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Methotrexate treatment in murine experimental systemic lupus erythematosus (SLE); clinical benefits associated with cytokine manipulation

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SUMMARY

The objective of this study was to determine the effects of Methotrexate (MTX) on the development and the course of experimental murine SLE, as well as on the cytokine profile involved in the disease. SLE was induced in naive BALB/c female mice by injection of the human anti-DNA MoAb bearing a common idiotype (16/6 Id). Six weeks following immunization, when high levels of autoantibodies were demonstrated, the mice were treated with MTX (2 mg/kg once a week) for a period of 10 months. MTX treatment had no effect on 16/6 Id-induced autoantibody production. However, MTX treatment had beneficial effects on the clinical manifestations of the experimental disease (i.e. leucocyte counts, levels of protein in the urine and immune complex deposits in the kidneys). Thus, only 20% of 16/6 Id-immunized BALB/c mice that were treated with MTX had immune complex deposits in their kidneys compared with 100% of SLE-afflicted BALB/c mice that were not treated. We have observed a significant elevation in IL-1, tumour necrosis factor (TNF) and IL-10 secretion in BALB/c mice afflicted with experimental SLE. IL-2, IL-4, IL-6 and interferon-gamma (INF-γ) levels were decreased in these mice compared with the levels detected in healthy controls. Treatment with MTX reversed the levels of all the above cytokines to normal levels observed in control mice. These studies demonstrate therapeutic effects of MTX on murine experimental SLE. The normal cytokine profile observed following treatment with MTX is suggested to play a role in the amelioration of the clinical manifestations of experimental SLE.

Keywords experimental systemic lupus erythematosus Methotrexate cytokines

INTRODUCTION

Methotrexate (MTX) has been shown to be an effective treatment for adult rheumatoid arthritis (RA), psoriasis and psoriatic arthritis. Evidence from several series and case reports demonstrated that it might also be useful in the treatment of other autoimmune or inflammatory conditions such as inflammatory myopathy, vasculitis, sarcoidosis, primary biliary cirrhosis, uveitis, asthma, polymyalgia rheumatica and giant cell arthritis [1,2]. SLE represents a prototype autoimmune disease with some clinical features for which MTX has been used with success. Recent reports have suggested that MTX treatment resulted in symptomatic improvement in SLE patients [3,4], in childhood SLE [5], and even in pregnant women with SLE [6].

We have demonstrated the induction of experimental SLE in naive BALB/c and C3H.SW mice (that do not develop SLE spontaneously) by immunization with a human anti-DNA MoAb which bears a common idiotype (Id) designated as the 16/6 Id [7]. Following immunization with the 16/6 Id or related MoAbs, the mice develop high titres of anti-DNA, nuclear extract (NE), 16/6 Id autoantibodies as well as SLE-related clinical manifestations including leukopenia, thrombocytopenia, proteinuria and immune complex glomerulonephritis. Using this model, which is similar in its manifestations to human SLE, we can easily investigate the effect of other exogenous factors, i.e. therapeutic agents. Recent studies using the same model have indicated that treatment of mice in which experimental SLE has been treated with the oestrogen antagonist Tamoxifen has been effective [8]. Similarly, treatment of mice with methimazole that was shown to down-regulate class I MHC expression, caused amelioration in all disease manifestations [9]. Administration of cyclosporin at an early stage of disease induction has also proved highly effective [10].

It is possible that many of the clinical manifestations seen in SLE might be due to an excess or absence of cytokine secretion and activity [11–14]. We have previously shown a cytokine dysregulation in mice afflicted with experimental SLE induced by immunization with the 16/6 Id [15]. Thus, the inflammatory

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related cytokines IL-1 and tumour necrosis factor-alpha (TNF-α) were shown to be significantly elevated in mice with experimental SLE, whereas a decrease in the levels of IL-2 and IL-4 was determined in the sick mice [15].

MTX has several known effects on cytokine production and activity. We have previously shown that MTX inhibits IL-1 activity [16]. Further, MTX was shown to cause a decrease in IL-6 activity [17,18] and IL-8 activity [19]. MTX has also been shown to lower soluble TNF receptor and IL-2 receptor levels [18,20].

The aim of the present study was therefore to follow the effects of MTX on the development and course of experimental murine SLE induced by immunization with the 16/6 Id and to determine its effects on the production of various cytokines which might play a role in the modulation of the experimental disease.

We demonstrate here the beneficial effect of MTX on the course of experimental SLE. Moreover, treatment with MTX reversed the abnormal secretion of cytokines to a profile shown in healthy mice.

MATERIALS AND METHODS

Mice

BALB/c female mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were used at 8–12 weeks old.

Antibodies

Human anti-DNA MoAbs bearing the 16/6 Id were isolated from the culture supernatant of a hybridoma secreting these antibodies, on a goat anti-human IgM–Sepharose 4B column [7]. For ELISA coating, MoAb 16/6 Id was used at a concentration of 10 μg/ml. Rabbit anti-16/6 Id serum that was used to assay levels of anti-anti-16/6 Id (16/6 Id^+) antibodies [7] was produced by immunization of rabbits with the 16/6 antibody. Its immunoglobulin fraction was used for ELISA coating at a concentration of 5 μg/ml.

Antigens

DNA. For identification of anti-DNA antibodies, plates were coated with 50 μl/well of 10 μg/ml methylated bovine serum albumin (BSA; Sigma, St Louis, MO). The plates were then washed and coated with 10 μg/ml calf thymus DNA (Sigma) for the detection of dsDNA.

HeLa nuclear extract. NE was prepared as described previously [7] and stored at −70°C. NE was used for coating in ELISA at a concentration of 10 μg/ml.

ELISA

Maxisorb microtitre plates (Nunc, Roskilde, Denmark) were coated with one of the above antigens or antibodies. Thereafter, the plates were blocked with 1% ovalbumin (OVA; Sigma), and the sera of the mice, diluted serially from 1:10 to 1:1280, were incubated for 90 min. Plates were then washed and incubated for 75 min with goat anti-mouse IgG (γ chain Fc-specific) conjugated to peroxidase (Jackson ImmunoResearch, West Grove, PA). After washing, plates were incubated with the substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS; Sigma) and read using an ELISA reader at 414 nm. Background optical density (OD) levels were substracted from the demonstrated data.

Immunization and treatment procedure

Groups of 5–10 mice were immunized intradermally into the hind footpads with 2 μg of the 16/6 antibody, in Freund's complete adjuvant (FCA; Difco Labs, Detroit MI). Three weeks later, booster injections, of the same amount of antibody in PBS, were administered in the same sites. Three weeks following the booster injection the mice were bled and their sera were tested for production of antibodies of various specificities. Upon confirmation of antibody responses in the immunized mice, MTX treatment commenced. Once a week 2 mg/kg were injected intraperitoneally. This dose correlates to the low-dose MTX used in clinical rheumatology (0·2 mg/kg per week) [21]. Mice received the last MTX injection 1 week before they were killed.

Detection of SLE-associated manifestations

The leucocyte counts were determined by mixing heparinized blood with 1% acetic acid in a ratio of 1:10, and the leucocytes were immediately counted. Proteinuria was measured in a semi-quantitative manner, using a Combistix kit (Ames-Miles, Slough, UK).

Immunohistology

Kidneys were removed and frozen immediately in liquid nitrogen. Frozen cryostat sections of 6 mm were air dried and fixed in acetone. For the detection of immunoglobulin deposits, sections were incubated with FITC-conjugated rabbit anti-mouse IgG (γ chain-specific; Jackson). Specific staining was visualized using a fluorescence microscope.

Stimulation of cells for cytokine production

Peritoneal macrophages were washed with cold PBS + fetal calf serum (FCS) and pooled from each mouse group. The cells were then incubated (10^6/ml) with or without lipopolysaccharide (LPS; 10 μg/ml) in RPMI with 10% FCS for 24 h. Supernatants were tested for the production of IL-1. Splenocytes (5×10^6/ml) pooled from each mouse group were incubated with or without concanavalin A (Con A; 2·5 μg/ml) in RPMI with 10% FCS for 24 h. Supernatants were tested for the presence of IL-2, IL-4, IL-6, interferon-gamma (IFN-γ), IL-10 and TNF-α.

Determination of cytokines

Measurement of cytokine levels was performed by either ELISA or bioassay, based on the availability of antibodies for ELISA and the sensitivity of the assay.

IL-1 activity was assessed using the LBRM-33(1A5) two-step assay [22] in which 1A5 cells were stimulated in the presence of phytohaemagglutinin (PHA), with the different supernatants at various concentrations to secrete IL-2. Following overnight incubation, supernatants of 1A5 cells were transferred to the IL-2-dependent cell line CTLL. Stimulation of the CTLL line by IL-2 was then measured following a 24-h incubation period by ^{3}H-thymidine uptake [23].

IL-2 was directly detected using the IL-2-dependent CTLL line, as described above. IL-4 activity was assessed using the CT4S cell line [24].

TNF-α levels in the supernatants were determined by the A-9 cell lytic assay using a tetrazolium salt MTT [25]. TNF-α activity was presented as ng/ml.

IL-6 secretion was assessed measuring IL-6-dependent proliferation of a murine B cell hybridoma cell line B9 [26].

IL-10 was determined by ELISA. Polystyrene microtitre plates were coated with the capture antibody, anti-mouse JES5–2A5 (rat IgG1; Pharminogen, San Diego, CA), and supernatants at different dilutions were added to the blocked plates. Specific binding was determined using the biotin-conjugated second antibody, anti-mouse SCC-1 (rat IgM; Pharminogen) followed by peroxidase-conjugated streptavidin and the appropriate peroxidase substrate. ODs were read at 414 nm using an ELISA reader [27].

IFN-γ levels were assessed by an ELISA assay using the combination of a capture rat anti-mouse IFN-γ MoAb (R4–6A2) and the biotinylated detection MoAb XMG1.2 [28]. Both antibodies were purchased from Pharminogen. Cytokine secretion was determined by the addition of streptavidin-peroxidase followed by incubation with the substrate ABTS (Sigma). The results were determined using an ELISA reader at 414 nm. Calculation of cytokine activities (except TNF-α) are presented as U/ml, calculated from standard curves.

Statistical analysis

Significance of differences between groups of mice was examined using Student's *t*-test or χ^2 with Yates' continuity correction. $P < 0.008$ was considered significant for the *t*-test. (This value was determined by dividing the P value of 0·05 that is used for two groups, by six, the possible number of combinations of pairs out of the four available groups.) $P < 0.05$ was considered significant for χ^2 analysis.

RESULTS

Autoantibody responses in 16/6 Id-injected and treated mice

BALB/c female mice were immunized and boosted with the 16/6 Id. Six weeks following immunization, when antibody titres in the injected mice were elevated, they were divided into groups (5–10 mice per group), that were not further treated, or were injected weekly with MTX 2 mg/kg, intraperitoneally. Control groups consisted of mice that were either not treated at all, or were injected with MTX only. The mice were bled monthly and their sera were tested for the presence of antibody and autoantibody titres. The above experiments were repeated twice. Table 1 demonstrates representative results of the antibody titres of one experiment, determined in the sera of BALB/c mice, following 2·5 months of treatment with MTX. Shown in the table are the high antibody titres specific to the 16/6 Id (immunizing antibody), anti-16/6 Id (16/6 Id$^+$), to dsDNA and to NE antigens. MTX treatment had no effect on antibody levels in the group immunized with the 16/6 Id. No significant antibody activity could be detected in either of the control groups (Table 1). To investigate further the effect of MTX treatment on humoral response, the kinetics of autoantibody production following 16/6 Id immunization was determined. No delay or decline in antibody titres as a result of MTX treatment could be observed. Furthermore, the treatment did not affect IgM and IgG autoantibody isotypes specific to DNA (data not shown). Thus, treatment with MTX did not affect antibody or autoantibody titres in mice immunized with the 16/6 Id.

Effect of MTX treatment on clinical manifestations in mice immunized with the 16/6 Id

It was of interest to determine whether treatment with MTX, although not affecting autoantibody responses in the experimental mice, is capable of modulating clinical manifestations characteristic of experimental SLE. Therefore, the experimental mice were tested for the development of leukopenia and proteinuria periodically, starting 5 months following immunization with the 16/6 Id. The results summarized in Table 2 indicate that whereas mice immunized with the 16/6 Id had significant leukopenia compared with leucocyte counts of the control groups, MTX-treated mice had normal levels of leucocytes. Most mice (12/15) that were injected with the 16/6 Id developed proteinuria ($\geqslant$ 100 mg/dl). In contrast, significant proteinuria was detected only in a minority (2/15) of the mice that were injected with the 16/6 Id and treated with MTX (Table 2).

Since kidney damage manifested by deposition of immune complexes in the kidneys of SLE-afflicted mice is one of the major characteristics of experimental SLE, the experimental mice were killed 8 months (first experiment) or 10 months (second experiment) following immunization, and their kidneys were evaluated for the presence of immune complex deposits. Table 2 also summarizes the results of kidney analyses of the experimental mice. Whereas 100% (15/15) of the 16/6 Id-immunized BALB/c mice that were not further treated had multiple immune complex deposits in their kidneys, immune complexes were found in only 20% (3/15) of the mice treated

Table 1. Antibody levels in the sera of mice with experimental SLE

Group	Immunization	Treatment	16/6 Id	Anti 16/6 Id (16/6 Id$^+$)	dsDNA	Nuclear extract antigens
1	16/6 Id	–	2·13 ± 0·28*	0·74 ± 0·09*	1·98 ± 0·24*	0·94 ± 0·21*
2	16/6 Id	MTX	1·95 ± 0·21*	0·82 ± 0·14*	2·05 ± 0·17*	0·89 ± 0·17*
3	–	–	0·13 ± 0·03	0·07 ± 0·01	0·21 ± 0·01	0·21 ± 0·01
4	–	MTX	0·10 ± 0·01	0·06 ± 0·05	0·19 ± 0·03	0·18 ± 0·02

Values are the results of ELISA of sera (diluted 1:100) obtained from five mice per group. Results are expressed as mean ± s.d. of OD at 414 nm. Determination of antibody titres was done 4 months following immunization with 16/6 Id and 2·5 months of Methotrexate (MTX) treatment.

* Titres of all antibodies in groups 1 and 2 were significantly higher ($P < 0.008$) than those of control groups 3 and 4. No statistically significant differences were found between OD values of group 1 and 2 (Student's *t*-test).

Table 2. Clinical manifestations of BALB/c mice with experimental SLE

Group	Immunization	Treatment	Leucocytes (cells/mm^3)	Proteinuria* >100 mg/dl†	Mice with* ICD‡
1	16/6 Id	–	3200 ± 1800§	12/15§	15/5§
2	16/6 Id	MTX	5800 ± 1300	2/15	3/15
3	–	–	6500 ± 1200	0/8	0/8
4	–	MTX	7200 ± 970	0/4	0/4

Leucocyte counts and proteinuria were determined in the experimental mice, 7 months following immunization with the 16/6 Id and 5·5 months of Methotrexate (MTX) treatment.

* Data on proteinuria and positive glomerular immune complex depositions (ICD) are of two independent experiments.

† Number of mice with proteinuria per number of animals tested.

‡ Number of mice with ICD per animals tested.

§ Significantly different ($P < 0.005$) compared with all groups (performed by Student's t-test for leucocyte counts and by χ^2 for proteinuria and ICD).

with MTX. These results were statistically significant (Table 2). Figure 1 shows a representative kidney section of an SLE-afflicted BALB/c mouse (a) in comparison with a kidney section of a mouse immunized with 16/6 Id and treated with MTX (b).

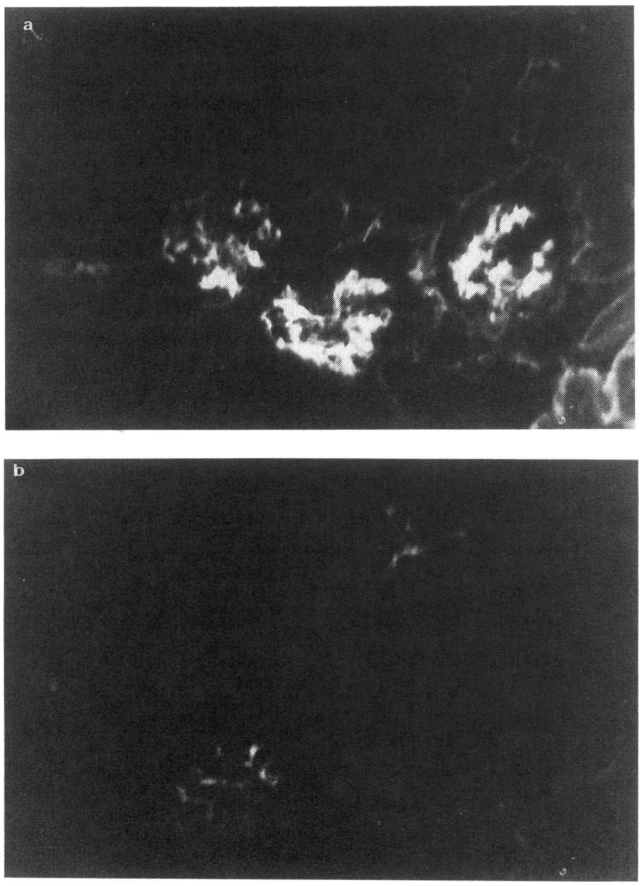

Fig. 1. Immunohistology of kidney sections 8 months after immunization with the human 16/6 Id. BALB/c female mice were immunized with the 16/6 Id before initiation of Methotrexate (MTX) treatment. Mice were killed and their kidney sections stained with FITC-labelled goat anti-mouse antibodies. Glomerular immunoglobulin deposits on the basal membrane, and mesangium in kidneys obtained from 16/6 Id-immunized treated mice (a) but not in kidneys from 16/6 Id-immunized and MTX-treated mice (b) are demonstrated (×400).

Cytokine profile in treated and non-treated 16/6 Id-immunized mice

As a dysregulation in cytokine production in mice afflicted with experimental SLE has been previously demonstrated [15], it was of interest to determine whether treatment with MTX could affect cytokine production. We therefore tested the cytokine profile in supernatants of either macrophages or spleen cells pooled from mice in the different experimental groups at the end of the trials, 8 or 10 months following immunization. Cytokine levels were evaluated in two different experiments and at least two assays for each cytokine were performed. Figures 2–4 and Table 3 demonstrate representative results of one experiment.

Figure 2 and Table 3 demonstrate the LPS-induced secretion of IL-1 by macrophages of BALB/c mice of the different experimental groups. It can be seen that IL-1 production was highly elevated in the 16/6 Id-immunized mice that were not further treated, and that treatment with MTX reduced IL-1

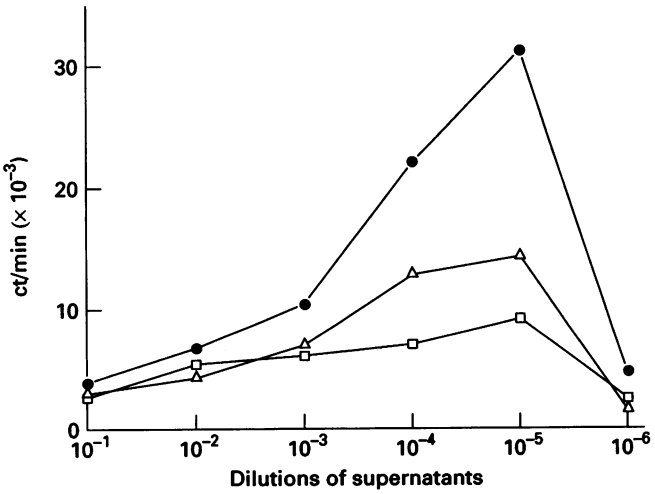

Fig. 2. Effect of Methotrexate (MTX) treatment on IL-1 production by pooled peritoneal macrophages in mice with experimental SLE. ●, 16/6 Id-injected mice; △, 16/6 Id-injected mice, MTX-treated; □, control mice. Each group consisted of five mice. IL-1 activity was measured using the 1A5-CTLD system. Values represent ct/min of ^{3}H-thymidine incorporation and are mean of triplicates. S.D. values did not exceed 10% of the mean of the ct/min values.

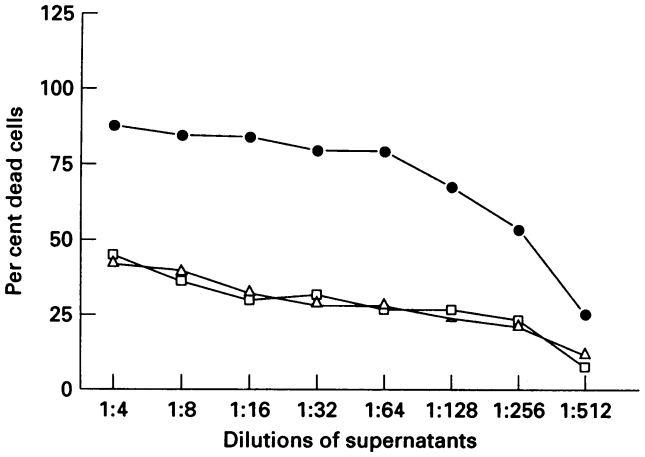

Fig. 3. Effect of Methotrexate (MTX) treatment on tumour necrosis factor (TNF) production by pooled splenocytes of mice with experimental SLE. ●, 16/6 Id-injected mice; △, 16/6 Id-injected mice, MTX-treated; □, control mice. Each group consisted of five mice. TNF-α levels in supernatants were determined by the A-9 cell lytic assay. Results are expressed as mean per cent of dead cells of triplicates. S.D. values did not exceed 10%.

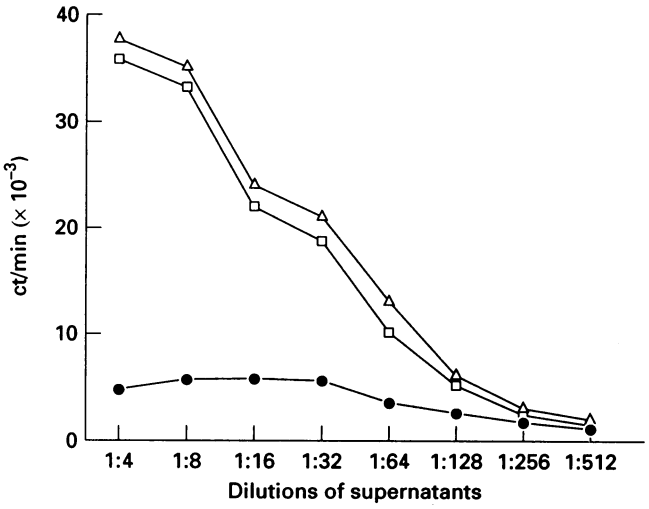

Fig. 4. Effect of Methotrexate (MTX) treatment on IL-2 production by pooled splenocytes of mice with experimental SLE. ●, 16/6 Id-injected mice; △, 16/6 Id-injected mice, MTX-treated; □, control mice. Each group consisted of five mice. IL-2 activity was evaluated by thymidine incorporation of CTLL cells. Results represent mean ct/min of triplicates. S.D. values did not exceed 10%.

secretion to levels comparable to those observed in the control group. A similar pattern for spontaneous secretion of IL-1 is also seen in Table 3.

TNF-α levels were also found to be highly elevated in the SLE-afflicted mice. Figure 3 and Table 3 show the activity of TNF-α in the supernatants of stimulated spleen cells of 16/6 Id-immunized mice. Similar to its effect on IL-1 secretion, treatment with MTX reduced the levels of TNF-α to baseline. TNF-α secretion of non-stimulated cells was similar to that shown in Con A-stimulated cells (Table 3).

Figure 4 and Table 3 show the levels of IL-2 secreted by spleen cells of the different experimental groups of BALB/c immunized and MTX-treated mice. Significantly reduced secretion of IL-2 could be observed in the 16/6 Id-immunized non-treated groups of mice. Treatment with MTX increased the capacity of lymphocytes obtained from these mice to secrete IL-2. Parallel to the findings for IL-2, splenocytes of SLE-afflicted mice produced very low levels of IL-4, IL-6 and IFN-γ (Table 3). MTX treatment reversed the production of the latter cytokines to levels comparable to those of healthy mice (Table 3).

IL-10 secretion by spleen cells in the SLE-afflicted mice was found to be increased. MTX treatment reversed the stimulated secretion of this cytokine to normal levels. It should be noted that MTX-treated control mice showed no differences in levels of any of the tested cytokines (Table 3).

Table 3. Effect of Methotrexate (MTX) on cytokine production in BALB/c mice with experimental SLE

Cytokine activity (U/ml)	IL-1 bioassay	IL-2 bioassay	IL-4 bioassay	IL-6 bioassay	IL-10 ELISA	IFN-γ ELISA	TNF-α* bioassay
Treatment							
Unstimulated cytokine secretion							
Control	989	ND	ND	4851	4	152	24
16/6 Id	11 643	ND	ND	520	10	5	120
16/6 Id + MTX	1520	ND	ND	5656	5	75	19
Control + MTX	925	ND	ND	4325	5	124	20
Stimulated cytokine secretion							
Control	5540	521	160	6041	45	254	30
16/6 Id	116 560	90	64	1150	103	75	145
16/6 Id + MTX	5234	560	134	9975	55	205	28
Control + MTX	4673	535	147	5530	52	223	25

IL-1 production by pooled peritoneal macrophages. All other cytokines by pooled splenic cells. Each group consisted of five mice. Macrophages were stimulated with lipopolysaccharide (LPS). Splenocytes were stimulated with concanavalin A (Con A).

*TNF activity is presented as ng/ml, all other cytokine activities are presented as U/ml. Cytokines were evaluated 8 months following immunization and 6·5 months of MTX therapy. ND, Not detectable.

DISCUSSION

The present study demonstrates the therapeutic effects of MTX on 16/6 Id-induced experimental murine SLE. MTX treatment resulted in an improvement in lupus-associated clinical manifestations including proteinuria, leukopenia and renal pathology, showing only minimal or absence of glomerular immune complex deposits. Our results do not favour humoral suppression as a major mechanism for the therapeutic effects of MTX, since in sharp contrast to its beneficial effects on clinical manifestations, MTX had no effect on the levels, kinetics or isotype distribution (IgM *versus* IgG) of anti-DNA or other SLE-related autoantibodies. In agreement with these results, treatment with the oestrogen antagonist Tamoxifen also did not affect autoantibody levels, although it had a positive clinical effect on experimental SLE [8]. Discordance between clinical manifestations and autoantibody levels has been reported among SLE patients as well [29]. Clinical trials with MTX in patients with RA also did not show significant changes in serum levels of immunoglobulins or rheumatoid factor [30].

The regulatory role of cytokines is exemplified in virtually all immune and immune-mediated responses. IL-1, IL-2, TNF-α, IL-6 and IFN-γ are involved in several autoimmune diseases such as RA, autoimmune thyroid diseases and SLE [12,31–33]. The *in vivo* expression and *in vitro* induction of these and other cytokines are aberrant in SLE patients [11–13]. B and T cell responses to different cytokines were also found to be defective in SLE [13]. Moreover, the presence of IL-1, IL-6 and IFN-γ in affected kidneys suggests that the latter may play a role in renal pathology [12]. Conflicting results are reported from different studies in SLE patients concerning the presence or production of different cytokines [12,13]. The latter might be due to the heterogeneity of disease activity in the patients, their treatment protocols, as well as different research approaches [12,13]. An aberrant production or reaction to certain cytokines was also found in mouse strains that develop SLE spontaneously [34]. However, the latter data have been incomplete due to the genetic nature of the disease in the SLE-prone strains and due to the inability to control the induction phase of the disease. It thus appears that the experimental model, in which an autoimmune disease is induced, is an appropriate tool for the study of the role of cytokines in the initiation and development of the disease, as well as of the effect of therapeutic means on the cytokine profile in treated mice.

We found that mice with experimentally induced SLE show a cytokine dysregulation ([15] and Table 3). These mice produced increased levels of the pro-inflammatory cytokines IL-1 and TNF-α by their peritoneal macrophages or splenocytes. The elevation of IL-1 and TNF-α (spontaneously and induced) is compatible with the inflammatory process associated with the disease, and reflects the hyperactivation of these cells which are responsible for the overproduction of IL-1 and TNF. Once induced, these cytokines act as pro-inflammatory effectors through their varied biological properties, including the initiation of a cascade of other cytokines that amplify the initial stimulus and lead to the persistence of disease and progression of pathological manifestations. The decreased production to normal levels of IL-1 and TNF in MTX-treated mice is compatible with its well known anti-inflammatory activity which is probably mediated by the regulation of these cytokines [35,36]. We have previously shown that MTX effectively suppresses IL-1 activity [16]. Others have demonstrated decreased production of IL-1 following MTX treatment in RA patients and in rats with adjuvant arthritis [36]. Limited information exists concerning the effect of MTX on TNF-α. However, reduced secretion of soluble TNF receptor was shown in RA patients following MTX treatment [18].

IL-10 has been reported to be a B cell stimulant on one hand, and on the other it was found to have suppressive functions on the production of several cytokines, e.g. IL-2, IFN-γ IL-1, IL-6 and TNF [37,38]. The high levels of secreted IL-10 determined in our experimental animal model and its reduction by MTX suggest that it plays a pro-inflammatory role in experimental SLE. The high levels of IL-10 may play a role in the inhibition of IL-2 and IFN-γ in SLE-afflicted mice.

Although all cytokines have some enhancing activities, most of them are also involved in down-regulation of the immune system and cytokine network. For example, IL-4 and IL-6, although primarily B and T cell stimulants, are capable of reducing IL-1 and TNF production [39,40]. IL-6 exerts some of its anti-inflammatory activity through the production of acute-phase proteins [39]. IFN-γ too has anti-inflammatory properties through suppression of IL-1 induction [32]. We have found a significant decrease in the production of several cytokines, i.e. IL-2, IL-4, IL-6 and IFN-γ, by the splenocytes of disease-afflicted mice. A decrease in *in vitro* production of IL-2 in SLE patients has been repeatedly reported [11,31,41,42]. Decreased *in vitro* production of IFN-γ was also reported in SLE patients [12]. The decreased production of IL-6 in our model does not correlate to other reports that found high levels of IL-6 in the sera of active SLE patients as well as increased *in vitro* production of IL-6 [14].

The inhibited production of the cytokines which have down-regulating activities in SLE patients and in our experimental model may be the cause for the persistence of inflammation in the disease process. In MTX-treated mice a restoration of the cytokine profile was observed to the range of secretion in control mice. It is possible that the positive influence of MTX on the clinical manifestations of SLE as well as of other rheumatic diseases is via modulation of the production or activity of those cytokines. The treatment protocol used in our study was not a preventive one, since it was initiated 2–3 weeks after the booster injection. At that time, the disease could already be detected by the presence of high autoantibody levels, although there were no overt clinical manifestations. A delayed MTX treatment, starting several months after immunization, when all clinical manifestations are present, may shed further light on the efficacy of MTX in this experimental disease. In addition, sequential evaluation of the cytokine profile at earlier stages of disease and treatment will provide more information concerning their role in disease pathogenesis. Our observations on the therapeutic effects of MTX in the experimental SLE model, and mainly on renal manifestations, should encourage further trials with MTX in SLE patients, even including those with renal involvement.

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