Rheumatology 2001;40:868–875

Systemic lupus erythematosus and rheumatoid arthritis patients differ from healthy controls in their cytokine pattern after stress exposure

R. Jacobs, C. R. Pawlak[1], E. Mikeska[1], D. Meyer-Olson, M. Martin[2], C. J. Heijnen[3], M. Schedlowski[4] and R. E. Schmidt

Division of Clinical Immunology,[1]Division of Medical Psychology, [2]Division of Clinical Molecular Pharmacology, Hannover Medical School, 30623 Hannover, Germany, [3]Division of Immunology, University Hospital for Children and Youth, 3501 CA Utrecht, The Netherlands and [4]Division of Medical Psychology, University of Essen, 45122 Essen, Germany

Abstract

Objective. To study whether patients with rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) differ from healthy individuals in their immune responses to acute psychological stress.

Methods. The phenotype and function of peripheral blood lymphocytes were analysed before and after stress exposure in patients and healthy subjects.

Results. Natural killer (NK) cell numbers increased transiently in all groups under stress. NK activity, however, increased in healthy controls only. We observed a stress-induced increase in interleukin (IL)-4-producing (IL-4$^+$) cells in SLE patients only, whereas interferon (IFN) γ^+ cell numbers increased due to stress in all three groups. An analysis of supernatants from phytohaemagglutinin (PHA) cultures revealed increased IFNγ and IL-10 levels in healthy subjects but not in SLE or RA patients after stress exposure.

Conclusions. These data demonstrate that RA and SLE patients differ in their immune response to stress from healthy controls. Changes in cytokine patterns might be responsible for stress-induced exacerbation of disease activity in RA and SLE patients.

KEY WORDS: Rheumatoid arthritis, Systemic lupus erythematosus, Stress, Natural killer cells, Cytokines.

The pathogenesis of chronic inflammatory autoimmune diseases such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) is still unknown. Both inflammatory diseases are characterized by the presence of autoantibodies which play a major role in these entities. In healthy individuals, antibody production is regulated by a well-controlled network of immunocompetent cells predominantly co-ordinated by cytokines.

Therefore, several studies have investigated the cytokine profile of SLE and RA patients *in vivo* and *in vitro*. Despite the inconsistent findings of either increased or decreased levels of interleukin (IL)-2, IL-4, IL-6, IL-10 or interferon (IFN) γ in RA and SLE patients in comparison with healthy controls, these studies confirm that SLE and RA patients display an altered cytokine profile [1–5].

Recent experimental data demonstrate that lymphocyte subpopulations and cytokine production are also controlled by factors of the neuroendocrine system [6, 7]. These data are paralleled by clinical observations suggesting an association between stress, life events, and disease activity in autoimmune patients [8–10]. In healthy individuals, acute psychological stress induces a transient increase in peripheral blood lymphocytes, with the most pronounced alterations in natural killer (NK) cell numbers [11–13]. Stress-induced changes are mediated mainly via sympathetic activation of β-adrenoceptors on lymphocytes [14].

Preliminary experimental findings support the hypothesis that in autoimmune patients the response of the sympathetic nervous system to stress differs from healthy controls [15–18]. Observations in SLE and RA patients demonstrated an altered number of β-adrenoceptors on peripheral blood mononuclear cells (PBMC) [19, 20]. Experimental observations in animals and humans suggest that both α- and β-adrenergic mechanisms modulate cellular immune functions and disease activity in chronic inflammation [21]. Treatment with a non-selective β-adrenergic receptor blocker in rats

Submitted 18 May 2000; revised version accepted 7 March 2001.
Correspondence to: R. Jacobs.

with experimentally induced arthritis significantly reduced disease symptoms [15]. However, whether and to what extent the neuroendocrine–immune communication is disturbed in autoimmune patients such as RA and SLE patients is unknown at present. Thus, in the present study we investigated whether SLE and RA patients differ from healthy individuals in the circulation of lymphocyte subpopulations, NK cell activity, and cytokine pattern before, immediately after, and 1 h after stress exposure, using public speech as a stress model [22, 23].

Patients and methods

Patients and subjects

Nine female RA patients (mean age 44.2 yr, range 31–60 yr), seven female patients with SLE (mean age 39.0 yr, range 20–61 yr), and 15 age-matched healthy female volunteers (mean age 39.0 yr, range 24–58 yr) participated in the study after informed consent. The SLE patients with active disease according to SLEDAI [24] were recruited from the out-patient clinic at Hannover Medical School and had been diagnosed according to the criteria of the American Rheumatism Association (ARA) [25, 26]. An assessment of disease activity in the RA patients during the last 14 days, according to the RADAR score [27, 28], revealed 14.63 ± 3.78 for pain/tenderness (scale: 0–60) and 1.87 ± 0.3 for level of function (scale: 0–4). The study was approved by the ethics committee of Hannover Medical School for human experimentation. Subjects with significant cardiovascular diseases, concurrent infections, or any history of other autoimmune disorders were excluded. All patients in this study were free of immunosuppressive medication. One patient was medicated with hydroxychloroquine (250 mg/day) and five patients took low doses of methotrexate (10–20 mg/week). There were no significant immunological differences comparing treated with untreated patients. The patients displayed no exacerbation of their disease activity at least 4 weeks prior to the onset of the experiment. In addition, for at least 4 weeks prior to the experiment, all subjects were free of medications such as anti-depressants, adrenoceptor antagonists, and benzodiazepines, which were suspected of altering immunological or affective responsiveness. Individuals with drug or alcohol abuse were excluded from this study.

Experimental design and procedure

The subjects were informed that the purpose of the study was to measure the effect of emotional state on immune responsiveness during their performance of a public talk. The experimental sessions were conducted between 8.30 and 11.30 a.m. The subjects were requested to avoid caffeine on the morning of the study. Upon arrival in the laboratory, the volunteers were seated in a chair facing a movable wall, and remained so throughout the entire experiment. After giving informed consent and resting for 10 min, an indwelling cannula was inserted into a prominent forearm vein. Thirty minutes after inserting the cannula the first blood sample (baseline) was drawn.

Acute psychological stress was induced by public talking. Three minutes after starting, the talk was interrupted and the second blood sample (stress) was drawn. The subjects were then asked to sit quietly and relax for the remaining time until the third blood sample was drawn 60 min after the stress exposure (follow-up).

White blood cells (WBC)

Blood was drawn using potassium-ethylene diamine tetraacetic acid (EDTA) Monovetten™ (Sarstedt, Nümbrecht, Germany) or in heparinized syringes for immunological analyses. WBC were counted automatically with STKS (Coulter Electronics Inc., Hialeah, USA).

Catecholamine concentrations

All blood samples were centrifuged at 4°C and the plasma stored at −80°C until assayed. All samples of one subject were analysed in the same assay. Catecholamine plasma levels were analysed using modified high-performance liquid chromatography (HPLC).

Monoclonal antibodies (mab)

Labelled mab directed against the respective human leucocyte antigens CD2 fluorescein isothiocyanate (FITC), CD4 FITC, CD8 phycoerythrin (PE), CD16 FITC (DAKO, Hamburg, Germany), CD3 FITC (Immunotech, Hamburg, Germany), CD3 PE, CD20 FITC, and CD56 PE (Becton Dickinson, Heidelberg, Germany) were used. The following mab for the detection of intracellular cytokines were purchased from Pharmingen (Hamburg, Germany): α-IFNγ, FITC, α-IL-2 PE, α-IL-4 PE, α-IL-6 PE, α-IL-10 PE. For all cytofluorometric experiments, appropriate isotype control antibodies (PE/FITC) were utilized.

Phenotypic analyses

Phenotypic analyses were performed using two-colour immunofluorescences in Ficoll-Hypaque-separated cell samples utilizing directly labelled mab as described previously [29]. Briefly, $1–3 \times 10^5$ cells/well were incubated with murine mab against the appropriate antigen at an optimal dilution for 30 min at 4°C. Non-specific binding was eliminated by mixing the samples with a 1 : 5 solution of a commercial human immunoglobulin G (IgG; Intraglobin, Biotest, Frankfurt, Germany). The samples were washed three times in phosphate-buffered saline/bovine serum albumin (PBS/BSA), and at least 10^4 cells per lymphocyte or monocyte gate were analysed using a FACScan (Becton Dickinson). The gates were set according to the forward scatter (FSC) and sideward scatter (SSC) properties of the cells.

Cell stimulation

After Ficoll separation, 2×10^6 PBMC were resuspended in 1 ml of culture medium [RPMI 1640

supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin solution]. The cells were then stimulated by the addition of 10 ng phorbol 12-myristate 13-acetate (PMA) and 1 mM ionomycin. One minute later the transport inhibitor monensin (2.5 mM) was added to prevent the secretion of the induced cytokines into the supernatant. After 4 h of culture at 37°C and 5% CO_2 the cells were harvested for further analyses.

Whole blood culture

Heparinized fresh blood from all three time points was diluted 1 : 10 with RPMI. These cell suspensions were cultured for either 24 h in the presence of phytohaemagglutinin (PHA) or for 36 h with lipopolysaccharide (LPS). After appropriate incubation times, cell-free supernatants of these cultures were carefully harvested and kept frozen in aliquots at − 20°C until analysis.

Staining of intracellular cytokines

After stimulation, the cells were washed with PBS/BSA and fixed for 10 min at room temperature in PBS containing 4% paraformaldehyde. The cells were then washed and resuspended in saponin buffer (PBS supplemented with 5 mM HEPES and 0.1% saponin). Subsequently, aliquots were stained simultaneously with mab against intracellular cytokines and surface antigens. Non-specific binding of the mab via Fc receptors was discriminated by adding human IgG solution. After 30 min incubation at 4°C the cells were washed once with saponin buffer and twice with PBS/BSA. After resuspension the cells were ready for FACS analysis.

Determination of soluble cytokines

Cytokines from supernatants of whole blood cultures were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits for IFNγ, IL-6 (Bender, Vienna, Austria), IL-2, IL-4, and IL-10 (Biosource, Ratingen, Germany) according to the manufacturers' recommendations.

NK assay

Standard 4 h ^{51}Cr release cytotoxicity assays were performed using cryopreserved PBMC. Frozen samples were rapidly thawed with three washes in RPMI 1640 and finally resuspended in medium supplemented with 10% FCS. One hour later the cells were counted and added in triplicate at four effector to target (E/T) ratios (60 : 1, 30 : 1, 15 : 1, and 7.5 : 1) in V-bottom microtitre plates with 5×10^3 ^{51}Cr-labelled K562 target cells per well, as previously described in detail [30]. The medium for the cytotoxicity assays was RPMI 1640 supplemented with 5% FCS and 1% penicillin/streptomycin. The assays were incubated for 4 h. Specific cytotoxicity was measured by determining released ^{51}Cr. Background values were determined by incubating target cells without effector cells. Maximal values were obtained by lysing target cells with Triton X-100 (Sigma, St Louis, MO, USA). Specific lysis was calculated by:

$$[(\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release})] \times 100$$

For a more precise analysis of cytotoxic capacity, lytic units were calculated according to the method of Bryant *et al*. [31]. Using this mathematical transformation the sigmoid dose–response relationship between the number of employed effectors and measured cytotoxicity can be linearized. Lytic units calculated in this way allow a comparison of cytotoxicity assays performed at different points of time.

Statistical analyses

Statistical analyses of the data were performed using ANOVAs with repeated measures. If not otherwise stated, the group × time interaction effect is given for the differences between SLE and healthy controls or RA and healthy controls.

Results

Catecholamine concentrations

The stress of public speaking significantly increased plasma concentrations of adrenaline and noradrenaline in patient groups and healthy individuals with no significant differences between groups (data not shown) [32].

WBC kinetics

Public speaking increased WBC numbers in all groups with significantly less pronounced changes in SLE patients compared with controls ($P < 0.005$). SLE patients displayed leucopenia reflected by significantly lower lymphocyte numbers compared with healthy controls ($P < 0.001$, group effect). In addition, stress-induced increases in lymphocyte numbers were observed in all three groups (Table 1).

Lymphocyte subsets

Due to lower absolute numbers of lymphocytes, SLE patients had reduced lymphocyte subset counts at all three time points (Fig. 1a–c). T lymphocyte ($CD3^+$)

Table 1. WBC counts before (baseline), immediately after (stress), and 1 h after (follow-up) stress exposure of healthy controls, RA and SLE patients (mean ± standard deviation). Public speaking increased WBC numbers in all groups with significantly less pronounced changes in SLE patients compared with controls ($P < 0.005$). Stress-induced increases in lymphocyte numbers were observed in all three groups ($P < 0.05$)

	Baseline	Stress	Follow-up
Leucocytes (/μl)			
RA ($n = 9$)	6600 ± 2163	8089 ± 1735	6811 ± 1902
Controls ($n = 15$)	6035 ± 1627	7635 ± 1718	6919 ± 1763
SLE ($n = 7$)	5443 ± 2224	6414 ± 1818	5500 ± 1414
Lymphocytes (/μl)			
RA ($n = 9$)	1647 ± 444	2351 ± 439	1811 ± 430
Controls ($n = 15$)	1792 ± 391	2392 ± 522	2035 ± 550
SLE ($n = 7$)	1117 ± 399	1499 ± 384	1177 ± 374

numbers increased after stress exposure in all groups (healthy controls $P < 0.01$, SLE and RA $P < 0.05$; Fig. 1a). The same was true for $CD8^+$ cells in all groups ($P < 0.05$, Fig. 1c). However, stress-induced changes in $CD4^+$ cell numbers were only observed in RA patients ($P < 0.05$), but not in SLE patients or healthy controls (Fig. 1b). The most pronounced stress-induced effect was observed in NK ($CD16^+/CD56^+$) cell numbers of patients and healthy controls (Fig. 2a). However, these stress-induced changes in NK cell numbers were significantly blunted in SLE patients ($P < 0.01$).

In healthy controls, the kinetics of NK cell numbers were paralleled by the cytotoxic capacity against K562 target cells. In contrast, SLE and RA patients did not exhibit significantly enhanced cytotoxicity after stress, despite an increase in NK cell numbers (Fig. 2b).

Cytokine production

To ascertain whether leucocytes from RA and SLE patients differed functionally from those of healthy controls, the cytokine-producing capabilities of WBC were analysed. Cytokine levels were determined from PHA (IFNγ, IL-2, IL-4, IL-6, IL-10) or LPS (IL-6, IL-10) culture supernatants by ELISA. IFNγ levels in healthy controls were 3-fold higher at baseline compared with both patient groups, increased during stress exposure, and returned to baseline values 1 h later ($P < 0.05$). In contrast, in SLE and RA patients, IFNγ levels were not affected by public speech (Fig. 3a).

Analyses of IL-2 levels revealed no differences between time points or patient groups and healthy controls (Table 2). PHA-driven supernatants from SLE and RA patients contained approximately 60% of the IL-6 (n.s.) and 25% of the IL-10 ($P < 0.05$) concentration that was measured from healthy individuals (Fig. 3b). The production of both cytokines was not affected by stress exposure.

Supernatants from LPS cultures from SLE and RA patients contained less IL-6 than cultures from healthy controls, without reaching statistical significance. These IL-6 levels did not change between the three time points in all three groups (Table 3). IL-10 levels from LPS-driven supernatants did not differ between patients and controls and were not affected by stress exposure.

Intracellular cytokines

To determine cytokine production on the single-cell level, intracellular cytokines were determined by FACS. To induce maximal cytokine synthesis, the cells were first stimulated with PMA and ionomycin. Under these conditions, patients and healthy volunteers displayed comparable numbers of $IFN\gamma^+$ cells at baseline. However, $IFN\gamma^+$ cell numbers subsequently increased by about 50% after stress exposure and returned to baseline values 1 h later (Fig. 4a). In contrast, SLE patients exhibited absolutely lower, and RA patients higher, numbers of IL-2-producing cells compared with controls, without reaching statistical significance

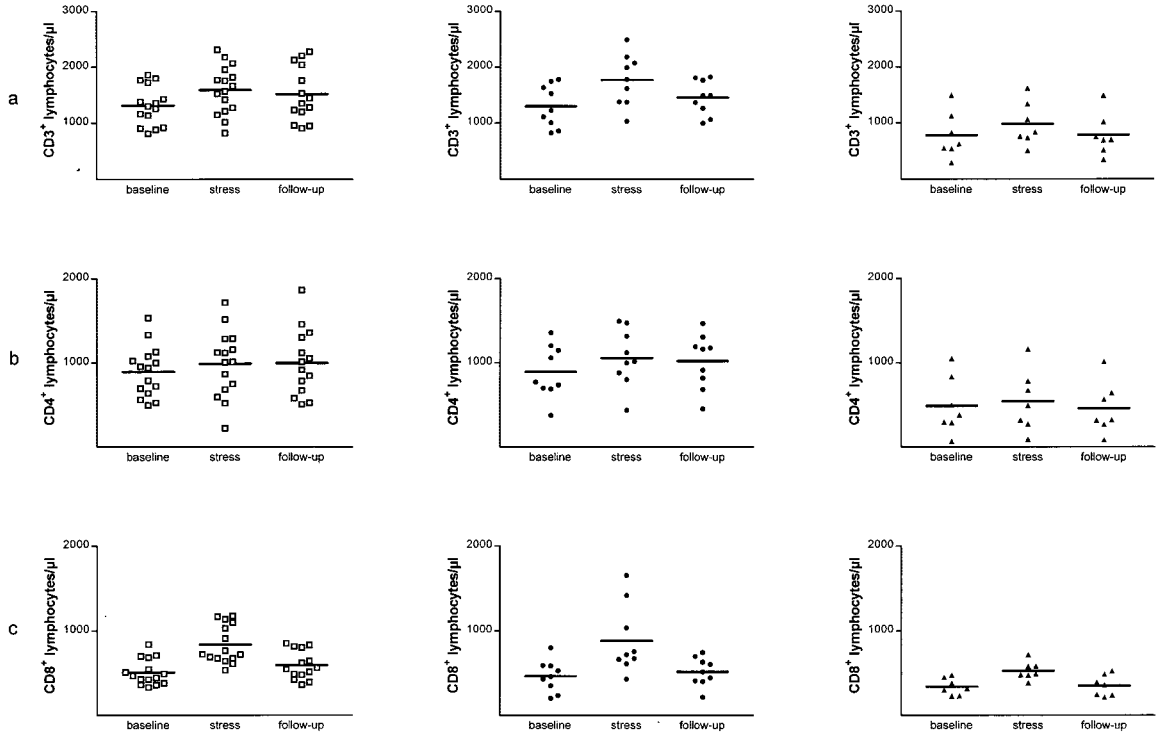

FIG. 1. Composition of lymphocyte subsets significantly changed after stress induction. (a) $CD3^+$ (healthy controls □, $P < 0.01$; SLE ▲ and RA ●, $P < 0.05$) and (c) $CD8^+$ ($P < 0.05$) lymphocytes increased after stress exposure in all groups. (b) Stress-induced changes in $CD4^+$ cell numbers were only observed in RA patients ($P < 0.05$), but not in SLE patients or healthy controls.

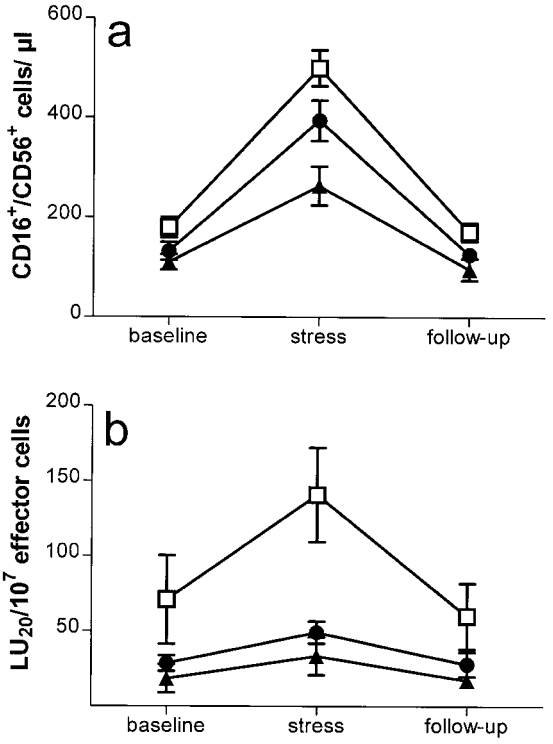

FIG. 2. (a) NK cells increased under stress in all three groups ($P < 0.01$). (b) In parallel, NK cytotoxicity assays revealed a significant increase under stress only in healthy controls (□, $n = 8$, $P < 0.05$). SLE (▲, $n = 7$) and RA (●, $n = 7$) patients displayed only mild changes without significance.

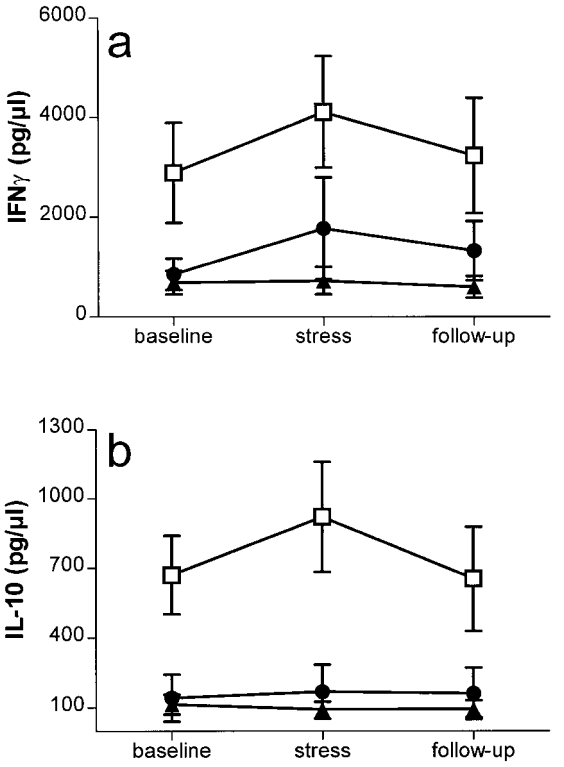

FIG. 3. IFNγ and IL-10 production was measured by ELISA from supernatants after 24 h of culture in the presence of PHA. (a) IFNγ levels in healthy controls (□, $n = 10$) were 3-fold higher at baseline compared with both patient groups, increased during stress exposure, and returned to baseline values 1 h later ($P < 0.05$). In contrast, in SLE (▲, $n = 4$) and RA (●, $n = 7$) patients, IFNγ levels were not affected by public speech. (b) Supernatants from SLE and RA patients contained approximately 25% of the IL-10 of healthy controls ($P < 0.05$). The production of this cytokine was not affected by stress exposure. Data are presented as mean ± standard error.

($P < 0.06$). However, in all groups, IL-2$^+$ cell numbers remained unchanged over time (Fig. 4b).

Analogous analyses performed for Th2 cytokines revealed that IL4$^+$ cells were not affected by stress in RA patients and healthy controls. IL4$^+$ cells of SLE patients, however, increased after stress by about 100% and subsequently dropped to baseline values ($P < 0.01$) (Fig. 5). In contrast, numbers of IL-6$^+$ and IL-10$^+$ cells did not differ between healthy controls and SLE and RA patients and remained unaffected by stress exposure (data not shown).

In addition, cell samples stained with αIL-10 antibodies were also gated on monocytes according to their properties in a FSC *vs* SSC display. However, IL-10$^+$ monocyte numbers did not differ between groups and did not change during the whole experiment (data not shown).

To assess whether stress can directly induce cytokine production, freshly isolated cells were analysed without prior stimulation by PMA/ionomycin or LPS. These experiments revealed no spontaneous cytokine production of non-stimulated cells at any time.

TABLE 2. IL-2 and IL-6 levels were analysed by ELISA (mean ± standard error of the mean). Whole blood samples from the indicated time points had been stimulated for 24 h with PHA. Differences between healthy controls and RA or SLE patients in regard to IL-2 and IL-6 production failed to reach statistical significance

	Baseline	Stress	Follow-up
IL-2 (pg/µl)			
RA ($n = 7$)	796 ± 233	717 ± 194	666 ± 334
Controls ($n = 10$)	409 ± 104	477 ± 132	400 ± 145
SLE ($n = 4$)	661 ± 250	618 ± 302	775 ± 429
IL-6 (pg/µl)			
RA ($n = 7$)	5141 ± 1728	5418 ± 1206	4317 ± 2135
Controls ($n = 10$)	7520 ± 1678	6831 ± 1484	4287 ± 1077
SLE ($n = 5$)	4646 ± 1525	4690 ± 2519	2148 ± 1000

Discussion

Acute psychological stress induced by a public talk induced a sympathetic activation with significant increases in heart rate, blood pressure, and blood catecholamine concentration [22, 32]. In parallel, leucocyte and lymphocyte counts, in particular of CD16$^+$/CD56$^+$ NK cell numbers, increased transiently in blood of patients and controls. This concurs with

TABLE 3. IL-6 and IL-10 levels were analysed by ELISA (mean ± standard error of the mean). Whole blood samples from the indicated time points had been stimulated for 36 h with LPS. Differences are not statistically significant

	Baseline	Stress	Follow-up
IL-6 (pg/μl)			
RA ($n = 7$)	2374 ± 482	3202 ± 489	2848 ± 563
Controls ($n = 10$)	7421 ± 1166	6806 ± 951	4692 ± 852
SLE ($n = 5$)	2990 ± 1371	2826 ± 1219	2290 ± 985
IL-10 (pg/μl)			
RA ($n = 7$)	144 ± 63	238 ± 69	248 ± 73
Controls ($n = 10$)	266 ± 75	281 ± 91	212 ± 83
SLE ($n = 7$)	165 ± 114	167 ± 130	121 ± 77

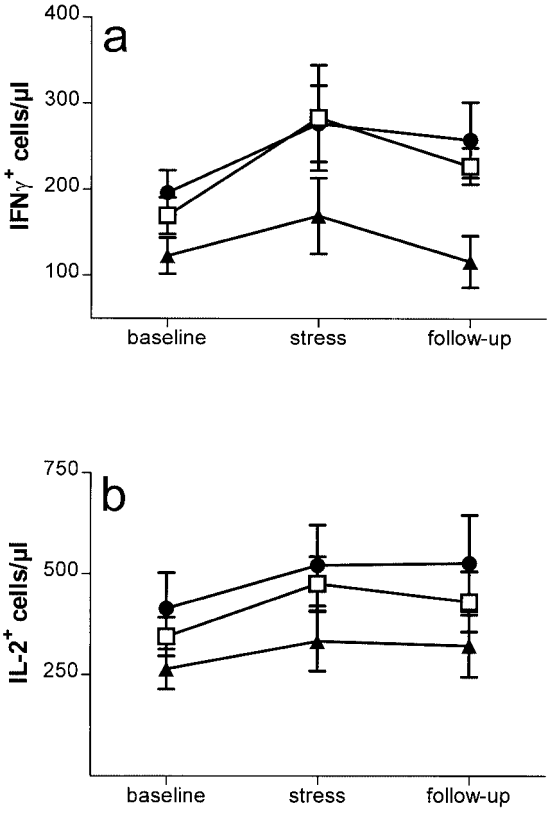

FIG. 4. (a) FACS analyses revealed that IFNγ^+ cells increased by about 50% after stress exposure and returned to baseline values 1 h later in all groups (SLE ▲, $n = 5$; RA ●, $n = 8$; healthy controls □, $n = 6$). (b) SLE patients (▲, $n = 4$) exhibited absolutely lower, and RA patients (●, $n = 7$) higher, numbers of IL-2-producing cells compared with healthy controls (□, $n = 10$) (nearly significant, $P < 0.06$). Data are presented as mean ± standard error.

the results of previous studies with healthy subjects employing either similar or different models of acute psychological stress [11, 14, 23, 33, 34].

SLE patients presented lower total lymphocyte counts and respective subset numbers, reflecting lymphopenia normally observed in these patients. Moreover, all stress-induced changes in lymphocyte subpopulations

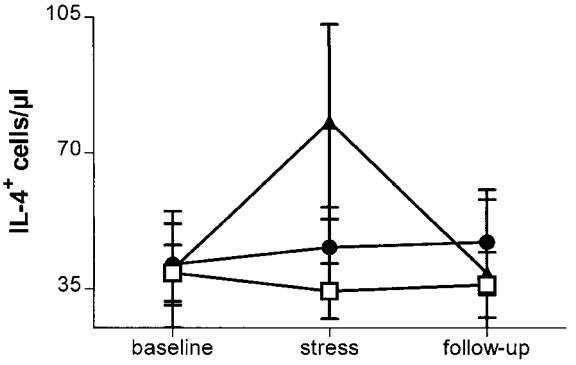

FIG. 5. Intracellular IL-4 expression from SLE (▲, $n = 7$) patients differed from healthy individuals (□, $n = 12$) and RA (●, $n = 9$) patients. IL4$^+$ cells were not affected by stress in RA patients and healthy controls. IL4$^+$ cells of SLE patients, however, increased after stress by about 100% and dropped to baseline values afterwards ($P < 0.01$). Data are presented as mean ± standard error.

(CD3$^+$, CD4$^+$, CD16$^+$/CD56$^+$) were less pronounced in SLE patients compared with controls or RA patients.

The only two studies so far involving SLE patients yielded inconsistent results for stress-induced alterations in lymphocyte subpopulations. Hinrichsen *et al.* [18] observed unchanged or reduced percentages of lymphocyte subpopulations despite elevated catecholamine concentrations after 2 h of neuropsychological testing in patients and healthy controls. In contrast, concurring with the present data, the same group reported a significant increase in percentages of lymphocyte subsets, in particular CD8$^+$ cells, after an acoustic stressor in both controls and SLE patients. However, two-colour fluorescence analyses in our study revealed that the changes in CD8$^+$ cells in patients and controls were caused predominantly by changes in CD8$^+$ NK cells (data not shown) [12].

The functional capacity of NK cells to kill target cells *in vivo* increased significantly after stress exposure in healthy controls, but not in SLE or RA patients. In standard NK assays there is a strong correlation between target number and effector ratio which allows a comparison of the killing capacity of different individuals after calculation of lytic units. Thus, at least in RA patients, an expected marked enhancement of NK cytotoxicity in parallel to the increases in NK cells under stress was not observed. This suggests an aberrant regulation of NK activity in RA patients. Previous data support this finding [35], where the activity of peripheral blood NK cells could be down-regulated by immune complexes isolated from synovial fluid. Therefore, the exhaustion of cytolytic activity in RA and the lack of increased activity may be due to prior *in vivo* activation of NK cells via CD16 [36]. We propose that NK cells in patients suffering from SLE or RA are less active due to permanent activation as a consequence of the inflammatory disease. Under these conditions, additional pre-activated NK cells that are recruited under stress may not be able to

enhance tumour killing efficiently. Furthermore, in healthy individuals and patients, NK cells may be recruited from different immune compartments where the functional capacity of NK cells is altered [14].

It has been shown that adrenaline is one of the main factors responsible for lymphocyte trafficking under acute psychological stress or physical exercise [12, 14, 37]. Other immune mediators that are potentially under the influence of acute stress are cytokines. Several studies which have already focused on this issue using different approaches have yielded inconsistent results.

In SLE patients and healthy controls we observed comparable levels of IL-2, but lower levels of IFNγ in SLE patients. Our experimental paradigm employed whole blood cultures without cell adjustment prior to the assays. Thus, it would be expected that all cytokine concentrations from SLE patients would be approximately half those of healthy controls, as SLE patients displayed only about half as many cells in peripheral blood compared with controls. Taking this into account, SLE patients displayed relatively elevated IL-2 after PHA stimulation. However, IFNγ was below control levels. In contrast to healthy volunteers, there were no changes in these cytokine concentrations throughout the whole experiment. RA patients exhibited increased IL-2 but diminished IFNγ levels compared with controls.

In general, measuring cytokines from plasma and serum samples or culture supernatants does not reflect real *in vivo* conditions. Cytokines do not effectively regulate immune functions systematically, but rather in low concentration near the effector site. In addition, *in vitro* analyses of cytokine levels in supernatants from activated cells are tempered by consuming cells within the bulk cultures. Furthermore, these culture conditions enable activated cells to proliferate. All these factors can affect the actual cytokine levels measured in the supernatant.

Thus, for a more accurate reflection of the *in vivo* situation we analysed intracellular cytokine production in mononuclear cells which were freshly isolated from peripheral blood. This method enables the direct identification of cytokines produced in single cells. The blockade of transport mechanisms in the cells inhibits the secretion of mediators, preventing any loss by cytokine consumption. In addition, due to the short culture time (4 h) of the assay, cell proliferation as an error source can be excluded.

Using this technique, analyses revealed that the number of IL-4$^+$ cells in SLE patients increased transiently under stress. IL-4 is known to drive powerful clonal proliferation and expansion of activated B cells which may lead to enhanced immunoglobulin secretion. Thus, it may be possible that stress exposure can induce exacerbation of SLE by activating autoreactive B-cell clones via an increase in IL-4-providing cells. In addition, the lack of immunosuppressive IL-10, as observed in culture supernatants from SLE and RA patients, may further contribute to increased disease activity. Analysing intracellular Th1 cytokines, we detected no significant differences in IL-2$^+$ and IFNγ^+ cells between healthy controls and patients.

With regard to Th2 cytokine-producing cells, there were neither significant differences between SLE or RA patients and healthy controls for IL-6$^+$ and IL-10$^+$ cell numbers, nor were these cell numbers affected by stress exposure. In addition, public speaking did not alter IL-4$^+$ cell numbers in healthy controls and RA patients.

The lack of spontaneous cytokine production by freshly isolated cells from different time points without prior *in vitro* stimulation indicates that stress-released mediators do not directly induce the production of cytokines.

In summary, we observed differences in cytokine patterns between healthy controls and both SLE and RA patients. The recruitment of IL-4$^+$ cells in SLE patients and the relatively decreased IL-10 production in SLE and RA patients in an acute stress situation may cause proliferation and expansion of autoreactive B-cell clones, subsequently increasing autoantibody levels.

In accordance with previous studies in our laboratory, it is likely that changes in the different cell types observed during stress are predominantly due to leucocyte trafficking and the cells may be mainly recruited via β-adrenergic mechanisms [12, 14]. Whether the changes in cytokine-producing cells are merely due to cell trafficking or whether lymphocytes can also be triggered directly by catecholamines needs to be shown in further *in vitro* studies.

Acknowledgements

The authors would like to thank Dr Jürgen Wollenhaupt for his help in recruiting patients and Dr Michael Exton for carefully reviewing the manuscript. This work was supported by the Volkswagen Foundation Germany, Grant I/72 032/031.

References

1. Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR. Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis. J Immunol 1991;147:117–23.
2. Cuadrado MJ, Marubayashi M, Ortega C *et al.* Relationship of IL-2, IL-2R (CD25$^+$), soluble IL-2R and IL-4 with disease activity in SLE patients. Lupus 1993;2:257–60.
3. Barcellini W, Rizzardi GP, Borghi MO *et al. In vitro* type-1 and type-2 cytokine production in systemic lupus erythematosus: lack of relationship with clinical disease activity. Lupus 1996;5:139–45.
4. Horwitz DA, Wang H, Gray JD. Cytokine gene profile in circulating blood mononuclear cells from patients with systemic lupus erythematosus: increased interleukin-2 but not interleukin-4 mRNA. Lupus 1994;3:423–8.
5. Richaud-Patin Y, Alcocer-Varela J, Llorente L. High levels of TH2 cytokine gene expression in systemic lupus erythematosus. Rev Invest Clin 1995;47:267–72.
6. Besedovsky HO, del Rey A. Immune–neuro-endocrine interactions: facts and hypotheses. Endocr Rev 1996; 17:64–102.

7. Kavelaars A, de Jong-de Vos VS, Kuis W, Heijnen CJ. The reactivity of the cardiovascular system and immunomodulation by catecholamines in juvenile chronic arthritis. Ann NY Acad Sci 1998;840:698–704.
8. Affleck G, Tennen H, Urrows S, Higgins P. Person and contextual features of daily stress reactivity: individual differences in relations of undesirable daily events with mood disturbance and chronic pain intensity. J Pers Soc Psychol 1994;66:329–40.
9. Thomason BT, Brantley PJ, Jones GN, Dyer HR, Morris JL. The relation between stress and disease activity in rheumatoid arthritis. J Behav Med 1992;15:215–20.
10. Dobkin PL, Fortin PR, Joseph L, Esdaile JM, Danoff DS, Clarke AE. Psychosocial contributors to mental and physical health in patients with systemic lupus erythematosus. Arthritis Care Res 1998;11:23–31.
11. Schedlowski M, Jacobs R, Stratmann G *et al*. Changes of natural killer cells during acute psychological stress. J Clin Immunol 1993;13:119–26.
12. Schedlowski M, Hosch W, Oberbeck R *et al*. Catecholamines modulate human NK cell circulation and function via spleen-independent beta 2-adrenergic mechanisms. J Immunol 1996;156:93–9.
13. Schedlowski M, Jacobs R, Alker J *et al*. Psychophysiological, neuroendocrine and cellular immune reactions under psychological stress. Neuropsychobiology 1993;28:87–90.
14. Benschop RJ, Jacobs R, Sommer B *et al*. Modulation of the immunologic response to acute stress in humans by beta-blockade or benzodiazepines. FASEB J 1996; 10:517–24.
15. Coderre TJ, Basbaum AI, Dallman MF, Helms C, Levine JD. Epinephrine exacerbates arthritis by an action at presynaptic B2-adrenoceptors. Neuroscience 1990;34:521–3.
16. Kuis W, Heijnen CJ. Rheumatoid arthritis and juvenile chronic arthritis: the role of the neuro-endocrine system. Clin Exp Rheumatol 1994;12(Suppl. 10):S29–34.
17. Hinrichsen H, Barth J, Ferstl R, Kirch W. Changes of immunoregulatory cells induced by acoustic stress in patients with systemic lupus erythematosus, sarcoidosis and in healthy controls. Eur J Clin Invest 1989;19:372–7.
18. Hinrichsen H, Barth J, Ruckemann M, Ferstl R, Kirch W. Influence of prolonged neuropsychological testing on immunoregulatory cells and hormonal parameters in patients with systemic lupus erythematosus. Rheumatol Int 1992;12:47–51.
19. Baerwald C, Graefe C, von WP, Krause A. Decreased density of beta-adrenergic receptors on peripheral blood mononuclear cells in patients with rheumatoid arthritis. J Rheumatol 1992;19:204–10.
20. Zoukos Y, Kidd D, Woodroofe MN, Kendall BE, Thompson AJ, Cuzner ML. Increased expression of high affinity IL-2 receptors and beta-adrenoceptors on peripheral blood mononuclear cells is associated with clinical and MRI activity in multiple sclerosis. Brain 1994;117:307–15.
21. Heijnen CJ, Rouppe van der Voort C, Wulffraat N, van der Net J, Kuis W, Kavelaars A. Functional alpha 1-adrenergic receptors on leukocytes of patients with polyarticular juvenile rheumatoid arthritis. J Neuroimmunol 1996;71:223–6.
22. Schmid-Ott G, Jacobs R, Jager B *et al*. Stress-induced endocrine and immunological changes in psoriasis patients and healthy controls. A preliminary study. Psychother Psychosom 1998;67:37–42.
23. Gerritsen W, Heijnen CJ, Wiegant VM, Bermond B, Frijda NH. Experimental social fear: immunological, hormonal and autonomic concomitants. Psychosom Med 1996;58:273–86.
24. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. Arthritis Rheum 1992;35:630–40.
25. Tan EM, Cohen AS, Fries JF *et al*. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
26. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus [letter] [see comments]. Arthritis Rheum 1997;40:1725.
27. Mason JH, Anderson JJ, Meenan RF, Haralson KM, Lewis-Stevens D, Kaine JL. The rapid assessment of disease activity in rheumatology (radar) questionnaire. Validity and sensitivity to change of a patient self-report measure of joint count and clinical status. Arthritis Rheum 1992;35:156–62.
28. Cocks RA, Rainer TH, Chan TY, Maycock PF, Lam NY. Increased plasma free cyclic-AMP levels following major trauma and their relevance to the immune response. Resuscitation 2000;45:105–9.
29. Jacobs R, Stoll M, Stratmann G, Leo R, Link H, Schmidt RE. CD16–CD56$^+$ natural killer cells after bone marrow transplantation. Blood 1992;79:3239–44.
30. Schmidt RE, MacDermott RP, Bartley G *et al*. Specific release of proteoglycans from human natural killer cells during target lysis. Nature 1985;318:289–91.
31. Bryant J, Day R, Whiteside TL, Herberman RB. Calculation of lytic units for the expression of cell-mediated cytotoxicity. J Immunol Methods 1992;146:91–103.
32. Pawlak CR, Jacobs R, Mikeska E *et al*. Patients with systemic lupus erythematosus differ from healthy controls in their immunological response to acute psychological stress. Brain Behav Immun 1999;13:287–302.
33. Mills PJ, Berry CC, Dimsdale JE, Ziegler MG, Nelesen RA, Kennedy BP. Lymphocyte subset redistribution in response to acute experimental stress: effects of gender, ethnicity, hypertension and the sympathetic nervous system. Brain Behav Immun 1995;9:61–9.
34. Naliboff BD, Solomon GF, Gilmore SL, Fahey JL, Benton D, Pine J. Rapid changes in cellular immunity following a confrontational role-play stressor. Brain Behav Immun 1995;9:207–19.
35. Hendrich C, Kuipers JG, Kolanus W, Hammer M, Schmidt RE. Activation of CD16$^+$ effector cells by rheumatoid factor complex. Role of natural killer cells in rheumatoid arthritis. Arthritis Rheum 1991;34:423–31.
36. Werfel T, Uciechowski P, Tetteroo PA, Kurrle R, Deicher H, Schmidt RE. Activation of cloned human natural killer cells via Fc gamma RIII. J Immunol 1989;142:1102–6.
37. Benschop RJ, Schedlowski M, Wienecke H, Jacobs R, Schmidt RE. Adrenergic control of natural killer cell circulation and adhesion. Brain Behav Immun 1997;11:321–32.