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DOI

[10.3109/09273949709085063](https://doi.org/10.3109/09273949709085063)

Publication date

1997

Published in

Ocular Immunology and Inflammation

[Link to publication](#)

Citation for published version (APA):

McMenamin, P. G., Crewe, J., & Kijlstra, A. (1997). Resident and infiltrating cells in the rat iris during the early stages of experimental melanin protein-induced uveitis (EMIU). *Ocular Immunology and Inflammation*, 5(4), 223-233. <https://doi.org/10.3109/09273949709085063>

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Ocular Immunology and Inflammation
0927-3948/97/US\$ 12.00

Ocular Immunology and Inflammation
–1997, Vol. 5, No. 4, pp. 223–233
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Buren (The Netherlands) 1997

Accepted 31 July 1997

Resident and infiltrating cells in the rat iris during the early stages of experimental melanin protein-induced uveitis (EMIU)

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Abstract Experimental melanin protein-induced uveitis (EMIU) is reported to be a model of anterior uveitis and choroiditis in which the retina is spared. In this study, we chose to compare EMIU with experimental autoimmune uveoretinitis (EAU), a well-recognised model of endogenous posterior uveitis, with regard to the nature and dynamics of the cellular infiltrate in the iris. Female Lewis albino rats were immunised with mixtures of crude retinal extract/complete Freund's adjuvant (CFA) (EAU), phosphate-buffered saline/CFA (controls), or iris melanin/CFA (EMIU) using established protocols. Animals were sacrificed on days 10 and 13 (around disease onset). Following whole body perfusion fixation, irides were dissected from the remainder of the globe. Iris wholemount preparations were then subjected to immunohistochemical analysis in order to investigate both the dynamics of infiltrating leukocytes and the effects of the inflammatory changes on resident immune cells in the iris. The nature of the cellular infiltrate in both EMIU and EAU models was essentially similar, namely there was a rich infiltrate of ED1⁺ mononuclear cells, Ox42⁺ neutrophils and T cells. Resident tissue macrophages (ED2⁺) were slightly below normal densities in the iris of EAU animals and marginally elevated in EMIU animals. MHC class II (Ia) staining, associated in the normal eye with dendritic cells (DC), was considerably elevated in EMIU. It is likely that this was due to both increased DC numbers and an influx of Ia⁺ exudate macrophages. No striking difference was found in the nature and phenotype of the cellular infiltrate in the iris at the onset of the disease in these two models of uveitis (EAU and EMIU). This suggests that the anterior segment inflammation in both models represents non-specific changes secondary to cytokine release associated with interaction of activated antigen-specific T cells and target antigens, namely retinal photoreceptors in EAU and uveal tract melanin-containing cells in EMIU. Alternatively, it may suggest that antigen-presenting cells resident in the iris and ciliary body in normal eyes have access to ocular antigens on both sides

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Acknowledgements: This work was supported by The Australian National Health and Medical Research Council and The Raine Foundation Visiting Professor Scheme funded Professor Kijlstra during a visit to The Department of Anatomy and Human Biology, The University of Western Australia, Perth, Western Australia.

of the blood-ocular barrier and are capable of activating circulating antigen-specific T cells in these models.

Key words Melanin; uveitis; iris; inflammation; immunohistochemistry

Introduction Until recently, the immunohistochemical analysis of immune cells in tissues bordering the anterior chamber (AC) was hampered by technical difficulties associated with the small amount of tissue available in conventional meridional sections of rodent eyes. A breakthrough initially came from studies which used planar or oblique sectioning to investigate the outflow pathways of the rat eye.¹ This was quickly and logically followed by the development of a method for performing immunostaining procedures on iris and ciliary body wholemounts²⁻⁴ and later on isolated choroidal wholemounts.^{5,6} Because of the advantages of this novel approach in providing 'aerial' or plan views of the entire tissue, we now have a detailed knowledge of the distribution and phenotype of immune cells in the uveal tract of the rodent and human eye. It is apparent that within the iris stroma there is a rich network of MHC class II (Ia)⁺ dendritic cells (DC) which co-exist alongside a distinct population of pleomorphic and dendriform resident tissue macrophages, many of which display a perivascular location. The ciliary body contains similar populations of DC and macrophages in the connective tissue stroma in addition to a network of DC interposed between the ciliary epithelial layers.^{3,4}

There have now been a number of immunohistochemical studies which have utilised the inherent advantages of wholemount techniques to examine the kinetics of neutrophil, monocyte, and lymphocyte infiltration and the response of resident immune cells (DC and macrophages) in the uveal tract in models of endotoxin-induced uveitis (EIU),^{7,8} experimental autoimmune uveoretinitis (EAU),⁶ and keratoplasty.⁹

Experimental melanin protein-induced uveitis (EMIU) is an experimental model of uveitis induced by a non-soluble melanin-associated protein derived from the choroid or iris and ciliary body. It has aroused considerable interest as an experimental model since the histopathological changes are restricted to the anterior segment and choroid with sparing of the retina.¹⁰⁻¹³ Thus, it has come to be regarded as a more representative model of non-infectious recurrent iridocyclitis and choroiditis in humans than previously available models.¹⁴

The only previous immunohistochemical investigation of EMIU was performed on conventional frozen sections.¹⁵ These authors reported a predominance of CD4 T cells over CD8 T cells and macrophages during the disease. For the purposes of the present investigation, we chose to compare and contrast the EMIU model with EAU with respect to the response of resident cells and the dynamics of cellular infiltration in the iris using the wholemount method. We chose EAU as a comparison for two main reasons. Firstly, although EAU is primarily considered a model of endogenous posterior uveoretinitis, it is well accepted that an important feature in the early phase of the disease is an acute anterior uveitis. Indeed, it is the anterior segment inflammatory changes which are largely used to detect the commencement of inflammation and grade the disease clinically.¹⁶ Therefore, we wished to determine whether the nature of the inflammatory cell infiltrate in EMIU was similar to that in EAU. Secondly, EAU was chosen for comparison because

we have recently completed a more comprehensive study of the immune cells in the uveal tract in the early and late stages of this model.⁶

Materials and methods

ANIMALS A total of 14 female Lewis rats (6-8 weeks old, specific pathogen-free), obtained from the Animal Resources Centre (Murdoch, Western Australia), were used in the present study. Throughout this study, all procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

EXPERIMENTAL AUTOIMMUNE UVEORETINITIS (EAU) Bovine crude retinal extract (CRE) (2 mg/ml) was prepared as described in our previous studies.⁶ Experimental animals received a 100 µl injection of an emulsion, consisting of equal proportions of CRE and complete Freund's adjuvant (CFA) (Bacto Adjuvant Complete H37 Ra, Difco, Detroit, MI, USA), subcutaneously into the right hind footpad. Animals received a concurrent 100 µl intraperitoneal injection of *Bordetella pertussis* toxin (1 µg). The footpad injection corresponded with a dose of 100 µg CRE, which in the present and previous studies has produced a mild-moderate form of EAU.^{17,18} Experimental animals were sacrificed on days 10 and 13 regardless of the presence or absence of clinical signs. Controls included animals treated with CFA/PBS and pertussis toxin alone. These were also sacrificed on days 10 and 13.

EXPERIMENTAL MELANIN PROTEIN-INDUCED UVEITIS (EMIU) Bovine iris melanin was prepared as described by Broekhuysse et al.¹⁹ Animals were immunised with a 100 µl emulsified mixture of 50:50 melanin antigen in PBS and CFA in the hind footpad. In addition, they received 100 µl of 50:50 mixture of melanin antigen and pertussis toxin (1 µg) intraperitoneally. The melanin preparation contained 1.4×10^9 granules/ml; therefore, the dose per animal was 0.7×10^8 .

CLINICAL GRADING OF EYE DISEASE All eyes were graded clinically with the aid of an operating microscope on a scale of 0-4 on days 10 to 13, and were sacrificed on days 10 and 13 (Table 1). Grade 1 disease (mild) is evident as discernible hyperemia with occasional cells adhering to the lens and pupil margin. Grade 2 disease (mild-moderate) is characterised by iris vessel dilation, flare, and iridocyclitis producing large numbers of inflammatory cells in the anterior chamber (AC); however, the pupil remains visible. Grade 3 disease (moderate) manifests as dense inflammatory cell infiltrates within the AC sufficient to obscure the pupil, hypopyon uveitis, and corneal haze. Grade 4 disease (acute-severe) is similar to Grade 3 with the additional feature of intraocular haemorrhage.

IRIS WHOLEMOUNT PREPARATION AND IMMUNOHISTOCHEMISTRY Tissues were fixed and processed as previously described.⁷ Briefly, animals were anaesthetised (sodium pentobarbitone, 100 mg/kg) prior to whole body perfusion via the left ventricle with cold heparinised PBS followed by cold 100% ethanol. Eyes were postfixed in ethanol after enucleation and for the period it took to dissect the eyes (total of approximately 15 min). Each eye was divided into anterior and posterior segments and the lens and capsule

were carefully removed. The iris was then separated from the cornea/sclera and divided by radial incisions into five equal portions.

IMMUNOHISTOCHEMISTRY Pieces of whole iris-ciliary body from each eye were incubated with a panel of primary monoclonal antibodies (mAbs). Negative controls included either PBS alone or use of an inappropriate mAb of the same isotype (e.g. anti-human macrophage marker, OKM1). The specificity of primary mAbs to rat macrophages, DC, and the methods for single immunohistochemical staining of wholemounts were as previously described.^{4,6,7} In the present study, we investigated the following mAbs: ED1 (lysosomal antigen in monocytes, tissue macrophages, and DC), ED2 (resident tissue macrophages), Ox6 (MHC class II), Ox42 (CD11b/18, present on neutrophils, macrophages, and DC), and Ox19/R73 (pan T cell). Wholemounts were first blocked with PBS/BSA/Tween and then incubated with the primary mAb followed by biotinylated sheep anti-mouse and streptavidin-horseradish peroxidase (Amersham Laboratories, Buckinghamshire, UK). All antibody incubations and other steps were performed at room temperature for 30-45 min. The horseradish peroxidase (HRP) was visualised by 3-amino-9-ethyl carbazole (0.2 mg/ml dissolved in dimethylformamide in acetate buffer pH 5; plus 0.35 µl/ml H₂O₂ [30% w/v]). Very few cells displaying endogenous activity were noted in negative controls. All visualisation agents and chemicals were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA). Stained iris/ciliary body wholemounts were mounted in aqueous medium on glass slides and coverslipped.

QUANTITATIVE ANALYSIS Immunopositive cells within the iris wholemounts were counted using a calibrated eyepiece graticule and a x40 objective lens as described previously.⁴ The mean area of tissue sampled for each mAb was 0.504 mm². The mean density of cells stained with each mAb per individual animal was calculated and pooled in each treatment group. As no significant difference was noted between days 10 and 13 in both EAU and EMIU groups, the data from both days were pooled for statistical purposes. All cell counts were performed on coded slides by a 'masked observer'. Differences between individual groups were analysed using t-test, where group numbers were three or above. Due to technical difficulties, obtaining consistent homogeneous immunostaining in wholemounts of inflamed eyes suitable for quantitative analysis was not always possible. Therefore, the number of animals per group for each mAb was not always identical (Table 1). Significance values indicate differences between experimental and control groups (Student's *t*-test).

Results

ED1-POSITIVE MONONUCLEAR CELLS IN THE IRIS Control irides are characterised by a network of ED1⁺ cells at a density of 891±80 cells/mm² (Fig. 1), which previous double colour immunohistochemical studies have shown include resident tissue macrophages and a few DC.^{4,6} The majority of ED1⁺ cells in control eyes are either dendriform or pleomorphic in shape (Fig. 2A). On days 10 and 13 post-immunisation, the iris from animals with grade 1 and grade 2 clinical disease (both EAU and EMIU) demonstrated extensive infiltration of rounded ED1⁺ mononuclear cells (approximately 3000

	ED1	ED2	Ox6	Ox42	Ox19/R73	Day of sacrifice	Clinical disease on day of sacrifice
Controls	891 (80)	595 (71)	417 (62)	838 (233)	6 (2)	D10 (n=3), D13 (n=1)	0 (n=4)
n	4	4	4	3	4		
EAU	2959 (57)**	303 (74)**	N.D.	2712 (714)	1714 (714)**	D10 (n=3), D13 (n=1)	2 (n=3), 3 (n=1)
n	4	4		2	3		
EMIU	2965 (983)**	871 (177)*	1248 (201)	2214 (928)	1045 (497)	D10 (n=3), D13 (n=3)	0 (n=1), 1 (n=4), 2 (n=1)
n	6	6	2	2	2		

cells/mm², $p < 0.01$) (Figs 1 and 2B). The resident ED1⁺ cells appeared to have lost their cytoplasmic extensions during the inflammatory response. In one non-responsive EMIU animal, ED1⁺ cell numbers were close to normal or only marginally elevated and cells had a typical 'resting' or normal morphology (pleomorphic and dendriform).

ED2-POSITIVE RESIDENT TISSUE MACROPHAGES IN THE IRIS Iris wholemounts from control animals demonstrated the network of ED2⁺ resident tissue macrophages (Fig. 2C), which corresponded in density (595 ± 71 cells/mm²) to those in normal animals as described in our previous studies.^{4,6,7} In EAU animals, the number of ED2⁺ resident tissue macrophages were marginally lower than in normals (303 ± 74 cells/mm²) ($p < 0.01$). By contrast, EMIU animals demonstrated marginally elevated ED2⁺ cell numbers (871 ± 177 cells/mm²) ($p < 0.05$), particularly in two of the day 13 animals, one of whom showed no overt clinical signs of disease other than excess Harderian gland secretions evident as a reddish staining of the fur and skin around the eyelids. In EMIU eyes with elevated numbers of ED2⁺ macrophages, the majority of which displayed rounded and pleomorphic morphology (Fig. 2D) suggesting either that the dendriform or bipolar resident cells had withdrawn their cytoplasmic extensions and/or that many of the cells were newly recruited mononuclear cells ('exudate macrophages') from the blood. The distribution pattern of these cells was similar to ED1 staining, although the extent of the infiltration was much less marked in nature (cf. Figs 2B and D).

MHC CLASS II (IA)⁺ CELLS IN THE IRIS The network of highly dendriform Ia⁺ DC in control animals corresponded with those in normal iris wholemounts in both morphology (Fig. 2E) and density (Fig. 1) (417 ± 62 cells/mm²).^{4,6,7} Density data from EAU animals was not available due to technical difficulties; however, a recent comparable study found that the overall density of iris Ia⁺ cells remained close to normal at this stage of EAU although there was an infiltration of small round Ia⁺ cells.⁶ In EMIU animals in which it was possible to quantitate Ia staining, there was a marked increase in cell numbers (1248 ± 201 cells/mm²), many of which displayed a more rounded or pleomorphic morphology (Fig. 2F) than in controls, suggesting recent recruitment from the blood.

TABLE 1. Immunohistochemical analysis of immune cell densities (cells/mm²) in iris in EAU and EMIU. Non-responders (clinically and histologically) were excluded from the calculations of group means. Numbers in parentheses are standard deviations.

* $P < 0.05$; ** $P < 0.01$.

Fig. 1. Quantitative analysis of immunopositive cells in rat iris wholemounts in control, EAU, and EMIU groups. Data are cells/mm².

OX42-POSITIVE CELLULAR INFILTRATE The Ox42 mAb recognises the β chain of the C3 receptor on neutrophils, monocytes/macrophages, and DC. In the normal non-inflamed iris wholemounts, there is weak but detectable staining of the resident tissue macrophage and DC populations in the iris and stronger staining in the ciliary body.⁷ In our laboratory, quantitation of small round multinucleate Ox42⁺ cells in inflamed eyes has acted as a useful indicator of the presence of infiltrating neutrophils and therefore the degree of inflammation in the uveal tract.^{6,7} In the present study, the pattern of Ox42 staining in control eyes (Fig. 2G) corresponded to that seen in normal specimens. A massive infiltrate of Ox42⁺ cells in the iris was identified in both EAU and EMIU animals (>2000 cells/mm²) (Figs 1 and 2H). Many of the infiltrating cells were either intravascular or perivascular, indicating recent

Fig. 2. Light microscopic views of iris wholemounts stained with a variety of mAb in control (A, C, E, G, and I) and EMIU animals (B, D, F, H, and J). p, pupil margin. A & B, ED1; C & D, ED2; E & F, Ox6; G & H, Ox42; I & J, Ox19/R73 (T cells). Original magnification of all micrographs, except for C (x220), is x150.

extravasation (Fig. 2H). On close examination, we concluded that these cells likely consisted of a mixture of mononuclear cells and neutrophils (not illustrated).

T CELLS IN THE IRIS As in previous studies of iris wholemounts from normal young animals, control irides in the present study were found to contain only very rare T cells (6 ± 2 cells/mm²) (Fig. 2I). In EAU (days 10 and 13) and EMIU (day 13) animals, there was massive influx of small round T cells (Ox19/R73⁺) throughout the iris (Figs 1 and 2J).

Discussion This study shows that two different models of uveitis, EAU and EMIU, are both characterised by a heavy infiltrate of ED1⁺ macrophages, neutrophils, and T cells and do not differ markedly from endotoxin-induced uveitis, indicating that the inflammatory response of the iris against different stimuli evolves via a common pathway.

Acute anterior uveitis is the most common form of uveitis, being responsible for 75% of all cases. Although single episodes of the disease do not generally result in significant visual damage, the recurrent nature of the disease may ultimately result in visual loss due to cataract, cystoid macular oedema, and glaucoma.²⁰ The close parallels between the pathogenesis of the EMIU model and human acute anterior uveitis, especially the lack of retinal involvement and its recurrent nature, makes it of considerable interest in uveitis research as an experimental paradigm of the clinical situation. There are also several links to suggest that melanocytes are the target antigen in some forms of uveitis. In the Vogt-Koyanagi-Harada syndrome, the patient usually progresses through three phases: initially, there are symptoms of meningitis; followed by the development of anterior and posterior uveitis; and as the uveitis begins to resolve, pigmentary disturbances of the skin (vitiligo, alopecia, and poliosis) and hearing deficiencies (dysacusis) may develop. This mixed group of findings may be due to damage to melanin-bearing cells in the meninges, uveal tract, skin, and ear.²¹

In the present study, we chose to compare the immunopathological changes in the anterior segment during the early phase of EMIU with EAU. Whilst the latter is a well-recognised model of endogenous posterior uveitis in which CD4 T cells are directed towards retinal photoreceptor antigens, it is characterised in the early stages by anterior uveitis. A comprehensive study of changes in resident immune cells and the phenotype of infiltrating cells in the rat uveal tract in the EAU model using wholemount preparations has recently been published.⁶

Although there have been a number of histopathological studies of EMIU,¹⁰⁻¹³ there has until now been only limited immunopathological study of this model.^{12,15} The more extensive study of Kim et al.¹⁵ differs from the present investigation in several respects. Firstly, they¹⁵ used conventional cryostat sections and investigated a large number of time points post-immunisation. In contrast, we chose to investigate only a few early time points in order to characterise the earliest inflammatory cells and we used iris wholemounts, the advantages of which have been outlined previously. Kim and co-workers¹⁵ reported only a moderate increase in ED2⁺ macrophage numbers in EMIU which is in agreement with our data. However, the choice of ED2 as a marker for monocytic/macrophagic infiltration by these authors is not ideal as this mAb recognises a membrane antigen on resident

tissue macrophages which is either absent or only weakly expressed on exudate or newly recruited macrophages.^{22,23} The antigen recognised by this mAb, presently unknown, is upregulated only after these cells have been in the tissue for some period, possibly as a consequence of interactions with collagenous elements of the extracellular matrix (C. Dijkstra, personal communication). Thus, Kim et al.,¹⁵ by choosing to study only ED2, may have underestimated the extent of macrophage infiltration in EMIU. In our investigation, we chose not only ED2, but also the mAb ED1, which recognises lysosomal proteins (CD68) and is strongly expressed on newly exudate macrophages.²⁴ We observed that ED1⁺ cells formed a major component of the infiltrating mononuclear cells in EMIU with densities exceeding 3000 cells/mm² compared to normal levels of 800-900 cells/mm². Chan et al.¹² reported macrophage infiltration in the uvea between days 7 and 10 with a peak at day 15; however, the method of macrophage identification was not clearly indicated.

The alteration in morphology of ED1⁺ and ED2⁺ macrophages from a largely dendriform and pleomorphic morphology to more regular cellular profiles together with the upregulation of ED1 was interpreted as evidence of activation, increased mobility, and phagocytic activity. It is likely that during such an acute inflammatory response in the iris and ciliary body, there will be considerable cell debris from apoptotic neutrophils and lymphocytes which will require destruction and removal. In addition, it is presumed that in EMIU as in EAU, macrophages would play a role in destruction of the target antigen.^{17,18,25,26} To date, there have been no ultrastructural studies of the EMIU model to indicate that macrophages or indeed T cells are actively responsible for attacking melanocytes (the presumed target cell) in the iris and ciliary body.

The present study revealed a change in morphology of MHC class II⁺ cells, predominantly DC, similar to that observed with iris macrophages. This may be indicative of activation and migration of these cells. Recent data has shown that in the normal eye the half-life of iris DC is around three days.²⁷ It is possible that the morphological changes and increased density observed in the current study could be a reflection of increased turnover of these sentinel cells in EMIU. Similar changes in morphology of Ia⁺ cells was observed in the early stages of EAU; however, the densities were not as high in EAU as those observed in EMIU.⁶ Specific experiments would be required to confirm whether traffic of DC increases during cell-mediated responses in the eye. Such an increase may be an advantage at a time when the likelihood of exposure to exogenous and altered endogenous antigens in the intraocular environment may be increased due to breakdown of the blood-ocular barrier and tissue damage.

A characteristic of both the EAU and EMIU models is an extensive infiltration of neutrophils. These cells constitute the majority of small round Ox42⁺ cells seen in iris wholemount preparations from diseased eyes. The involvement of neutrophils is in some respects surprising since both models are immune-mediated responses directed at 'self antigens' and not acute inflammatory responses, where the presence of neutrophils is an important characteristic feature. However, the involvement of neutrophils in these models is to some extent dependent on the choice and use of adjuvant.²⁶ The influx of neutrophils has been noted in previous studies of EMIU,^{11,12,19} while others have stated they are rare in active lesions.¹³ We would postu-

late that neutrophilic infiltrates in EMIU are likely a non-specific event mediated by the local production of chemokines and cytokines in the eye following interaction of T cells with target antigen and partly due to the use of pertussis and CFA as adjuvant agents. Cytokine release will in turn upregulate adhesion molecule expression on local vascular endothelium thus facilitating margination and extravasation of neutrophils. Bamworth and co-workers²⁸ have presented good evidence that neutrophil influx may significantly contribute to the breakdown of the blood-ocular barrier following experimental intraocular cytokine injections. An upregulation of adhesion molecules in iris and ciliary body (stroma and epithelium) prior to disease onset in EMIU has been reported;^{12,15} however, no specific observations regarding vascular endothelium were mentioned.

The influx of T cells into the iris and ciliary body noted at the early stages of the disease were similar in both EAU and EMIU models. Kim and co-workers¹⁵ also reported a massive T cell infiltrate which was noted to be too difficult to accurately quantify in sections. We would have expected the T cell influx in the anterior segment in EAU animals to have been less marked than EMIU as the former is a model of posterior uveitis where the target antigen is located in the photoreceptor layer of the retina.

The observations in the present study together with similar findings in endotoxin-induced uveitis⁷ lead us to present two main postulates. Firstly, inflammation of the iris/ciliary body may be a non-specific response mediated by cytokines which, regardless of the triggering stimulus within the eye (e.g. LPS, T cell-target antigen interaction), easily cascades into non-specific acute inflammation whose cellular complexion, particularly the monocytic and neutrophilic components, is largely similar in most situations. Secondly, that DC or macrophages in the iris and ciliary body may under normal conditions possess ocular-specific antigens or peptides (e.g. S-antigen or melanin-associated protein) in association with their surface MHC class II molecule. This could easily result in antigen presentation to autoreactive T cells trafficking through these tissues and consequent clonal expansion. Both hypotheses require further experimental investigations to clarify these issues.

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