TUMOUR NECROSIS FACTOR ALPHA AND ITS SOLUBLE RECEPTORS
PARALLEL CLINICAL DISEASE AND AUTOIMMUNE ACTIVITY IN
SYSTEMIC LUPUS ERYTHEMATOSUS

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SUMMARY

Cytokines are believed to play an important role in the pathogenesis of systemic lupus erythematosus (SLE). However, for tumour necrosis factor alpha (TNF-α) both beneficial and deleterious effects have been reported. To obtain information about the involvement of this cytokine in the pathophysiology of SLE, serum levels of TNF-α, the soluble forms of the 55 and 75 kDa tumour necrosis factor receptors (TNF-R55 and TNF-R75), and interleukin-6 (IL-6) were measured by ELISA in nine female patients over a period of 2 yr. Compared to healthy controls, levels of TNF-α (median 47 pg/ml, range <15–222 pg/ml), TNF-R55 (median 1.9 ng/ml, range 0.8–10.8 ng/ml), TNF-R75 (median 4.7 ng/ml, range 1.5–15 ng/ml) and IL-6 (median 3.5 pg/ml, range <3.5–52 pg/ml) were significantly elevated in SLE patients (P < 0.0001 vs controls in all cases). There were strong correlations between TNF-α and its soluble receptors (P < 0.0001). Moreover, TNF-α and both TNF-Rs strongly correlated with clinical and serological parameters of disease activity, such as the European Consensus Lupus Activity Measurement (ECLAM) score, anti-dsDNA antibodies, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and anaemia (P < 0.0001 for all comparisons). TNF-α and TNF-R75 also correlated with IL-6 (P < 0.0001). However, no correlation between IL-6 and ECLAM was found, and the correlation of IL-6 with anti-dsDNA was relatively weak; in contrast, IL-6 correlated strongly with CRP and ESR (P < 0.0001). Although these data do not allow us ultimately to discriminate between beneficial and deleterious effects of TNF-α, they nevertheless suggest a central role for the TNF system in the pathophysiology of SLE.

KEY WORDS: SLE, Cytokines, TNF-α, TNF receptors, IL-6.

SYSTEMIC lupus erythematosus (SLE) is an autoimmune disease of as yet unknown aetiology. Aberrant immune regulation, B-cell hyperactivity and the production of autoantibodies are cornerstones of its pathophysiology [1]. Studies of lymphokines in patients with SLE have suggested that these messenger peptides might play an important role in the pathogenesis of this disease [2]. In fact, T-cell products such as interferon gamma (IFN-γ) [3, 4] or the soluble interleukin-2 (IL-2) receptor [5, 6] have been found to be increased in sera of SLE patients, and hyperproduction of IL-2 in vivo has also been suggested [7].

The role of monokines is less clear. Thus, studies on tumour necrosis factor alpha (TNF-α) have so far led to some controversial results. TNF-α administered at high doses to lupus-prone (NZB × NZW)F1 mice before apparent disease manifestation seemed to have a protective effect [8], whereas TNF-α administration in low amounts at a later stage led to the development of nephritis [9]. In other lupus-prone strains (MRL/l, BXSB), the administration of TNF-α had no beneficial effect or even accelerated disease [10]. Moreover, transcripts of TNF-α were detected in the kidneys of mice with SLE [9–11] and glomerular basement membrane-containing immune complexes were shown to induce the formation of TNF-α in vitro [12].

Controversial observations have also been reported in patients with SLE. In one study, slightly elevated TNF-α serum levels were found only during chronic infections [13], whereas another study failed to detect TNF-α while finding serum levels of the soluble TNF-receptors (TNF-R) to be increased [14]. Other investigators reported elevated TNF-α levels, but did not see any clear-cut correlation with disease activity or the acute-phase response [15]. Interestingly, TNF genes are located within the major histocompatibility complex (MHC) and differences in TNF-α production were seen in patients with different MHC class II genotypes [16]. Moreover, TNF genes are polymorphic and significant associations between certain TNF alleles, MHC haplotypes and SLE have been found (reviewed in [17]). Based on these findings, an involvement of TNF-α in the genetic predisposition to autoimmune disease and severity of lupus has been suggested.

The effects of TNF-α can be inhibited in vitro by soluble receptors which are formed by proteolytic cleavage of cell surface TNF-R [18]. The production of these soluble receptors is enhanced by TNF-α itself [19] and results in downregulation of the membrane-expressed receptors. The soluble forms of the receptors compete with membrane-bound forms for TNF-α and thus may block its function [20]. However, there is also evidence that binding of TNF-α by soluble receptors in vivo might protect the cytokine from proteolytic inactivation, thereby increasing its half-life in the circulation [21]. Increased TNF-R levels have been

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observed in several diseases, including SLE and rheumatoid arthritis (RA), and may correlate with disease activity [14, 22-24].

In contrast to the discrepant results obtained for TNF-α, there have been consistent reports on elevated IL-6 levels in SLE [25-29]. IL-6 influences B-cell differentiation and is a potent inducer of immunoglobulin production, including anti-double-stranded (ds) DNA antibody [30, 31]. Thus, IL-6 could be one of the important factors in SLE inducing B-cell hyperactivity and autoreactivity. However, controversy exists regarding the correlation of the acute-phase protein levels with IL-6 [15, 25-27].

In order to elaborate further the role of monokines in the pathophysiology of SLE, the TNF-α/TNF-R system as well as IL-6 were analysed in SLE patients over a period of 2 yr. Levels of TNF-α, TNF-Rs and IL-6 were found to be increased, and correlated with clinical disease activity and serological abnormalities.

**RESULTS**

**Patients and controls**

Over a period of 2 yr, 7-9 serum samples (a total of 70) were collected from each of nine female patients with SLE. All patients met the American College of Rheumatology 1982 revised criteria for the classification of SLE [32]. Patients' ages were between 20 and 68 yr (median 42 yr), and disease duration ranged from <1 to 14 yr (median 7 yr). Informed consent was obtained from all patients. During the observation period, patients were subjected to different therapeutic regimens which included no treatment (two patients/eight samples), 2.5-50 mg prednisolone/day (six patients/22 samples), prednisolone plus hydroxychloroquine (two patients/eight samples), hydroxychloroquine only (one patient/two samples) and prednisolone plus azathioprine (two patients/14 samples). Three patients received prednisolone plus monthly cyclophosphamide pulse therapy (nine samples), two of them starting during the observation period. Two patients were subjected to haemodialysis, one of them intermittently (seven samples). None of the samples was taken during an episode of a severe bacterial infection requiring hospitalization. One patient (no. 5) acquired a cytomegalovirus infection and was treated adequately.

Seventeen age-matched healthy individuals served as controls for TNF-R and IL-6 determinations; 70 samples from 35 healthy controls (two samples per person, 1 month apart) were used for TNF-α determination.

**Measurement of cytokines and soluble receptors**

TNF-α was measured by ELISA (Médgenix, Fleurus, Belgium; detection limit 15 pg/ml). This ELISA detected both free and receptor-bound TNF-α. The soluble 55 and 75 kDa TNF-Rs (TNF-R55 and TNF-R75) were measured by ELISAs (R&D Systems Inc., Minneapolis, MN, USA; detection limit 200 pg/ml). IL-6 was measured by ELISA (R&D Systems Inc., Minneapolis, MN, USA; detection limit 3.5 pg/ml). All samples were measured in duplicates. The specificity and sensitivity of cytokine measurements were assessed by determining the recovery of exogenously added cytokines (20, 50, 200 pg/ml) in serum samples, and by pre-incubation of cytokine-containing sera with increasing amounts of neutralizing antibodies (Endogen, Boston, MA, USA); these antibodies were able to decrease cytokine levels down to the detection limit, demonstrating the specificity of the assay.

Both intra- and inter-assay reproducibility were tested by using five selected serum samples as internal controls. Intra-assay variation was better than 10%; inter-assay variation was better than 20%. In addition to internal validation, all assays were validated within the Cytokine Consensus Study Group of the European Workshop for Rheumatology Research [34].

**Statistical analysis**

Owing to the skewed distribution of the data, summary statistics are given as medians and 5-95% ranges. The Kendall correlation coefficient was used to assess correlation between disease activity and cytokine levels, and for comparisons between different strata of subjects the Wilcoxon test was used. Data were processed using the SAS package.

**Assessment of clinical and immunological parameters**

At each visit, patients were examined clinically and disease activity was determined using the European Consensus Lupus Activity Measurement (ECLAM) score [33]. For all patients, the following laboratory parameters were recorded: ESR, erythrocyte (RBC) and leucocyte (WBC) cell counts, and serum creatinine levels. Proteinuria was assessed by dipstick. CRP and complement C3 and C4 levels were measured by nephelometry. Anti-dsDNA antibodies were determined by indirect immunofluorescence on *Crithidia lucilae* (Bios, München, Germany) and by radioimmunoassay (Amersham, UK). ANA were analysed by indirect immunofluorescence using Hep2 cells (Whittaker Bioproducts, Walkersville, MD, USA). Autoantibodies to Sm, U1-RNP, Ro and La were determined by double immunodiffusion using bovine spleen extracts (Immunogenesis, Sacramento, CA, USA), and by immunoblotting using HeLa cell nuclear and cytoplasmic extracts as antigenic source; autoantibodies to ribosomal RNP and RA33/hnRNP-A2 were determined by immunoblotting.

**RESULTS**

**Serum levels of TNF-α, TNF-R55, TNF-R75 and IL-6**

Serum levels of TNF-α in SLE patients ranged from <15 to 222 pg/ml (Fig. 1). Fifty-nine (84%) samples had TNF-α levels >30 pg/ml, a value never observed in healthy controls (range <15-23 pg/ml). Patients with inactive disease throughout the observation period (ECLAM <2.5) had levels between 30 and 50 pg/ml (patients 1 and 2). Patients experiencing flares of disease, invariably and irrespectively of therapy, had
serum levels of TNF-α > 50 pg/ml. These increased levels coincided with clinical symptoms: fever and polyarthralgia in patient 3; fatigue and pleurisy in patient 5; fatigue, fever and arthralgia in patient 8; active renal disease in patients 4, 6 and 7. Patient 5, who acquired a cytomegalovirus infection and was treated with gancyclovir, did not have elevated TNF-α levels at this point in time. There was no correlation of TNF-α with corticosteroid therapy.

Serum levels of TNF-R55 ranged from 0.8 to 10.8 ng/ml (median 1.9 ng/ml) for patients, and from 0.9 to 1.4 ng/ml (median 1.1 ng/ml) for control subjects (Fig. 2a). Levels of TNF-R75 ranged from 1.5 to 15 ng/ml (median 4.7 ng/ml) for patients and from 1.6 to 4.3 ng/ml (median 2.6 ng/ml) for controls (Fig. 2b). The differences between patients and healthy controls were highly significant ($P < 0.0001$), and there was a strong correlation between the two receptors ($r = 0.72; P < 0.00001$).

Serum levels of IL-6 ranged from <3.5 to 52 pg/ml (Fig. 3). This cytokine was detected in 50% of all sera and in 8/9 patients tested, but remained undetectable in the sera of controls.

Correlations between cytokines and TNF-Rs

Levels of TNF-α correlated strongly with those of TNF-R55 ($r = 0.56, P < 0.0001$) and TNF-R75 ($r = 0.66, P < 0.0001$; Fig. 4a); furthermore, there was also a good correlation between TNF-α and IL-6 (Fig. 4b). Interestingly, a similar correlation was seen between IL-6 and TNF-R75 ($r = 0.42, P < 0.0001$), whereas the correlation of IL-6 with TNF-R55 was less pronounced ($r = 0.24, P < 0.005$).

Correlations of cytokines and TNF-Rs with clinical and serological parameters of disease activity

These correlations are summarized in Table I. There were significant correlations of TNF-α, TNF-R55 and TNF-R75 with the ECLAM disease activity score and with anti-dsDNA levels. In contrast, correlations with ANA titres were rather weak. TNF-α and both TNF-Rs also correlated well with the acute-phase response (ESR and CRP), and inversely with RBC. However, there were no significant correlations with creatinine serum levels or clearance and proteinuria. For IL-6, good correlations were found only with CRP and with ESR, whereas the correlation with anti-
ds-DNA was rather weak. The correlations of TNF-α and IL-6 with CRP are also shown in Fig. 5a and b.

To demonstrate the association between TNF-α and clinical disease activity in individual patients, the time courses of TNF-α and ECLAM are shown for two patients (Fig. 6a and b). These data further indicate that changes in the levels of TNF-α may parallel or even antedate changes in the clinical course as measured by the ECLAM score. Similar results were obtained for both TNF-Rs (not shown).

DISCUSSION

In the present study, we have shown that serum levels of TNF-α and its soluble receptors were increased in patients with SLE, and that these levels correlated with clinical disease activity (ECLAM score) as well as with anti-dsDNA and other laboratory measures related to disease activity. These findings contrast previous reports on low or undetectable TNF-α in SLE [13, 14] and on a lack of correlation with disease activity [15]. However, as far as TNF-Rs are concerned, our data are in good agreement with those reported by Aderka et al. [14].

Like in most recent investigations on cytokine levels in biological fluids, we used commercial ELISAs for our measurements. Such assays have proven to be much more reproducible and more specific than bioassays [34–36]. Moreover, in contrast to bioassays, they are not influenced by therapeutically administered immunosuppressive drugs such as steroids or cyclophosphamide, which may be contained in patients’ sera. Thus, although not measuring bioactivity per se, immunoassays are generally considered to be very useful for the determination of bioactive substances such as hormones, cytokines or neurotransmitters in body fluids.

A comparison between SLE and RA patients [24] showed that TNF-α levels in the circulation were higher in SLE. Interestingly however, TNF-α levels in synovial fluids of RA patients were comparable to serum levels of SLE patients. In contrast, TNF-R levels in RA synovial fluids were ~5-fold higher than those in SLE sera. On the other hand, serum concentrations of TNF-Rs were similar in both diseases. Taking into consideration the current view that an imbalance between TNF-α and its soluble receptors may be critical in certain disease states such as RA [22–24] or septicemia [37], one may speculate that TNF-R levels in sera of SLE patients might actually be too low to neutralize the cytokine efficiently. Thus, it may be of particular significance that the highest levels of TNF-α and TNF-Rs were measured in patients with nephritis; these levels did not depend on renal function, as has been reported for non-SLE patients with chronic renal failure [38]. Remarkably, increased expression of TNF-α mRNA was found in kidney tissue of several lupus-prone mouse strains, suggesting an involvement of this cytokine in renal disease [9, 11].

The role of TNF-α as a pro-inflammatory cytokine is well documented, whereas the biological functions of its soluble receptors are not yet understood. Although they can block TNF function, they could also prevent its inactivation, thus increasing the half-life of TNF-α in the circulation [20, 21, reviewed in 39]. Since TNF-α

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<td>Correlation of TNF-α, soluble TNF receptors and IL-6 with parameters of disease activity</td>
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can induce expression and shedding of its own receptors [19, 40, 41], the strong correlation of TNF-α with both receptors observed by us and many other investigators may be considered an indication of the cytokine's biological activity. Nevertheless, one must bear in mind that TNF-R expression and shedding is not only regulated by TNF-α, but also by other cytokines, including IL-1, IFN-γ and IL-4, in a very complex and still poorly understood manner [42, 43]. Distinct functions have been attributed to the two receptors; thus, there is strong evidence for an involvement of TNF-R55 in TNF cytotoxicity, whereas TNF-R75 may be particularly important in the immune and inflammatory response [44, 45]. In this context, it was interesting to note that TNF-R75 correlated somewhat better than TNF-R55 not only with TNF-α, but also with IL-6.

In accordance with previous investigations [15, 25–28], we found increased serum levels of IL-6 in our patients. Despite its good correlation with TNF-α and TNF-R75, IL-6 did not correlate with the ECLAM score and the correlation with anti-dsDNA levels was rather weak. However, we could find a close correlation of IL-6 with CRP and ESR, which is at variance with reports suggesting that in SLE patients IL-6 does not correlate with the acute-phase response [15, 27]. Similar correlations were found for TNF-α, indicating that TNF-α may be involved—presumably via its effects on IL-6 induction [28]—in the regulation of the acute-phase response in patients with SLE. IL-6 is not only the main inducer of hepatic acute-phase protein synthesis [46], but also a potent regulator of B-cell activity and can even induce mesangial cell proliferation, a pathological event in the course of glomerulonephritis [47]. Furthermore, IL-6 has been reported to play an important role in the pathophysiology of several murine lupus strains [48]. Thus, chronic overproduction of IL-6 may be responsible for several of the immune abnormalities characteristic of SLE [49]. Since there is evidence that TNF-α can (directly or indirectly) induce expression of IL-6 [28], the correlations observed between IL-6 and TNF-α (and also TNF-R75) seem to indicate further that TNF-α may be one of the factors responsible for IL-6 overproduction.

To date, the importance of TNF-α in the pathogenesis of SLE is unclear. Looking at experimental models, contradictory results have been reported on...
the effect of TNF-α administration on murine lupus: low-dose application apparently accelerated disease in (NZB × NZW)F1 mice [9], whereas high doses retarded it [8, 50]. Moreover, the administration of anti-TNF-α monoclonal antibodies to mice treated with anti-IL-10 antibodies was shown to reverse the protective effects of the anti-IL-10 treatment [51]. In other animal models, however, TNF-α was ineffective or even worsened the disease [10]. Obviously, the effects of TNF-α did not only depend on the disease model investigated, but also on the dosage of the cytokine. Therefore, data from animal models must be interpreted with some caution and cannot easily be extrapolated to the human situation.

Thus, do the results obtained in this study allow any conclusions to be drawn about a potential role of TNF-α in the pathogenesis of SLE? Taken together, we have provided evidence for the presence of significant amounts of TNF-α in the sera of SLE patients and of this cytokine’s association with a variety of other variables: clinical disease activity, immune abnormalities including anti-dsDNA autoantibodies and elevated serum IL-6, anaemia, and the acute-phase response. Even though these associations are highly indicative of TNF-α bioactivity, they do not unambiguously prove a pathogenic role. In RA, the effects of TNF-α are clearly deleterious, whereas in SLE a protective role is currently being discussed [52, 53]. Although serum levels alone cannot provide definite answers, we feel that our data are more compatible with a pathogenic rather than a protective role of TNF-α in human SLE. Since immune complexes have been shown to induce TNF-α [12], it is conceivable that this cytokine is activated particularly once immune complexes have been generated. It would then be important by virtue of its pro-inflammatory activity and could be involved in the generation of vascular injury and organ damage, as suggested recently [54]. Moreover, by its ability to induce IL-1 and particularly IL-6, but also by virtue of its own effects on B and T cells, TNF-α could be involved in the immunological dysregulation characteristic of SLE. On the other hand, it cannot be excluded that TNF-α might also exert some protective effects, especially in early disease, as reported for NZB/W mice [8, 50], or via its effects on induction of IL-6 and, as a consequence, of the acute-phase response.

Thus, the detailed role of TNF-α in the pathophysiology of SLE is not yet fully resolved, and requires further studies in man and experimental animals. Nevertheless, since TNF-α plays a key role in the cascade of pro-inflammatory cytokines, the correlations seen in our patients suggest that TNF-α is one of the important factors in human SLE.

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