

BREAKTHROUGH OF IMMUNE SELF-TOLERANCE TO CALRETICULIN INDUCED BY CpG-OLIGODEOXYNUCLEOTIDES AS ADJUVANT

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Abstract: Reportedly, bacterial DNA containing unmethylated cytosine-guanosine dinucleotide motif-containing oligodeoxynucleotides (CpG-ODNs) can induce Th1-type adjuvant effects. We produced autoantibodies and induced hepatitis in mice using extracted proteins from human hepatocytes with CpG-ODNs as adjuvant. Western blot analysis was performed of sera from immunized mice and two patients with autoimmune hepatitis (AIH). When a common band was detected, N-terminal amino acid sequencing was performed to determine its site. For detection of antibodies against the identified protein (calreticulin), ELISA was performed of sera of 50 patients with AIH: 45 with primary biliary cirrhosis (PBC), 24 with chronic hepatitis C (CH), and 24 healthy controls. Mice were immunized with calreticulin protein with CpG-ODNs as adjuvant. Several reacted bands were detected in their sera; in addition, a common band to the sera of patients with AIH was detected at 60 kDa. Subsequent N-terminal amino acid sequencing revealed that the protein was human calreticulin. ELISA showed that, of patients with AIH, PBC, and CH, 30.0% (15/50), 17.8% (8/45), and 12.5% (3/24), respectively, were positive for anti-calreticulin antibodies. Splenocytes from immunized mice produced IFN- γ after they were pulsed with calreticulin protein. Histological analyses of liver specimens taken from mice immunized with calreticulin protein together with CpG-ODNs showed spotty and focal necrosis. Immunofluorescence analysis showed increased expression of calreticulin in the liver treated with CpG-ODNs. These results suggest that a breakthrough of immune self-tolerance to calreticulin is induced with CpG-ODNs as adjuvant and that calreticulin protein might be a target antigen in this model.

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic liver disease that is associated with hypergammaglobulinemia and circulating autoantibodies, such as antinuclear antibodies (ANA), anti-smooth muscle antibodies (aSMA), anti-liver-kidney microsomal antibodies (aLKM), and anti-asialoglycoprotein receptor antibodies (aAS-GPR). Because no disease-specific autoantibodies have been discovered^{1,2}, international diagnostic criteria for AIH are now generally used³. It is hoped that AIH-specific autoantibodies will be identified in the near future.

It has been reported that bacterial DNA containing unmethylated cytosine-guanosine dinucleotides (CpG) activate Th1-type immune response^{4,5}. The CpG motif-containing oligodeoxynucleotides (CpG-ODNs) activate macrophages, lymphocytes, natural killer cells, and dendritic cells to secrete various inflammatory cytokines, including interferon (IFN) α/β , interleukin (IL)-6, IL-12, tumor necrosis factor- α (TNF- α); they also induce Th1-type adjuvant effects⁶⁻¹⁰.

Recently, interactions between CpG-ODNs, which have Th1-type adjuvant effects, are attracting attention in relation to autoimmune disorders. Some investigators have reported that immunization of autoantigens with CpG-ODNs engenders the breakthrough of immune tolerance and subsequent production of autoantibodies^{11,12}. Other studies have also indicated that CpG-ODNs, which have immunostimulatory functions, are present in the mammalian genome and activate self B-cells to produce autoantibodies¹³.

Therefore, it is possible that autoantibodies are induced by immunization of hepatocyte proteins together with CpG-ODNs, considering that CpG-ODNs participate in the production of autoantibodies in autoimmune liver diseases, such as AIH. Target antigens participating in the onset of immune-mediated hepatitis might be identified if these proteins induced hepatitis with CpG-ODNs as adjuvant.

In this study, we induced anti-calreticulin antibodies in the sera of mice by immunizing human hepatocyte proteins with CpG-ODNs and induced hepatitis in mice using calreticulin proteins. Anti-calreticulin autoantibodies have been identified in sera from patients with various autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, mixed connective tissue disease, and inflammatory bowel diseases¹⁴⁻¹⁶. Recently, the presence of anti-calreticulin antibodies was revealed in sera of patients with AIH, PBC, and alcoholic liver cirrhosis¹⁷. It has been suggested that the high prevalence of IgA anti-calreticulin antibodies in patients with PBC reflects stimulation of the intestinal immune system¹⁷. Our data allow us to conclude that a breakthrough of immune self-tolerance to calreticulin is induced with CpG-ODNs as adjuvant and that

calreticulin protein might be a target antigen in this model.

MATERIALS AND METHODS

All animal experiments were performed according to national guidelines for use of animals in biochemical research.

Preparation of antigens

Cytosol proteins were extracted from normal human hepatocytes (5×10^6 /cells, Cryo hNHpes; Sanko Junyaku Co., Japan) using cell fraction kits (BioVision Research Products, CA, USA) according to the manufacturer's instructions. Preparation proteins were stored at -20°C until use.

Preparation of ODNs

The CpG-ODNs were synthesized (Bex Co. Ltd., Tokyo, Japan) and used for immunization: CpG-ODN 1668, 5'-TCC-ATG-ACG-TTC-CTG-ATG-CT-3'; and non-CpG-ODN 1720, 5'-TCC-ATG-AGC-TTC-CTG-ATG-CT-3' (analogous to CpG-ODN 1668). These ODNs were dissolved in phosphate-buffered saline (PBS) before use in experiments.

Immunization of mice with hepatocyte cytosol and CpG-ODNs

Eight-week-old female BALB/c mice, obtained from Kumagai Farm (Sendai, Japan), were housed in filtertop cages. Water and food were provided *ad libitum*.

A total of 25 female BALB/c mice were assigned randomly to five groups of five mice each. Mice in each group were injected intradermally at the base of the tail with (1) PBS (50 μl /mouse) as control (Group 1-1), (2) extracted proteins (250 μg /mouse) diluted with PBS (50 μl) (Group 1-2), (3) CpG-ODNs (50 μg /mouse) diluted with PBS (50 μl) (Group 1-3), (4) extracted proteins (250 μg /mouse) and CpG-ODNs (50 μg /mouse) combined (Group 1-4), or (5) extracted proteins (250 μg /mouse) and non-CpG-ODNs combined (Group 1-5). Mice were sensitized three times at 0, 10, 20 days, and sera were obtained at 25 days and stored at -20°C until use.

Immunoblotting

Preparation proteins from human hepatocytes or calreticulin proteins (2 μg , Sigma Chemical Co., St. Louis, MO) were resuspended in 250 μl of sample buffer (125 mmol/l Tris-HCL (pH 6.8) containing 4% SDS, 20% glycerol, and 5% 2-mercaptoethanol), boiled for 5 min, and resolved by SDS-polyacrylamide gel electrophoresis using 1.5-mm-thick slab gels with a 5% stacking gel and a 10% separating gel. Proteins were either stained with Coomassie brilliant blue R or transferred electrophoretically to PVDF membranes at 200 mA for 90 min.

Immunoblotting was performed at room temperature, and all dilutions were made using Tween-PBS. The PVDF membranes were treated for 1 h at room

temperature with 3% bovine serum albumin in PBS (BSA/PBS) and probed for 1 h with sera (1:100) from immunized mice and from two patients with AIH, who fulfilled the generally accepted diagnostic criteria³. Membranes were washed five times with Tween-PBS and incubated with alkaline phosphatase-conjugated goat F(ab')₂ anti-mouse IgG or goat F(ab')₂ anti-human IgG (BioSource International Inc., Camarillo, CA) diluted 1:1,000. After washing five times with Tween-PBS, color was developed by placing the membrane in a solution of naphthol FAS phosphate and fast blue BB salt (Sigma Chemical Co., St. Louis, MO).

N-terminal amino acid sequencing and computer search for protein identification

Western blot analysis was performed for IgG antibodies in sera from immunized mice and two patients with AIH. It showed a common band of 60 kDa. Proteins were transferred electrophoretically onto PVDF membrane. The membrane was cut at the band of 60 kDa; then the selected protein was subjected to amino acid sequencing by Edman-degradation using an automatic protein/peptide sequencer (Applied Biosystems 492; Foster City, CA). Search for similarity of amino acid sequence was performed using BLAST computer programs of the Genetics Computer Group (Madison, WI, USA) to screen protein databases. The analysis indicated that this protein was calreticulin. Similar immunoblotting was also performed using purified calreticulin protein (Sigma-Aldrich Japan K.K.) using the sera of immunized mice and the sera of patients with AIH.

Patients and sera

Sera were obtained from 50 patients with AIH (1 man and 49 women, age 21–77 years), 45 patients with primary biliary cirrhosis (PBC) (5 men and 40 women, age 34–78 years), 24 patients with chronic hepatitis (CH) caused by hepatitis C virus (12 men and 12 women, age 36–70 years), and 24 healthy controls. Diagnoses of AIH were based on criteria established by the International Autoimmune Hepatitis Group in 1999³. Furthermore, PBC was diagnosed using either histology of liver biopsy specimens or clinical findings of anti-mitochondrial antibodies (AMA) and cholestatic dysfunction of the liver followed by jaundice or pruritus, based on 'Criteria for Diagnosis of PBC in Japan' by the Study Group for Autoimmune Hepatitis, a subdivision of the Research Group for Intractable Hepatitis, sponsored by the Ministry of Health, Labour and Welfare of Japan¹⁸. Blood was taken from the cubital vein; sera were prepared by clotting each specimen. The sera were stored at –20°C until use.

ELISA for detection of anti-calreticulin antibodies in patients with AIH

Each well of 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) was coated with 500 ng of calreticulin in 50 mM sodium carbonate buffer (pH 9.5) and left at 4°C overnight. After aspiration of supernatants and subsequent washing of the plates three times with PBS, the plates were blocked to prevent non-

specific binding with 100 μ l of 3% bovine serum albumin in phosphate-buffered saline (BSA/PBS) for 3 h. Then, 100 μ l of serum sample diluted 1 : 100 in 1% BSA/PBS was added, and the plates were incubated for 1 h at room temperature. After three washes with Tween-PBS, 100 μ l of 1 : 1,000 diluted alkaline phosphatase-conjugated goat anti-human IgG (γ) (Tago Inc., Burlingame, CA) was added. Then the plates were incubated for 50 min at room temperature. The wells were washed three times with Tween-PBS and treated with *p*-nitrophenyl phosphate substrate (Sigma Chemical Co., St. Louis, MO). Optical density (OD) at 405 nm was measured using an ELISA spectrometer (3550 reader; Bio-Rad Laboratories Inc., Hercules, CA). In evaluating the positive frequency of anti-calreticulin antibodies, a titer above the mean+3SD of healthy controls was defined as positive.

Immunization of mice with calreticulin and CpG-ODNs

Eight-week-old female C57BL6 mice, obtained from Kumagai Farm (Sendai, Japan), were housed in filtertop cages. Water and food were provided *ad libitum*.

A total of 25 female C57BL6 mice were assigned randomly to five groups of five mice each. Mice in each group were injected intradermally at the base of the tail with (1) PBS (50 μ l/mouse) as control (Group 2-1), (2) calreticulin protein (20 μ g/mouse) diluted with PBS (50 μ l) (Group 2-2), (3) CpG-ODNs (50 μ g/mouse) (Group 2-3), (4) calreticulin protein (20 μ g/mouse) and CpG-ODNs (50 μ g/mouse) combined (Group 2-4), and (5) calreticulin protein (20 μ g/mouse) and non-CpG-ODNs combined (Group 2-5). Mice were sensitized three times, at 0, 4, 8 weeks; sera were obtained at 10 weeks and stored at -20°C until use.

Biochemical and histological analysis

The extent of hepatocellular injury was monitored histologically and biochemically by measuring serum alanine aminotransferase (ALT) activity using a standard clinical automatic analyzer. For histopathological evaluation, the liver samples were fixed in 10% buffered formalin and embedded in paraffin, then sectioned and stained with hematoxylin and eosin (Sakura Finetechnical Co., Ltd., Tokyo, Japan). In addition, for the indirect immunofluorescence method, live tissues were embedded in OCT compound and frozen. From frozen tissues, 8- μ m-thick sections were prepared, which were fixed in cold acetone for 20 s then washed with PBS. Nonspecific reactions were blocked and the sections were incubated for 1 h at room temperature with a 50-fold-diluted solution of anti-calreticulin antibody (Upstate Group, Inc., Lake Placid, NY). After washing with PBC, the sections were incubated for 30 min at room temperature with a 50-fold-diluted solution of FITC-conjugated anti-rabbit IgG (MP Biomedicals, Aurora, Ohio). After washing with PBC, expression of calreticulin proteins was observed using a fluorescence microscope (AX80; Olympus Optical Co. Ltd., Tokyo, Japan).

Levels of IFN- γ and interleukin (IL)-4 in the supernatants from splenocyte culture

Spleens were harvested from sacrificed mice 25 days after immunization with calreticulin protein and CpG-ODNs for cytokine assay. Mice ($n=5$) were sacrificed by CO₂ inhalation. Their spleens were harvested and teased to produce single cell preparations. Splenocytes were resuspended in RPMI 1640 supplemented with 10% FBS, 2 mM penicillin/streptomycin, and 50 μ M β -mercaptoethanol at a concentration of 2×10^6 /ml. Culture supernatants were harvested after incubation with or without calreticulin protein (10 μ g/ml) for 72 h at 37°C in 5%CO₂. Levels of IFN- γ and IL-4 in the supernatants were analyzed using ELISA (Pierce Biotechnology Inc., Rockford, IL). As control specimens, splenocytes from normal mice ($n=5$) were used.

Statistical analysis

Results are expressed as mean \pm SD. Differences were compared using the Mann-Whitney *U*-test; $p < 0.05$ was considered significant.

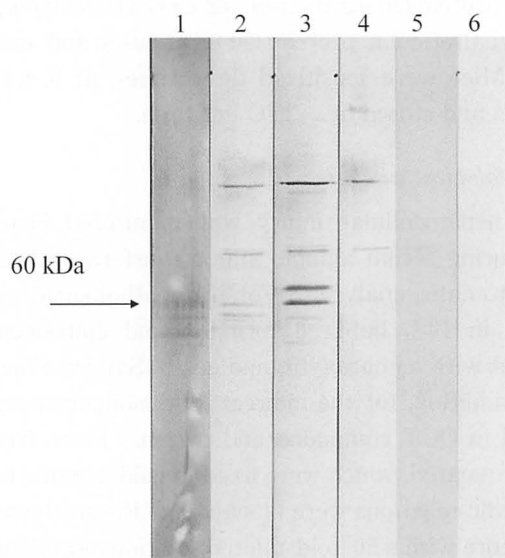


Fig. 1. Results of Western blot analysis using extracted protein from normal human hepatocytes as an antigenic substrate: lane 1, reaction with serum of a patient with AIH; lanes 2, 3 and 4, reaction with sera from mice injected with extracted proteins and CpG-ODNs (Group 1-4 mice); lane 5, reaction with serum from mice injected with extracted proteins alone; lane 6, reaction with serum from mice injected with CpG-ODNs alone. Several reacted bands were detected in sera from Group 1-4 mice (lanes 2, 3 and 4). Several reacted bands were also detected in serum from a patient with AIH (lane 1). A common band for Group 1-4 mice was detected at 60 kDa.

RESULTS

Immunoblotting of preparation proteins from human hepatocytes

Figure 1 shows results of Western blot analysis of cytosol proteins prepared from human hepatocytes. Several reacted bands were detected in the sera from mice injected with extracted proteins and CpG-ODNs combined. Sera from mice injected with extracted proteins alone, CpG-ODNs alone, and extracted proteins and non-CpG-ODNs combined showed no reactions. Several reacted bands were also detected in the sera of patients with AIH. Among all reactions with patients' sera and mouse sera, a common band was detected at 60 kDa.

N-terminal amino acid sequencing

Using N-terminal amino acid sequencing, a 10-amino acid sequence was identified as DPAIYFKENL, corresponding exactly to the known N-terminus of human calreticulin.

Immunoblotting using calreticulin proteins

Figure 2 shows results of Western blot analysis of sera using purified calreticulin

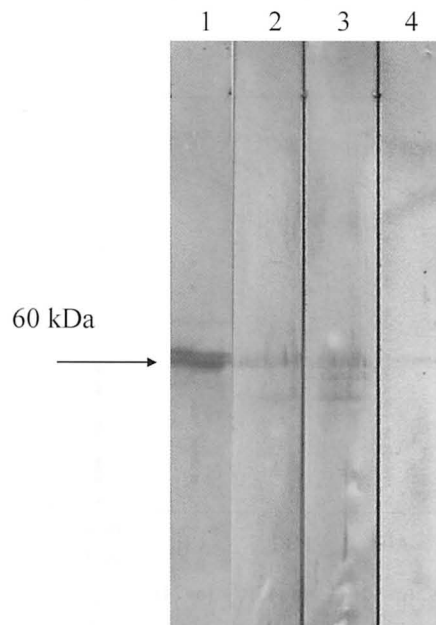


Fig. 2. Results of Western blot analysis using calreticulin protein: lane 1, protein staining with Coomassie brilliant blue PR-250; lanes 2 and 3, reaction with sera from patients with AIH; lane 4, reaction with sera from Group 1-4 mice. The positive band of 60 kDa that coincided with the expected size of calreticulin protein was found in sera from patients with AIH (lanes 2, 3) and serum from Group 1-4 mice (lane 4).

protein (60 kDa). The reacted band at 60 kDa was detected in the respective sera of mice injected with extracted proteins and CpG-ODNs combined and patients with AIH. No reaction with calreticulin protein was apparent in sera from other groups of mice.

Anti-calreticulin antibodies in ELISA

Figure 3 shows that the mean OD value on ELISA of anti-calreticulin antibodies was significantly ($p < 0.01$) higher in patients with AIH (197 ± 121.2) than in healthy controls (71.8 ± 28.9). In evaluating the positive frequency of anti-calreticulin antibodies in ELISA, 30.0% (15/50) of patients with AIH, 17.8% (8/45) of patients with PBC, and 12.5% (3/24) of patients with CH were positive for anti-calreticulin antibodies.

Immunization by calreticulin proteins

Figure 4 shows results of Western blot analysis of sera from mice immunized with calreticulin protein. The reacted band at 60 kDa was detected only in the sera from mice injected with calreticulin protein and CpG-ODNs combined. Serum ALT levels were increased in these mice (80.1 ± 85.9 IU/ml) compared with mice injected with PBS alone (18.3 ± 1.5 IU/ml), calreticulin diluted with PBS (18.0 ± 1.0 IU/ml), CpG-ODNs alone (22.0 ± 5.2 IU/ml), or calreticulin and non-CpG-ODNs combined

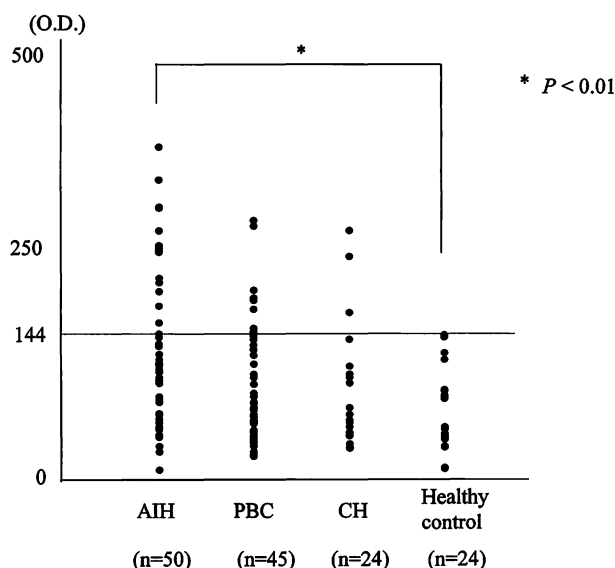


Fig. 3. Anti-calreticulin antibody titers, determined by ELISA, of sera from patients with AIH ($n=50$), PBC ($n=45$), or CH ($n=24$) and from healthy controls ($n=24$). The mean titer was significantly ($p < 0.01$) higher in patients with AIH than in healthy controls. Of patients with AIH, PBC and CH, 30.0% (15/50), 17.8% (8/45) and 12.5% (3/24), respectively, were positive for anti-calreticulin antibodies, defined as titer above the mean+3SD of healthy controls. * $p < 0.01$. AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; CH, chronic hepatitis.

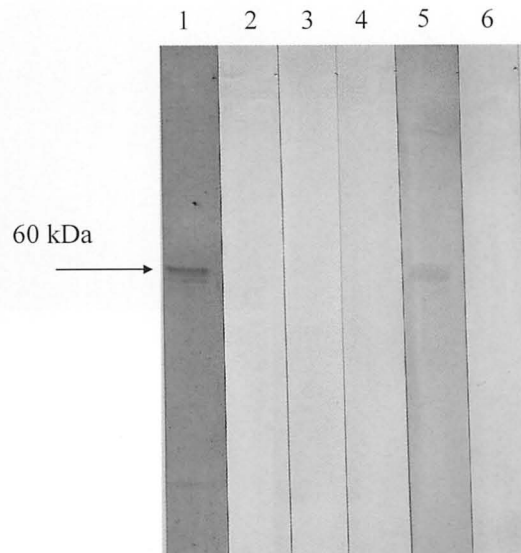


Fig. 4. Results of Western blot analysis in sera of mice immunized with calreticulin protein and CpG-ODNs combined (Group 2-4 mice): lane 1, calreticulin protein staining with Coomassie brilliant blue PR-250; lane 2, reaction with sera from normal mice as control; lane 3, reaction with sera from mice injected with calreticulin proteins alone; lane 4, reaction with sera from mice injected with CpG-ODNs alone; lane 5, reaction with sera from Group 2-4 mice; lane 6, reaction with sera from mice injected with calreticulin proteins and non CpG-ODNs combined. Reacted band at 60 kDa was detected only in sera from Group 2-4 mice (lane 5).

(17.3 ± 2.1 IU/ml); the differences, however, were not statistically significant.

In histological analysis, liver specimens taken from mice treated with CpG-ODNs alone showed extramedullary hemopoiesis but not hepatitis. The livers of mice immunized with calreticulin protein and CpG-ODNs combined showed spotty or focal necrosis (Fig. 5). These changes were observed in all mice immunized with calreticulin protein and CpG-ODNs combined, but not in any of the other groups. In addition, the expression of calreticulin in the liver was increased in mice treated CpG-ODNs alone and calreticulin protein with CpG-ODNs (Fig. 6). Demonstrably, CpG-ODNs treatment enhances the expression of calreticulin in the liver. However, it remains unclear whether the expression of calreticulin increased in cytoplasm or on the cell surface.

Levels of IFN- γ and IL-4 in the supernatants from splenocytes culture

To investigate T helper cell differentiation induced by immunization by calreticulin protein with CpG-ODNs, production of IFN- γ and IL-4 was evaluated from splenocytes pulsed with or without calreticulin *in vitro*. Splenocytes pulsed with calreticulin produced significantly more IFN- γ ($24,075.4 \pm 138.3$ pg/ml) than splenocytes that had not been pulsed with calreticulin ($4,512.4 \pm 3,921.7$ pg/ml) (Fig. 7). Splenocytes cultured with or without calreticulin produced no detectable IL-4.

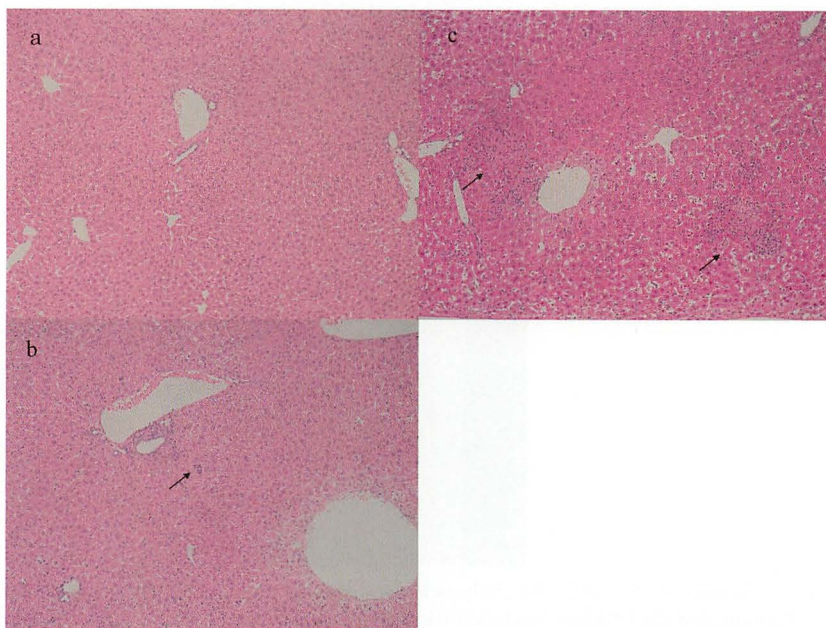


Fig. 5. Liver histology in (a) a normal mouse (Group 2-1), (b) a mouse treated with CpG-ODNs alone (Group 2-3), and (c) a mouse treated with calreticulin protein and CpG-ODNs combined (Group 2-4). A liver specimen taken from a mouse treated with CpG-ODNs alone (b) shows large immature blasts and erythroblasts that are indicative of extramedullary hemopoiesis (arrows). The liver of a mouse immunized with calreticulin protein and CpG-DNAs combined (c) shows focal necrosis (arrows) (hematoxylin and eosin staining; original magnification $\times 130$).

These findings show that Th1-biased cellular immune response is induced in this model.

DISCUSSION

In this study, we induced anti-calreticulin antibodies in the sera of mice by immunizing human hepatocyte proteins with CpG-ODNs. Anti-calreticulin antibodies were detected in sera of patients with AIH and PBC. The mean titer was higher in patients with AIH than in healthy controls. Moreover, hepatitis was induced in mice that were immunized with purified calreticulin protein and CpG-ODNs. In this model, calreticulin protein might be a target antigen; CpG-ODNs might participate in autoantibody production.

No reports describe how autoantibody production is induced by the CpG sequence in the patients with AIH. However, many studies have been made of autoantibodies in AIH^{19,20}. The CpG-ODNs can induce autoantibodies that are undetectable by conventional methods because CpG-ODNs are three times more efficient in producing autoantibodies than complete Freund's adjuvant²¹. The CpG-

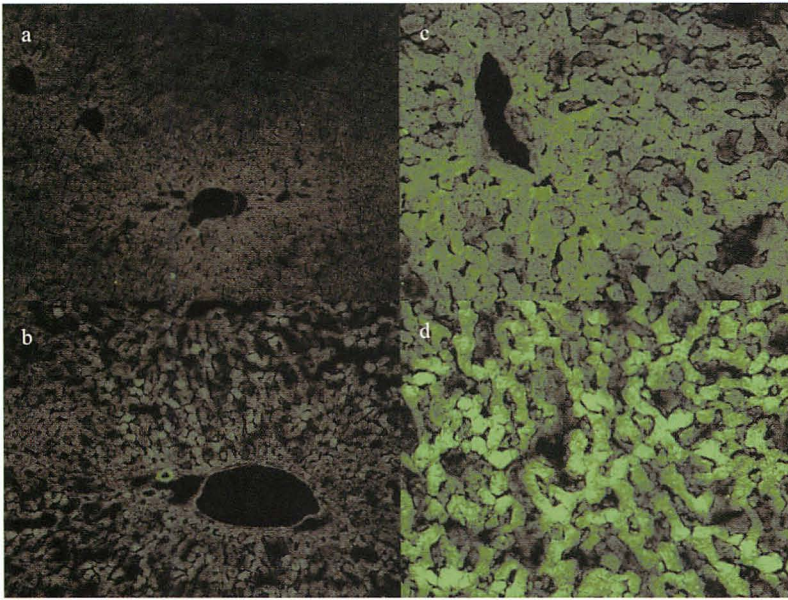


Fig. 6. An indirect immunofluorescent micrograph using anti-calreticulin antibody in (a) a normal mouse (Group 2-1), (b) a mouse treated with calreticulin protein (Group 2-2), (c) a mouse treated with CpG-ODNs alone (Group 2-3) and (d) a mouse treated with calreticulin protein and CpG-ODNs combined (Group 2-4). Expression of calreticulin in the liver was increased in mice that had been treated using CpG-ODNs alone and using calreticulin protein with CpG-ODNs.

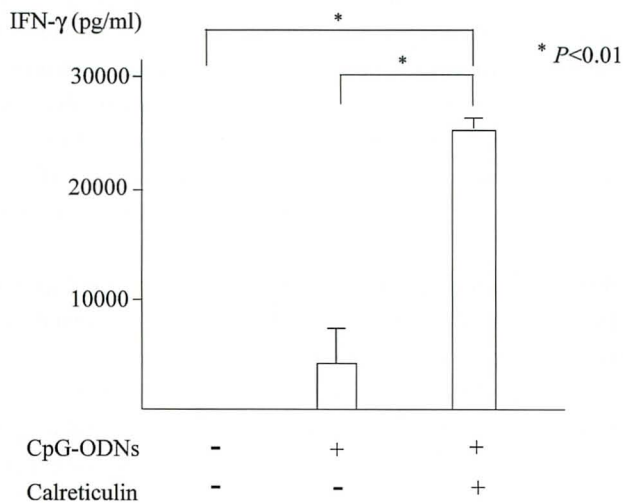


Fig. 7. IFN- γ production from splenocytes from immunized calreticulin protein with CpG-ODNs.

Results are representative mean values for five mice per group. Splenocytes pulsed with calreticulin protein produced IFN- γ (24,075.4 \pm 138.3 pg/ml) that was significantly ($p < 0.01$) higher than that of splenocytes that had not been pulsed with calreticulin (4,512.4 \pm 3,921.7 pg/ml). IFN- γ was not detected in supernatant of splenocytes cultured from normal control mice.

ODNs activate innate immunity through Toll-like receptor 9, one pattern recognition receptor that recognizes pathogen-associated molecular patterns²²). Activation of the innate immunity by CpG-ODNs particularly engenders production of IL-12 from antigen-presenting cells, thereby fostering a Th1-biased immune response in adaptive immunity²³). The Th1 dominant response generated by immunization with antigen and adjuvant containing CpG-ODNs has been applied to vaccination or immunotherapy for infectious diseases, neoplasms, and allergic diseases²³). In this model, mice that had been immunized with CpG-ODNs with calreticulin protein had anti-calreticulin antibodies; furthermore, their splenocytes showed Th1-immune response.

Recently, breakthroughs in immune tolerance by autoantigens with CpG-ODNs were reported^{11,12}). In the present study, breakthrough of immune tolerance was also induced by immunizing liver antigens, including calreticulin protein, with CpG-ODNs as a Th1-inducing adjuvant, suggesting that CpG-ODNs participate in the pathogenesis of hepatitis. Splenocytes from immunized mice produced IFN- γ after treatment of calreticulin protein. Therefore, Th1-immune response was induced in this model. Moreover, it is possible to induce response by activated T cells because the expression of calreticulin was increased in immunized mice. An additional inference is that liver injury is induced by anti-calreticulin antibody via antibody-dependent cellular cytotoxicity. However, the mechanisms of liver injury are unclear because calreticulin expression on the cell surface was not confirmed in our study. The major localization site of calreticulin is the ER. In addition, expression of calreticulin is up-regulated in the activated T cells, and the protein is targeted to the cytotoxic T-cell granules²⁴). Interaction between calreticulin and anti-calreticulin autoantibody might be possible if calreticulin is expressed on the cell surface. In this study, liver from CpG-ODNs treated mouse showed up-regulation of expression of calreticulin by indirect immunofluorescent staining. The mechanism by which hepatitis was induced in this study is unknown. However, results of this study strongly suggest that calreticulin protein plays an important role in hepatitis.

The CpG-ODNs occasionally trigger extramedullary hemopoiesis²⁵). In fact, the liver specimen treated with CpG-ODNs alone showed extramedullary hemopoiesis. However, no hepatic injury was found, although the histological findings in this study differed from typical histological features of AIH: interface hepatitis or plasma cell infiltration in portal tracts. Generation of a chronic disease model by immunizing mouse with calreticulin and CpG-ODNs is needed for future study.

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