European Journal of Ophthalmology / Vol. 11 no. 3, 2001 / pp. 252-260

Allopurinol has immunomodulating activity following topical and systemic application in experimental autoimmune uveitis

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PURPOSE. *Allopurinol has beneficial effects in the systemic treatment of lens-induced uveitis and experimental autoimmune uveitis (EAU). This is believed to be due to a reduction of oxidative tissue damage through its dose-dependent free radical scavenging ability, and to an immunomodulating effect. The purpose of this study was to demonstrate the immunological effects on the IgG-antibody repertoire in EAU after topical and systemic allopurinol and steroids.*

METHODS. *We assigned 43 male Lewis rats to 6 different groups: healthy rats (BASE, n=3), EAU without therapy (CTRL, n=9); systemic treatment with allopurinol (ALSYS, n=9, 50 mg/kg body wt.i.v., given every three days for two weeks), topical allopurinol (ALLOC, n=6, 8 times/day), systemic methylprednisolone (STSYS, n=10, 7.5 mg/kg body wt. i.v. (Hoechst, Frankfurt, Germany)), and topical treatment with prednisolone acetate 1% (Inflanefran Forte®) (STLOC, n=6). Sera were tested against Western blots (WB) of SDS-PAGE (sodium-dodecyl-sulfate polyacrylamide-gelelectorphoresis) of retinal proteins. Based on digital image analysis, discriminance was analysed.*

RESULTS. *The analysis of discriminance indicated that all therapy groups were significantly different from untreated controls (Wilks' lambda = 0.174; $p<0.01$). Comparing only the number of peaks and the intensities, the WB of allopurinol treated animals showed a much stronger, significant, immunomodulating effect than those treated with steroids ($p>0.05$), even after topical application ($p<0.01$).*

CONLCUSIONS. *Allopurinol had an immunomodulating effect in EAU. Surprisingly, the topical application had more effect than systemically applied allopurinol. Allopurinol had stronger effects than systemic or topical steroids. Allopurinol appears to offer promise in the treatment of uveitis. Topical application of the drug helps to reduce possible complications such as the allopurinol hypersensitivity syndrome. (Eur J Ophthalmol 2001; 11: 252-60)*

KEY WORDS. *EAU, Allopurinol, Immunology*

Accepted: January 4, 2001

INTRODUCTION

Oxidative reactions are thought to play a major role in the tissue damage caused by lens-induced uveitis (LIU). Allopurinol shows beneficial effects in the treatment of LIU because it can reduce this damage through its dose-dependent free radical scavenging ability (1-3). In addition, it has a strong immunomodulating effect, more pronounced than that of steroids (4-6).

Experimental autoimmune uveitis (EAU) is an established

1120-6721/252-09$04.50/0

model for human posterior uveitis (7, 8). The aim of this study was to examine the immunomodulating effects of allopurinol using the EAU model and to compare the effects of allopurinol and steroids applied either topically or systemically.

Western blotting (WB) has evolved as one of the most important tools for investigating autoantibodies (AAB). Since normal sera contain complex repertoires of naturally occurring antibodies against many different autoantigens (9-11), pathogenically relevant AAB may be eclipsed by such natural AABs. We therefore developed a new technique (Mega Blot) to compare and analyze these complex AAB repertoires. This technique has been successfully used in the analysis of AAB in myositis, myopathy and systemic lupus erythematosus (12, 13). The present investigation employed an improved WB technique which allows the simultaneous quantitative analysis of complex banding patterns in EAU in animals.

METHODS

Animals

The treatment of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Lewis rats were used (n=43). Six groups were investigated: healthy rats without EAU (BASE, n=3), EAU with no treatment (CTRL, n=9), EAU with systemic allopurinol (ALSYS n=9, 50 mg/kg body wt. i.v., given every three days for two weeks), EAU with topical allopurinol (ALLOC n=6, 8 times/day (14)), EAU with systemic methylprednisolone (STSYS n=10, 7.5 mg/kg body wt. i.v. (Hoechst, Frankfurt, Germany)), and EAU with a topical steroid (STLOC n=6, prednisolone acetate 1% (Inflanefran forte®), 8 times/day). ALLOC and STLOC received the last treatment 6 hours before they were killed.

EAU was induced with crude bovine retinal extract (CRE, 1.5 mg/ml) prepared as described before (15-17). The rats were injected subcutnaeously in the right hind footpad with 250 µl CRE (375 µg) and 100 µl Freund's adjuvant containing up to 8 mg/ml *M. tuberculosis* (modified from Sigma, München, Germany). At the same time, the animals received an i.v. injection of 1 µg pertussis toxin (Sigma, München, Germany) in 0.4 ml saline. The BASE group received saline injections according to the protocol. Treatment was started at the time of immunization.

The rats were killed two weeks after the immunization, by which time the severity of the disease in this modified model of EAU (15) reaches its maximum.

Tissue sampling

Two weeks after immunization, the rats were deeply anesthetized with ether and a blood sample was taken by heart puncture. The samples were centrifuged and the supernatants stored at –70 °C. All animals were killed afterwards by exsanguination.

For histological examination and biochemical study, non-traumatic enucleation was done and the eyes were fixed by immersion in cold Karnovsky's fixative (8% paraformaldehyde, 25% glutaraldehyde buffered in 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4 (3, 18).

SDS-PAGE of CRE

The retinal proteins were redissolved in sample buffer (0.08 M Tris-HCl, pH 6.8, 1% (v/v) 2-mercaptoethanol, 10% (w/v) saccharose, 1% SDS, 0.005% bromphenol blue) and separated by SDS-PAGE on discontinous slab gels ((19); stacking gel T=6%, C=2.5%, 0.05 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS; separating gel (T=12.8%, C=2,5%, 0.0375 M Tris-HCl, pH 8.8, 0.1% SDS, electrode buffer: 0.188 M glycine, 0.188 M Tris, pH 8.8, 0.1% SDS; approx. 25 µg retinal protein/lane.

Western blotting

The SDS-PAGE preparations were electroblotted onto a nitrocellulose membrane using a Semi-Dry Blotter (Biometra, Germany) (20). The quality of transfer was checked by staining the membrane with avidin-biotin (BioRad, Munich, Germany). The membrane (Western blot, WB) was cut into strips approx. 0.4 cm wide, which were incubated for 12 h with rat serum diluted 1:40 in 5% bovine serum albumin (BSA), in Tris-buffered saline (TBS). After incubation, the blots were washed, blocked with 10% BSA in TBS (1 h), incubated with peroxidase-conjugated rabbit anti-rat-IgG serum (diluted 1:2000 in 5% BSA in TBS, 1 h), and washed. The reaction product was visualized using 0.05% 4-chlorol-naphthol with 0.015% hydrogen peroxide in

20% methanol in TBS. Molecular weights were estimated using marker proteins (Pharmacia, Freiburg, Germany, MW standards "High" and "Low").

Digital image analysis of western blots

The data were acquired using a color flat-bed scanner (EPSON GT-9000, Epson Germany, Düsseldorf, Germany). Digital image analysis and evaluation of densitometric data of the WB were done using ScanPacK software (Biometra, Göttingen, Germany) as described in detail elsewhere (21-23). For each WB strip, ScanPacK creates densitometric data files showing the gray-intensity values against the Rf (relative mobility) (x-axis). ScanPacK evaluates the height, area, molecular weight, Rf, etc. for all peaks in each file.

MegaBlot procedure

Each individual densitograph was reduced to 100 extinction values by first calculating the mean extinction values from the original 8-bit gray-values of the densitometric file for each 1/100th of the region of interest, either across the entire Rf range, i.e., Rf=0 through Rf=1, or within a relevant part. The background level was then substracted and the resulting maximum extinction (i.e., band intensity of an individual blot) was set to 100%. The other extinction values of this blot were established as percentages, thus reducing the influence of different absolute staining intensities of individual blots. For each individual WB, a vector containing 100 mean extinction values was created. These vectors represented the six clinical groups. Thus, from all blots of each group, a mean data vector was calculated, reflecting the general staining pattern of each group. Multivariate analysis of discriminance was done an these data vectors. This analysis not only tests the null hypothesis that mean data vectors of the different groups derive from a multivariate normally distributed population, but also shows which of the various groups are statistically different. Discriminant function analysis can thus be used to determine which variables (Rf ranges) caused significant differences, or which variables can discriminate between groups. Additionally, the analysis allows classification of blots; it can be used to test whether an individual blot pattern is similar to the pattern of a particular known group or to which of several group patterns it shows the greatest similarity. Only variables whose mean value was greater than a chosen cut-off value were used in the analysis. The MegaBlot procedure has been described in detail elsewhere (13, 12, 24).

Histological analysis

Numbers were randomly assigned to the enucleated globes by a "masked" technician. Then the globes were divided and one half was embedded in paraffin. Four-µm sections from different levels (serial section 0,10, and 20) were stained with hematoxylin-eosin (H&E), and adjacent sections were stained with Giemsa. Two independent "masked" investigators used a 0 to 4 score (3) to rate the degree of damage due to intraocular inflammation. The inflammation was graded according to the number of lymphocytes, macrophages and polymorphonuclear granulocytes, as well as the tissue destruction. To assess intraobserver variability, the sections were evaluated twice by each investigator on separate days, and a combined uveitis score per specimen was calculated as the average of 12 values (three sections, x2 investigators, x2 runs).

Allopurinol and oxypurinol

Allopurinol and oxypurinol were assayed by HPLC using Bio-Rad instrument (Munich, Germany) (1).

Retina samples: the retina sample was suspended in 0.6 N perchloric acid, homogenized using an Ultra Turrax R blender (Janke & Kunkel, Staufen, Germany) and centrifuged at 3000 g for 5 min. Twenty microliters of the clear supernatant was injected into the HPLC apparatus.

Aqueous humor samples and sera: before injection into the HPLC apparatus the aqueous humor samples was measured and diluted to 25 µl using 0.6 N perchloric acid, mixed on a Vortex mixer, and centrifuged at 3000 g for 5 min; 20 µl of the clear supernatant were injected into the HPLC apparatus.

The following columns were used: (a) Spherisorb 50 x 4.6 mm S5 ODS2, 80A, octadecyl, 20 cm, and (b) precolumn Spherisorb 50 x 4.6 mm S5 ODS2, 80A, octadecyl, 5 cm (Promochem, Wesel, Germany). The upper pressure limit of the high-pressure pump was set at 2600 psi. An injection valve with a 20-µl loop and a 50-µl syringe were used. The UV detector was set at 235 nm. Before injecting the sample about 50

ml of eluent [KH_2PO_4 – buffer (30 mmol; pH = 4.5) + 0.5% methanol] was used to equilibrate the column. Flow rate was 1 ml/min.

Allopurinol and oxypurinol were identified on the basis of the retention times of known standards (Sigma, Deisenhofen, Germany).

The values of the retina samples were expressed as means (± SE) of allopurinol and oxypurinol/g tissue. Allopurinol and oxypurinol in aqueous humor were expressed as means ± SE/ml.

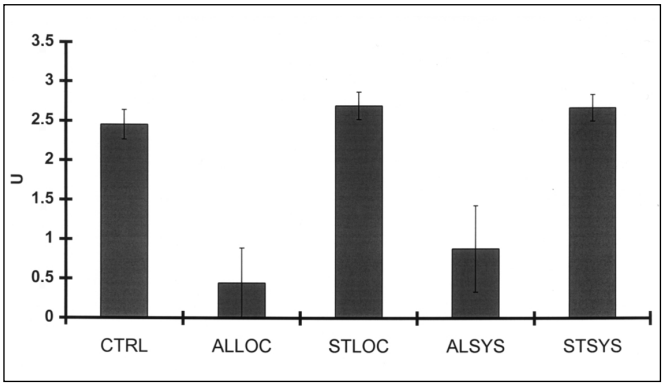

Fig. 1 - *Uveitis scores (mean and SE) of the histological study of the groups CTRL (untreated EAU rats), ALLOC (topical allopurinol), ALSYS (systemic allopurinol), STLOC (topical steroid), and STSYS (systemic steroid). The animals receiving allopurinol had significantly less inflammatory changes (P<0.05) than untreated controls, whereas the animals treated with steroids showed no significant reduction in the histological parameters.*

RESULTS

All was successfully induced in all rats. Histological examination showed a significant reduction in inflammatory changes (P<0.05) in ALLOC and ALSYS

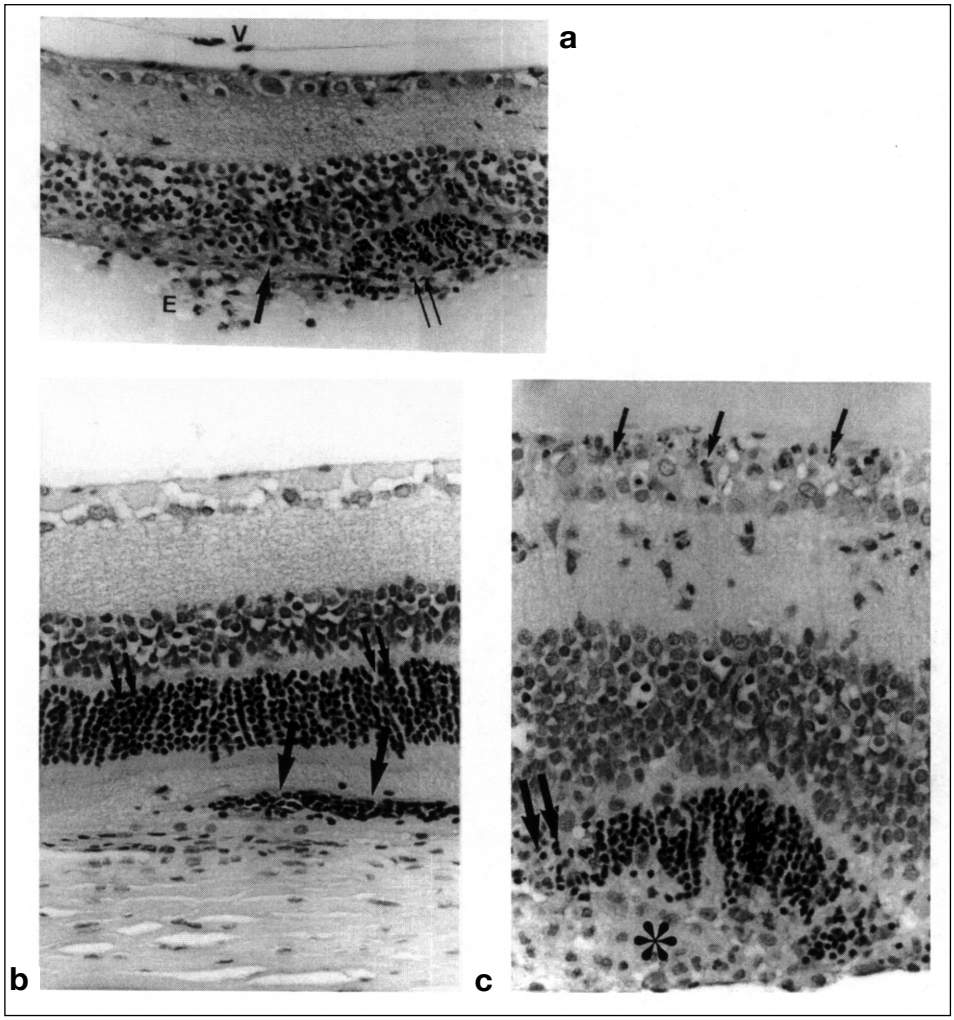

Fig. 2 - ***a)*** *Retina from untreated rat with EAU. The retina is completely detached, and the photoreceptor layer – apart from focal remnants (double arrow) – is replaced by a subretinal inflammatory membrane (arrow). Note inflammatory cells in the subretinal exudate (E) as well as in the vitreous (V) (L: lens) (Hematoxylin-eosin, original magnification x40).* ***b)*** *Retina from a rat treated with topical allopurinol. Most of the retinal architecture is well preserved without any inflammatory changes; only in a few places is there a subretinal infiltrate of inflammatory cells (arrows). Note that even here the photoreceptor layer (double arrow) is unremarkable with intact outer segments (Hematoxylin-eosin, original magnification x40).* ***c)*** *Retina from rat treated with topical steroid. The retina is detached, and there is a marked inflammatory cell infiltrate throughout the retina (at this site most obvious in the nerve fiber and ganglion cell layer, arrows) with partial loss of the photoreceptor layer (double arrow) and formation of a subretinal membrane (asterisk) (Hematoxylin-eosin, original magnification x40).*

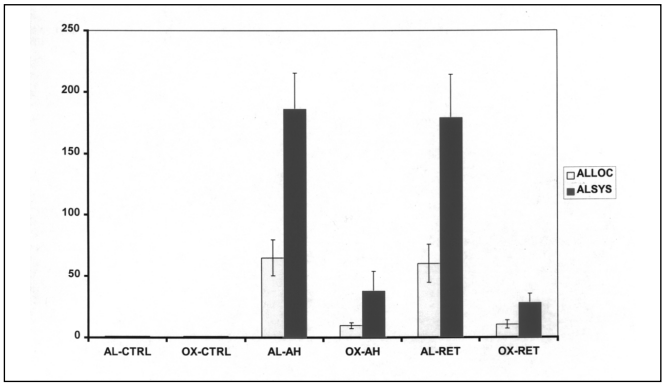

Fig. 3 - *Levels of allopurinol and oxypurinol in aqueous humour (µmol/L) and retinal tissue (nmol/g) after systemic or topical treatment. AL-CTRL and OX-CTRL: concentrations of allopurinol and oxypurinol in aqueous humour of untreated controls; AL-AH and OX-AH: concentrations of allopurinol and oxypurinol in aqueous humour; AL-RET and OX-RET: concentrations of allopurinol and oxypurinol in retina samples.*

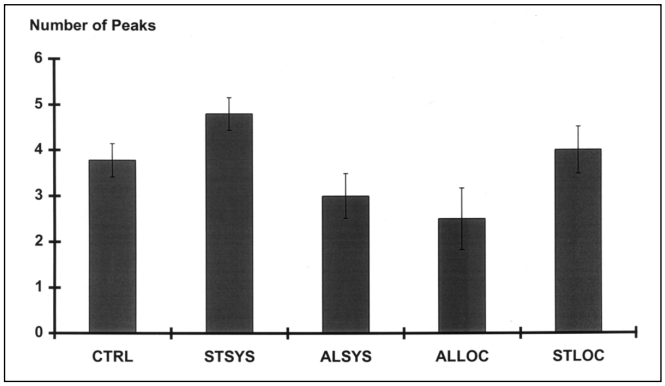

Fig. 4 - *Mean numbers of peaks detected in the WB of each group (CTRL, untreated EAU animals), ALLOC, topicall allopurinol, ALSYS, systemic allopurinol, STLOC, topical steroid, and STSYS, systemic steroids. The animals receiving allopurinol had fewer peaks than CTRL. A slight increase was found in the animals treated with steroids.*

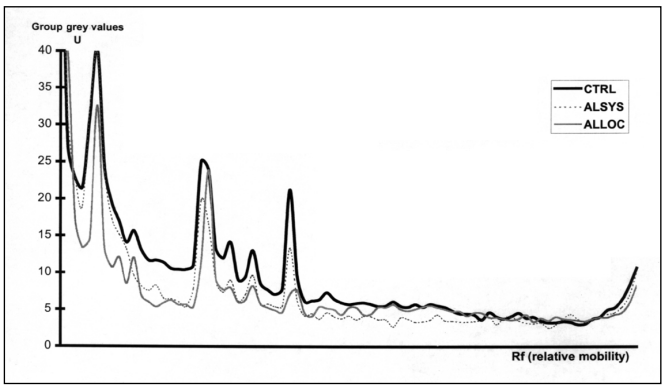

Fig. 5 - *Mean group grey values of EAU (untreated controls), ALSYS (systemic allopurinol), and ALLOC (allopurinol) were plotted against the relative mobility (Rf). The grey values of the allopurinol treated animals were lower, in a wide Rf range.*

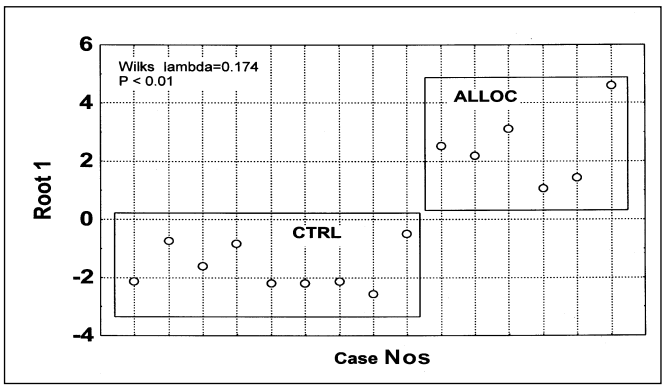

Fig. 6 - *Canonical roots of the discriminance analysis of the ALLOC (topical allopurinol) and CTRL (untreated controls) groups. The graph shows the very good discrimination between groups, with no overlaps.*

compared to untreated controls (CTRL). In STSYS and STLOC there was no such reduction (Fig. 1). Figure 2 shows the histological pictures of rats from the CTRL, ALLOC, and STLOC group.

Allopurinol and oxypurinol in aqueous humour were identified and quantified by HPLC. After topical application, allopurinol reached a level of approx. 64 µmol/ml in aqueous humour and approx. 60 nmol/g in the retinal tissue, compared to respectively approx. 186 µmol/ml and 180 nmol/g after systemic admistration (Fig. 3).

Complex staining was detected in the WB of SDS-PAGE preparations of bovine retinal proteins. Counting the bands in each WB, the ALSYS and ALLOC groups had fewer peaks ($P<0.05$) than CTRL and a slight increase was observed in the STSYS ($P<0.05$) and STLOC ($P>0.05$) groups (Fig. 4). WB incubated with sera from healthy rats (BASE) did not show any bands.

The mean peak intensity of WBs was lower in ALSYS than CTRL and was even lower in ALLOC ($P<0.05$). In STSYS and STLOC the mean peak intensity ($P>0.05$) was higher than CTRL. Figure 5 shows the mean gray values against the relative mobility (Rf) of the CTRL, ALSYS, and ALLOC groups. Mean gray values of ALSYS and ALLOC were lower than CTRL.

Analysis of discriminance indicated that autoantibody patterns against retinal proteins in sera of untreated EAU rats were significantly different from those of EAU rats receiving treatment (Wilks' lambda = 0.174; $P < 0.01$). A multiple group discriminant analysis establishes several different discriminant functions. The maximum number of functions will be equal to either the number of groups minus one, or the number of variables in the analysis, depending on which is smaller. The first function provides the best overall discrimination between groups, the second provides the second best, etc. This calculation is done by canonical correlation analysis. The successive functions are also called canonical roots (25). One can also visualize how these two roots discriminate between groups by plotting the individual scores of each WB. Figure 6 is a plot of the first canonical root resulting from analysis of discriminance, against the case numbers for the CTRL and ALLOC groups. This graph illustrates the quality of discriminance between the individual WBs used in the discriminant space.

DISCUSSION

Histological examination clearly demonstrated the beneficial effect of allopurinol, given either systemically or topically. Steroids, had no significant effect on the histological parameters. To further analyze and interpret these results, we studied the immunomodulating effect of both drugs. The MegaBlot technique found changes in the autoantibody repertoire of EAU rat sera after treatment and was able to discriminate between the different treatment modalities used in this study. The two drugs had very different immunological effects.

Methylprednisolone

STSYS and STLOC gave a larger number of peaks and greater peak intensity in WB, similar to the results of a previous study using the model of lens-induced uveitis (LIU) (4, 5). In contrast, however, to these previous studies, STSYS and STLOC had no significant effect on the histological parameters in EAU. Similar results were found in (17). The antiinflammatory effect of glucocorticoids is based on protection of the integrity of the cell and plasma membrane and stabilization of the lysosomal membranes, thus preventing the extravasation of lysosomal enzymes (26). These drugs also reduce the synthesis of prostaglandins, thromboxane and leukotrienes (27, 28), and the accumulation of neutrophils and macrophages in inflamed tissue. Even though they increase the number of neutrophils in the blood (29), and they inhibit the migration of neutrophils through adhesion molecules (30). Another therapeutic mechanism involves their action on immunological processes: they alter the response of T-lymphocytes on antigen stimuli (31).

Methylprednisolone is believed to suppress the generation of cytotoxic lymphocytes and to suppress specifically sensitized cytotoxic lymphocytes (natural killer cells). The depletion of natural killer cells (NK) is probably a major mechanism in NK depression of patients receiving immunosuppressive drugs (32, 33). After high-dose steroid therapy, the induction of peripheral blood T-cell apoptosis seems to be an important mechanism in the immunosuppression (34). However, despite the suppression of T-lymphocytes, methylprednisolone does not affect the activity of B-lymphocytes; nor does it affect the synthesis of IgG and IgE anitbodies (30).

In several animals the number and intensity of peaks was slightly increased. This might be due to the fact that physiologically regulated antibody production is not affected by methylprednisolone. However, steroids might be effective during other steps of the immunoregulatory pathway such as antigen-antibody binding (35), the reaction of the migration inhibitory factor on macrophages, or the depression of NK, leading to a positive feedback mechanism (i.e. increased antibody production).

Allopurinol

Allopurinol had imunological effects in both EAU and LIU (4). Analysis of discriminance showed a significant difference between the CTRL and treatment groups. Allopurinol overwhelmed the effects of steroids. The number and intensity of peaks in WB decreased, even after topical application. The histological parameters were significantly reduced ($p<0.01$) in both ALSYS and ALLOC.

All parameters evaluated in the present study (histological biochemical, and immunological) were affected more by topical allopurinol than by systemic administration.

Systemic allopurinol gave a concentration of 186 µmol/l in aqueous humour and 179 mmol/g in retinal tissue; after topical application, the concentrations were 64 µmol/l and 60 nmol/g. Oxypurinol could be detected in all tissues.

Augustin et al. (1) showed that allopurinol has beneficial effects on the inflammatory processes in LIU by demonstrating that in ocular tissues allopurinol reaches the levels necessary for scavenger activity after a systemic dose of not less than 50 mg/kg body weight. They concluded that the xanthine oxidase mechanism at this concentration of allopurinol played a minor role in the oxidative tissue damage in LIU (1, 36). They further demonstrated that oxidative reactions play a major role in this tissue damage (2, 3). Allopurinol and oxypurinol were believed to act as direct scavengers of free radicals and hypochlorous acid, reducing tissue inflammation and oxidative tissue damage (1).

In the present study, the allopurinol level necessary for antioxidative activity was never reached after topical applications (> 180 µmol/L are necessary for antioxidative properties, but only about 60 µmol/L were reached in the aqueous humour). Nonetheless, this treatment gave better results than all the other groups. This might be due to the fact that, at the very low allopurinol levels obtained after topical application, the immunomodulating effect of this drug becomes more important and exceeds the antioxidative effect, which can only play a minor role at these concentrations (36). Consiering the half-life of allopurinol in biological tissues of approx. 3 hours, systemic application of allopurinol every three days results in an initially very high level, which then decreases followed by the next high peak at the next injection. After topical application, the level of allopurinol is certainly low – much lower than with a systemic dose – but constant. This constant low level may have a positive effect on the immunomodulating property of the drug.

Both allopurinol and methylprednisonole had immunological effects. However, they influence immunological functions in very different ways. In contrast to the effects of steroids (see above), allopurinol caused changes in the autoantibody repertoire. We could prove this in both EAU and LIU (4).

The LIU model is ideal for studying the pathophysiology of immune complex-mediated uveitis. LIU is a severe Arthus reaction with pronounced tissue inflammation and leads to ocular oxidative tissue damage (37, 38). The EAU model is a T-cell-mediated disease (15, 8) and is a useful model for human posterior uveoretinitis. The immunological intervention of the drug may be at various levels such as the process of antigen presentation, the stage of specific homing of autoreactive T cells to the target organ, or the activation of the effector cells in the tissues (8).

Allopurinol showed immunological effects in both, animal models, which were immunologically very different. Thus, it can be conlcuded that the drug interferes with the immunological processes at various levels though the mechanism is still unclear. In both models the autoantibody repertoire is changed and the number of neutrophils in the inflamed tissue is reduced. This might be due to depression of the activation of effector cells and antibody production. A T-cell specific action alone is not possible, because LIU is not T-cell mediated.

Although allopurinol is widely prescribed for primary and secondary hyperuricemia (gout), side effects have been described in about 1% of recipients. The majority of these effects are mild and include pruritus, diffuse or maculo-papular erythema, urticaria and ichthyosis. More severe reactions are well recognized and include exfoliative dermatitis, toxic epidermal necrolysis and a generalized hypersensitivity syndrome (39). Patients developing the allopurinol hypersensitivity syndrome were mostly middle-aged men with hypertension and/or renal failure, receiving excessive doses. Although the pathophysiological pathway leading to the development of the allopurinol hypersensitivity syndrome is unknown, it probably involves an immune mechanism following accumulation of the drug in patients with poor renal function (40). The fact that mainly patients with renal failure, receiving high allopurinol doses, develop the hypersensitivity syndrome is a further advantage of topical allopurinol in uveitis. The systemical levels of allopurinol reached after topical application are so low that the risk of hypersensitivity is minimized.

In view of its broad therapeutic range and minor side effects, allopurinol appears to offer a useful new approach in the treatment of uveitis. In addition, its effects on antibody production could be exploited in allergic diseases.

ACKNOWLEDGEMENTS

Supported by "Deutsche Forschungsgemeinschaft" DFG Au-126-2.

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