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NUCLEAR RIBONUCLEOPROTEINS RECOGNIZED BY HUMAN ANTINUCLEAR ANTIBODIES IN RETROVIRUS-INFECTED CELLS

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SUMMARY : Antibodies present in sera of patients with auto immune diseases (systemic Lupus erythematosus, mixed connective tissue disease) were used to react with nuclear ribonucleoproteins (HnRNPs) from normal cells and cells infected with retroviruses. Only antibodies directed against Sm and RNP antigens precipitated particles with definite spectra of small nuclear RNAs (SnRNA) and proteins. No difference could be found between infected and uninfected cells, suggesting that virus replication is dependent on normal cellular fonctions.

INTRODUCTION

It is generally assumed that viral mRNA synthesis in cells infected with retroviruses is very similar to that of host cellular mRNAs, as viral messenger RNAs are transcribed from the integrated genome (1). In eukariotic cells, mRNA is generated by processing and cleavage of heterogeneous high molecular weight nuclear RNA (HnRNA), which is associated to proteins and small nuclear RNAs or SnRNAs in the form of HnRNPs (2, 3, 4, 5, 6). Recently, several antibody activities found in sera from patients with Systemic Lupus Erythematosus (SLE) were reported to specifically recognize distinct classes of HnRNPs containing several SnRNAs and proteins (7, 8). Since preliminary data in our laboratory indicated the presence of viral RNA in HnRNPs of murine cells productively infected with retrovirus, several human sera with defined specificities found in auto-immune diseases were used to form immune complexes with HnRNPs, in order to possibly detect virus-related ribonucleoproteins. Our results, in agreement with those of Lerner et al. confirmed that some SLE antinuclear antibodies precipitate HnRNPs containing characteristic SnRNAs. No evidence for the existence of viral specific structures was found.

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MATERIALS AND METHODS

Cells : TF-P10 erythroleukemia Friend cells producing Friend Polycythemia virus (FV-P), fibroblastic Friend-Eveline cells producing the non defective lymphatic leukemia virus and D-55 uninfected mouse fibroblasts were grown as described precendently (9). Conditions for labeling RNA and proteins have been described (9). Cells were labeled for 48 h.

Cell fractionation : Nuclei were isolated as previously described (6) and suspended in Tris-HCl 0.01M, pH 7,4, 0.01M NaCl and 0.002M $MgCl_2$, 0.007M mercaptoethanol and 0.25mM Phenyl Methyl Sulfonyl Fluoride. They were disrupted by sonication (6). Extracts were layered onto a 30% sucrose cushion in the same buffer and centrifuged for 25 mn at 3,700 rpm in a RPI centrifuge. The band of material concentrating at the buffer-sucrose interphase was harvested, diluted with buffer and centrifuged for several hours at 49,000 rpm in a Beckman 50 rotor. This material has been shown to contain a heterodisperse spectrum of $HnRNP_S$ between 20 and 200S, when analyzed by velocity sedimentation in sucrose gradients. Initially, purification of HnRNPs was persued by submitting the post nuclear extract to sedimentation through a triple sucrose cushion (4). Since no improvement was observed in the results, it was not used in the next experiments.

Immune precipitation : HnRNPs were thoroughly homogenized in Tris HCl 0.01M, pH 8.5, 0.1M NaCl, 0.001M $MgCl_2$. To get rid of the aggregates, which could have introduced artefacts, the suspension was centrifuged at 12,000 g for 2 minutes in a Janewski microcentrifuge. For the tests, 5.10^5 to 10^6 cpm of ^{3}H-uridine or ^{3}H-aminoacid-labeled HnRNPs were put into Eppendorf 1.5 ml plastic tubes and diluted to a final volume of 0.5 ml. Appropriate sera (10 ul) were added and the tubes were incubated overnight at 4°C. Then, 100 ul of a 10% staphylococcus aureus suspension (Cowan I strain) were added and incubation was continued for 2 more hours. The complexes were harvested by centrifugation and treated as previously reported (9).

RNA and protein isolation. Analysis in polyacrylamide gels : RNA was extracted from immune complexes by 1% sodium dodecylsulfate and phenol (10). It was recovered by ethanolic precipitation and analyzed by electrophoresis in 10% polyacrylamide slab gels. Proteins were analyzed as previously described (9). In both cases, the gels were processed for fluorography (9).

Sources of sera : Three sera (two contained anti RNP* antibodies and one anti Sm*) were obtained from patients diagnosed as systemic lupus erythematosus or related diseases. Another one contained anti SS-B* antibodies (otherwise called anti La*) and was drawn from a patient with Sjögren syndrome. These four sera showed an antinuclear activity as detected by indirect immunofluorescent technic on rat liver sections with a speckled pattern at a minimal dilution of 1/100. Two kinds of control sera without antinuclear antibodies were used. First, a rhumatoïd arthritis patient's serum (FR) which contained anti human IgG activity detected by hemagglutination techniques. Secondly, a so-called normal serum consisting of a pool of 25 different sera obtained from healthy blood donors not exceeding 40 years.

A monospecific goat antiserum directed against the structural retrovirus p30 protein was obtained from the National Cancer Institute, Bethesda, Maryland.

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*RNP, Sm and SS-B (otherwise called La) are distinct serologically defined antigens (réf.11).

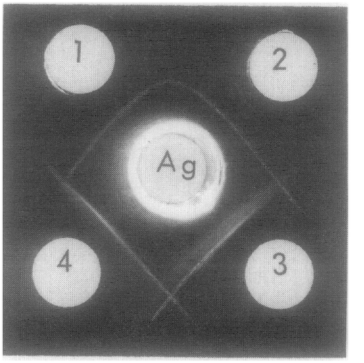

Fig. 1 : Screening for anti-extractable nuclear antigen (ENA) activity : The central well contained 60 ul of saline-soluble acetone extract of rabbit thymus at the protein concentration of 12 mg/ml. Peripheric wells contained 40 ul of four undiluted sera. Well 1 and 2 : anti RNP antibodies. Well 3 : anti SS-B. Well 4 : anti Sm. The precipitation reactions were observed at 48 hours. Precipitin lines of the anti RNP system (wells 1 and 2) did not appear when the antigen was pretreated with Pancreatic Ribonuclease.

Screening for anti-extractable nuclear antigen (ENA) activity : Double immunodiffusion method was performed in 0,5% agarose dissolved in Phosphate Buffer Saline, pH 7.2, containing 0,1 °/₀₀ Na Azide and 3% polyethylene glycol 6,000, using a saline-soluble acetone extract of rabbit thymus (Pel-freeze Biologicals, INC. Rogers A.R.) as antigen. Each of the four sera that had an antinuclear activity gave only one precipitin line with the thymus extract (fig. 1). Two of these lines (wells 1 and 2) fused completely, implying identical antigen-antibody systems. The three ENA antibody specificities were identified, using the same technic and distinct reference sera kindly given by Dr. E.M. Tan (Division of Rheumatic diseases, University of Colorado Medical Center, Denver, Colorado).

RESULTS

1) SnRNA content of immune complexes precipitated from HnRNPs of retrovirus-producing cells by SLE human sera : Balb/c mouse erythroleukemia cells producing the polycythemia Friend virus complex were labeled for 48 hours with ^{3}H-Uridine and fractionated into cytoplasmic and nuclear fractions. Nuclear HnRNPs were prepared and treated with different sera. Immune precipitates were deproteinized and their SnRNA content was analysed by electrophoresis in 10% polyacrylamide gels (fig. 2). Both anti RNP 1 and 2 and anti Sm sera precipitated several SnRNAs, with mobilities identical to those of previously identified SnRNAs (10, 12, for comparison, the SnRNAs of untreated HnRNPs are presented in the lane 1). Anti Sm antibodies precipitated principally RNA in the region of the H species (or U_6) and some G' (or 5S) and D (or U_1) species (lane 3). Under the same conditions,

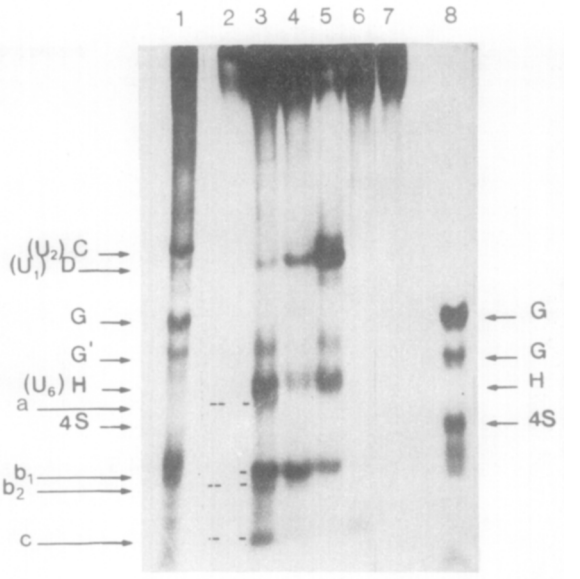

Fig. 2 : Gel electrophoresis of SnRNAs precipitated by different human sera. Lane 1 : control HnRNPs : lane 2 : normal serum ; lane 3 : anti Sm ; lane 4 : anti RNP1 ; lane 5 : anti RNP2 ; lane 6 : anti SS-B ; lane 7 : FR ; lane 8 : 5S and 4S RNA markers.

anti RNP_1 and anti RNP_2 sera precipitated mostly D and H species and to a lower extent, C (U_2), and G' (lanes 4 and 5). Besides these known SnRNAs, the presence in immune precipitates of species with a smaller size was also observed (see fig. 2 and 3, bands a, b_1, b_2 and c).A certain degree of specificity depending on the sera was noticed, as the a and c species were recognized only by the anti Sm serum and b_1 was precipitated by both.

In that series of tests, only the anti Sm and anti RNP antibodies exhibited a reactivity towards nuclear HnRNPs. Neither normal sera nor other pathologic sera did react with SnRNA-containing structures (fig. 2). Interestingly, the antibodies to SS-B (or La) antigen did not precipitate any RNA but very faint bands at the position of a, b and c species (fig. 2, lane 6).

To ascertain the validity of these results, another experiment was performed in which the SnRNA content of the supernatants and pellets resulting from treatment of nuclear HnRNPs with respectively normal serum, anti Sm and anti RNP_2 antibodies were compared (fig. 3). With normal serum, all the SnRNAs were entirely located in the supernatant (lane 2s). No radioactivity was found in the pellet (lane 2p). On the contrary, pellets

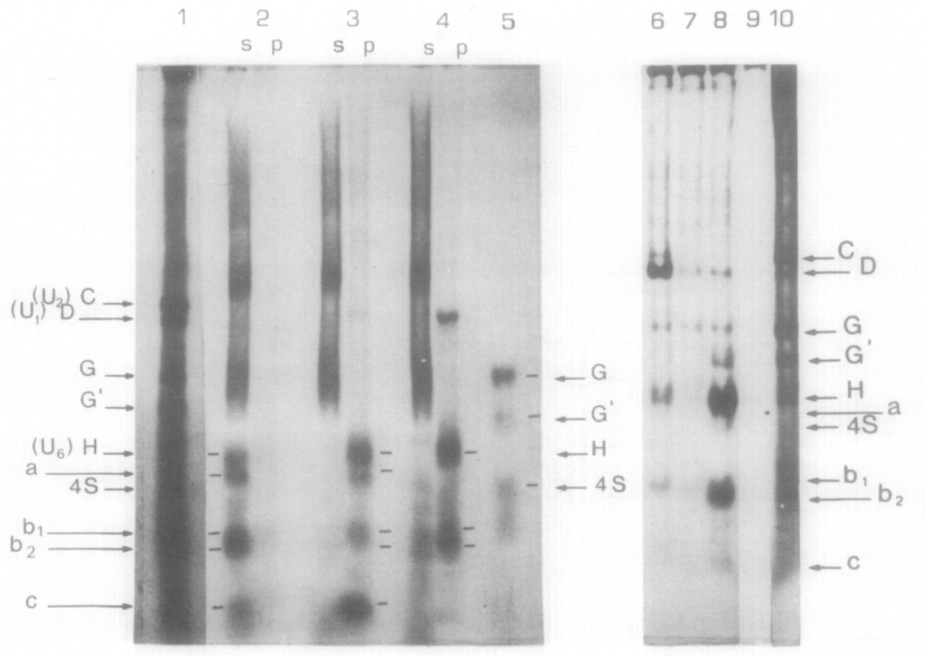

Fig. 3 : Gel electrophoresis of SnRNAs precipitated by human sera from TF-P10 virus-producing cells (lanes 1 to 4) and D-55 uninfected cells (lane 6 to 10). HnRNP extracts were treated by sera and supernatant (s) and pellets (p) were analyzed for their SnRNA content. Lane 1 : TF-P10 control HnRNPs ; lane 2 : normal serum ; lane 3 : anti Sm ; lane 4 : anti RNP2 ; lane 5 : 5S and 4S RNA markers ; lane 6 : D-55 cells, anti RNP2 ; lane 7 : anti RNP1 ; lane 8 : anti Sm ; lane 9 : normal serum ; lane 10 : control.

of materials precipitated by anti Sm and anti RNP antibodies gave patterns which superimposed to those of fig. 2 (lanes 3 and 4).

Since no SnRNA with smaller size than tRNA was found in immune complexes from uninfected HeLa cells (7), we speculated that b_1, b_2 and c species could reflect a retrovirus expression in the cells that were employed in these experiments. To clarify this point, the tests were repeated with uninfected mouse fibroblasts (D-55 cells). The electrophoregram presented in fig. 3 (lanes 6 to 10) did not reveal any difference with those of infected cells. It was concluded that the presence of actively-replicating viral structures in the infected cells did not modify the composition of SnRNAs present in HnRNPs, which were precipitated by the human antinuclear antibodies.

2) Analysis of polypeptides contained in precipitable HnRNPs : To examine protein composition of the HnRNP fractions precipitated by SLE antisera, Friend-Eveline and TF-P10 cells were labeled with ^{3}H-aminoacids for 48 hours and equal samples of the HnRNP extract were treated with anti Sm

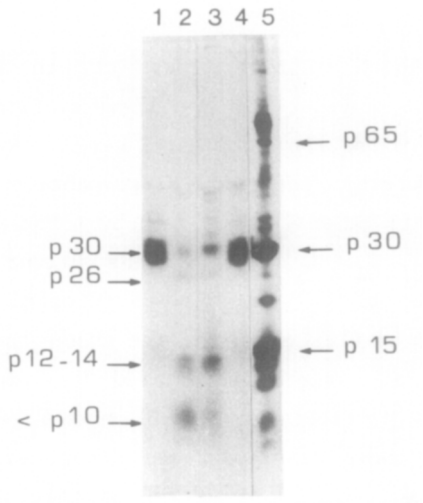

Fig. 4 : Gel electrophoresis of polypeptides precipitated by anti Sm, anti RNP and virus anti p30 antibodies. Lane 1 : anti p30 ; lane 2 : supernatant of the HnRNP precipitate analysed in lane 1 then treated with anti Sm ; lane 4 : virus anti p30 ; lane 3 : supernatant of HnRNP precipitate analysed in lane 4. Lane 5 : viral polypeptides used as markers.

and RNP antibodies and also with a monospecific anti p30 goat serum directed against the major structural protein of the retroviruses. It can be seen in fig. 4, that anti RNP and anti Sm sera both precipitated four main bands of polypeptides with average molecular weights of 30,000, 26,000, 12-14,000 and less than 10,000 daltons. The band p12-14 was actually a doublet with at least two molecular species in it. The wide band corresponding to light polypeptides (less than 10,000) was certainly specific, because it was not precipitated by normal serum, but it was probably composed of several species. None of these bands occured in precipitates resulting from treatment of HnRNPs with an antiserum directed against the viral p30 structural protein. This point was confirmed by direct treatment of HnRNP with the virus antiserum or by secondary precipitation of the supernatants previously treated with anti Sm and anti RNP sera. In both cases, the patterns were not modified. The band p30 precipitated by SLE antisera was not due to the presence of residual virus p30 in the supernatants, as an excess of antibodies was utilized.

The same results were obtained with uninfected cells, suggesting again that specific viral structures if they exist in the HnRNPs of infected cells were not precipitated by SLE sera or were not distinguishable from the host cell HnRNPs.

DISCUSSION

The results presented in this report are in agreement with those of Lerner et al. (7,8), in so far as they confirm that antibodies from patients with SLE or related diseases have a capacity for complexing nuclear ribonucleoproteins. Yet, some discrepancies were noted. Among them, was the fact that anti Sm antibodies used by these authors precipitated six SnRNA (U_{1a} + U_{1b}, U_2, U_4, U_5, U_6), whereas in our conditions, only bands running at the place of U_1 and U_6 were detected. Moreover, we detected other SnRNAs with a smaller size. On the other hand, anti RNP antibodies used by Lerner at al. only precipitated U_1, while our anti RNP_1 and anti RNP_2 antibodies precipitated U_1, U_6 and b_1 (fig. 2 and 3). Several reasons may explain these discrepancies. First, since we have not used a postnuclear supernatant, it could be argued that some RNP structures were lost in our conditions. However, patterns of the total extract show that most of the SnRNAs are present in our preparations. Another important technical point concerns use of Staphylococcus aureus suspension for collecting immune complexes. This procedure has been reported not to precipitate with an equal efficiency all classes of human immunoglobulins. Furthermore, we have observed that non specific structures can cosediment with the bacterial suspension of Staphylococcus aureus. It should also be noted that the various human sera containing antibodies directed against the so-called Sm and RNP specificities may well recognize distinct antigenic sites on the same molecular complex.

To date, SnRNA species with a smaller size than t-RNA have not been characterized using these experimental conditions. A recent report mentioned that RNP antigen from calf thymus nuclei, purified by affinity chromatography, contains 2 polynucleotides with 40 and 60 nucleosides in length (13). These two molecular species could be equivalent to the b_1-b_2 and c species we have observed in mouse cells. These molecules can represent true entities, existing in HnRNPs or are generated by cleavage of longer molecules. Such a relationship could exist for example between D and b_1, as the intensities of the bands corresponding to these two molecules were inversely related in the immune precipitates generated by RNP_1 and RNP_2 antisera (see fig. 2, lane 4 and 5). Because of the low amounts presently available, we have not still been able to test this point.

It appears that the polypeptide composition of HnRNPs precipitated by anti Sm or anti RNP antibodies is very similar. Although our patterns present notable differences with those published by Lerner (7), they are in agreement with results of Takano et al. and Douval et al. (13, 14), who

both described recently the existence of two quantitatively major polypeptides with molecular weight of 30,000 and 13,000 in the purified RNA antigen.

Finally, in this work we have not remarked any differences in the electrophoregram of SnRNA from infected and uninfected cells. If so, the expression of the viral genome could proceed via mechanisms which are indistinguishable from those existing in the normal cells. That is to say, that HnRNPs carrying either viral RNA or cellular HnRNA are respectively associated with the same SnRNAs. Alternatively if a specific population of HnRNPs is involved in the virus replications, our results imply that this class does not react with antibodies directed against HnRNPs of normal cells.

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