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PAPER

AVEMAR (a new benzoquinone-containing natural product) administration interferes with the Th2 response in experimental SLE and promotes amelioration of the disease

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The potential of oral treatment with AVEMAR (AVEMAR), a new benzoquinone-containing fermentation product of wheat germ, on features of experimental systemic lupus erythematosus (SLE) in naive mice, induced by idiotypic manipulation, was studied. We assessed the effect of AVEMAR on the profile of autoantibody production and the response of Th1/Th2 related cytokines as well as the clinical picture of experimental SLE in the SLE-induced mice.

When the product was given in the pre-immunization period, down-regulation of autoantibody production (anti-dsDNA, mouse 16/6 Id, and anti-histones) following treatment with AVEMAR was noted (eg anti-dsDNA decreased from 0.898 ± 0.097 OD at 405 nm to 0.519 ± 0.103 OD following treatment). This effect was sustained for at least 4 weeks after discontinuation of the therapy. Serological manifestations associated with a delay in Th2 response (IL-4 and IL-10) were recorded (eg IL-4 decreased from 91.7 ± 8.11 to 59.55 ± 7.78 ng/ml in splenocyte condition media). The mice showed normal ESR, WBC and less than 100 mg/dl of protein in the urine in comparison to > 300 mg/dl protein in the SLE non-treated mice.

In conclusion, oral intake of AVEMAR can ameliorate the clinical manifestations of experimental SLE, via affecting the Th1/Th2 network inhibiting Th2 response. *Lupus* (2001) **10,** 622–627.

Keywords: AVEMAR; benzoquinone; wheat germ; experimental model; autoantibodies; cytokines

Introduction

Animal models of systemic lupus erythematosus (SLE) and of antiphospholipid syndrome (APS), are of a great value in assessing novel therapies and approaches for the treatment of these conditions, due to the obvious limitations of evaluating new treatment modalities on patients. A few years ago, we and others introduced a novel method for the induction of experimental autoimmune conditions[1–8] including SLE and APS, in naive mice, based on the idiotypic immunization. Thus immunization of naive mice with a specific autoantibody (Ab_1; eg anti-dsDNA, anti-cardiolipin, or anti-proteinase-3 Abs), led to the generation of Ab_2 (anti-Id), namely an anti-autoantibody, and 2–3 months later to the generation of mouse Ab_3 (anti-anti-autoantibody), which simulates the original autoantibody (human or mouse origin) in its binding properties. This antibody network in naive mice leads to the production of specific pathogenic autoantibodies (eg Ab3), followed by emergence of a spectrum of serological, immunohistochemical and clinical manifestations of the respective autoimmune disease (eg immunization with anti-dsDNA led to experimental SLE, immunization with anti-cardiolipin resulted in experimental APS). The idiotypically induced model showed an upregulation of Th1 cytokine (IL-2, IFNγ) production at the early stage of the disease, while the increase in the Th2 cytokine (IL-4, IL-10) expression was ascendant as the autoimmune status progress.[9] Amelioration of the clinical manifestation of the disease was achieved by immunomodulation of the cytokine profile via reversing the Th2 response to Th1.[10]

These idiotypically induced experimental models of autoimmune diseases enabled a series of studies evaluating novel therapeutic modalities.[11–14] The studied treatments entailed hormonal manipulation (androgens, anti-prolactin agents), intravenous

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immune globulins (IVIG), Id-specific suppressor T cells, anti-idiotypic antibodies, and cyclosporin-A (CsA). The recently introduced novel approaches include anti-$CD4^+$ antibodies, oral tolerance, bone marrow transplantation, methimazole, tamoxifen, ciprofloxacin, IL-3 and dietary manipulation. Diet enriched with long-chain polyunsaturated fats has a suppressing effect on the expression of experimental SLE in BALB/c mice,[15] and linseed oil (enriched with omega-3 unsaturated fatty acid) prevented the appearance of clinical manifestations of experimental antiphospholipid syndrome.[16] Obviously, the main advantage of dietary manipulation is the lack of undesired side effects, normally associated with various immunosuppressive treatment modalities in SLE.

Wheat is one of the staple foods of mankind. Its kernel also contains the germ, which has been considered as an animal feed so far. Only a small portion of this milling by-product has been marketed for human consumption. Remarkable non-nutrients of wheat germ include the methoxy-substituted benzoquinones, which are present as glycosides of the corresponding methoxyhydroquinones. These compounds have been reported to exert anticancer effects in experimental systems.[17] A group of chemists led by one of us (MH) succeeded in developing a biotech process to manufacture a wheat germ extract (AVEMAR) standardized to the content of methoxy-substituted benzoquinones (0.04%). The extract has shown unexpectedly interesting biological activities, the experiments were not entirely reproducible but with either the pure benzoquinones or with the otherwise biologically active wheat germ lectin (WGA) present in the extract. A highly significant anti-metastatic effect of AVEMAR has also been observed.[18–20]

AVEMAR was found to cause an increase in the T cell response to Concanavalin A which resulted in an increase of blastic transformation. An immune restoring effect in a skin graft system by shortening the survival time of skin grafts in a co-isogenic mouse skin transplantation model was also shown to be a result of AVEMAR administration.[20]

Based on these biological characteristics of AVEMAR, we have investigated the potential of oral treatment on the features of experimental SLE in naive mice.

Materials and methods

Mice

Female BALB/c mice, aged 10–12 weeks, were purchased from Tel-Aviv University animal house, Israel. All the mice were kept in a standard condition of temperature (24°C) and 12 h light cycle. The mice were kept in plastic cages (five animals per cage) and were fed with rodent pellets and tap water.

Induction of experimental SLE

BALB/c mice were immunized intradermally in the hind footpads with 10 μg of anti-ssDNA human mAb, which carries the 16/6 idiotype, in complete Freund's adjuvant. Three weeks later a booster injection of the immunoglobulin (10 μg) in phosphate buffered saline (PBS) was given at the same site, as previously detailed.[2,3] The mice were bled every 2 weeks, and the levels of autoantibody titers were determined in the sera by ELISA for mouse 16/6 Id, anti-ssDNA, anit-dsDNA, anti-histones, anti-cardiolipin (aCL), and for anti-phosphatidylcholine (aPC) as a negative control. Established parameters of experimental SLE, as described previously (increased erythrocyte sedimentation rate, leukopenia and proteinuria),[1–5] were evaluated.

Treatment of experimental SLE with AVEMAR

AVEMAR was introduced daily *per os*, 7 days before disease induction and during a period of 9 weeks, to the BALB/c mice (5 mg/mouse) at a rate of 0.1 ml/mouse by biomedical needle (Thomas Scientific, Popper & Sons Inc, NY, USA). The groups of mice that were included in the study were: mice induced by anti-ssDNA mAb to develop lupus like disease with or without AVEMAR supplementation, and non-immunized mice with or without AVEMAR feeding.

Spot ELISA to determine autoantibody secreting cells

Mouse splenocytes (1×10^7 cells/ml) were assayed for their ability to secrete *in vitro* mouse 16/6, anti-ssDNA and aCL antibodies.[10] The preparation of splenocytes was done by teasing the spleen and passing the splenocytes through 0.45 μ nylon-mesh. The erythrocytes were lysed with 0.08 M Tris-buffered ammonium chloride. The cells were seeded in RPMI 1640, into 24-well tissue culture plates (Nunclon, Dalta, Denmark) precoated with dsDNA, cardiolipin or anti-16/6, phosphatidylcholine (using the same methods as coating ELISA plates), and blocked for 2 h with 3% BSA. The cells were incubated overnight. The secreted immunoglobulins (anti-DNA, 16/6, aCL) were bound during this time to the antigen and probed by enzymatic reaction. Anti-mouse polyvalent alkaline phosphatase was added for 2 h at 37°C. Following extensive washings, BCIP (5-bromo-4-chloro-3-indolyl phosphate; Sigma Chemical Co.

St Louis, MO, USA) was added in 2-aminopropranolol Triton X-405 $MgCl_2$ buffer to 3% Agar (type I, low electroendosmosis; Sigma) heated and diluted in BCIP buffer at 4:1 ratio, resulting in a 0.6% agar solution. Overnight incubation in 37°C resulted in blue spots. The specific ELISPOTS were evaluated in comparison to ELISPOT of total IgG secreting cells.

Preparation of spleen conditioned medium (SCM) for cytokine detection

Spleen cells from individual mice in each group were prepared as a single-cell suspension at a concentration of 1×10^7 cells/ml in tissue culture medium with or without addition of 10% FCS. The cells were coculture in a 24-well plate (Nunclon, Dalta, Denmark) at 5×10^6 cells/well/500 µl with or without Con A (Sigma Chemical Co. St Louis, MO, USA; 2 µg/ml). Plates were incubated for 24–48 h at 37°C and 5% CO_2. Cell-free supernatants were collected, centrifuged at 600 *g* for 15 min, filtered (0.22 µm) and stored at −70°C until tested.

Cytokine assay

The concentration of a studied cytokine produced by splenocytes *in vitro* was determined by ELISA according to the manufacturer protocol (R&D System Europe Ltd, Oxon, UK).

Results

Effect of AVEMAR on sera autoantibody titer in SLE mice

Oral administration of AVEMAR to lupus mice resulted in moderate levels of autoantibody titers (eg anti-dsDNA, anti-histones, mouse 16/6 Id, aCL) in the sera when compared to the titers of autoantibodies in the non-treated mice, as shown in Figure 1. The titers of anti-dsDNA decreased from OD 1.0175 ± 0.162 at 405 nm to OD of 0.5966 ± 0.106 ($P < 0.02$). The *P*-values between the treated and non-treated groups of mice, for all the tested autoantibodies were between 0.01 and 0.02.

Comparison of the titers of all autoantibodies tested, in the treated *vs* the untreated group revealed decreased titers of autoantibodies (42–56% less) in the AVEMAR-treated group of mice as compared to the non-treated lupus group of mice.

Table 1 demonstrates the antibody-forming cell activity (AFC) of splenocytes derived from the experimentally induced SLE mice following treatment with AVEMAR. The data shows a marked and significant inhibition in the number of anti-dsDNA and anti-16/6 Id AFC activity following treatment with AVEMAR (eg the number of anti-dsDNA AFC dropped from 73.7 ± 4.2 to 32.3 ± 4.2 upon treatment with AVEMAR). Moderate inhibition of the number of aCL AFC was documented, but was non-significant ($P > 0.05$), when compared to anti-phosphatidylcholine AFC activity (Table 1).

The long duration of the downregulatory effect of AVEMAR on autoantibody titers is demonstrated in Figure 2. Anti-dsDNA titers dropped from an OD of 0.898 ± 0.097 at 405 nm to 0.596 ± 0.106 OD and remained decreased 0.591 ± 0.103 OD, 4 weeks after the end of treatment. Non-significant changes in the titers of the tested autoantibodies in the plasma of the

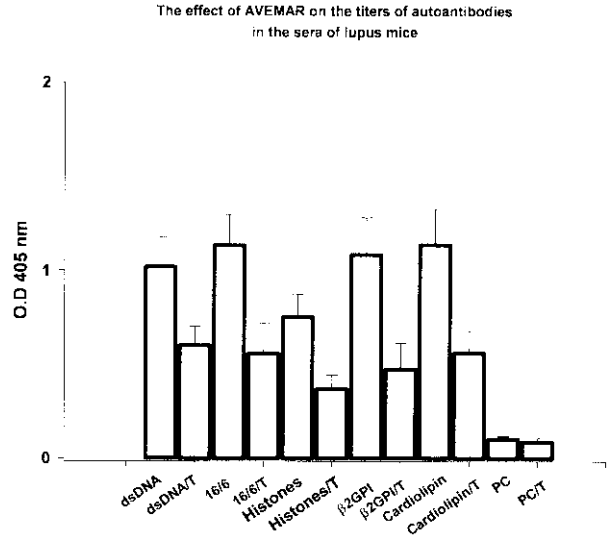

Figure 1 The effect of AVEMAR on the titers of autoantibodies in the sera of lupus. The sera were tested at dilution of 1:200. dsDNA/T, anti-dsDNA in AVEMAR treated mice; Hist/T, anti-total histones in treated mice; 16/6/T, mouse 16/6 idiotype in treated mice; cardiolipin-CL/T, in treated mice; CL/T, anti-cardiolipin in treated mice; PC/T, anti-phosphatidycholin in treated mice. The levels of autoantibodies in normal BALB/c mice with or without treatment ranged between 0.059 and 0.115 OD at 405 nm. The data are presented as means ± s.d. in OD at 405 nm of three experiments, 10 mice in each group.

Table 1 Antibody-forming cell activity (AFC) of splenocytes derived from lupus mice treated with AVEMAR

Ab to:	*Non-treated lupus mice*	*Treated lupus mice*	*Non-immunized*
dsDNA	73.7 ± 4.2	32.3 ± 4.2 $P < 0.037$	4 ± 2
16/6	65.3 ± 5.9	30.3 ± 3 $P < 0.04$	2 ± 1
Cardiolipin	47 ± 4.6	38.3 ± 3 $P > 0.05$	4 ± 1
PC	8 ± 1	8 ± 1	1 ± 1

The data are presented as means ± s.d. of AFC per 10^5 cells of three separate experiments.

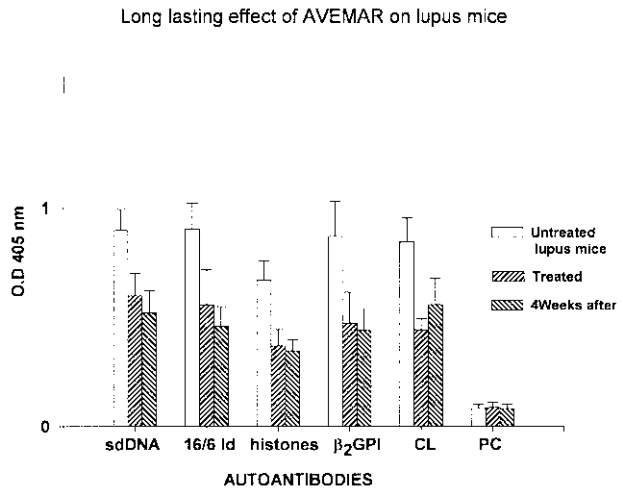

Figure 2 Long-lasting effect of AVEMAR on the titers of autoantibodies in the sera of lupus mice upon oral administration of AVEMAR. Data are presented in OD at 405 nm, as mean ± s.d. of 10 mice in three different experiments.

different groups of mice were observed during the tested time points ($P > 0.05$).

Th1/Th2 in vitro cytokine production

In vitro production of IL-2, IFNγ, IL-4 and IL-10 by splenocytes derived from experimentally induced SLE mice treated with AVEMAR is shown in Figure 3. A non-significant change in the level of IL-2 and IFNγ production ($P > 0.05$ for both groups), and a pronounced decrease in the production of IL-4 ($P < 0.01$) and IL-10 ($P < 0.01$), upon exposure of lupus mice to AVEMAR, can be clearly seen. The

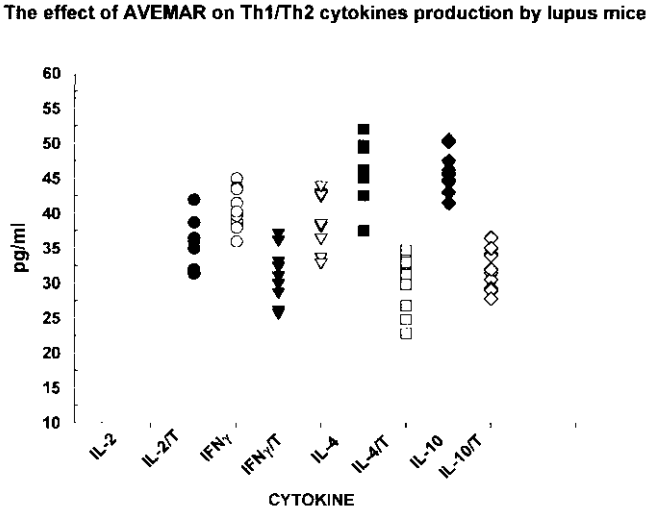

Figure 3 Th1/Th2 response in experimental SLE upon exposure to AVEMAR. The levels of cytokines IL-2, IFNγ, IL-4, IL-10 produced *in vitro* by splenocytes derived from lupus mice treated with AVEMAR.

concentration of IL-4 in the splenocyte condition media decreased from 91.7 ± 8.11 to 59.55 ± 7.78 ng/ml, while IL-10 dropped from 91.65 ± 5.8 to 63.95 ± 5.8 ng/ml, upon AVEMAR administration to the lupus mice.

The ESR (mm/6 h), the WBC count (cells/mm^3) and the amount of proteinuria (mg/dl) are shown in Table 2. AVEMAR-treated lupus mice showed a normal ESR, normal WBC (as compared to the leukopenia observed in the non-treated group of lupus mice), and developed only a very moderate proteinuria (< 100 *vs* > 300 mg/dl in the untreated group of lupus mice or less than 30 mg/dl in the non-immunized mice).

Discussion

Nutritional status is now recognized as having a significant impact upon normal immunocompetence. Among the many therapeutic modalities which are currently being tested in experimental as well as human SLE are dietary immunomodulations.[10–16,22–28] Dietary manipulation may provide the most cost-effective and least toxic therapy for patients with SLE, saving the patient from the undesired side effects normally associated with various immunosuppressive treatments.

AVEMAR, is an orally applied fermentation product of wheat germ containing 0.04% substituted benzoquinone (AVEMAR). Preliminary studies have shown that the product has antimetastatic effects as well as immunomodulating properties.[18–20]

It has also been recently shown that AVEMAR significantly enhanced the degree of blastic transformation of mice splenic lymphocytes caused by Concanavalin A.[20] Oral supplementation of AVEMAR was also found to have an immune-restoring effect in a skin graft system by shortening the survival time of skin grafts in a co-isogenic mouse skin transplantation model.[20] In the current study, the material was found to possess immunosuppressive capabilities.

Table 2 Clinical manifestations in naive mice with experimental lupus, treated orally with AVEMAR

Feeding of lupus mice with AVEMAR	*Treated* n = *10*	*Non-treated* n = *10*	*Non-immunized* n = *10*
Findings			
ESR (mm/6 h)	2.6 ± 1 $P < 0.02$	7.3 ± 0.9	1.7 ± 0.67
WBC (cells/mm^3)	5888 ± 378 $P < 0.04$	2958 ± 325	5997 ± 367
Proteinuria (mg/dl)	< 100 $P < 0.02$	> 300	< 30

ESR, erythrocyte sedimentation rate; WBC, white blood cell count.

Compared to control mice which were not fed with AVEMAR and developed experimental SLE, the mice which were exposed to the treatment, showed a significantly lower humoral response (approximately 50%), as shown by production of autoantibodies (eg anti-ssDNA, anti-dsDNA, mouse 16/6 Id, anti-histones). The antibody response was associated with normal values of the ESR, WBC, and by moderate proteinuria when compared to mice which did not receive AVEMAR and developed high titers of autoantibodies in the sera, increased ESR, leukopenia and significant proteinuria.

Analysis of the Th1/Th2 cytokine response in the AVEMAR-treated mice compared with the untreated mice with experimental lupus showed an interdependence between the balance in Th1/Th2 cytokine response and the clinical picture. AVEMAR caused a decrease in the Th2 cell response (IL-4, IL-10 production) in the mice which were exposed to the treatment, as compared to the non-treated group of lupus mice.

Our data are in line with other groups of researchers who have tried to improve the clinical manifestations of SLE by diet in mouse models as well as in human, by immunomodulating the Th1/Th2 cell dynamic balance.[21]

Th1 T cells produce IL-2 and IFNγ cytokines while Th2 cells generally produce IL-4 and IL-10 cytokines which immunomodulate an immune reaction.[28] Previous studies demonstrated that the expression of IL-4 and IFNγ play a prominent role in the pathogenesis of murine lupus.[29,30] Administration of anti-IL-4 antibody was effective in preventing the onset of lupus nephritis in these mice. Furthermore, mice with constitutive transgenic expression of IL-4 developed experimental lupus nephritis which could be abrogated by IL-4 neutralizing antibody.[31] Kinetics of cytokine production in experimental SLE model, induced by idiotypic manipulation — immunization with a human anti-ssDNA mAb (16/6 Id) — revealed dysregulation of Th1/Th2 cytokine network at different stages of disease development.[9] At the early stage of the disease, a preferential increase in Th1 cytokine IL-2, IFNγ production was documented, while IL-4 and IL-10 (Th2 cytokines) production predominated later in the disease course.[9] Our results are in line with the above data. AVEMAR was found to affect preferentially the very early stage of the disease induction by prolonging the Th1 response and reversing the early stage of the Th2 response towards Th1 (eg enhancing IL-2 and IFNγ production and decreasing IL-4 and IL-10 production). Diet manipulation in $NZB \times NZWF_1$ mice, which develop lupus, showed that fish oil administration delayed the onset of autoimmune kidney disease by suppressing both Th1 (IL-2, IFNγ) and Th2 (IL-5, IL-10) cytokine production.[32] High IL-4 and IL-10 gene expression was observed in lupus patients in the active stage of disease.[33] More interesting are the observations that SLE patients produced abnormally large amounts of IL-10,[34–36] and serum levels of IL-10 correlated with disease activity.[37–39] Treatment of lupus patients with IL-10 antagonist (IL-10 mAb) resulted in a decrease in SLE disease activity index, and emphasized the importance of IL-10 involvement in the pathogenesis of the disease.[40] Furthermore, employing gene therapy, IL-4 and IL-10 genes were found to be most frequently protective.[41,42] Referring to our results, administration of AVEMAR starting prior to disease induction, delayed the Th2 response. Taken together, the results of administration of AVEMAR are encouraging enough to propose a diet based on AVEMAR for a high-risk population to develop lupus, as a prevention measure.

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