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Biological Expression of Antigenic Determinants of Murine Leukemia Virus Proteins gp69/71 and p30

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Antisera to purified structural proteins of Rauscher murine leukemia virus, the major envelope glycoprotein, gp69/71, and the major internal protein, p30, were studied by immunofluorescence of viable and fixed virus-infected cells and by virus neutralization. Group-specific and type-specific determinants of gp69/71 were demonstrated by immunofluorescence and virus neutralization tests, indicating that these determinants are located in the cytoplasm and probably on the cell surface as well as on virus envelope. Antisera against p30 showed anti-group and anti-interspecies activities by immunofluorescence with no virus-neutralizing activity. Both antigenic determinants of gp69/71 were sensitive to guanidine-hydrochloride and to a lesser degree to ether treatment, whereas the group-specific determinants of p30 were relatively stable to these treatments.

One of the principle approaches to the analysis of murine C-type viruses has been through immunochemical characterization of isolated viral proteins (6, 12, 16, 19, 22). Two of the major structural components of these viruses, the envelope glycopeptides of apparent molecular weight 69,000 and 71,000, gp69/71, and the core protein of 30,000, p30, have been extensively studied and shown to have multiple antigenic determinants, including those, limited to a particular virus (type-specific); common to many or all murine C-type viruses (group-specific); common to C-type viruses of several species (interspecies) (5, 17, 21, 22, 23).

Although much information is available regarding C-type virus structure and classification from the use of viral proteins as immunochemical reagents, little is known concerning the biology of the protein antigenic determinants in the intact virus and virus-infected cells. Accordingly, the studies reported here were undertaken to learn whether these antigenic determinants can be detected utilizing monospecific antisera in immunofluorescence tests on virus-infected cells; whether there is significant virus-neutralizing activity in a monospecific antiserum directed at the purified glycopeptides; and whether techniques employed in disruption of viruses prior to protein purification alter known antigenic determinants.

MATERIALS AND METHODS

Viruses and cells. Cells infected with murine leukemia virus (MuLV) used in immunofluorescence studies include: rat cells infected with Rauscher MuLV, RTC (R-MuLV) (Microbiological Associates, Huebner-Price 5033); NIH Swiss embryo fibroblasts infected with Moloney MuLV, NIH (M-MuLV); the E_3G2 leukemia cells, a passaged C57BL/6 leukemia which provides the standard test cell for the Gross cell surface antigen (GCSA) (15) E_3G2 (G-MuLV); and feline leukemia virus infected feline lung fibroblasts, FLF (FeLV) provided by W. D. Hardy, Jr. of the Sloan-Kettering Institute.

Tissue culture-grown Rauscher, Moloney, and Gross viruses were generously provided by J. W. Hartley and W. P. Rowe of the National Institute of Allergy and Infectious Diseases. Two cells were used for tissue culture MuLV neutralization studies, NIH secondary embryo fibroblasts in the standard procedure (7) and the III6A, SC-1 line (J. E. Officer and J. W. Hartley, unpublished data), sensitive to both N- and B-tropic viruses (8) provided by J. Hartley and M. Lander of the National Institute of Allergy and Infectious Diseases.

Antisera. Rabbit and goat antisera to gp69/71 and p30 of R-MuLV in JLS-V9 cells were prepared utilizing immunogens purified by phosphocellulose column chromatography and Sephadex gel filtration (22). Rabbit antisera to RTC (R-MuLV) p30 and FLF (FeLV) p27 were prepared from fractions purified in guanidine-hydrochloride gel filtration (4, 12) by E. Fleissner and W. D. Hardy, Jr. of the Sloan-Kettering Institute and kindly provided by them. The preparations used for immunization were homogenous by

polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS)-containing gels, and the respective antisera formed single precipitin lines in immunodiffusion against whole disrupted virus.

Immunofluorescence tests. Indirect immunofluorescence of fixed cells was performed as described by Hilgers et al. (9) with minor modifications. Cells derived from mice were incubated at 37 C for 20 min and tissue culture cells were incubated for 14 to 18 h prior to acetone fixation. Slides were stored at −70 C for later use. Fluorescein-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG were purchased from Hyland Laboratories, Inc. The antiserum titer (end point) is expressed as the highest dilution showing bright cytoplasmic fluorescence.

Immunofluorescence absorption tests were performed with a concentration of antiserum two dilutions below the end point as determined from indirect immunofluorescence. Test antigens were prepared from a single cell suspension of 2×10^7 cells per ml or a 20% tissue homogenate suspended in phosphate-buffered saline, pH 7.2, and disrupted by sonic oscillation for 3 to 5 s with a Biosonic IV. After centrifugation at 3,000 rpm for 30 min at 4 C, the supernatant was collected and stored at −70 C for later use. A 20-μliter amount of this antigen was incubated with the same volume of antiserum overnight at 4 C, and residual immunofluorescence activity was tested on prepared fixed cells. The results are expressed as the absorption index, i.e., the maximal dilution of antigen capable of absorbing immunofluorescence activity.

Membrane immunofluorescence and immunofluorescence absorption tests were performed as described previously (25).

For all immunofluorescence tests, the slides were examined in a Leitz Orthoplan microscope equipped with an Osram HBO 200 mercury lamp, and BC38 Excitor, KP490 Interference, and K530 Barrier filters.

Virus neutralization. Various dilutions of antisera were added to different concentrations of MuLV in a total volume of 0.6 ml in Eagle medium with 10% fetal calf serum, glutamine, penicillin, and streptomycin. The mixture was incubated at 37 C in a 5% CO_2 atmosphere, and then at 4 C for 30 min each. Virus-antibody mixtures were inoculated on cells in the standard UV-XC procedure (11, 18).

Disruption of virus by guanidine-hydrochloride and ether. Guanidine-hydrochloride treatment was performed by incubating purified virus or a sonically treated preparation for 45 min at 56 C with 8 M guanidine-hydrochloride in a buffer of 50 mM Tris (pH 8.8), 10 mM EDTA, and 2% β-mercaptoethanol, and then dialyzed against 2.5 mM Tris buffer (pH 7.4) at 4 C (4). Ether treatment consisted of exposing materials to ether three times for 5 min each.

RESULTS

Immunofluorescence titration of antisera. Monospecific antisera to R-MuLV gp69/71, R-MuLV p30, and FeLV p27 showed specific granular cytoplasmic fluorescence with all MuLV-infected cells, characteristic of infection with C-type virus (Fig. 1); uninfected cells were immunofluorescence negative (Table 1).

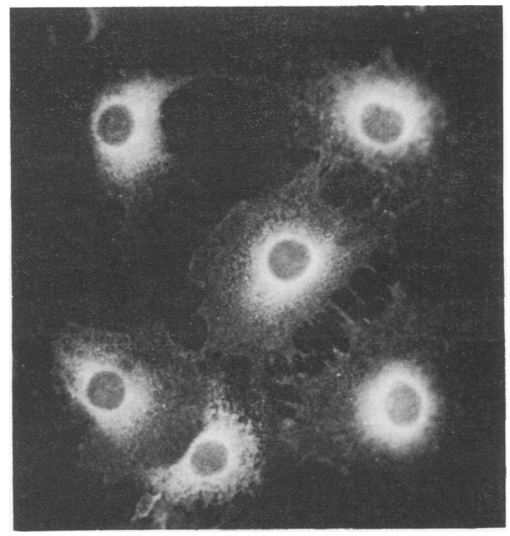

FIG. 1. *Indirect immunofluorescence of RTC (R-MuLV) with monospecific rabbit anti gp69/71.*

Whereas the end point of the goat serum was two- to eightfold higher than that of the rabbit serum, titers of both goat and rabbit anti-gp69/71 were significantly higher against cells infected with R-MuLV, in contrast to G-MuLV and M-MuLV. The results suggest either that these antisera recognize type-specific determinants unique to R-MuLV, as well as group-specific determinants common to murine C-type viruses, or that there are quantitative differences in gp69/71 content of the different MuLV types. Anti-gp69/71 sera showed no reactivity with FeLV-infected cells, indicating the absence of detectable interspecies activity by immunofluorescence testing; such activity has been detected by use of radioimmunoassay (22, 23).

Antisera to R-MuLV p30 showed similar immunofluorescence activity with R-MuLV-, G-MuLV-, and M-MuLV-infected cells, indicating strong group reactivity. Type-specific determinants of p30 were not detected in immunofluorescence; apparently the presence of these determinants was masked by the higher titer of group-specific determinants. The reactivities of rabbit and goat antisera were quite similar with regard to p30 (data not shown). Interspecies reactivity was detected with antisera to MuLV p30 and FeLV p27; the group (species) reactivity was greater, indicating a higher titer in recognition of the group-specific as compared to interspecies determinants.

Immunofluorescence absorption analysis. Rabbit antisera were incubated with sonically treated antigen preparations and tested for residual immunofluorescence activity against

TABLE 1. *Immunofluorescence titers of antisera with fixed infected cells*[a]

Virus	Cells	Antiserum			
		Rabbit anti R-MuLV gp69/71	Goat anti R-MuLV gp69/71	Rabbit anti R-MuLV p30	Rabbit anti FeLV p27
R-MuLV	RTC	320	5,120	640	40
R-MuLV	NIH	640	>2,560	1,280	80
M-MuLV	NIH	160	320	640	80
G-MuLV	$E_{\delta}G2$	40	320	1,280	80
FeLV	FLF	<10	<20	80	320
None	RLF	<20	<20	<40	<20
None	NIH	<20	<20	<40	<20
None	FLF	<10	<20	<10	<20

[a] Virus-infected cells showed strong fluorescence with dilutions of antiserum shown, and then rapidly lost the brightness beyond those dilutions; their uninfected counterparts were invariably immunofluorescence-negative at the dilutions indicated.

TABLE 2. *Immunofluorescence absorption analysis of specificities recognized by three rabbit antisera*[a]

Viral antigen used for absorption	Anti R-MuLV gp69/71 Tested on:			Anti R-MuLV p30 Tested on:				Anti FeLV p27 Tested on:	
	R-MuLV RTC	M-MuLV NIH	G-MuLV $E_{\delta}G2$	R-MuLV RTC	M-MuLV NIH	G-MuLV $E_{\delta}G2$	FeLV FLF	G-MuLV $E_{\delta}G2$	FeLV FLF
R-MuLV-RTC	+	+	+	+	+	+	ND	+	−
M-MuLV-NIH	−	+	+	+	+	+	ND	+	−
G-MuLV-$E_{\delta}G2$	−	+	+	+	+	+	+	+	−
FeLV cat spleen	−	−	−	−	−	−	+	+	+
C57L spleen, RLF, normal cat spleen	−	−	−	−	−	−	−	−	−

[a] Viral antigens were prepared by sonication of infected cells or extracts, as described in Materials and Methods, and mixed at various dilutions with antiserum at a concentration fourfold higher than the end point. The antisera were then incubated with test cells in the standard indirect immunofluorescence procedure. A positive result (+) indicates that antigen diluted eightfold yielded positive absorption of the specific immunofluorescence reaction. A negative result (−) indicates that the undiluted viral antigen preparation showed no absorbing activity. ND, Not done.

R-, M-, G-MuLV-, and FeLV-infected cells (Table 2). Antibodies to gp69/71 purified from R-MuLV were absorbed by R-MuLV, but not by G-MuLV or M-MuLV when tested on R-MuLV-infected cells, indicating recognition of type-specific determinants unique to R-MuLV. By contrast, antibodies reactive with test cells infected with M-MuLV and G-MuLV were absorbed with all the murine C-type viruses because of shared group-specific determinants. These experiments indicate that the higher titer of monospecific antisera when measured against R-MuLV-infected cells was due to qualitative differences in the antigenic determinants, rather than to quantitative differences in the amount of gp69/71, confirming the presence of type-specific determinants. Both type-specific and group-specific activities of antiserum to gp69/71 were absorbed by intact cells with a pattern similar to that seen with sonically treated preparations (data not shown); thus the antigenic determinants appear to reside on the cell surface as well as in the cytoplasm. As with indirect immunofluorescence, interspecies determinants are not detected in absorption tests; FeLV-infected cells do not absorb activity against MuLV-infected cells.

Absorption tests with anti R-MuLV p30 showed that reactivities were absorbed equally by all MuLV cell preparations, indicating exclusively recognition of group-specific determinants. The interspecies determinants were specifically demonstrated by the reciprocal absorption of activity of antisera to MuLV p30 and FeLV p27 by both viruses.

Virus neutralization. A study of virus neutralization by use of the XC assay revealed strong neutralizing activity of monospecific goat antisera to gp69/71 against each of the viruses

tested: R-MuLV, M-MuLV, and G-MuLV. No neutralizing activity was observed with antisera prepared to p30 (Table 3). The titer of anti-R-MuLV gp69/71 was greater against R-MuLV than against G-MuLV and M-MuLV, suggesting that type-specific as well as group-specific determinants of gp69/71 are recognized in neutralization; quantitative differences in gp69/71 content of the three viruses are unlikely in view of immunofluorescence absorption data. Analysis of the neutralization curves (Fig. 2) indicates that the three viruses can be distinguished from one another. Residual infectious R-MuLV is lower than residual G-MuLV at all dilutions of virus and antiserum. At high concentrations of anti-gp69/71, M-MuLV is neutralized as efficiently as R-MuLV, whereas with dilution of antiserum the titer is similar to that seen with G-MuLV. Perhaps M-MuLV and R-MuLV share an identical region of gp69/71 moiety not shared by G-MuLV, or there is cross-reaction between closely related determinants in these two viruses. The significant neutralization of G-MuLV by an antiserum prepared against R-MuLV gp69/71 indicates the powerful role of the group-specific determinants of this protein in virus neutralization.

Membrane immunofluorescence reactivity of anti-gp69/71. The presence of cell surface viral antigenic determinants was examined with antiserum to gp69/71 on viable E$_\delta$G2 cells. A typical fluorescence reaction was observed (Fig. 3) with titers of antiserum similar to those seen in the test with fixed cells. This cell surface reactivity was removed by absorption of the antiserum with either viable cells or sonically disrupted G-MuLV, R-MuLV, or AKR spleen cells. Absorption was not observed with virus-free tissue culture cells, sheep erythrocytes

TABLE 3. *Titer of goat antisera in virus neutralization*[a]

Antiserum	Cells	Test virus		
		R-MuLV	G-MuLV	M-MuLV
Anti gp69/71[b]	NIH	320	80	160
	SC-1	640	160	160
Anti p30[c]	NIH	<10	<10	<10
	SC-1	<10	<10	<10

[a] Various dilutions of antiserum were mixed with different dilutions of virus. In the case of the antisera to gp69/71, the results are expressed as the dilution of antiserum giving 90% reduction of XC plaques. In the case of the antisera to p30, there was less than 50% reduction in plaques at the higher concentration tested (1:10 dilution).

[b] Antiserum dilution showing 90% neutralization.

[c] Antiserum dilution showing 50% neutralization.

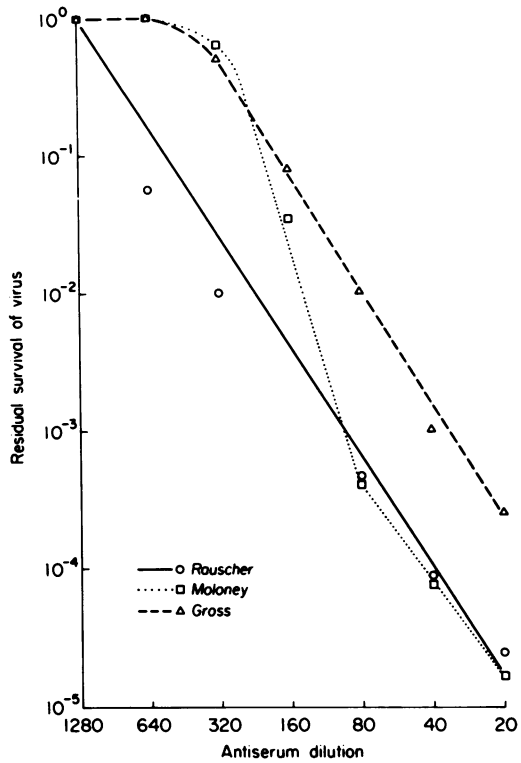

FIG. 2. *Virus neutralization curves of goat anti gp69/71 against various dilutions of R-, G-, and M-MuLV.*

representing Forssman antigen and RTC (R-MuLV) cell sonicate treated with guanidine-hydrochloride. The group-specific determinant of gp69/71 is available at the cell surface for reaction with the corresponding antibody.

Susceptibility of antigenic determinants to treatment with guanidine-hydrochloride and ether. Disruption of MuLV with guanidine-hydrochloride or ether has been used as an initial step in viral protein purification for preparation of antisera (4, 12). Accordingly, it was of interest to learn if such treatment affected the capacity of infected cells to absorb immunofluorescence activity of antisera to R-MuLV gp69/71 and p30 prepared by use of phosphocellulose chromatography and gel filtration (22). Residual activities of absorbed antisera were tested against cells infected with G-MuLV for group-specific reactivity, and R-MuLV for type-specific reactivity (Table 4). Both group-specific and type-specific antigens of gp69/71 are sensitive to guanidine-hydrochloride and to a lesser degree to ether treatment, whereas the group-specific antigen of p30 is relatively stable in the presence of both agents.

These experiments confirm the nonidentity of the group-specific determinants of gp69/71 and p30 detected by other methods (22, 23) and also indicate that the renaturation of protein after treatment with guanidine-hydrochloride apparently leaves residual changes in some antigenic determinants of the glycoprotein moieties.

DISCUSSION

Analysis of the character and cellular location of viral antigenic determinants performed in this study by immunofluorescence may be viewed as complementary to radioimmunoassay and other procedures in the characterization of C-type viral proteins. Immunofluorescence techniques have been used to demonstrate type-specific and group-specific determinants of MuLV gp69/71, as well as group-specific and interspecies determinants of p30 in the cytoplasm of fixed infected cells. Furthermore, group-specific determinants of gp69/71 have been found on the surface of unfixed cells. The interspecies determinants of gp69/71 and type-specific determinants of p30 were not detected by immunofluorescence; these determinants are known to be less prominent by radioimmunoassay (21, 23).

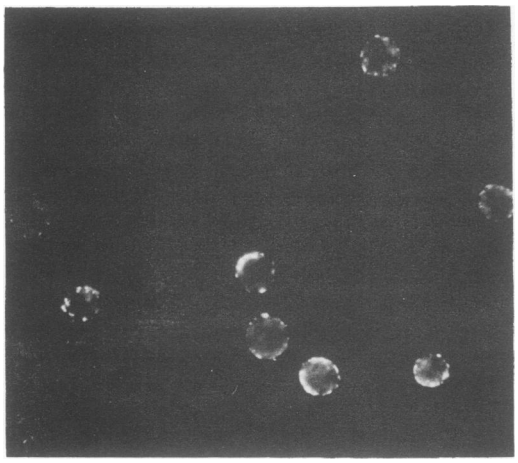

FIG. 3. *Membrane fluorescence with goat anti-gp69/71.*

The potent neutralization activity of a monospecific goat antiserum to gp69/71, and the envelope localization of viral glycoproteins (2, 10, 12, 19, 20, 24), indicate the importance of gp69/71 in viral envelope function. Neutralization of several murine C-type viruses by antiserum to gp69/71 has also been demonstrated by Steeves et al. using the Friend MuLV spleen focus assay (20). The XC test confirms these results and allows direct measurement of neutralizing activity to C-type viruses which grow on mouse cells, while eliminating possible problems of host-induced modifications inherent in an in vivo system. Type-specific discrimination in virus neutralization provide a means of further identification of murine C-type viruses, augmenting the original classification of the serological subgroups G and FMR (14), as well as more recent subclassifications of this group of viruses (1, 3). The group-specific reactivity in virus neutralization suggests that use of a purified gp69/71 or specific protein fragments in vaccination programs might be a realistic possibility, particularly as antisera to gp69/71 has significant neutralizing activity for all naturally occurring viruses tested. (T. Pincus, M.

TABLE 4. *Sensitivity of MuLV antigenic determinants to treatment with guanidine-hydrochloride or ether*[a]

Absorbing antigen	Treatment	Anti gp69/71 tested on:		Anti p30 tested on:	
		R-MuLV RTC	G-MuLV $E_\partial G2$	R-MuLV RTC	G-MuLV $E_\partial G2$
R-MuLV-RTC	Sonication	>16	16	16	32
	Sonication + ether	2	0	16	16
	Sonication + GuHCl	0	0	16	32
Purified R-MuLV	Sonication	>16	>16	>16	>16
	Ether	2	0	>16	>16
	GuHCl	0	0	16	NT
G-MuLV-$E_\partial G2$	Sonication	0	32	16	32
	Sonication + ether	0	4	16	16
	Sonication + GuHCl	0	2	16	>32
RLF	Sonication	0	0	0	0

[a] Infected cells and purified virus were treated with guanidine hydrochloride or ether as described in Materials and Methods prior to standard immunofluorescence absorption tests. Results are expressed as the absorption index, i.e., the titer of antigen which inhibits a specific immunofluorescence reaction. GuHCl, guanidine-hydrochloride.

Strand, and J. T. August, unpublished data).

It is not unexpected that the viral envelope gp69/71 is accessible to antibody at the surface of productively infected cells as indicated by immunofluorescence. A viral glycoprotein with properties similar to gp69/71 has been described on the surface of infected cells by the immunoprecipitation method (10). Whether the glycoprotein is located on the viral envelope or cell surface, or both, is not yet clear. Immunofluorescence of viable cells has also revealed group-specific determinants of p30 on the cell surface (26); immunoelectron microscopy indicated focal labeling of the cell surface, but not of the virions. These data are consistent with an earlier study of Nowinski et al. (12), in which it was shown by immunoelectron microscopy that an antiserum to viral glycoprotein reacted with the viral envelope, whereas an antiserum to p30 reacted with the cell surface but not with the viral envelope.

Modification of gp69/71 by treatment with guanidine-hydrochloride is important because this method has been widely applied in the separation of viral protein (13). Rabbit antiserum prepared to R-MuLV gp69/71 after guanidine-hydrochloride chromatography is not nearly as effective in virus neutralization as the goat anti-gp69/71 used in this study (H. Ikeda, T. Pincus, and E. Fleissner, unpublished data). Although it has been difficult in general to obtain good MuLV neutralizing antisera through immunization of rabbits, which may account for the latter findings, the alteration of antigenic determinants by guanidine-hydrochloride must also be considered a possible difficulty.

Murine C-type viruses show complex polymorphism due to genetic variation. The viral proteins show variable regions, recognized as type-specific determinants, and common regions, recognized as group-specific and interspecies determinants. The experiments presented here indicate that antigenic determinants of the various viral components serve as probes not only in identification and study of isolated proteins, but also in characterization of intact C-type viruses and virus-infected cells.

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