European Journal of Ophthalmology / Vol. 13 no. 2, 2003 / pp. 185-191

Immunological effects of allopurinol in the treatment of experimental autoimmune uveitis (EAU) after onset of the disease

F.H. GRUS[1], A.J. AUGUSTIN[1], K. LOEFFLER[3], J. LUTZ[2], N. PFEIFFER[1]

[1] Department of Ophthalmology, University of Mainz
[2] Department of Physiology, University of Würzburg
[3] Department of Ophthalmology, University of Bonn - Germany

PURPOSE. *Allopurinol reduces oxidative tissue damage and exerts immunomodulating effects in the treatment of experimental autoimmune uveitis (EAU). However, the mechanism of the immunologic pathway remains unclear. In previous studies, treatment was started at the time of immunization. Therefore, whether allopurinol prevents the onset of the disease (i.e., acts in a protective manner) is not known.*

METHODS. *Sixteen male Lewis rats were used: 6 EAU without therapy [control]; 4 EAU with allopurinol treatment starting 7 days after immunization [AL7]; and 6 EAU with allopurinol treatment starting 11 days after immunization [AL11]. Their sera were tested against Western blots of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of retinal proteins. Based on digital image analysis, analysis of discriminance was done.*

RESULTS. *There were significant immunomodulating effects in both therapy groups (Wilks' lambda 0.001, $P < 0.008$) compared to controls. However, the effects were more pronounced in the AL7 group, where peak intensities and the number of peaks were markedly more reduced.*

CONCLUSIONS. *Immunomodulating effects of allopurinol can be detected even if the therapy starts after the onset of the disease. Thus allopurinol strongly influences the immunologic mechanism in this model of autoimmune disease. In view of its minimal side effects, the drug could be a promising alternative for the therapy and prophylaxis of uveitis and other autoimmune diseases. (Eur J Ophthalmol 2003; 13: 185-91)*

KEY WORDS. *Allopurinol, Immunology, Autoimmune, Autoantibody, Uveitis*

Accepted: September 5, 2002

INTRODUCTION

Allopurinol is well known as a treatment of gout as it is an xanthine oxidase inhibitor. The drug also has antioxidative properties and can act as a scavenger of oxygen free radicals (1). In previous studies, allopurinol showed therapeutic effects in the treatment of lens-induced uveitis (1, 2). At first, these effects were interpreted as antioxidative effects only. However, an additional immunologic effect was shown in the animal model of lens-induced uveitis (3-6). Further studies were carried out using another animal model, experimental autoimmune uveitis (EAU).

EAU is an established animal model of human posterior uveitis (7, 8), with a T-cell-mediated mechanism (9). EAU can be induced in Lewis rats by retinal S-antigen or complete retinal extract. Uveitis starts with immigration of polymorphonuclear granulocytes and mononuclear leukocytes and leads to a loss of the photoreceptor layer within two weeks. Allopurinol al-

1120-6721/185-07$03.50/0

so had antioxidative effects in EAU: the therapy significantly reduced the lipid peroxides and the myeloperoxidase activity in the retina and aqueous humor (10). Allopurinol also showed some immunologic effects in EAU (11). It modifies the autoantibody repertoire of animals. This effect was even superior to the effect of steroids (4-6, 11) in all immunologic studies with EAU reported until now. Other groups demonstrated an immunologic effect of allopurinol on T-cells in mouse models (12).

Allopurinol treatment was started at the time of immunization in these EAU studies. Thus, it is unclear whether allopurinol only prevents the outbreak of the disease or is also therapeutically active. Any such activity would be due to immunologic effects after the outbreak of the disease and therefore after the formation of the autoantibodies.

In the present study, treatment was started at different times after the immunization. As in previous studies, the immunologic effects were quantified by Western blot (WB) analysis. In WB, the antigens are separated according to their molecular weight. The method allows the simultaneous comparison of a variety of antigen-antibody reactions. The differences in these complex autoantibody repertoires were quantified using multivariate statistical methods based on a digital image analysis of the blots. This method has been tested successfully with other diseases (e.g., myositis, myopathy, Graves disease, and Tourette syndrome) (13-15).

METHODS

The treatment of animals conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Four groups of male Lewis rats (n = 19) were investigated: healthy animals (BASE, n = 3), rats with EAU without treatment (EAU, n = 6), rats with EAU given systemic allopurinol starting on day 7 after immunization (AL7, n = 4, 50 mg/kg body weight intravenously), and rats with EAU with systemic allopurinol treatment starting on day 11 after immunization (AL11, n = 6, 50 mg/kg body weight intravenously). The AL7 and AL11 groups received allopurinol every three days after starting treatment (AL7, three treatments; AL11, two treatments).

The BASE group received injections of saline according to the protocol, starting at the time of immunization.

The animals were killed two weeks (14 days) after the immunization. At this time, the severity of the disease in this modified model of EAU (16) reaches a maximum.

Tissue sampling

Two weeks after immunization, the animals 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 histologic examination and biochemical study, nontraumatic enucleation was done and the eyes were fixed by immersion in cold Karnovsky fixative (8% paraformaldehyde, 25% glutaraldehyde buffered in 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4) (3, 17).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of crude retinal extract

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 discontinuous slab gels [(18), 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; approximately 25 µg retinal protein/lane].

Western blots

The SDS-PAGE preparations were electroblotted onto a nitrocellulose membrane using the Semi-Dry Blotter (Biometra, Germany) (19). The quality of transfer was checked by staining the membrane with avidin-biotin (BioRad, Munich, Germany). The membrane (WB) was cut into strips approximately 0.4 cm wide, which were incubated for 12 hours 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-immunoglobulin G serum (diluted 1:2000 in 5% BSA in TBS, 1 h), and washed. The reaction product was visualized using 0.05% 4-chloro1-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 flatbed scanner (EPSON GT-9600, 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 (20, 22). For each WB strip, ScanPacK creates densitometric data files by showing the gray-intensity values versus the Rf (relative mobility) values (x-axis). ScanPacK evaluates height, area, molecular weight, Rf value (relative mobility) etc., for all peaks in this densitometric data file.

Statistics

Each individual densitograph was reduced to 100 extinction values by first calculating the mean extinction values from the original eight-bit gray values of the densitograph file for each 1/100 of the region of interest (across the entire Rf range; i.e., Rf = 0 through Rf = 1). Then the background level was subtracted from these data 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 transformed into 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. Each WB belongs to one of the three experiment groups (EAU, AL7, AL11). Thus, from all blots of each group, a mean data vector of this group was calculated, reflecting the general staining pattern of each group.

From these data vectors of each blot, a multivariate analysis of discriminance was done. This 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 groups are statistically different. Based on this, discriminant function analysis can be used to determine which variables (Rf ranges) caused the mean value comparison to become significant or which variables can discriminate between groups. This statistical procedure has been described in detail elsewhere (13, 23).

Histologic analysis

Numbers were randomly assigned to the enucleated globes by a blind-folded technician. The globes were then divided and one half was embedded in paraffin. Four-micrometer sections from different levels (serial sections 0, 10, and 20) were stained with hematoxylin-eosin (H-E), and adjacent sections were stained with Giemsa. Two independent blind-folded investigators evaluated the results, using a 0 to 4 score, described in detail in Augustin et al (3), reflecting the degree of damage by intraocular inflammation. The severity of the inflammation was graded according to the number of lymphocytes, macrophages, and polymorphonuclear granulocytes, and the tissue destruction. To assess intra-observer variability, the sections were evaluated twice by each investigator on separate days.

RESULTS

The histologic examination (Fig. 1) found milder inflammation in the animals treated with allopurinol (AL7 and AL11). This stands in agreement with the results of Augustin et al (10). The histologic inflammation scores were significantly ($p < 0.05$) lower in the animals receiving allopurinol starting after 7 days (AL7).

The rat sera were tested against the WB of bovine retinal proteins. Antigen antibody reactions (staining) could be found in all groups. (Fig. 2). Only a very few bands were found on the blots of the BASE group.

Analysis of discriminance showed a significant immunologic effect of both allopurinol groups compared to the control group (Wilks lambda 0.001, $p < 0.008$). These effects were stronger in the AL7 group. Figure 3 illustrates the canonical roots derived from the analysis of discriminance. These give a measure of the location; i.e., the separation of each single WB in the discriminant space. Every point in the figure corresponds to a single WB of one rat. The nearer the points are to each other, the more similar are the patterns

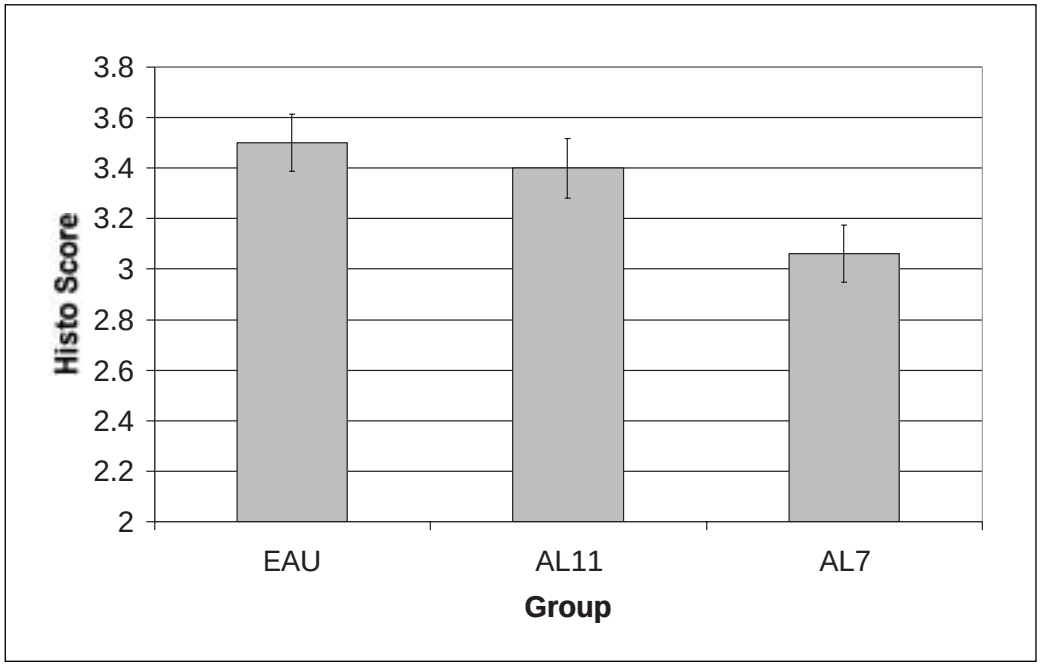

Fig. 1 - *Histologic uveitis scores. The uveitis was less pronounced in animals receiving allopurinol. The decrease was significant in the group starting treatment seven days after immunization (AL7, p < 0.05).*

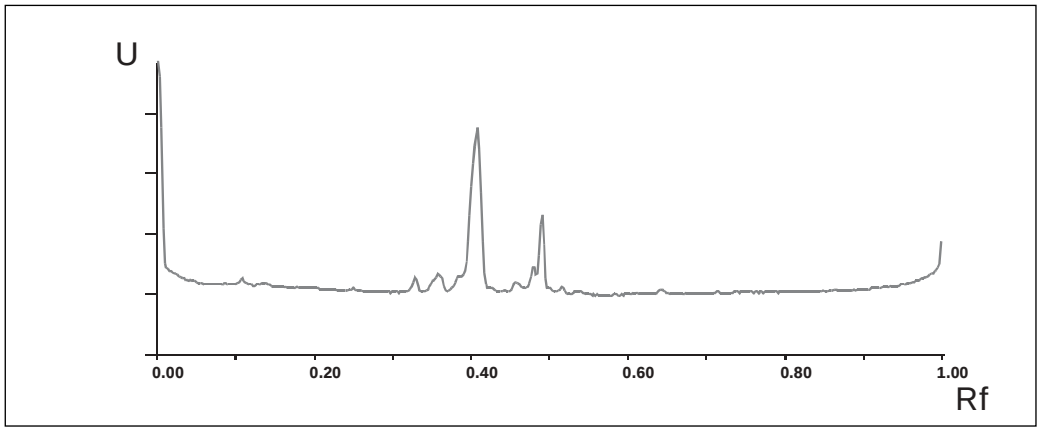

Fig. 2 - *A densitograph of a Western blot of the control group is shown. The gray values (scanner-units) are plotted against the relative mobility (Rf-value). The relative mobility is correlated to the molecular weight of the antigens.*

of the underlying WB. The patterns are very similar within every experimental group (Fig. 3). However, both allopurinol groups are quite clearly separated from the control group (EAU). The autoantibody patterns of the rats in which treatment started after seven days are even more clearly different from the control group. The AL7 group showed a stronger immunologic effect than the AL11 group.

The mean numbers of peaks/blot are shown in Figure 4 for each group. Allopurinol treatment resulted in a decrease of the average number of peaks in AL7 ($p < 0.05$) and AL11. However, the effect was more pronounced when treatment was started on day 7.

Figure 5 shows the mean gray intensities plotted against the relative mobility (Rf value) for the control (EAU) and treatment groups (AL7 and AL11). Treatment led to a reduction in the staining intensity (antigen antibody reaction) over wide Rf ranges ($p < 0.01$). This was, however, substantially more expressed in the animals whose treatment was started on day 7.

DISCUSSION

In EAU allopurinol has more pronounced effects than methylprednisolone on the reduction of oxidative tis-

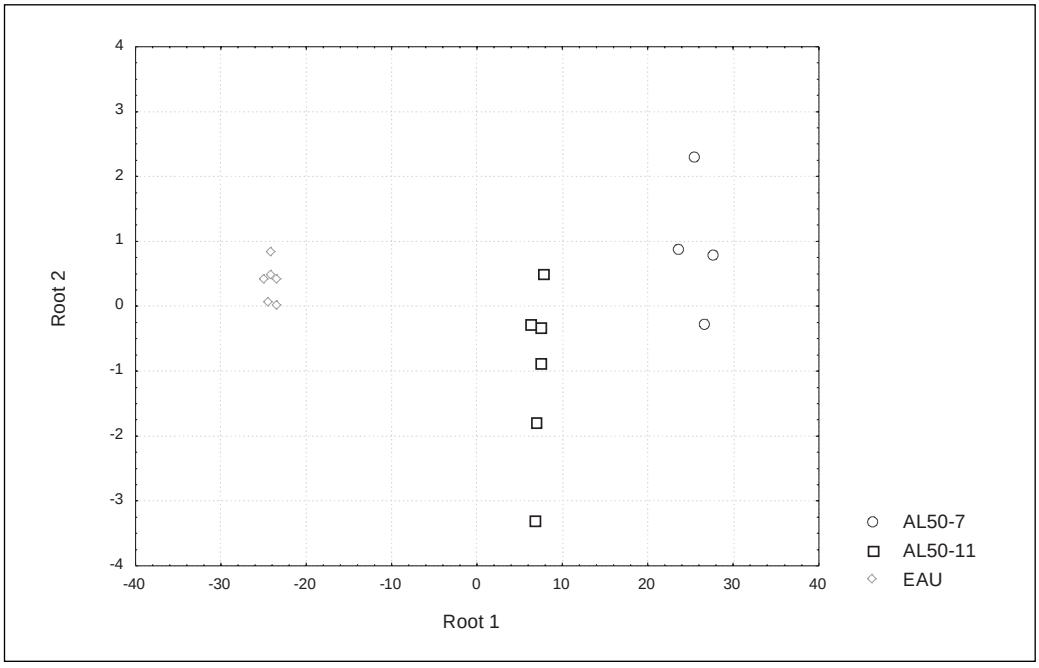

Fig. 3 - *The first and second canonical roots derived from the analysis of discriminance are plotted against each other. A clear-cut separation is visible between the three experiment groups-suggesting the significant discrimination between the autoantibody patterns of the groups and therefore the extent of the drug's immunologic effects. However, the blots of the AL11 group stay much closer to the control group in the discriminant space than the blots of the AL7 group.*

sue damage and on histologic parameters (10).

At histologic examination, some decrease in inflammatory cells was found in about one third of the animals after allopurinol treatment. The biochemical experiments showed a decrease to a quarter (myeloperoxidase activity) or a third (lipid peroxides) of the values of the control group after allopurinol.

In previous studies, beside its antioxidative effects allopurinol had strong immunologic effects in EAU (11). In the present study, allopurinol significantly changed the rats' autoantibody repertoires. However, in all studies carried to date, allopurinol treatment was started at the beginning of the immunization. Therefore, it cannot be excluded that allopurinol already influences the induction phase of the autoimmune process and thus works in a protective manner on development of uveitis.

We found an immunologic effect of allopurinol even when the treatment was started 7 or 11 days after immunization. The shorter the period between immunization and starting treatment , the greater the success of the treatment. It can be interpreted that allopurinol simply needs more than the remaining 3-4 days to fully exert its immunologic effects in the AL11 group. Furthermore, there was no significant reduction in the severity of the disease when the drug was given on day 11. On the other hand, it could also be concluded that the inflammation was not so strongly developed after seven days (AL7) as after 11. Nevertheless, in both groups (AL7 and AL11) immunologic effects were observed after treatment with allopurinol.

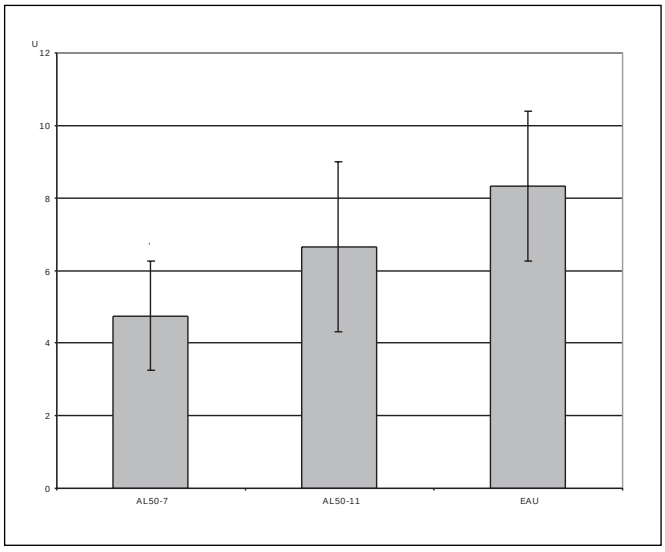

Fig. 4 - *The mean numbers of peaks were plotted for each experiment group (EAU, AL7, and AL11). The groups receiving allopurinol (AL7, AL11) show a definite decrease in the number of peaks.*

The hallmarks of EAU are ocular inflammation, disruption of the retinal architecture, and partial to com-

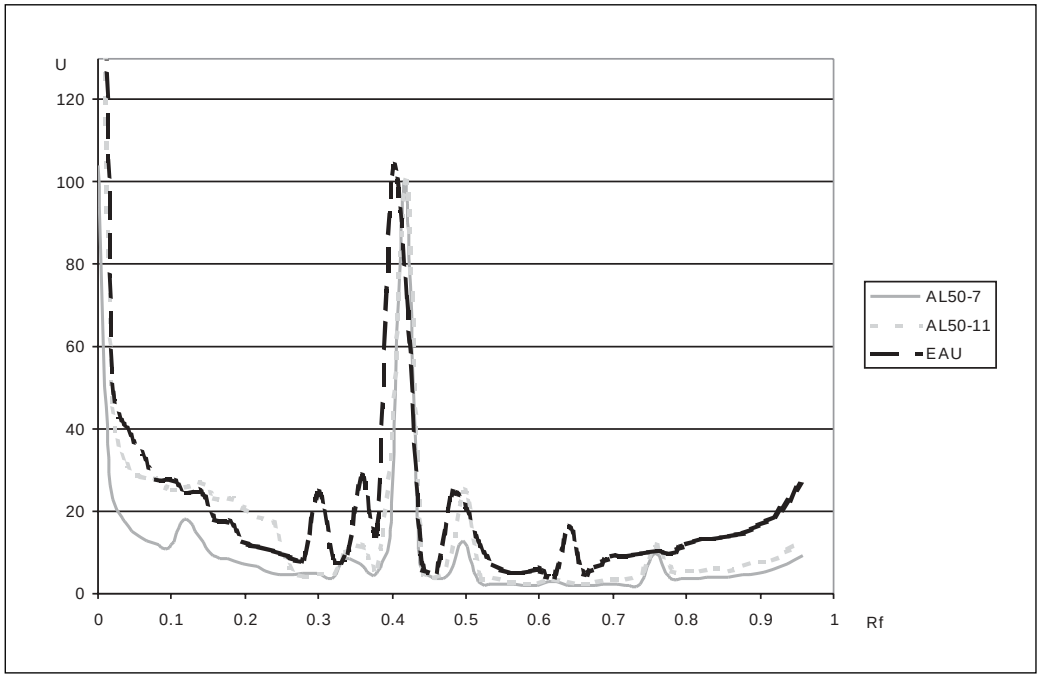

Fig. 5 - *The mean gray values (scanner units) of each experiment group (EAU, AL7, and AL11) are plotted against the relative mobility (Rf-value). Allopurinol strongly reduced the intensity of the antigen antibody reaction over a wide range of Rf values. This effect is more pronounced in the AL7 group.*

plete destruction of the photoreceptor cell layer. The disease typically appeared within 6 to 8 days, usually with minimal anterior chamber involvement with a peak of inflammation on day 14 (9, 16, 24).

The study does not exclude that allopurinol protects against the outbreak or the deterioration of the disease. The prophylactic use of the drug has been attributed to its antioxidative effect. However, this study found that besides its possible protective mechanisms, allopurinol also exerts therapeutic immunologic effects after the outbreak of the disease. It probably influences the autoimmune mechanisms of the disease at very different steps. Whether this interaction occurs during the initial priming phase, during differentiation to Th1 and/or Th2 cells, or in the effector phase remains unclear. Nevertheless immunologically, allopurinol has very different effects in animal models. Lens-induced uveitis (Arthus reaction) has a different mechanism from EAU. Because allopurinol shows significant immunologic effects in both models, one can assume that these are not caused by T cells only. This conclusion is in accordance with a recent study by Kato et al (12), which found that allopurinol affected the response to ovalbumin in immune- deficient mice. Kato et al concluded that allopurinol should influence the responses of both B and T cells (12).

Considering the large number of patients with gout treated with allopurinol every day, its minimal side effects, and the strong immunologic action shown in our studies, allopurinol could be an alternative to drugs with more troublesome side effects for the treatment of autoimmune and allergic disorders.

ACKNOWLEDGEMENTS

Supported by "Deutsche Forschungsgemeineschaft" DFG AU-126-2.

Reprint requests to:
F.H. Grus, MD, PhD
Universitäts-Augenklinik
der Universität Mainz
Langenbeckstr. 1
55101 Mainz, Germany
f@grus.de

REFERENCES

1. Augustin AJ, Böker T, Blumenröder SHH, Lutz J, Spitznas M. Free radical scavenging and antioxidant activity of allopurinol and oxypurinol in experimental lens-induced uveitis. Invest Ophthalmol Vis Sci 1994; 35: 3897-904.
2. Augustin AJ, Spitznas M, Koch F, Meller D, Sekundo W. Effekte von Allopurinol und Steroiden auf den oxidativen Gewebsschaden und die Entzündungsparameter bei linseninduzierter Uveitis. Ophthalmologe 1995; 92: V173.
3. Augustin A, Spitznas M, Sekundo W, et al. Effects of allopurinol and steroids on inflammation and oxidative tissue damage in experimental lens-induced uveitis: A biochemical study. Br J Ophthalmol 1996; 80: 451-7.
4. Grus FH, Augustin AJ, Zimmermann CW, Spitznas M, Sekundo W, Lutz J. Immunodulating activity of allopurinol in experimental lens-induced uveitis. Graefes Arch Clin Exp Ophthalmol 1997;235: 118-23.
5. Grus FH, Augustin AJ, Koch F, Zimmermann CW, Lutz J. Autoantibody repertoires in animals with lens-induced uveitis after different therapies using megablot technique: A comparison. Adv Ther 1996; 4: 203-15.
6. Grus FH, Augustin AJ, Zimmermann CW. Computer-supported analysis (MegaBlot) of allopurinol-induced changes in the autoantibody repertoires of rats suffering from experimental lens-induced uveitis. Electrophoresis 1997; 18: 516-9.
7. Forrester JV, Liversidge J, Towler H, McMenamin PG. Comparison of clinical and experimental uveitis. Curr Eye Res 1990; 9: 75-84.
8. Forrester JV, Liversidge J, Dua HS, Dick A, Harper F, McMenamin PG. Experimental autoimmune uveoretinitis: A model system for immunomodulation. Curr Eye Res 1992; 11: 33-40.
9. Nussenblatt RB. Experimental autoimmune uveitis: Mechanisms of disease and clinical therapeutical indications. Invest Ophthalmol Vis Sci 1991; 32: 3131-41.
10. Augustin AJ, Loeffler KU, Sekundo W, Grus FH, Lutz J. Effects of systemically applied allopurinol and prednisolone on experimental autoimmune uveitis. Graefes Arch Clin Exp Ophthalmol 1999; 237: 508-12.
11. Grus FH, Augustin AJ, Sekundo W, Loeffler K. Immunological action of systemically applied allopurinol in experimental autoimmune uveitis. Klin Monatsbl Augenheilkd 2000; 216: 165-71.
12. Kato C, Sato K, Wakabayashi A, Eishi Y. The effects of allopurinol on immune function in normal and BALB/c and SCID mice. Int J Immunopharmacol 2000; 22: 547-56.
13. Zimmermann CW, Grus FH, Dux R. Multivariate statistical comparison of autoantibody repertoires (Westernblots) by discriminant analysis. Electrophoresis 1995; 16: 941-7.
14. Zimmermann CW, Grus FH, Dux R. Examination of complex autoantibody patterns by discriminance analysis. Med Genet 1993; 5: 327.
15. Grus FH, Augustin AJ. Analysis of the IgG autoantibody repertoire in endocrine ophthalmopathy using the MegaBlot technique. Curr Eye Res 1998; 17: 636-41.
16. Butler TL, McMenamin PG. Resident and infiltrating immune cells in the uveal tract in the early and late stages of experimental autoimmune uveoretinitis. Invest Ophthalmol Vis Sci 1996; 37: 2196-210.
17. Karnovsky M. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 1965; 27: 137-8.
18. Laemmli UK. Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature 1970; 227: 680-5.
19. Towbin H, Staehlin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 1979; 76: 4350-3.
20. Grus FH, Nuske JH. Densitometrie mit dem EPSON Scanner GT-6000. In: Schemel-Trumpfheller CK, ed. GIT-Laborjahrbuch 1991. Darmstadt: GIT Verlag; 1991: 234-5.
21. Nuske JH, Grus FH. Moderne Densitometrie. BioTec 1993; 2: 33-5.
22. Nuske JH, Grus FH. Quantitative Densitometrie. BIO-Forum 1993; 11: 436-7.
23. Grus FH. Ein schnelles und einfaches Verfahren zur Analyse komplexer Autoantikörper-Muster; Dissertation Universität-Gesamthochschule Essen; 1994.
24. Caspi RR, Roberge FG, McAllister CG, et al. T cell lines mediating experimental autoimmune uveoretinitis (EAU) in the rat. J Immunol 1986; 136: 928-33.