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Hiroshi Fujinaga · Ken Takeuchi · Kazuhiko Kaneda
Yoshinari Takasaki · Hiroshi Hashimoto

Analysis of autoantibodies to cell cycle-associated antigens

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Abstract To determine the specificities of autoantibodies targeting cell cycle-associated antigens in rheumatic diseases, we studied 30 sera which were obtained from patients visiting our hospital and which exhibited a variegated speckled pattern in indirect immunofluorescence (IF). The immunoreactivities of the sera were analyzed by Western blotting (WB) and IF. Various reactivities to cellular components were observed in IF. The sera reacted with proteins of various molecular weights in WB. Serum OH from a patient with rheumatoid arthritis showed fine speckled nucleoplasmic and nucleolar staining at interphase, discrete dot staining associated with chromosomes and the midbody at mitosis, and reacted with a 34-kD polypeptide in WB. The target antigen was different from proliferating cell nuclear antigen (PCNA). The 34-kD antigen was characterized by IF using double staining procedures and cell synchronization. The results showed that the expression of the antigen was mainly observed between the late G1 and M-phases. This study indicated that various cell-cycle-associated antigens were recognized in sera from patients with rheumatic diseases, and suggested that a 34-kD antigen recognized by serum OH was a novel cell cycle-associated autoantigen.

Key words Autoantibody · Cell cycle · CENP-F · Na antigen · Proliferating cell nuclear antigen (PCNA)

H. Fujinaga · K. Takeuchi · K. Kaneda · Y. Takasaki (✉) · H. Hashimoto
Department of Internal Medicine and Rheumatology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
Tel. +81-3-3813-3111; Fax +81-3-5800-4893
e-mail: tyoshi@med.juntendo.ac.jp

H. Fujinaga
Department of Japanese Oriental Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan

Introduction

Antinuclear autoantibodies (ANA), which are useful for diagnosing and understanding the clinical features of systemic autoimmune diseases, are invaluable probes of the cell biology of the nucleus.[1] Recent studies have revealed that patient sera often recognized cell cycle-associated autoantigens. In indirect immunofluorescence (IF) for screening antinuclear antibody, the staining patterns of these autoantibodies are characterized by both positively and negatively staining cells coexisting in same field, which is called the variegated speckled staining pattern.

One of the representative cell cycle autoantibodies, the antibody to proliferating cell nuclear antigen (PCNA), was first described as an autoantibody detected in sera from patients with systemic lupus erythematosus (SLE).[2] Several studies have revealed that the target antigen is a polypeptide with a molecular weight of 34000, whose expression increases between the late G1 and S phases during the cell cycle.[3] PCNA is known to be an auxiliary protein of DNA polymerase-δ and essential for both DNA replication and repair.[4] The incidence of anti-PCNA antibody in lupus sera is approximately 3%, but the detection of anti-PCNA antibody is useful for diagnosis because the appearance of the autoantibodies is restricted to lupus patients and is associated with thrombocytopenia, seizure, and nephritis.[5,6]

Other cell cycle-associated antigens recognized by autoimmune sera have been identified by several investigators, such as Cyclin B,[7] p330d/CENP-F,[8] SG2NA,[9] RMSA-1,[10] and NuMA.[11] Autoantibodies to CENP-F were detected in sera from patients with various types of cancer,[12,13] and the clinical correlation of autoantibodies to Cyclin B with hepatocellular carcinoma has been suggested.[7] However, the clinical usefulness of these autoantibodies has not definitely been established because the specificities of the diseases and clinical manifestations associated with these autoantibodies have not been clarified.

In the present study to determine the incidence and clinical usefulness of autoantibodies to cell-cycle-associated antigens in connective tissue diseases, the immunoreactivities

of these sera were studied using IF and Western blotting (WB). We further focused on one of the cell cycle-associated autoantibodies showing a unique staining pattern in IF, and characterized the target antigen.

Materials and methods

Patients and antibodies

Forty-five samples of sera which exhibited a variegated speckled pattern in IF using commercially available HEp-2 slides (MBL, Japan) were screened in this study. These sera were obtained from patients who visited the Juntendo University Hospital and underwent routine ANA analysis. From these 45 sera samples, 30 with titers higher than 1:80 in IF were selected and used for further studies.

Diagnoses of SLE,[14] rheumatoid arthritis (RA),[15] and systemic sclerosis (SSc)[16] were decided using the criteria for diagnosis proposed by the American Rheumatism Association. A diagnosis of Sjögren's syndrome (SjS) was decided using the criteria proposed by the European Community.[17] Diagnoses of polymyalgia rheumatica (PMR) and adult Still's disease were determined using the criteria proposed by Bird et al.[18] and Yamaguchi et al.,[19] respectively. Ulcerative colitis (UC) was diagnosed by colonofiberscope and rectal biopsy.

Anti-PCNA standard serum and other standard sera to nuclear antigens, such as double-stranded DNA, Sm, Sjögren's syndrome antigen A (SSA), Sjögren's syndrome antigen B (SSB), U1 RNP, Jo-1, and scleroderma antigen (Scl-70) were kindly provided by Dr. Eng M. Tan (The Scripps Research Institute, La Jolla, CA, USA) and used in IF and double immunodiffusion (DID).

The mouse monoclonal antibody to cyclin B (Transduction Laboratories, Lexington, KY, USA) was used as a marker for the G2 and M phases.[20]

A murine serum containing antifibrillarin antibodies was produced in female SJL/J mice by subcutaneous injection of $HgCl_2$ as previously described.[21]

Cell culture and synchronization

MOLT-4 cells and HeLa cells (American Type Culture Collection, Rockville, MD, USA) were grown in RPMI 1640 and DMEM, respectively, supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics (streptomycin/penicillin G).

For synchronization, cells were arrested at the G1/S phase using a double thymidine block procedure as reported previously, with minor modification.[22] Briefly, growth medium on slide cultures (Nunc, Naperville, IL, USA) was replaced by medium containing 2.5 mM thymidine (Sigma, St. Louis, MO, USA). After 16 h, the cultures were washed three times with normal medium and incubated with fresh normal medium at 37°C for 10 h, followed by a second incubation with medium containing 2.5 mM thymidine. After incubation for 16 h, the cells were washed three times with normal medium, and the medium was replaced with fresh normal medium. Cells were analyzed by IF at 0, 2, 4, 6, 9, 12, 18, and 24 h after removal of the second thymidine block.

Antigen source

MOLT-4 cells grown in culture dishes were solubilized directly in Laemmli's sample buffer.[23] The extracts were passed several times through 26-gauge needles, heated at 100°C for 5 min, and used for 15% SDS-PAGE.

Rabbit thymus extract (RTE) was prepared as an antigen source to purify PCNA as previously reported.[24] Briefly, 1 ml PBS (0.15 M NaCl, 0.01 M phosphate buffer, pH 7.4) was added to 90 mg rabbit thymus acetone powder (Pel-Freez, Rogers, AR, USA), and the saline-soluble antigen was extracted. After centrifugation of the mixture, the supernatant was purified by 40%–65% ammonium sulfate fractionation and Sephadex G200 (Pharmacia, Uppsala, Sweden) gel filtration as previously described.[25] This partially purified antigen was further purified by affinity chromatography using the IgG fraction of anti-PCNA standard serum AK coupled to CNBr-activated Sepharose 4B (Pharmacia).

Indirect immunofluorescence

For the screening of ANA by IF, commercially available HEp-2 slides (MBL, Japan) were used as substrates. HeLa cells grown on chamber slides were also prepared. HeLa cells were fixed in 50% methanol and 50% acetone at −20°C for 5 min. After fixation, the cells were incubated with the primary antibody at the following dilutions in PBS: human sera, 1:100; mouse anticyclin B antibody, 0.25 μg/ml; murine serum containing antifibrillarin antibody, 1:100. Fluorescein isothyocyanate (FITC)-conjugated goat anti-human IgG or rhodamine-conjugated goat antimouse IgG antibody (KPL, Gaithesburg, MD, USA) was used as a secondary detecting reagent.

Double immunodiffusion

The DID assay was conducted on plates of 0.4% agarose (Sea Kem, Rockland, ME, USA), 0.01% sodium azide in PBS as previously described.[26] Precipitation reactions were allowed to develop for 48 h at room temperature. Calf thymus extract (CTE) and RTE were used as antigens to identify the precipitin reaction of the sera.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli.[23] The slab gels consisted of 15% acrylamide and 0.1% SDS in Tris HCl, pH 8.8, with 15 mm stacking gels containing 5% acrylamide and 0.1% SDS in Tris HCl, pH

6.8. After electrophoresis, proteins were transferred to nitrocellulose.[27] After blocking with PBS-0.1% Tween 20 (PBS-T) containing 3% nonfat dry milk (PBS-T/milk) at room temperature for 60 min, the nitrocellulose was cut into strips and incubated with sera diluted 1:100 in PBS-T/milk and with alkaline phosphatase-labeled goat antihuman IgG (KPL, Gaithesburg, MD, USA) diluted to 0.5 μg/ml in PBS-T/milk. The bound conjugate was detected by incubation with alkaline phosphatase substrate (KPL, Gaithesburg, MD, USA).

Affinity purification of antibodies

Nitrocellulose membranes containing the protein band of interest were cut into small pieces and blocked overnight in PBS-T/milk. After the pieces of membrane had been incubated for 2 h in the autoimmune serum at a 1:50 dilution, antibodies were eluted from the membrane by low pH for 4 min in 200 mM KPO_4, pH 2.5, 150 mM NaCl, 0.1% BSA, followed by neutralization with 1 M Tris, pH 8.7. This affinity purification procedure was repeated several times, and eluted antibodies were concentrated using Centricon-30 microconcentrators (Amicon, Beverly, MA, USA) and used for IF studies and WB.[9]

Results

Disease associations and immunoreactivities of cell cycle-associated autoantibodies

Among the 30 sera tested in this study, the majority were obtained from patients with SLE, RA, and SjS (12, 5, and 5 sera, respectively). Other cell cycle-associated antibodies were detected in sera from patients with SSc, PMR, adult Still's disease, and UC. Six sera samples were obtained from patients who were not diagnosed with any rheumatic disease (two patients with eruption, two with arthralgia, one with oral ulcer, and one with a family history of connective tissue disease). To test for the presence of any well-established ANAs in these sera, DID assays were performed. Eight sera were positive in at least one assay and five sera contained multiple antibodies. Anti-PCNA antibody was detected in three sera, all of which were from lupus patients. Other antibodies were detected in some sera (anti-DNA antibody in one serum, anti-RNP antibody in one serum, anti-SSA antibody in seven sera, anti-SSB antibody in one serum, anti-Scl-70 antibody in one serum).

To study the immunoreactivities of these sera to cellular components, IF was first performed. Using logarithmically growing HEp-2 cells as the IF assay substrate, all 30 sera showed a variegated speckled pattern (Fig. 1A). In IF, various reactive patterns of intracellular staining were observed, including reactivities to the nucleolus (Fig. 1A, panels 2 and 5), mitotic spindle (Fig. 1A, panels 3, 4, 6, and 8), and midbody (Fig. 1A, panels 2 and 5). Reactivity to mitotic cells was observed in all sera except for the anti-PCNA antibody preparation (Fig. 1A, panel 1).

To further study the specificities of the cell cycle-associated antibodies, the immunoreactivities of these sera were compared in WB using MOLT-4 whole cell extract as antigen (Fig. 1B). Reactive bands were observed in 20 of 30 sera, and reactivity at a molecular weight of 89 000 was most frequently detected (seven sera). The observation of several fluorescence patterns in IF and reactivities against proteins of different molecular weights in WB suggested the presence of various cell cycle-associated autoantigens.

Five sera reacted with a polypeptide at a molecular weight of 34 000, and three of them were shown to contain anti-PCNA antibody by DID (data not shown). One of these five sera, serum OH, was obtained from a 62-year-old female with RA. Since serum OH showed unique reactivity in IF and WB (Fig. 1A, panel 2; Fig. 1B, lane 2), we attempted to analyze the autoantibodies in the serum and characterize the target antigen recognized by the serum (OH antigen).

Specificity of the antigen recognized by serum OH in IF and WB

In IF, serum OH showed a variegated speckled pattern, fine speckled nucleoplasmic staining and nucleolar staining in interphase cells, and discrete dots staining in the central area of the cell, like anticentromere antibody, in addition to midbody staining in metaphase cells (Fig. 2). In WB, serum OH showed reactivity to a 34 kD polypeptide, whose molecular mass was almost identical to that of PCNA (Fig. 1B, lane 1, 2). In DID, serum OH did not produced any precipitin line (data not shown).

To determine whether the 34-kD antigen recognized by serum OH was different from PCNA, the immunoreactivity of serum OH was compared with that of anti-PCNA serum in WB (Fig. 3). In WB, using crude RTE as the antigen, both serum AK and serum OH reacted with a 34-kD polypeptide. When PCNA antigen was purified from RTE and used for WB, serum OH did not react with the purified PCNA, while serum AK showed specific reactivity with the 34-kD PCNA polypeptide. This result indicated that the antigen recognized by serum OH was different from PCNA.

For the characterization of the 34-kD antigen recognized by OH, the antibody to the 34-kD band was eluted from the blot and used for IF and WB (Fig. 4). Antibody to 34-kD antigen purified from the blot showed the same staining pattern observed in IF using serum OH, and the purified antibody reacted with 34-kD polypeptide in WB. Purified anti-PCNA antibody, which was used as a control, also showed a specific staining pattern, as did prepurified serum AK. In contrast, samples purified from a different area from 34 kD on the blot showed no reactivity in IF (data not shown). These results indicated that the antibodies to 34-kD polypeptide was specific for the cell cycle-associated antigen recognized by serum OH, which was different from anti-PCNA antibody.

To determine whether the OH antigen is localized in the nucleolus, HeLa cells were double-stained with serum OH and a murine serum with antifibrillarin antibody. There was

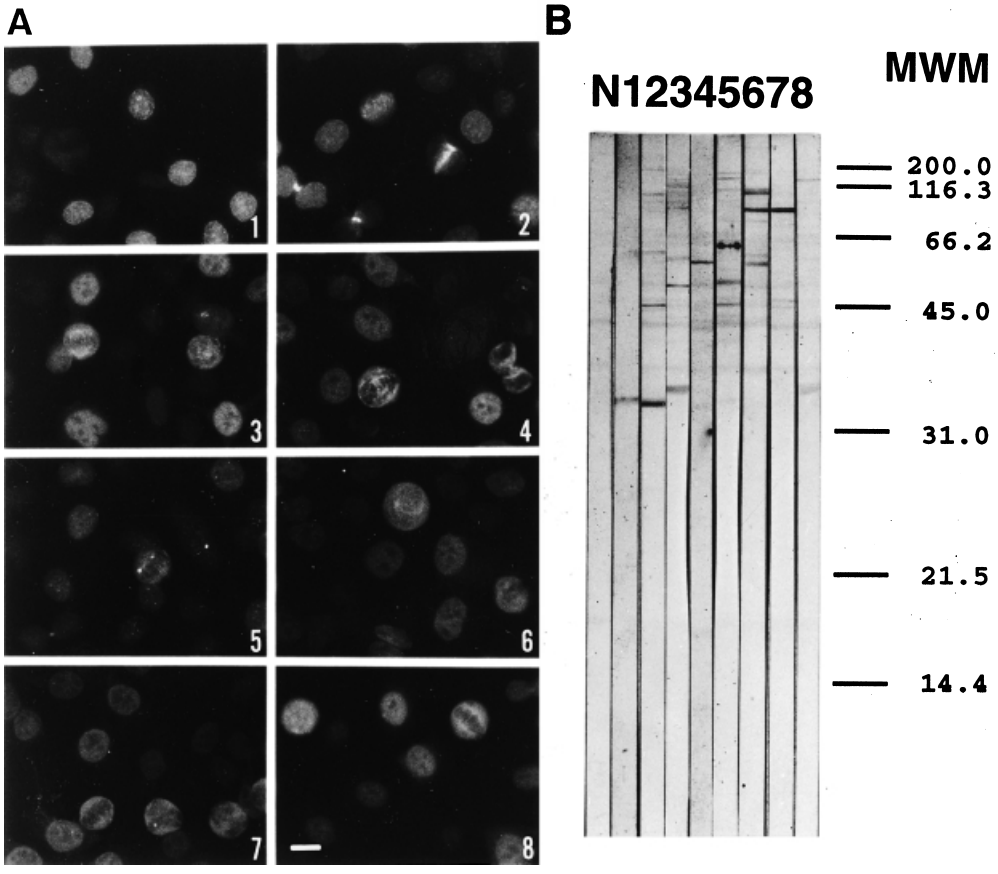


Fig. 1. Representative reactive patterns of patient sera in immunofluorescence (IF) using HEp-2 cells (**A**), and in Western blotting (WB) using MOLT-4 whole cell extract (**B**). The panel numbers in IF and lane numbers in WB are identical. The immunoreactivities of anti-proliferating cell nuclear antigen (PCNA) serum AK and serum OH are shown in panels *1* and *2*, respectively. *Bar* 20 μm

a marked correlation between the coarse granular staining in IF using serum OH and the nucleolar staining in IF using antifibrillarin antibody (Fig. 5, indicated by single arrows). This result suggested that the target antigen recognized by serum OH was partially located in the nucleolus. However, there were some interphase cells showing nucleoplasmic staining without nucleolar staining (Fig. 5, indicated by double arrows), suggesting that nucleolar localization of OH antigen was also dependent upon the cell cycle.

Localization of OH antigen during the cell cycle

To further study the expression of OH antigen during the cell cycle, HeLa cells were double-stained with serum OH and mouse anticyclin B antibody as a marker for the G2 and M phases. In IF, cyclin B is known to accumulate in the perinuclear region of the cytoplasm during G2, enter the nucleus at prophase, accumulate at the spindle caps, and then be degraded at the metaphase/anaphase boundary. All positive cells stained by anticyclin B antibody were also stained by serum OH (Fig. 6A and B, indicated by single arrows). On the other hand, most cells negative for staining by anticyclin B antibody were not stained by OH. However, some negative cells were stained by OH. In contrast, none of the cells positive for staining by anticyclin B antibody were stained by anti-PCNA antibody, and all cells negative for staining by anticyclin B antibody were stained by anti-PCNA antibody. Consequently, it was suspected that OH antigen, like cyclin B, appeared during the G2 and M phases.

For further analysis of the expression of OH antigen during the cell cycle, HeLa cells were synchronized by a double thymidine block and used as substrates for IF (Fig. 7). The synchronized cells were double-stained with a mixture of serum OH and a mouse anticyclin B antibody, and the immunoreactivities were compared with that of anti-PCNA antibody. When most cells were in G1/S at 2 h after release of the thymidine block, no expression of cyclin B was detected. Both serum OH and anti-PCNA antibody stained the nucleus. Serum OH showed fine speckled staining throughout the nucleus, and nucleolar staining in some cells. At this stage, anti-PCNA antibody showed no nucleolar staining. In G2 at 9 h, serum OH stained the nucleus and nucleolus in most cells. Cyclin B was detected in the cytoplasm at this time, and the reactivity to PCNA had almost disappeared. Most cells entered mitosis at 12 h. Serum OH reacted with chromosomes and the midbody, and anticyclin B antibody showed positive metaphase staining. In G1 at 18 h, no reactivity to either OH antigen or cyclin B was detected. When most cells reentered the S phase at 24 h, OH antigen appeared again, as did PCNA. These results suggested that OH antigen appeared between G1/S and M, mainly in the nucleoplasm at G1/S, in the nucleoplasm and nucleolus at G2, and in the chromosome and midbody at the M phase.

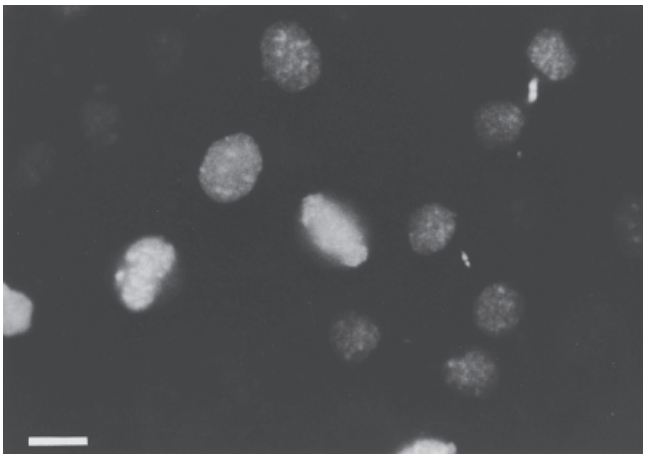


Fig. 2. IF pattern characteristic of serum OH using HEp-2 cells. *Bar* 20 μm

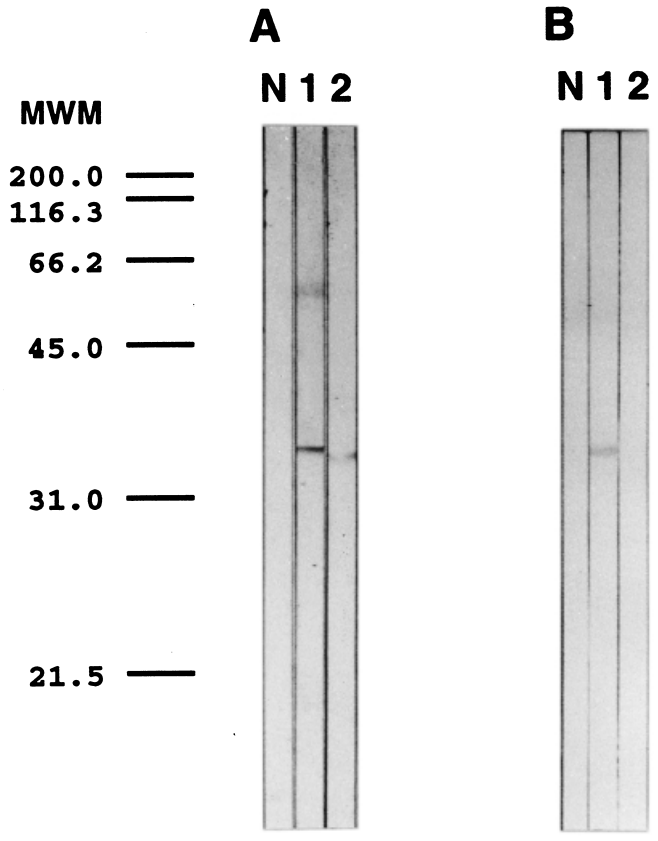
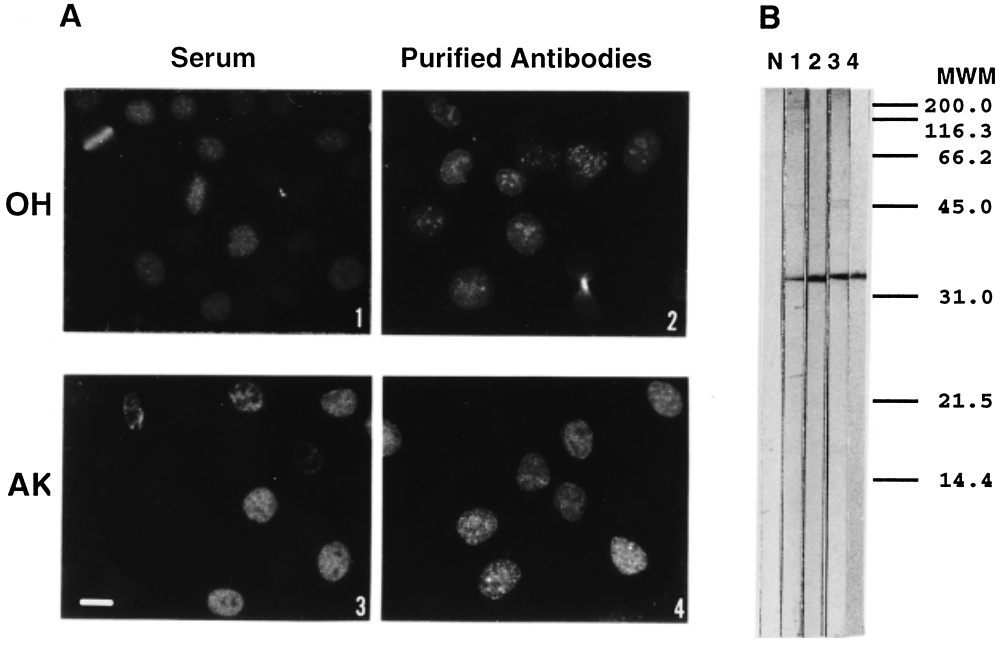


Fig. 3. Comparison of the immunoreactivity of antibodies from sera AK and OH in WB using rabbit thymus extract (RTE) (**A**) and using PCNA purified by TOB 7 (**B**). Lane *N*, normal human serum; lane *1*, serum AK; lane *2*, serum OH

Fig. 4. Comparison of immunoreactivities of sera without purification and affinity-purified antibodies from sera AK and OH in IF (**A**) and WB (**B**). *1*, serum OH; *2*, antibodies to 34-kD band purified from serum OH; *3*, serum AK; *4*, antibodies to 34-kD band purified from serum AK. Lane *N*, normal human sera. *Bar* 20 μm



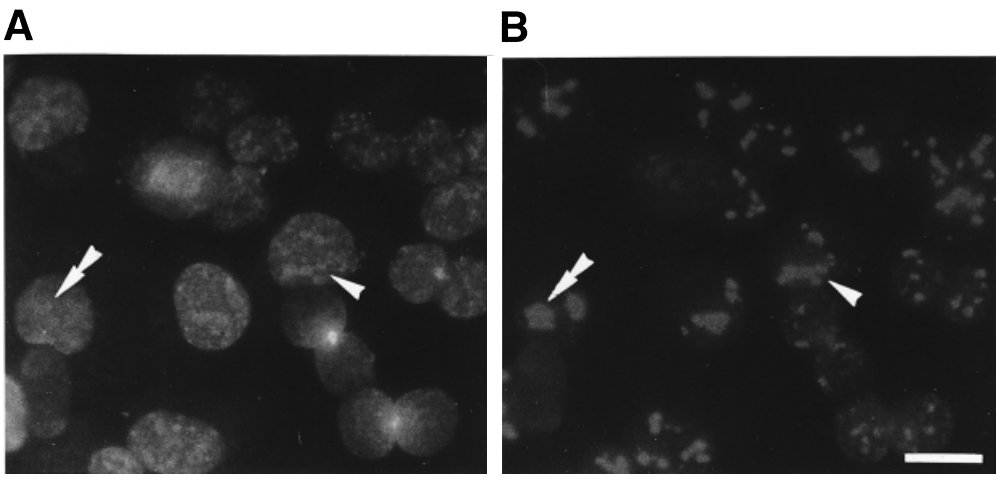


Fig. 5. Localization of OH antigen in the nucleolus. HeLa cells were stained with a mixture of serum OH (fluorescein isothiocyanate (FITC), **A**) and mouse antibody to fibrillarin (rhodamine, **B**). A *single arrow* indicates correlation of the coarse granular staining in IF using serum OH and nucleolar staining; a *double arrow* indicates an interphase cell showing nucleoplasmic staining without nucleolar staining in IF using serum OH. *Bar* 20 μm

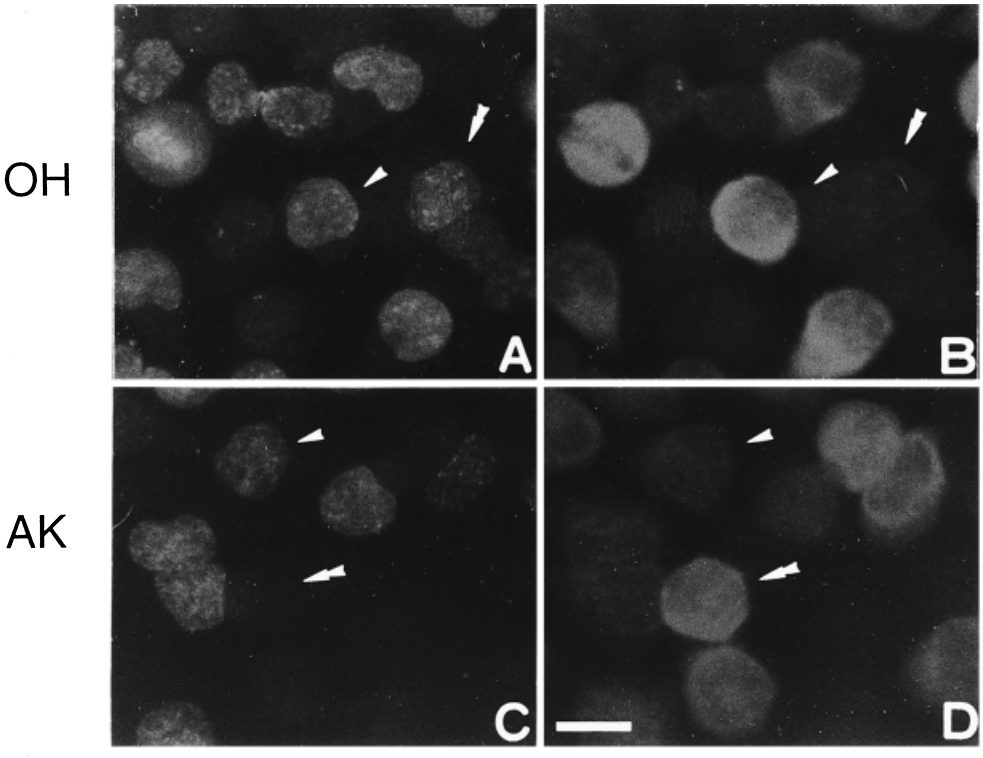


Fig. 6. Double staining with human sera (FITC) and anticyclin B antibody (rhodamine). HeLa cells were stained with a mixture of serum OH (panel *A*) and anticyclin B antibody (panel *B*), or a mixture of serum AK (panel *C*) and anticyclin B antibody (panel *D*). *Single arrows* in panels *A* and *B* indicate a positive cell double-stained by serum OH and anticyclin B antibody. *Double arrows* in panels *A* and *B* indicate a cell stained only by OH. *Bar* 20 μm

Discussion

In this study, we analyzed the immunoreactivity of autoimmune sera with cell-cycle-associated antibodies. All 30 sera examined showed a variegated speckled pattern in IF. The serum samples were mainly obtained from lupus patients (12 of 30). However, the remaining 18 sera were obtained from patients with other connective tissue diseases or several nondefined diseases. These results suggested that cell cycle-associated autoantibody was not a marker for the diagnosis of SLE, even though the majority of sera were obtained from lupus patients.

Autoantibodies against PCNA, RMSA-1, NuMA, and CENP-F are known to be representative autoantibodies against cell cycle-associated antigens detected in rheumatic

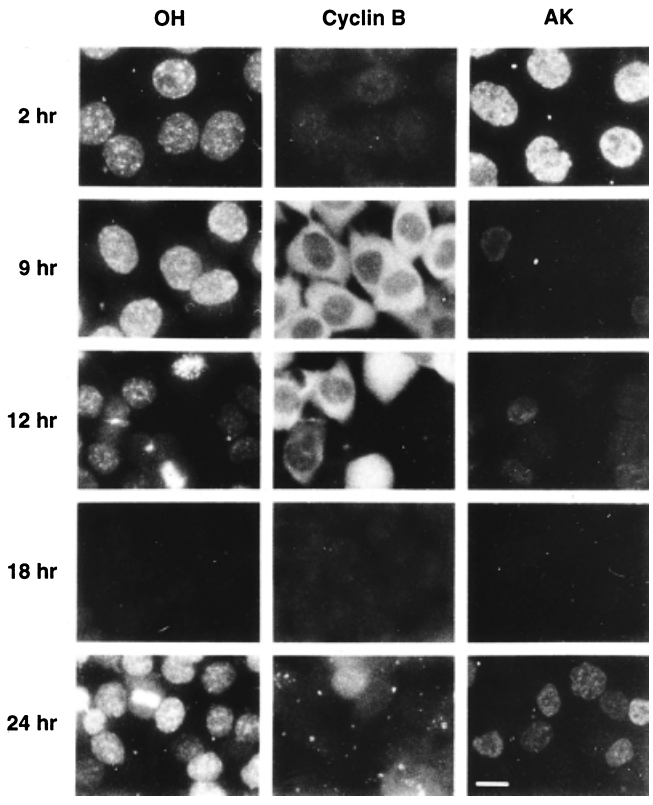


Fig. 7. The expression of OH antigen during the cell cycle. HeLa cells synchronized by excess thymidine block were stained by a mixture of serum OH (FITC) and a mouse anticyclin B antibody (rhodamine), or by serum AK with anti-PCNA antibody. *Bar* 20 μm

diseases. Among these autoantibodies, only anti-PCNA antibody has been established as being clinically useful in SLE by several investigators.[28] Anti-PCNA antibody is specifically detected in lupus sera and the presence of this antibody is strongly associated with thrombocytopenia, seizure, and nephritis.[5,6] In this study, immunoreactivity to PCNA was confirmed in three sera by DID, and all of these sera were from lupus patients. However, our results indicated that nine other lupus sera which showed variegated speckled patterns in IF were negative for anti-PCNA antibody and showed different immunoreactivities from anti-PCNA antibody in IF and WB. These results suggested that the variegated speckled pattern in lupus sera is not specific for anti-PCNA antibody, and that further analysis for the identification of anti-PCNA antibody will be required for an understanding of the clinical features.

In the IF and WB studies, several reactivities were observed, suggesting the presence of various cell cycle-associated antigens recognized by autoimmune sera. Only anti-PCNA antibody did not show reactivities with mitotic cells in IF, suggesting that most cell cycle-associated antigens appeared during mitosis.

The autoantibody reacting with a polypeptide with a molecular weight of 89000 that was detected in seven sera was the most frequently detected autoantibody in this analysis. Three of these sera were obtained from lupus patients, one serum was from an RA patient, and the other sera were obtained from patients who were not diagnosed with rheumatic disease. Autoantibodies reacting with other polypeptides were also obtained from various rheumatic diseases, but we could not identify the disease specificities or clinical significance of the cell cycle-associated autoantigens. Further studies using sera from a large number of patients will be needed to determine the clinical relevance of these cell cycle autoantibodies.

Among the sera in this study, we found an autoantibody that recognized an antigen (OH antigen) which was localized in various cell components during the cell cycle. The purification of this autoantibody revealed that the target antigen was a single 34-kD polypeptide. Anti-OH antibody showed fine speckled staining in interphase cells, and discrete dot staining in the central area in the cell and midbody in M-phase cells, which seems to resemble those of Na antigen[29,30] and CENP-F.[8,31] In addition, serum OH did not show any precipitation lines in DID, and the same result was also reported in DID using anti-Na antibody.[29]

The disease associations of these autoantibodies were also studied. Anti-OH antibody was obtained from a patient with RA. In contrast, anti-Na antibody has been reported to be specific for SLE.[30] Anti-CENP-F antibody is rarely found in rheumatic diseases, but is often detected in cancers of various types, chronic liver disease, chronic rejection of renal allografts, and Crohn's disease.[12,13] Further, the target antigen recognized by serum OH was different from the Na and CENP-F antigens because (i) the target antigen appeared in the nucleolus during the S and G2 phases, whereas the Na and CENP-F antigens did not appear in the nucleolus, (ii) the target antigen also appeared in the G1/S phase, whereas the Na antigen appeared from the S/G2 to the M phase, but did not appear in the G1/S phase, (iii) anti-OH antibody specifically reacted with a 34-kD polypeptide, while the molecular weight of CENP-F has been reported as 330000.

Anti-Na antibody was reported to be present in 83% of the sera showing a variegated speckled pattern.[30] However, similar staining patterns were rarely detected by IF in our study. Since we used sera with titers higher than 1:160, and most titers of anti-Na antibody are less than 1:160,[29] anti-Na antibody might have been underestimated in our study.

From the results demonstrated here, we conclude that many cell cycle-associated autoantigens are targeted by autoimmune sera from patients with various rheumatic diseases. We also characterized OH antigen as a newly identified cell cycle-associated autoantigen. Further definitive work will be needed to define the specificities of these antigens and to determine the clinical importance of these antibodies.

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