Innate Phagocytosis and P2X7 Receptor Function: a Comparison of Endophthalmitis Patients and Population Controls

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Abstract

Background Endophthalmitis is an uncommon and serious intraocular infection. Endophthalmitis most commonly follows on from cataract surgery or intravitreal injections. The rate of endophthalmitis has fallen in recent years with improved surgical techniques, and infection control. However, despite all precautions, cases of endophthalmitis continue to present. It is possible that poor intraocular immune function makes some patients susceptible to endophthalmitis. We propose that innate phagocytic function is one determinant of the development of endophthalmitis. In order to investigate this, we measured the innate phagocytic function and P2X7 receptor function acutely and at intervals through convalescence for patients who developed endophthalmitis, and compared it with the function of a published population control group. **Methods** Patients presented to a tertiary eye hospital emergency department with acute endophthalmitis. Informed consent was obtained, and peripheral blood taken. Innate phagocytosis of ethidium and fluorescent latex beads was then quantified using a validated flow cytometric-method at the Florey Institute. Patients had repeat bloods taken at 1 week and 3 months after recruitment. **Results** P2X7 receptor function was significantly reduced in the endophthalmitis cohort compared to controls. P2X7 function improved through convalescence as patients recovered from endophthalmitis but did not improve to the level of controls. Phagocytic function was reduced in the endophthalmitis cohort compared to controls at all time points. There was no improvement in phagocytic function observed as patients recovered from endophthalmitis. **Conclusions** The data from this study showed greatly lowered phagocytic function and P2X7 function in patients who developed endophthalmitis, as compared to population controls. This important finding may allow us to predict susceptibility or guide future therapeutics through enhancement of innate phagocytic function.

Background

Endophthalmitis is an uncommon and sight threatening infection of the internal eye. It is precipitated by intraocular surgery and other insults that introduce microbes to the vitreous/aqueous humour of the eye (1). Endophthalmitis can be devastating with lower than 6/60 visual acuity reported in 55% of cases. Some eyes become chronically painful and enucleation (removal of the globe) is required (2). Endophthalmitis risk has been associated with several factors. Intraoperative complications, diabetes mellitus, older age and communication between the vitreous and conjunctival flora have been associated with higher rates of endophthalmitis (3) (4).

Although there have been advances in understanding ocular immunity, exactly how the eye maintains an appropriate balance between immune privilege and fighting infections is unknown. One trial conducted by Mislberger et al. measured anterior chamber microbiological contamination by taking bacterial cell cultures at the beginning and end of cataract surgery. Surprisingly it found that 14.1% of patients were culture positive at the beginning and 13.7% at the end (5). Despite these positive cultures, no patients in the study developed endophthalmitis. This finding indicates that there are immune factors which prevent the intraocular spread of bacteria in most patients. It led us to investigate whether there are quantifiable deficiencies in the innate immune system in endophthalmitis patients.

The Florey Institute Phagocytosis Laboratory has developed a validated flow cytometric assay which quantifies patient phagocytic function from blood samples. Phagocytosis forms a key component in the innate immune response to infections and is analysed in this study. Another relevant assay available at the Phagocytosis Laboratory is the P2X7 receptor uptake assay. The P2X7 receptor (P2X7R) is an ionotropic receptor found on immune cells (6). The receptors are expressed in the conjunctiva and the both anterior and posterior segments of the eye. P2X7 has received attention in the field of ophthalmology as a therapeutic target because of its involvement in ocular diseases. P2X7 is implicated in age related macular degeneration, diabetic retinopathy and glaucoma (7-9). The role of the P2X7 receptor in endophthalmitis has not been investigated.

Methods

Recruitment

Patients were recruited at a tertiary eye hospital emergency department. Patients were included who had a clinical diagnosis of endophthalmitis. Informed consent was obtained for recruitment into the study. Peripheral blood was taken from patients within two hours of presentation and follow up bloods were obtained at one week and three months.

Laboratory method

The testing of P2X7 receptor function and innate phagocytic function takes approximately 5 hours utilising flow cytometric analysis methods developed by Ben Gu and James Wiley of the Florey Neuroscience Institute at the University of Melbourne (10). All laboratory testing was conducted by the same research student.

The blood samples were diluted with Phosphate Buffered Saline, PBS. Ficoll® Paque Plus was added to the solution. The samples were then spun in a centrifuge for 35 minutes. Following the centrifuge, the lymphocyte layer was extracted from the samples using transfer pipettes and then diluted with PBS. The new samples were then placed in centrifuge for 10 minutes.

After this centrifuging a small pellet of condensed cells formed. To ensure that no residual red blood cells remained with the lymphocytes remained, 10mL of Red Blood Cell Lysis solution (A combination of NH_4Cl , KHCO_3 , EDTA at pH 8.0) was added. The cells were left at room temperature for 10 minutes to allow the Lysis solution to eliminate all red blood cells. The remaining lymphocytes were diluted with PBS and put in centrifuge for 10 minutes.

Following the separation of lymphocytes from whole blood a highly concentrated lymphocytes cell pellet remained. A small amount (approximately 130 μL) of sodium solution was added to keep the cells healthy. 10 μL of the cell pellet was combined with 990 μL of PBS. Invitrogen Countess Automated Cell Counter was used to count the concentration of cells in the diluted solution and therefore the

concentration of the undiluted solution. This concentration value to determine the volume of solution required to provide 5 million lymphocytes.

Antibodies were added so that the cells can be recognised on flow cytometry and specific subgroups of CD14 and CD16 monocytes to be analysed (Figure 1). Two different batches are created, the ethidium uptake batch and the fluorescent bead uptake batch (Figure 2). After pipetting the antibodies into two separate tubes for ethidium uptake and fluorescent bead uptake, the cells are left on ice for 20 minutes.

Flow cytometric analysis

Prior to beginning data acquisition, the flow cytometer function was checked with calibration beads. This ensured that the machine was operating consistently across all tests.

Ethidium is added to the baseline tube immediately prior to acquisition. Similarly, ethidium is added to the ATP tube just prior to acquisition. After 20 seconds of capturing data on the cytometer the ATP tube is removed and 10µL of ATP is added. Flow cytometry was conducted for 5 minutes and 40 seconds for each tube before the data was saved.

Three tubes were made for fluorescent bead uptake. The baseline tube was incubated in a 37°C bath for five minutes before acquisition. The CPX tube was incubated for 10 minutes with 5µL of Copaxone added and the ATP tube was incubated for 15 minutes with 10µL of ATP added. Fluorescent beads were added to each tube 20 seconds into each acquisition. The fluorescent bead uptake was measured in each tube for 7 minutes.

Data collection

The results were analysed using FlowJo® Version 7.6.5 software. This software is compatible with Windows operating systems.

Each assay creates a density scatter plot. To record the ethidium uptake assay I gated CD 14+ monocytes using the forward scatter and side scatter plot.

Calculating arbitrary units

For the ethidium uptake, the arbitrary area between the ATP uptake curve and the baseline curve was the end outcome measured. To calculate this area, the results from FlowJo® software to excel spreadsheets containing the formulas required to measure the arbitrary area. These formulas were devised by Dr Ben Gu.

For fluorescent bead uptake, the function was measured by area under the uptake curve for each test (baseline, ATP and CPX). The outcome measured was the function of CD 14+ monocytes. Like the ethidium uptake, the results obtained from the FlowJo® were imported to excel spreadsheets containing Dr Ben Gu's formulae for determining arbitrary area units.

Statistical analysis

Results were tested for statistical significance on GraphPad Prism 7.02 using un-paired t-tests. Statistical significance was determined using the Holm-Sidak method, with $\alpha = 0.05$.

Results

Cohort demographics

The endophthalmitis patient cohort had an average age of 78.1 years and an even gender ratio. A total of 8 patients were recruited, 5 of them had bloods taken at day 3, and 4 had follow up bloods taken at 3 months.

Data for the control cohort was obtained from the Dr Ben Gu's 2016 Innate Phagocytosis in Alzheimer's disease study (11). The healthy control cohort ($n=19$) had an average age of 74.4 and $n=9$ female participants and $n=10$ male participants.

Ethidium uptake

Mean ethidium uptake of CD14+ monocytes improved from Day 1 through to 3 months (Figure 7). The comparison between Day 1 and 3 months was statistically significant ($p=0.0425$), while the other comparisons, Day 1 to Day 3 and Day 3 to 3 months, were not statistically significant. (Day 3 to 3 months $p=0.476$, Day 1 to Day 3 $p=0.243$).

Ethidium uptake was significantly reduced at all time points compared to control data ($p<0.05$). The Day 1 group had a mean of 5392 (standard deviation 4461), the Day 3 group had a mean of 9644 (sd 6981) and the 3-month group had a mean of 13507 (sd 21688). The healthy control group had a mean of 28076 (sd 5357).

Fluorescent bead uptake

Baseline uptake

Baseline fluorescent bead phagocytic uptake was significantly reduced in the cohort at all timepoints compared to the control group ($p<0.05$). There was no statistically significant difference between the timepoints through convalescence (Figure 8). The day 1 cohort had a mean of 53208 (sd 28326), the day 3 cohort had a mean of 52429 (sd 17676), the 3 month cohort had a mean of 41252 (sd 22403) and the control group had a mean of 134398 (sd 32260).

CPX uptake

The corpaxone boosted fluorescent bead uptake showed no statistically significant change between the different endophthalmitis time groups (Fig. 9). At each time point the phagocytic uptake was significantly lower than the control group. The day 1 cohort had a mean of 70739 (sd 22863), the day 3 cohort had a

mean of 81972 (sd 23629), the 3 month cohort had a mean of 89662 (sd 20262) and the control group had a mean of 149474 (sd 41336).

ATP uptake

ATP phagocytic uptake was significantly reduced in each endophthalmitis cohort compared to the control group (Fig. 10). There was no statistically significant difference between the Day 1, Day 3 and 3 month data sets. Day 1 had a mean of 58823 (sd 25580), Day 3 had a mean of 67449 (sd 30522), the 3 month group had a mean of 69386 (sd 10199) and the control group had a mean of 121937 (sd 40289) (Fig. 10).

Discussion

The primary finding of this study is that there was a significant reduction in both phagocytic function and P2X7 function in the endophthalmitis cohort (Fig. 7,8). Interestingly the cohort's P2X7 receptor function recovered over the 3-month time period after the initial infection, but not to the level of controls.

Phagocytic function was significantly lower at all time points. As phagocytic function and the P2X7 receptor are key components in the innate immune response, these patients are demonstrating that their innate immune system was unable to eliminate the invading pathogens. They are therefore unlike the 14% of patients identified in the Mistlberger's trial who were had positive intraocular bacterial cultures but did not develop endophthalmitis (5).

This finding could be interpreted in different ways. The observed reduction in innate immune function could be due to underlying patient traits or it could be a normal physiological response to infection. The former seems more compelling when the results at three months convalescence are considered, as the patients no longer have an intraocular infection at this time. Both explanations are interesting when considered in both endophthalmitis prevention and the broader context of how the immune system responds to infection and what prevents the body from mounting an effective response.

Quantifying the phagocytic function of patients before they contract endophthalmitis would clarify how phagocytic function responds in these patients, however, as endophthalmitis is an uncommon outcome of intraocular surgery, this is impractical. Future trials could recruit more patients and have a longer follow up period through convalescence to see if phagocytic function recovers with time, matched controls rather than population controls would also allow for more robust conclusions.

Conclusions

Endophthalmitis is an uncommon infection of the internal eye that is a sight threatening emergency. Infection control measures employed during ophthalmic surgery can reduce the risk of contracting endophthalmitis, but they cannot eliminate the risk entirely. This study demonstrated that a patient cohort who contracted endophthalmitis had reduced P2X7 receptor function and reduced phagocytic function compared to population controls. It indicates that certain patients have poor immune function which

renders them uniquely susceptible to infection when undergoing ophthalmic procedures such as cataract surgery. This is an interesting finding which can be further investigated to improve our understanding of endophthalmitis pathophysiology, and may potentially allow for future determination of suseptability and / or therapeutic targetting.

List Of Abbreviations

APC	Allophycocyanin
ATP	Adenosine Triphosphate
CPX	Copaxone (also known as glatiramer acetate)
FITC	Fluorescein isothiocyanate
mAb	Monoclonal antibodies
PFA	Paraformaldehyde
YO	Yellow-orange

Declarations

Ethics approval and consent to participate

Human Research and Ethics Committee approval was obtained from Royal Victorian Eye Ear Hospital Human Research Ethics Committee. HREC number: 11/988H/16. Written consent to participate was obtained from each participant.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the author on reasonable request.

Competing interests

The authors declare they have no competing interests

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Author's contributions

AH collected blood, ran laboratory tests, analysed and synthesised information. XH assisted with laboratory work. BG, PA and RD supervised the study and were involved in writing this paper. The study design was created by PA, RD and BG in conjunction with AH. All authors have read and approved this manuscript.

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Figures

Figure 1

Schematic representation of the contents of each uptake batch: The Ethidium uptake tube is used for P2X7 receptor function and the Fluorescent bead uptake tube is used for innate phagocytic function

Ethidium tube contents	Fluorescent bead tube
~5 million lymphocytes in ~ 100μL of solution	~10 million lymphocytes in ~ 100μL of solution
10μL of CD14 FITC monoclonal antibody.	10μL of CD 16 FITC monoclonal antibody.
	20μL of CD14 APC.

Figure 2

The contents of the uptake tubes

Figure 3

The two Ethidium uptake tube contents. Ethidium was added to the baseline tube immediately before acquisition. Ethidium was also added to the ATP immediately before acquisition and ATP was added 20 seconds into data acquisition. Flow cytometric analysis was conducted for 5 minutes 40 seconds for each tube.

Figure 4

The three fluorescent bead uptake tube contents. Yellow-Orange (YO) Fluorescent Beads were added to each tube 20 seconds into acquisition. Flow cytometric analysis was conducted for 7 minutes for each tube.

Figure 5

Example of an ethidium uptake assay. The area between the two curves is the arbitrary unit calculated

Figure 6

An example of fluorescent bead uptake curves. Arbitrary units of function are measured by the total area under each curve

Figure 7

Ethidium uptake of CD14+ monocytes compilation.

Figure 8

Baseline uptake collation

Figure 9

CPX boosted uptake collation

Figure 10

ATP inhibited uptake collation