Antigenic Epitopes on Human P-glycoprotein Recognized by Autoimmune Hepatitis Autoantibody as a Case Study

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Multiple drug resistant protein 1 (MDR1), P-glycoprotein, plays a role in the blood-brain barrier, preventing drug distribution into the brain, and in cancer chemotherapy. MDR1 is composed of two repeated fragments and there are six transmembrane domain (TMD) on the N-terminal of each repeat and a nucleotide-binding domain (NBD) on the C-terminal. We reported that sera from autoimmune hepatitis patients well reacted with MDR1 by enzyme-linked immuno-sorbent assay (ELISA) [Shinoda *et al*., (2004) *Autoimmunity*, **37**, 473–480]. In this study, to determine antigenic sites, peptides on the extra-cellular loop (ECL), TMD and NBD of MDR1 were applied to ELISA with sera from 4 autoimmune hepatitis (AIH) patients and 4 normal individuals. The results showed that serum from Patient 3 reacted well with peptide 314–328 and weakly with peptide 957–971. Meanwhile, serum from Patient 4 reacted well with peptide 850–857 and weakly with peptide 741–755 and 957–971. All the five peptides reacted with sera from Patients 3 and 4 were located on ECL. Normal sera did not react with those peptides and the reactions of sera from Patients 1 and 2 were marginal. Sera from 4 patients and normal individuals did not react with peptides of TMD and NBD. These results suggest that some ECL on MDR1 play a role of antigenic determinants, and TMD and NBD do not. Personal specificity and diversity of antibodies from the AIH patients (such as Patients 3 and 4) against antigenic determinant were found.

Key words —— multiple drug resistant protein 1, autoimmune hepatitis, autoantibody, P-glycoprotein

INTRODUCTION

Autoimmune hepatitis (AIH) is a disease of unknown etiology characterized by hypergammaglobulinemia, the presence of liver-related autoantibodies,[1] and a good response to immunosuppressive treatment. The current classification of AIH and several autoantibodies/target autoantibodies found in this disease have been reported previously. AIH is subdivided into two major types: AIH type 1 (AIH-1) and AIH type 2 (AIH-2). AIH-1 is characterized by the detection of smooth muscle autoantibodies (SMA) and/or antinuclear antibodies (ANA). Antineutrophil cytoplasmic autoantibodies (ANCA), antibodies against the asialoglycoprotein receptor (anti-ASGP-R) and antibodies to soluble liver antigens or liver-pancreas (anti-SLA/LP),[2–4] which binds selenocysteine tRNA,[5,6] may be useful for the identification of individuals who are seronegative for ANA/SMA. AIH-2 is characterized by the presence of specific autoantibodies against liver and kidney microsomal antigens (LKM), which contain drug-metabolizing enzymes, and/or autoantibodies against liver cytosol 1 antigen. There have been several reports about autoantibodies against drug-metabolizing enzymes in AIH-2 sera.[7] Eukaryotically expressed cytochrome P450 (CYP) 2D6 is the universal target of LKM-1 in AIH-2 and chronic hepatitis C (HCV) infection.[8] CYP2D6 is considered an immunodominant epitope and target of virus/self cross-reactivity in LKM-1 autoantibody-positive liver disease.[9] An autoantibody against CYP2D6 has been reported[10–15] and is located on the cellular surface.[16,17] We reported the presence of autoantibodies against various drug-metabolizing enzymes in AIH sera.[18]

Multiple drug resistant protein 1 (MDR1) is the most valuable transporter among the phase III transporters, such as CYP3A4 among the phase I drug-metabolizing enzymes.[19] Human MDR1 pumps a wide variety of cytotoxic compounds out of the cell, and many therapeutic compounds are also substrates

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of MDR1. The drug efflux transporter MDR1 was originally discovered by its ability to confer multidrug resistance to tumor cells. Observations have confirmed that many tumor cell lines overexpressing MDR1 exhibit reduced intracellular drug concentration secondary to enhanced drug efflux. The main role of MDR1 is that of a transporter of drug excretion, and it acts in the liver, excreting large compounds. In the liver, organic anion transporting peptides (OATPs) mainly pump drugs from blood to hepatocytes, and those drugs in the hepatocytes are excreted by MDR1, multidrug resistance associated protein (MRP)2/3 and bile salt export pump (BSEP) to bile. Meanwhile, in the kidney, some organic anion transporters (OATs) and organic cation transporters (OCTs) act to excrete small compounds from blood to urine. MDR1 also plays a role in the blood-brain barrier, preventing drug distribution in the brain, and also acts to excrete various drugs into the intestinal lumen. MDR1 has a nucleotide binding domain (NBD) and uses adenosine triphosphate (ATP) to excrete drugs, and the ATPase activity of MDR1 is increased by drug binding and inhibited by vanadate. Single-point mutations at the key lysine residue in Walker A and at the key aspartate in Walker B abrogate, as assessed, ATPase activity, by the vanadate-induced trapping of nucleotides.[19)]

MDR1 is composed of two repeated fragments and there are six transmembrane domains (TMDs) on the N-terminal of each repeat and an NBD on the C-terminal. These two repeats are dependent and cooperate as one functional molecule having one pocket for excreting drugs. The twelve TMDs form a funnel facing the outside of cells, and NBD is contained in cytosol as a dimer. One NBD is composed of Walker A, Q-loop, ATP-binding cassette (ABC)-signature and Walker B for phosphate binding of nucleotide. This tertiary structure of MDR1 is suggested from the structure of NBD of histidine permease (HisP), clarified by X-ray crystallography.[20)] On the model of HisP, the NBD positions described above make a functional domain, and the same NBD structure is found on many other ABC transporters. An experiment with *mdr1* gene knockout mice showed the high plasma area under the curve (AUC) of drugs in mdr null mice [*mdr1a*(−/−)] and a high level in the brain, indicating that *mdr1* has an efflux function (prevention of absorption) in the intestinal lumen and acts as a drug uptake barrier in the brain, as well as having the function of urinary and biliary drugs excretion.[21−23)] The transcription of MDR1 is dependent on two sites, the promoter site (−105/−100)(−245/−141) and the enhancer site (−7864/−7817).[24, 25)] The rough three-dimensional structure of MDR1 with a resolution of 8Å was reported and the transmembrane regions adopted an asymetric configuration in the nucleotide-bound state.[26)] We reported the presence of autoantibodies against MDR1 in AIH sera.[18)]

In this report, we show antigenic epitopes on MDR1 recognized by autoantibodies in the sera of two out of four patients with AIH-1.

MATERIALS AND METHODS

Sera from AIH Patients —— The four samples of AIH type 1 sera used, numbered 1, 2, 3 and 4, corresponded to female patients aged 57, 50, 63 and 48 years old, respectively. These four sera having high level of aminotransferase, hypergammaglobulinemia and ANA of key AIH-1 indicator (autoantibody) were supplied from Dr. T. Matsufuji of Jikei University in Tokyo. In four AIH-1 sera, serum from patient 1 indicated Sjogren syndrome with AIH of double pathology, and serum from patient 2 indicated Hashimoto disease of double pathology. Patients 3 and 4 showed only AIH symptom of ANA positive. Patients 1, 2 and 4 had been treated with a corticosteroid, prednisolone. Sera from Patients 3 and 4 also contained anti-tRNA antibodies. All four patients were negative for hepatitis viruses B and C. Permission to use the sera was given by each patient in 1995. We also used sera from healthy volunteers as a control: numbered 7–10, from women aged 59, 52, 50 and 55, respectively. Our four AIH-1 patients are female and about 50 years old. Therefore we selected four healthy volunteers of female and the same years old.

Enzyme-linked Immuno-sorbent Assay (ELISA) for the Estimation of Autoantibody Levels in Sera —— ELISA for the estimation of autoantibody levels in sera was performed using the standard method.[27)] Peptides were supplied from Thermo Electron GmbH, Ulm, Germany. Those sequences are shown in Table 1 and the positions on MDR1 are shown in Fig. 1. These peptides were dissolved in 0.1 M $NaHCO_3$ at pH 9.8 at 10 mg/ml. 50 µl antigen solution at 2.5 µg/ml in 0.1 M $NaHCO_3$ was added to each well of the flexible polyvinyl chloride assay plate (Falcon 353912; BD Biosciences, Cowley, U.K.), and the plate was left overnight at 4°C. The plate was then washed 3 times with phosphate-buffered saline (PBS) con-

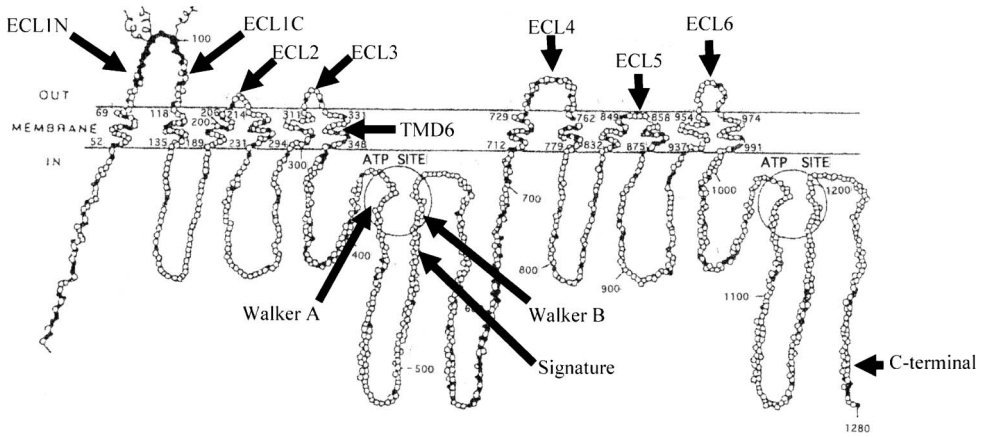

Fig. 1. Secondary Structure Model of MDR1 on the Membrane. ATP Site is a Complex of Walker A and Walker B

Table 1. Peptides used in This Study

Name	Position	Sequence
ECL1N	71–85	VFGEMTDIFANAGNL
ECL1C	104–116	FMNLEEDMTRYAY
ECL2	207–214	GFTRGWKL
ECL3	314–328	FWYGTTLVLSGEYSI
TMD6	335–346	FFSVLIGAFSVG
Walker A	427–434 (1070–1077)	GNSGCGKS
Signature	530–538 (1175–1183)	QLSGGQKQR
Walker B	543–551 (1188–1196)	RALVRNPKI
ECL4	741–755	RIDDPETKRQNSNLF
ECL5	850–857	SFIYGWQL
ECL6	957–971	FRFGAYLVAHKLMSF
C-terminal	1257–1270	HQQLLAGKGIYFSM

taining 0.05% Triton X-100, 50 μl of blocking solution (1% bovine serum albumin) was added and the plate was left for 30 min. The blocking solution was discarded and the plate was washed 3 times with PBS containing 0.05% Triton X-100. Human serum (50 μl) of a primary antibody was added sequentially at a dilution of 1/2, starting from 1/5000. The plate was incubated at 37°C for 30 min and then washed. The secondary antibody (goat) conjugated with a horseradish peroxidase (HRP) against human immunoglobulin G was used after 10000-fold dilution and 50 μl was added to each well. The plate was left for 30 min at 37°C and then washed 5 times with PBS. The level of the secondary antibody bound in each well was detected by a color reaction with a solution (100 μl) containing *0*-phenylenediamine (1 g/l) and 30% H_2O_2 (1 ml/l) in 0.1 M citrate buffer at pH 5.2. The colored plate was measured with an ARVO1420 plate reader at 492 nm. In the study, no coloring at dilution point $(1/2)^{12}$ was observed for sera from all the four healthy volunteers, while coloring, $OD_{492} > 0.05$, was observed for sera from some AIH patients. Thus, when OD_{492} at dilution point $(1/2)^{12}$ is higher than 0.05, the sera are defined "positive" to the peptide tested. We previously reported adsorption of protein to glass surfaces *etc.* as a monolayer.[28] According to the result, the adsorption amount of the peptides in this study was estimated ca. 50 ng/well. Although solubility was different among the peptides, the adsorption amount to the well surface would be an almost same level of 50 ng/well.

Measurement of MDR1 Activity —— MDR1 activity was generally measured by determination of the concentration of inorganic phosphate generated from ATP hydrolysis with the standard molybdate method[29] or the continuous cycling method,[30] under conditions without ATP decomposition. MDR1 consumes ATP when its substrate binds to the active site and liberates inorganic phosphate. In this report, the radioactivity of [^{32}P]inorganic phosphate was separated in the supernatant from [^{32}P]ATP, which is adsorbed onto charcoal by centrifugation.[31] The reaction mixture (20 μl) contained 2.5 μg MDR1 membrane protein (Gentest, MA, U.S.A.) and 1 mM [^{32}P]ATP (1 Ci/mol) (Institute of Isotopes Co. Ltd., Budapest, Hungary) in a buffer (pH 7.4) made of 50 mM 2-amino-2-hydroxymethyl-1,3-propanediol(Tris)-2-(N-morpholino)ethanesulfonic acid (MES), 2 mM ethylene glycol-bis(*β*-amino ethylether)-*N*, *N*, *N′*, *N′*-tetraacetic acid tetrasodium (EGTA), 50 mM KCl, 2 mM 1,4-dithiothreitol and 5 mM NaN_3. The reaction was started by the addition of verapamil at a final concentration of 20 μM. The mixture was incubated for 30 min at 37°C. For the inhibition

experiment, anti-serum was added to the reaction mixture and kept in an ice bath for 10 min before the addition of verapamil. For standard inhibition, vanadate was added at a concentration at 0.5 mM before the addition of verapamil. The reaction was stopped by the addition of 40 μl of 10% charcoal (Norit A) in 0.1 M phosphate buffer, followed by centrifugation at 8000 rpm. Charcoal binding ATP was removed by this centrifugation and the supernatant containing ^{32}P-inorganic phosphate was applied to a filter paper and dried. The radioactivity of ^{32}P on the filter paper was counted by Cherenkov or analyzed using the radio-image analyzer Fuji BAS2500.

RESULTS

We specified antigenic epitopes on MDR1 against sera from AIH patients by ELISA. The reactivity of sera from 4 AIH patients and normal volunteers were tested against 12 selected peptides on MDR1, as shown in Fig. 1 and Table 1. Each peptide was selected as a structurally characteristic domain, such as extra-cellular loop (ECL), TMD, Walker A, Signature, Walker B and the C-terminal side, known as an antigenic site for the preparation of antiserum in animals. Figure 2 shows titration patterns for autoantibodies in Patient 3 against the twelve peptides. In Fig. 2(A), autoantibodies in Patient 3 reacted well with peptide ECL3 (314–328), moderately with peptide ECL2 (207–214) and TMD6 (335–346), and weakly with Signature. Other peptides, such as ECL1N, ECL1C, Walker B and C-terminal, did not react with serum from Patient 3 as shown in Fig. 2(B). Figure 2(C) also shows the titration patterns for Patient 3 serum against Walker A, ECL4, ECL5, and ECL6. These results are summarized in Table 2.

Figure 3(A) shows titration patterns for autoantibodies in Patient 4 against the twelve peptides. ECL4 (741–755), ECL5 (850–857), and ECL6 (957–971) reacted well with autoantibodies in Patient 4. However Walker A did not react. On the other hand, reactivity was low against peptide ECL1N (71–85), ECL1C (104–116), Signature (530–538), Walker B (543–551), ECL4 (741–755), and extremely low against peptide C-terminal (1257–1270), as shown in Fig. 3(B).

Figure 4 shows titration patterns for sera from AIH patients and normal individuals against peptide ECL3 (314–328). The serum of Patient 3 well

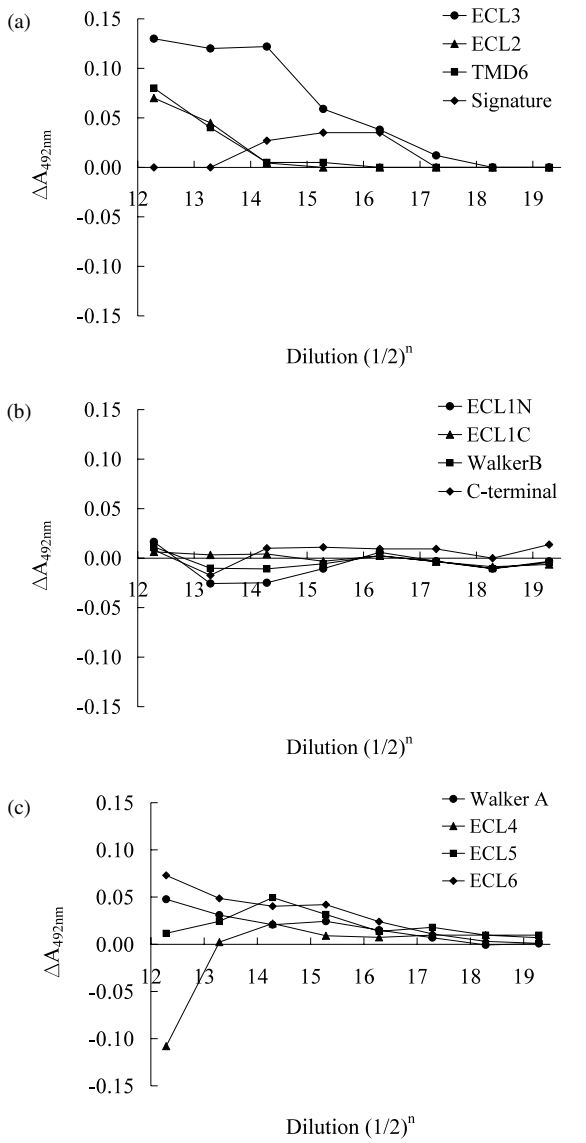

Fig. 2. Titration Patterns of Anti-MDR1 in Patient 3 with Some Peptides by ELISA

A shows the patterns of ECL2, ECL3, TMD6, and Signature. B shows the patterns of ECL1N, ECL1C, Walker B, and C-terminal. C shows the patterns of ECL4, ECL5, ECL6, and Walker A.

reacted with ECL, but there was no other reaction. As shown in Table 2, the reactivity of sera from AIH patients and normal volunteers against ECL1N were marginal. Sera from all four AIH patients reacted with some of the peptides tested. On the other hand, sera from normal individuals did not react against these peptides at all. Sera from Patients 1 and 2 weakly reacted with a few peptides, whereas sera from Patients 3 and 4 reacted well with some peptides. The primary reactions for Patients 3 and 4 were ECL3 and ECL5, respectively, and serum from Patient 4 weakly reacted with ECL4 and ECL6.

Table 2. Summary of ELISA for Antigenic Epitope on MDR1 for AIH Autoantibody

Serum	Peptide							
	ECL1N 71	ECL1C 104	ECL2 207	ECL3 314	TMD6 335	Walker A 427	Signature 530	Walker B 543
Patient 1	−[a)]	−	−	±	−	−	−	−
Patient 2	−	±	−	−	−	−	−	+
Patient 3	−	−	±	+++	±	−	−	−
Patient 4	±	±	−	±	−	−	−	−
Normal 7	−	−	−	−	−	−	−	−
Normal 8	−	−	−	−	−	−	−	−
Normal 9	−	−	−	−	−	−	−	−
Normal 10	−	−	−	−	−	−	−	−

Serum	Peptide						
	ECL4 741	ECL5 850	ECL6 957	Walker A 1070	Signature 1175	Walker B 1188	C-terminal 1257
Patient 1	−	−	−	−	−	−	−
Patient 2	−	−	−	−	−	+	−
Patient 3	−	+	++	−	−	−	−
Patient 4	++	+++	++	−	−	−	−
Normal 7	−	−	−	−	−	−	−
Normal 8	−	−	−	−	−	−	−
Normal 9	−	−	−	−	−	−	−
Normal 10	−	−	−	−	−	−	−

a) −: the difference of the values at A_{492} between sample (+antigen) and blank (−antigen) is < 0.05, ±: ~ 0.05, +: 0.05–0.10, ++: 0.10–0.15, +++: 0.15–0.20.

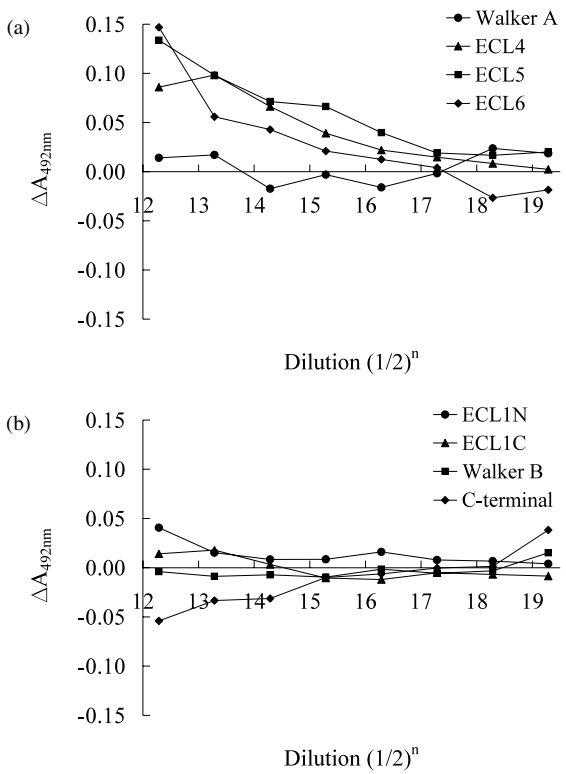

Fig. 3. Titration Patterns of Anti-MDR1 in Patient 4 with Some Peptides by ELISA

A shows the patterns of ECL4, ECL5, ECL6, and Walker A. B shows the patterns of ECL1N, ECL1C, Walker B, and C-terminal.

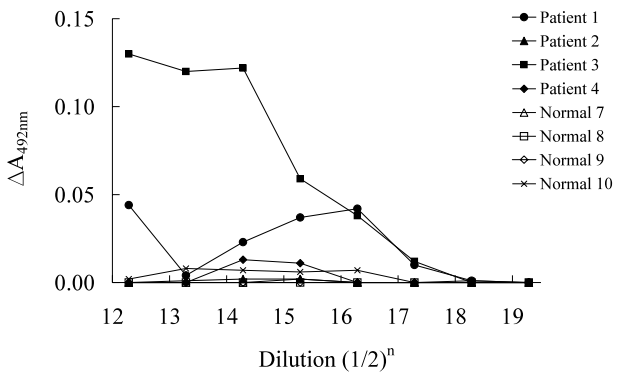

Fig. 4. Titration Patterns of Anti-MDR1 with Sera from 4 AIH and 4 Normal Volunteers against ECL3 by ELISA

In order to specify a significant region on ECL3 for autoantibodies in Patient 3, further divided peptides were tested in ELISA (Table 3). As shown in Fig. 5, the C-terminal portion of ECL3 (314C) reacted well with serum from Patient 3, compared with the result with the N-terminal of ECL3 (314N). For further investigation, three synthetic peptides (Table 3), each with two contiguous alanine substitutions across the length of ECL3, were employed in ELISA. As shown in Fig. 6, peptides 314CM1 and 314CM2, which have the alanine substitutions at amino acids (aa) 323–324 and aa 325–326 re-

Table 3. The Sequences of Mutants of MDR314 (ECL3) and MDR850 (ECL5)

Name	Sequence
ECL3 (314–328)	
314wt	FWYGTTLVLSGEYSI
314N	FWYGTTLV
314C	VLSGEYSI
314CM1	VL**AA**EYSI
314CM2	VLSG**AA**SI
314CM3	VLSGE**AA**I
ECL5 (850–857)	
850wt	SFIYGWQL
850A1	**AA**IYGWQL
850A3	SFI**AA**WQL
850A5	SFIYG**AA**L

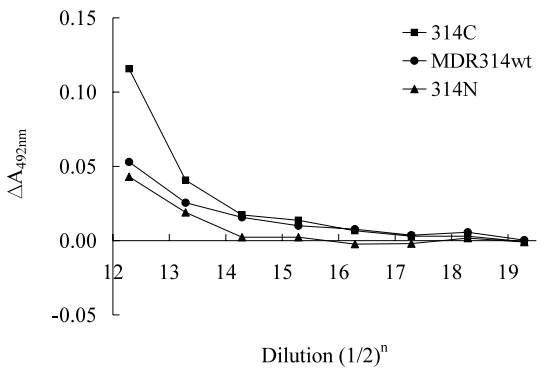

Fig. 5. Titration Patterns of Anti-MDR1 in Patient 3 with N-terminal or C-terminal Peptides of ECL3 by ELISA

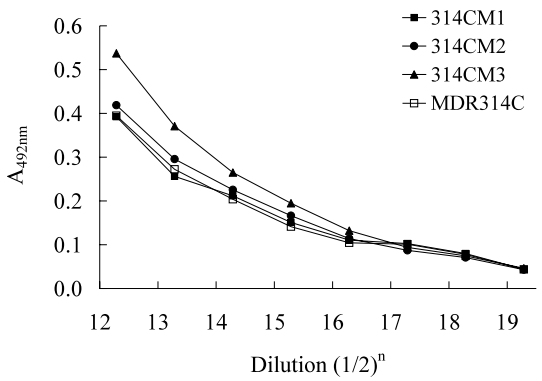

Fig. 6. Titration Patterns of Anti-MDR1 in Patient 3 with Some Peptides Derived from ECL3 by ELISA

spectively, weakly reacted with serum from Patient 3 compared to 314CM3. These findings suggest that the antigenic epitope is aa 323–325, Serine-Glycine-Glutamic acid (Ser-Gly-Glu). Meanwhile, as shown in Table 2, serum from Patient 4 reacted

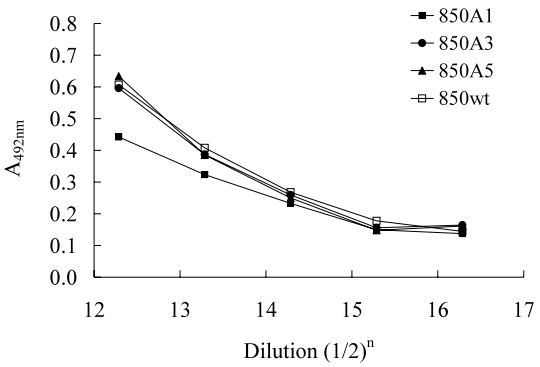

Fig. 7. Titration Patterns of Anti-MDR1 in Patient 4 with Some Peptides Derived from ECL5 by ELISA

well with ECL5. The mutant peptides for ECL5 were constructed by two contiguous alanine substitutions as shown in Table 3. Figure 7 shows that peptide 850A1, which has the alanine substitutions at aa 850–851, weakly reacted compared to 850A3 and 850A5. This suggests that aa 850–851 Serine-Phenylalanine (Ser-Phe) in ECL5 is an antigenic epitope.

We investigated the inhibitory effect on MDR1 activity of sera from 4 AIH patients and 4 controls. MDR1 activity was determined by measuring the release of inorganic phosphate from ATP using verapamil as a substrate. However, we could neither detect inhibition of MDR1 activity by sera from AIH patients nor find any significant difference in inhibition of MDR1 activity between AIH patient and control sera (data not shown). This may be because the antigenic epitope on ECL of MDR1 is not related with its activity. The active site is present on NBD, the inner site of MDR1, when the drug is exposed, and AIH sera did not react with NBD regions, such as Walker A, Signature and Walker B.

DISCUSSION

Our previous study showed that sera from autoimmune hepatitis patients well reacted with MDR1 by ELISA.[18] In this study, we identified antigenic sites on MDR1 recognized by autoantibodies from four AIH patients. The results showed that serum from Patient 3 reacted well with peptide ECL3 and weakly with ECL6. Meanwhile, serum from Patient 4 reacted well with peptide ECL5 and weakly with peptide ECL4 and ECL6. Normal sera did not react with those peptides, and the reactions of sera from Patients 1 and 2 were marginal. Sera

from 4 patients and normal individuals did not react with peptides of TMD and NBD. These results clarified that some ECL on MDR1 play the role of antibodies in patients (such as Patients 3 and 4). This kind of specificity and diversity of autoantibodies in patients was also observed in the antigenic epitope on CYP2D6 against sera from AIH type 2 patients.[9)]

Every epitope on MDR1 against sera from AIH patients existed on ECL, and are thought to play an important role in constructing an exit for substrates. MDR1 is present in various human tissues, such as the liver, intestine and brain. So far, the localization of MDR1 in these cell tissues has been clarified as shown in Fig. 8. MDR1 is localized on the biliary canalicular front of hepatocytes and on the brush border membrane of intestine enterocytes. On the other hand, MDR1 localized on the endothelial cells of the brain capillary faces blood. Thus, lymphocytes are accessible to the ECL region of MDR1 expressed on the brain capillary. Under topological circumstances, the immune system in AIH patients should recognize MDR1 in the brain capillary, not in the liver or intestine, followed by the generation of autoantibodies. Our finding that MDR1 epitopes against autoantibodies exist on ECL coincides with the topology of MDR1, though it is possible that the auto-immune system in AIH patients recognizes MDR1 in unknown tissues where MDR1 exist. MDR3 (ABCB4) has a similar epitope, described later, but its expression is low in the brain, where the immune system recognizes it as an autoantigen.[32)]

In the above section, we discussed the distribution of MDR1 on the plasma membrane of cells. However, recently, some reports have been published that MDR1 is predominantly intracellular, largely in Golgi vesicles and Golgi cisternae of the site of glycosphingolipid synthesis, and not in the plasma membrane.[33)] Thus, it was reported that

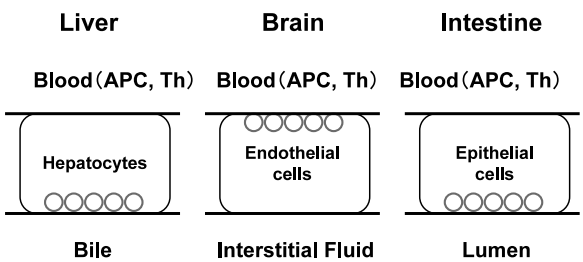

Fig. 8. Position of MDR1 in Liver, Brain, and Small Intestine Cells
Circles show position of MDR1.

MDR1 plays the role of glucosylceramide translocase within the Golgi complex and functions as a glucosylceramide translocase, flipping glucosylceramide to the inner Golgi surface. Meanwhile, it has been reported that some glucosylceramide inhibitors failed to cause any reversal of multidrug resistance, despite depleting glycolipids to some extent.[34)]

MDR1 is a member of the ATP-binding cassette superfamily of transporters, and inevitably, the degree of homology of amino acids at the ATP-binding cassette, such as Walker A and Walker B, is high among ABC transporters. Meanwhile the degree of homology on the extra-cellular loop is low compared to on the ATP-binding cassette. Our experiments with mutant peptides suggested that the key amino acids of MDR1 for the recognition of autoantibodies should be Ser-Gly-Glu of 323–325 on ECL3 for Patient 3, and Ser-Phe of 850–851 on ECL5 for Patient 4. From an amino acid sequence homology search on the Swissprot database (http://www.ncbi.nlm.nih.gov/BLAST), MDR3 (ABCB4) and ABCB11 (bile salt export pump) were found as ABC transporters with moderate homology (identities 11/15) against the ECL3 region of MDR1; however, the key amino acid of Ser-Gly-Glu on ECL3 of MDR1 is not retained in either MDR3 or ABCB11. Therefore, ECL3 on MDR1 would be a specific region for autoantibodies from Patient 3. On the other hand, amino acids on ECL5 are identical between MDR1 and MDR3, suggesting that autoantibodies from Patient 4 could bind to both MDR1 and MDR3. In humans, MDR3 encodes a subtype of P-glycoproteins as well as MDR1.[35,36)] Deleuze *et al.* reported that MDR3 plays an important role in the biliary secretion of phospholipids and that the MDR3 defect leads a subtype of progressive familial intrahepatic cholestasis due to a low level of phospholipids in bile[37)] (http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html).

In conclusion, we determined the antigenic epitopes of MDR1 against sera from AIH patients. Sera from 4 AIH patients and 4 normal volunteers were used in this study. The results showed that serum from Patient 3 reacted well with peptide 314–328 and weakly with peptide 957–971. The antigenic epitope on peptide 314–328 was Ser-Gly-Glu of 323–325. Meanwhile, serum from Patients 4 reacted well with peptide 850–857 and weakly with peptides 741–755 and 957–971. The key amino acids in peptide 850–857 for recognition by autoan-

tibodies from Patient 4 were Ser-Phe of 850–851. Both antigenic epitopes exist on ECL. Normal sera did not react with those peptides and the reactions of sera from Patients 1 and 2 were marginal. Sera from AIH patients did not inhibit MDR1 activity. These results suggested that ECL on MDR1 dose not play a role in MDR1 activity but antigenic determinants do. MDR1 recognized by the immune system would be on endothelial cells in the brain capillary, not on hepatocytes and epithelial cells in the intestine.

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