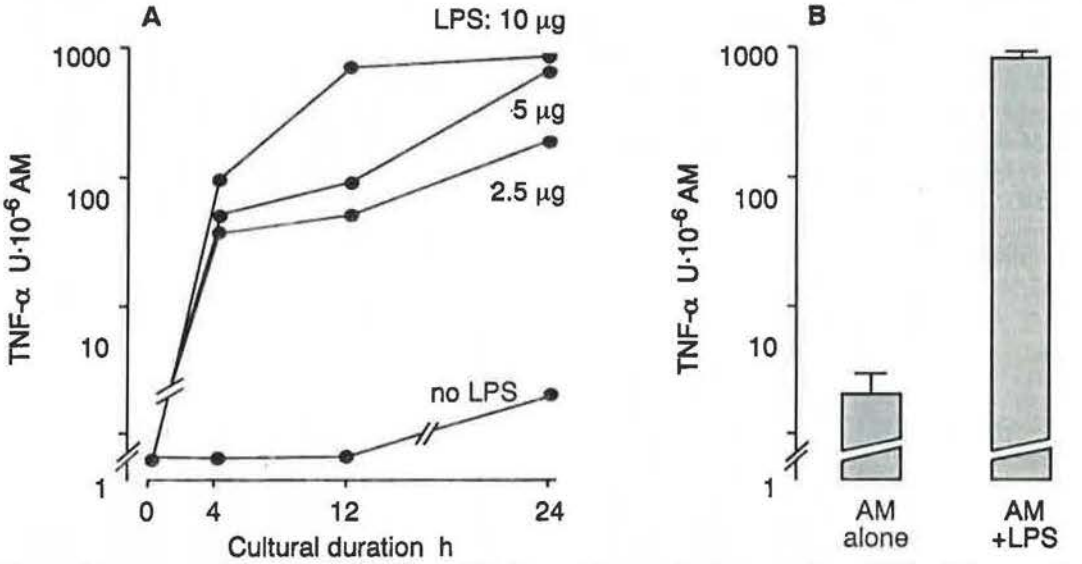

Fig. 2. – Release of tumour necrosis factor-α (TNF-α) by activated normal human alveolar macrophages (AM). AM were cultured alone or with addition of lipopolysaccharides (LPS). The amounts of TNF present in the supernatants after culture were quantified as described in Materials and Methods. A) Time-dependent and LPS dose-dependent production of TNF by AM: each point represents the mean for two evaluations; the data shown correspond to one representative experiment using alveolar macrophages of one subject. B) TNF release over 24 h in culture by AM from all subjects with or without activation by LPS (10 μg·ml^{-1}).

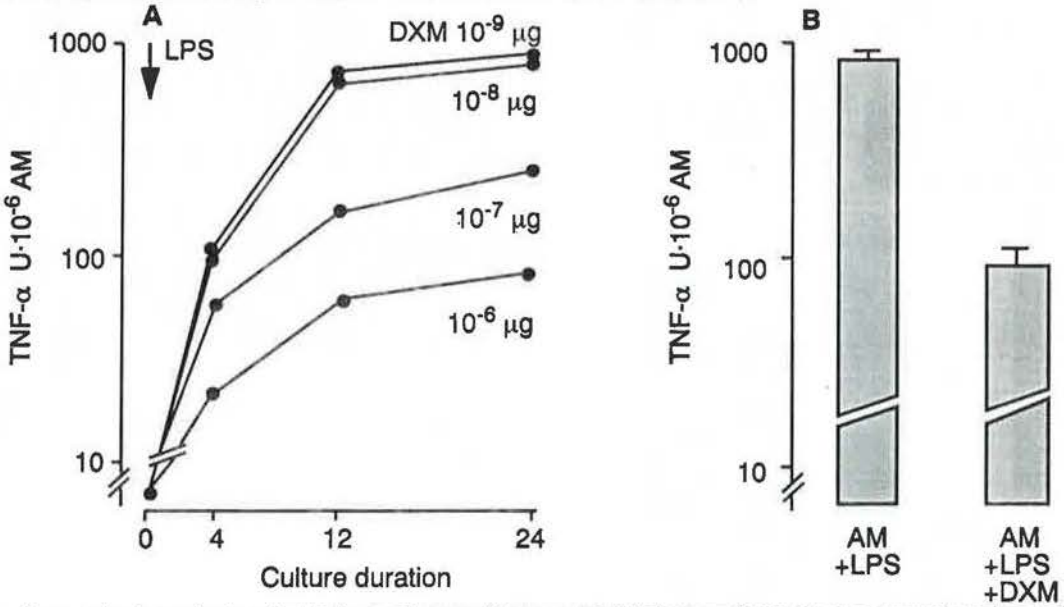

Fig. 3. – Suppression by pretreatment with dexamethasone of tumour necrosis factor-α (TNF-α) release by activated normal human alveolar macrophages (AM) AM were pretreated by serial dilutions of dexamethasone (DXM) before being activated by addition of lipopolysaccharides (LPS, 10 μg·ml^{-1}) and cultured for various durations of time. The amounts of TNF present in the supernatants after culture were quantified as described in Materials and Methods. A) Dose-dependent inhibition by dexamethasone of TNF release by AM: each point represents the mean for two evaluations; the data shown correspond to one representative experiment using AM of one subject. B) Dexamethasone (10^{-6} M) inhibition of TNF release by LPS (10 μg·ml^{-1}) activated AM.

Results

In order to define TNF optimal release by activated human AM, AM from normal subjects were cultured alone or in the presence of increasing concentrations of LPS (fig. 2A). Whilst normal AM did not spontaneously release any significant amount of TNF, the addition of LPS induced a dose-dependent and time-dependent release of TNF. Maximum release of TNF was observed with an LPS concentration of 10 μg·ml^{-1} without cell toxicity and without effect on TNF quantification by L-929 test. As a result of this

To address the question of DXM effects on TNF release, normal AM were pretreated by incubation with DXM and, after removal of DXM, the same AM were activated by LPS and cultured. TNF release was suppressed in a dose-dependent fashion with a maximal suppression, without cytotoxicity and without effect on TNF assay, for a concentration of DXM of 10^{-6}M (fig. 3A), and concentrations lower than 10^{-8}M were without effect on TNF release. As a result of this preliminary observation, AM from all subjects were pretreated with 10^{-6}M of DXM before LPS activation. This procedure resulted in a constant and marked suppression of TNF release (fig. 3B): 86±13 *vs* 839±72 TNF units, p<0.01.

To evaluate whether this suppression was only specific for optimally activated AM (10 $\mu g \cdot ml^{-1}$ of LPS) or was also observed with AM cultured in the presence of lower concentrations of LPS, AM from several normal subjects were pretreated with 10^{-6}M DXM before activation with dilutions of LPS (fig. 4). Interestingly, for each concentration of LPS, there was a suppression of TNF release in relation with DXM pretreatment: LPS 10 $\mu g \cdot ml^{-1}$: 839±72 *vs* 86±13 TNF units, p<0.01; LPS 5 $\mu g \cdot ml^{-1}$: 666±36 *vs* 58±23 TNF units, p<0.01; LPS 2.5 $\mu g \cdot ml^{-1}$: 224±57 *vs* 53±26 TNF units, p<0.01; and LPS 1 $\mu g \cdot ml^{-1}$: 127±51 *vs* 23±14 TNF units, p<0.05.

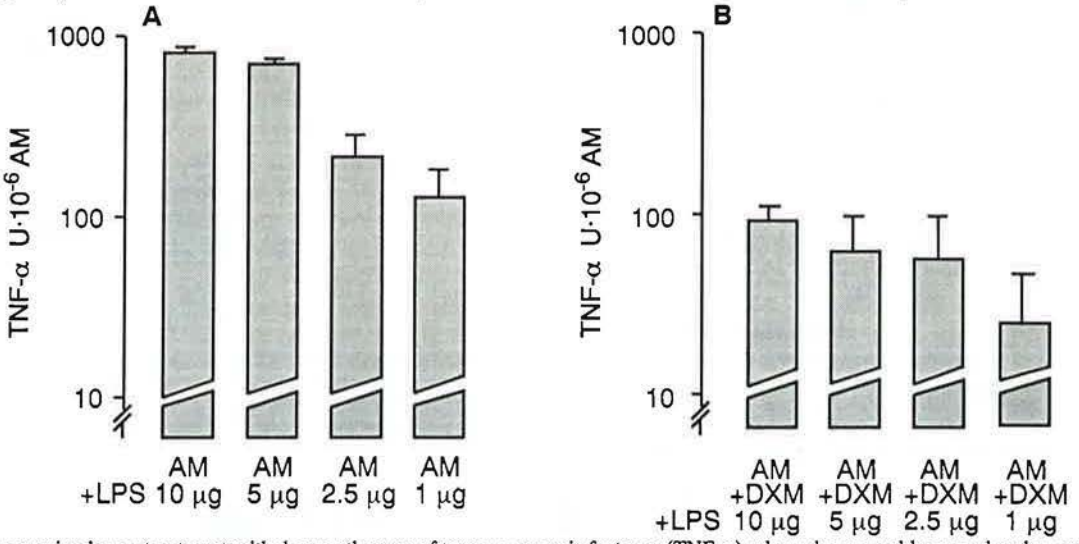

Fig. 4. – Suppression by pretreatment with dexamethasone of tumour necrosis factor-α (TNF-α) release by normal human alveolar macrophages (AM) activated with several concentrations of lipopolysaccharides. AM obtained from normal subjects (n=5) were cultured (24 h) after activation by addition of dilutions of lipopolysaccharides (LPS). The amounts of TNF present in the supernatants after culture were quantified as described in Materials and Methods. A) Absence of pretreatment with dexamethasone. B) AM were pretreated with dexamethasone (DXM; 10^{-6} M) before LPS activation.

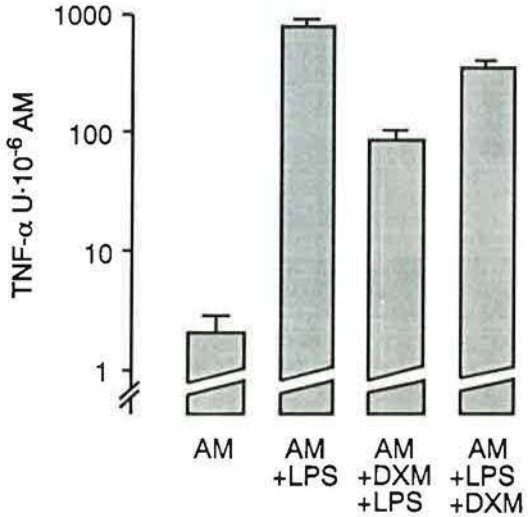

Fig. 5. – Limited suppression of tumour necrosis factor-α (TNF-α) release by normal alveolar macrophages (AM) activated with lipopolysacchrides before dexamethasone treatment. AM from normal subjects (n=6) were cultured for 24 h, alone (AM), with addition of lipopolysaccharides (10 $\mu g \cdot ml^{-1}$) (AM+LPS), with addition of LPS after pretreatment with dexamethasone (10^{-6} M) (AM+DXM+LPS), or with treatment with DXM after preactivation with LPS (AM+LPS+DXM). The amounts of TNF present in the supernatants after culture were quantified as described in Materials and Methods.

Finally, in order to define whether DXM treatment could suppress TNF release only when applied before LPS activation or also when applied after LPS activation, normal AM were isolated and cultured in parallel either with DXM treatment before LPS activation or with LPS activation before DXM treatment. As a result (fig. 5), DXM treatment was shown to have a limited effect on TNF release by AM when applied after LPS activation: 530±47 TNF units *vs* 839±72 TNF units with LPS alone (p<0.01), and *vs* 86±13 TNF units with DXM treatment prior to LPS activation (p<0.001), suggesting that AM, once activated, are less sensitive to DXM treatment.

Discussion

Activated human AM release high amounts of TNF, and the suppression of this release by pretreatment with DXM is of interest in respect to this cytokine's biological activities and the current understanding of its role in lung disorders.

TNF is a monokine initially purified as a factor inducing *in vitro* and *in vivo* cell lysis of some cancer cell lines [1–4], and characterized by other major

activities involved with: cachexia, anti-infectious defences, regulation of acute phase protein gene expression, cartilage and bone resorption, and the modulation of endothelial cell properties [1–4]. In the lung, activated normal AM produce elevated amounts of TNF in comparison to autologous blood monocytes, suggesting that high *in situ* levels of TNF can be present in the lung when AM are activated [15, 16]. Furthermore, TNF release has been suggested to play a role in several lung disorders including: the adult respiratory distress syndrome (ARDS) complicating the endotoxic shock [21], sarcoidosis [22], and pneumoconiosis [23].

Corticosteroids exert their effects by interacting with specific receptors present on target cells. In this respect, AM express receptors for corticosteroids [24], and DXM modulation of TNF release has previously been shown to be exerted at both transcriptional and post-transcriptional levels for mouse macrophages, and human blood monocytes [25, 26]. Furthermore, DXM, at concentrations above 10^{-5}M, has been suggested to reduce TNF production by normal human AM [16].

Our observations can be related to the alteration of the anti-infectious defences induced by chronic corticosteroid treatment [17], and to the pathogenicity of the endotoxic shock. TNF is likely to play a role in lung protection against infections by eliciting neutrophils and by activating them by induction of their phagocytosis, cytotoxic activities, degranulation, and release of reactive oxygen radicals in parallel with a stimulation of macrophage cytotoxicity in an autocrine fashion [8–12, 27, 28]. Furthermore, TNF can induce an antiviral state in some target cells [5–7]. Thus, the absence of a high local release of TNF by AM activated by infectious bodies present in the lower respiratory tract can lead, in parallel with the steroid-induced suppression of a number of biological processes, to a decrease of lung defences and the subsequent development of an infectious condition.

This information is also relevant to the pathogenicity of the endotoxic shock, knowing that, at least in experimental models, TNF has been shown to be able to induce a shock [1–4] and that pretreatment by anti-TNF antibody prevents the animal from developing a toxic shock after endotoxin injection [29]. Furthermore, pretreatment with corticosteroids can prevent the endotoxic shock while, once the endotoxin-induced pathological state has been started, corticosteroid treatment is unable to stop the evolution [30]. These data and clinical observations [21] suggest that TNF release may play a central role in the pathogenicity of the ARDS complicating the endotoxic shock. In this respect, the marked suppression by DXM of TNF release by AM, only before their activation, is consistent with the fact that corticosteroids would be efficient in preventing an endotoxic shock with ARDS only when prescribed as a preventive treatment in patients likely to develop this condition.

References

1. Ruff MR, Gifford GE. – Tumor necrosis factor. *Lymphokines*, 1981, 2, 235–272.
2. Beutler B, Greenwald D, Hulmes JD, Chang M, Pan Y-CE, Mathison J, Ulevitch R, Cerami A. – Identity of tumor necrosis factor and the macrophage-secreted factor cachectin. *Nature*, 1985, 316, 552–554.
3. Beutler B, Cerami A. – Cachectin and tumor necrosis factor as two sides of the same biological coin. *Nature*, 1986, 320, 584–588.
4. Dinarello CA. – Cytokines: interleukin-1 and tumor necrosis factor (cachectin). *In*: Inflammation: Basic Principles and Clinical Correlates. J.I. Gallin, I.M. Goldstein, R. Snyderman, eds., Raven Press Ltd, New York, 1988, pp. 195–208.
5. Mestan J, Digel W, Mittnacht S, Hillen H, Blohm D, Müller A, Jacobsen H, Kirchner H. – Antiviral effects of recombinant tumor necrosis factor *in vitro*. *Nature*, 1986, 323, 816–819.
6. Wong GHW, Goeddel DV. – Tumor necrosis factors α and β inhibit virus replication and synergize with interferons. *Nature*, 1986, 323, 819–822.
7. Koff WC, Fann AV. – Human tumor necrosis factor-α kills herpes virus-infected but not normal cells. *Lymphokine Res*, 1986, 5, 215–221.
8. Klebanoff SJ, Vadas MA, Harlan JM, Sparks LJ, Gamble JR, Agosti JM, Waltersdorph AM. – Stimulation of neutrophils by tumor necrosis factor. *J Immunol*, 1986, 136, 4220–4225.
9. Tsujimoto M, Yokota S, Vilcek J, Weissman C. – Tumor necrosis factor provokes superoxide anion generation from neutrophils. *Biochem Biophys Res Commun*, 1986, 137, 1094–1100.
10. Djeu JY, Blanchard DK, Halkias D, Friedman H. – Growth inhibition of *Candida albicans* by human polymorphonuclear neutrophils: activation by interferon-gamma and tumor necrosis factor. *J Immunol*, 1986, 137, 2980–2984.
11. Ming WJ, Bersani L, Mantovani A. – Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. *J Immunol*, 1987, 138, 1469–1474.
12. Philip R, Epstein LB. – Tumor necrosis factor as immunomodulator and mediator of monocyte cytoxicity induced by itself, gamma-interferon, and interleukin-1. *Nature*, 1986, 323, 86–89.
13. Hocking WG, Golde DW. – The pulmonary-alveolar macrophage. *New Engl J Med*, 1979, 301, 580–587, and 639–645.
14. Bowden DH. – The alveolar macrophage. *Environ Health Perspect*, 1984, 55, 327–341.
15. Martinet Y, Yamauchi K, Crystal RG. – Differential expression of the tumor necrosis factor/cachectin gene by blood and lung mononuclear phagocytes. *Am Rev Respir Dis*, 1988, 138, 659–665.
16. Strieter RM, Remick DG, Lynch JP III, Genord M, Raiford C, Spengler R, Kunkel SL. – Differential regulation of tumor necrosis factor-α in human alveolar macrophages and peripheral blood monocytes: a cellular and molecular analysis. *Am J Respir Cell Mol Biol*, 1989, 1, 57–63.
17. Siegel SC. – Corticosteroid agents; overview of corticosteroid therapy. *J Allergy Clin Immunol*, 1985, 76, 312–320.
18. Klech H, Pohl W. – Technical recommedations and guidelines for bronchoalveolar lavage (BAL). *Eur Respir J*, 1989, 2, 561–585.

19. Aggarwal BB, Kohr WJ. – Human tumor necrosis factor. *Methods Enzymol*, 1985, 116, 448–456.
20. Onozaki K, Matsushima K, Aggarwal BB, Oppenheim JJ. – Human interleukin-1 is a cytocidal factor for several tumor cell lines. *J Immunol*, 1985, 135, 3962–3968.
21. Marks JD, Marks CB, Luce JM, Montgomery AB, Turner J, Metz CA, Murray JF. – Plasma tumor necrosis factor in patients with septic shock. Mortality rate, incidence of adult respiratory distress syndrome, and effects of methylprednisolone administration. *Am Rev Respir Dis*, 1990, 141, 94–97.
22. Bachwich PR, Lynch JP III, Larrick J, Spengler M, Kunkel SL. – Tumour necrosis factor production by human sarcoid alveolar macrophages. *Am J Pathol*, 1986, 125, 421–425.
23. Borm PJA, Palmen N, Engelen JJM, Buurman WA. – Spontaneous and stimulated release of tumor necrosis factor-α (TNF) from blood monocytes of miners with coal workers pneumoconiosis. *Am Rev Respir Dis*, 1988, 138, 1589–1594.
24. Werb Z, Foler R, Munck A. – Interaction of glycocorticoids and macrophages. Identification of glycocorticoid receptors in monocytes and macrophages. *J Exp Med*, 1978, 147, 1684–1694.
25. Beutler B, Krochin N, Milsark IW, Luedke C, Cerami A. – Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. *Science*, 1986, 232, 977–980.
26. Sariban E, Imamura K, Leubbers R, Kufe D. – Transcriptional and posttranscriptional regulation of tumor necrosis factor gene expression in human monocytes. *J Clin Invest*, 1988, 81, 1506–1510.
27. Shalaby MR, Aggarwal BB, Rinderknecht E, Sverdesky LP, Finkle BS, Palladino MA Jr. – Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors. *J Immunol*, 1985, 135, 2069–2073.
28. Larrick JW, Graham D, Toy K, Lin LS, Senyk G, Fendly BM. – Recombinant tumor necrosis factor causes activation of human granulocytes. *Blood*, 1987, 69, 640–644.
29. Beutler B, Milsark IW, Cerami A. – Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science*, 1985, 229, 869–871.
30. Brigham KL, Bowers RE, McKeen CR. – Methylprednisolone prevention of increased lung vascular permeability following endotoxemia in sheep. *J Clin Invest*, 1981, 67, 1103–1110.