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DETECTION AND ANALYSIS OF ANTIBODIES AGAINST RETROVIRUS DETERMINANTS IN THE SERA OF HAEMATOLOGIC PATIENTS

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Summary. — A panel of 11 retroviruses including animal retroviruses types B, C, D and human retroviruses HTLV-I and HTLV-III was used as antigen for detection of antibodies in 265 sera of patients suffering from haematologic and autoimmune diseases and in 98 control sera. In contrast to the control sera, the 39 patients sera contained antibodies against internal proteins of animal retroviruses types B and C. No antibodies against human retroviruses proteins or type D retrovirus core proteins were detected. The antibodies from positive sera immunoprecipitated the RNA from polyribosomal fraction of R-MuLV infected spleen cells which also showed positive hybridization with a specific cDNA probe. The antibodies in patients' sera were directed mainly against RNA-binding proteins p15 of type C or p14 type B virus. The retrovirus antigenic determinants were expressed also in human embryo haemopoietic cells. The possible interpretation of antibody formation against the determinants of animal retroviruses in humans is discussed.

Key words: *retroviruses*; *antigens*; *genome expression*; *antibodies*

Introduction

In a previous study of the specificity of autoantibodies against erythroid cells the authors described a group of antigenic determinants expressed predominantly on human embryo erythrokaryocytes and probably also on mouse embryo and R-MuLV induced erythroid cells (Etkin *et al.*, 1984). This phenomenon probably accounts for by the fact that the sera of patients with haematologic and autoimmune diseases contained antibodies against retrovirus antigens. The presence of antibodies against animal retrovirus antigens in the sera of such patients has been reported by a number of authors (Maeda *et al.*, 1985; Mellors and Mellors, 1978; Toth *et al.*, 1982). Antibodies against retrovirus determinants in patients' sera were directed against a

broad spectrum of viral proteins including the products of *gag*, *pol* and *env* genes (Anh-Tuan *et al.*, 1984; Derks *et al.*, 1982; Okamoto *et al.*, 1983; Rucheton *et al.*, 1985). In this paper we describe the analysis of the specificity of antibodies against retrovirus antigens in the sera of haematologic patients in order to elucidate the probable cause of the occurrence of such antibodies.

Materials and Methods

Viruses. Purified preparations of Mo-MuLV, R-MuLV, FeLV, RD-II4B, SSV, GaLV, MPMV and MMTV viruses (National Cancer Institute) were obtained due to the U.S.A.—U.S.S.R. Cooperation Agreement in Oncology. HTLV-1 from the HUT-102 cell culture supernatant and BLV from the supernatant of a continuous leukaemic bovine lymphocyte culture were isolated by isopycnic ultracentrifugation in the sucrose density gradient.

Cells. Human embryo erythrokaryocytes were isolated from embryo liver (10—12 weeks) by careful pipette dispersion of the tissue followed by differential centrifugation at 400 g in 0—8 % Ficoll 400 (Pharmacia F.C.) concentration gradient at a 1.09 density. Erythroid cells were isolated from rabbit bone marrow in a similar fashion on day 4 after subcutaneous injection of phenylhydrazine (Merck) at a dose of 15 mg/kg. The cells of spontaneous mammary tumour of mice C3H were isolated by pipetting the fragmented tissue. The tissue was pretreated with collagenase (Calbiochem) purified by gel-filtration on Sephadex G-200 at a concentration of 100 μg/ml and by hyaluronidase (Serva) at the same concentration for 1 hr at 37 °C. After that stroma and cellular aggregates were sedimented; the virus-induced erythroblasts of R-MuLV infected mice were prepared from spleens of BALB/c mice with marked splenomegalia.

The cells rinsed with Hanks' solution in the form of 10 % suspension were fixed with glutaraldehyde (Merck) at a final concentration of 0.05 % for 20 min. The rinsed cells were then consecutively incubated in 0.2 mol/l glycine solution pH 7.2, and in bovine serum diluted at 1 : 10. For isolation of antibodies against cell membrane antigens the fixed and treated cells were incubated at 37 °C for 1 hr with a ten-fold volume of the tested serum diluted with bovine serum at a ratio of 1 : 10. The cells were washed and the antibodies were eluted with 0.2 mol/l solution of glycine-HCl-buffer pH 2.8. For maximum purification the eluate was immediately neutralized with 1 mol/l Tris-HCl-buffer pH 8.0 and the antibodies were once more absorbed to the Protein A Sepharose 4B column. The antibodies were eluted from the column and neutralized as described above. Then they were dialyzed against saline and used immediately.

Serologic tests. The enzyme-linked immunosorbent assay (ELISA) was conducted according to a conventional procedure (Voller *et al.*, 1976). To control the specificity of the method, the material tested was pre-neutralized with an excess of antigen. For neutralization test the antigen was titrated. Horseradish peroxidase conjugate (RZ-3, Serva) against human immunoglobulins was prepared by periodate oxidation of the enzyme (Wilson and Nakane, 1978). The working dilution of the conjugate was adjusted to detect 5—10 ng/ml of antibodies. 5-Aminosalicylic acid was used as chromogen. For detection of antibodies against HTLV-III virus the diagnostic kit Vironostika anti HTLV-III (Organon) was used. Specificity of antibodies in patients' sera was identified by immunoblotting (Towbin *et al.*, 1979).

RNA isolation. For isolation of virus-specific mRNA from infected splenic cells of BALB/c mice immunoprecipitation of polyribosomal fraction with purified antibodies from patients' sera was carried out (Shapiro and Young, 1981). The antibodies were added to polyribosomal preparation containing 10 μg of total RNA. The sample was then incubated for 30 min at 4 °C and passed through a Protein A-Sepharose column. The column was washed and the sorbed material was eluted with 0.5 % solution of sodium dodecyl sulphate (Serva). RNA was isolated from the eluate by phenol extraction followed by ethanol precipitation. Viral RNA was detected in the preparation by dot-hybridization on nitrocellulose membrane (Millipore) using α-^{32}P-deoxyribonucleotide phosphate-labelled cDNA. The probe was prepared using the endogenous revertase of R-MuLV isolated from infected BALB/c mice plasma by isopycnic ultracentrifugation (Maniatis *et al.*, 1982).

A panel of 281 sera was collected from patients with autoimmune diseases, pre-leukaemia. The sera were stored in aliquotes at —70 °C. Each aliquote was used only once.

Results

Sera of 265 patients were tested for the presence of antibodies against proteins of 12 detergent-lysed retroviruses of type B (MMTV), type C (Mo-MuLV, R-MuLV, FeLV, RD-II4B, GaLV, SSV, BLV, HTLV-I), type D (MPMV) and HTLV-III; from these, 39 sera presumably contained antibodies against given antigens as detected by ELISA. These sera were demonstrated to be positive in one or more assays in comparison to 98 sera of healthy persons. Fig. 1 shows the results of primary screening for each of the detected sera in each assay system employed. The areas marked in Fig. 1 correspond to the results of antibody detection in the sample group of donor sera at a significance level $p = 0.05$.

The specificity of the screening technique was assessed with the help of preliminary neutralization of sera against the virus antigens. This enabled to select 32 sera that were proved to contain antibodies against antigens of ether-disrupted retroviruses Mo-MuLV, R-MuLV, FeLV, RD-II4B, SSV,

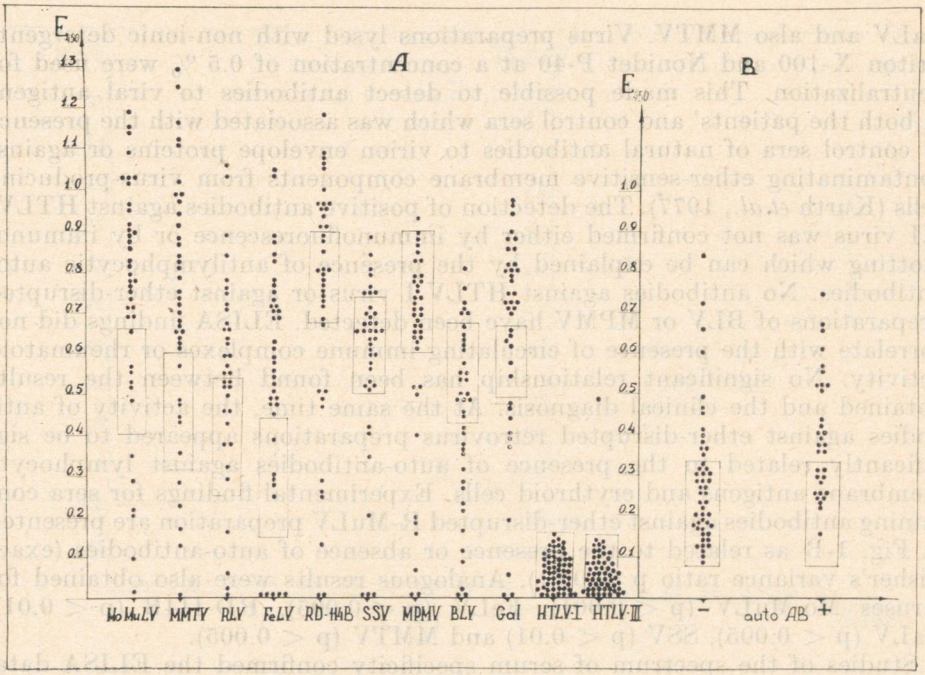

Fig. 1.

Activity of antibodies against retrovirus antigens in the patients' sera

A — ELISA results of 39 positive sera with a panel of retrovirus antigens.

B — comparison of activities of antibodies against R-MuLV in the sera as related to the activities of autoantibodies. The areas indicated with quandrangles show the results for 98 donor sera.

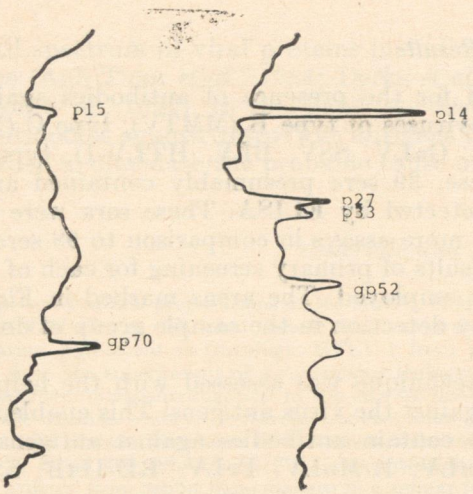

Fig. 2.
Densitograms of immunoblotting of serum containing antibodies against MMTV (above) and lacking antibodies against SSV (below)

GaLV and also MMTV. Virus preparations lysed with non-ionic detergents Triton X-100 and Nonidet P-40 at a concentration of 0.5 % were used for neutralization. This made possible to detect antibodies to viral antigens in both the patients' and control sera which was associated with the presence in control sera of natural antibodies to virion envelope proteins or against contaminating ether-sensitive membrane components from virus-producing cells (Kurth *et al.*, 1977). The detection of positive antibodies against HTLV-III virus was not confirmed either by immunofluorescence or by immunoblotting which can be explained by the presence of antilymphocytic auto-antibodies. No antibodies against HTLV-I virus or against ether-disrupted preparations of BLV or MPMV have been detected. ELISA findings did not correlate with the presence of circulating immune complexes or rheumatoid activity. No significant relationship has been found between the results obtained and the clinical diagnosis. At the same time, the activity of antibodies against ether-disrupted retrovirus preparations appeared to be significantly related to the presence of auto-antibodies against lymphocyte membrane antigens and erythroid cells. Experimental findings for sera containing antibodies against ether-disrupted R-MuLV preparation are presented in Fig. 1-B as related to the presence or absence of auto-antibodies (exact Fisher's variance ratio $p = 0.01$). Analogous results were also obtained for viruses Mo-MuLV ($p < 0.001$). FeLV ($p < 0.005$), RD-II4B ($p < 0.01$), GaLV ($p < 0.005$), SSV ($p < 0.01$) and MMTV ($p < 0.005$).

Studies of the spectrum of serum specificity confirmed the ELISA data. Neutralizing activity of serum antibodies was predominantly directed to internal RNA-binding virion proteins or against antigens having the same molecular weight as these proteins. According to the data of immunoblotting, the major antigens of retrovirus preparations tested are p15 of FeLV, Mo-MuLV, and R-MuLV and also MMTV p14. Fig. 2 shows immunoblotting den-

sitograms of MMTV and SSV with a patient's serum containing antibodies against MMTV and containing no significant antibodies against SSV as revealed in ELISA. It can be seen in the densitogram that, in addition to the major antigen showing a molecular weight equal to that of p14, the patient's serum detected a number of antigens with a molecular mass close to those of proteins p27, gp33, gp52, and probably representing precursor proteins. However, these additional components can be detected in different ways by different sera of patients; the antigens corresponding to retrovirus envelope proteins were also detected by donor sera.

Regression analysis (Fig. 3) of ELISA data allowed to establish significant regression relationships of 3 types: direct correlation between activity of sera against type C viruses, direct correlation between the activities of sera against type C viruses and MMTV and a negative correlation between activities of sera against type C virus and MMTV.

The results of regression analysis are ambiguous. Therefore, cross-specificity of antibodies isolated by sorption-elution from the cells expressing the R-MuLV and MMTV antigens was analysed in ELISA by quantitative

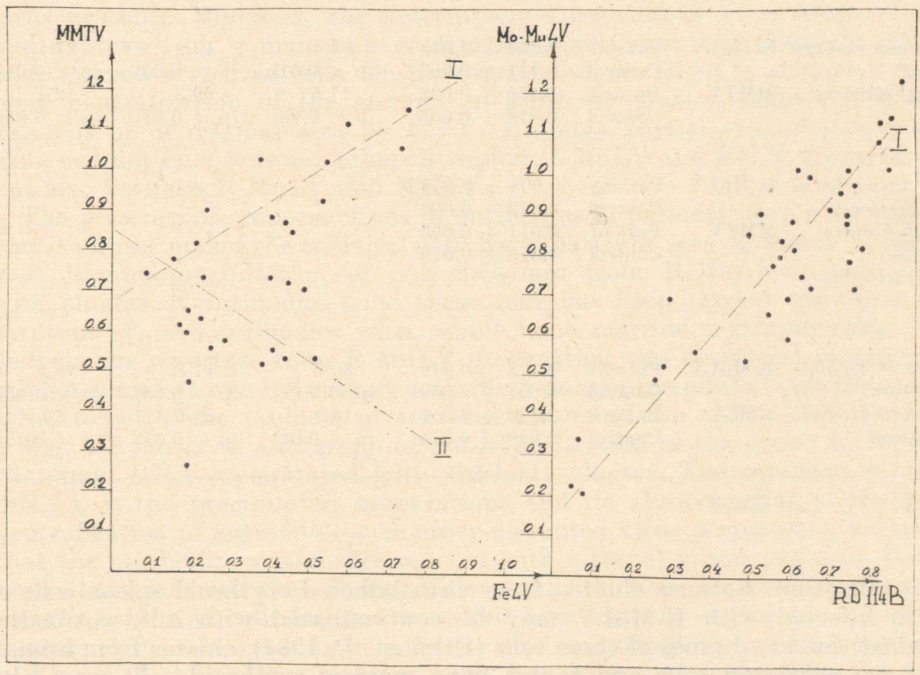

Fig. 3.

Correlation analysis of ELISA data

A — correlation between retroviruses of types B and C.

B — correlation between the type C retroviruses.

Table 1. Neutralization analysis of eluates from cytosorbents containing antibodies from the serum of a preleukaemic patient

Cytosorbent for antibody isolation	Antigen used in ELISA	Serum source	Eluate activity in ELISA E_{450}, n = 4	Neutralization of eluate activity in ELISA (%) with virus proteins		
				R-MuLV	MMTV	FeLV
Cells of spontaneous mammary tumour of C3H mice	R-MuLV	Patient	0.023 ± 0.008	—	—	—
		Control	0.025 ± 0.009			
	MMTV	Patient	0.156 ± 0.023	8.0	78.2	20.3
		Control	0.032 ± 0.018	($p < 0.05$)	($p < 0.01$)	($p < 0.05$)
Spleen erythroblasts of Balb/c mice infected with R-MuLV virus	R-MuLV	Patient	0.237 ± 0.011	95.1	24.8	28.7
		Control	0.012 ± 0.010	($p < 0.01$)	($p < 0.05$)	($p < 0.05$)
	MMTV	Patient	0.015 ± 0.006	—	—	—
		Control	0.005 ± 0.005			
Human embryo liver erythroblasts	R-MuLV	Patient	0.253 ± 0.028	88.4	13.2	27.1
		Control	0.011 ± 0.007	($p < 0.01$)	($p < 0.05$)	($p < 0.05$)
	MMTV	Patient	0.150 ± 0.022	17.5	99.3	12.4
		Control	0.013 ± 0.005	($p < 0.05$)	($p < 0.01$)	($p < 0.05$)
Erythrocytes of C3H mice, Balb/c mice, human erythrocytes	R-MuLV	Patient	0.008 ± 0.006	—	—	—
		Control	0.012 ± 0.006			
	MMTV	Patient	0.011 ± 0.008			
		Control	0.019 ± 0.010			
Sera at 1 : 200 dilution	R-MuLV	Patient	0.321 ± 0.046	80.5	17.5	31.1
		Control	0.024 ± 0.008	($p < 0.01$)	($p < 0.05$)	($p < 0.05$)
Sera at 1 : 200 dilution	MMTV	Patient	0.189 ± 0.051	18.1	80.6	8.5
		Control	0.029 ± 0.014	($p < 0.05$)	($p < 0.01$)	($p < 0.05$)

Note: (—) — not tested

neutralization. Because eluates from virus-infected erythroid spleen cells of mice infected with R-MuLV may be contaminated with auto-antibodies against the membranes of these cells (Etkin *et al.*, 1984), eluates from human embryo erythroid cells and rabbit bone marrow erythroid cells were also used as controls. Table 1 shows the results of analysis of a patients' serum containing antibodies against viruses Mo-MuLV, R-MuLV, FeLV, RD-II4B, GaLV and MMTV. For neutralization ether-disrupted virus preparations were used. The results presented in Table 1 together with the regression

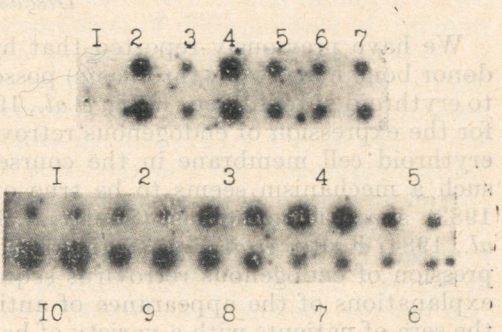

Fig. 4.
Hybridization of immunoprecipitated polyribosomal RNA from R-MuLV-virus-infected mouse erythroblasts with labelled viral cDNA
I — immunoprecipitation of polyribosomes with eluate of antibodies from virus -infected cells (2, 3), human embryo erythroid cells (4, 5) and rabbit bone marrow erythroid cells (6, 7). Antibodies are isolated from a pool of patients' sera. 1 — control with donor serum immunoglobulins; 3, 5, 7 — preliminary neutralization of eluates with a disrupted virus. II — immunoprecipitation of polyribosomes with immunoglobulins of patients' sera (3—10) and with donor sera (1—2).

analysis data indicate the presence in the patients' sera of cross-reacting antibodies directed against proteins of type C viruses as well as of type B virus proteins. Moreover, the determinants detected in virus preparations and expressed on human embryo erythroid cells were not found in rabbit bone marrow erythroid cells. On the other hand, we failed to achieve a complete neutralization of the activity of antibody eluates against R-MuLV antigens or of original sera by FeLV, although partial cross-reaction was observed not only between ether-disrupted R-MuLV and FeLV preparations but also between R-MuLV and MMTV.

The presence of cross-reactions of antibodies in patients' sera with type C and B viruses makes the antiviral activity of patients' sera doubtful. To settle this, immunoprecipitation of polyribosomes from R-MuLV-infected cells with eluates of antibodies from these cells has been carried out after the sorption of auto-antibodies with rabbit bone marrow erythroid cells. The endogenous revertase from R-MuLV preparation was employed to generate a DNA probe; it was hybridized with the immunoprecipitated polyribosomal RNA to settle the problem of antiviral nature of the analysed antibodies.

Fig. 4-I shows an autograph of dot-hybridization of the probe with polyribosomal RNA precipitated with antibody eluates. The presence of viral mRNA in the precipitated preparations and its absence after preliminary neutralization of antibodies with ether-disrupted virus preparation indicates that the antibodies specifically reacted with internal virion proteins. It has been also confirmed that R-MuLV determinants were expressed on human embryo erythroid cells but not on rabbit bone marrow erythroid cells. Analogous results obtained with patients' sera immunoglobulins are presented in Fig. 4.-II. Although a rigorous proof has yet to be provided for the antiviral nature of patients' serum antibodies with respect to other type C retroviruses tested and MMTV, still the results obtained seem to suggest that immunoblotting findings are generally sufficient to proof the antiviral nature of antibodies.

Discussion

We have previously reported that human embryo erythroid cells (unlike donor bone marrow erythroblasts) possess determinants which do not belong to erythroid autoantigen (Etkin *et al.*, 1984). These data can probably account for the expression of endogenous retrovirus sequences on the human embryo erythroid cell membrane in the course of embryogenesis. The existence of such a mechanism seems to be true of embryonal thymocytes (Ono *et al.*, 1983), syncytiotrophoblast (Suni *et al.*, 1981) and human placenta (Jerabek *et al.*, 1984; Kalter *et al.*, 1973). If this phenomenon really exists, then the expression of endogenous retrovirus sequences may be one of the most likely explanations of the appearance of antibodies against retrovirus antigens in the sera of patients with a variety of haematologic and autoimmune diseases. This point of view is indirectly confirmed by the reported presence of virus-like particles of C type and by the expression of determinants of retrovirus antigens in the cells of leukaemic patients (Chan *et al.*, 1986; Derks *et al.*, 1982) and also in tumour cell lines (Boller *et al.*, 1983; Bronson *et al.*, 1984; Smith *et al.*, 1977).

Our screening experiments demonstrated in the sera of tested patients, but not in those of controls, the presence of antibodies against determinants of types C and B retrovirus antigens mainly directed against RNA-binding virion proteins and *gag* gene products (Figs 1 and 2). Regression analysis of ELISA results in the sera of haematologic and autoimmune disease patients in the search of antiviral antibodies taken together with the data on cross-neutralization by these antibodies of viral antigens (Table 1) suggests independent specificities of antibodies against determinants of type C retroviruses and MMTV. It should be noted, however, that not only cross-reacting antigenic determinants were detected by patients' sera between type C retroviruses, but also a cross-specificity was observed between virus type C and B. Although the employment of heterologous antisera does not allow one to establish such intertype determinants, their presumable existence was mentioned in an earlier paper (Segal-Eiras *et al.*, 1983), whereas the existence of cross-reacting determinants in type C retrovirus proteins has been acknowledged in general (Barbacid *et al.*, 1980).

The presence in human embryo erythroblasts of R-MuLV and MMTV determinants detected by antibodies in patients' sera indicates the existence of endogenous DNA sequences encoding for proteins cross-reacting with the antigens of known animal retroviruses. The employment of genetic material from exo- and endogenous animal retroviruses for detection and cloning of presently known human endogenous retroviruses (Bonner *et al.*, 1982; Callahan *et al.*, 1985; Martin *et al.*, 1984; Repaske *et al.*, 1985) also implies that there may be immunochemical "crossings" of endogenous human retroviruses with animal retroviruses. The expression of such cross-reacting proteins during various pathologies may cause the appearance of antibodies against retrovirus determinants in patients' sera.

The comparison of antibody activities against retrovirus determinants in patients' sera suggests 2 alternatives. On the one hand, a predominant immu-

ne response against common type C retrovirus determinants cannot be ruled out, and on the other, simultaneous expression of determinants of different antigenic retrovirus sequences is also possible. The latter suggestion is supported by the existence in the sera of patients and controls of a natural level of antibodies against a broad spectrum of envelope retrovirus proteins, BLV including. In addition, no antibodies against HTLV-I were seen in the patients' and control sera. This virus is relatively new for human population. It is endemic in a number of regions (Blattner *et al.*, 1984) and the human genome probably does not possess its sequences outside these areas.

The detection of antibodies against MMTV determinants in the sera of haematologic and autoimmune disease patients deserves special discussion. The available data have not established a correlation between clinical diagnosis and the presence of antibodies against antigens of certain retroviruses of types C and B in the patients' sera. The studies on mouse retroviral leukemogenesis, however, have demonstrated not only a large contribution of type C retroviruses to the development of pathology, but also the involvement of MMTV (Ball *et al.*, 1984; Dudby and Risser, 1984) associated with expresion of virus proteins (Joshi and Karande, 1980).

Possible mechanisms of activation of endogenous retroviral sequences are of undoubted interest. Both the infection with various DNA- and RNA-containing viruses (Ivarson *et al.*, 1985; Lasky and Tray, 1984) and immunologic phenomena (Ono *et al.*, 1983) may play an important role in this process.

The data presented here suggest a leading role of internal RNA-binding retrovirus proteins in the induction in patients of humoral immune response against retroviral determinants. Synthesis and production of such proteins may be regarded as a probable cause of immune deficiency in patients suffering from haematologic and autoimmune diseases (Denner *et al.*, 1985).

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