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Human Liver Nucleolar Antigens[1] (41246)

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Abstract. In an extension of previous studies on the antigens in rat liver nucleoli (R. K. Busch, R. C. Reddy, D. H. Henning, and H. Busch, Proc. Soc. Exp. Biol. Med. **160**, 185 (1979); R. K. Busch and H. Busch, Tumori **63**, 347 (1977); F. M. Davis, R. K. Busch, L. C. Yeoman, and H. Busch, Cancer Res. **38**, 1906 (1978)), rabbit antibodies were elicited to human liver nucleoli isolated by the sucrose–Mg^{2+} method (10). Fluorescent nucleoli were found in liver cryostat sections treated with rabbit anti-human liver nucleolar antibodies followed by fluorescein-conjugated goat anti-rabbit antibodies. In HeLa cells, fluorescence was distributed throughout the nucleus and in a nuclear network but was not localized to the nucleolus. In placental cryostat sections, an overall nuclear fluorescence was observed with some localization to nucleoli. Immunodiffusion analysis revealed two immunoprecipitin bands which appeared to be liver specific. Other immunoprecipitin bands were common to liver, placenta, and HeLa nuclear extracts. Rocket immunoelectrophoresis revealed two liver-specific antigens, one migrating to the cathode and the other to the anode. Other rockets exhibited identity to antigens of other nuclear extracts. These results demonstrate the presence of human liver nucleolar-specific antigens which were not found in the HeLa and placental cells.

A recent report from this laboratory (1) presented data in which rabbit antibodies to rat liver nucleoli were used to detect specific rat liver antigens. It was found that two antigens, Ln-1 and Ln-3, were liver specific and one antigen Ln-2 was shared by liver and other tissues. Studies in which antibodies to normal rat liver and rat tumor nucleoli were used to evaluate rat liver and tumor antigens were reported previously (2, 3).

Recent studies from this laboratory have used human tumor nucleoli to elicit antibodies in rabbits (4). Of particular importance was the finding that a human tumor nucleolar antigen was present in a wide variety of human tumors (5, 6). This study led to the present report in which normal human liver nucleoli were used to immunize rabbits and the resultant antibodies were employed to study antigens in human liver and other tissues (7).

Although enzyme functions of human liver have been studied (8), and human liver tissues have been used as antigens (9), few studies have been done on human liver nucleolar antigens. Inasmuch as the possibility exists that the neoplastic state results from loss of a normal protein, information on normal human liver nucleolar and nuclear antigens may aid in the detection of specific proteins lost in neoplasia.

Materials and Methods. *Isolation of nuclei and nucleoli.* Nucleoli were isolated from human liver by the sucrose–Mg^{2+} method (10). Human liver specimens were obtained from autopsy (Harris County Medical Examiner's Office) of accident victims approximately 7 hr postmortem as approved by the Baylor Human Research Committee. The tissue was passed through a tissue grinder, washed twice in NKM (0.13 *M* NaCl/0.005 *M* KCl/0.008 *M* $MgCl_2$), and homogenized in 2.2 *M* sucrose/0.01 *M* $MgCl_2$. The nuclear pellet obtained after centrifugation was suspended in 0.34 *M* sucrose/0.0005 *M* $MgCl_2$ and sonicated to yield nucleoli or alternatively the nuclei were used for preparation of antigens.

The human placental nuclei were isolated in a similar manner from placentas obtained shortly after delivery (4). The HeLa nuclei were prepared from HeLa S3 cells grown in

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Spinner culture bottles and purified as described previously (4).

Immunization of rabbits. As previously reported (11) rabbits were immunized with whole nucleoli suspended in complete Freund's adjuvant. Each rabbit received three weekly immunizations and monthly boosters with incomplete Freund's adjuvant (11). The serum was collected and converted to Ig by 50% $(NH_4)_2$ SO_4 precipitation. The Ig was absorbed with human serum.

Immunofluorescence of nucleoli. The fluorescence of nucleoli was produced by the indirect immunofluorescence method (11) on human liver and placenta cryostat sections and on HeLa cells fixed in acetone.

Rocket immunoelectrophoresis. The procedure employed was that described by Laurell (12). The antigens were placed in wells and electrophoresed into a 1% agarose gel buffered with barbital, pH 8.4. Electrophoresis was carried out for 3 hr at 10 V/cm or overnight at 1.5–2 V/cm. The agarose gels were stained with Coomassie blue and destained with 13% acetic acid and 30% alcohol.

Immunodiffusion technique. The immunodiffusion plates were prepared with 0.8% agarose dissolved in PBS (0.01 *M* sodium phosphate/0.14 *M* NaCl, pH 7.4). The Ig fraction was placed in wells at a concentration of 15–20 mg/ml Ig and the nuclear extracts were placed in wells at a concentration of approximately 5–10 mg/ml. The antigens and antibodies were allowed to diffuse into the agarose gel at room temperature. The immunoprecipitin bands appeared in 18–36 hr and usually the plates were photographed 1 week later.

Preparation of nuclear antigens. The liver, placenta, and HeLa nuclei were extracted three times with 0.075 *M* NaCl/0.025 *M* EDTA, pH 8 (13, 14). The supernatants were concentrated in an Amicon with a UM 10 membrane (mol wt cutoff—10,000). The nuclei were subsequently extracted three times with 0.01 *M* Tris–HCl, pH 8. These supernatants were also concentrated as described above. The extracts were used as antigens for the rocket immunoelectrophoresis and for the immunodiffusion studies. All the extraction solutions used in this study contained 1 m*M* PMSF (phenylmethylsulfonyl fluoride).

Results. The localization of human liver antigen(s) in the nucleoli of cryostat sections of human liver tissue is illustrated in Figs. 1A and B. The acetone-fixed sections were initially treated with rabbit anti-human liver nucleolar antibodies and then with fluorescein-conjugated goat anti-rabbit antibodies. The nucleoli were visible as bright fluorescent organelles (arrows) against a less fluorescent nuclear background. In most tissue sections a limited number of fluorescent nucleoli were observed, presumably because of their small size and limited section thickness. In addition, nuclear membrane fluorescence (arrowheads) was frequently observed.

In HeLa cells, the localization of fluorescence was less restricted. As shown in Fig. 1C, there was nucleolonemal fluorescence (arrows) and fluorescence in a nuclear network (arrowheads). In the placental sections (Fig. 1D, arrow) fewer nucleoli were found and more background fluorescence was noted.

The results of the immunodiffusion studies are shown in Figs. 2A and B. The anti-human liver nucleolar antibodies (Ab) in the center well formed several immunoprecipitin bands with the liver NaCl–EDTA antigen (LNa). The small arrowhead (Fig. 2A) points to a distinct fine band designated L1 which appeared to be liver specific. A second immunoprecipitin band L2 (arrow, Fig. 2A) exhibited identity with the NaCl–EDTA antigens of HeLa nuclei (HNa). A comparable band (arrow) was found in the NaCl–EDTA placental nuclear extract (PNa) of Fig. 2B. A third immunoprecipitin band L3 (double arrowheads) formed between liver nucleolar antibodies (Ab) and the NaCl–EDTA antigens of liver (LNa), placenta (PNa), HeLa (HNa), and 0.01 *M* Tris–HCl antigens of liver (LTr) and placenta (PTr, double arrowheads). This precipitin band was not found in the HeLa Tris extracts. A dense liver immunoprecipitin band L4 (large arrowhead, Figs. 2A, B) formed be-

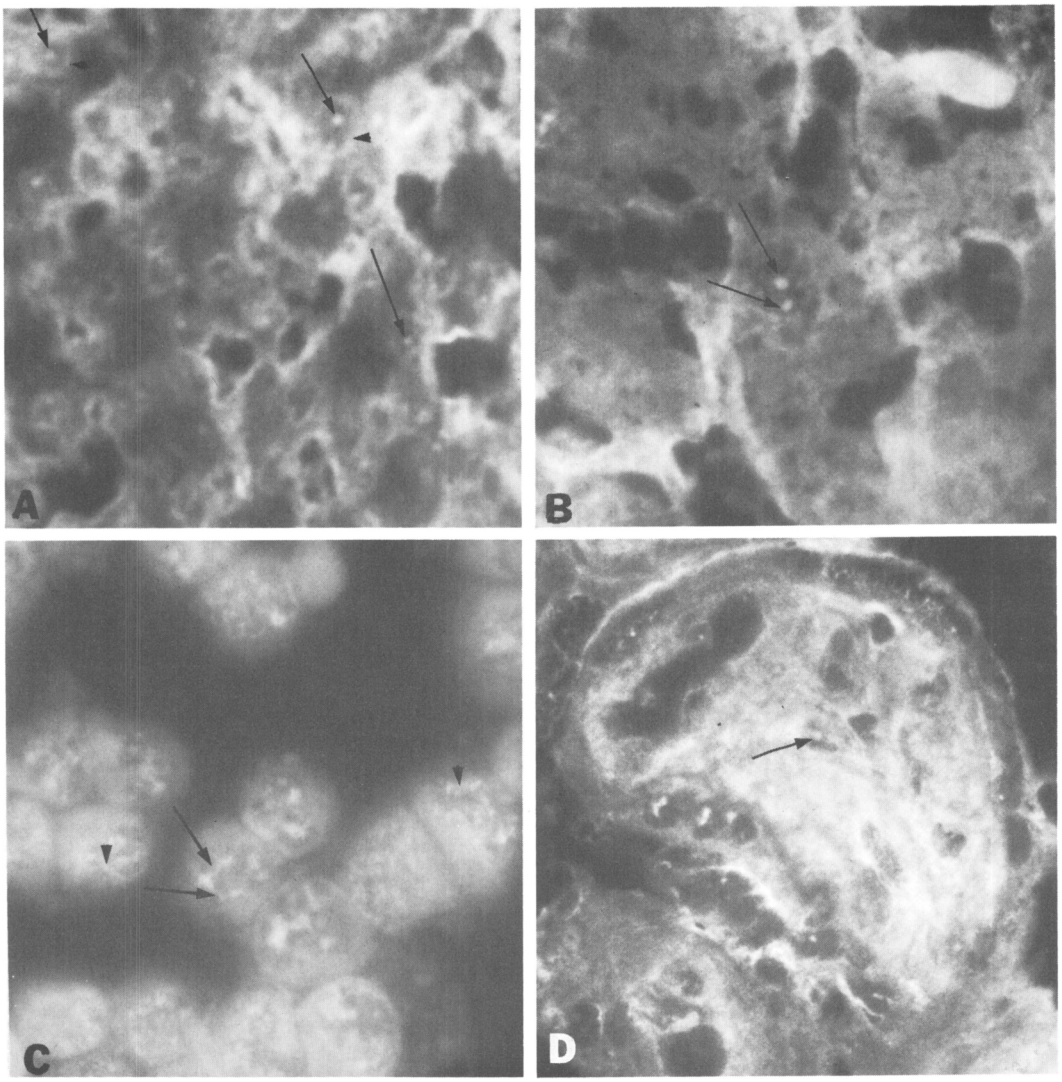

FIG. 1. (A) Immunofluorescence of human liver nucleoli in acetone fixed cryostat section of human liver. The sections were treated with rabbit anti-human liver nucleolar antibodies and then fluorescein-conjugated goat anti-rabbit antibodies. The arrows point to fluorescent nucleoli and arrowheads point to the nuclear envelope (×400). (B) Same as Fig 1A (×630). (C) Immunofluorescence of HeLa cells (fixed in acetone). The cells were treated with rabbit anti-human liver nucleolar antibodies and then fluorescein-conjugated goat anti-rabbit antibodies. The arrows point to the nucleolonemas and arrowheads point to nuclear network (×400). (D) Immunofluorescence of human placenta cryostat sections fixed in acetone. The tissue was treated with rabbit anti-human liver nucleolar antibodies and then fluorescein-conjugated goat anti-rabbit antibodies. The arrow points to a nucleolus (×400).

tween liver nucleolar antibodies (Ab) and liver NaCl–EDTA antigens (LNa). In Fig. 2A this band may contain an antigen that reacts only with liver extracts and another antigen that forms a connecting precipitin line between the HeLa antigen (HNa) and the liver antigen (LNa). In Fig. 2B the band which forms only with the liver antigen

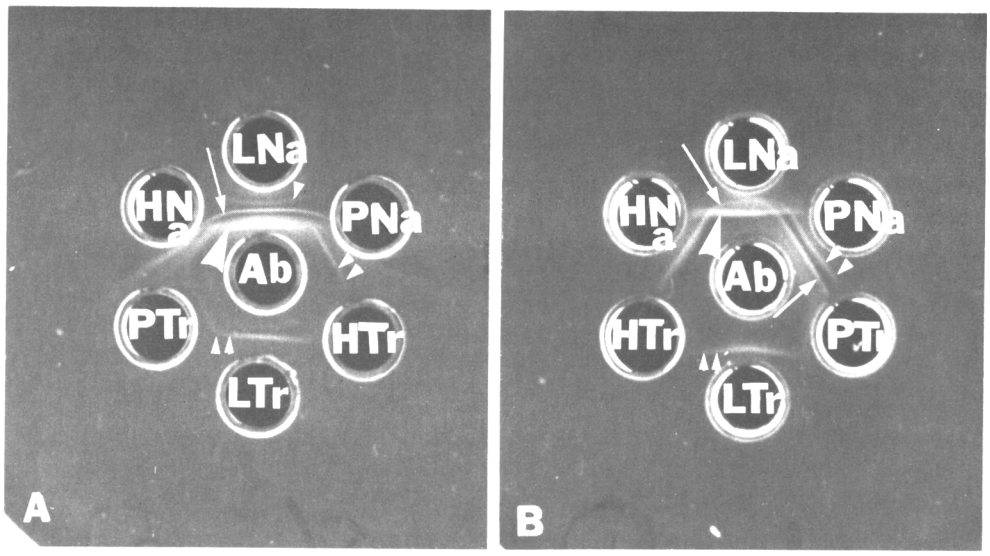

FIG. 2. (A) Immunodiffusion plate with liver nucleolar antibodies (Ab) in the center well. The liver nuclear NaCl–EDTA extract (LNa) formed a distinct precipitin band L1 (small arrowhead) with the antibodies (Ab). The precipitin band L4 (large arrowhead) may share partial identity with the HeLa extract (HNa) and may also have a distinct component (see Fig. 2B). A common precipitin band L2 (arrow) formed between the liver nucleolar antibodies (Ab) and nuclear antigen extracts of liver and HeLa NaCl–EDTA (LNa and HNa). A fourth precipitin band L3 (double arrowheads) was shared by three NaCl-EDTA extracts (LNa, HNa, and PNa) and two Tris-HCl extracts (LTr and PTr). The HeLa Tris extract (HTr) did not form immunoprecipitin bands with the antibodies. (B) Immunodiffusion plate with rabbit anti-human liver nucleolar antibodies (Ab) in the center well. This is similar to Fig. 2A but different nuclear preparations were used as antigens.

(LNa) extends out sufficiently to show lack of cross-reactivity with the HeLa antigen (HNa).

Figure 3 shows the rockets obtained with the rabbit anti-human liver nucleolar antibodies and the nuclear extracts. In Fig. 3A extracts were placed in wells cut from an agarose zone which did not contain antibodies and were electrophoresed into agarose that contained human liver nucleolar antibodies (from cathode to anode). This gel (Fig. 3A) had two groups of rockets; one group was formed by antigens that migrated further into the agarose (F); the second group migrated slowly into the agarose (S). The rockets in wells 1 through 9 fused to form common S rockets indicating they had common antigenic determinants. The S antigens were found in the wells which contained either 0.01 *M* Tris–HCl nuclear extracts of HeLa (1, 7), liver (3, 8), and placenta (6, 9) or NaCl–EDTA nuclear extracts of HeLa (2), liver (4), and placenta (5). There were four common rockets in the F group of antigens. This series of rockets (F) was formed by 0.01 *M* Tris–HC1 nuclear extracts of liver (wells 3, 8) and the NaCl–EDTA nuclear extracts in wells containing liver (4), HeLa (2), and placenta (5).

In addition to the shared or common rockets (S) and (F) there appeared to be a liver-specific rocket (A) formed between NaCl–EDTA liver nuclear extracts in well 4 (arrow) and liver nucleolar antibodies present in the agarose.

In Fig. 3B the anti-human liver nucleolar antibodies were distributed throughout the agarose gel to test for antigens migrating toward the cathode.

The NaCl–EDTA nuclear extracts of liver (wells 1, 4) produced three rockets (arrowheads), referred to as R1–R3 (Table I) that migrated toward the anode and a rocket (B) that migrated toward the cathode. In NaCl–EDTA nuclear extracts

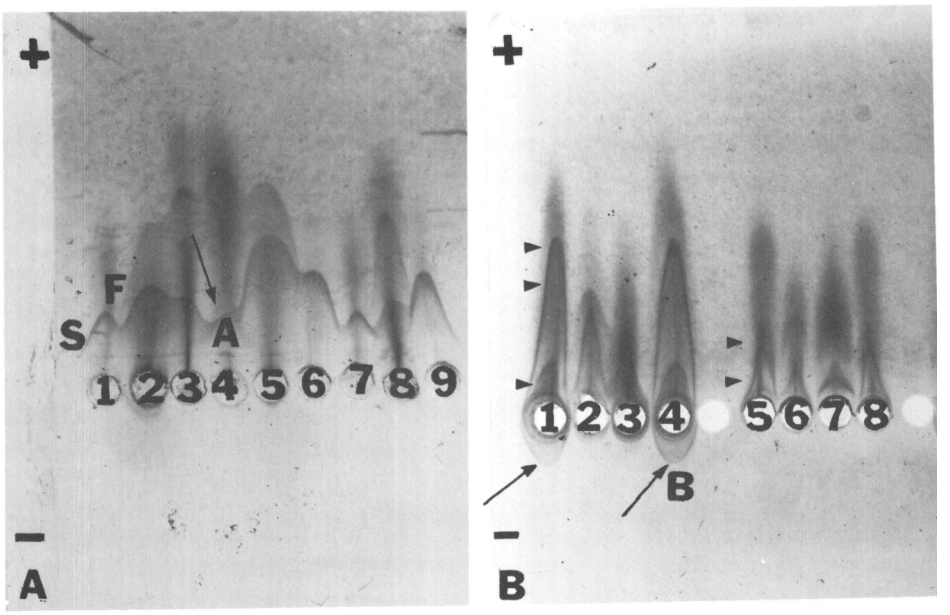

FIG. 3. (A) Rocket immunoelectrophoresis in which nuclear extracts were electrophoresed into 1% agarose which contained anti-human nucleolar antibodies, (approx 1 mg/ml). The electrophoresis was at 1.5–2 V/cm for 18 hr. The nuclear extracts were in the following wells: 0.01 *M* Tris–HCl, HeLa (1, 7), liver (3, 8), placenta (6, 9); and NaCl–EDTA, HeLa (2), liver (4), and placenta (5). There were common rockets (S) in wells 1–9 and (F) in wells 2–5 (and 8). The arrow points to a liver-specific rocket (A). (B) Rocket immunoelectrophoresis in which anti-human liver nucleolar antibodies were added to 1% agarose matrix and the nuclear extracts were placed in wells. The electrophoresis was at 1.5–2 V/cm for 18 hr. There were three rockets R1–R3 (arrowheads) formed between liver NaCl–EDTA extracts (wells 1, 4) and anti-human nucleolar antibodies. An additional rocket B (arrows) moved toward the cathode and appears to be liver specific. There were three rockets R1–R3 formed between NaCl–EDTA extracts of placenta (well 2) and the antibodies and two rockets R1 and R2 formed between HeLa (well 3) and the antibodies. The 0.01 *M* Tris–HCl extracts were added to wells: liver (5, 8), placenta (6), and HeLa (7). Each extract gave rise to two rockets none of which appears to be liver specific (R4, R5, arrowheads).

from placenta (well 2), three rockets (R1–R3, Table I) were found; none migrated toward the cathode. In HeLa NaCl–EDTA nuclear extracts (well 3), two rockets (arrowheads) were identified that migrated toward the anode (R1, R2; Table I).

The 0.01 *M* Tris–HCl nuclear extracts (liver, wells 5, 8; placenta, well 6; HeLa, well 7) each formed at least two rockets (arrowheads R5, R6; Table I). No antigen exhibited migration toward the cathode.

Discussion. Table I summarizes the results obtained with the immunodiffusion and rocket immunoelectrophoresis techniques. With each method, two liver-specific antigens were found. L1 and L4 were identified as immunoprecipitin bands and A and B were found as rockets. Each of these antigens was found in the NaCl–EDTA extracts of liver nuclei. In addition, this extract had several antigens in common with placenta and HeLa cells (L2, L3, S, R1, R2, F) or placenta alone (R3).

In the 0.01 *M* Tris–HCl nuclear extracts, the antigens were either common to liver, placenta, and HeLa (S, R4, R5) or common only to liver and placenta (L3). Antigen (F) which was common to the NaCl–EDTA extracts of liver, placenta, and HeLa was found only in 0.01 *M* Tris–HCl nuclear extract of liver.

Further studies are needed to determine whether the liver antigens L1 and L4 are the same as A and B. Since rockets are

TABLE I. LIVER ANTIGENS FOUND IN NUCLEAR EXTRACTS BY THE USE OF IMMUNODIFFUSION AND ROCKET IMMUNOELECTROPHORESIS

Nuclear extracts	Immunodiffusion antigens	Rocket antigens	
		Fig. 3A	Fig. 3B
NaCl–EDTA			
Liver	L1–L4	S,F,A	R1–R3,B
Placenta	L2, L3	S,F	R1–R3
HeLa	L2, L3	S,F	R1, R2
0.01 *M* Tris–HCl			
Liver	L3	S,F	R4, R5
Placenta	L3	S	R4, R5
HeLa	—	S	R4, R5

formed by immunoprecipitation (12), each of these antigens is apparently related but their identity needs to be ascertained. The nuclear antigens which were common to liver, placenta, and HeLa extracts as shown by immunodiffusion could not be definitively correlated with the common nuclear antigens obtained by the rocket immunoelectrophoretic technique. Accordingly two sets of nomenclature were used (Table I).

The immunofluorescence studies showed a correlation between the nucleoli and nuclear membranes of liver and the nuclear network of the HeLa cell. Each of these had positive fluorescence. The placental nucleoli were partially obscured by the overall fluorescence of the placental tissue. The relationship between the antigens observed by fluorescence and the immunoprecipitin method requires further analysis.

The present study represents an initial approach in the evaluation of the numbers and types of human liver nucleolar antigens. In studies on human liver nucleolar antigens, some extracts apparently contained proteases; accordingly, it was necessary to use several fresh extracts for these studies. In addition, it was difficult to obtain identical matched samples and accordingly some variability was anticipated. The significance of particular antigens, especially those that are liver specific will be determined by isolation and purification of the antigens (1); these studies are now in progress. Several proteins of the rat liver were isolated and studied in this laboratory, e.g., BA (15). It will be of interest to compare these with the human liver proteins recently analyzed by two-dimensional gel electrophoresis (16).

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