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ARTICLE

Development and Characterisation of a Monoclonal Antibody Family Against Aquaporin 1 (AQP1) and Aquaporin 4 (AQP4)

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Recent studies have discovered the existence of water-channel molecules, the so called aquaporins (AQP) presumably involved in active, ATP dependent water transport between the intracellular and extracellular compartments. Both genetic and protein sequences and structures of the AQPs are known and crystallographic analyses of some members of the AQP family have been performed. Specific antibodies are required to examine their histological locations and analyse their roles in physiological and pathological pathways of water transportation and osmotic regulation. Until recently some polyclonal antibodies have been developed against certain members of the AQP family. However, to date highly specific monoclonal antibodies against aquaporins do not exist. We have developed a monoclonal antibody family against the aquaporin 1 (AQP1) and aquaporin 4 (AQP4) molecules. Well-conserved epitop sequences of AQP1 and AQP4 proteins were selected by computer analysis and their synthetic peptide fragments were used as the antigens of immunisation and the following screening. Antibodies were characterised by immunoserological methods (ELISA, dot-blot and immunoblot), flow cytometry and immunohistochemistry of formaldehyde-fixed and paraffin-embedded tissue samples. RT-PCR tests controlled the specificity of the immune reactions. Our monoclonal antibodies recognised AQP1 and AQP4 in all the techniques mentioned above and apparently they are useful both in various quantitative and qualitative measurements of the expressions of AQP1 and AQP4 in several species (human, rat, mouse, invertebrates, even plants). According to preliminary immunohistochemical studies our monoclonal anti-AQP1 and anti-AQP4 antibodies are appropriate tools of patho-morphological examinations on routine formol-paraffin tissue samples. (Pathology Oncology Research Vol 8, No 2, 115–124, 2002)

Keywords: aquaporin 1, aquaporin 4, anti-aquaporin antibodies, monoclonal antibody, immunohistochemistry

Introduction

Prokaryotic and eukaryotic cells regulate their volumes through transporting ions and water molecules via the cell membrane by the concerted action of different mechanisms. Mercury sensitive and resistant channels participate in the net transport of water as a part of this osmotic regulation.[1,11,12] The family of water channelling molecules - named aquaporins (AQP) – was discovered during the last decade.[1,2,14,6] The molecular and genetic structures, cellular expression patterns, levels and functions of these molecules have been extensively studied recently.[4,7,20,28]

A 28 kDa channel forming membrane integrated protein (MIP) in the brain was described as the first member of the AQP family (AQP1 or CHIP 28).[2] AQP1 has been measured in different tissues, mostly in various membrane organelles in direct contact with capillary endothelial cells.[9,25] A highly homologous structure is characteristic for all members of the aquaporin family.[7] The polypeptide chain forms six putative transmembrane domains called H1-H6 constructing a definitive channel for water transport.[2,8]

AQP2 that plays a major role in the concentration of urine is a vasopressin-regulated water channel. Nucleic acid techniques specified AQP4 as the regulator of water balance in the central nervous system found in the ependymal cells lining the aqueduct and glial elements of the

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cerebral cortex and brainstem and the Purkinje cells of the cerebellum.[4,26] AQP3 and AQP5 were described in human salivary glands expressed by the acinar secretory cells located in the basolateral and in apical membrane.[9,10,25] The AQP6, 7, 8, and 9 molecules have been described only recently, their functions are less specified.[7, 14]

The major characteristics, the genetic and protein structures of the AQP family members are well known, however, functional investigations of the different AQPs participating in the osmotic regulation both at cellular and tissue levels require further pathomorphological and pathophysiological investigations. Polyclonal antibodies have been developed against some members of the AQP family, but no data with specific monoclonal antibodies have been published yet.[9,10,24]

Based on computer modelling and analysis of genomic databases and the protein structures of aquaporins we have selected the most conserved primary sequences of the two pathologically most important members of the AQP family. Monoclonal antibodies against AQP1 and AQP4 were developed and characterised by immunohistochemistry (including formaldehyde fixed and paraffin embedded materials), flow cytometry and immunoserology.

Materials and methods

Peptide fragments

We have selected the most characteristic and well-conserved sequences of the AQP1 and AQP4 molecules based on their amino acid sequence analysis. To avoid cross-reaction between the chosen AQP1 and AQP4 peptid fragments or other members of the AQP family we have analysed the entire amino acid sequences of all the known AQPs with the Clustal W multiple sequence alignment software (Swiss Prot, ExPASy server). The immunogenity and biological activity of the chosen not cross-reactive, conserved antigenic peptides of AQP1 and AQP4 were predicted by the ProtParam tool prediction software (SwissProt, ExPASy server) and analysed by the criteria described in the appropriate literature.[5,13,19]

Against rat AQP1 we had chosen two peptides. The peptide fragment called AQP1/22 is an intracellular part of the AQP1 protein. The amino acid sequence of AQP1/22 is as follows: 249-GQVEEYDLDDDINSRVEMKPK-269. The second peptide fragment called AQP1 is an extra-cellular part of the AQP1 protein and functions as a water-channel. The amino acid sequence of AQP-1 is: 198-GSAVLTRNFSN-208.

Against rat AQP4 also two peptides were selected. The peptide fragment called AQP4/18 is an intracellular part of the AQP4 protein. The amino acid sequence of AQP-4 is the following: 301-CIDIDRGDEK KGKDSSEG-318. The second peptide fragment called AQP4 is an extracellular, water-transporting part of the AQP4 protein. The amino acid sequence of AQP-4 is: 206 GPAVIMGNWEN 231.

Immunisation and hybridoma development

Synthetic peptide fragments (produced by Department of Medical Chemistry, University of Szeged, Hungary) were coupled to thyreoglobulin (TG) and bovine serum albumin (BSA) (both from Sigma Chem. Co., USA) serving as carrier molecules and were then used to immunise female Balb/c mice Charles River Laboratories, Raleigh, USA).[18,25] The animals were injected with 100 μg peptide-TG conjugates in complete Freund adjuvant, followed by two intra-peritoneal boosts in incomplete Freund adjuvant 28 and 78 days later. The specific immunoglobulin content of sera from immunised mice were controlled regularly by simple binding ELISA.[17]

Antigen specific B lymphocytes prepared from the spleens of high responder animals were preselected using a method developed in our department previously.[23] The fusion partner was the Sp-2/0 Ag14 (ATCC, USA) mouse myeloma cell line and the hybridoma cells were prepared and cloned as usual. [23]

Preparation of monoclonal antibodies

The selected clones of hybridoma cells were cultured in DMEM (Sigma Chem. Co., USA) containing 10% FCS (Gibco, USA). The mass production of antibodies was performed by hybridoma fermentation (HarvestMouse, Serotec, UK). Monoclonal antibodies were purified by protein-G or protein-A based Sepharose4B affinity chromatography (Pharmacia, Uppsala, Sweden). The antibodies were concentrated by Amicon ultrafiltration (Millipore, Badford, MA, USA) and the final concentrations were measured by the Micro-Bradford assay.[15,21,22,30]

Preparation of protein fractions containing aquaporins

Different organs (brain, stomach and kidney from rat and mouse) and cultured cells (Hep-G2) were used for the characterisation of the monoclonal antibodies.

The following protocol was used for the isolation of aquaporins from different tissues. Raw tissue pieces were mechanically dispersed in 0.15 M PBS pH 7.2 completed with sucrose (0.25 M), EDTA (0.01 M) and protease inhibitors (8.5 μM leupeptin and 1 mM o-phenyl methane sulphonyl fluoride /PMSF/). Samples were ground with the IKA-WERK Ultraturax (Germany) homogenisator on ice for 3 minutes (2 x 10^4 rpm). The samples were sonicated with (Cole-Parml Inst. Co. USA) for 2 minutes on ice at 90% duty circle using an US microtip. After homogenisation fats were removed with tri-chloro-tri-fluoro-acetic acid (Sigma Chem. Co, USA) and the samples were centrifuged at 2.5 x 10^3 rpm, for 20 minutes at 4 C. The protein content was determined by the Micro-Bradford assay as usual.[21,30] The quantitative results were mea-

sured by a microphotometer (Dynatech MR7000, USA) and were given as a concentration of optical density (OD 490) over protein mass. Samples of 1mg/ml protein concentration were stored at –20 °C.

In order to isolate membrane bound aquaporins the tissues were dispersed in a homogenisation buffer (0.25 mM Tris HCl, pH 7.4 completed with 5 mM EDTA, 5 mM $MgCl_2$, 40 ug/ml Bacitracin, 250 mM sucrose and protease inhibitors such as 0.1 mM PMSF, 25 mM Aprotinin, 8.5 µg/ml leupeptin and 1 µg/ml Pepstatin). Samples were ground by the IKA-WERK Ultraturax machine for 3 minutes (2 x 10^4 rpm) on ice. The homogenate was centrifuged at 1 x 10^4 g for 5 min at 4 °C. The pellet was homogenised in cold homogenisation buffer and centrifuged as before. The supernatant was combined with the samples with from the previous step. The supernatant was centrifuged at 7 x 10^4 g for 50 minutes at 4 °C. The pellet was resuspended in cold homogenisation buffer and centrifuged as before. Then the pellet was resuspended again in homogenisation buffer and the protein concentration was measured by the Bradford assay. Samples were stored at –20 °C.

Total protein fraction of tissues and cultured cells was isolated with TRI-Reagent (Sigma Chem. Co, USA) following the producer's instructions.

ELISA

The synthetic peptides or their carrier-coupled forms and the isolated protein fractions of different tissues were incubated for 12 hours at 4 °C followed by 60 min incubation at 37 °C on a 96-well polystirol plate (NUNC, France). Following the saturation of non-specific binding sites with 5% gelatin (Sigma Chem. Co, USA) three washing steps with PBS containig 0.1% Tween 20 (Sigma Chem. Co, USA) were performed. Then samples were incubated with different dilutions of purified anti-AQP1 and anti-AQP4 antibodies for 60 min; finally the plate was incubated with anti-mouse Ig HRPO labelled antiserum (Dakopatts, Denmark) for 60 min. The colour reaction was developed with ortho-phenylene diamine (Sigma Chem. Co, USA). In some cases biotin labelled anti-AQP antibodies were used. The colour reaction was developed with streptavidin-HRPO amplifying system (Dakopatts, Denmark). Results were measured by the Dynatech MR7000 (USA) microphotometer and calculated as optical density (OD 492).

Dot blot

The synthetic peptides or their carrier-coupled forms and the isolated protein fractions of different tissues were incubated for 60 min at 37 °C on a 96-well dot-blot block (Bio-Rad, Hercules, USA) containing a nitro-cellulose membrane (Bio-Rad, Hercules, USA). Then the nitro-cellulose was washed, covered with TBS and 5% low fat milk in order to saturate non-specific binding sites. The nitrocellulose sheets were incubated with monoclonal antibodies of different dilutions and the reactions were developed by the anti-mouse Ig HRPO labelled secondary antiserum or the streptavidin-HRPO amplifying system. The colour reaction was developed with amino-ethyl-carbasol (Sigma Chem. Co, USA).

Western blot

The cytosolic, membrane-bound or total protein fractions of different tissues were boiled for 5 min in SDS sample buffer and run in 8-25% gradient gel using the PhastSystem automated electrophoresis system (Pharmacia, Sweden) or 10% SDS-poly-acrylamide gel using Mini Protean 3 device (Bio-Rad, Hercules, USA). The gel was transferred to a nitrocellulose-membrane (Sartorius, Germany or Bio-Rad, Hercules, USA) using the same equipments. The immunereactions were applied as described above. The colour reaction was developed with a Renaissance WB chemi-luminescence amplifying system (PerkinElmer, UK) using Kodak X-ray film.

Immunohistochemistry

Conventional formaldehyde-fixed (4%) and paraffin-embedded tissue samples from the tissue bank of the Histopathology Ltd. (Hungary) were used for the characterisation of the anti-aquaporin monoclonal antibodies. The deparaffinated and rehydrated tissue sections were heated with microwaves (3 x 5 minutes at 750 W) in citrate buffer (0,016 M citric acid, 0,084 M sodium citrate) adjustment of the buffer between each heating session. The endogenous peroxidase activity was blocked with 0.05% hydrogen peroxide in absolute methanol for 30 min. The slides were washed 3 x 5 minutes in phosphate-buffered saline (PBS) at pH 7.4 after each incubation step. The slides were pre-incubated in 1-5 % bovine serum albumin (BSA) dissolved in PBS, for 30 min at 37°C, in order to prevent the non-specific binding of the primary antibodies. Then the sections were incubated with the anti-aquaporin antibodies overnight at 4°C. Following a rinse, the samples were incubated for 1 hour with biotinylated an anti-mouse Ig specific secondary antibody (Vector Laboratories, Burlingame, USA) diluted to 1:50 in 1% BSA PBS. After washing the sections were treated with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, USA) for 1 hour at room temperature following the manufacturer's guidelines. In order to detect reactions the slides were incubated in 0.03% H_2O_2 and 3,3-diaminobenzidine (DAB) (0,5 mg/ml in PBS) for 5 minutes. In some case we have used the EnVision and System Peroxidase Kits (Dakopatts, Den-

mark). Finally the sections were washed in distilled water, dehydrated with ethanol, cleaned in xylene and mounted in DePeX (BDH Laboratory Supplies, Poole, UK).

Flow cytometry

Cell surface and intracellular labelling of *in vitro* cultured Hep-2 (human epidermoid carcinoma cell line ATCC, CCL23) Hep-G2 (human hepatocellular carcinoma (ATCC HB 8065), Caco-2 (human adenocarcinoma, ATCC HTB 37) Jurkat (human acute leukemia, ATCC TIB 152) and Sp-2/0-Ag14 (mouse myeloma, ATCC CRL 1581) cells or isolated peripheral human blood mononuclear cells (PBM) were performed on ice. Cell surface staining was performed with FITC labelled antibodies on $5x10^5$ cells in 100 µl PBS/0,1%BSA/0,1%NaN_3. After two washing steps in PBS the cells were fixed in 1.0% buffered formaldehyde and measured for fluorescence. For intracellular staining the cells were fixed in buffered 4% paraformaldehyde on ice for 20 min then washed 3 times in PBS and permeabilised with 0.1% saponin containing PBS/NaN_3/BSA buffer. The cells were incubated with FITC-mAb conjugates or isotype controls in permeabilisation buffer for 30 min, washed two times in permeabilisation buffer, one time in PBS, and fixed in 1.0% buffered formaldehyde. For indirect labelling FITC labelled sheep anti-mouse IgG was used after incubation with the first antibody in permeabilisation buffer. The samples were measured and analysed in a FACSCalibur type Becton Dickinson device using the CellQuest software.

Controls for immunoassays

We have compared our monoclonal antibodies with an indifferent negative control monoclonal antibody; the anti-FITC IgG1 developed in our laboratories previously. Identical isotype controls (Beckton Dickinson, USA) were used in flow cytometry. We also compared our monoclonal antibodies with anti-AQP1 (3391/2353) and anti-AQP4 (LL182) polyclonal antisera developed by other laboratories.[24] We used these negative and positive controls in all immunohistochemical and immunoserological methods.

Nucleic acid techniques

RNA ISOLATION. Tissue samples of animals were immediately snap-frozen in liquid nitrogen and stored at –70°C until RNA isolation. All downstream applications were performed in a nuclease-free environment. Evenly sized pieces of approx. 40mg were ground mechanically in liquid nitrogen to get a homogenous dry powder of cells. Total RNA was then isolated using the GenElute mammalian total RNA kit (Sigma). The quality of the RNA isolates was confirmed by gel-electrophoresis in formaldehyde containing denaturing gel. The presence of the two major rRNA bands without significant smear approved RNA integrity. RNA quantity and purity was determined by a UV spectrophotometer. In all cases relative purity was 1.7-1.9 (OD 260nm/280nm).

REVERSE TRANSCRIPTION. RT was performed using the SuperScript II kit following the attached instructions (Gibco). For all samples 2 µg total RNA served as template for RT. Briefly, cDNA was synthesised by the SuperScript II (M-MLV) enzyme utilising oligo-(d)-T_{12-18} primers in order to reverse-transcribe all poly-A tailed mRNA molecules under RNase protection provided by the RNasOUT inhibitor enzyme. All reagents were provided as part of the kit and were used at the recommended final concentrations. The thermal protocol of cDNA synthesis was performed in a Hybaid Sprint thermal cycler device following regular guidelines optimised for oligo-(d)-T_{12-18} primers.

OLIGONUCLEOTIDES. The following rat cDNA specific primer pairs (synthesised by Genosys Ltd.) were used for polymerase chain reaction at a final concentration of 0.5 µM. The sequence of the used primers were: β-actin forward primer: 5'-ATC ATG TTT GAG ACC TTC AAC

Table 1. **Main features of monoclonal antibodies against AQP1 and AQP4**

Antibody	*Clone*	*Isotype*	*Epitope specificity*
Anti-AQP 1	AQP 1/22, 1/A_5	IgG2b	Amino acid sequence 249-269 Active water channel loop of AQP1 molecule
Anti-AQP 1	AQP 1, 7/D_{11}	IgG1	Amino acid sequence 198-208 C terminal domain of AOP1 molecule
Anti-AQP 4	AQP 4/18, 1/E_3	IgG3	Amino acid sequence 301-318 C terminal domain of AQP4 molecule
Anti-AQP 4	AQP 4/18, 3/D_2	IgG1	Amino acid sequence 301-318 C terminal domain of AQP4 molecule

Table 2. **Optimal working dilutions of anti-AQP1 and anti-AQP4 monoclonal antibodies**

	dilutions of affinity purified antibodies used (1 μg/ml)			
Clone name	*ELISA*	*Western Blot*	*dot blot*	*Immunohisto-chemistry*
AQP 1/22, 1/A5	1:2000	1:500	1:500	1:200
AQP 1, 7/D11	1:1000	1:200	1:200	1:200
AQP 4/18, 1/E3	1:2000	1:500	1:500	1:200
AQP 4/18, 3/D2	1:2000	1:500	1:500	1:200

AC-3', β-actin reverse primer: 5'-TCT GCG CAA GTT AGG TTT TGT C-3', AQP1 forward primer: 5'-AGC GAA ATC AAG AAG AAG CTC-3', AQP1 reverse primer: -CCT CTA TTT GGG CTT CAT CTC-3', AQP4 forward primer: 5'-TCC CTT TGC TTT GGA CTC AG-3', AQP4 reverse primer: 5'-TTC CCC TTC TTC TCC TCT CC-3'. The melting points of the utilised primers are as follows: β-actin: 56°C, AQP1: 62°C, AQP4: 57°C. The sizes of the resulting amplicons are: β-actin: 825 bp, AQP1: 807 bp, AQP4: 719 bp.

POLYMERASE CHAIN REACTION. All PCR was performed in a Hybaid Sprint thermal cycler device. Typically cDNA from 50ng of total RNA was used as sample for each PCR using Taq polymerase (Clontech) at a final concentration of 1 unit/reaction (1 unit/10 μl). In all cases careful control reactions validated our data. For β-actin PCR 20-25 thermal-cycles, for AQP PCR 30-35 thermal-cycles were performed. These cycle numbers are the results of earlier optimisation reactions in order to find the exponential range of all the target cDNAs. Each thermal protocol included an initial 3 minute denaturation and a final 10 minute elongation step. In addition, all thermal protocols began with a 5°C thermal "touchdown" through 5 cycles and ended with an elongation time increment of up to 50% through the last 10 cycles, to ensure both high specificity and yield.

VISUALISATION. Gel electrophoresis was performed in 0.5x TBE buffer. In all cases half of the reaction mixture (5?l) was visualised over UV light in 2% agarose gels containing ethidium-bromide. Digital and analogue pictures were both taken.

Results and Discussions

The participation of water-channels in immediate water distribution among the intracellular and extracellular compartments under physiologic and pathologic conditions have been analysed in different cells and tissues previously.[6,14,24] Some results suggest the active participation of aquaporins in ATP dependent water-transport driven by inorganic ion-gradient under physiologic circumstances.[9,10,25,29] On the other hand, the prompt generation of certain pathologic phenomena (e.g. mucosal or brain oedema) in which rapid extracellular water accumulation develops independent from the actual intracellular ion concentration may also be linked to the functions of aquaporin proteins. Aquaporins have significant functions in different body fluid secretions as liquor, urine, saliva, gastric juice or gastro-intestinal mucus production. Aquaporin expression in salivary glands were recently analysed with nucleic acid techniques and immunohistochemistry.[9,10] Koyama et al. evaluated the localisation of different aquaporins (AQP1, AQP3, AQP4 and AQP8) in rat gastrointestinal tract.[17] Since the secreted hydrochloric acid is isotonic to the plasma fluid within the stomach, its transport should be accompanied by water transport across the membranes of parietal cells managed by aquaporins.

The literature contains several contradictions concerning the localisation of aquaporins due to differences in the inves-

Table 3. **Antigens recognised by monoclonal anti-AQP1 and anti-AQP4 antibodies in ELISA, dot-blot and Western blot methods**

Antigen	*Clone name*			
	AQP 1/22, 1/A5	*AQP 1, 7/D11*	*AQP 4/18, 1/E3*	*AQP 4/18, 3/D2*
Synthetic AQP1 peptid 249-269	+	–	–	–
Synthetic AQP1 peptid 198-208	–	+	–	–
Synthetic AQP4 peptid 301-318	–	–	+	+
Cytoplasmic protein fraction of rat brain	+	+	+	+
Membrane bound protein fraction of rat kidney	+	+	+	+
Total protein fraction of rat stomach	+	+	+	+
Cytoplasmic protein fraction of mouse brain	+	+	+	+
Membrane bound protein fraction of mouse kidney	+	+	+	+
Total protein fraction of in vitro cultured Hep-G2	+	+	+	+
Total protein fraction of in vitro cultured Jurkat	–	–	–	–

tigation methods used. Molecular biological techniques focus on mRNA expression of different aquaporins but cannot clarify the localisation of the water channel proteins in the living structures. Polyclonal antibodies are suitable for the immunoserological and immunohistochemical mapping of the expressed aquaporins. However, the high number of cross-reactive epitopes result technical difficulties and severely limit the identification of water channel molecules. Our main goal was to develop monoclonal antibodies against characteristic epitopes of AQP1 and AQP4 molecules.

We have selected well-conserved and characteristic epitopes following complex computer analysis and modelling of AQP1 (amino acid sequences 198-20, and 249-269) and AQP4 (amino acid sequences 301-318) with close homology among different species. The selected peptide fragments have over 95% sequence homology in vertebrates and approx. 85% in invertebrates and plants. The chosen epitopes did not show sequence homology with other members of AQP family or other known proteins.

We have developed four different libraries of monoclonal antibodies against AQP1 and AQP4. The clones were characterised by different immunological techniques and selected for multipurpose micro-analytical applications. The best clones selected by different immunoserological and immunohistochemical techniques are referred to as AQP1/22 $1/A_5$ and AQP1 $4/D_{11}$ against aquaporin 1 molecules, and AQP4/18 $1/E_3$ and AQP4/18 $3/D_2$ against aquaporin 4 molecules. (See details in *Table 1.)* These monoclonal antibodies recognised both the synthetic peptide fragments and the natural AQP1 or AQP4 in total protein fractions of rat brain, kidney and stomach tissue lysates in ELISA, dot-blot, Western blot and immunohistochemistry with high specificity and optimal sensitivity. (See details in *Table 2*.) The selected clones exhibited no cross-reactivity. We possess only one monoclonal antibody against the sequence 206-231 of AQP4 and the technical features of this antibody (IgM isotype) do not suite further immunological techniques due to its high non-specific binding capacity. A new set of antibodies against this sequence is being development.

No differences have been found between ELISA and dot-blot results concerning the recognition of synthetic and native antigens. ***Table 3.*** summarises the results of immunoserological characterisation. A quantitative immu-

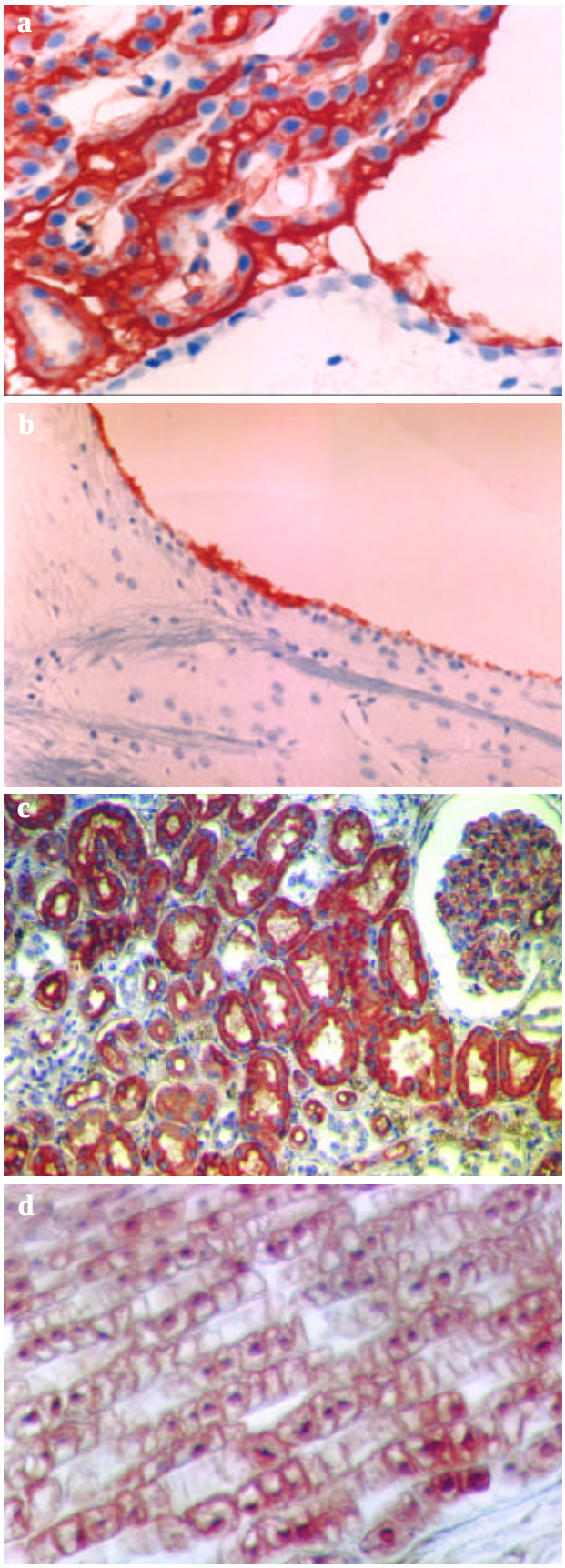
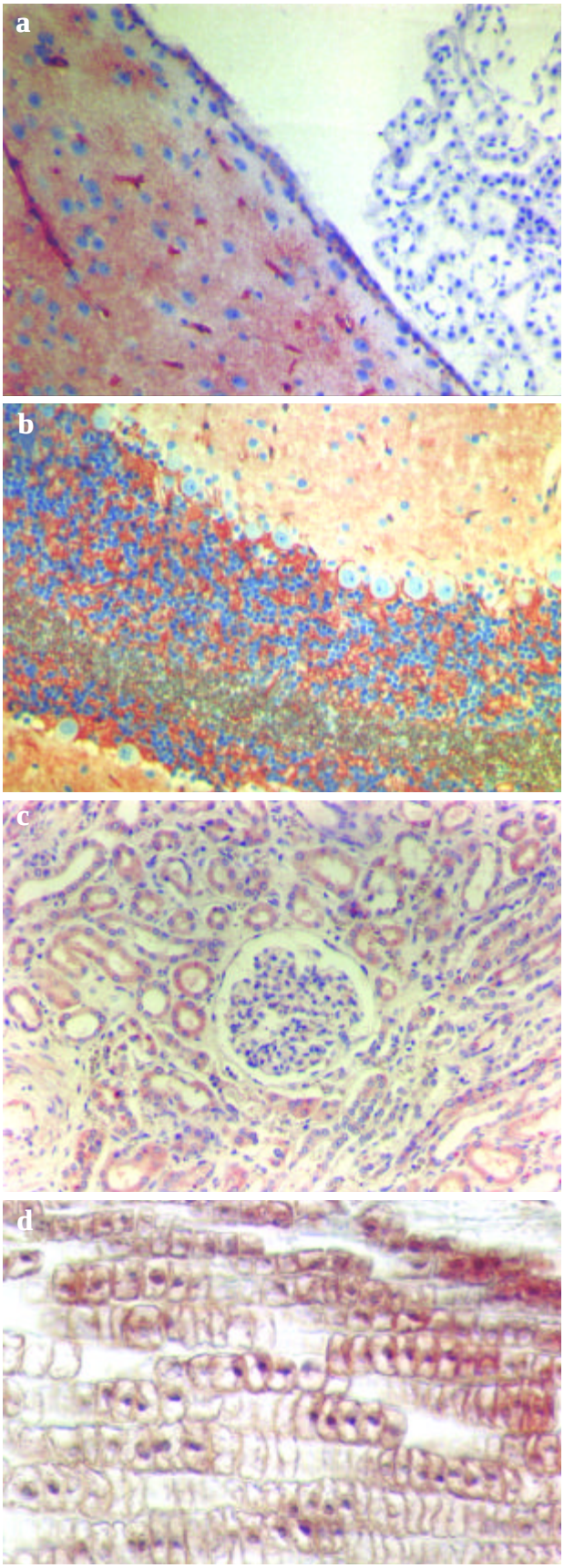


*Figure 1. AQP1 staining in paraffin embedded tissue section. **a**: rat brain choroid plexus, stained by anti-AQP1clone AQP1/22 1/A5 (IgG_{2b}), 200 X; **b**: rat brain wall of lateral chamber, stained by anti-AQP1 clone AQP1/22 7/D11 (IgG_1), 200 X; **c**: rat kidney, stained by anti-AQP1clone AQP1/22 1/A5 (IgG_{2b}), 200 X; **d**: been root, differentiation zone, anti-AQP1 clone AQP1/22 7/D11 (IgG_1), 300 X. In all sections amino-ethil-carbasol and the EnVision amplifying system were used. The tissues were counterstained with hematoxylin.*



noserological method based on our monoclonal antibodies is under construction.[3]

In accordance with mainstream literature data all monoclonal antibodies were specific to their appropriate target epitopes by immunohistochemistry, but some altered and new details have been found.

AQP1 has been described previously to be present in the choroid plexus of the brain.[4] We have detected AQP1 expression in the epithelial cells of the choroid plexus and ependyma of human (data not shown) and rat brain. (*Figure 1a and 1b*) The labelling patterns of the anti-AQP1 monoclonal antibodies (clones 1/A5 and 4/D11) were similar to those of literature.[1,2,4] No immunoreactions have been detected in either the cortical or the medullar regions of the brain.

We have found AQP1 expression in human (data not shown) and rat kidney. AQP1 is abundantly expressed in the capillary endothelium of glomeruli and the apical brush-border of the epithelial cells of the proximal tubules similarly to previous literature data.[4] (*Figure 1c)*

We have detected intensive immunostaining with anti-AQP1 antibodies in different plant tissues such as corn (data not shown) and bean root. (*Figure 1d)* We have examined the proliferation-, differentiation- and absorption zones of the root. AQP1 is expressed in the cambium cells of the proliferating zone and the forming primary phloem and primary xylem (inner bark) of the differentiation zone. Epidermal cells express AQP1 only at low intensity in the absorption zone. No similar data have been found in literature.

We have detected AQP4 expression with anti-AQP4 monoclonal antibodies in human (data not shown) and rat brain cortex in association with glial end-feet close to the capillaries. No labelling was found in the plexus choroideus or in the ependyma. (*Figure 2a)* AQP4 expression has been previously characterised in astroglial end-feet surrounding capillaries and forming the glia limitans in the cortical region of the brain.[4] The staining pattern of the anti-AQP4 monoclonal antibodies were the same as with polyclonal antibodies described previously.

A novelty is the detection of AQP4 in human (data not shown) and rat cerebellum in strong association with Purkinje cells and the basket synapses, surrounding cerebellum capillaries on the glia end-feet membranes. The staining

*Figure 2. AQP4 staining in paraffin embedded tissue section. **a**: rat brain plexus chorioideus, stained by anti-AQP4 clone AQP4/18 1/E3 (IgG_3), 200 X; **b**: rat cerebellum, stained by anti-AQP4 clone AQP4/18 3/D2 (IgG_1), 100 X; **c**: rat kidney, stained by anti-AQP4 clone AQP4/18 3/D2 (IgG_1), 200 X; **d**: been root, differentiation zone, anti-AQP4 clone AQP4/18 1/E3 (IgG_3), 300 X. In all section EnVision amplifying system was used and visualised with amino-ethil-carbasol. The tissues were counterstained with hematoxylin.*

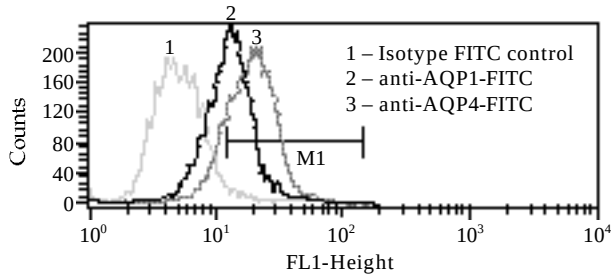


Figure 3. AQP1 and AQP4 detection on Hep-G2 cells with FITC labelled anti-AQP1 and anti-AQP4 monoclonal antibodies. Flow cytometric histogram, the FL1 height (fluorescence intensity of the FITC molecule) is plotted against the counts (numbers of cells with a given fluorescence activity). 1: FITC labelled IgG_1 isotype control, 2: FITC labelled anti-AQP1 clone AQP1/22 7/D11 (IgG_1), 3: FITC labelled anti-AQP4 clone AQP4/18 3/D2 (IgG_1). M_1: range of the specific labelling.

patterns with the anti-AQP4 monoclonal antibodies (clones 1/E3 and 3/D2) in the cerebellum were very characteristic. No AQP1 was found in this tissue localisation. (*Figure 2b)* This type of AQP4 expression could be related to the disruption of the blood-brain barrier and in the generation of cerebral oedema and suggests the participation of this water-channel structure in the reestablishment of the osmotic equilibrium of the brain.[24,26]

We have detected AQP4 in human (data not shown) and rat kidney. AQP4 has been described in the basolateral membranes of the medullar collecting duct cells.[4] The labelling pattern of our anti-AQP4 monoclonal antibodies (clones 1/E3 and 3/D2) correspond to this, however the labelling intensity is weak, compared to AQP1 staining. (*Figure 2c)* The glomeruli and the epithel of the proximal tube were not recognised by the anti-AQP4 monoclonal antibodies.

AQP4 expression has been detected in corn (data not shown) and bean roots from the early developmental

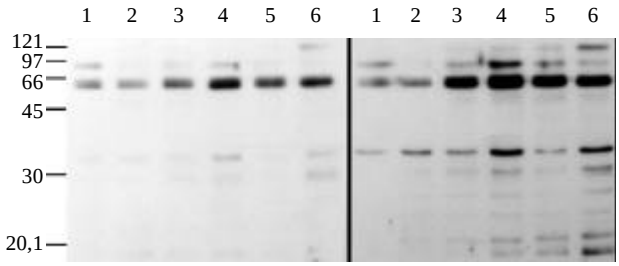


Figure 4. Western Blot analysis of AQP1 and AQP4 molecules in the membrane fraction of 1: VLSS positive control and membrane protein fractions of brain, 2: Hep-G2, 3: mouse brain, 4: rat brain, 5: mouse kidney, 6: rat kidney. The left block developed with anti-AQP1 (clone AQP 1/22, 1/A5) monoclonal antibody; the right block developed with anti-AQP4 (clone: AQP 4/18, 3/D2) monoclonal antibody.

stages. AQP4 is expressed in the cambium cells of the proliferating zone and in the forming primary phloem and primary xylem elements of the differentiation zone. In the absorption zone primary and secondary phloem and xylem elements do not stain with the anti-AQP4 antibodies, but the root hairs are intensely stained (data not shown). This labelling pattern of plant roots for AQP has never been published before. (*Figure 2d)*

Most tumors have been shown to exhibit high vascular permeability and high interstitial fluid pressure, but the transport pathways for water within tumors remain unknown. In breast carcinomas, AQP1 was localised in correlation with vascular structures.[6,14] The cell membranes of normal epithelial cells do not express AQP1, whereas the presence of AQP1 was described in the membranes of some tumor cells.[6]

We have examined AQP1 and AQP4 expression in the isolates of *in vitro* cultured standard tumor cell lines by flow cytometry. We did not find any cell surface AQP1 or AQP4 staining on these cell lines, which is consistent with the intramembrane situation of the peptide fragments used for immunisation. We have found intracellular AQP1 and AQP4 expression on some cultured cell lines. Hep-G2 (human hepatocellular carcinoma) cells and Caco-2 (human adenocarcinoma) cells express both AQP1 and AQP4, while Hep-2 (human epidermoid carcinoma) cells express only AQP4. Lymphoid cell lines like the human Jurkat cells and the murine Sp-2/0-Ag14 myeloma cells did not express any of the aquaporins (data not shown). In our experiments 73% of Hep-G2 cells express AQP1 and 95% of Hep-G2 cells express AQP4. The flow cytometric histograms show that the mean fluorescence value for AQP1 staining was 15.37, whereas in the case of AQP4 the mean fluorescence value was 24.31, which means that Hep-G2 cells express twice as much AQP4 as AQP1. (*Figure 3*.)

We have had two controls for the specificity of our results. Western blot analyses have been performed to verify the appropriate molecular weight of the positive immunoreactions and RT-PCR detection has proved the AQP1 and AQP4 expression at the mRNA level.

We aimed to detect AQP1 and AQP4 protein expression in different organs of rats and mice and in cultured Hep-G2 and Jurkat cells with dot-blot (*Table 3*) and Western Blot techniques. We have found that all anti-AQP1 and anti-AQP4 monoclonal antibody clones recognised AQP1 and AQP4 molecules from total, membrane- and cytoplasmic protein fractions at the appropriate molecular weight. Monoclonal antibodies stained mainly the H3-H6 domains resulting signals at 63 Kd. The bands above at 84 Kd were the H2-H6 domains, at 115 Kd size are the H1-H6 domains. The bands below at 30 Kd are due to the Le loop and the H6 domain. Lighter bands are degradation products of the H6 domain possi-

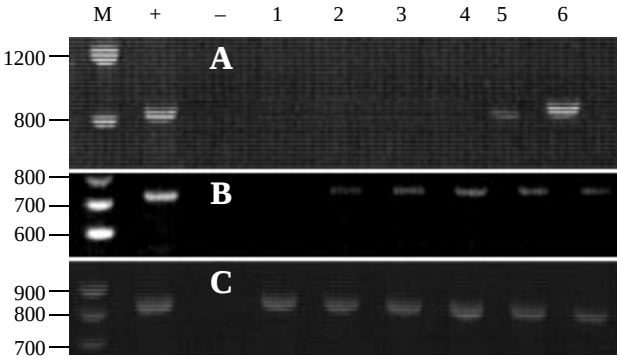


Figure 5. Gel electrophoresis of polymerase chain reaction with A: AQP1, B: AQP4, 3: β-actin. In all cases half of the reaction mixture (5 μl) was visualised under UV light on 2% agarose gels containing ethidium-bromide The sizes of the resulting amplicons are, A: AQP1: 807 bp, B: AQP4: 719 bp, C: β-actin: 825 bp. Samples are M: DNA standard ladder, +: positive control cDNA, - genomic control cDNA, 1: cDNA sample after 5 minutes from oedema inducing treatment, 2: 30 minute sample, 3: 60 minute sample, 4: 120 minute sample, 5: 240 minute sample

bly produced by ultrasonic treatment. (*Figure 4.)* Compared to the publications with polyclonal anti-AQP1 and anti-AQP4 sera, the bands were exceptionally specific, there was only minimal background staining and monoclonal antibodies stained significantly less fragmented bands.[2,8,24]

We aimed to measure AQP transcription, AQP mRNA levels in different tissues of known positive immunoreactions with our monoclonal antibodies. Our model of pathophysiology was alcohol induced gastric mucosal oedema.[3] With our monoclonal antibodies we have found AQP1 expression in strong correlation with mucosal capillary and AQP4 staining in the parietal cells of rat gastric mucosa and we have followed the changes during the oedema induction (data not shown).[3] AQP4 was mostly localised in the basal membrane of parietal cells in direct contact with the surrounding mucosal glands similarly to literature data.[3,8] We have compared the immunoserological and immunohistochemical data with the results of RT-PCR analysis.

We have found that both AQP1 and AQP4 mRNA levels were elevated during oedema development but there was a significant difference concerning the onset of the increase. AQP1 mRNA level has increased significantly later compared to AQP4 mRNA level, the former rising after only two hours compared to just a matter of minutes in the case of the latter. These relative changes are validated by the even -actin signals of all samples at a sensitive (exponential range) PCR cycle number. (*Figure 5.)* The kinetics follows our protein level results – but with an approximately one-hour time delay – to prove functionally the specificity of our monoclonal antibodies.

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