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Improvement of herpetic stromal keratitis with fumaric acid derivate is associated with systemic induction of T helper 2 cytokines

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Summary

Fumaric acid derivates have been shown to stimulate T helper-2-cytokines (interleukin (IL)-4, -5) without affecting the T-helper-1-cytokine (IL-2, interferon (IFN)-γ)-response. Herein, the influence of systemic treatment with the fumaric acid derivate dimethylfumarate (DMF) on the secretion of T helper-cytokines and the development of HSV-1 stromal keratitis (HSK) was studied in mice. The corneas from BALB/c mice were infected with 10^5 PFU of HSV-1 (KOS strain). While one group of mice was treated intraperitoneally with PBS, another group of mice received DMF at 15 mg/kg of body weight. Expression of IL-2, -4, -10 and IFN-γ was analysed in HSV-1 activated lymphocytes by ELISA. The severity of epithelial and stromal herpetic keratitis was investigated clinically. Corneas were studied for the inflammatory cell infiltration, and the CD3-, CD4- and CD8-positive cells were analysed by immunohistochemistry. The IL-2, -4, 10 and IFN-γ content was measured in the corneas. Virus replication in the eyes was analysed by a plaque-assay. The DTH-response, the HSV-specific T cell proliferation and the serum neutralizing antibody-titres were investigated. DMF increased IL-4 and IL-10, but not IL-2 and IFN-γ, secretion in activated lymphocytes from the spleen. Incidence and severity of stromal HSV-1 keratitis was reduced in the DMF group ($P < 0{\cdot}01$). In the corneas from DMF-treated mice, the numbers of CD3+ and CD4+ cells were decreased and IL-4 was increased. Severity of epithelial disease and the virus-clearance from the eyes did not differ between the PBS and DMF group of mice. DTH, HSV-specific T cell proliferation and the neutralizing antibody-titres were not impaired. DMF increased the T helper-2-cytokine secretion in activated lymphocytes. After corneal HSV-1 infection, corneas from DMF treated mice had increased IL-4 content. This is associated with an improvement of herpetic stromal keratitis and reduced corneal T cell infiltration. DMF did not impair the systemic antiviral response.

Keywords: fumaric acid esters, dimethylfumarate, immunomodulation, herpes simplex virus, keratitis, T cells, neutrophils

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Introduction

Herpetic stromal keratitis (HSK) is a disease that may cause corneal scars and neovascularization during recurrent episodes of inflammation. There is substantial evidence that the type-1 T lymphocytes that predominantly secrete interleukin (IL)-2 and interferon (IFN)-γ are pathogenic in the evolution of the disease. Cells isolated from eyes in the acute stage of HSV-1 keratitis produce IL-2 and IFN-γ, but not IL-4 or IL-10 [1]. In addition, the incidence and severity of herpetic corneal disease is improved through intraperitoneal injection of anti-IFN-γ and anti-IL-2 [2]. Neutralization of IL-2 or IFN-γ caused a remission of HSK [3,4].

In contrast, typical Th2 cytokines have been detected during the healing phase of HSK [5,6]. Furthermore, the topical administration of plasmid DNA encoding IL-4 or IL-10 suppressed the development of HSK [7,8]. Recombinant IL-10 injected into the corneas of mice suppressed the development of HSK [9]. Local IL-10 administration reduced macrophage inflammatory protein-2 (MIP-2), MIP-1a and

monocyte chemoattractant protein (MCP)-1 and also reduced the T cells and neutrophil-infiltration of the cornea after HSV-1 infection [10].

One of the major interests in cytokine research in HSK is to modify the complex immune-response in order to prevent or improve corneal immune-pathology. For reducing corneal lesions, two different possibilities for immune modulation are conceivable. A decrease in the Th1 cytokine response may have a beneficial effect on the course of the disease. However, this approach might be a major disadvantage for the host's antiviral defence, as IL-2 is involved in the elimination of herpes simplex virus [11], and anti-IL-2 and IFN-γ treatment led to an exacerbation of periocular skin lesions in mice after corneal HSV-1 infection [2]. Therefore, an attempt to increase Th2 cytokine expression may be another reasonable therapeutic option.

Psoriasis vulgaris is a chronic skin disease that is type-1 cytokine mediated [12]. The therapeutic benefit of fumaric acid derivatives for the systemic treatment of patients with psoriasis vulgaris has been well established [13]. It has been demonstrated recently that one of the important action mechanisms of the fumarates is through its modulation of the Th2 cytokine-release. Whereas the IL-4 and IL-5 secretion was enhanced in activated T-helper cells, the Th1-cytokines were not affected [14].

These studies prompted us to use fumaric acid derivatives also in an experimental model of herpetic keratitis. Our data suggest that fumaric acid derivatives cause an immunomodulation by increasing the Th2 cytokine release in cultured lymphocytes. The systemic DMF treatment increased the IL-4 expression in the cornea and improved the course of HSK, while the systemic antiviral immune responses were not diminished.

Materials and methods

Mice

Six- to 8-week-old female BALB/c mice were obtained from Charles River Laboratories. Animals were used in accordance to the 'Principles of laboratory animal care' (NIH publication no. 85–23, revised 1985) and to the protocols approved by the Institutional Animal Care and Use Committee.

Virus, viral infection and clinical examinations

Herpes simplex virus (KOS strain) was passaged on Vero cells (American Type Culture Collection, ATCC, CCL 81, Rockville, MD, USA), and the virus-containing supernatant was aliquoted and stored at −80°C [6,15].

Following intraperitoneal anaesthesia with ketamin HCl (2 mg) and mepivacain HCl (400 ng), the epithelium of the right eye was scratched and 10^5 PFU of HSV-1 in a volume of 5 µl were instilled onto the cornea [6,15].

The mice were then evaluated under an operation-microscope for the clinical signs of herpetic keratitis. Epithelial disease was graded dependent on the geographical area of the lesions on the epithelial surface: 0, no epithelial lesions; 1+, less than 25%, 2+, less than 50%, 3+, less than 75%, 4+, 75% to 100%. Stromal disease was graded from 0 to 4+, with a score of 1+ consistent with less than 25%, 2+ less than 50%, 3+ less than 75% and 4+ between 75 and 100% corneal opacity with corneal neovascularization, oedema, and thinning [6,16].

Plaque assay

Vero cells were grown to confluence in RPMI with 5% heat-inactivated fetal bovine serum (Sigma, Germany) in 24-well tissue-culture plates (Becton Dickinson, USA). The virus-containing tissues were homogenized and serially (1 : 10) titrated in RPMI. Each dilution was cultured for 1 h at 37 °C on mono-layers after discarding the serum-containing supernatant. After incubation, the supernatants were removed and each well was covered with 0·6% RPMI/agarose medium and incubated for 2 days. Finally, all wells were fixed with 0·5 ml of 37% formalin solution. On the next day, the RPMI/agarose was removed, the mono-layers in each well were stained with 2% crystal violet, and the plaques were counted ($n = 5$ each time point and group) [15].

Study design

Under sterile conditions, 0·5 mg of the lyophilized DMF was dissolved daily in 0·5 ml of PBS. Mice in the DMF-group were injected intraperitoneally with DMF at 15 mg/kg of body-weight. This DMF-dosage has been determined as nontoxic for mice. Mice were treated daily for 28 days before, and for 14 days after the corneal HSV-1 infection. No toxic side-effects or intolerance reactions occurred in any of the mice. Mice in the control group were injected daily with PBS.

Histological staining

At day 14 after corneal HSV infection ($n = 8$, each group), the HSV-infected globes were enucleated, fixed in McDowels-fixative, dehydrated by ascending ethanol concentrations and paraffin-embedded. Five µm-sections were cut and were stained with haematoxylin and eosin [6]. The sections were studied histopathologically. In two separate serial sections, the numbers of total infiltrating cells and of PMN were enumerated within the central cornea in high-power fields (250x).

Immunohistochemical staining

Other HSV-infected eyes were removed at day 14 following infection ($n = 6$ each group) and snap-frozen in liquid

nitrogen. Specimens were stored at −80 °C after embedding in OCT compound (Ames Company, Miles Laboratory, Elkhart, IN, USA). Four μm cryostat-sections were treated with an immunoperoxidase staining protocol [6,15] and were incubated with the primary monoclonal antibodies (30 min): rat anti-mouse CD3 mAb (dilution 1 : 20 in PBS, Pharmingen) for the detection of T cells; rat anti-mouse CD4 mAb (dilution 1 : 20 in PBS, Pharmingen) for the detection of T helper lymphocytes; rat anti-mouse CD8 mAb (dilution 1 : 20 in PBS, Pharmingen) for the detection of T suppressor/cytotoxic T cells. Negative controls were included that were processed without the primary antibodies.

Three separate high-power fields (250×) of two serial sections were studied for the number of positively stained cells in a 10×10 mm grid. The counting was performed in a masked fashion by two observers independently.

Cytokine expression in the corneas and spleens

Corneas were excised from the DMF-and PBS-treated animals after removing the limbal tissue. Samples were stored at −80 °C until assayed. The corneas were thawed, minced, sonicated for 30 s in 1 ml PBS, and centrifugated at 10 000 *g* for 10 min. The cell homogenates were assayed for IFN-γ, IL-2, IL-4 and IL-10 with the use of commercially available ELISA kits (Pharmingen, Hamburg, Germany) ($n = 15$ each group).

Lymphocytes from single cell suspension from the spleens were harvested from mice ($n = 12$) on day 14 after corneal HSV-1 infection. 5×10^6 cells resuspended in 1 ml of RPMI-1640 with hepes/mercaptoethanol and 10% heat-inactivated bovine serum were added to each well of 24-well plates. Then 100 μl UV-inactivated HSV-1 (2×10^7 PFU HSV-1) was placed in each well. After 48 h incubation with medium, 25 μM DMF or ConA at 37 °C in 5% CO_2, cell supernatants were harvested. The concentrations of IL-2, IFN-γ, IL-4 and IL-10 were determined [16].

In another set of experiments, spleen cells were obtained from mice that were treated *in vivo* with DMF or PBS for 28 days before and 14 days after corneal HSV-1 infection. 5×10^6 cells were incubated for 48 h with UV-HSV-1 in the absence of DMF, and then the cytokine concentrations were measured in the cell supernatants.

Delayed type hypersensitivity reaction

Delayed type hypersensitivity reaction (DTH)-responsiveness was determined using the footpad-swelling assay as described previously [16]. Fifty μl of UV-inactivated virus solution was inoculated in the right hind footpad using a 30-gauge needle, and 50 μl of RPMI in the left hind footpad was used as control. Footpad swelling was measured after 24 h with a micrometer. Results were expressed as specific footpad swelling in mm. ($n = 8$ each group).

Proliferation assay with ^{3}H-thymidine

The isolated spleen cells were resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS, 5×10^{-4} M mercaptoethanol and 5 mM HEPES. A 100 μl aliquot containing 1×10^5 cells was immediately added to each well of a 96-well microtitre flat-bottom plate. UV-inactivated virus-solution at a final concentration of 2×10^7 PFU was added to each well in triplicate, and this was followed by an incubation at 37 °C in 5% CO_2 for 3 days. One μCurie of tritiated thymidine was added to each well, the cells were incubated for 12 h at 37 °C, and the amount of incorporated tritiated thymidine was measured in a beta plate reader. Spontaneous count was determined with 10% FCS, which served as an irrelevant protein control. 5 μg/ml of Concanavalin A (*Canavalia ensiformis*, Sigma) were used as a positive control [16] ($n = 5$ each time point).

Antibody titres against HSV-1

Equal volumes of serial 1 : 2 serum-dilutions with RPMI 1640 were added to tubes each containing 10^3 PFU/ml of HSV-1 (KOS strain). The serum-virus mixtures were incubated at 37 °C for 1 h before aliquots of 0·2 ml were analysed with a plaque assay [16]. The neutralizing antibodies were counted in that dilution that reduced the mean PFU value by 50% in comparison to the controls.

Statistics

The entire experiment was performed twice with an identical design. Fisher's protected least significant difference test was used to analyse the statistical significance of differences between keratitis-incidences, mean values of clinical keratitis-scores. Student's *t*-test was used to determine the differences between the experimental groups with respect to the cell numbers in the histological and immunohistochemical studies, plaque assays, DTH, cytokine expression, serum antibody-titres and lymphocyte proliferation assay. $P < 0{\cdot}05$ was considered statistically significant.

Results

DMF influences the cytokine expression in spleen cells obtained after HSV-1 infection

To analyse the effects of DMF on Th1-cytokine expression, the concentrations of IL-2 and IFN-γ were determined in supernatants from lymphocytes that had been harvested from the spleens from mice 14 days after corneal HSV-1 infection. Cytokine release was studied in cells that were stimulated with UV-HSV Ag. There was a minimal baseline secretion of IL-2 and IFN-γ in the medium control. The results show that the secretion of IL-2 and IFN-γ did not change with DMF as compared to the medium control. The

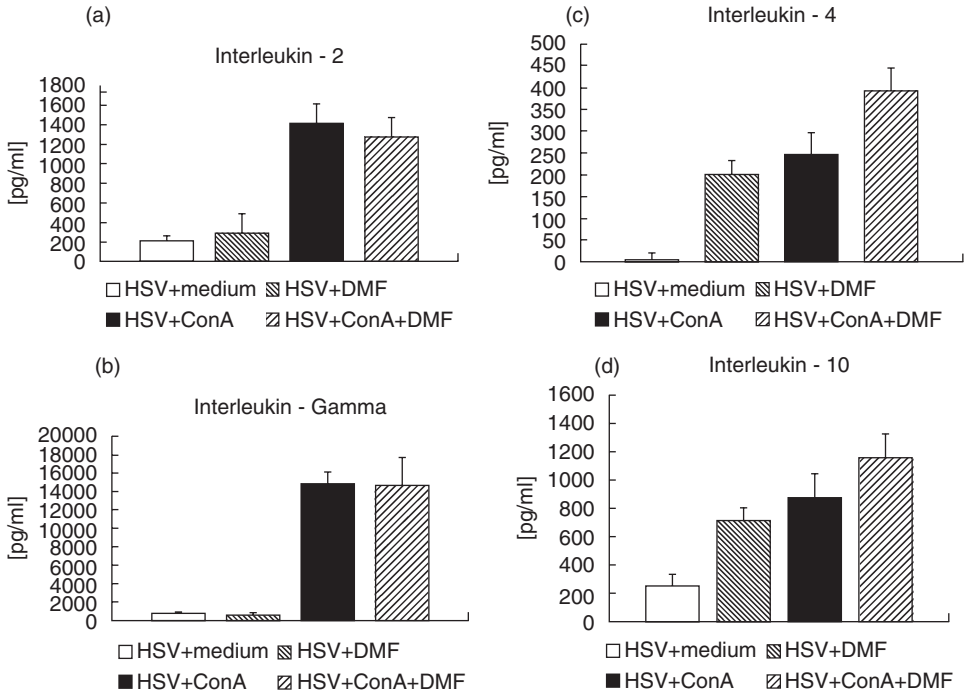

Fig. 1. Influence of dimethylfumarate (DMF) on the secretion of T helper cytokines in splenic cells. ELISA data from cell culture supernatants of cells harvested at day 14 p.i. from the spleen. Up-regulation of IL-4 and IL-10 but not IL-2 and IFN-γ secretion after 24 h incubation with DMF. $P < 0{\cdot}05$.

expression was highly accelerated with concanavalin A (ConA) (Fig. 1). In contrast, the cytokine release for IL-4 and IL-10 showed a strong increase in cells treated with 25 μM DMF as compared to the medium control (Fig. 1). Higher DMF concentrations did not change this pattern of cytokine secretion (data not shown). These findings show that DMF stimulated the IL-4 and IL-10, but not IFN-γ and IL-2 secretion of HSV-1 activated lymphocytes from the spleen.

Additional experiments were performed with splenocytes that were obtained from corneally HSV-1 infected mice that were treated with or without DMF. The cells were stimulated *in vitro* by HSV in the absence of DMF. Lymphocytes from the DMF treated group of mice had increased secretion of IL-4 and IL-10, but not of IL-2 or IFN-γ (Table 1).

DMF improves the course of stromal HSV keratitis

Further studies analysed whether or not DMF treatment might modulate the development of herpetic stromal keratitis. While stromal keratitis has developed by day 14 p.i. in 10 out of 14 mice (72%) in the HSV-1 infected animals that were PBS treated, only 4 out of 19 mice in the DMF-group (22%) showed the clinical signs of stromal keratitis ($P = 0{\cdot}004$). While profound oedema, stromal inflammation and neovascularization was noted in the PBS-treated mice, stromal inflammation, oedema and neovascularization were less severe in the mice that were treated with DMF ($P = 0{\cdot}004$, Fig. 2).

Table 1. Influence of dimethylfumarate (DMF) on the secretion of cytokines in splenic cells obtained from mice that were treated with DMF or PBS 28 days before and 14 days after corneal HSV-1 infection. Up-regulation of IL-4 and IL-10 *in vitro* by stimulation with HSV-1 in the absence of DMF.

Cytokines	PBS treatment	DMF treatment	P-value
IL-10	619·9 ± 49·3	890·2 ± 37·7	<0·001
IL-4	36·4 ± 4·3	59·6 ± 4·0	<0·001
IFN-γ	1078·3 ± 86·5	1096·5 ± 49·2	0·79
IL-2	167·8 ± 7·5	160·3 ± 8·4	0·29

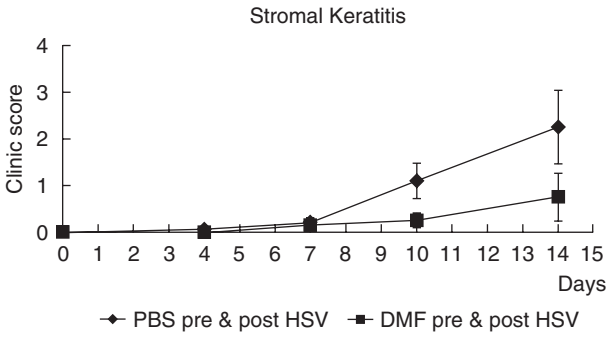

Fig. 2. Influence of dimethylfumarate (DMF) on the development of herpetic stromal keratitis mice. Groups of mice were treated daily intraperitoneally with DMF or PBS 28 days before and 14 days after corneal HSV-1 infection. The severity of stromal keratitis on day 14 after infection was graded as: 0 clear cornea; +1 mild corneal haze; +2 moderate corneal opacity or scarring; +3 severe corneal opacity, iris visible; +4 opaque cornea, iris not visible, necrotizing stromal keratitis with ulceration. Mean score ± SD of all mice in each group. DMF reduced the severity of keratitis ($P < 0{\cdot}01$).

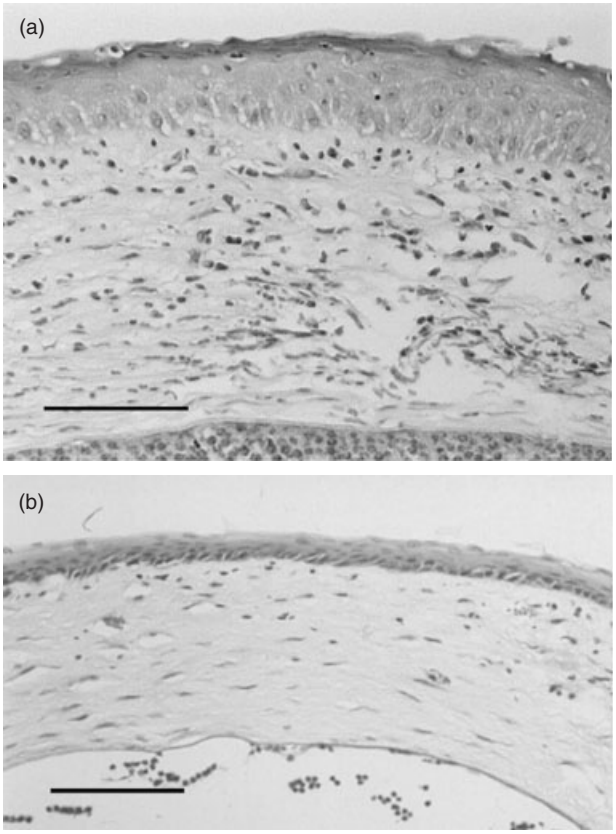

Fig. 3. Influence of dimethylfumarate (DMF) on the histopathological appearance of the cornea after HSV-1 infection. Groups of mice were treated daily intraperitoneally with DMF or PBS 28 days before and 14 days after corneal HSV-1 infection. Corneal sections representative for (a) PBS- or (b) DMF-treated mice; haematoxylin-eosin staining. Magnification ×120. Scale bar indicates 100 μm.

In order to further support the idea that DMF may improve the course of stromal HSV-1 keratitis, the corneas harvested at day 14 after infection were analysed histologically. The corneas from the PBS-treated mice had a marked oedema and inflammatory cell infiltration in the epithelium and stroma. Stromal tissue necrosis and a profound neovascularization was detected (Fig. 3a). In contrast to this, HSV induced stromal inflammation, corneal neovascularization and tissue destruction were profoundly reduced in the mice that had been treated with DMF (Fig. 3b). The numbers of the total inflammatory cells and the PMNs infiltrating the central cornea in the PBS treated mice at day 14 p.i. were 409·1 ± 91·4 and 296·6 ± 59·7, respectively, and were reduced to 144·3 ± 51·4 ($P = 0·027$) and 109·1 ± 37·2 ($P = 0·021$) in the DMF treated mice.

Influence of DMF on T cells infiltrating the cornea

The further experiments were designed to investigate the influence of DMF treatment on the number of cells from various T cell populations in the corneas of mice after HSV-1 infection. Corneal specimens were immunohistochemically stained with antibodies directed against CD3, CD4 or CD8. The results summarized in Table 2 show that the number of T cells in the cornea comprises only a small percentage of the total inflammatory cells and as compared to the PMN numbers. In addition, the data show that higher numbers of CD3 and CD4 positive cells were present in the central cornea of PBS-treated mice than in the corneas of DMF-treated mice. The numbers of CD8 positive cells in both groups of mice were very small, and no differences were found between the groups.

Expression of Th1 and Th2 cytokines in corneas after HSV-1 infection

In order to investigate the influence of DMF on the expression of T cell cytokines in the cornea, the cytokine levels in the corneas at day 14 after corneal HSV-1 infection were analysed by ELISA technique. The level of IL-4 was increased markedly after the DMF treatment ($P < 0·05$), while no significant differences were noted between the PBS and DMF treated mice with respect to the level of another typical Th2 cytokine IL-10 (Fig. 4). Furthermore, the expression of IL-2 and IFN-γ, which are representing characteristic Th1 cytokines, were not influenced by the DMF treatment (Fig. 4).

DMF is not influencing infectious epithelial HSV keratitis

This set of experiments was performed in order to study the influence of DMF on the course of infectious epithelial keratitis that occurs in the first few days after corneal HSV-1 infection. Our data show that all mice developed a mild and transient epithelial keratitis within the first day after infection. This healed completely within 5–7 days without any scar-formation or neovascularization. The severities and course of the epithelial keratitis did not differ between the PBS and DMF treated mice. The mean (± SD) scores were in the PBS treated mice 3·4 ± 0,4, 2·8 ± 0·7 and 0·1 ± 0·2, and in the DMF treated mice 3·6 ± 0·2, 3·2 ± 0·9 and 0·2 ± 0·1 on the days 1, 3 and 5 p.i., respectively.

Table 2. Influence of dimethylfumarate (DMF) on the corneal inflammatory infiltration in the central cornea. Groups of mice were treated daily intraperitoneally with DMF or PBS 28 days before and 14 days after corneal HSV-1 infection. Immunohistochemical staining with antibodies directed against CD3, CD4 or CD8. Numbers of positively stained cells in a high-power field. Cell numbers were reduced in mice treated with DMF. Cell numbers, mean ± SEM.

	Groups		
Antibodies	PBS	DMF	P-value
CD3	37·3 ± 7·5	6·8 ± 4·5	0·007
CD4	30·6 ± 4·2	6·8 ± 2·7	0·001
CD8	1·0 ± 0·5	0·8 ± 0·4	0·7

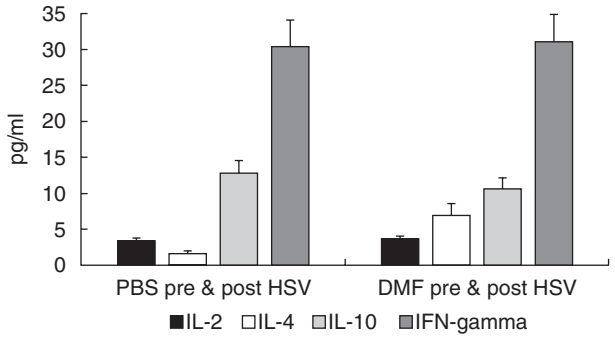

Fig. 4. Influence of dimethylfumarate (DMF) on the secretion of certain T helper cytokines in the HSV-1 infected corneas. Groups of mice were treated daily intraperitoneally with DMF or PBS 28 days before and 14 days after corneal HSV-1 infection. ELISA data are shown. Up-regulation of IL-4 but not IL-2 and IFN-γ after 24 h incubation with DMF ($P < 0{\cdot}05$).

Plaque assay examinations were performed from eye homogenates in order to study the influence of DMF treatment on the virus-titres from the HSV-1 infected eyes. The data reveal that the virus was nearly cleared from the eyes of both groups by day 7 p.i., and the DMF treatment did not influence these patterns (Fig. 5). Together with the clinical data this shows that DMF treatment does not impair the viral clearance from the eye and the course of infectious epithelial keratitis after corneal HSV-1 infection.

Influence of DMF treatment on the systemic immune response

This series of experiments was performed to study the influence of the DMF treatment on the HSV-1 specific immune response.

The DTH-response is substantially mediated by CD4+ T cells. Although DMF treatment had an influence on the CD4 + population and the cytokine profile in the cornea, the results herein now show that DMF did not change the DTH response at the days 7 and 14 after infection. In the PBS treated mice, mean (± SD) foot pad swelling was $0{\cdot}41 \pm 0{\cdot}26$ and $0{\cdot}65 \pm 0{\cdot}2$; and in the DMF treated mice it was $0{\cdot}47 \pm 0{\cdot}27$ and $0{\cdot}59 \pm 0{\cdot}1$ on the days 7 and 14 p.i., respectively.

The HSV antigen-specific T cell-proliferation was determined in cell suspensions from the spleen. While HSV-specific cell proliferation was detected on the days 7, 10 and 14 p.i., the responses did not differ between the PBS and the DMF treated mice.

On the days 10 and 14 p.i., HSV neutralizing antibodies were present in serum specimens. The capacity of the serum to neutralization of the HSV-1 was measured. No significant antibody-production was detected in both groups on day 7 p.i. The titres determined on day 14 p.i. were increased as compared to the day 10 specimens. However, there were no significant differences in the antibody titres between the PBS and DMF treated mice.

Discussion

Recent cell culture experiments have provided evidence that fumaric-acid derivates exert a selective increase of the cytokine production of T cells towards a Th2 profile. Monomethylfumarate (MMF) modulated the cytokine secretion of activated peripheral blood mononuclear cells in a dose dependent manner, as it increased the production of IL-4 and -5. Interestingly, the production of IL-2 and IFN-γ was not affected [14]. Others have reported that DMF enhanced the production of the Th2 cytokine IL-10 from cultured HUT T cells [17] and in human peripheral blood mononuclear cells [18], but it had no effect on the IL-12 secretion [18], a known inducer of a Th1 response.

Our *in vitro* experiments with lymphocytes that were obtained from the spleens of mice 14 days after corneal HSV-1 infection show that the expression of IL-4 and IL-10 was increased when the HSV-1 activated cells were cocultured with DMF as compared to the medium control. Similar results were obtained with regional lymph node cells (data not shown). Also, spleen cells isolated from DMF treated mice that were infected corneally with HSV-1 had increased levels of IL-4 and IL-10 when they were activated *in vitro* by HSV-1 in the absence of DMF than cells from the PBS treated mice in the control group. In agreement with previous observations, the IFN-γ and IL-2 levels in DMF treated lymphocytes had not been influenced by the DMF treatment. Taken together, these observations suggest a selective up-regulation

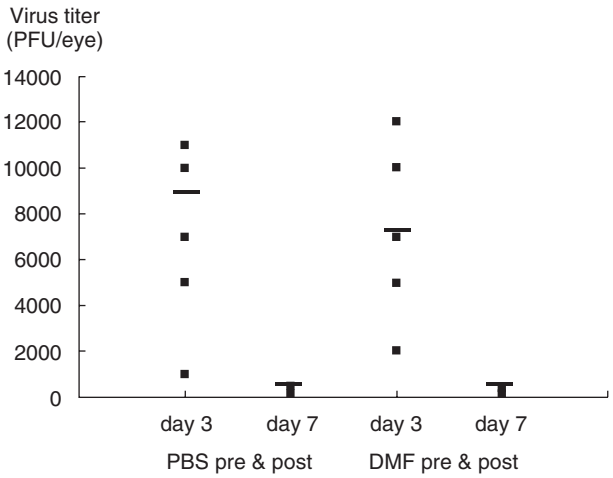

Fig. 5. Influence of dimethylfumarate (DMF) on the virus replication in eyes after corneal HSV-1 infection in mice. Groups of mice were treated daily intraperitoneally with DMF or PBS 28 days before and 14 days after corneal HSV-1 infection. DMF treatment did not alter the clearance of infectious virus particles from the infected eyes ($n = 5$, each time point and group).

of Th2 cytokines by fumaric acid esters, and this notion is in correspondence with previous studies [14,17,18].

It has been repeatedly shown that patients that suffer from psoriasis can be successfully treated with fumaric acid esters [13,19]. The lesions typically have monocytes and CD4 + cell infiltration with preferential expression of IFN-γ and IL-2 that are typical for type 1-cytokine mediated disease [12,20,21]. Fumaric acid esters also suppressed the type-1 mediated acute and chronic rejection in an animal model of kidney transplantation [22]. Our previous observations [23] and these herein that systemic DMF treatment reduced the incidence and severity of T cell mediated HSK, is in agreement with these previous findings.

Recent studies have shown that systemic fumaric acid ester treatment suppresses the number of peripheral CD4+ and CD8+ T cells [24] and the number of mononuclear cells, ODP4 + T-helper cells and CD15+ granulocytes in the psoriatic lesions [25]. DMF treatment reduced the corneal infiltration with CD3, CD4 + cells in our model, and this is in agreement with the effect of systemic fumaric ester treatment in patients with psoriasis [25].

Previously, effects of fumaric acid esters on granulocytes were suggested as mechanisms anti-inflammatory actions mechanisms of these drugs [26]. The vast majority of inflammatory cells infiltrating the cornea after HSV-1 infection consist of PMNs. This is associated with tissue destruction and scar formation [27,28]. It is interesting to note that the infiltration of the cornea with PMNs was reduced after DMF treatment [23].

Previous experiments showed that DMF increased the IL-10 secretion in cocultures of psoriatic keratinocytes with HUT78 T cells [17]. Simultaneously, the IFN-γ secretion was inhibited under these cell culture conditions. This suggested that DMF is able to cause an immmunomodulation away from Th1 cytokine to the Th2 cytokine secretion. In the HSV-1 infected mouse corneas, an increased IL-4 cytokine secretion was detected in the DMF treated mice as compared to the PBS group. However, the IL-10 expression was not enhanced in the corneas from DMF treated mice. It might be speculated that the differences between the previous cell culture experiments and our observations is caused by the different experimental settings used. The IL-2 and IFN-γ secretion was not altered in the cornea of the DMF treated mice. It has been previously shown that fumaric acid esters suppress the production of chemokines that may be critical for the recruitment of T cells [29]. DMF has also been reported to inhibit intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule-1 expression in endothelial cells [30]. We speculate that these mechanisms may also be of relevance for the DMF effects on experimental HSK.

In previous investigations, the monomethylfumarate stimulated increase of Th2 cytokines in human peripheral blood mononuclear cells was not associated by a decrease in Th1 secretion [14]. No significant influence on the Th1 cytokine production was noted in our experiments. This suggests that the effect of the drugs is directly on the Th2 cells and not influenced by a paracrine effect of the Th1 cells, and this is in accordance with earlier observations [20]. We can only speculate about the mechanisms that influence the Th2 cells. Others have shown that Th1 cells can be transversed into IL-4 producing cells upon exposure to IL-4 [31]. Specific membrane-binding sites for methylated fumarate compounds that stimulate the signal transduction pathway are expressed by T lymphocytes [32].

It is an important finding in our experiments that the course of epithelial keratitis has not been altered by the DMF treatment. Infectious superficial keratitis appears in the first days after corneal infection and results from viral replication. It may be speculated that this effect is due to an unimpaired Th1 response by DMF, as the HSV-specific delayed-type hypersensitivity-reaction has not been altered. Both, IL-2 and IFN-γ are pleotropic cytokines that are essential for the antiviral defense and they synergize in various respects. Beside other effects, IL-2 influences NK cells, antibody-dependent cellular cytotoxicity, and cytotoxic T cell proliferation and IFN-γ affects macrophage activation, CD8 function and MHC expression. Treatment regiments of immune mediated HSK with inhibition of the Th1 response may therefore diminish the antiviral immune defense, while reducing the immune mediated stromal keratitis. The unaffected Th1 cytokine secretion might be a substantial benefit of DMF in comparison to other approaches. This is further supported by the notion that neither the HSV-1 antigen specific T cell proliferation nor the virus neutralizing antibody production was impaired after systemic DMF treatment in mice after HSV-1 infection.

Previously, recombinant IL-4 treatment of IL-4 knockout mice significantly increased ocular HSV-1 replication [11]. Our data now show that although IL-4 levels were increased in corneas and spleens of DMF treated mice after HSV-1 infection, healing of epithelial keratitis and elimination of virus from the corneally HSV-1 infected mice was not impaired.

The ability of fumaric-acid derivates to alter the balance between cytokines away from the pathogenic Th1 responses, as described in the present investigation, may have a beneficial effect on the course of experimental HSK. However, the acute HSK analysed in these experiments differs in various important aspects from the chronically recurrent disease in human. The observations herein must be proven in the clinical situation.

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