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PERMEABILITY OF THE BLOOD-BRAIN BARRIER AND TRANSPORT OF NANOBODIES ACROSS THE BLOOD-BRAIN BARRIER

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ABSTRACT

The presence of a blood-brain barrier (BBB) and a blood-cerebrospinal fluid barrier presents animmense challenge for effective delivery of therapeutics to the central nervous system. Many potential drugs, which are effective at their site of action, have failed due to the lack of distribution in sufficient quantity to the central nervous system (CNS). In consequence, many diseases of the central nervous system remain undertreated. Antibodies, IgG for example, are difficult to deliver to the CNS due to their size (~155 kDa), physico-chemical properties and the presence of Fc receptor on the blood-brain barrier. Smaller antibodies, like the recently developed nanobodies, may overcome the obstacle of the BBB and enter into the CNS. The nanobodies are the smallest available antigen-binding fragments harbouring the full antigen-binding capacity of conventional antibodies. They represent a new generation of therapeutics with exceptional properties, such as: recognition of unique epitopes, target specificity, high affinity, high solubility, high stability and high expression yields in cost-effective recombinant production. Their ability to permeate across the BBB-makes thema promising alternative for central nervous system disease therapeutics. In this review, we have systematically presented different aspects of the BBB, drug delivery mechanisms employed to cross the BBB, and finally nanobodies — a potential therapeutic molecule against neuroinfections.

Key words: blood-brain barrier; carrier; nanobody; permeability; shuttle; transport

Barriers of the brain

Signalling within the central nervous system (CNS) is carried out by neuronsthat communicate with each other using a combination of chemical and electrical signals. For reliable neural signalling, regulation of the ionic microenvironment is critical [2]. There are three major interfaces (barriers) in the brain and spinal cord of mammals that keep the microenvironment stable (homeostasis) [3]. The principal barrier sites between blood and brain are listed below:

A) The blood-brain barrier (BBB) is created by tight junction (TJ) formation at the level of the cerebral capillary endothelial cells. It is by far the largest surface area for blood — CNS exchange, and it covers between 12 and 18 m^2 in an average human adult [36]. No brain cell lies further than about 25 μm from a capillary, so once the solutes or drugs cross the BBB, diffusion distances to neurons and glial cell bodies are short. For that reason, drugs with the ability to cross the BBB arecurrently the most used method for global delivery of drugs to all brain cells.
B) The blood-cerebrospinal fluid barrier (BCSFB) located at the choroid plexuses in the lateral, third and fourth ventricles of the brain.
C) The arachnoid barrier enveloping the brain, which is avascular and lying under the dura.

A combination of physical barrier (TJs between cells reducing flux), transport barrier (specific transport barrier mediating solute flux) and metabolic barrier (enzymes metabolizing molecules in transit) represents the function of the barrier at all three interfaces. Modulation and regulation of the barrier function is possible both physiologically and pathologically [4].

The blood-brain barrier in brief

A close inductive association of several cell types, especially the end feet of astrocytic glial cells, maintain the differentiation of the endothelium into a barrier layer [4, 49]. The supporting roles in barrier induction, maintenance and function are performed by pericytes, microglia and neural terminals, which are also closely associated with the endothelium [4, 37]. The hyperaemia of brain is accomplished by a group of cells, closely related to each other, called the neurovascular unit (NVU). The NVU is composed of neurons, astrocytes, endothelial cells of the BBB, myocytes, pericytes and extracellular matrix components [32].

The role of TJs (zonulae occludentes) is a significant reduction of the permeation of ions and polar solutes, primary to maintain the ionic homeostasis in the brain. This permeation is carried through paracellular diffusional pathways between the endothelial cells from the blood plasma to the brain extracellular fluid [7, 49].

TJs consist of a complex of proteins spreading across the intercellular cleft, such as occludin and claudins, and junctional adhesion molecules (JAM) [48, 49]. Cytoplasmic scaffolding and regulatory proteins ZO-1, ZO-2, ZO-3 link the junctional molecules occludin and claudins to intracellular actin and the cytoskeleton via cingulin [31, 48, 49].As Abbott and colleagues [3] reviewed, the disappearance of either claudin-3 or claudin-5 from the tight junctional complexes might result in a compromised BBB.

Adherent junctions (AJs) and TJs are part of the junctional complexes between endothelial cells. In AJs, cadherin proteins spread across the intercellular cleft and are linked into the cell cytoplasm by alpha, beta and gamma scaffolding proteins. The AJs give structural support to the tissue by holding the cells together, and are essential for formation of TJs. The disruption of AJs gradually leads to the disruption of the barrier [48].

Permeability of the BBB

As Saunders et al. [44] reviewed, several studies observed that parenteral injections of trypan blue and other acidic dyesin animal models stained almost all tissues except the brain. These experiments were followed by subsequent studies,which were using embryos or the newborn of various species. Most of them gave the same result as in adults, whereas most of the brain was not stained aside from the circumventricular organs, which led to the concept of the brain being protected by the BBB. Despite similar results both in embryos and adults, it is still widely believed that the BBB is immature and poorly formed in embryos, foetus and the newborn, leaving the developing brain more vulnerable to drugs or toxins entering the foetal circulation from the mother. However, new evidence show that many adult mechanisms, such as functionally effective TJs, are present in the embryonic brain. Furthermore, some transporters are even more active during development than in the adult.

The TJs block the penetration of macromolecules by restriction of paracellular diffusional pathways between the endothelial cells to ions and other polar solutes. The restriction of ion movement results in the high *in vivo* electrical resistance of the BBB, which is estimated to be 8000 Ω cm^2 [45]. The BBB keeps optimal ionic composition for synaptic signalling function by a combination of specific ion channels and transporters, and thus provides a stable environment for the function of neurons [4, 10]. Since the central and peripheral nervous system both use many of the same neurotransmitters, separating the neurotransmitter pools controls "cross-talk" interference between the two signalling networks [1]. As a typical example serves gluta-

mate, a neuroexcitatory amino acid which blood plasma levels significantly fluctuate after the ingestion of food [4, 10]. Similar to neurotransmitter levels, the protein content in the cerebrospinal fluid (CSF) is much lower than that of the plasma, with a different protein composition. Plasma proteins such as albumin, plasminogen or prothrombin are damaging to neural tissue, which in the final analysis can lead to apoptosis [23, 35]. If the BBB is damaged, these large serum proteins are able to leak into the brain and can cause serious pathological consequences.

The BBB also contributes to the brain homeostasis by protecting the CNS from various neurotoxic substances circulating in the blood, such as endogenous metabolites, xenobiotics or exogenous substances otherwise acquired from the environment. The level of neurogenesis is relatively low compared to the continuous steady rate of neuronal cell death throughout life, therefore any acceleration in the natural rate of cell death resulting from an increased access of neurotoxins into the brain would become prematurely impairing [27].

The neural tissue requires a low passive permeability of the BBB to many essential water-soluble nutrients and metabolites, while lipid soluble substances are able to cross the barrier passively by diffusion. Therefore, to ensure an adequate supply of water soluble substances, specific transport systems are expressed in the BBB [4, 49]. In addition to the unidirectional and bidirectional transport of small molecules, some substances are able to enter the brain tissue from the blood by other ways, e. g. facilitated diffusion (glucose via GLUT-1) or a receptor-mediated endocytosis (transferrin or insulin) [25].

Changes in BBB permeability

The BBB as a dynamic system, is capable of responding to local changes and requirements. Its regulation serves for the adjustment of nutrient supply, protection from circulating agents, or modification to ease local repairs [4]. A number of mechanisms and cell types are able to regulate the BBB in both physiological and pathological conditions. For example, apical cell-cell junction interactions participate in the regulation of gene expression, cell proliferation, polarity and apoptosis using different types of proteins. The TJs are one type of such cell-cell junctions and associate with several signalling complexes. Expression of TJ components allows cell differentiation by suppressing proliferation. These components affect several signalling and transcriptional pathways, and changes in the expression of TJ proteins are associated with several disease conditions. The aforesaid regulation includes changes in the function ofTJs [6], and in the expression and activity of many transporters and enzymes [4, 17]. It appears that intracellular scaffold proteins ZO-1, ZO-2 and ZO-3 regulate the effectiveness of the TJs [48, 49]. Furthermore, many of the cell types associated with brain microvessels, such as astrocytes and microglia, release cytokines and vasoactive agents, which can modify the TJ assembly and barrier permeability [4, 41]. Also, alterations in both intracellular and extracellular calcium concentration can modulate the electrical resistance across the cell layer, thus modifying the effectiveness of the TJs as a barrier. A rise in intracellular calcium may initiate activation of the actin cytoskeleton and may change the configuration of claudins and occludin [3].

Physiological ways of transport across the BBB

There are several potential routes for permeation across the BBB. The majority of large blood-borne molecules are physically prevented from entering the brain by the presence of the BBB and TJs. To ensure the supply of essential substances into the brain, there are specific and some non-specific transcytotic mechanisms used. Transport of macromolecules across the BBB *via* transcytosis allows solutes with large molecular weight, such as proteins and peptides, to enter the CNS intact. Transcytosis may be either receptor-mediated (RMT) or adsorptive-mediated (AMT) [3].

The BBB endothelium must contain a number of specific solute carriers (transporters) to supply the CNS with essential polar substances, such as amino acids and glucose necessary for metabolism. When penetration of the BBB is considered, bases which carry a positive charge have an advantage over acids. It is probably caused by the cationic nature of these molecules, and their interaction with the negatively charged glycocalyx and phospholipid head groups of the outer leaflet of the cell membrane that ease their entry [3]. Many polar essential molecules such as glucose, amino acids and nucleosides are transported by carrier mediated influx via solute carriers (SLCs), which may be passive, or active. Active transport is further differentiated to primarily active (energy is derived directly from the breakdown of ATP) or secondarily active (energy comes from the electrochemical gradient created by pumping ions out of the cell) [3]. The solute carriers may be unidirectional either into or out of the cell, bi-directional, they may involve an exchange

of one substrate for another, or be driven by an ionic gradient. In the last case, the direction of transport is reversible depending upon the electrochemical gradient [3].

A large spectrum of lipid-soluble molecules are able to passively diffuse through the BBB and enter the brain [28]. There is a general interrelation between the rate at which a solute enters the CNS and its lipid solubility. It is usually defined as a distribution coefficient expressed in terms of log D (logD octanol/buffer partition coefficient at pH 7.4) [14]. These passively penetrating solutes are captured by ATP-binding cassette (ABC) transporters, multidomain integral membrane proteins, and translocated across the endothelial cells. Some of the more important ABC transporters are Pgp (transporter P-glycoprotein) and BCRP (breast cancer resistance protein), which are placed in the luminal membrane of the BBB endothelium, and MRP (Multidrug resistance-associated protein) placed in either luminal or abluminal membranes [8, 9].

The movement of the blood gases, oxygen and carbon dioxide, across the BBB is diffusive as well, and the dissolved gases move down their concentration gradients [3].

Mononuclear cells appear to be able to penetrate directly through the cytoplasm of the endothelial cells by a process of diapedesis, which enables them to cross the BBB without disruption of TJs [19, 50]. During diapedesis, the fluid-filled channel through the cell is never created. The leukocyte enters the endothelial cell with the luminal membrane closing over it before it creates an opening in the abluminal membrane [12, 50].

Nanobodies

Antibodies or immunoglobulins are glycoproteins produced by B-cells, which play a central role in the host immune defense. Conventional antibodies are multimers of heavy (H) and light (L) chains, each chain consisting of constant (C) and variable (V) domains [28, 40]. In a conventional antibody, the variable region of the heavy chain (VH) and the variable region of the light chain (VL) combine to make the antigen binding site, although it was discovered that the heavy chain alone can also bind antigens [46].

Immunoglobulin G fragmented by proteolytic enzyme papain produce 3 fragments of similar molecular weight (50 kDa), but of different charge. Two out of three fragments are identical and keep their antigen binding ability, which is why they are called fragments of antigen-binding (Fab). The third fragment does not bind the antigen and crystalizes, therefore it's called fragment crystallis able (Fc) [18, 38].

Since the constant domains of antibodies are not involved in the recognition of antigen, a range of smaller antibody fragments such as Fab, F(ab´)2, Fv and scFv have been designed. In comparison with the conventional antibodies, smaller antibody formats are more cost- effective to produce, have a faster organ clearance [30, 51], penetrate the solid tumours more efficiently [53], and are more suitable for structural analysis [29, 42].

In the early 1990s it was discovered that the antibody repertoire of camelids contains antibodies consisting of heavy chains only, which are referred to as heavy-chain antibodies (HCAbs) [24]. Despite the absence of light chains in camelid HCAbs, these antibodies display an extensive antigen-binding repertoire and their binding affinities for their cognate antigens are comparable to conventional antibodies. Structurally, the antigen-binding domains of camelid HCAbs are composed of the antigen-binding variable domain termed VHH (variable domain of the HCAbs), followed by a hinge region and two constant domains CH2 and CH3, while the CH1 domain known from conventional antibodies is missing [24]. With approximately 15 kDa VHHs are the smallest naturally derived antigen-binding antibody fragments. Recombinantly produced VHH fragments are also called "nanobodies" [34]. The advantages of nanobodies include: small size (2.5 nm in diameter and about 4 nm height), recognition of unique epitopes, high affinity, high solubility, high stability, and high expression yields in heterologous expression systems [15, 22].

As Ghassabeh et al. [22] reviewed, HCAbs have also been described in humans as a pathological disorder termed "heavy chain disease." However, these human HCAbs devoid of light chain fail to bind antigen, and are consequently non-functional. Camelid HcAbs, on the other side,have evolved to be fully functional even in the absence of light chains, while harbouring the full antigen-binding capacity of the conventional antibodies.

Transport of nanobodies across the BBB

The BBB is only permeable to lipophilic molecules of up to 400 Da in size [39], therefore conventional antibodies are unable to spontaneously cross, given their average size is approximately 155 kDa [13].The delivery of conventional antibodies to the brain is especially tiresome, due to

Fc-receptor mediated efflux to the blood [16], thus nanobodies lacking an Fc-part represent a promising alternative to brain targeting antibodies. Therapeutic application of nanobodies to CNS is difficult because the BBB restrain the delivery of intravenously injected nanobodies to the brain. Various strategies have been developed and tested to overcome the BBB; for example, antibodies against receptors that undergo transcytosis across the BBB have been used as vectors to target drugs or therapeutic peptides into the brain [5].

In a therapeutic experiment using the Hargreaves model of inflammatory pain (injection of inflammatory agents into the rat or mouse hind paw), Farrington et al. [20] tested the ability of FC5 nanobodies as a drug deliverer. It was shown that FC5 conjugated with opioid peptide Dal could be deployed as a drug delivery shuttle *in vivo* to induce a significant analgesic response in contrast to unconjugated Dal peptide. The FC5 is a nanobody, which selectively recognizes human cerebromicrovascular endothelial cells (HCEC) and transmigrates across them *in vitro* and across the BBB *in vivo* [33]. The same group later suggested that FC5 binds to a putative α (2,3)-sialoglycoprotein receptor (Fig. 1A) and is transcytosed *via* clathrin vesicles [5]. The potential of the nanobody FC5 as a shuttling-nanobody can be used to transfer other therapeutics, e.g. proteins or therapeutic nanobodies through the BBB [43].

Receptor-mediated transcytosis for brain targeting was utilized also by Wang et al. [47]. They showed that a fusioncomplex of a peptide derived from apolipoprotein E and a model therapeutic protein (α-L-iduronidase) could be transferred to the brain via binding to the LDL receptor expressed on cells of the BBB. Apolipoprotein E (ApoE) binds to alow density lipoprotein receptor-related protein 1 (LRP1) (Fig. 1B) inducing transcytosis, which can be used as a shuttle for therapeutic nanobodies in the future [43].

Another research group studied the transferrin receptor and the insulin receptor (Fig. 1C) in receptor-mediated transcytosis of small molecule drugs and therapeutic proteins [11, 52]. Both receptors can be found on the luminal membrane of brain capillary endothelial cells. These results indicate that triggering transcytosis through nanobodies targetting these receptors could also be a promising alternative to ligand-based delivery of drugs to the brain [51, 52]. Molecular Trojan horse by fusing the therapeutic proteins to the monoclonal antibodies (MAb) against human insulin or transferrin receptor have been demonstrated as an efficient strategy for the delivery of therapeutic protein to the brain [11].

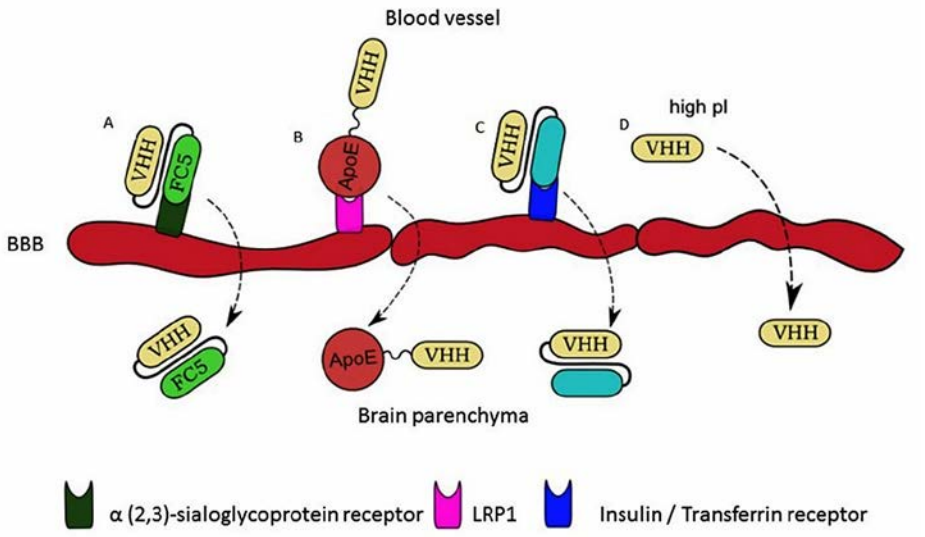

Fig. 1. Ways of transport of nanobodies across the BBB
(A) Nanobody FC5 binding to α(2,3)-sialoglycoprotein receptor can be used as a drug deliverer for other nanobodies;
(B) Transcytosis induced by the binding of Apolipoprotein E (ApoE) to low density lipoprotein receptor-related protein 1 (LRP1);
(C) Transcytosis triggered by nanobody againstan Insulin/Transferrin receptor;
(D) Spontaneous crossing of nanobodies with high isoelectric point through the BBB
Source: An original drawing

Other studies reported that nanobodies with a high isoelectric point (pI)~9,5 [26, 33] spontaneously cross the BBB (Fig. 1D). Such nanobodies easily gain access to the brain and even penetrate cells and bind to intracellular proteins. In a mouse study, Li et al. [26] used a recombinant nanobody E9 (pI = 9.4) directed against glial fibrillary acidic protein (GFAP), a specific marker of astrocytes. This nanobody crossed the BBB *in vivo,* diffused into the brain tissue, and was able to bind to intracellularly expressed GFAP in astrocytes. The FC5 nanobody described above has a basic pI 9.2 which might contribute to its transcytosis into the brain parenchyma [43].

There are other possible routes of transport used in other therapeutics, which were not tested yet with nanobodies. Gaillard et al. [21] have tested the use of CRM197, a nontoxic mutant of diphtheria toxin, as a targeting vector for drug delivery to the brain. CRM197 was tested for its brain delivery potential as it has been shown to endocytose after binding the membrane-bound precursor of heparin binding epidermal growth factor-like growth factor (HB-EGF), also known as the diphtheria toxin receptor.

CONCLUSIONS

Conventional antibodies are unable to spontaneously cross the BBB, due to the Fc-receptor mediated efflux to the blood. Because of that, novel approaches of therapy of neurodiseases or neuroinfections have been researched. Nanobodies are a promising alternative to conventional antibodies and other CNS therapeutics, given the lack of an Fc-part and recognition of unique or hidden epitopes, which gives them a possibility to succeed where conventional antibodies commonly fail. Thanks to their unique features, such as small size while preserving antigen binding capacity, the recognition of hidden epitopes and the ability to penetrate through biological barriers, they appear to be ideal candidates for therapeutic purposes. Several possible routes for nanobody translocation through the BBB have been already described and tested. Furthermore, there are other successful ways of transport of therapeutics through the BBB, which have not been tested with nanobodies yet. In addition to their therapeutic function, nanobodies can also be used as a drug delivery shuttle, given their ability to trigger a receptor-mediated transcytosis. Despite all of the advantages of the therapeutic use of nanobodies, their translocation across the BBB and successful utilization in the treatment of neurodiseases is not yet thoroughly researched.

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