Autoantibody to DNA Topoisomerase II in Primary Liver Cancer[1]

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ABSTRACT

A patient with chronic hepatitis associated with hepatitis C virus infection was observed to convert from antinuclear antibody-negative to antinuclear antibody-positive status at the time when liver cancer was detected. The newly recognized antibodies reacted with a nuclear protein doublet of 170 and 180 kDa in immunoblotting, and in fluorescence-activated flow cytometry the antigens were shown to vary in expression level in a cell cycle-related manner: minimum in G_1, increasing in S, and maximum in G_2 and M. In synchronized HeLa and HEp-2 cells, immunofluorescence microscopy showed uniformly distributed staining of the nucleoplasm in S-phase, with increased intensity of nucleoplasmic staining in G_2, at which time nucleolar staining was also present. In M, condensed chromosomes were uniformly stained. Using previously characterized polyclonal antibodies to DNA topoisomerase II (topo II) as reference markers, the antigens recognized by the patient's serum were shown in Western blotting to have the same mobilities as DNA topo IIα (170 kDa) and β (180 kDa) isoforms. The patient's serum was also highly efficient in inhibiting DNA topo II in an *in vitro* functional assay. Antibody to DNA topo II appeared *de novo* in close association with transformation to cancer, and since dysregulation of DNA topo II is considered to be involved in some forms of tumorigenesis, the observed antibody response in this patient could conceivably be an immune reaction to the abnormally regulated protein.

INTRODUCTION

Autoantibodies in systemic autoimmune diseases such as systemic lupus erythematosus, polymyositis, and scleroderma have been used to identify antigens and to characterize the molecular structure and function of novel cellular components (for review, see Ref. 1). The antigens include small nuclear ribonucleoprotein particles engaged in pre-mRNA splicing (2), proliferating cell nuclear antigen involved in DNA replication (3), and Ku involved in a DNA-dependent protein kinase reaction (4). From studies in systemic autoimmune diseases, it has been suggested that an antigen-driven mechanism might be the basis of autoantibody responses.

In breast and lung cancer, antibodies to the tumor suppresser gene product *p53* have been detected (5–7) and missense mutations of the *p53* gene which lead to aberrant expression of p53 has been suggested to be responsible for the antibody response (6, 7). Recently we reported the finding of autoantibodies to nuclear and nucleolar antigens (ANA[5]) in patients with HCC (8, 9). Retrospective longitudinal study in patients with chronic liver disease who developed HCC revealed that one third of the HCC patients showed changes in ANA manifested as seroconversion from negative to positive antibody status, increase in ANA titers, or change in specificities (9). These manifestations were observed in close temporal relationship with diagnosis of HCC in several patients.

We now describe one patient with hepatitis C-related chronic liver disease who developed an autoantibody targeting a cell cycle-related nuclear antigen which was identified as DNA topo II. Seroconversion and increase in titers of ANA were found to be closely associated with a rise in AFP levels and diagnosis of liver cancer.

MATERIALS AND METHODS

Patient and Sera. A Japanese male who had a history of blood transfusions during an operation for osteomyelitis at age 10 had been healthy until he was found to have abnormal liver functions during a routine health examination at age 56. He was referred to Shinshu University Hospital for evaluation of the liver disease at age 59 (in 1978) and after clinical studies, including liver biopsy, was diagnosed as having non-A, non-B chronic hepatitis. Since then, he has been seen at the hospital at regular intervals. Later he was found to have antibody to hepatitis C virus determined by methods recently described (10). He was not treated with IFN for his chronic hepatitis. At age 69 (in 1988) a space-occupying lesion in the liver was detected by ultrasonography. Hepatic angiography revealed a small tumor stain in the right hepatic lobe and computed tomographic radioimaging after iodized poppyseed oil (Lipiodol) infusion showed at least three high density areas (each ~3 cm in diameter) in the right hepatic lobe. At this time the serum AFP level was 73 ng/ml (normal <20 ng/ml). He was diagnosed as having HCC and was given transhepatic arterial chemoembolization therapy. In spite of repeated treatments, tumor progression was not

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[5] The abbreviations used are: ANA, autoantibody to nuclear antigens; HCC, hepatocellular carcinoma; AFP, α-fetoprotein; topo II, topoisomerase II.

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arrested, and he died at age 72 (in 1990). Postmortem was restricted to needle biopsy of the liver which showed cholangiocarcinomatous tumor of the liver. Clinical information was obtained from the patient's charts and attending physicians. Sera were collected at several time points when liver function tests were performed and kept frozen (Hi sera).

Cell Culture. HEp-2, HeLa, 3T3, and MOLT-4 cells were obtained from American Type Culture Collection (Rockville, MD) and were cultured in DMEM supplemented with 10% FCS, 1% L-glutamine, and 2.5 mg/ml gentamicin sulfate in a humidified atmosphere containing 5% CO_2 at 37°C.

Immunofluorescence Microscopy. Serum was diluted in PBS and titration of ANA titers was performed on commercially prepared HEp-2 cells (Bion, Park Ridge, IL). Other cell lines were grown in this laboratory, fixed in 100% methanol for 5 min at −20°C, and permeabilized in 100% acetone for 3 min at −20°C. To rule out the possibility that patterns of immunolocalization might be caused by certain fixation conditions, cells were also fixed in 2% formaldehyde buffered in PBS for 20 min at room temperature followed by permeabilization in 100% acetone for 5 min at −20°C, or fixed and permeabilized in 100% acetone for 10 min at −20°C. All of these different cell fixation procedures produced the same immunofluorescent patterns. FITC-conjugated goat anti-human IgG (Caltag, San Francisco, CA) was used as the secondary detecting reagent.

Western Blotting. Cells grown in culture dishes were scraped off in ice-cold PBS. After centrifugation, cell pellets were lysed in Laemmli's sample buffer (11) and sheared by passage through fine needles. Samples were boiled for 5 min and cellular debris was removed by centrifugation. Protein samples were electrophoresed in 7.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose. Western blotting procedures were performed as described (12). Primary antibody was diluted 1:100 in PBS containing 3% nonfat dried milk and 0.05% Tween 20. Bound antibodies were detected by using ^{125}I-labeled protein A (ICN, Irvine, CA) followed by autoradiography.

Flow Cytometry. Flow cytometry using methanol-fixed MOLT-4 cells has been described previously (13). Briefly, cells were incubated with sera diluted 1:50 in PBS containing 0.1% BSA for 30 min at room temperature. After rinsing twice with PBS containing 0.1% BSA, samples were reacted with FITC-conjugated goat anti-human IgG (Caltag). After two additional washes, cells were resuspended in a DNA staining solution containing propidium iodide (10 mg/ml) and RNase (1.8 K units/ml) and kept cold and dark for at least 30 min until flow cytometry analysis. Cells were analyzed in a FACScan flow cytometer equipped with an argon laser (488 nm; Becton Dickinson Immunocytometry Systems, Mountain View, CA) as described previously (14).

Cell Synchronization. HeLa cells were arrested at S-phase by using double thymidine block (15), and were harvested at closely spaced time intervals after release from thymidine block as described in "Results."

Polyclonal Antisera to DNA Topo II and Topo II Functional Assay. Antibodies to DNA topo IIα and β isoforms were raised in rabbits by immunization with synthetic peptides from the predicted sequences of the enzymes and were kindly provided by Dr. F. H. Drake (SmithKline Beecham, King of Prussia, PA). R22 selectively reacted with DNA topo IIα in Western blots and R29 raised against a highly conserved peptide common to both α and β isoforms recognized both equally well (16). Rabbit antiserum A10 from the laboratory of one of the co-authors (L. F. L.) was raised against biochemically purified DNA topo II (17) and was reactive with both α and β forms. All of these rabbit antisera were used in Western blots.

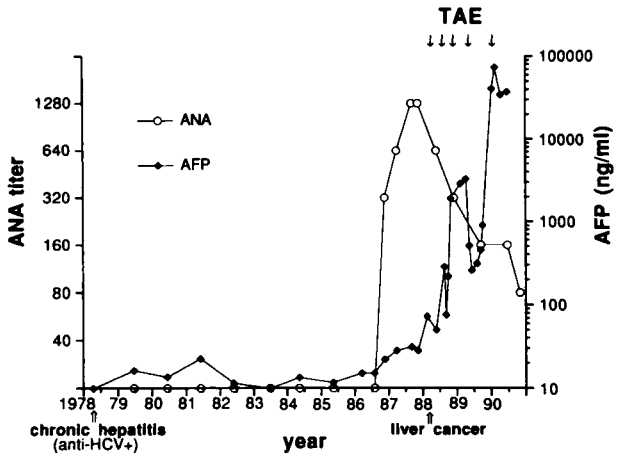

Fig. 1 Clinical course and changing titers of ANA. ♦, serum AFP levels; ○, ANA titers determined by indirect immunofluorescence; ⇑, time points when chronic hepatitis and liver cancer were diagnosed; ↓, time points when transhepatic arterial embolization therapy (*TAE*) was performed.

A functional assay for DNA topo II was used to determine whether the antibody in cancer serum had inhibitory activity. Naturally knotted P4 phage DNA was used to assay for inhibition of the strand-passing activity of topo II by various sera (18). Reaction systems (20 μl each) contained 40 mM Tris (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 μg BSA, 1 mM ATP, and 30 ng/ml human topo II. Sera from the cancer patient or control serum (undiluted and at 1:10–1:1000 dilutions) were added to the system and allowed to react for 30 min at 37°C. Reactions were stopped by the addition of 5 μl 20% Ficoll, 5% Sarkosyl, and 50 mM EDTA and then analyzed in 0.8% agarose gel in Tris-phosphate electrophoresis (0.08 M Tris-phosphate, 0.002 M EDTA, pH 8.0) buffer.

RESULTS

The clinical course and changing titers of ANA in the patient are depicted in Fig. 1. Seroconversion from ANA-negative to ANA-positive status was observed between June and November 1986. ANA titers subsequently increased dramatically and reached 1:1280 in October 1987. During the period of ANA seroconversion from negative to positive, progressive elevation in AFP levels was detected. Primary liver cancer was diagnosed in February 1988 by abdominal sonography, serum chemistries, and hepatic angiography, and the patient was given five courses of doxorubicin by transhepatic arterial chemoembolization. In spite of the treatment, the patient's condition deteriorated and he died approximately 3 years after the diagnosis was made.

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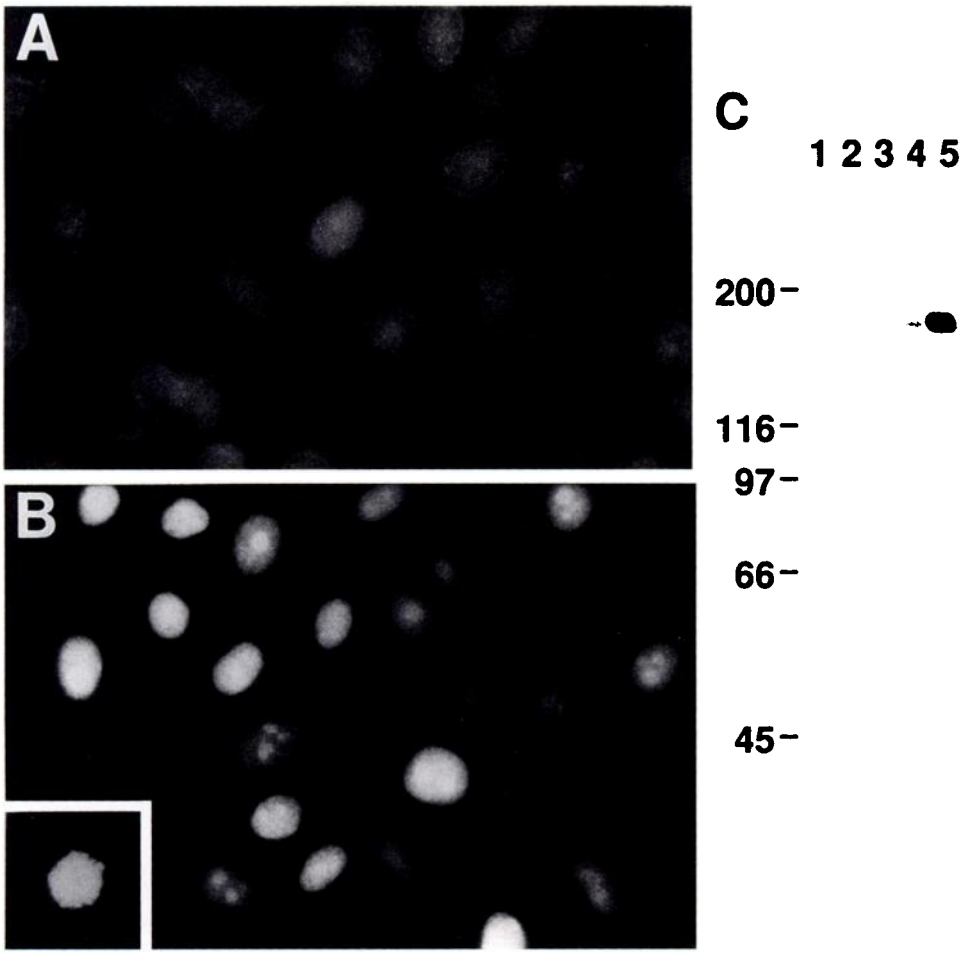

Fig. 2 Immunolocalization and Western blotting analysis with Hi sera. Indirect immunofluorescence microscopy on HEp-2 cells with Hi serum obtained in 1979 (*A*) and October 1987 (*B*). *Inset*, Metaphase cell. × 500. Western blotting analysis of sequential Hi sera using whole cell extract from HeLa cells (*C*). *Lane 1*, normal human serum; *Lanes 2–5*, probed with Hi sera from 1979, 1985, March 1987, and October 1987, respectively. *Numbers in margins*, relative molecular mass of proteins in kDa.

Fig. 2 depicts representative immunofluorescence microscopy on HEp-2 cells with serum from 1979 showing low background immunofluorescence in all cells (Fig. 2*A*), whereas serum from 1987 showed cell to cell variability with weak to strong densely speckled nucleoplasmic staining and in some cells association with granular staining of nucleolar regions (Fig. 2*B*). The variability in intensity and pattern of nuclear and nucleolar staining suggested a cell-cycle relationship since cells in various phases of the cell cycle were present in this nonsynchronized culture. This immunofluorescence pattern did not differ with different fixation methods, and was therefore not due to fixation artifact. In mitotic cells, uniform staining of condensed chromosomes was observed (Fig. 2*B*, *inset*). When sequential sera from the patient were tested by Western blotting (Fig. 2*C*), reactivity with a 170/180-kDa band was found in the 1987 sera but not in earlier samples and the signal intensity of the October 1987 sample (*Lane 5*) was clearly much stronger than that of the March 1987 sample (*Lane 4*) in keeping with increasing titer of immunofluorescent ANA (Fig. 1). It was also noteworthy that no other reactive bands were detected in Western blotting against whole cell extract (Fig. 2*C*), suggesting that this serum was relatively monospecific by this assay.

To determine whether variation in nuclear and nucleolar immunofluorescence staining with Hi serum was related to the cell cycle, MOLT-4 cells were analyzed by fluorescence-activated flow cytometry. Dot plot from 5000 cells reacted with normal human serum (Fig. 3*A*) or Hi serum of October 1987 (Fig. 3*B*) is presented in Fig. 3. Staining with Hi serum increased in cells with S-phase DNA content reaching maximum levels in G_2-M cells (Fig. 3*B*), which supports the observations in immunofluorescence microscopy.

Synchronized HeLa cells were used to analyze in more detail the distribution of this cell cycle-related antigen. With Hi serum of October 1987 as the antibody probe, weak nuclear staining was detected after release of cells from thymidine block when they were in the G_1-S boundary (Fig. 4*A*). Progressively increasing immunofluorescence in the nucleoplasm was observed 3 h later in mid-S-phase (Fig. 4*B*) and 6 h later in late S-phase (Fig. 4*C*). Eight h later in late S-G_2, there was strong nucleoplasmic immunofluorescence and nucleolar staining was

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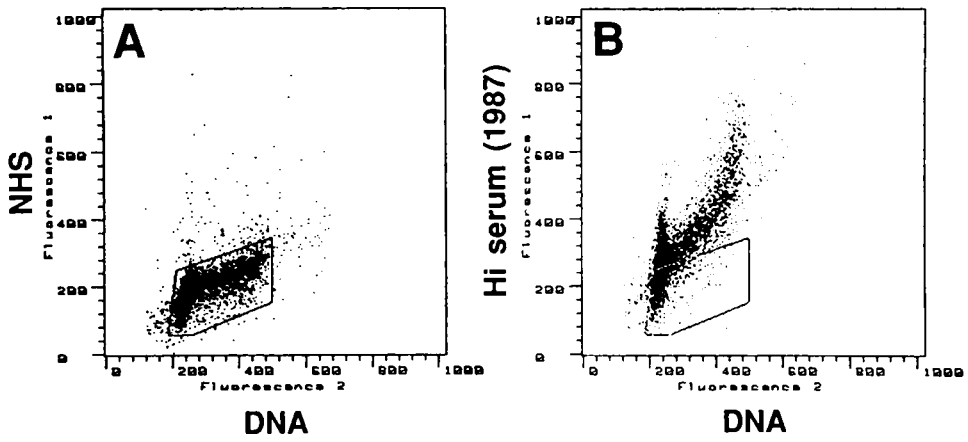

Fig. 3 Flow cytometric analysis of immunostaining with Hi serum using MOLT-4 cells. Dot plot from 5000 cells stained with normal human serum (*A*) or Hi serum from October 1987 (*B*). Intensity of immunostaining with antibody is presented in the vertical axis and related to DNA content detected by propidium iodide in the horizontal axis. The *boxed area* in *B* is the equivalent location of the *boxed area* in *A*.

also observed at this time (Fig. 4*D*). Ten h later many cells were in M and there was uniform staining of condensed chromosomes (Fig. 4*E*). Immunofluorescence intensity reverted to lower levels after M, and during G_1 only weak nucleoplasmic staining with absence of nucleolar staining was observed (Fig. 4*F*). Western blotting using extracts from synchronized HeLa cells gave results which corresponded with immunofluorescence studies. Increasing intensity of the 170/180-kDa signal was detected in extracts from early S, mid-S and late S-phase cells, reaching the maximum in extracts from G_2-M cells (data not shown).

The molecular size of the 170/180-kDa antigen and the immunolocalization patterns in the cell cycle were somewhat similar to the reported properties of DNA topo II. Fig. 5 shows an immunoblotting study using mouse 3T3 cell extracts as the source of antigen. The single thick band found in immunoblotting against HeLa cell extracts was now resolved into a doublet (Fig. 5, *Lane 2*) and migrated with the same mobility as that of DNA topo IIα and β isoforms, using previously characterized rabbit antisera from two different sources as reference reagents. Studies of antibodies from autoimmune sera have shown that autoimmune antibodies are highly efficient in blocking function. In a P4 unknotting assay (Fig. 6), serum from October 1987, which was antibody positive in Western blotting and immunofluorescence, was capable of inhibiting P4 bacteriophage unknotting at a dilution of 1/100 whereas a serum of an earlier date (May 1985), which was antibody negative, was incapable of inhibition even with undiluted serum. This functional inhibition assay also helped to confirm the identity of the antigen.

DISCUSSION

DNA topo II is a nuclear enzyme with several functions. Its role in DNA replication (19, 20) and in chromosome segregation during mitosis (21, 22) has been clearly demonstrated and it is proposed to play a role in DNA transcription by participation in the regulation of transcription-dependent torsional stress (23, 24). Human DNA topo II is expressed in at least two different isoforms called α (170 kDa) and β (180 kDa; Ref. 25) and they are differentially expressed in the cell cycle. In MSB-1 cells (chicken lymphoblastoid cells), the 170-kDa form of the enzyme begins to increase during DNA replication and continues to increase through S and G_2, peaking in late G_2-M (26). As cells progressed from mitosis into G_1, much of the enzyme was degraded. These findings have been extended to studies of both the 170- and 180-kDa enzymes in mouse 3T3 cells (27). Expression of the 170-kDa enzyme was similar to that described in MSB-1 cells but expression of the 180-kDa enzyme was constant throughout the cell cycle but in a much lower amount. It was proposed that these different patterns of expression might suggest different functional properties.

The human serum reported in this study reacted with both α and β forms of topo II in mouse 3T3 cells. With HeLa cell extracts as the substrate in Western blotting, it was difficult to resolve the antigen into a doublet, whereas with 3T3 cell extracts, this could be readily observed (Fig. 5, *Lane 2*). It is possible that the autoimmune response is induced by only one of the isoforms (*e.g.*, topo IIα) but this epitope(s) is shared by both isoforms. Alternatively, the serum reported here represents a mixture of antibodies with specificity against each individual isoform of topo II. In immunofluorescence localization, the serum showed a cell cycle distribution highly suggestive of the α form (170 kDa) of topo II, with low to undetectable nuclear fluorescence in G_1, increasing in S, and peaking in G_2-M, confirmed also with fluorescence-activated flow cytometry. The immunofluorescence results obtained in HeLa cells could be explained by the high abundance of topo IIα and the low to undetectable amount of topo IIβ in HeLa cells (28). The localization of DNA topo II in nucleoli was observed in this study in the late S-G_2 of the cell cycle (Fig. 4*D*), and nucleolar localization of topo II has also been detected in *Drosophila* and HeLa cells (22, 29). The focal and heterogeneous nuclear staining patterns observed with the Hi serum have also been observed previously by others using anti-topo II antiserum (30).

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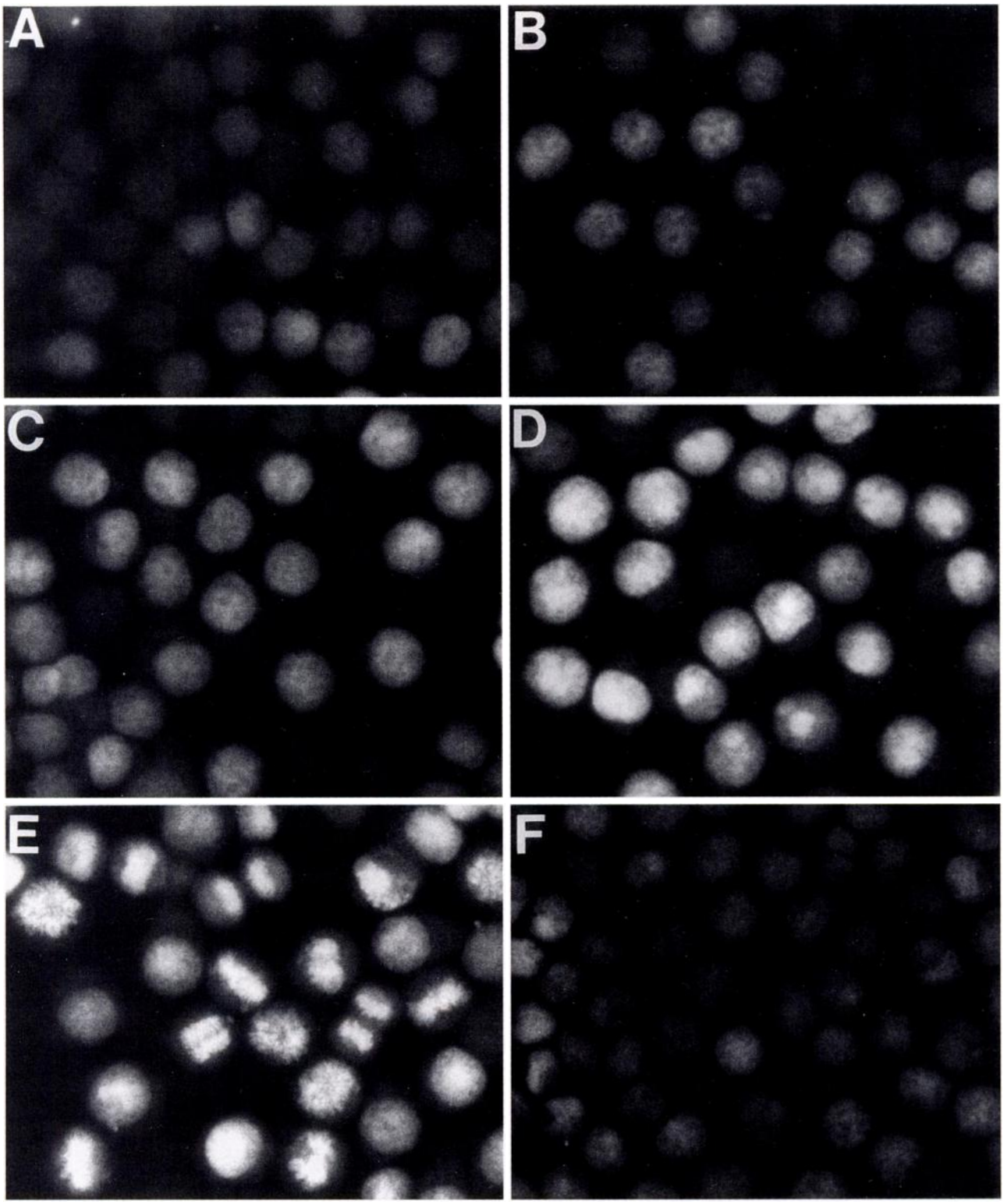

Fig. 4 Immunolocalization in different cell cycle phases with Hi serum of October 1987. HeLa cells were arrested at G_1-S boundary by double thymidine block. After release from thymidine block, cells were harvested at several time points and processed for immunofluorescence including immediately after release from thymidine block when most of the cells were in G_1-S transition (*A*), 3 h later in mid-S-phase (*B*), 6 h later in late S-phase (*C*), 8 h later in G_2 (*D*), 9 h later in M (*E*), and 14 h later in G_1 (*F*). × 500.

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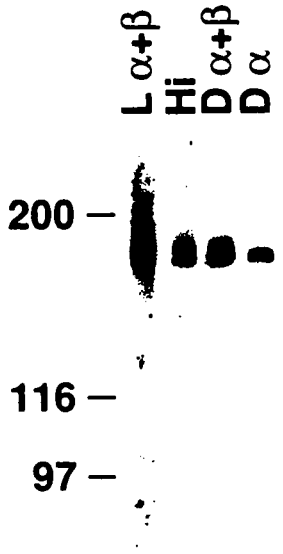

Fig. 5 3T3 cell extracts were used in Western blots to compare patient serum Hi with rabbit antisera to DNA topo II. Lα + β is antiserum to biochemically purified topo II (17) and Dα + β and Dα are antisera to synthetic peptides (25). Serum Hi reacted equally well with the α (170-kDa) and β (180-kDa) forms of topo II.

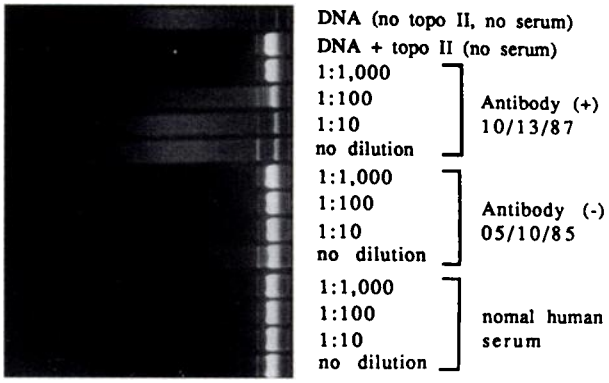

Fig. 6 Inhibition of DNA topo II function in a bacteriophage P4 unknotting assay (18). Serum containing anti-topo II (October 13, 1987) showed inhibition of topo II function up to 1:100 dilution whereas serum 2 years earlier had no inhibitory activity even undiluted.

The observations reported here are part of a study of patients with chronic liver disease (chronic hepatitis or liver cirrhosis) who progress to hepatocellular carcinoma and show changes in ANA responses in the process (8, 9, 31). The patient reported here had hepatitis C virus-associated chronic hepatitis, a condition with a high predisposition for progression to HCC. The postmortem needle biopsy of the liver showed cholangiocarcinoma although concomitant HCC could not be ruled out because of the limited size of the liver specimen obtained. In several other patients with HCC, we have not detected antibody to topo II and whether it might be more prevalent in cholangiocarcinoma remains to be determined. Autoantibody to topo II has been reported in 1 patient with systemic lupus erythematosus of a total of 68 patients studied (32). Others have reported detecting antibodies to topo II in 4 of 11 patients with systemic lupus erythematosus, but since antibody activity could be removed by absorption with DNA, the significance of this finding is uncertain (33). This group also found antibody to topo II in 6 of 16 patients with cryptogenic fibrosing alveolitis. This latter finding has not yet been confirmed by other reports and it appears that the real prevalence of this autoantibody in different disease conditions has to be ascertained.

The efficient inhibition of *in vitro* topo II function by patient serum is a recognized feature of autoantibodies found in systemic autoimmune diseases like lupus and scleroderma (1, 34) where autoantibodies were shown to inhibit functions such as pre-mRNA splicing and DNA replication (for lupus autoantibodies) and RNA polymerase I transcription (for scleroderma autoantibodies). Studies to characterize the epitopes recognized by autoantibodies have shown that these regions of the antigens are highly conserved and composed of discontinuous amino acid sequences which shape a conformation-dependent determinant (35, 36). In contrast, many experimentally induced monoclonal or polyclonal antibodies to these antigens recognize continuous primary structure determinants (35). It would be interesting to define the epitope(s) on DNA topo II recognized by autoantibodies since if analogy with other autoantibodies holds true, this region might be one of the active sites of the enzyme.

Studies of spontaneous autoantibody responses to nuclear antigens such as DNA, histones, and nuclear proteins involved in synthetic or regulatory functions give compelling reasons to believe that these autoimmune reactions are antigen driven (1, 37). The relatively monospecific immune response to DNA topo II in this patient and its close relationship with conversion to cancer raise the intriguing possibility that the *de novo* immune response was being driven by molecular events involved in malignant transformation. It has been observed from previous studies that intranuclear molecules involved in different functions are targets of such autoimmune reactions. An antigen was identified recently with another HCC serum which appears to belong to a family of splicing factors (31). Many other cellular antigens can be shown to be targets of autoantibodies in HCC and other cancers and elucidation of the identity of these antigens might help to clarify their possible roles in the transformation process. However the immune responses to such intracellular antigens should not be taken to mean that the antibodies are participating in pathogenesis, since there is little evidence to show that these antibodies are internalized into the cell.

One of perhaps several immune response pathways which might be implicated in the autoantibody response to DNA topo II in this patient is the possible overexpression of topo II in the patient's tumor cells. There was evidence to support this notion in two studies showing that antibodies to p53 were correlated with overexpression of p53 in breast and lung cancer patients (6, 7). The chromosomal location of topo IIα is in the region of 17q21–22 (38) as is the location of the oncogene *erb*B-2/HER-2/neu. The latter is often amplified in primary breast cancer (39) and it has recently been shown that topo IIα was found to be coamplified in 12% of c-*erb*B-2-amplified cases (40). Antibodies to *erb*B-2 were detected in breast cancer patients (41), but no

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studies were designed to look for antibodies to topo II. In the patient in this report, no cryopreserved tissues were available to determine whether amplification of these molecules were present in cancer tissue.

REFERENCES

1. Tan, E. M. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. Adv. Immunol., *44:* 93–151, 1989.

2. Yang, V. W., Lerner, M. R., Steitz, J. A., and Flint, S. J. A small nuclear ribonucleoprotein is required for splicing of adenoviral early RNA sequences. Proc. Natl. Acad. Sci. USA., *78:* 1371–1375, 1981.

3. Prelich, G., Kostura, M., Marshak, D. R., Mathews, M. B., and Stillman, B. The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication *in vitro*. Nature (Lond.), *326:* 471–475, 1987.

4. Gottlieb, T. M., and Jackson, S. P. The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. Cell, *72:* 131–142, 1993.

5. Crawford, L. V., Pim, D. C., and Bulbrook, R. D. Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. Int. J. Cancer., *30:* 403–408, 1982.

6. Davidoff, A. M., Iglehart, J. D., and Marks, J. R. Immune response to p53 is dependent upon p53/HSP70 complexes in breast cancers. Proc. Natl. Acad. Sci. USA, *89:* 3439–3442, 1992.

7. Winter, S. F., Minna, J. D., Johnson, B. E., Takahashi, T., Gazdar, A. F., and Carbone, D. P. Development of antibodies against p53 in lung cancer patients appears to be dependent on the type of *p53* mutation. Cancer Res., *52:* 4168–4174, 1992.

8. Imai, H., Ochs, R. L., Kiyosawa, K., Furuta, S., Nakamura, R. M., and Tan, E. M. Nucleolar antigens and autoantibodies in hepatocellular carcinoma and other malignancies. Am. J. Pathol., *140:* 859–870, 1992.

9. Imai, H., Nakano, Y., Kiyosawa, K., and Tan, E. M. Increasing titers and changing specificities of antinuclear antibodies in patients with chronic liver disease who develop hepatocellular carcinoma. Cancer (Phila.), *71:* 26–35, 1993.

10. Kiyosawa, K., Sodeyama, T., Tanaka, E., Gibo, Y., Yoshizawa, K., Nakano, Y., Furuta, S., Akahane, Y., Nishioka, K., Purcell, R. H., and Alter, H. J. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology, *12:* 671–675, 1990.

11. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.

12. Chan, E. K. L., and Pollard, K. M. Autoantibodies to ribonucleoprotein particles by immunoblotting. *In:* N. R. Rose, E. Conway de Macario, J. L. Fahey, H. Friedman, and G. M. Penn (eds.), Manual of Clinical Laboratory Immunology, pp. 755–761, Washington, DC: American Society for Microbiology, 1992.

13. Landberg, G., Tan, E. M., and Roos, G. Flow cytometric multiparameter analysis of proliferating cell nuclear antigen/cyclin and Ki-67 antigen: a new view of the cell cycle. Exp. Cell Res., *187:* 111–118, 1990.

14. Landberg, G., and Roos, G. Antibodies to proliferating cell nuclear antigen as S phase probes in flow cytometric cell cycle analysis. Cancer Res., *51:* 4570–4574, 1991.

15. Bootsma, D., Budke, K., and Vos, O. Studies on synchronous division of tissue culture cells initiated by excess thymidine. Exp. Cell Res., *33:* 301–309, 1964.

16. Chung, T. D. Y., Drake, H. F., Tan, K. B., Per, S. R., Crooke, S. T., and Mirabelli, C. K. Characterization and immunological identification of cDNA clones encoding two human DNA topoisomerase II isozymes. Proc. Natl. Acad. Sci. USA, *86:* 9431–9435, 1989.

17. Miller, K. G., Liu, L. F., and Englund, P. T. A homogeneous type II DNA topoisomerase from HeLa cell nuclei. J. Biol. Chem., *256:* 9334–9339, 1981.

18. Liu, L. F., Davis, J. L., and Calender, R. Novel topologically knotted DNA from bacteriophage P4 capsids: studies with DNA topoisomerases. Nucleic Acids Res., *9:* 3979–3989, 1981.

19. Wang, J. C. DNA topoisomerases. Annu. Rev. Biochem., *54:* 665–697, 1985.

20. Liu, L. F. DNA topoisomerase poisons as antitumor drugs. Annu. Rev. Biochem., *58:* 351–375, 1989.

21. Earnshaw, W. C., Halligan, B., Cooke, C. A., Heck, M. M. S., and Liu, L. F. Topoisomerase II is a structural component of mitotic chromosome scaffolds. J. Cell Biol., *100:* 1706–1715, 1985.

22. Swedlow, J. R., Sedat, J. W., and Agard, D. A. Multiple chromosome populations of topoisomerase II detected in vivo by time-lapse, three-dimensional wide-field microscopy. Cell, *73:* 97–108, 1993.

23. Liu, L. F., and Wang, J. C. Supercoiling of the DNA template during transcription. Proc. Natl. Acad. Sci. USA, *84:* 7024–7027, 1987.

24. Hirose, S., and Suzuki, Y. *In vitro* transcription of eukaryotic genes is affected differently by the degree of DNA supercoiling. Proc. Natl. Acad. Sci. USA, *85:* 718–722, 1988.

25. Drake, F. H., Hofmann, G. A., Bartus, H. F., Mattern, M. R., Crooke, S. T., and Mirabelli, C. K. Biochemical and pharmacologial properties of p170 and p180 forms of topoisomerase II. Biochemistry, *28:* 8154–8160, 1989.

26. Heck, M. M. S., Hittelman, W. N., and Earnshaw, W. C. Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. Proc. Natl. Acad. Sci. USA, *85:* 1086–1090, 1988.

27. Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K., and Drake, F. H. Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. Cell Growth & Differ., *2:* 209–214, 1991.

28. Hsiang, Y-H., Wu, H-Y., and Liu, L. F. Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. Cancer Res., *48:* 3230–3235, 1988.

29. Zini, N., Martelli, A. M., Sabatelli, P., Santi, S., Negri, C., Astaldi Ricotti, G. C. B., and Maraldi, N. M. The 180-kDa isoform of topoisomerase II is localized in the nucleolus and belongs to the structural elements of the nucleolar remnant. Exp. Cell Res., *200:* 460–466, 1992.

30. Wolverton, J. S., Danks, M. K., Granzen, B., and Beck, W. T. DNA topoisomerase II immunostaining in human leukemia and rhabdomyosarcoma cell lines and their responses to topoisomerase II inhibitors. Cancer Res., *52:* 4248–4253, 1992.

31. Imai, H., Chan, E. K. L., Kiyosawa, K., Fu, X-D., and Tan, E. M. Novel nuclear autoantigen with splicing factor motifs identified with antibody from hepatocellular carcinoma. J. Clin. Invest., *92:* 2419–2426, 1993.

32. Hoffmann, A., Heck, M. M. S., Bordwell, B. J., Rothfield, N. F., and Earnshaw, W. C. Human autoantibody to topoisomerase II. Exp. Cell Res., *180:* 409–418, 1989.

33. Meliconi, R., Bestagno, M., Sturani, C., Negri, C., Galavotti, V., Sala, C., Facchini, A., Ciarrocchi, G., Gasbarrini, G., and Astaldi Ricotti, G. C. B. Autoantibodies to DNA topoisomerase II in cryptogenic fibrosing alveolitis and connective tissue disease. Clin. Exp. Immunol., *76:* 184–189, 1989.

34. Tan, E. M. Autoantibodies in pathology and cell biology. Cell, *67:* 841–842, 1991.

35. Huff, J. P., Roos, G., Peebles, C. L., Houghten, R., Sullivan, K. F., and Tan, E. M. Insights into native epitopes of proliferating cell nuclear antigen using recombinant DNA protein products. J. Exp. Med., *172:* 419–429, 1990.

36. Muro, Y., Tsai, W-M., Houghten, R., and Tan, E. M. Synthetic compound peptide simulating antigenicity of conformation-dependent autoepitope. J. Biol. Chem., *269:* 18529–18534, 1994.

37. Shlomchik, M., Mascelli, M., Shan, H., Radic, M. Z., Pisetsky, D., Marshak-Rothstein, A., and Weigert, M. Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation. J. Exp. Med., *171:* 265–297, 1990.

38. Tsai-Pflugfelder, M., Liu, L. F., Liu, A. A., Tewey, K. M., Whang-Peng, J., Knutsen, T., Huebner, K., Croce, C. M., and Wang, J. C.

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Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21–22. Proc. Natl. Acad. Sci. USA, *85:* 7177–7181, 1988.

39. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science (Washington DC), *244:* 707–712, 1989.

40. Smith, K., Houlbrook, S., Greenall, M., Carmichael, J., and Harris, A. L. Topoisomerase IIα co-amplification with *erb*B2 in human primary breast cancer and breast cancer cell lines: relationship to *m*-AMSA and mitoxantrone sensitivity. Oncogene, *8:* 933–938, 1993.

41. Disis, M. L., Calenoff, E., McLaughlin, G., Murphy, A. E., Chen, W., Groner, B., Jeschke, M., Lydon, N., McGlynn, E., Livingston, R. B., Moe, R., and Cheever, M. A. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res., *54:* 16–20, 1994.

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