Original Article

An experimental study to evaluate safety/toxicity of intravitreal natalizumab

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Purpose: The purpose of this prospective experimental study was to evaluate the safety/toxicity of α4β1 integrin blockade in rabbit retina using its monoclonal antibody (Natalizumab). **Methods:** Twelve New Zealand albino rabbits were divided into three groups ($n = 4$). Unilateral intravitreal injections of three different concentrations of natalizumab were performed in every rabbit of each group (Group A: 0.625 mg, Group B: 1.25 mg, and Group C: 2.5 mg). Baseline electroretinogram (ERG) and fundus photography were performed prior to injection. At days 1, 7, and 21 postinjection, ERG and fundus photography of each eye were performed. At last follow-up, Group C animals with highest drug concentration were sacrificed and the enucleated eyes were evaluated for retinal toxicity using transmission electron microscopy (TEM). **Results:** No difference in ERG responses was observed in eyes injected with low and intermediate concentration of natalizumab between day 0 and day 21. Furthermore, rabbits injected intravitreally with highest dose showed reduction in amplitude of "a" wave ($P = 0.0017$) and a reduction in amplitude of "b" wave of ERG at day 21 ($P = 0.0117$). TEM revealed changes in the outer plexiform layer and inner nuclear layer, suggestive of toxicity primarily to the photoreceptor synaptic terminals and bipolar cells. **Conclusion:** Low-dose (0.625 mg) and intermediate-dose (1.25 mg) intravitreal injection of natalizumab appears safe for rabbit retina. However, functional and anatomical changes were observed in rabbit retina following a high-dose (2.5 mg) intravitreal injection of a monoclonal antibody blocking α4β1 integrin.

Key words: α4 integrin intravitreal injection, electroretinogram, natalizumab, uveitis

The process of lymphocytes gaining access into the posterior segment of the eye in the experimental models of autoimmune uveitis is governed by the activation of inflammatory cells and expression of adhesion molecules by the local vascular endothelium.[1] Alpha-4 (α4) integrins are the extracellular membrane receptors that facilitate extracellular matrix adhesion during homing of cells to the inflammatory site in inflammatory conditions. Integrin alpha-4 beta-1 (α4β1) can mediate both cell–cell and cell–extracellular matrix adhesion by binding to either vascular cell adhesion molecule 1 (VCAM-1) or fibronectin.[2]

The physiological role of α4β1 integrin and fibronectin has also been reported in the developing retinal neurons and their survival. Integrins in the β1 family play an important role in neuroblast migration and axon outgrowth during neuronal development in the retina.[3] In the developing chicken retina model, the α4 antagonist has been reported to cause increased apoptosis leading to thinning of the retina and reduction in numbers of retinal ganglion cells.[4]

Presently, intraocular inflammatory conditions such as vasculitis, posterior uveitis, and papillitis are treated with steroids or immunosuppressive agents. According to anatomical location, pan-uveitis has been considered as a generalized inflammation of all three parts of uvea: iris, ciliary body, and choroid as per the definition of International Uveitis Study Group.[5] Considering the involvement of α4β1 integrin in ocular inflammatory disorders, its blockade could be beneficial for the development of newer treatment modalities for pan-uveitis. As the α4β1 integrin has been reported to play a crucial physiological role in the neural retina,[4] studying the effect of its blockade on functions of the retina was felt as important.

Hence, the present study was carried out to evaluate the safety of α4β1 integrin blockade following intravitreal injection of its monoclonal antibody (Natalizumab) in an animal model.

Methods

Animals
Twelve New Zealand rabbits, weighing 1.5 to 2.0 kg each, were included in the study after obtaining due permission from Institute Animal Ethics Committee (870/IAEC/15). These rabbits were divided into three groups ($n = 4$) (Group A = 0.625 mg, Group B = 1.25 mg, and Group C = 2.5 mg natalizumab). All experimental procedures followed the ARVO guidelines for the use of animals in ophthalmic and vision research.

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Procedure

New Zealand rabbits (*n* = 4) in each group were anesthetized by an intramuscular injection using ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg) solution. Proparacaine (0.5%) ophthalmic solution was used as topical anesthetic agent. Natalizumab (α4-integrin blocker) powder for injection (Abbott Laboratories, Chicago, IL, USA) was reconstituted with sterile water for injection. Rabbit's eye was washed with 5% povidone–iodine ophthalmic solution, following which 0.625, 1.25, and 2.5 mg natalizumab solution was injected intravitreally in one eye (experimental eye) in each rabbit of each group, respectively.

The electroretinogram (ERG) study was carried out using a standardized protocol in our laboratory. Topical instillation of phenylephrine (2.5%) and tropicamide (0.5%) was done for dilating rabbit's pupil. The clinical examination was performed by fundus imaging and ERG recordings using MICRON III rodent imaging system enabled with LabScribe software (Phoenix Laboratory, USA). The baseline ERG was recorded prior to the intravitreal injection and then ERG recordings were performed on day 1, 7, and 21 after injection. Funduscopic examinations were performed in all animals till the 21-day period for signs of infection, inflammation, or toxicity. Post the last ERG recording, the rabbits of Group C (highest dose 2.5 mg) were sacrificed by an excess of carbon dioxide, and their retina was prepared for examination. All animals were evaluated prior to the experiment for any media opacities or retinal damage.

Intravitreal injection of natalizumab

All procedures were performed using standardized protocols under sterile conditions. Rabbits were restrained, and the ocular surface was anesthetized using 0.5% proparacaine hydrochloride ophthalmic solution. Prior to the intravitreal injection, eyes were washed with 5% povidone–iodine ophthalmic solution. A 30-gauge needle attached to a 1.0 ml tuberculin syringe was inserted into the vitreous cavity perpendicular to the sclera, approximately 1 mm posterior to the limbus through the pars plana route. The syringe was directed toward the center of the vitreous and the drug was then slowly injected. To avoid post injection drug reflux, sterile cotton-tip applicator over the injection site was applied immediately after the removal of the injection needle. Post injection, the rabbit eyes were instilled with topical antibiotics.

Electroretinogram

The ERG study protocol for evaluating the retinal toxicity was standardized in our laboratory according to the International Society for Clinical Electrophysiology of Vision (ISCEV) guidelines 2015. Scotopic ERG was recorded using MICRON III rodent imaging system (Phoenix Inc., USA) in the rabbits after adequate dark adaptation. Eyes of the rabbits were dilated using tropicamide 0.8% and phenylephrine 5%. Using artificial tears contact was made between the gold electrode (active) of the optics by placing it gently on the cornea, the reference electrode was placed on the forehead, and the tail of the rabbit was connected to ground electrode. The full-field light-evoked ERG response was obtained using the rabbit adaptor adjusted to the axial length. The retina was stimulated using a white light of 1 cds/m^2 intensity; the mean of 20 sweeps was taken for the calculation of the amplitude and latencies of "a" and "b" waves of the ERG using the LabScribe software (Phoenix Inc., USA).

The amplitude and latency were expressed in microvolt and millisecond, respectively.[6]

Light and ultramicroscopy studies

Ultramicroscopy studies were conducted using transmission electron microscope (TEM). The rabbits injected with the highest dose of natalizumab were sacrificed using an excess of carbon dioxide. The eyes were enucleated, and the eyeballs were fixed for 2 h at 4°C in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Small pieces of the retina were cut and post-fixed in 1% osmium tetroxide in phosphate buffer for 2 h at 4°C. The materials were dehydrated in cold-graded concentrations of acetone to propylene oxide and finally embedded in Epon 812. The sections were cut on a Leica UC7 ultramicrotome. Semi-thin sections were stained with toluidine blue and observed under a light microscope. Ultrathin sections were mounted on copper grids, double stained with a saturated solution of uranyl acetate in 50% alcohol for 15 min and with alkaline lead citrate for 15 min, washed and viewed with a FEI Tecnai™ G20 TEM (FEI, Eindhoven, The Netherlands).[7]

Statistical analysis

A commercially available statistical software package (GraphpadPrism ver. 5 for Windows, GraphPad Software, Inc., USA) was used to perform statistical analysis of the data. Data are presented as mean ± SEM. Intergroup analysis was performed using two-way analysis of variance with *post hoc* test (Bonferroni test) and a *P* ≤ 0.05 was considered as significant.

Results

Retinal imaging after intravitreal injection of α4β1 integrin blocker

After intravitreal injection with all three doses of natalizumab, the vitreous of the experimental eyes appeared clear. During the follow-up period, no changes were observed in the groups. Fundus imaging showed no signs of retinal detachment, media opacity, inflammation, vitreous hemorrhage, or optic atrophy. Fig. 1 represents the fundus images of natalizumab injected eyes: (A) Dose: 0.625 mg: baseline and 1, 7, and 21 days; (B) Dose: 1.25 mg: baseline and 1, 7, and 21 days; and (C) Dose: 2.5 mg: baseline and 1, 7, and 21 days.

Electroretinogram

The effect of low, intermediate, and high doses of natalizumab after intravitreal administration was assessed on the rabbit retina at day 0, 1, 7, and 21 by using ERG.

The "a" wave amplitude of the low, intermediate, and high doses of natalizumab is shown in Fig. 2a. No statistical difference was observed in the amplitude of low and intermediate groups at different time intervals. However, a significant fall in "a" wave amplitude was observed between day 0 and day 21 in the high-dose group (*P* = 0.0017).

The "b" wave amplitude of the groups at different time intervals are shown in Fig. 2b. The 'b' wave amplitude of high-dose natalizumab group was significantly reduced (*P* = 0.0117) at day 21 when compared to day 7 of this group. No difference in amplitude of "b" wave was observed in the low and intermediate dose groups of natalizumab at different time intervals.

The "a" wave and "b" wave latency of all experimental groups are shown in Fig. 3a and b, respectively. No significant difference in latencies was observed among the groups and also at different time intervals within a group.

Transmission electron microscopy

Electron microscopy of the retina was only performed on Group C (highest dose) animals, which showed changes on ERG. The normal ultrastructural features of rabbit retinal cells were consulted in the literature.[8] We did not observe any significant changes in the retinal pigment epithelium, photoreceptors, and outer nuclear layer. However, there were significant changes in the outer plexiform layer that showed degenerative synaptic ribbons and loss of synaptic vesicles in photoreceptor synaptic terminals [Fig. 4a]. The synaptic ribbons were either deformed or appeared smaller or partly dissolved into amorphous materials. A rough count through the outer plexiform layer revealed damage or disorganization in one-third of the total synaptic terminals present in a unit distance of 100 μm length, suggesting significant alterations in photoreceptor output terminals. Besides, there were appreciable changes in the inner nuclear layer that showed loss of essential organelles and vacuolation in bipolar neurons [Fig. 4b] with damaged (loss of cristae) and swollen mitochondria in them. However, the horizontal cells were apparently devoid of any such fine structural changes: their cytoplasm was replete with organelles as depicted in Fig. 4c. A mild, insignificant hypertrophy was observed in Müller cells that surrounded the disorganized bipolar cell somata.

Discussion

Adhesion molecules play a pivotal role in the recruitment of inflammatory cells to the sites of inflammation. It has been well studied that integrins are involved in making bridges between several adhesion molecules expressed on the vascular endothelial cells and immune cells. Among them, α4β1 integrin receptor molecules are primarily involved in the transfer of immune cells, namely lymphocytes into the site of inflammation.[9,10] Integrin α4β1 can mediate both cell–cell and cell–extracellular matrix adhesion by binding to either VCAM-1 or fibronectin.[2] Leucocyte migration across the endothelium is well known in uveitis. In noninfectious pan-uveitis, vitrectomy is one of the therapeutic options to remove the lodged lymphocytes in the vitreous, inflammatory debris, immune complexes, and autoantigens.[11] Intraperitoneally injected peptide-based small molecule inhibitors of α4 integrins have been reported to be therapeutically beneficial in experimental
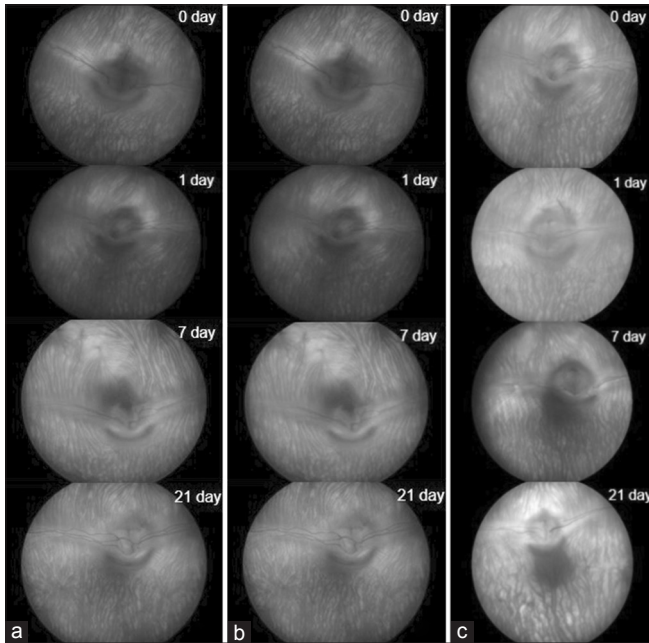

Figure 1: Representative images of the fundus of the natalizumab injected eye. (a) Dose: 0.625 mg: baseline and 1, 7, and 21 days; (b) Dose: 1.25 mg: baseline and 1, 7, and 21 days; and (c) Dose: 2.5 mg: baseline and 1, 7, and 21 days showing no significant morphological alterations in the fundus between different groups
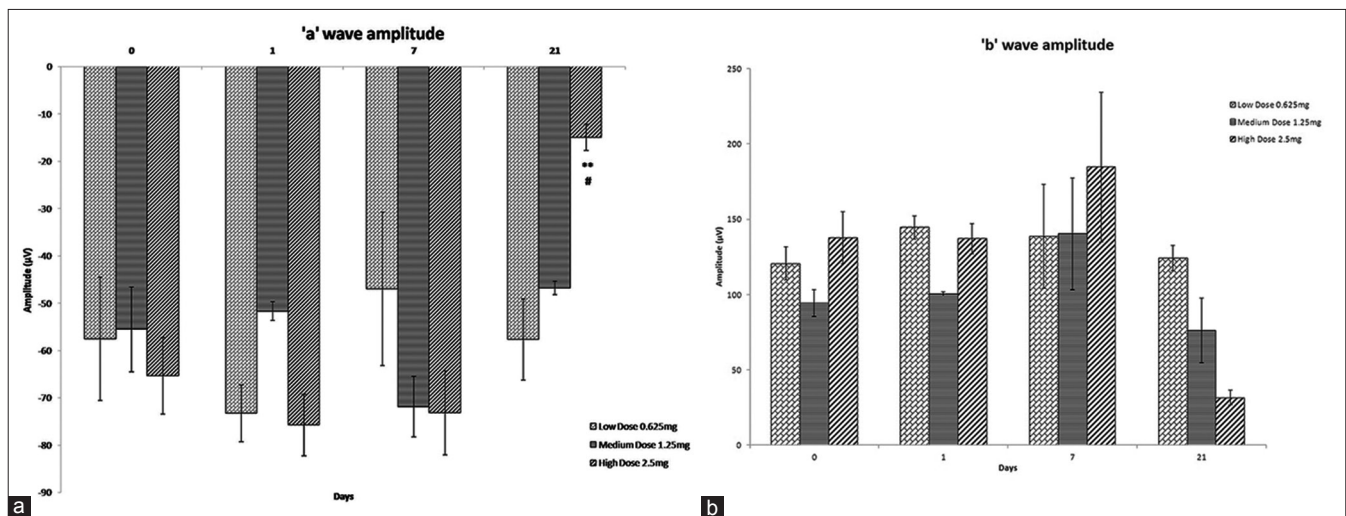
Figure 2: (a) Representative graph for "a" wave amplitudes. No statistical difference observed in amplitude of low and intermediate groups at different time intervals, but a significant fall in "a" wave amplitude was observed at day 21 for the high-dose group as compared to the baseline (*P = 0.0017). (b) Representative graph for "b" wave amplitudes. The "b" wave amplitude of high-dose natalizumab group was also significantly reduced (*P = 0.0117) at day 21 when compared to day 7 of its group. No difference in amplitude of "b" wave was observed in low and intermediate dose group of natalizumab
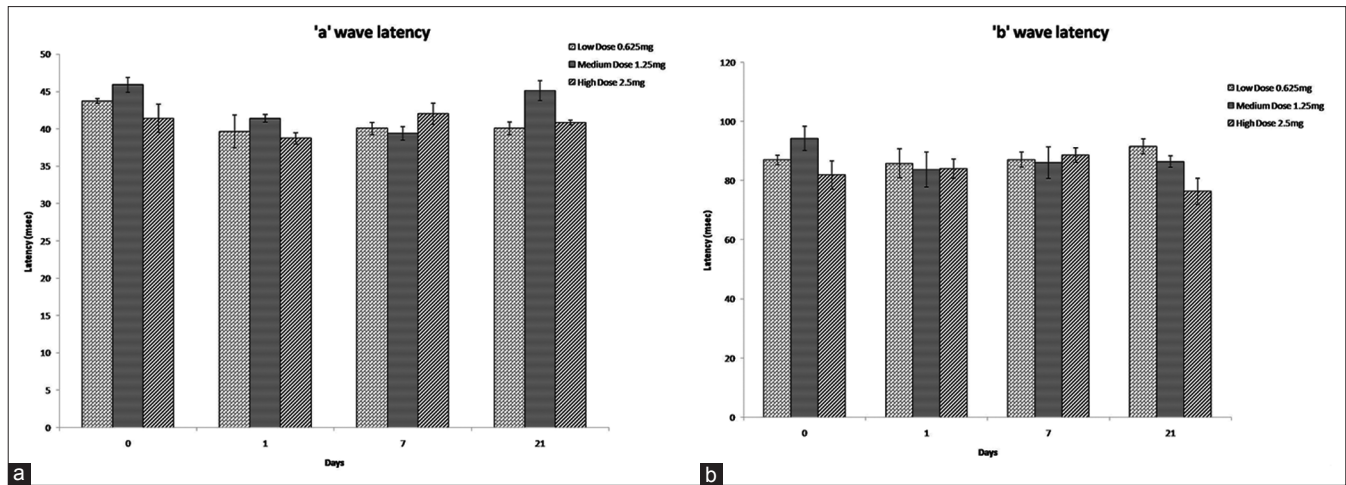
Figure 3: (a) Representative graph for "a" wave latency of all experimental groups showing no significant difference in latencies observed among the groups and at different time intervals within a group. (b) Representative graph for "b" wave latency of all experimental groups showing no significant difference in latencies observed among the groups and at different time intervals within a group
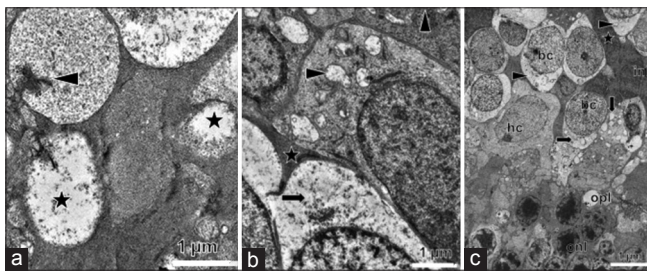
Figure 4: Electron micrographs of 2.5 mg dose group. (a) Disorganized synaptic ribbons (arrowheads) and depleted synaptic vesicles (stars) in photoreceptor synaptic terminals (b) Inner nuclear layer shows damage in bipolar cells, paucity (arrows), and disorganization of organelles, especially mitochondria (arrowheads). Star denotes Müller cell cytoplasm. (c) Another section showing damage in inner nuclear layer (inl), vacuolation (arrows), and paucity of organelles in bipolar cells (bc). Horizontal cells (hc) appeared normal in cytoplasmic content. Star denotes Müller cell cytoplasm

autoimmune uveitis.[1] Therefore, blocking the α4β1 integrin is expected to be beneficial as an adjunct therapy for pan-uveitis. In an inflamed eye the blood retinal barrier is compromised. Thus, even an intravitreally injected α4β1 integrin blocker may gain access to the α4β1 integrin adhesion molecules. Whether an intravitreally injected α4β1 integrin blocker would have clinical benefits is not within the scope of this paper. The expression of VCAM-1 in the prevascular glial cells and the internal limiting membrane has been documented in the autoimmune model of experimental uveitis.[12] Considering the anatomical presence of integrin adhesion molecules in the retina,[3] studying blocker-induced functional and structural changes of the retina was felt important.

Natalizumab is the first drug of its kind developed under the class of selective adhesion molecule inhibitors. It has been approved by Food and Drug Administration for the treatment of multiple sclerosis (MS) and Crohn's disease.[13] By binding to α4β1 integrin receptors expressed on lymphocytes, it inhibits the interaction of these receptors with endothelial receptors such as VCAM-1 and mucosal addressin cell adhesion molecule-1, subsequently arresting their translocation and

cellular inflammation.[14] Natalizumab has been reported to help in attenuating the inflammatory response in relapsing MS, inflammatory bowel disease, and ulcerative colitis.[10,14,15]

α4β1 blockade using monoclonal antibody (natalizumab) has been reported to be involved in preventing the recruitment of immune cells into the parenchyma of the brain across blood–brain barrier. Natalizumab has been reported to inhibit the migration of inflammatory cells across the blood–brain barrier in MS.[16,17]

A similar effect on the blood–ocular barrier may help in treating intraocular inflammatory disorders. Therefore, there might be a possibility of using natalizumab as a new therapeutic modality for the treatment of noninfectious pan-uveitis. To enable this objective and establish safety of an intravitreal injection of this drug, three different doses of natalizumab (0.625, 1.25, and 2.5) were injected intravitreally in rabbits and further evaluation was performed post dosage at day 1, 7, and 21. In this study, we did not find any functional changes up to 21 days on injecting 0.625 and 1.25 mg of natalizumab.

Anterior and posterior ocular examination using MICRON-III revealed no signs of retinal detachment, media opacity, inflammation, vitreous hemorrhage, or optic atrophy at all the dose levels. Whereas, at a higher dose of 2.5 mg of natalizumab, we observed significant changes in ERG. Ultrastructural analysis using a TEM revealed the presence of intact retinal pigment epithelium, photoreceptor layer, and outer nuclear layer, whereas degenerative changes were observed in the outer plexiform layer and inner nuclear layer at the highest dose of the drug. This analysis has also revealed the loss of synaptic vesicles and synaptic ribbon integrity in photoreceptor synaptic terminals along with a marked loss of cytoplasmic organelles from bipolar neurons at the highest intravitreal dose of natalizumab in this study.

Natalizumab-induced EM changes were well correlated with the altered "a" and "b" wave ERG amplitude seen at day 21 of the highest dose (2.5 mg) of natalizumab. The reduction in amplitude of "a" and "b" waves at day 21 in the highest dose group indicates that there were changes in the photoreceptor synaptic terminals and inner nuclear layer.

There are relatively few reports on the documented intravitreal use of monoclonal antibodies in uveitis. In recently published studies, Hamam *et al.* (2016) have reported a pilot study on the efficacy of intravitreal adalimumab at a dose of 1.5 mg in patients suffering from active noninfectious uveitis.[18] The intravitreal adalimumab showed promising results by decreasing the macular edema and improving the best corrected visual acuity in the majority of eyes.[19] Manzano *et al.* (2011) in their study reported that intravitreally injected adalimumab in rabbit eyes at a high dose of 10 mg exhibited mild clinical changes as well as ERG amplitude changes reflecting early toxicity.[20] As compared to tumor necrosis factor-α blockade by adalimumab (showed ERG changes at 10 mg), the present study of α4β1 integrin receptor blockade by natalizumab (showed ERG changes in 2.5 mg) showed a four times lower threshold for toxicity. This could be due to its physiological relevance in the retina.

There are few case reports of acute retinal necrosis following systemic use of natalizumab.[21-23] These have been ascribed to viral causes secondary to immunosuppression. We found retinal toxicity (not related to infection) following intravitreal injection at a dose of 2.5 mg in our study. Thus, the possibility of development of a secondary viral retinitis and judicious use of a low dose of natalizumab would have to be considered in further studies.

Conclusion

This study for the first time reports the ocular toxicity profile of intravitreally injected α4β1 monoclonal blockade using antibody (natalizumab) in rabbit eyes. Significant functional and ultrastructural changes were seen in the retina of rabbits at a dose of 2.5 mg, whereas no toxicity was seen at a dose of 0.625 and 1.25 mg. It remains to be determined whether an intravitreal dose of natalizumab up to 1.25 mg is able to neutralize migrating lymphocytes into neural retina or not. Therefore, further studies using uveitic animal models are required to confirm the safe dose and clinical utility of α4β1 monoclonal blockade as an adjunctive therapy in pan-uveitis.

Conflicts of interest

There are no conflicts of interest.

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