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## Serum-derived immunoglobulins alter amyloid $\beta$ transport across a blood-brain barrier *in vitro* model

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Since passive immunization with serum-derived immunoglobulins (intravenous immunoglobulins) showed several positive effects in some patients with Alzheimer's disease (AD), intravenous immunoglobulins (IVIg) are discussed as a possible treatment option. IVIg, an antibody product derived from human plasma, contains natural antibodies against amyloid  $\beta$  (A $\beta$ ) peptide. Until now it is not known, how IVIg interferes with pathogenesis in AD, but several proposed mechanisms are in discussion. Receptor types which are involved in transport processes at the BBB are LRP, RAGE and hFcRn. We were looking for an *in vitro* BBB model expressing these receptors and studied the alteration of transport of A $\beta$  peptides across this model under the influence of immunoglobulins. Cell line ECV304 was found to be suitable for our experiments. We found evidence for involvement of an improved clearance of A $\beta$  across the BBB as well as a decreased A $\beta$  influx from blood to the brain probably following complex formation of immunoglobulins with free A $\beta$  in the periphery. Furthermore, we were able to confirm the activity of IVIg preparations which acted the same way but showed slightly less efficacy in comparison to monoclonal anti-A $\beta$  antibodies. Based on these results we suggest multiple mechanisms responsible for the efficacy of immunotherapy in Alzheimer's disease.

### 1. Introduction

Alzheimer's disease (AD) is a neuropathological degenerative condition with ongoing decline in cognitive functions and memory. It is one of the most common causes of dementia. Pathological hallmarks are neuronal cell loss, neurofibrillary tangles, senile plaques and cerebrovascular amyloid deposits as well as neuroinflammation.

The major constituents of senile plaques and deposits are amyloid  $\beta$  (A $\beta$ ) peptides. There are different forms of the peptide and these differ in length and in their neurotoxic properties. Amyloid  $\beta$  1-42 (A $\beta$ (1-42)) is the major component of senile plaques and the more amyloidogenic form of the peptide (Tanzi et al. 2004), whereas soluble amyloid  $\beta$  1-40 (A $\beta$ (1-40)) is the major form of circulating A $\beta$  peptides in the blood (Marco and Skaper 2006). In Alzheimer's disease the concentration of both peptides in the CNS is elevated and therefore they are common targets of AD therapy.

The mechanism by which A $\beta$  levels in the CNS are increased in AD is not clarified up to now. One suggestion is an increased endogenous secretion of the peptide by neurons in contrast to disturbances in the clearing mechanisms of the peptide out of the brain and accumulation and deposition in brain parenchyma (Deane et al. 2004).

In this context, the blood-brain barrier (BBB) possesses different receptors which interact with A $\beta$  peptides and maintain the transport of these peptides from brain to blood and vice versa. The low-density lipoprotein receptor-related protein (LRP) is nowadays known to mediate the clearance of A $\beta$  peptides out

of the brain into the plasma via transport across the BBB. In contrast to LRP, the receptor for advanced glycation end products (RAGE), a multiligand receptor in the immunoglobulin superfamily, binds soluble A $\beta$  peptides in the periphery and transports them into the CNS (Zlokovic 2004). Except for these transport mechanisms, A $\beta$  is not able to pass the blood-brain barrier in healthy individuals (Clifford et al. 2007). In Alzheimer patients and in transgenic animal models of AD there is an imbalance in the expression of LRP and RAGE at the blood-brain barrier. RAGE is upregulated whereas LRP is downregulated, which favors the accumulation of A $\beta$  peptides in the brain (Shibata et al. 2000).

Recently, another receptor is discussed to play an important role in A $\beta$  transport mechanisms at the BBB. The Fc receptor neonatal (FcRn) (Deane et al. 2005; Schlachetzki et al. 2002), which is a Fc gamma receptor, is usually involved in the placental transfer of maternal IgG into the fetal circulation, as well as in the regulation of serum levels of IgG. It is composed of two subunits: an integral membrane glycoprotein, the alpha-chain (MW ~ 45 kDa), and  $\beta$ 2-microglobulin. The FcRn is functionally and structurally distinct from the other Fc gamma receptors and is an MHC class I related receptor (Simister and Mostov 1989).

Schlachetzki et al. (2002) found evidence for the expression of the FcRn at the brain microvascular endothelium and the choroid plexus epithelium and suggested it to play a crucial role in antibody-based therapies in the case of Alzheimer's disease. The BBB FcRn mediates transport of IgG molecules only in brain to blood direction (Zhang and Pardridge 2001).

Under physiological conditions IgG transport across the BBB is restricted (Bouras et al. 2005) and how antibodies gain access to the brain is unclear. Some antibodies use extracellular pathways to enter the brain (Banks et al. 2002) or undergo adsorptive-mediated transcytosis (Bickel 1995). However, once they are in the brain, anti-A $\beta$ -antibodies bind to soluble A $\beta$  peptides and these A $\beta$ -IgG complexes can be eliminated through the BBB via the FcRn (Deane et al. 2005). This provides one possible explanation how immunotherapy in AD is able to enhance clearance of brain A $\beta$  peptides.

Both active (Janus et al. 2000; Morgan et al. 2000; Schenk et al. 1999) and passive (Bacskai et al. 2001; Bard et al. 2000; Bredza et al. 2005) immunization in mouse models of AD resulted in significant reduction in plaque burden, neuritic dystrophy and gliosis and improved cognitive performance and functional outcome of the disease. Since active immunization approaches resulted in some severe adverse inflammatory symptoms in the brains of volunteers (Schenk 2002), the importance of passive immunotherapy increased regarding the issue of safety.

For passive immunization specified monoclonal antibodies (Bard et al. 2000; Bredza et al. 2005) as well as intravenous immunoglobulins (IVIG) (Dodel et al. 2004; Hack and Scheltens 2004; Istrin et al. 2006) are under investigation. The effectiveness of serum-derived or intravenous immunoglobulins (IVIG) has been evaluated in a small number of patients with AD and they were shown to stabilize cognitive decline and improve cognition in treated patients (Dodel et al. 2004; Weksler et al. 2004). IVIG preparations, which are prepared from plasma of healthy individuals, contain natural antibodies against A $\beta$  peptides (Dodel et al. 2002; Du et al. 2003). These antibodies are able to reduce neurotoxic effects and fibril formation of A $\beta$  peptides (Du et al. 2003). Similar results were shown by Istrin et al. (2006), who studied the interaction of A $\beta$  peptides and IVIG *in vitro* using a microglial cell line. They found that IVIG increased cellular tolerance to A $\beta$  aggregates, enhanced microglial migration and mediated phagocytosis of A $\beta$  peptides. Dodel et al. (2004) investigated the impact of IVIG on A $\beta$  levels in CSF and serum of AD patients and confirmed a reduction of total A $\beta$  levels in the brain.

The aim of the presented study was to investigate transport mechanisms of A $\beta$  peptides at the BBB using an already established *in vitro* BBB model based on the human cell line ECV304 cultured in astrocyte conditioned medium (ACM). Under the influence of glial factors, BBB properties are inducible in this model, as the upregulation of the glucose transporter GLUT-1 and  $\gamma$ -glutamyl transpeptidase. Additionally, it exhibits increased transendothelial electrical resistance (TEER) and restricted permeability (Neuhaus et al. 2008). This indicates that ECV304 layers are able to form tight junctions (Kiessling et al. 1999) and thus they can be used for BBB permeability studies (Garberg et al. 2005; Neuhaus et al. 2008).

In regard to examine A $\beta$  transport, the cells should exhibit the main receptors interfering with transport processes at the BBB. Therefore, ECV304 cells were analyzed for the expression of RAGE, LRP and human FcRn.

After assessing the transport rate of A $\beta$  peptides alone, it should be investigated whether immunoglobulins (monoclonal anti-A $\beta$ - antibodies or IVIG) are able to alter A $\beta$  permeation across the *in vitro* BBB in both directions (blood to brain and brain to blood).

We found that both immunoglobulin preparations interfered with A $\beta$  transport processes, whereas monoclonal antibodies exerted a stronger impact than IVIG regardless of transport direction. Influx of A $\beta$  peptides from blood to the brain compartment of the *in vitro* system was markedly decreased when antibodies were added. Moreover, we were able to show an enhanced clearance of A $\beta$  peptides, when transport experiments were per-

formed in the opposite direction, from brain to blood. These results are in concordance with the often stated “sink” hypothesis, which suggests an interaction of A $\beta$  peptides with specific antibodies in plasma creating a concentration gradient across the BBB and therefore promoting efflux of A $\beta$  peptides from brain into blood and on the other hand lead to a reduction of A $\beta$  permeation into the brain because of a limited present amount of free A $\beta$  peptides. Additionally, as already shown in former studies (Bard et al. 2000; Deane et al. 2005), we propose an active receptor mediated clearance of A $\beta$  peptides after passover of circulating antibodies across the BBB.

## 2. Investigations and results

In order to study the transport rate of A $\beta$  peptides across the blood-brain barrier the ECV304 BBB *in vitro* model was used.

### 2.1. Detection of LRP, RAGE and hFcRn

To investigate whether the ECV304 BBB *in vitro* model is suitable to study the transport of A $\beta$  peptides, the expression of the three main receptors interfering with these transport processes at the BBB was assessed using immunofluorescence and western blot analysis.

Indirect immunofluorescence was used to detect LRP, RAGE and hFcRn. Cells were grown on glass coverslips until confluence, permeabilized with cold methanol, washed and incubated with the primary and afterwards the FITC-conjugated secondary antibody solution. For the detection of LRP and RAGE monoclonal primary antibodies were used and for hFcRn a polyclonal affinity-purified anti-hFcRn antibody (Leitner et al. 2006) was used. In ECV304 cells, a bright fluorescent staining was consistently present within the cytoplasm regarding especially the receptor types LRP and RAGE (Fig. 1A and B). When staining hFcRn, the labelling mainly appeared as fluorescent dots, which leads to the suggestion of an association of that receptor type with vesicular structures (Fig. 1C and D). This is consistent with earlier observations concerning expression of FcRn in endothelial as well as epithelial cells (Borvak et al. 1998; Ellinger et al. 1999). A similar staining pattern was obtained when staining BeWo/hFcRn cells. BeWo/hFcRn is a Trophoblast-derived cell line transfected with hFcRn (Ellinger et al. 2005) and was used as a positive control for hFcRn expression (Fig. 1E).

No labeling was obtained in control experiments in which the primary antibodies were omitted (data not shown).

To verify these results, another technique to control the expression of LRP, RAGE and hFcRn in ECV304 cells was applied. For this purpose SDS-Page and subsequent Western blotting was performed. For the detection of hFcRn polyclonal anti-hFcRn antibodies were used. As shown in Fig. 2A, the antibody reacted with a protein of about 45 kDa in ECV304 and BeWo/hFcRn cells. In ECV304 cells another band with somewhat lower molecular weight was detected, probably representing either a degradation product or a precursor of hFcRn (Ellinger et al. 2005).

When applying anti-RAGE antibodies an immunoreactive band was detected in ECV304 cells at about 43 kDa, corresponding to the molecular weight of the receptor protein (Fig. 2B). No positive control was available. Tubulin was used as a loading control and bands appeared at the molecular weight of about 55 kDa (Fig. 2A and B).

ECV304 cells were found to be negative in LRP expression using western blotting because no band could be detected at the corresponding molecular weight of the receptor protein (data not shown).

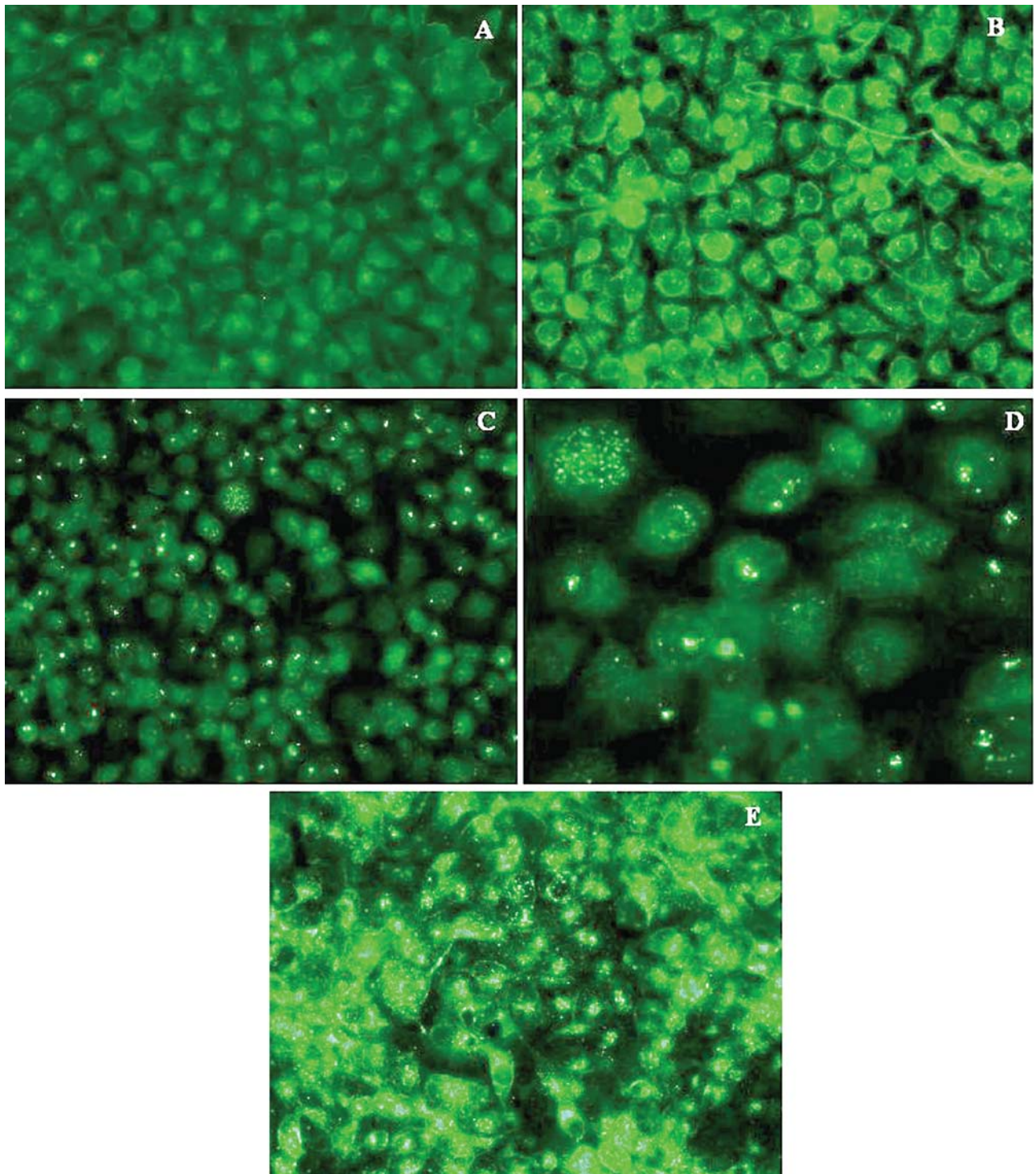


Fig. 1: Detection by immunofluorescence of LRP (Fig. 1A), RAGE (Fig. 1B) and hFcRn (Fig. 1C and D) in ECV304 cells. Transfected BeWo/hFcRn cells (Ellinger et al. 2005) were used as a positive control for the expression of hFcRn (Fig. 1E). No positive control was available for the expression of RAGE and LRP. Control staining where the first antibody was omitted resulted in no detectable staining (images not shown)

In conclusion, the expression of RAGE and hFcRn was confirmed using two independent techniques, immunofluorescence and western blotting. LRP was not detected when western blot analysis was applied, but its expression in ECV304 could be confirmed with the slightly more sensitive immunofluorescence technique, which indicates a lower expression level of LRP than of the other two receptor types in ECV304 cells.

## 2.2. $A\beta(1-40)$ transport experiments

In order to study transport properties of  $A\beta$  peptides across the *in vitro* BBB model, the ECV304 cell layers were cultured in

PBMEC growth medium for 14 days to achieve a tight barrier, according to the results published by Neuhaus et al. (2008). Permeability of  $A\beta(1-40)$  was investigated over a time period of 5 h (sampling after 60, 120, 300 min), and the flux to the acceptor compartment was analyzed by ELISA.

A slight but no significant increase of permeability over time was detectable. Thus, it was confirmed, that the integrity of the cell layers was stable over the experimental time (Fig. 3).

Experiments were performed bidirectionally, either from the apical to the basolateral (influx studies) or the basolateral to the apical compartment (efflux studies).

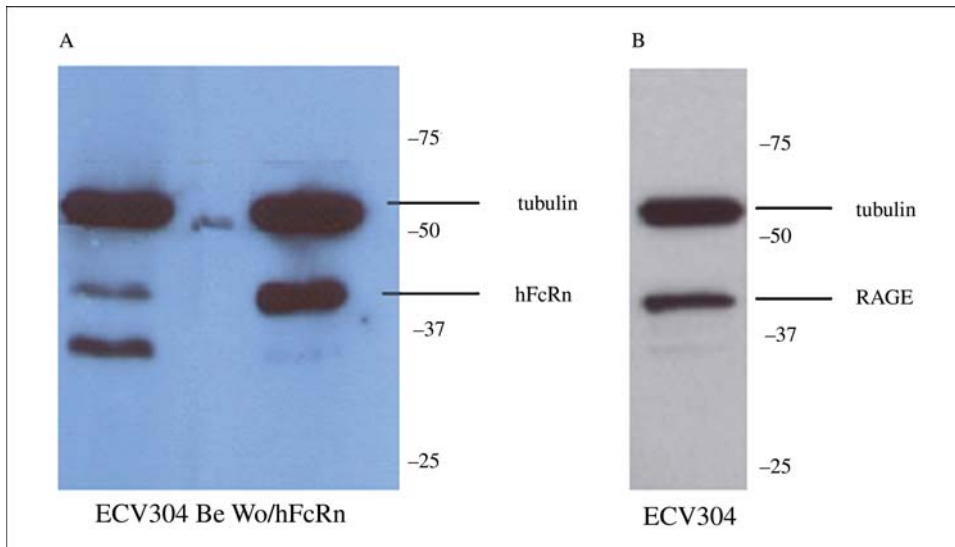


Fig. 2: Western blot analysis of hFcRn and RAGE in ECV304 cells. Fig A: Transfected BeWo/hFcRn cells (Ellinger et al. 2005) were used as a positive control for the expression of hFcRn and a band was detected in ECV304 at the same MW as shown in BeWo/hFcRn Fig B: No positive control was available for the expression of RAGE, but a immunoreactive band appeared at about 43 kDa, corresponding to the molecular weight of the receptor protein

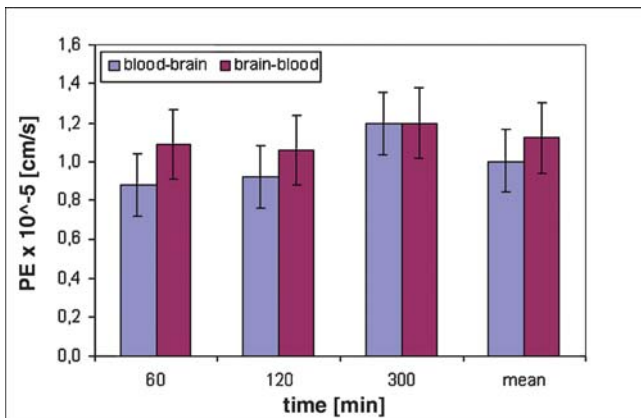


Fig. 3: Permeability coefficients (Pe) of Aβ(1-40) across the blood-brain barrier ECV304 *in vitro* model either in blood-to-brain or brain-to-blood direction. Permeability of Aβ(1-40) to the acceptor compartment was investigated over a time period of 5 h and Pe after 60, 120, 300 min as well as the mean permeability coefficient were calculated (mean ± SD, n = 6)

The difference between influx and efflux of Aβ(1-40) was not significant and the mean permeability coefficient (Pe) was  $1,00 \pm 0,16 \times 10^{-5}$  cm/s from blood to brain and  $1,12 \pm 0,18 \times 10^{-5}$  cm/s in the opposite direction (Fig. 3).

In order to see whether immunoglobulins are able to interfere with Aβ transport, the above mentioned experiments were performed in the same manner but with addition of either IVIG or monoclonal anti-Aβ-antibodies. For this reason, IVIG (5 μM, final concentration) or the monoclonal anti-Aβ antibody 6E10 (100 nM, final concentration) were added to the apical side (blood side) of the *in vitro* system at the same time as Aβ(1-40). Aβ(1-40) was either applied together with the immunoglobulins at the apical side of the system, or when performing efflux experiments, immunoglobulins were added to the apical and Aβ(1-40) to the basolateral compartment. Then Aβ transport studies were performed as already described.

As a control, Aβ(1-40) permeability across cell layers without addition of immunoglobulins was determined with each transport experiment and the mean relative differences of Aβ(1-40) permeation coefficients were calculated (Fig. 4). These experimental settings resulted in significant alterations in Aβ(1-40) transport, showing enhanced clearance of Aβ peptides out of the brain and a decline in Aβ blood to brain transport. In detail,

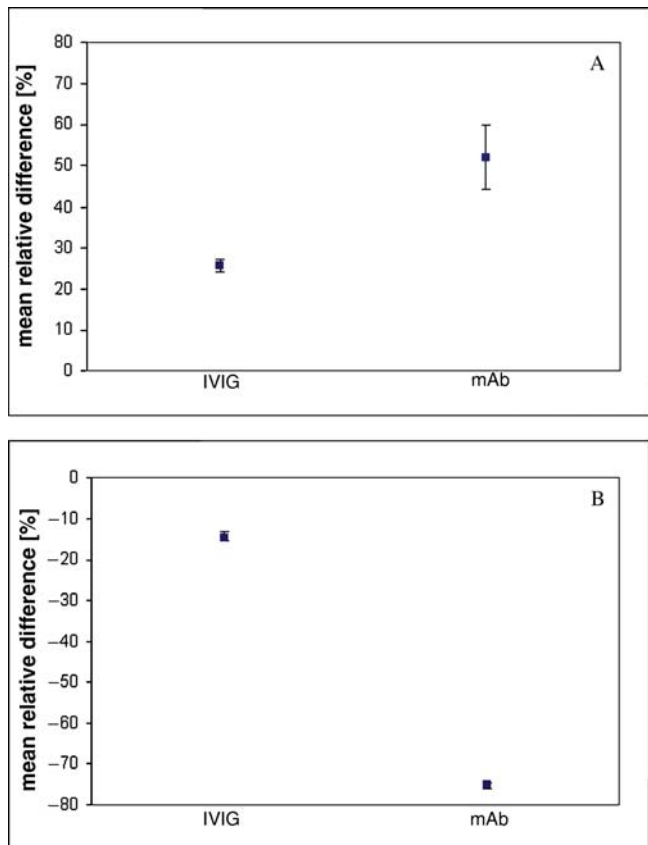


Fig. 4: Mean relative difference of Aβ(1-40) permeation alone in comparison to permeation after co-incubation with either Intravenous Immunoglobulins (IVIG) or monoclonal anti-Aβ(1-40) antibodies (mAb) across the blood-brain barrier ECV304 *in vitro* model in brain-to-blood (Fig A) and blood-to-brain (Fig B) direction (mean ± SEM, n = 3). Fig A shows a high increase in Aβ(1-40) transport out of the brain after addition of immunoglobulins. The opposite was observed when transport was performed in blood-to-brain direction, where inward transport of Aβ(1-40) was declined after addition of immunoglobulins

Fig. 4A shows an increase in Aβ(1-40) permeation from brain to blood of  $25.76 \pm 2.67\%$  when IVIG was added. With the monoclonal antibody 6E10 the permeability was elevated by  $52.20 \pm 13.48\%$ . The opposite phenomenon was observed when Aβ(1-40) blood to brain transport was measured. Here the

immunoglobulins caused a reduction in A $\beta$ (1–40) permeation across the *in vitro* BBB. IVIG addition resulted in a decline of  $14.36 \pm 1.83\%$  and the application of the monoclonal antibodies in a reduction of  $75.25 \pm 1.29\%$ , respectively (Fig. 4B).

### 3. Discussion

Immunotherapy is nowadays a promising approach to reverse cognitive signs and diminish plaques, which are consequence of the degenerative Alzheimer's Disease (AD), in transgenic animals as well as AD patients (Hack and Scheltens 2004; Janus et al. 2000; Schenk et al. 1999; Weksler et al. 2004). Two strategies are under investigation, the active and the passive immunization approach. Active immunization, where A $\beta$  peptides are administered to generate an anti-A $\beta$  antibody response in treated individuals, appeared to be problematic because of safety concerns about autoimmune reactions in humans (Orgogozo et al. 2003). This phenomenon was not observed after passive immunization using generated monoclonal anti-A $\beta$ -antibodies or human intravenous immunoglobulins (IVIG). Another advantage of passive immunization is that treatment can be discontinued if needed (Sigurdsson et al. 2002) and in case of IVIG, a safe use has been confirmed in treating many immune and inflammatory diseases since a long time (Jordan et al. 2009).

For this reason, we investigated the ability of IVIG as well as monoclonal anti-A $\beta$  antibodies to interfere with A $\beta$  transport processes at the BBB. We were able to confirm a strong influence of both types of immunoglobulins on A $\beta$  brain levels, using a BBB *in vitro* model expressing the three main receptors regarding A $\beta$  transport (LRP, RAGE, hFcRn).

That antibodies have the efficacy to reduce the amyloid burden in the brain was confirmed in several other studies (Bacsakai et al. 2001; Bard et al. 2000; Janus et al. 2000). Bacsakai et al. (2001) who used a novel technique, the *in vivo* multiphoton microscopy, which allows visualization of distinct brain structures in living anesthetized mice, applied anti-A $\beta$ -antibodies directly to the cortex of these transgenic AD mice and showed a remarkable clearing of amyloid-deposits within a few days.

How antibodies might act in reducing the amyloid level in the brain and how they get access into the CNS is not clarified by now but there are several theories. The main proposed mechanisms are either a direct interaction of immunoglobulins and A $\beta$  peptides in the brain followed by rapid clearance of the complex across the BBB or an interaction of the antibodies with circulating A $\beta$  peptides in the blood and so prevent them from entering the brain. A reduced influx of A $\beta$  after administration of specific immunoglobulins was confirmed in a couple of studies (Banks et al. 2005; Deane et al. 2005; Pan et al. 2002).

This is in concordance with our results, which showed a diminished transport rate of A $\beta$ (1–40) across the *in vitro* BBB in blood-to-brain direction after simultaneous application of anti-A $\beta$  antibodies to the blood compartment of the *in vitro* system. This effect was significantly high when monoclonal antibodies were used, but a measureable activity was also exerted when using IVIG.

Whether circulating antibodies, normally restricted by the blood-brain barrier, are able to enter the brain or not, is of great importance regarding the hypothesis of facilitated A $\beta$  efflux by antibody entry into the CNS, binding of A $\beta$  within the CNS and efflux of the ligand–antibody complex from brain to the blood. Banks et al. (2002) suggested that the ability of antibody to first enter and then exit the brain would provide an effective mechanism for removing ligands from the brain.

The study showed that antibodies directed against A $\beta$  peptides crossed the BBB at a very low rate by way of the extracellular

pathways, but in amounts that are therapeutic for other substances (about 0.11% of an intravenous dose by 1 h). Over time the role of efflux mechanisms increased but the antibody was still detectable 3 days after administration.

A passive immunization approach in a mouse model with AD-like amyloid pathology supported both mechanisms and at the one hand showed prevention of blood borne A $\beta$  transport across the BBB into the brain and at the other hand resulted in increased clearance of brain A $\beta$  to the blood after systemic antibody administration (Deane et al. 2005). This is in agreement with our *in vitro* results, where immunoglobulins based at the blood side of our model system enhanced A $\beta$  efflux across the cell layer.

Deane et al. (2005) demonstrated that IgG-assisted A $\beta$  efflux was mainly mediated via the neonatal Fc receptor (FcRn), in contrast to LRP clearance, which decreased with aging. These findings support the use of our *in vitro* model, which expresses LRP at a lower extent than the human FcRn and therefore reflects similar conditions of receptor expression at the BBB in Alzheimer's disease *in vivo*.

In contrast to our results, another *in vitro* approach could not find evidence that antibodies inhibit reuptake of A $\beta$  peptides which has before been effluxed from brain (Banks et al. 2005). Here a murine BBB *in vitro* model using freshly isolated mouse brain endothelial cells was employed. The expression pattern of FcRn, LRP and RAGE was not tested. It can be suggested it was altered due to the isolation procedure.

Our model, although cells are not of cerebral origin, clearly reveals advantage over the above mentioned murine model, because it is of human origin and shows an expression pattern of the main A $\beta$  related transporters similar to that seen in Alzheimer diseased brains, which means upregulation of RAGE, downregulation of LRP and expression of human FcRn.

In summary, the present work introduces an already known *in vitro* model (Neuhaus et al. 2006; Youdim et al. 2003) which might be useful in studying A $\beta$  transport processes across the BBB *in vitro* in order to discover detailed mechanisms and participation of specific transporters. Based on our results, for efficacy of Alzheimer's immunotherapy we suggest an assumption of multiple mechanisms, involving an improved clearance of A $\beta$  across the BBB as well as a decreased A $\beta$  influx from blood to the brain following complex formation of immunoglobulins with free A $\beta$  in the periphery.

Furthermore, we were able to confirm the activity of IVIG which acted the same way but showed slightly less efficacy in comparison to monoclonal anti-A $\beta$  antibodies. In consideration of safety, IVIG might provide a useful and rather secure vaccination strategy in AD because it is widely used in other indications and an assured safety profile is established.

## 4. Experimental

### 4.1. Materials

Iscove's modified Dulbecco's medium (IMDM), Ham's F-12, newborn bovine serum, L-glutamine and penicillin/streptomycin were obtained from Invitrogen Life Technologies (Gibco, Carlsbad, CA, USA). Amphotericin B, transferrin and gelatin were purchased from Sigma (St. Louis, MO, USA). Heparin was purchased from MP Biomedicals (Irvine, CA, USA) and Collagen from ICN Biomedicals Inc. (Aurora, OH, USA). Transwell<sup>®</sup> inserts (12-well, PTFE-membrane 0.4  $\mu$ m) and culture plates (Falcon, Bedford, MA, USA) were obtained from Costar and BD Biosciences. A $\beta$ (1–40) and A $\beta$ (1–42) peptides were purchased from American Peptide. Acrylamide, ammonium persulfate, DTT research grade, TEMED research grade, TRIS research grade and glycine anal. Grade was obtained from Serva Electrophoresis GmbH (Heidelberg, Germany). Acetonitrile was purchased from Malinchröd Baker B.V. (Deventer, Holland) and molecular weight standard (Precision Plus Protein<sup>™</sup>) from Biorad Laboratories (Hercules, CA, USA).

#### 4.2. Antibodies

Anti-RAGE (rabbit polyclonal) and anti-LRP (5A6, mouse monoclonal) antibodies were purchased from Abcam (UK). Secondary FITC-conjugated goat anti rabbit IgG and goat anti-mouse IgG were obtained from Invitrogen (Vienna, Austria) and secondary HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Santa Cruz and purchased from Szabo-Scandic (Vienna, Austria).

A rabbit polyclonal anti-hFcRn antibody was kindly provided from the Medical University of Vienna, Austria (Leitner et al. 2006). A monoclonal anti-A $\beta$  antibody 6E10, which recognizes the amino terminus of A $\beta$ , was purchased from Signet (SIG 39320-99). IVIG was obtained from Baxter (Vienna, Austria) as Gammagard liquid.

#### 4.3. Cell culture

The ECV304 cell line was purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). The rat glioma cell line C6 was obtained from the German Cancer Research Center (DKFZ, Heidelberg, Germany). C6 cells were used to produce astrocyte conditioned medium (ACM). They were grown in 175 cm<sup>2</sup> tissue flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) and maintained in the so-called C6-medium, which consisted of IMDM and Ham's F-12 (1:1 mixture), 7.5 vol % new born calf serum, 1.0 vol % penicillin/streptomycin (10<sup>4</sup>U/ml), 7 mM L-glutamine, 5 mg/mL transferrin, 0.5 U/mL heparin and 0.25 mg/mL amphotericin B. ECV304 cells were grown in gelatin coated 25 cm<sup>2</sup> tissue culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) and maintained in PBMEC medium, which consists of C6 medium mixed in equal amounts with ACM, which was obtained from the supernatant of culturing C6 cells (Neuhaus et al. 2006). All cells were cultured at 37 °C in a 95% humidified atmosphere containing 95% air and 5% CO<sub>2</sub>. Subcultivation was performed via trypsinisation every 4 to 5 days.

#### 4.4. Immunofluorescence

Cells were seeded on cover slips (diameter 10–13 mm) and grown for 5–10 days until they reached confluence in PBMEC growth medium.

Thereafter, cells were fixed with –20 °C methanol for 20 min on ice and then washed 3 times with PBS. For rehydration cells were incubated in PBS for 20 min at RT.

Afterwards cells were incubated with the primary antibody solution for 1 h at 37 °C (anti-RAGE, anti-LRP or anti-hFcRn diluted 1:100 in blocking buffer, 1% BSA in PBS). Cell layers were washed with PBS three times and then incubated with a FITC-conjugated secondary antibody (anti-mouse antibody for LRP or anti-rabbit antibody for hFcRn and RAGE, 1:100 diluted in blocking buffer) for 30 min at 37 °C.

After washing with PBS, cells were embedded using Gel Mount<sup>TM</sup> Aqueous Mounting Medium (Sigma) and viewed under the microscope (Nikon Eclipse 50i microscope).

#### 4.5. SDS-PAGE and Western blotting

In brief, samples were electrophoresed on 10% SDS gels and transferred onto PVDF (polyvinylidene difluoride) membranes using a semi dry blotter at 50 mA/gel for 1 h. PVDF membranes were washed twice with aqua bidest. and blocked with 5% dry milk powder in TBS (25 mM Tris-HCl, pH 7.8, containing 145 mM NaCl) and Tween 20 0.1% at RT for at least 1 h. After washing the membranes with TBS-T (TBS and 0.1% Tween20) three times, they were incubated with the primary antibody solution for 1 h at RT (anti-LRP 1:1000, anti-RAGE 1:1000 and anti hFcRn 1:500 in milk powder solution). Blots were washed, followed by incubation with a secondary HRP-conjugated goat-anti-rabbit (1:10000) or goat-anti-mouse antibody (1:5000) for 1 h at RT. Thereafter, blots were washed again using TBS-T and were developed using the ECL plus western blotting detection system (Amersham Bioscience).

#### 4.6. A $\beta$ (1–40) quantification

Human  $\beta$  Amyloid 1–40 Immunoassay Kits were purchased from Biosource and performed according to the manufacturer's instructions. Reaction products were quantified using a spectrophotometer (Platereader 400ATX, SLT Labinstrument Austria) measuring the difference in absorbance at 450 nm. All ELISA detections were conducted in duplicate.

#### 4.7. Transport studies

ECV304 cells were seeded at a density of ~83 000 cells/cm<sup>2</sup> in collagen coated 12-well Transwell<sup>®</sup> inserts and grown until they built up a confluent cell layer with appropriate barrier properties.

This was controlled by assessing the TEER every 2 to 3 days after seeding. TEER measurement was performed with a Millipore Millicell Electrical

Resistance System (ERS, Millipore, Vienna, Austria) after changing the medium. Additionally, TEER of coated inserts without cells was also determined. These blank values were subtracted from cell values to calculate the TEER of the cell layer only (Neuhaus et al. 2006).

Transport studies were performed on day 14 after seeding. At this time point cell layers showed sufficient barrier properties according to Neuhaus et al. (2008) and were used for permeability experiments. For transport experiments growth medium without serum was used. The medium was exchanged shortly before the beginning of the experiment. 500  $\mu$ l were added in the apical and 1500  $\mu$ l in the basolateral compartment. Then, the system was allowed to equilibrate for 30 min at 37 °C before the test compound was added. To measure transport of A $\beta$  peptides, A $\beta$ (1–40) was used at a final concentration of 50 nM (250 ng/ml).

To start the transport experiment in blood-brain direction the medium of the apical chamber was removed and the A $\beta$ (1–40) solution (1:100 of aqueous 5  $\mu$ M A $\beta$ (1–40) stock solution in serum free medium) was added immediately. To perform experiments in brain-blood direction, the medium of the basolateral compartment was exchanged against the A $\beta$ (1–40) solution.

Samples (150  $\mu$ l or 50  $\mu$ l) were taken after 60, 120 and 300 min from the acceptor compartment, which was either the basolateral or the apical compartment, dependent on transport direction (blood to brain or brain to blood). The taken volume was immediately replaced with the same volume of fresh serum free medium. For blank values, inserts without cells were used and the experiment was performed in the same way as already described.

To address whether IVIG or monoclonal anti-A $\beta$  antibodies can influence A $\beta$  transport properties, immunoglobulins were added to the apical side (blood side) of the *in vitro* system at the beginning of the transport experiment and A $\beta$  transport studies were performed as already described above. IVIG and the monoclonal anti-A $\beta$  antibody 6E10 were used at a final concentration of 5  $\mu$ M and 100 nM.

As control, A $\beta$  transport without the presence of immunoglobulins was additionally determined with each transport experiment in order to enable direct comparison and hence avoid influence of daily variations.

#### 4.8. Calculation of the permeability coefficient

The permeability of A $\beta$ (1–40) across the *in vitro* BBB was expressed as permeability coefficient (Pe, cm/s) and calculated as described in more detail before (Neuhaus et al. 2006) with some slight modifications. A $\beta$  concentration of the stock solution and of samples was assessed by ELISA (Human  $\beta$  Amyloid 1–40 Immunoassay Kits, Biosource).

During the sampling procedure 150  $\mu$ l or 50  $\mu$ l were removed from the basolateral or apical chamber, dependent on transport direction, and the same amount of fresh medium was immediately added. Consequently, substance concentration was diluted at every sampling step and the concentration gradient from donor to the acceptor side was artificially increased. Considering these changes the data points had to be corrected by means of Eq. (1).  $c_t$  values were calculated for each raw data:

$$c_t = c_{raw\ data} * \frac{V_{acc} - V_{sample}}{V_{acc}} + c_{medium} * \frac{V_{sample}}{V_{acc}} \quad (1)$$

$c_{raw\ data}$  was the determined concentration of the donor chamber at time  $t$ ,  $c_{medium}$  represented the substance concentration in fresh medium, which was usually zero.  $V_{acc}$  (1500  $\mu$ l or 500  $\mu$ l) and  $V_{sample}$  (150  $\mu$ l or 50  $\mu$ l) meant the volumes of the acceptor chamber and the sample.

After calculating  $c_t$  values using Eq. (1), a corrected permeation curve was obtained by subtracting  $c_m$  values from  $c_{raw\ data_{n+1}}$  for each time point. Clearance was then calculated using these new corrected data points according to Eq. (2):

$$clearance[\mu L] = \frac{C_A * V_D}{C_D - \left(\frac{V_D}{V_A} * \sum C_{A-1}\right)} \quad (2)$$

$C_A$  referred to the substance concentration in the acceptor chamber,  $C_D$  was the concentration in the donor compartment.  $V_A$  and  $V_D$  were the corresponding volumes of the chambers. Since the amount of the test compound in the donor insert decreases over time under conditions of unidirectional flux, the  $C_D$  value for each time point (each well) had to be corrected. Thus, the summed up total amount of substance found in each acceptor compartment before the actual one ( $\sum C_{A-1}$ ) was related to the donor volume and subtracted from  $C_D$ .

The slope of clearance vs. time was determined by linear regression analysis and the permeability coefficient (PE) across the cell layer was calculated using Eq. (3).

$$\frac{1}{PE_{cell}} = \frac{1}{PE_{all}} - \frac{1}{PE_{blank}} \quad (3)$$

$PE_{cell}$  refers to the permeability coefficient of the ECV304 cell layer, whereas  $PE_{all}$  describes the permeability across the membrane filter and

the cell layer.  $PE_{blank}$  represents the permeability coefficient through the filter without cells (blanks).

Values were divided by the surface area of the filter ( $=0.9\text{ cm}^2$ ) to generate the cell permeability coefficient in centimeters per second.

The permeability coefficient for the cell layer only ( $PE_{cell}$ ) can be calculated with Eq. (3). Thus, the reciprocal value of the average permeability coefficient without cell layer ( $PE_{blank}$ ) was subtracted by the permeability coefficient of the experiment with cell layer ( $PE_{all}$ ) to gain the reciprocal value for the cell layer only.

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