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Cloning and Functional Expression of a New Aquaporin (AQP9) Abundantly Expressed in the Peripheral Leukocytes Permeable to Water and Urea, but Not to Glycerol

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A new member (AQP9) of the aquaporin family was identified from human leukocytes by homology cloning using PCR. A full length clone was obtained by screening human liver cDNA library. AQP9 encodes a 295-amino-acid protein with the amino acid sequence identity with AQP3 (48%), AQP7 (45%), and other aquaporins (~30%), suggesting that AQP3, AQP7, and AQP9 belong to a subfamily of the aquaporin family. Injection of AQP9-cRNA into *Xenopus* oocytes stimulated the osmotic water permeability 7- folds with a low activation energy (4.2 kcal/mol) which was inhibited by 0.3 mM mercury chloride by 48 %. AQP9 also facilitated urea transport 4-folds. However, in contrast to AQP3 and AQP7, AQP9 did not stimulate the glycerol permeability, suggesting a unique permeability character. Northern blot analysis revealed the high expression of 3.5-kb messages in peripheral leukocytes ⩾ liver > lung = spleen, but not in thymus. The possible role of AQP9 in the immunological function of leukocytes is intriguing and the identification of AQP9 with unique permeability profile may expand our understanding of water and small solute transport in the body.

Key Words: **water transport; urea; leukocytes; liver; *Xenopus* oocyte.**

The human red blood cells have specialized transport pathways for the water and the urea permeations. The water pathway was identified as a water channel, aquaporin 1 (AQP1)[1]. The urea pathway was identified as the urea transporter B (UTb)[2]. However, the pathways for the water and the urea transports in the human leukocytes have not been identified. Because the leukocytes are subjected to the same osmotic gradients as are red blood cells during the circulation through the kidney medulla, it is possible that specialized water and urea transporters are also present in the leukocytes. Previous functional studies of leukocytes, lymphocytes, and macrophages examining the specialized water permeability are controversial. A relatively low osmotic water permeability with a high activation energy (13-18 kcal/mol) was reported for the human leukocytes and leukemic cells [3]. A similar result was also reported for human lymphocytes [4]. The high activation energy of the water transport speaks against the presence of a water channel. However, these studies were conducted more than 20 years ago and more thorough examinations using the recent advanced technology may be needed. More recent work with dog alveolar macrophages [5] reported the ratio of 3.1 for the osmotic water permeability coefficient (P_f) over the diffusional water permeability coefficient (P_d). The result is compatible with the presence of aqueous pore in the membrane.

In last five years, many water channels (aquaporins) which belong to the MIP family have been identified [reviewed in 6]. In mammals, nine members have been reported [reviewed in 1, 7]. AQP0 (originally named MIP26) is present exclusively in lens epithelium with minimum water permeability. AQP1 is present in many tissues including red blood cells, kidney, eye, lung, choroid plexus, bile duct, and vascular endothelium. AQP2 is solely present in the apical region of kidney collecting duct cells. AQP3 is present

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at the basolateral membranes of kidney, colon, trachea, urinary bladder, skin, and sclera of eye with permeability to glycerol and urea as well as water. AQP4 is also present at the basolateral membranes of kidney, colon, trachea, stomach, and at the plasma membranes of skeletal muscle, spinal chord, brain, and retina. The water permeability of AQP4 is mercury-insensitive, and this characteristics is shared only by AQP7. AQP5 is present at the apical membranes of exocrine tissues. AQP6 (originally named WCH3 or hKID) is present only in kidney with low water permeability [8]. AQP7 is present in testis, adipose tissue, kidney, and heart with the similar functional character as AQP3 [9, 10]. AQP8 is present in testis, liver, pancreas, placenta, and salivary gland [11, 12, 13].

As more than 23 MIP family proteins are identified in *Arabidopsis thaliana* [14], more members will be expected in mammals. We examined the presence of aquaporin family in leukocytes with PCR-based approach through which we have cloned several aquaporins [9, 11, 15, 16]. Here we report the cloning and the functional expression of a new aquaporin (AQP9) from human leukocytes and liver.

EXPERIMENTAL PROCEDURES

Reverse transcribed PCR. One microgram of human leukocytes total RNA was reverse-transcribed and used for PCR with 5 μM of a set of degenerative primers as previously reported [16]; sense strand, 5′-CAYATNAAYCCNGCNGT-3′, and antisense strand, 5′-AARTCNCKNGCNGGRTT-3′ (the abbreviation recommended by the IUPAC=IUB). The primers were derived from the consensus amino-acid sequences of the MIP family [6](His-Ile-Asn-Pro-Ala-Val, Asn-Pro-Ala-Arg-Asp-Phe, respectively). The PCR was conducted in the following profile: 94°C for 1 min, 46°C for 1 min, 72°C for 3 min for 30 cycles. The PCR products were ligated into TA cloning vector (PCRII, In Vitrogen, San Diego) and sequenced.

Screening the cDNA library. A random-primed human liver cDNA library in λgt 11 (cat # HL1115b, Clontech) was screened under a stringent condition (6×SSPE, 5×Denhardt's solution, 0.2%SDS, 100 μg/ml salmon sperm DNA, 50% formamide at 42 °C) with a PCR clone labeled with [α-^{32}P]dCTP (random priming; Amersham). Several positive clones (AQP9) were isolated, subcloned into Bluescript plasmid vector, and sequenced by a fluorescence DNA sequencer (Applied Biosystems 373A).

Expression of AQP9 in Xenopus oocytes. *Eco*RI fragment (1.3 kb) of AQP9 cDNA (containing open reading frame and the untranslated sequences) was blunt-end-ligated into the *Bgl*II site of a pSP64T-derived Bluescript vector containing 5′ and 3′ untranslated sequences of β-globin gene of *Xenopus* (pXBG-ev1; a generous gift from Peter Agre, Johns Hopkins University). The capped cRNA was synthesized using T3 RNA polymerase after a digestion with *Not*I to linearize the plasmid. The defoliculated *Xenopus* oocytes were injected with 50 nl of water or of AQP9 cRNA (10 ng) and incubated at 18 °C for 48 h in modified Barth's buffer.

Osmotic water permeability assay. Water permeability was measured as described before [16]. In brief, oocytes at stage V-VI were transferred from 200 mOsm to 70 mOsm of the modified Barth's buffer at 25 °C and the oocyte swelling was monitored by videomicroscopy. The coefficient of osmotic water permeability (P_f, μm/s) was calculated from the initial 15-s response of oocyte swelling as previously reported [16]. To examine the effect of mercury on oocyte P_f, oocytes were incubated in the Barth's buffer containing 0.3 mM $HgCl_2$ for 5 min before the assay. To determine the reversibility of the mercurial effect on the oocyte P_f, the oocytes were exposed to a reducing agent, 2-mercaptoethanol, at 5 mM for 15 min after the $HgCl_2$ treatment. The Arrhenius activation energy was calculated by measuring P_f at 5 and 25 °C.

Glycerol and urea uptake assay. The oocytes were incubated in the Barth's solution either with [^{14}C]glycerol (specific activity, 5.59 GBq/mmol; Amersham) or [^{14}C]urea (specific activity, 2.02 GBq/mmol; Amersham) at room temperature for 10 min. The oocytes were then rapidly washed four times with the ice-cold Barth's solution. The individual oocytes were lysed in 200 μl of 10% SDS solution overnight for the liquid scintillation counting.

Northern blots. The human multiple tissue Northern blots were obtained from Clontech (Palo Alto, CA). The filters were hybridized together under high stringency condition with a 2.9 kb AQP9 cDNA labeled by random priming with [α-^{32}P]dCTP. Each lanes has 2 μg of poly(A)$^+$ RNA from human tissues. The filters were washed in the high stringency condition.

RESULTS

Cloning of AQP9 cDNA and analysis of the amino acid sequence. We exploited the two highly conserved NPA boxes of MIP family proteins for designing a set of degenerative oligonucleotide PCR primers. The searches for new aquaporins from human leukocytes cDNA using these primers led to the identification of a new clone. As our preliminary Northern blot analysis using this PCR clone as a probe revealed its relatively high expression in liver, we screened a human liver cDNA λgt 11 library with the PCR clone as a probe. We obtained the almost full length clone with a polyadenylation signal and a poly (A)+ tail at the 3′ end. The cDNA clone of AQP9 (Fig. 1A) has 2,900 nucleotides. As the message of AQP9 was ~3.5 kb in Northern blot analysis, the clone may miss some 5′ noncoding end sequence. The translation initiation site was assigned to the first ATG triplet that is downstream of nonsense codons found in-frame. The cDNA consists of a 5′ untranslated region of 216 bp and a long 3′ untranslated region of 1799 bp. The 3′ non coding sequence contains a consensus polyadenylation signal (double underlined). An open reading frame encodes a protein of 295 amino acids with a relative molecular mass of 31.4 kDa. The hydropathy analysis predicts six transmembrane regions with N-terminus and C-terminus localized in the cytosol similar to other MIP family members (Fig. 1B). There is a N-linked glycosylation site (142-144 N-A-T) at the second extracellular loop. No potential consensus protein kinase A phosphorylation site is present in the predicted amino-acid sequence. There is a protein kinase C phosphorylation site (11-13 S-F-K), and a casein kinase II phosphorylation site (26-29 T-L-S-E) at

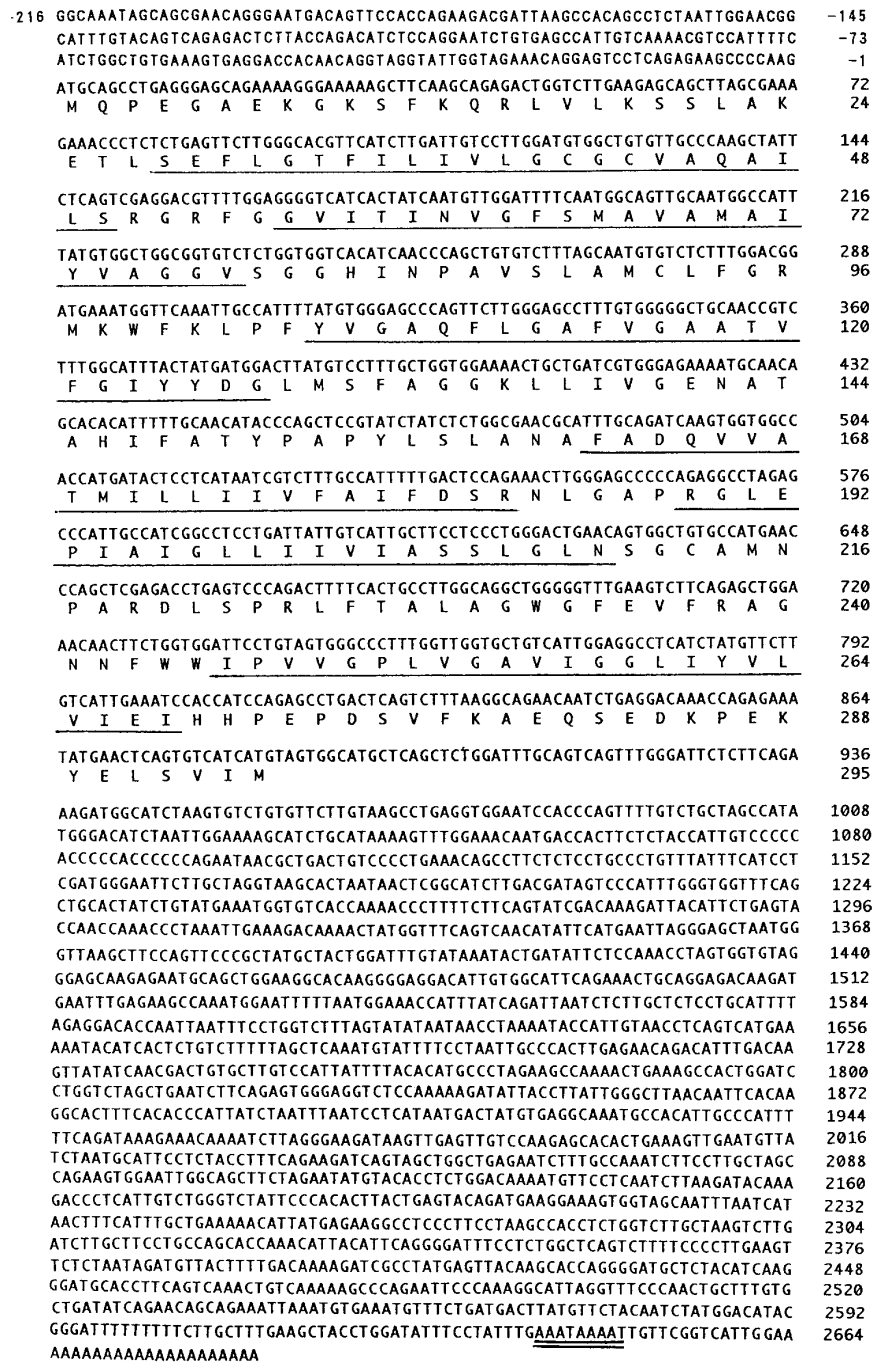

FIG. 1. Sequence analysis of human AQP9. **A,** Nucleotide sequence and deduced amino acid sequence of the clone isolated from a human liver cDNA library. Probable transmembrane domains are underlined. A polyadenylation consensus is double-underlined. **B,** Hydropathy analysis of deduced amino acid sequence using a 13-residue window [26]. The average local hydrophobicity at each residue was plotted on the vertical axis and the residue number on the horizontal axis. The probable transmembrane regions are numbered. **C,** Alignment of the amino acid sequences of *E. coli* GlpF (Acc. No: M55990), human AQP3 (Acc No: AB001325), human AQP7 (Acc. No: AB006190), and human AQP9. Gaps are inserted to maximize matching. White letters in black boxes denote the amino acid residues conserved at least two of them. The predicted transmembrane domains of AQP9 are underlined. The conserved NPA motifs are double-underlined.

the intracellular segment of amino-terminus. The searching the protein data base revealed highest amino-acid sequence identity with AQP3 (48%) and AQP7 (45%), but lesser identity with other aquaporins including AQP1 (33%). For comparison, AQP3, AQP7 and GlpF (glycerol facilitator of *E. coli*.) were

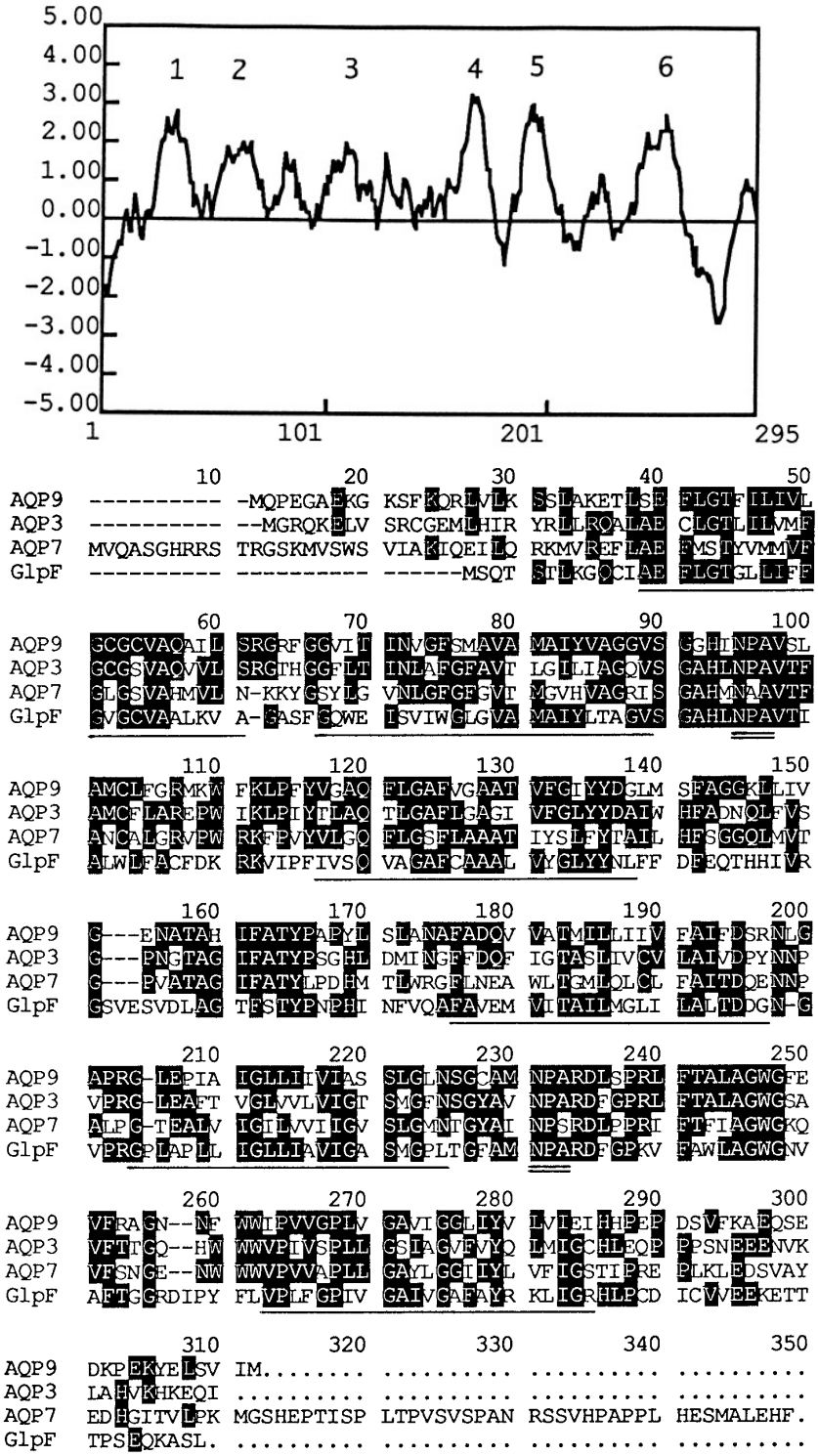

FIG. 1—*Continued*

aligned with AQP9 in Fig 1C. Two highly conserved areas (NPA boxes) are evident as previously indicated. The phylogenetic tree of ten mammalian aquaporins using Clustal method is shown in Fig. 2. The subgroup composed of three aquaporins (AQP3, AQP7, and AQP9) is evident.

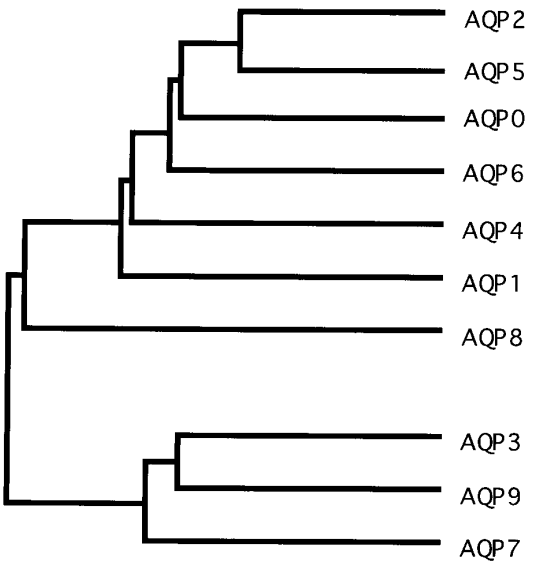

FIG. 2. Phylogenetic tree of the ten mammalian aquaporins demonstrating two subgroups (Clustal method).

The functional characterization of AQP9. We examined the function of AQP9 when expressed in *Xenopus* oocytes. The osmotic water permeability coefficient (P_f) of AQP9-cRNA(10 ng)-injected oocytes was 7 times higher than P_f of water-injected oocytes at 25 °C (Fig. 3A)(123±7μm/s; n=10 vs. 18±3μm/s; n=12, mean ±SEM). The induction was slightly lower than the levels observed in other aquaporins. The stimulated water permeability was inhibited by 0.3 mM $HgCl_2$ by 48% (64±6μm/s; n=10). The effect of $HgCl_2$ was not the toxic effect as its effect was reversed by a reducing agent, 5 mM of 2-mercaptoethanol (108±6μm/s; n=18). As AQP9 has cysteine residue at three residues prior to the second NPA sequence similar to AQP1, this Cys-213 may be responsible for mercury inhibition as demonstrated in AQP1 [17, 18]. To examine the temperature dependency of P_f, P_f at 5 °C and 25 °C were measured (n=12 each). The determined activation energy from the Arrhenius equation of P_f was 4.2 kcal/mol, a value in the range expected for a water channel.

As AQP3 transport glycerol [16, 19, 20] and urea [16, 21], we examined the glycerol and the urea transports in the AQP9-expressing oocytes. The oocytes were incubated in Barth's buffer containing 92 KBq/ml of [^{14}C]glycerol. Surprisingly, AQP9 cRNA (10 ng) injection did not stimulate the glycerol uptake (Fig. 3B)(3.7±0.8×10^{-6} cm/sec; n=9 in AQP9 vs. 3.4±0.6 ×10^{-6} cm/sec; n=10 in control), which was in contrast to the previous reports of the glycerol uptake by AQP3 [16, 19, 20] and AQP7 [9, 10]. The result was confirmed by repeated study with different batches of oocytes. On the other hand, the urea uptake was stimulated by AQP9 expression (Fig. 3B). The incubation in 22.6 μM[^{14}C] urea for 2 min resulted in the increase of the urea uptake through AQP9 by 4-folds (5.5±0.8 ×10^{-6} cm/sec; n=12 in AQP9 vs. 1.3±0.3 ×10^{-6} cm/sec; n=12 in control). The degree of stimulation of urea uptake by AQP9 was higher than that of AQP3 [16, 20] but lower than that of AQP7 [9, 10]. Thus, AQP9 is a unique aquaporin which permeates water and urea, but not glycerol.

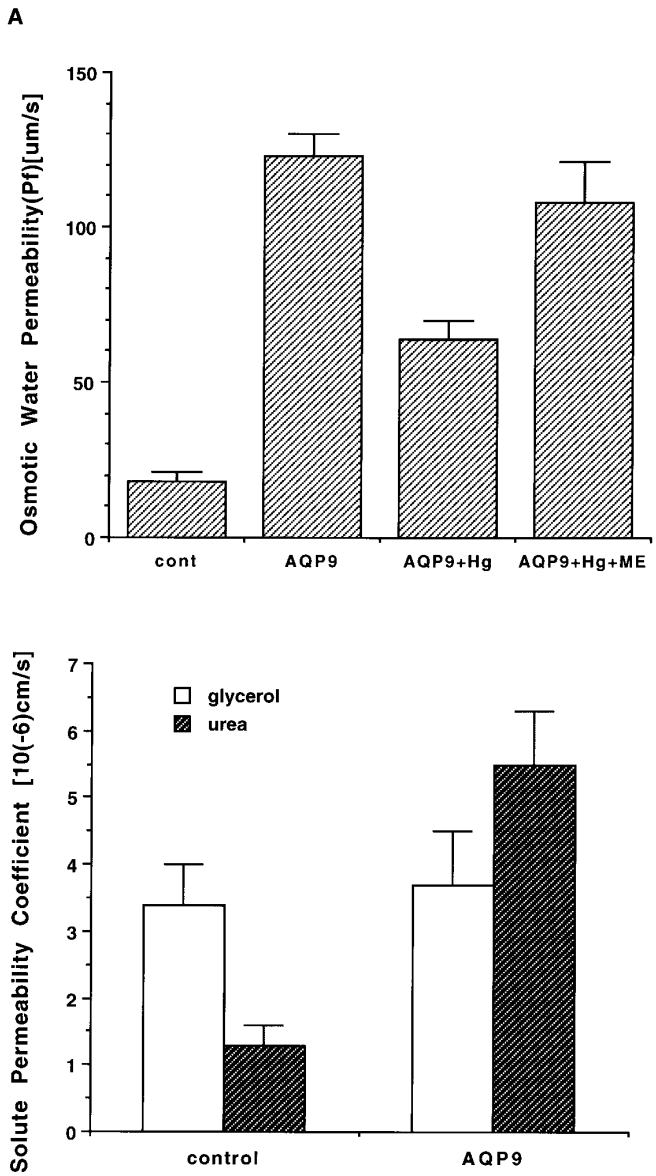

FIG. 3. Functional expression of AQP9 in *Xenopus* oocytes. **A,** Osmotic water permeability (Pf) of oocytes injected with 50 nl of water or 10 ng of AQP9 cRNA. Bars show mean±SEM. Hg indicates that the assay was performed after 5 min incubation in 0.3 mM mercury chloride. ME indicates the another incubation in 2-mercaptoethanol (5mM) for 15 min. **B,** The permeability coefficients of glycerol and urea determined by [^{14}C]glycerol and [^{14}C]urea uptake into the oocytes injected with water (control) or 10 ng AQP9 cRNA(AQP9).

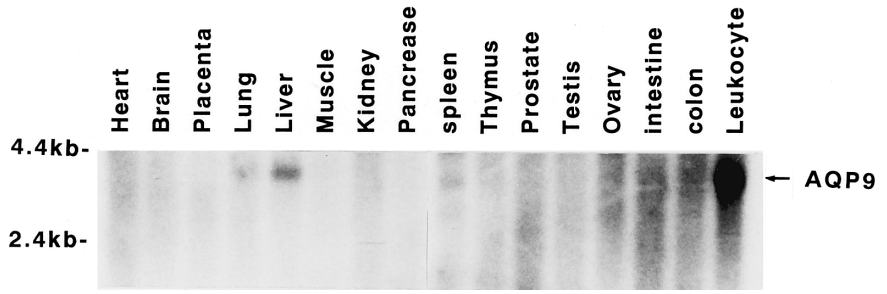

FIG. 4. Northern blot analysis of AQP9. 2 μg poly(A)$^+$RNA from various human tissues (Clontech) was hybridized with ^{32}P-labeled probe of AQP9 cDNA (Fig. 1A). The positions of the RNA markers (kb) are indicated.

The tissue distribution of AQP9 expression. Northern blot analysis revealed that the AQP9 mRNA (~3.5 kb) was expressed as a single band predominantly in peripheral leukocytes (Fig. 4). AQP9 was also expressed in liver at lower amount, and in lung and spleen with much lower amount. However, the other leukocytes-abundant tissues such as thymus and intestines, did not express AQP9.

DISCUSSION

We have cloned a new water channel (AQP9) from human leukocytes and liver. AQP9 has the sequence similarity to AQP3 and AQP7 (Fig. 2). Thus, AQP3, AQP7, and AQP9 belong to a new subfamily in aquaporins. On the other hand, AQP0, 1, 2, 4, 5 and 6 have amino acid homology with each other in the order of 40-50% and belong to the other subfamily (Fig. 2). AQP8 may belong to the latter subfamily although it has a lower similarity (Fig. 2). The latter group seems to be selective for water permeation, while the former group seems to be less selective and permeable to small neutral solutes such as glycerol and urea. Among the former group, AQP9 is functionally unique in that it has no glycerol permeability while AQP3 and AQP7 have a high glycerol permeability. As the molecular size of glycerol is slightly larger than that of urea, the pore size of AQP9 may be narrower than those of AQP3 and AQP7. Alternatively, the glycerol permeability requires some glycerol binding site which is missed in AQP9. The mutagenesis and swapping experiments may reveal responsible residues for glycerol permeation. This unique permeation profile suggests that the pore structure of aquaporins is heterogeneous and invites further investigations.

The previous results on water permeability of leukocytes and hepatocytes have been controversial. The recent examination of rat hepatocyte water permeability concluded the absent of a functional water channel in hepatocytes and the absence of messages of AQP1 and AQP4 [22]. However, a previous study with rat hepatocytes reported the presence of a functional water channel [23]. Moreover, the recently cloned AQP8 was localized at hepatocytes shown by in situ hybridization [12]. The present study also revealed the presence of another aquaporin in liver. Therefore, a thorough examination of water channel function in liver is necessary. As AQP9 permeates urea, it may function as urea channel [24] in liver which produces abundant urea. The previously cloned two urea transporters (UTa and UTb) are not expressed in liver [2]. Third type of urea transporters is expected to be present in liver. Whether AQP9 is the major transporter of urea in hepatocytes remains to be clarified, although its expression in liver is not impressive in Northern blot analysis (Fig. 4). The role of AQP9 in leukocytes is more speculative. The physiological significance of AQP1 at human red blood cells is totally unknown as Colton-null people who have no functional AQP1 show no phenotypic changes [25]. The identification of another aquaporin in blood cells, namely leukocytes, may provide some clue to the role of aquaporins in blood cells. Alternatively, AQP9 may have some roles in some specialized leukocyte functions such as immunological response and bactericidal activity. Such functions of the aquaporins are intriguing and must be tested in future studies.

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