

Differences in the composition of inflammatory cell infiltrate in lens-induced uveitis under therapy with allopurinol or steroids

W. SEKUNDO¹, A.J. AUGUSTIN²

¹Department of Ophthalmology, University of Marburg, Marburg

²Department of Ophthalmology, University of Mainz, Mainz - Germany

PURPOSE. *The aim of this study was to compare the qualitative changes in the composition of inflammatory cell infiltrate in lens-induced uveitis (LIU) under treatment with allopurinol (Allo), methylprednisolone (Pred) or the two drugs combined (Allo/Pred).*

METHODS. *Twenty male Wistar rats were sensitized with lens proteins for eight weeks. Intravenous (IV) therapy was started after anterior capsule disruption in one eye of each animal. Five rats were randomly assigned to each of the four groups: controls, Allo (50 mg/kg bw), Pred (7.5 mg/kg bw) and Allo/Pred (50 mg/7.5 mg per kg bw). Eyes were enucleated 24 hours later and fixed in paraformaldehyde/glutaraldehyde. Sections at three levels were stained with Giemsa and examined using a 0 to 4+ score for each type of inflammatory cell. Granulocytes were seen as neutrophils and eosinophils. Neutrophils were divided into polymorphs and "others", and graded with lymphocytes.*

RESULTS. *In all therapy groups there was a significant reduction of polymorphs ($p < 0.05$) in comparison to the control group. There was also a significant reduction in lymphocytes ($p < 0.05$) in the Pred and Allo/Pred groups as compared to the control group and the Allo group.*

CONCLUSIONS. *Single-dose IV allopurinol significantly reduced the overall number of polymorphonuclear leucocytes in LIU. Unlike methylprednisolone, allopurinol did not have any significant impact on lymphocytes. (Eur J Ophthalmol 2001; 11: 264-8)*

KEY WORDS. *Uveitis, Experimental, Rat, Allopurinol treatment, Morphology, Pathology*

Accepted: July 3, 2000

INTRODUCTION

Oxygen free radicals such as superoxide anion, hydroxyl radical and hydrogen peroxide are known for their toxic and destructive nature to the tissue components in the body (1). In the eye, free radicals cause tissue damage in several conditions, such as chemical burns (2), infectious keratitis (3), light-induced corneal (4) or retinal (5) damage, and uveitis (6). Augustin et al showed that in the model of lens-induced uveitis (LIU), allopurinol significantly reduced inflammatory parameters (7, 8). In addition, Grus et al recently re-

ported that repeated doses of systemic allopurinol changed the autoantibody repertoire in LIU (9).

In one of these studies (8), where the overall number of inflammatory cells was calculated as a so-called "inflammatory score", the composition of the inflammatory cell infiltrate in LIU was rather unsophisticated. We therefore decided to assess and compare the qualitative changes in the composition of the inflammatory cell infiltrate in LIU under treatment with a steroid or allopurinol alone, or the combination of these two drugs, using a simple, easily reproducible histological technique such as Giemsa stain.

MATERIALS AND METHODS

Treatment of experimental animals conformed to the ARVO resolution on the use of animals in research. Twenty male Wistar rats were randomly assigned to four groups (5 animals in each group): controls, allopurinol (Allo), methylprednisolone (Pred) and the two drugs combined (Allo/Pred).

Experimental lens-induced uveitis (LIU) was induced as described by Rao et al (10). Briefly, the animals were sensitised every two weeks over a two-month period with four subcutaneous injections of 10 mg bovine lens protein in complete Freund's adjuvant (Sigma, Deisenhofen, Germany). One week after the last injection the lens of the right eye was ruptured using a tip of a 30-gauge bent needle. Therapy was started immediately after rupture of the lens. The Allo group received a 50 mg/kg body weight bolus of intravenous (IV) allopurinol, the Pred group received 7.5 mg/kg body weight IV methylprednisolone and the Allo/Pred group both drugs, as in our previous studies (7, 8).

Twenty-four hours later the animals were deeply anesthetized using ether. The right eye was marked laterally by a 4.0 silk suture, and non-traumatic enucleation of the globes was done after which the rats were killed by exsanguination. The eyes were immediately fixed by immersion in cold Karnovsky's fixative (4% paraformaldehyde, 2.5% glutaraldehyde buffered in 0.1 M Na₂HPO₄ and 0.1 M KH₂PO₄). Numbers were randomly assigned and masked by a technician. After 48 hours of fixation the globes were divided in the horizontal plane. One half was embedded in paraffin for histopathology; 4-µm thick sections were cut on a sliding microtome. Three levels were prepared, with 20 and 10 serial sections inbetween. The sections were stained with Giemsa and examined by light microscopy in a blinded fashion using a 0 to 4 score applied to each type of inflammatory cell seen 0, no cells; 1, ~ 20 cells per high power field (HPF: x400); 2, ~ 40 cells per HPF; 3, ~ 60 cells per HPF; 4, massive.

In practical terms, standards were established by

TABLE I – INDIVIDUAL SCORES (0 TO 4) FOR DIFFERENT INFLAMMATORY CELL TYPES AS SEEN BY GIEMSA STAIN IN 20 GLOBES

Group	Neutrophils		Eosinophils	Lymphocytes
	PMN	Others		
Controls n=5	4 4 4 2 2	3 2 1 2 2	2 2 2 1 2	1 1 1 1 1
Total	16	10	9	5
Allopurinol n=5	0.5 1 3 2 1	1 2 1 1 1	1 1 0.5 0.5 0.5	1 1 1 1 1
Total	7.5	6	3.5	5
Allopurinol + Prednisolone n=5	1 2 1 1.5 2	0.5 2 2 1.5 2	0 1 1 1 0	0 1 0 0.5 0
Total	7.5	8	3	1.5
Prednisolone n=5	1 3 1.5 1 1	1 2 1 1 1.5	0.5 2 1 1 0.5	0.5 1 0.5 0.5 1
Total	7.5	6.5	5	3.5

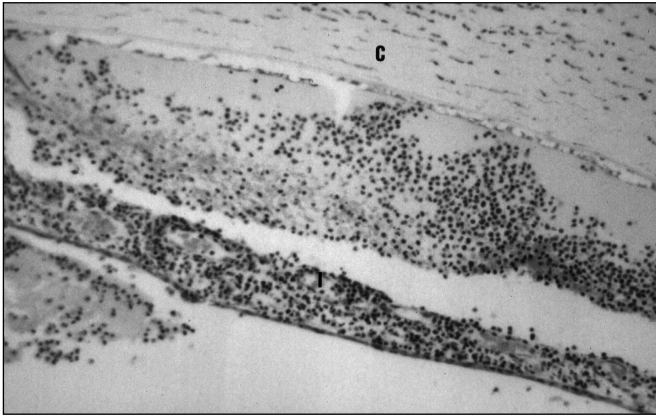


Fig. 1 - Control group. The anterior chamber exudate, posterior cornea and iris stroma contain vast numbers of neutrophils. Eosinophils and lymphocytes are sparse. (C = Cornea, I = Iris; Giemsa, original magnification 200 x).

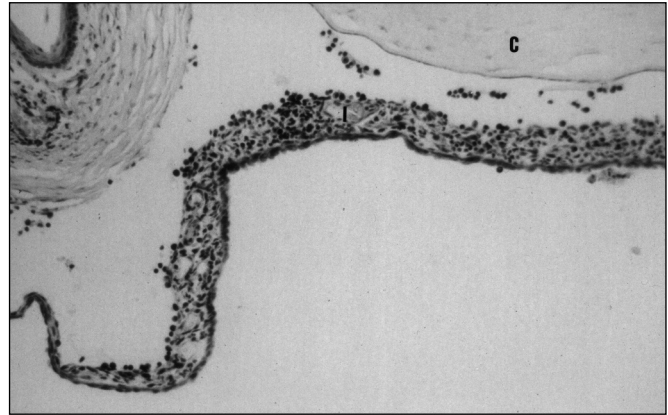


Fig. 2 - Allopurinol treatment group. The overall number of inflammatory cells is reduced, with most marked reduction for neutrophils. (C = Cornea, I = Iris; Giemsa, original magnification 200 x).

counting the cells in 12 high-power fields (four in each structure: cornea, anterior chamber, iris/anterior lens) when the first level (20 sections) was examined. The remaining 40 sections were graded by comparison with established standards. If the cell density was midway between individual scores a half point was assigned in order to increase the precision of the grading system (e.g. 50 cells/HPF would be graded as 2.5). Only unequivocally identifiable cells stained with Giemsa were graded. After evaluation the random numbers were re-assigned to the original treatment groups.

Statistical analysis for each inflammatory cell type and treatment group was carried out using the Mann-Whitney U test ("Statistica" software package).

RESULTS

Light microscopy shown pathological changes were confined to the anterior segment of the globe. There was marked inflammatory infiltration of the iris, ciliary body, and limbus, and a proteinaceous and inflammatory cellular anterior chamber exudate in all control eyes where the axial cornea was also often affected (Fig. 1). Treated eyes presented fewer inflammatory changes (Figs. 2 and 3).

On Giemsa-stained sections lymphocytes and granulocytes were easily noted from their staining characteristics and morphology. The latter were seen as neutrophils and eosinophils. Depending on the ap-



Fig. 3 - Methylprednisolone treatment group. Some polymorphonuclear leukocytes, other neutrophils and a few red blood cells are observed within the anterior chamber exudate. The iris stroma appears thin because of a modest inflammatory infiltration. (C = Cornea, I = Iris; Giemsa, original magnification 200 x).

pearance of the nucleus, neutrophils could be further divided into polymorphonuclear leucocytes (PMN) and "other neutrophils".

Table I shows the individual scores in each group. In comparison to the controls all treatment groups showed significant ($p < 0.05$) reductions of granulocytes and particularly PMNs. All therapies exerted a similar effect on PMNs, but not on lymphocytes. With regard to the overall number of lymphocytes (but not their subsets, which are not accessible with the technique used), only methylprednisolone regimens, namely the Pred and Allo/Pred groups, had a significant ($p < 0.05$) effect, compared to controls. The com-

bination of both drugs reduced lymphocytes more than prednisolone alone, though this effect was not statistically significant.

DISCUSSION

Lens induced uveitis (LIU) is a well established model of Arthus-type endophthalmitis in rats (10). Augustin et al showed allopurinol was an effective treatment (7, 8). Allopurinol is widely used for the treatment of hyperuricemia-associated disorders such as gout, reducing uric acid by inhibiting the enzyme xanthine oxidase. Allopurinol's effect in LIU was believed to be mainly due to its free radical and hypochlorous acid scavenging properties (7, 8). Not only allopurinol, but also its metabolite oxypurinol, is a powerful scavenger of hypochlorous acid and hydroxyl radicals (11, 12). However, the same group has also shown that allopurinol has immunomodulating activities (9, 13).

In ophthalmology, the mainstem therapy for uveitis is based on topical and/or systemic steroids. Their broad spectrum of anti-inflammatory effects is related to inhibition of arachidonic acid release from phospholipids, and therefore reduction of prostaglandin and thromboxane synthesis. Vascular permeability and monocyte migration to inflamed tissues are reduced (14). Some dose-dependent scavenger properties of steroids have been reported (15, 16), but these doses were not employed in the present study.

In this study, methylprednisolone and allopurinol both significantly reduced the density of PMNs in the anterior segment of the eye. The domination of PMN in the inflammatory infiltrate is typical of the Arthus reaction which is an immune-complex mediated disease (17). When PMNs undergo a "respiratory burst" hydroxyl radicals are deliberated via superoxide formation (18). Further tissue injury produced by reactive oxygen metabolites includes direct cytotoxic effects, promotion of proteolytic enzyme activity by inactivation of alpha-1-antiprotease, alteration of tissue substrates by the hydrolysis of basement membrane collagen, and amplification of the inflammatory response as mediated by the lipoxygenase pathway and leukotriene B4 production (19).

We assume that in the present experimental setting allopurinol reduces the amount of free radicals thus

stopping the vicious circle of tissue self-destruction with further attraction of new PMNs and subsequent leukocytoclasia. The reduction of PMNs by the steroid is most likely due to its stabilising effect on lysosomal membranes, thus preventing the excretion of the lysosomal enzymes which would otherwise attract further inflammatory cells (20).

We also noted that methylprednisolone, and methylprednisolone in combination with allopurinol had a significant impact on lymphocytes. This underlines the different mechanism of action of these two drugs at the dosage used and in the setting of the present experiment. It also suggests that the impact on lymphocytes in this experiment is mostly related to the steroid. It was suggested that methylprednisolone affected lymphoid cells through binding "glucocorticoid-responsive elements" to genes of cytokine synthesis (21).

Our findings with a single dose of allopurinol confirm the results of Grus et al who clearly showed the immunoregulating effects of allopurinol, with repeated dose of the drug, but did not detect any immunomodulating effect with a single dose (9). Interestingly allopurinol also showed immunomodulating activity in experimental autoimmune uveitis (EAU) after daily intravenous doses for 14 days (13). Although this activity appears to be dose-dependent, further immunohistochemical studies using monoclonal antibodies against different subsets of lymphocytes may be of interest in order to find out the lowest immunoeffective dose of this drug.

In summary, the present morphological study found single-dose IV allopurinol had a potent antiinflammatory effect, significantly reducing PMN numbers in LIU. However, unlike methylprednisolone, allopurinol had no significant effect on lymphoid cells.

ACKNOWLEDGEMENTS

Supported by Deutsche Forschungsgemeinschaft (DFG) grant Au 126/1-1.

Reprint requests to:
Walter Sekundo, MD
MZ für Augenheilkunde der Philipps-Universität
Robert-Koch-Strasse 4
35037 Marburg, Germany
sekundo@mail.uni-marburg.de

REFERENCES

1. Blake DR, Allen RE, Lunec J. Free radicals in biological systems: A review oriented to inflammatory processes. *Br Med Bull* 1987; 43: 371-85.
2. Marx DW, Burris TE, McCay PB, Rowsey JJ, Gavin M. Evidence for free radical activity in corneal alkali burns. *Invest Ophthalmol Vis Sci* 1984; 25 (suppl): S329.
3. Alio JL, Artola A, Serra A, Ayala MJ, Mulet ME. Effect of topical antioxidant therapy on experimental infectious keratitis. *Cornea* 1995; 14: 175-9.
4. Cejkova J. Allopurinol prevents severe corneal damage by UVB-rays. *Invest Ophthalmol Vis Sci* 1998; 39 (suppl): S4706.
5. Delmelle M. Retinal damage by light: possible implication of singlet oxygen. *Biophys Struct Mechanisms* 1977; 3: 195-8.
6. Ishimoto S, Wu G, Hayashi S, Zhang J, Rao NA. Free radical tissue damages in the anterior segment of the eye in experimental autoimmune uveitis. *Invest Ophthalmol Vis Sci* 1996; 37: 630-6.
7. Augustin AJ, Böker T, Blumenröder S H-H, 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.
8. Augustin AJ, Spitznas M, Sekundo W, et al. Effects of allopurinol and steroids on inflammation and oxidative tissue damage in experimental lens-induced uveitis: a biochemical and morphological study. *Br J Ophthalmol* 1996; 80: 451-7.
9. Grus FH, Augustin AJ, Zimmermann CW, Spitznas M, Sekundo W, Lutz J. Immunomodulating activity of allopurinol in experimental lens-induced uveitis. *Graefes Arch Clin Exp Ophthalmol* 1997; 235: 118-23.
10. Rao NA, Calandra AJ, Sevanian A, Bowe B, Delmage JM, Marak GE. Modulation of lens-induced uveitis by superoxide dismutase. *Ophthalmic Res* 1986; 18: 41-6.
11. Grootvelt M, Halliwell B, Moorhous CP. Action of uric acid, allopurinol and oxypurinol on the myeloperoxidase-derived oxidant hypochlorous acid. *Free Radic Res Commun* 1987; 4: 69-76.
12. Moorhouse CP, Grootvelt M, Halliwell B, Quinlan G, Gutteridge JMC. Allopurinol and oxypurinol are hydroxyl radical scavengers. *FEBS Lett* 1987; 213: 23-8.
13. Augustin AJ, Löffler 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.
14. Harrison RN, Lippman SS. How steroid hormones work. *Hosp Prac* 1989; 24: 63-76.
15. Hall ED. Lipid antioxidants in acute central nervous system injury. *Ann Emerg Med* 1993; 22: 1022-7.
16. Hall ED. The effects of glucocorticoid and nonglucocorticoid steroids on acute neuronal degeneration. *Adv Neurol* 1993; 59: 241-8.
17. Riede UN. Pathogene Immunreaktionen. In: Riede UN, Schaefer HE eds. *Allgemeine und spezielle Pathologie*. 4th ed. Stuttgart, New York: Georg Thieme Verlag 1995; 194-5.
18. Rao NA, Fernandez MA, Sevanian A, Romero JL, Till GO, Marak GE. Treatment of experimental lens-induced uveitis by dimethyl thiourea. *Ophthalmic Res* 1988; 20: 106-11.
19. Klebanoff S. The iron-H₂O₂-iodide cytotoxic system. *J Exp Med* 1982; 156: 1262.
20. Weissman G, Sessa G, Bevans V. Effect of DMSO on the stabilisation of lysosomes by cortisone and chloroquin *in vitro*. *Ann NY Acad Sci* 1967; 141: 326-32.
21. Beato M. Gene regulation by steroid hormones. *Cell* 1980; 56: 335-44.