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ELISA for the Detection of Antigens Cross-Reacting with Primate C-Type Viral Proteins (p30, gp70) in Human Leukemic Sera*

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A number of methods have been applied in the past to the serologic identification and characterization of C-type viral proteins. The most specific and most sensitive system used has been the competition radioimmunoassay (RIA). This assay detects group specific as well as interspecies activity of the major viral proteins (Parks and Scolnick 1972; Strand and August 1973). Disadvantages of this assay include the necessity of radioactive isotopes, false positive results from contaminating proteases, and the limited stability of the radioactively labeled reagents.

Recently, the enzyme immunoassay (EIA) technique has been developed (Engvall and Carlsson 1976). This technique has also proved to be highly specific and sensitive in detecting antigens and antibodies in a variety of systems (Voller et al. 1976). It avoids the biohazards of radioactivity and is simple and cheap. A further advantage is the stability of the coupled reagents. Low cost and simplicity of the EIA technique allow the screening of large numbers of samples.

We report here the application of the enzyme linked immunosorbent assay (ELISA) to the detection and quantification of the core protein p30 of the murine leukemia viruses (MuLV), the baboon endogenous virus (BaEV), and the simian sarcomavirus (SiSV) and of the envelope glycoprotein gp70 of SiSV. We further report attempts to detect with this technique the presence in human leukemic sera of antigens cross-reacting with primate viral structural proteins.

* Conducted, in part, as study of the Süddeutsche Hämoblastosegruppe (SHG).

A. Methods and Materials

I. Antigens

MuLV p30, BaEV p30, and SiSV p30 were purified by a two step electrofocusing procedure (Schetters et al. 1980). Homogeneity of the purified viral proteins was demonstrated by SDS gel electrophoresis. SiSV gp70 was the gift of Dr. R. Gallo, NCI.

II. Antibodies

Antibodies against viral p30 proteins were prepared from immunized rabbits. The specificity of the antisera was controlled by precipitation of disrupted virus and subsequent gel electrophoretic analysis of the precipitated proteins. The activities of the sera were directed almost exclusively against the respective p30s. Antiserum against SiSV gp 70 as the gift of Dr. R. Gallo, NCI. IgG was purified as described (Schetters et al. 1980).

III. Coupling Procedure of Peroxidase to IgG

In essence the procedure of Nakane and Kawaoi (1974) as modified by Mesa-Tejada et al. (1978) was followed which involves blocking of the amino groups of the peroxidase with phenylisocyanate, introduction of aldehyde groups with Na-periodate, and formation of a Schiff's base with the IgG by incubation of the IgG with the activated peroxidase.

IV. ELISA Procedure

In essence, the procedure as described by Schetters et al. (1980) was followed. Microtiter plates were coated with 200 µl per well of 5–10 µg IgG/ml 50 mM carbonate-bicarbonate buffer, pH 9.6, at 37° for 2 h. After washing, the antigen of interest was added

in phosphate buffered saline (Dulbecco's PBS) containing 5% normal rabbit serum, 5% aprotirin (Trasylol), 0.1 mM thimerosal, and 0.1% tween 20, and plates were incubated at 4° overnight. After extensive washing the peroxidase-coupled IgG was added and incubated at 37° for 2 hours. After washing 200 µl of substrate solution (20 mg o-phenylenediamine · 2HCl and 0.005% H_2O_2 per 50 ml substrate buffer) were added, and the colour reaction was followed photometrically with a Titertek-Multiscan (Flow) at 450 nm. Backgrounds generally ranged from 0.02 to 0.1 A_{450}.

V. Glycosidase Treatment

The procedure as described by Ohno et al. (1979) was followed and adapted to the ELISA technique. The glycoprotein was bound to microtiter plates under standard conditions. The glycoprotein-coated plates were washed, and 200 µl of a glycosidase mixture (Miles, Frankfurt) was added at a concentration of 100 µg/ml in 50 mM citrate buffer, pH 4.0, containing 100 µg BSA/ml. The plates were incubated at 37°C overnight, washed, and then used under standard conditions. The efficacy of the glycosidase treatment was controlled by gel electrophoresis of radioactively labeled glycoproteins with and without glycosidase treatment on parallel gels.

B. Results

I. Detection and Quantification of p30

The ELISA test system was standardized and optimized with purified viral antigens of three viral systems: p30 of MuLV, p30 of SiSV, and p30 of BaEV. Figure 1 depicts representative standard concentration curves with the three viral p30s. As can be seen, p30 is detected specifically and sensitively down to quantities of 0.01 to 0.5 ng per 200 µl reaction volume in all three systems tested. For better comparison of different tests, maximal absorbance of the reaction was designated 100% representing A_{450} values ranging from 0.7 to 1.7. Fetal calf serum, normal goat and rabbit sera, extracts of uninfected cells of the cell lines in which the viruses were grown, viral glycoprotein, and human lipoprotein did not react. The reaction was abolished by pronase digestion, but not by extraction with ether or digestion with nucleases.

The ELISA was successfully applied to the detection and quantification of p30 in tissue culture cells, in tumor tissue, and in sera (Schettler et al. 1980). Parallel determinations

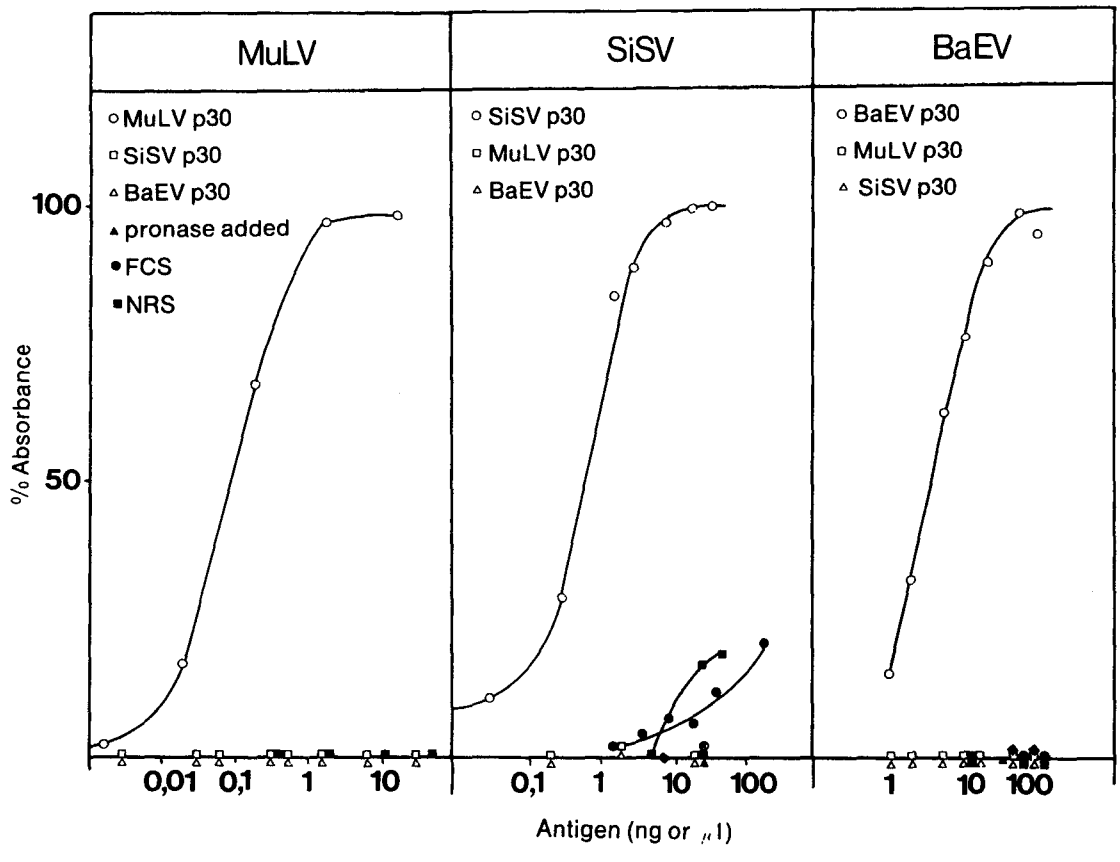

Fig. 1. ELISA standard concentration curves with purified homologous p30 from MuLV, SiSV, and BaEV

by competition RIA yielded virtually identical results (data not shown).

II. Detection and Quantification of gp 70

The ELISA technique was applied to the viral glycoprotein SiSV gp70 in a similar way as described for p30 (Schetters et al., in press). Figure 2 depicts a representative standard concentration curve similar to those observed with p30. Sensitivity and specificity were comparable to those obtained with the RIA. Fetal calf serum, normal rabbit serum, normal goat serum, extracts of uninfected cells, and components of normal human sera did not react. To exclude unspecific reactions with the carbohydrate moieties of glycoproteins the reactivity of the anti-SiSV gp70 antisera with the carbohydrate and protein moieties of SiSV gp70 was determined. For this purpose the reactivity of anti gp70 IgG with gp70 antigen was determined before and after treatment with a mixture of different glycosidases. The reactivity with gp70 was not changed by treatment with glycosidases. It therefore is concluded that the protein part and not the sugar part of the glycoprotein is recognized by the antiserum.

III. Detection in Human Leukemic Sera of Antigens Cross Reacting with SiSV p30 and with BaEV p30

The ELISA technique was then applied to the search in human leukemic sera for the presence of antigens reactive with antisera against BaEV p30 and SiSV p30 (Hehlmann et al., to be published). Proteins reacting with antibodies against p30 of BaEV and SiSV were specifically detected in leukemic sera, but not, or at very low levels only, in sera from non-leukemic patients or from healthy laboratory workers (Fig. 3). The reaction was abolished by pronase treatment and by preabsorption of the antiserum with insolubilized positive human sera (done for anti-BaEV p30). Cross-reacting antigens were detected with anti-SiSV p30 IgG in 15 out of 49 leukemic sera (30.6%) and with anti-BaEV-p30 IgG in 19 out of 48 leukemic sera (39.6%) (Fig. 3). Reactivity was also detected in some normal sera, but this reactivity was little above background and considerably less than that detected in leukemic sera as can be seen from Fig. 3. Attempts to confirm these data by competition RIA were successful only in a small number of cases (data not shown), and the nature of these antigens is therefore presently under investigation.

IV. A. Detection in Human Sera of Antigens Cross-Reacting with SiSV gp70

We then examined with the ELISA technique human sera for the presence of antigens cross-reacting with the viral envelope glycoprotein of SiSV. Cross-reacting antigens were detected in human leukemic sera, but similar reactions were obtained with a number of normal human sera (H. Schetters, V. Erfle, R.

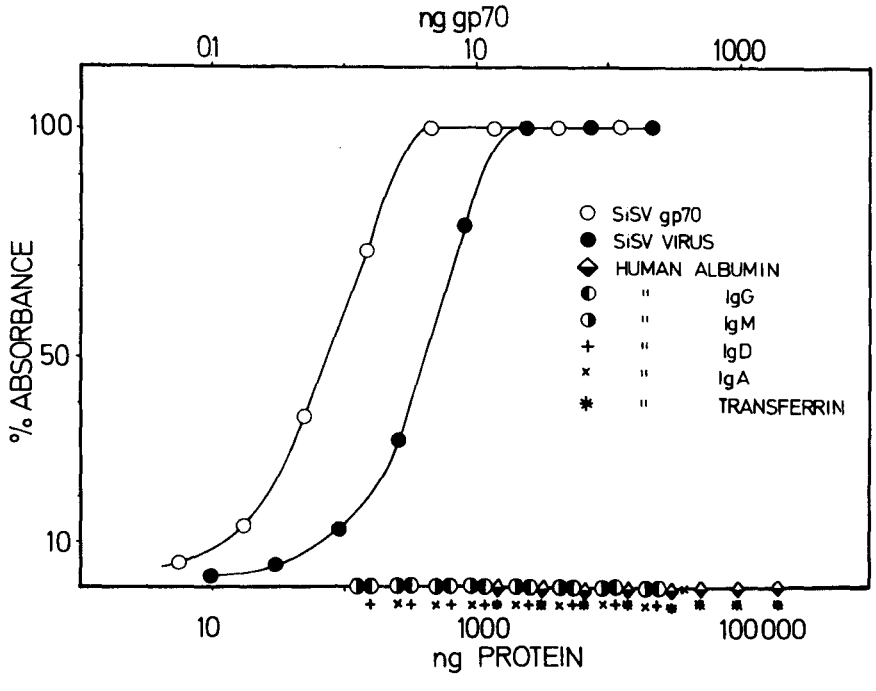

Fig. 2. ELISA standard concentration curve with purified SiSV gp70

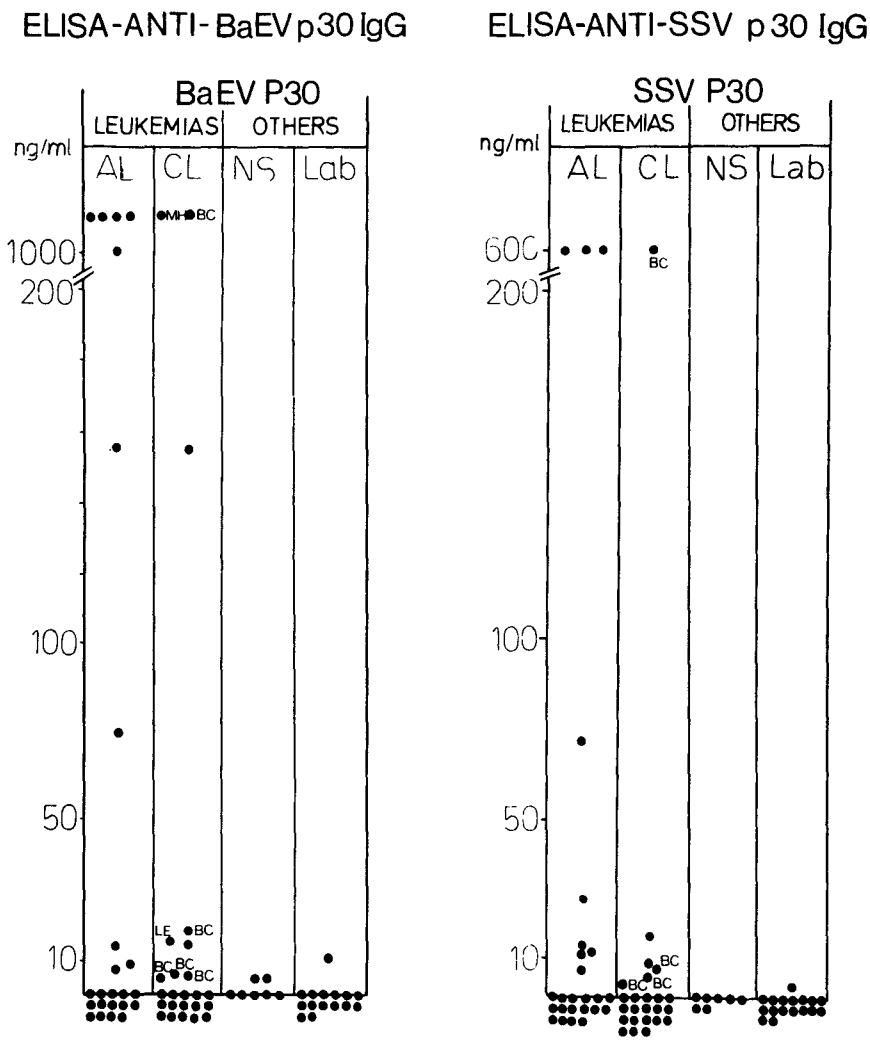

Fig. 3. Detection in human leukemic sera of antigens reactive with antisera against SiSV p30 and BaEV p30 by ELISA. AL = acute leucemias, CL = chronic leucemias, NS = normal sera, Lab = sera of laboratory workers, BL = CML blast crisis

Hehlmann, manuscript in preparation). The reactions were not due to recognition of carbohydrate moieties as demonstrated by prior treatment with glycosidases. Absorption experiments to increase specificity and to diminish possible unspecific reactivities are under way.

In order to examine a possible physiological or prognostic role of antigens cross-reacting with SiSV gp70 for human disease, we examined the sera from 23 patients with chronic myelogenous leukemia (CML) in blast crisis for cross-reacting antigens, antibodies, and immune complexes and correlated the presence and absence of these parameters with survival (Hehlmann et al., in manuscript). As can be seen in Fig. 4, about half of the sera are positive for one or more structures cross-reacting with SiSV gp70, whereas the other half is negative. Clinical data are available on 16 of the 23 patients. Of these 16 patients those patients with cross-reacting antigens or antibodies (nine patients) had a median survival after the diagnosis of blast crisis of 2.5 months, whereas those patients negative for SiSV gp70 (seven patients) had a median survival of 7.4 months. This difference of survival in both groups is significant ($P<0.005$, Fisher test). The nature of the cross-reacting antigens is under further investigation. A summary of the results with human sera for which more than one cross-reacting antigen has been determined is presented in Table 1.

C. Discussion

We conclude from our studies that the ELISA technique can specifically and quantitatively detect retroviral proteins in cells, in tumor tissue, and in sera. Since the ELISA is a binding assay and no competition is involved, we would expect a somewhat broader specificity of the ELISA than of a competition assay with respect to interspecies reactivity. However,

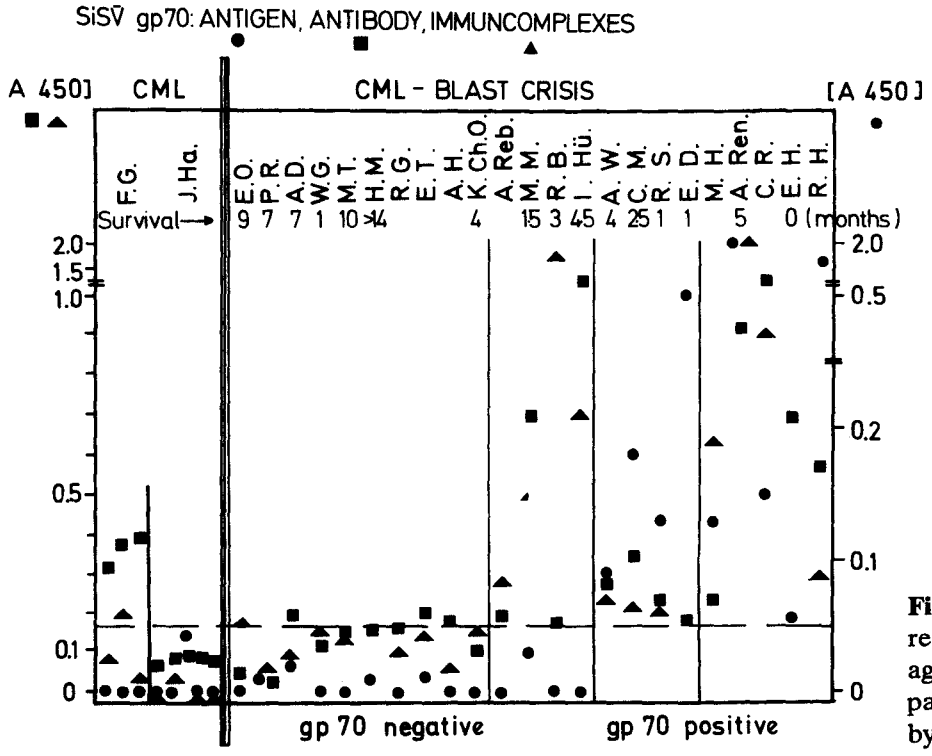

Fig. 4. Detection of antigens reactive with antiserum against SiSV gp70 in sera of patients with CML blast crisis by ELISA

Table 1. Cross-reacting antigens in human leukemias

Leukemias		Cross-reacting with SiSV		With BaEV p30 (ng equivalent/ml)
		p30	gp70	
AML	Mee.	>600 (72)	0 (2.2)	0
	Sut.	>600	16.5	>1840
	Scha.	8.5	0	1040
	Schm.	12.4 (13.2)	8 (6.8)	N.D.
	Mi.	0	0	8
AMML	Wi.	>600	0	>1840 (×2)
	Kli.	30	4.3	16
	W.-C.	80 (9.6/0)	0 (1.8/0)	>1840 (78/9.6)
	Mai.	14	0	170
ALL	Gic.	14	0.55	0
CML-BC	A.W.	15	0.8	>1840
	A.Ren.	>600	37	>1840
	J.Hü.	0	0	4.8
	E.O.	6.8	0	18.4
	C.R.	5.6	1.2	6.8
	M.H.	0	0.9	0
	P.R.	0	0	6.4
	M.M.	0	0.5	6
CML	J.Ha.	14 (8.4/18/6.4/10)	0 (0/0/5.6/0)	0
CLL	Pas.	0	0	168
Sézary	Kast.	8.8	0	N.D.
	Kö.	6.0/0	10.0/4.4	N.D.
Paraproteinemia	Scheit.	130	6	N.D.

although interspecies reactivity has been detected with the ELISA in some cases, it has not been demonstrated so regularly by ELISA as by competition RIA. It has not yet been determined whether this peculiarity is a property of the ELISA test itself or a consequence of our non-denaturing purification procedure of the proteins tested (electrofocusing, no SDS).

The detection with the ELISA-technique of antigens cross-reacting with primate C-type viral proteins in human leukemias is consistent with the earlier detection of viral structures (reverse transcriptase, RNA, virus-like particles, C-type viruses) in human leukemic cells with other methods (Gallo et al. 1975; Hehlmann 1976; Gallo et al. in this volume), although previous reports on antigens cross-reacting with viral structural proteins have been sporadic and not convincing (Sherr and Todaro 1975) and have been challenged (Stephenson and Aaronson 1976). Reports on the presence of anti-p30 antibodies in human sera (Herbrink et al. 1980) and of anti-reverse transcriptase antibodies on the surface of leukocytes from patients with AML and CML blast crisis (Jacquemin et al. 1978) have to be judged cautiosly because of the known existence of heterophil antibodies (Barbacid et al. 1980; Kurth and Mikschy 1978; Snyder and Fleissner 1980).

The failure to reproduce the human ELISA results with human sera with the RIA competition technique points to the possibility that some unrelated but cross-reacting antigens have been detected. Alternatively, the ELISA binding assay might recognize p30-related antigens of low avidity not detected by the more stringent RIA competition assay. To decide on these alternatives the nature of the detected antigens is under further investigation.

The observation that the presence of cross-reacting antigens is associated with leukemia or with shorter survival suggests that regardless of their etiologic role, these antigens might be of prognostic use.

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