Analysis of IL-6 levels in human vitreous fluid obtained from uveitis patients, patients with proliferative intraocular disorders and eye bank eyes

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ABSTRACT

Several studies suggest a role for IL-6 in the pathogenesis of uveitis. Earlier we have shown that aqueous humour obtained from patients with uveitis contained raised levels of IL-6. In the study described here we investigated the IL-6 levels in vitreous fluid samples obtained from 75 uveitis patients with different uveitis entities. Vitreous samples from 14 patients with proliferative intraocular disorders (PID) and 29 eye bank eyes were used as controls. All the samples were tested in the IL-6 B9 bioassay as well as in a sensitive ELISA for IL-6.

Raised IL-6 levels were detected in the vitreous fluid of uveitis patients as well as patients with PID, implicating IL-6 as a common inflammatory mediator. The highest mean level of IL-6 was found in the vitreous fluid of patients with acute retinal necrosis. The mean IL-6 levels measured by the ELISA were higher compared to the levels measured by the B9 bioassay. This may be caused by the presence of B9 bioassay inhibitory factors in the vitreous fluid of these patients.

mental models, whereby raised IL-6 levels were detected in aqueous humour of rats undergoing endotoxin induced uveitis (8).

For a better understanding of the immunological response in the eye we analyzed whether raised IL-6 levels can also be detected in vitreous fluid samples of uveitis patients, undergoing a therapeutic vitrectomy. Especially in posterior uveitis, cytokines in the vitreous may play a role in mediating the inflammatory response. Another question we addressed is whether IL-6 is related to certain uveitis entities. We observed that IL-6 levels were indeed raised in vitreous fluid of uveitis patients but similar findings were observed in patients with proliferative intraocular disorders (PID), implicating IL-6 as a common inflammatory mediator.

INTRODUCTION

IL-6 is an important factor in host defense mechanisms. It plays a role in many diseases with a different pathogenesis: infection, autoimmunity, and plasma cell malignancies (1). Raised IL-6 levels can be detected in sera as well as in other body fluids, depending on the site of inflammation. For example, raised IL-6 levels can be detected in cerebrospinal fluid in bacterial or viral meningitis (2), in synovial fluid in different types of arthritis (3), in sera in septic shock or severe burns (4,5), in urine before kidney transplantation rejection (6), and in aqueous humour in Fuch's heterochromic cyclitis (FHC) and toxoplasma chorioretinitis (TC) (7). The two last examples suggest a role for IL-6 during intraocular inflammation. Further evidence that IL-6 might play a role in uveitis was obtained in experi-

MATERIALS AND METHODS

Samples

Samples from uveitis patients (N=75) were obtained during therapeutic vitrectomies. The samples were obtained from patients with different uveitis entities. We categorized the uveitis patients into 6 groups: cause unknown (N=30), noninfectious cause (N=14), infectious cause (N=11), immunocompromised patients (N=8), ARN (N=11) and ocular lymphoma (N=1). The noninfectious causes included patients with pars planitis (N=8), sarcoidosis (N=1), HLA-B27 positive uveitis (N=2), juvenile chronic arthritis (N=1), Behçet's disease (N=1) and Fuch's heterochromic cyclitis (N=1). The group with infectious causes of uveitis contained patients with *Toxoplasma* (N=7), *Borrelia* (N=1),

HSV (N=1), EBV (N=1) and one patient with *Toxocara* uveitis. The immunocompromised group consisted of patients with AIDS (N=2) or patients treated with immunosuppressive or cytotoxic medication, 4 in case of malignancy and two in case of kidney transplantation. Two patients with immunosuppressive or cytotoxic medication had an intraocular infection, CMV in one case and *Toxoplasma* in the other. Vitreous fluid samples of patients with proliferative intraocular disorders (N=14), and postmortem eye bank eyes (N=29) were used as controls. All samples were stored at -20°C until use.

IL-6 measurements

All samples were tested in the B9 bioassay (9) as well as in a sensitive ELISA (10) for IL-6. Standard curves of both tests using purified human recombinant IL-6 (kindly provided by Dr. L.A. Aarden from the Central Laboratory of the Netherlands Bloodtransfusion Service) are shown in figure 1. As can be seen from the standard curves the B9 bioassay is approximately 50 times more sensitive than the ELISA. In monocyte supernatants and synovial fluids, there is a good correlation between both tests (10).

B9 bioassay

The IL-6 bioassay was performed using the murine hybridoma cell line B9 (kindly provided by Dr. L.A. Aarden) which is dependent upon IL-6 for its proliferation. Human IL-6 and murine IL-6 are both active in the B9 assay with a similar specific activity (11). The B9 cells have been shown not to proliferate in response to endotoxin (12). The vitreous fluid samples to be tested were diluted 1:50 in assay medium (RPMI 1640 supplemented with 5% foetal calf serum, 50 µM 2-mercaptoethanol and penicillin/streptomycin). 50 µl of the sample dilutions were incubated for 72 hours with 50 µl of cell suspension (5×10^3 cells) in a 96 wells plate (Nunc, Roskilde, Denmark). The proliferation of cells was measured by determining the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma no. M-2128) conversion by the cells (13). 10 µl of MTT at a concentration of 5mg/ml, was added to each well. After four hours, the reaction was stopped by addition of

Figure 1: Standard curves of purified human recombinant IL-6 in the B9 bioassay and ELISA. The B9 bioassay is 50 times more sensitive than the ELISA.

Figure 2: IL-6 levels in vitreous fluid samples obtained from uveitis patients, patients with PID and eye bank eyes, analyzed in the B9 bioassay. The horizontal bars indicate the mean level of IL-6 and the standard deviation of each group.

100 µl of acidic-isopropanol. The formed MTT-formazan crystals were resuspended and the absorbance was measured at 570 nm. The amount of IL-6 was calculated using a dose response curve obtained with human recombinant IL-6 (0.25-8 pg/ml). In earlier experiments where we measured the IL-6 levels in aqueous humour, we could neutralize the B9 bioactivity with anti-human IL-6, in a dose related way (7).

ELISA

A sensitive sandwich ELISA exactly as described by Helle et al (10) was also used to detect IL-6. The mono-

clonal and polyclonal antibody for this ELISA were a gift from Dr. L.A. Aarden from the Central Laboratory of the Netherlands Bloodtransfusion Service. The vitreous fluid samples were diluted 1:10 in PTG (PBS supplemented with 0.02% Tween and 0.2 gr/l gelatin). The samples dilutions were incubated for two hours at room temperature in a 96 wells plate (Nunc immunoplate, Maxisorb, Denmark) that had been coated overnight with a monoclonal anti-IL-6 antibody (mAB CLB.IL-6/16). Polyclonal biotinylated sheep anti-IL-6 antibodies were added in the presence of 1% normal sheep serum to avoid false positive reactions. After washing, streptavidin-horseradish peroxidase (strep-HRP; Amersham) was added. The signal was amplified with biotin-tyramin and strep-HRP. Tetramethylbenzidin (Merck, Germany) was used as a substrate and the reaction product was read in a Titertek Multiskan Plus at 450 nm. The amount of IL-6 was calculated from a standard curve using various concentrations of human recombinant IL-6 (5-75 pg/ml).

Miscellaneous

Statistical analysis was performed using the Kruskal-Wallis test and correlation was measured using the Spearman test; both are nonparametric tests.

RESULTS

B9 bioassay

IL-6 was detected (> 10 pg/ml) in vitreous fluid samples of 38 out of 75 uveitis patients tested (51%; mean 109 pg/ml). Of the patients with proliferative intraocular disorders, 9 out of 14 had a detectable IL-6 level (64%; mean 76 pg/ml) in the aspirated vitreous fluid (fig.2). In the eye bank group, the IL-6 levels were raised in two out of 29 samples (7%; mean 23 pg/ml). In one of these samples, the IL-6 level was more than 100 pg/ml. This donor died of a malignancy.

Comparison of the different uveitis entities showed that the highest mean IL-6 levels in vitreous fluid were observed in patients with ARN (mean 164 pg/ml) (Fig.3). High mean levels of IL-6 were also seen in the group of

Figure 3: IL-6 levels in the vitreous fluid of patients with different uveitis entities analyzed in the B9 bioassay. The horizontal bars indicate the mean level of IL-6 and the standard deviation of each group.

Figure 4: IL-6 levels in vitreous fluid samples obtained from uveitis patients, patients with PID and eye bank eyes, analyzed in the IL-6 ELISA. The horizontal bars indicate the mean level of IL-6 and the standard deviation of each group.

patients with an unknown cause of uveitis (mean 159 pg/ml). In the immunocompromised group, the IL-6 levels in vitreous fluid were low (mean 18 pg/ml). In the patient with ocular lymphoma, no IL-6 could be detected.

ELISA

Using the ELISA, IL-6 was detected in more vitreous fluid samples and higher levels were obtained as compared to the bioassay. In the uveitis group, raised IL-6 levels were

Figure 5: IL-6 levels in the vitreous fluid of patients with different uveitis entities analyzed in the IL-6 ELISA. The horizontal bars indicate the mean level of IL-6 and the standard deviation of each group.

Figure 6: The correlation between vitreous fluid IL-6 levels measured in the ELISA and B9 bioassay ($r=0.70$; $t=10.6$; significant when $t>2$).

found in 41 out of 75 samples (81%; mean 272 pg/ml). In samples of patients with PID, IL-6 was detectable in 10 out of 14 samples (71%; mean 131 pg/ml). In the eye bank eyes 12 out of 29 samples contained IL-6 (41%; mean 47 pg/ml) (fig.4). In 4 of these samples (14%) the IL-6 level was larger than 100 pg/ml. The causes of death of these donors were cardiac (N=2), malignancy (N=1) and unknown

(N=1) respectively. The donor who died of malignancy (see also B9 results) had the highest IL-6 level (386 pg/ml). The difference between the IL-6 levels in uveitis samples and postmortem eye bank eyes was statistically significant. The IL-6 levels in the different uveitis entities are shown in figure 5. The mean level in the ARN samples (mean 495 pg/ml) is higher as compared to the other uveitis groups. Raised IL-6 levels could also be detected in four samples of immunocompromised patients (mean 276 pg/ml). No IL-6 was detected in the vitreous of the patient with an intraocular lymphoma.

B9 bioassay versus ELISA

The correlation between the vitreal IL-6 levels as measured by the ELISA or the B9 bioassay is shown in figure 5 ($r=0.70$; $t=10.5$; significant when $t>2$). In a number of samples, there is only a signal in the ELISA (fig.6) and no detectable activity in the bioassay using the B9 cells. This suggests the presence of an inhibitory factor in the bioassay. Heat inactivation (30 min. incubation at 56°C) did not affect the IL-6 bioassay indicating that the inhibitory factor is heat stable. A better correlation between IL-6 levels as measured in the bioassay versus the ELISA was obtained in the PID group ($r=0.86$; $p<0.001$).

DISCUSSION

This study shows that IL-6 is detectable in vitreous fluids from patients with various uveitis entities as well as proliferative intraocular disorders. This suggests that IL-6 is a common inflammatory mediator, not related to a specific ocular disorder. The highest mean levels, found in the ARN group, are probably due to the severity of inflammation and the tissue damage in this disease. The mean IL-6 levels in vitreous fluids are low (109 pg/ml; B9 bioassay) compared to the reported mean levels measured in the B9 bioassay in other body fluids including aqueous humour. The highest levels were found in sera of patients with a septic shock (4) and in cerebrospinal fluid of patients with bacterial meningitis (2). In FHC the mean IL-6 level in aqueous humour was 543 pg/ml and for TC it was 19,228 pg/ml (7).

Although IL-6 is generally considered to be an important mediator of inflammation (1), several studies provided evidence that it also has a downregulatory effect on inflammation (14,15). At the moment it is not yet clear if IL-6 in the eye has a proinflammatory or a protective role. Studies using experimental animal models may clarify this issue.

A discrepancy was found between IL-6 levels measured in the B9 bioassay and the ELISA. In the majority of the samples IL-6 levels measured in the B9 bioassay were much lower compared to the levels measured in the ELISA. Only 3 samples clearly showed a higher signal in the B9 bioassay than in the ELISA (figure 6). This may be due to the presence of unidentified cytokines other than IL-6 which could also stimulate the proliferation of the indicator cell line. Neutralisation of these samples with anti-IL-6 antibodies might clarify this issue. Previously we could neutralize B9 cell proliferation activity of aqueous humour with anti-human IL-6 antibodies (7). A similar discrepancy between an immunochemical test and a bioassay was recently described by Feldmann et. al., who could not observe a correlation between interleukin-8 levels in synovial fluid measured by radioimmunoassay and chemotactic activity (16).

The explanation for the lower values observed in the bioassay can be divided into IL-6 activity inhibition or B9 cell proliferation inhibition. In case of IL-6 inhibition, one can speculate about suppressive cytokines (17), soluble IL-6 receptor (18,19), functional different forms of IL-6 (20) or IL-6 binding factors. Other growth factors in vitreous, such as TGF- β (21) have not been shown to interfere with the bioassay (22). Furthermore it has been shown that IL-6, bound to its soluble receptor is still bioactive (19). Recently Planck et al provided evidence for the production of IL-6 inhibitory factors after stimulation of human retinal pigment epithelial cells with human recombinant IL-1 α (23). Structural different forms of IL-6 have been demonstrated (20), and may functionally differ in binding to the receptor and capacity to induce proper signal transduction. For

instance the biological activity of high molecular weight IL-6 is less active in the B9 bioassay than low molecular weight IL-6 (20). Another possibility is that some IL-6 molecules have lost their bioactivity due to post-translational processing but can still be detected in the ELISA. Gel electrophoresis and immunoblots of the vitreous fluid samples with different IL-6 levels in both tests may clarify this issue.

Another explanation for the discrepancy between ELISA and bioassay may be the interference of immunosuppressive or cytotoxic drugs present in vitreous fluid of treated uveitis patients. Most of the uveitis patients we analyzed were treated with steroids (eyedrops, parabulbar injection or systemic prednisone 5-60 mg/day) at the time of the vitrectomy. None of the patients with proliferative intraocular disorders, however, used steroids at the time of the vitrectomy. In the PID group, the inhibition in the bioassay seems to be less as compared to the uveitis group, although the PID group is much smaller. The discrepancy between the IL-6 levels measured in the B9 bioassay and ELISA in vitreous fluid samples of four of the immunocompromised patients might be caused by the immunosuppressive/cytotoxic medication levels in their vitreous fluid. The possible inhibitory effect of therapeutic drugs in vitreous fluid needs further investigation. Treatment with immunosuppressive drugs might cause a decrease in intraocular cytokine levels due to their anti-inflammatory properties. We have not yet performed a comparative study concerning IL-6 levels in treated versus untreated patients. In view of the points mentioned above, an ELISA may be preferable to the B9 bioassay to detect IL-6. On the other hand a comparison between immunoreactive and bioactive IL-6 may lead to the discovery of regulatory proteins.

This study supports the current opinion that cytokines play a role in mediating intraocular inflammation (7,8,24) whereby highest mean levels of immunoreactive IL-6 was found in the vitreous fluid of patients with acute retinal necrosis. Manipulation of the cytokine network may provide important therapeutic tools for the future management of clinical uveitis.

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