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## Abstract

Anti-idiotypic antibodies (Ab2) play an important role in the homeostasis of immune responses and are related to the development and the disease activity of certain autoimmune diseases. The asialoglycoprotein receptor (ASGPR) is considered one of the target antigens in the pathogenesis of autoimmune chronic active hepatitis (AIH). We previously developed a mouse monoclonal antibody (clone 8D7) which recognizes rat and human ASGPR. In this study, to help investigate the anti-ASGPR antibody-anti-idiotypic antibody network in patients with AIH, we developed a syngeneic mouse monoclonal Ab2 to the 8D7 anti-ASGPR antibody (Ab1). One clone, designated as 3C8, tested positive for specific reactivity to 8D7-Ab1 and did not bind to other irrelevant immunoglobulins. By competitive inhibition assays, the binding of 8D7-Ab1 to liver membrane extracts, i.e., the crude antigen preparation, was inhibited by 3C8-Ab2 in a dose-dependent manner, and the binding of 8D7-Ab1 to 3C8-Ab2 was inhibited by the liver membrane extracts. In the immunohistochemical analysis, 3C8-Ab2 blocked the specific staining of sinusoidal margins of rat hepatocytes by 8D7-Ab1. These results suggest that 3C8 anti-idiotypic antibody recognizes the specific idiotypic determinants within the antigen-binding site of 8D7-Ab1.

**KEYWORDS:** anti-idiotypic antibody, autoimmune hepatitis, asialoglycoprotein receptor, monoclonal antibody

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*Original Article*

## Development of Syngeneic Monoclonal Anti-idiotypic Antibodies to Mouse Monoclonal Anti-asialoglycoprotein Receptor Antibody

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Anti-idiotypic antibodies (Ab2) play an important role in the homeostasis of immune responses and are related to the development and the disease activity of certain autoimmune diseases. The asialoglycoprotein receptor (ASGPR) is considered one of the target antigens in the pathogenesis of autoimmune chronic active hepatitis (AIH). We previously developed a mouse monoclonal antibody (clone 8D7) which recognizes rat and human ASGPR. In this study, to help investigate the anti-ASGPR antibody-anti-idiotypic antibody network in patients with AIH, we developed a syngeneic mouse monoclonal Ab2 to the 8D7 anti-ASGPR antibody (Ab1). One clone, designated as 3C8, tested positive for specific reactivity to 8D7-Ab1 and did not bind to other irrelevant immunoglobulins. By competitive inhibition assays, the binding of 8D7-Ab1 to liver membrane extracts, *i.e.*, the crude antigen preparation, was inhibited by 3C8-Ab2 in a dose-dependent manner, and the binding of 8D7-Ab1 to 3C8-Ab2 was inhibited by the liver membrane extracts. In the immunohistochemical analysis, 3C8-Ab2 blocked the specific staining of sinusoidal margins of rat hepatocytes by 8D7-Ab1. These results suggest that 3C8 anti-idiotypic antibody recognizes the specific idiotypic determinants within the antigen-binding site of 8D7-Ab1.

**Key words:** anti-idiotypic antibody, autoimmune hepatitis, asialoglycoprotein receptor, monoclonal antibody

**T**he idiotype of an antibody is a unique structure in the antigen-binding site of the antibody molecule (Ab1). In the humoral immune responses, anti-idiotypic antibodies to the idiotype structures (Ab2) are also produced because the idiotype itself often displays immunogenicity. Anti-idiotypic antibodies play an important role in the homeostasis of immune responses, for instance, by modifying the binding of antibodies to their

target antigens [1]. It has been suggested that a disruption of this idiotype-anti-idiotypic antibody network is related to the development and the disease activity of autoimmune diseases such as systemic lupus erythematosus [2-4].

In the sera of patients with autoimmune chronic active hepatitis (AIH), a major autoimmune liver disease, autoantibodies to asialoglycoprotein receptor (ASGPR) have been found [5-8]. ASGPR is a hepatocyte-specific antigen and is present on the sinusoidal surface of hepatocytes [9, 10]; this receptor is considered a target antigen in the pathogenesis of AIH [11]. It is possible

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that the ASGPR antibody and its anti-idiotypic network are involved in the development and the regulation of hepatocyte injury in patients with AIH.

We previously developed a mouse monoclonal antibody (clone 8D7) which recognizes rat and human ASGPR [12–14]. For the present study, we developed a mouse monoclonal Ab2 to the 8D7 anti-ASGPR antibody in order to help investigate the anti-ASGPR antibody-anti-idiotypic antibody network in cases of AIH.

## Material and Methods

**Antibodies.** The mouse monoclonal antibody to rat ASGPR (clone 8D7, IgG1) developed by immunization of BALB/c mice with extracts of rat liver plasma membranes (LME) has been described previously [12, 13]. The antibody was purified from the ascitic fluids of pristane (Aldrich, Milwaukee, WI, USA)-treated BALB/C mice bearing a 8D7 hybridoma using protein G-agarose gel affinity chromatography (MAb Trap Kit; Amersham Pharmacia Biotech, Buckinghamshire, UK). Irrelevant mouse IgG1 monoclonal antibodies (clones 5E8 and J4-48), normal mouse IgG (DAKO Japan, Tokyo, Japan), and mouse IgM monoclonal antibody (clone 8C7) were used as controls. The 5E8 IgG1 monoclonal antibody recognizes the bile canalicular membrane antigen of rat hepatocytes [13]; the J4-48 IgG1 monoclonal antibody recognizes the membrane cofactor protein, a complement regulatory protein (IMMUNOTECH S.A., Marseilles, France) [15]; the 7C8 IgM monoclonal antibody reacts with the undefined cell surface antigen of rat hepatocytes [13]. IgM mouse monoclonal antibodies were purified by affinity chromatography (ImmunoPure IgM Purification Kit; PIERCE Chemical Company, Rockford, IL, USA) from the ascitic fluids of pristane-treated BALB/C mice bearing hybridomas.

**Immunization Protocols and Ab2 Production.** The 8D7-Ab1 was coupled to keyhole limpet hemocyanin (KLH) in order to enhance its immunogenicity by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (PIERCE Chemical Company), according to the manufacturer's instruction. Five week-old BALB/C mice were immunized by intraper-

itoneal injection of 100  $\mu$ g of 8D7-Ab1 coupled to KLH precipitated with 60  $\mu$ l of aluminum hydroxide gel every 2 weeks; this was repeated 5 to 7 times. Sera were taken after the final injection and assayed for antibody binding to 8D7-Ab1 by an enzyme-linked immunosorbent assay (ELISA) (see below). The animal with the highest titer was chosen, and a booster injection of KLH-conjugated 8D7-Ab1 was given 3 days before fusion. Spleen cells from the animal were fused with nonsecretory myeloma cells P3X63-Ag8.613 by using 30% polyethyleneglycol 1000, as described [16, 17]; hybrids were selected in hypoxanthine-aminopterin-thymidine medium. Culture supernatants were screened by binding to 8D7-Ab1 by ELISA (see below); hybridoma cells whose supernatants reacted with 8D7-Ab1 were expanded and cloned by a limiting dilution method. The immunoglobulin class and subclass of the monoclonal antibodies were determined by a commercially available ELISA (MonoAb-ID EIA kit; Zymed, San Francisco, CA, USA).

**ELISA assays.** For the screening of Ab2, the wells of microtiter plates (Immuno Plate II; Nunc, Denmark) were coated with 8D7-Ab1 (100  $\mu$ l of a 20  $\mu$ g/ml solution), and the culture supernatants of hybridoma cells were added to the wells. After washing, biotin-labeled 8D7-Ab1, prepared as described [18], was added. After washing, bound 8D7-Ab1 was detected with horseradish peroxidase (HRP)-labeled streptavidin (GIBCO BRL, Gaithersburg, MD, USA) using 2,2'-azino-di-3-ethylbenzo-thiazoline-6-sulphonic acid as substrate. Optical densities at 415 nm were measured on an automated ELISA plate reader.

The specificity of the Ab2 was determined by the following ELISAs: (a) the reactivity of the Ab2 was screened on plates coated with either 8D7-Ab1, irrelevant IgG1 mouse monoclonal antibodies, or normal mouse IgG (100  $\mu$ l of a 20  $\mu$ g/ml solution). Serially diluted Ab2 (100  $\mu$ l of a solution diluted at 0.01 to 10  $\mu$ g/ml) was added to the plates, and the binding of Ab2 antibody was revealed with biotin-labeled 8D7-Ab1 and HRP-labeled streptavidin; (b) the ability of the anti-idiotypic antibody to prevent the binding of Ab1 to the crude antigen was evaluated on plates coated with LME (100  $\mu$ l of a 0.1 mg/ml solution). Biotin-labeled 8D7-Ab1 (50  $\mu$ l of a 5  $\mu$ g/ml solution) was incubated with Ab2 (50  $\mu$ l of a solution diluted to 0.01  $\mu$ g/ml to 1 mg/ml) or with irrelevant mouse monoclonal antibody. The mixture was added to the plates, and the binding of biotin-labeled Ab1 was revealed with HRP-labeled streptavidin; (c) the ability of

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### Abbreviations

AIH, autoimmune hepatitis; ASGPR, asialoglycoprotein receptor; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; LME, extracts of rat liver plasma membranes.

the crude antigen to prevent the binding of Ab1 to Ab2 was evaluated on plates coated with purified Ab2 (100  $\mu$ l of a 20  $\mu$ g/ml solution). Biotin-labeled 8D7-Ab1 (50  $\mu$ l of a 5  $\mu$ g/ml) was incubated with LME (50  $\mu$ l of a solution diluted to 0.16 to 5 mg/ml) or rat kidney membrane extracts prepared from rat kidney acetone powder (Sigma, St. Louis, MO, USA) as control. The mixture was added to the plates, and the binding of biotin-labeled Ab1 was revealed with HRP-labeled streptavidin.

**Immunohistochemistry.** We immunohistochemically examined the ability of the anti-idiotypic antibody to prevent the staining of the plasma membrane of rat hepatocytes by 8D7-Ab1. Cryostat sections of fresh Sprague-Dawley rat liver fixed with cold acetone were reacted with biotin-labeled 8D7-Ab1 preincubated either with Ab2 or with irrelevant mouse monoclonal antibody, followed sequentially by HRP-labeled streptavidin, then with diaminobenzidine solution containing hydrogen peroxide.

## Results

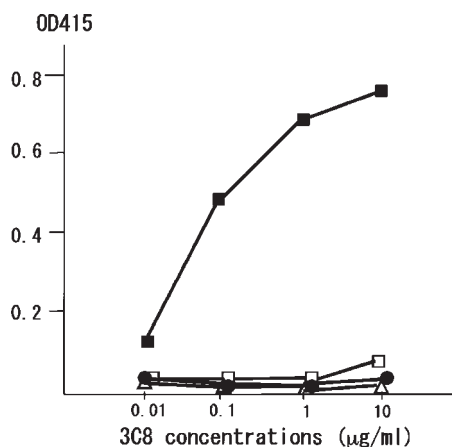
Of a total of 425 hybrids generated, only one clone tested positive for specific reactivity to 8D7-Ab1. This clone remained stable after cloning and continued to

produce an antibody specific to 8D7-Ab1. This clone was expanded and designated as 3C8. The 3C8 antibody was characterized as an IgM and purified from ascitic fluids of pristane-treated BALB/C mice bearing a 3C8 hybridoma using affinity chromatography. To assess its specificity, 3C8-Ab2 was tested by ELISA against 8D7-Ab1, irrelevant IgG1 5E8 and J4-48, and normal mouse IgG; 3C8-Ab2 bound solely to 8D7-Ab1 (Fig. 1). The results suggest that 3C8-Ab2 is specific to the variable domains of 8D7-Ab1.

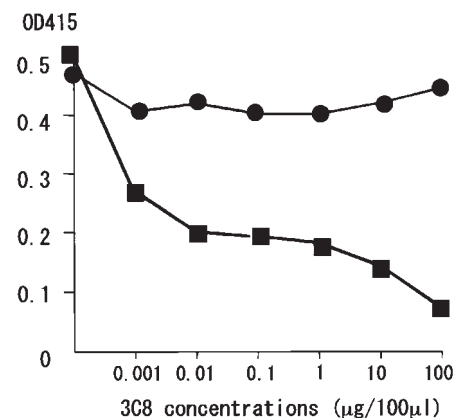
Further characterization of 3C8-Ab2 was performed by competitive inhibition assays. As shown in Fig. 2, the binding of biotin-labeled 8D7-Ab1 to the crude antigen, LME, was inhibited by 3C8-Ab2 in a dose-dependent manner, whereas the binding was unaffected by irrelevant 7C8 IgM monoclonal antibody. Furthermore, the binding of biotin-labeled 8D7-Ab1 to 3C8-Ab2 was inhibited by LME, as shown in Fig. 3. In the immunohistochemical analysis, 3C8-Ab2 blocked the specific staining of sinusoidal margins of rat hepatocytes by 8D7-Ab1 (Fig. 4).

## Discussion

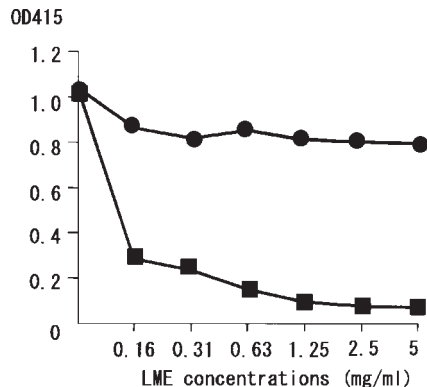
In this study, we described the generation of a mouse monoclonal anti-idiotypic antibody (clone 3C8) against 8D7



**Fig. 1** Binding of 3C8-Ab2 to 8D7 mouse monoclonal anti-ASGPR antibody. ELISA plates were coated with either 8D7-Ab1 (■), irrelevant IgG1 5E8 (□), J4-48 (●) mouse monoclonal antibodies or normal mouse IgG (△). Serially diluted 3C8-Ab2 was added to the plates, and the binding of Ab2 antibody was revealed with biotin-labeled 8D7-Ab1 and HRP-labeled streptavidin.



**Fig. 2** Inhibition of the binding of 8D7-Ab1 to LME by 3C8-Ab2. ELISA plates were coated with LME. Biotin-labeled 8D7-Ab1 was incubated with 3C8-Ab2 (■) or irrelevant 7C8 IgM monoclonal antibody (●). The mixture was added onto the plates, and the binding of biotin-labeled Ab1 was revealed with HRP-labeled streptavidin.

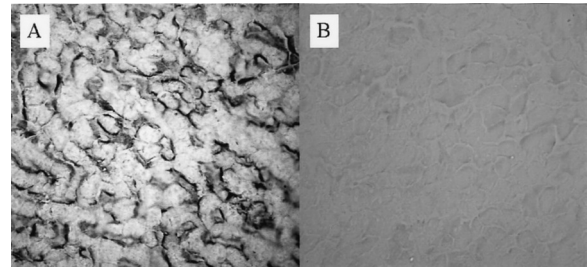


**Fig. 3** Inhibition of the binding of 8D7-Ab1 to 3C8-Ab2 by LME. ELISA plates were coated with 3C8-Ab2. Biotin-labeled 8D7-Ab1 was incubated with LME (■) or rat kidney membrane extracts (●). The mixture was added to the plates, and the binding of biotin-labeled Ab1 was revealed with HRP-labeled streptavidin.

anti-ASGPR mouse monoclonal antibody. To avoid the production of xenogeneic or allogeneic antibodies, we intended to generate syngeneic anti-idiotypic antibodies. For this purpose, the coupling of Ab1 with KLH was required to enhance the immunogenicity of Ab1 [19]. In the preliminary experiments, immunization with Ab1 without KLH did not produce antibody responses to Ab1. By using KLH-coupled Ab1 and repeated immunization, we were able to generate a hybrid (clone 3C8) that produced anti-idiotypic antibody.

Characterization of the 3C8 antibody has shown the following: 3C8-Ab2 is specific for 8D7-Ab1; (b) 3C8-Ab2 is capable of inhibiting the binding of 8D7-Ab1 to LME (as shown by ELISA); (c) 3C8-Ab2 is capable of inhibiting the staining of the sinusoidal plasma membrane of rat hepatocytes by 8D7-Ab1 (as shown by immunohistochemistry); (d) the binding of 8D7-Ab1 to 3C8-Ab2 can be blocked by LME. Taken together, these results suggest that 3C8-Ab2 recognizes specific idiotypic determinants within the antigen-binding site of 8D7-Ab1.

Anti-idiotypic antibodies (Ab2) elicited by the variable region of the inducing antibody (Ab1) can be divided into 2 categories: (1) Ab2 that recognizes an epitope in the binding site (paratope) of Ab1, *i.e.*, Ab2 $\beta$ , (2) Ab2 recognizes an idiopeptide in the non-binding sites of Ab1, *i.e.*, Ab2 $\alpha$ . The results of our study suggest that 3C8-Ab2 is an Ab2 $\beta$  anti-idiotypic antibody of the 8D7 anti-ASGPR antibody. Certain Ab2 $\beta$  can mimic the structure of the antigen that originally generated Ab1 (*i.e.*, implying the concept of the so-called “internal image” or



**Fig. 4** Blocking of the staining of rat hepatocytes. Cryostat sections of rat liver were reacted with biotin-labeled 8D7-Ab1 preincubated either with 7C8 irrelevant mouse IgM monoclonal antibody (A) or 3C8-Ab2 (B), followed sequentially by HRP-labeled streptavidin, then with diaminobenzidine solution containing hydrogen peroxide. Dark reaction products indicating the sites of ASGPR are present on the sinusoidal margins of the hepatocyte (A).

“functional mimicry” [1]). In such an instance, it is suggested that Ab2 $\beta$  can be used instead of the primary antigen. Ab2 $\beta$  offers promise as a “vaccine” against infectious agents [20, 21] and might also be used to induce antitumor immune responses to tumor antigens [22–25]. In the event that our 3C8-Ab2 is an “internal image” antibody, it might eventually be used as a surrogate antigen for the detection of anti-ASGPR antibody in AIH patients or to induce specific immune responses to ASGPR in the creation of AIH animal models.

However, the biochemical criteria for identification of the presence of an “internal image” determinant on an anti-idiotypic antibody are not definitive. By sequencing the variable regions of the antibodies, some authors have attributed the antigen mimicry of Ab2 to shared structures of the Ab2 and the antigen [26–28]. In contrast, others have found that Ab2s functionally mimicking the antigen have no obvious structural similarities to the antigens [29]. Accordingly, structural analysis alone cannot predict the functional mimicry properties of an Ab2; functional and immunological analyses are necessary in order to determine if an Ab2 is truly an “internal image” antibody. In this study, we showed that 3C8-Ab2 recognizes specific idiotypic determinants within the antigen-binding site of 8D7-Ab1. However, further studies are needed to clarify whether the 3C8 anti-idiotypic antibody is an “internal image” antibody and if it can truly be used as a surrogate antigen for ASGPR.

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