

## RELATION OF $G_{IX}$ ANTIGEN OF THYMOCYTES TO ENVELOPE GLYCOPROTEIN OF MURINE LEUKEMIA VIRUS\*

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The cell-surface antigen  $G_{IX}$  is expressed on the thymocytes of certain strains of mice and not on the thymocytes of others (1). Its inheritance has been studied most extensively in crosses of the  $G_{IX}^+$  strain 129 with C57BL/6 (B6)<sup>1</sup> and other  $G_{IX}^-$  strains. Its expression has been shown to depend, in 129 mice, on genes at two loci, on chromosomes 7 and 17 (1, 2). In company with other T-lymphocyte surface components (TL, Ly-1, etc.) expression of  $G_{IX}$  depends on the T-lymphocyte pathway of differentiation (3, 4) and it does not occur on other cells (with the single exception of sperm, on which  $G_{IX}$  has been found by serological absorption, although no comprehensive study of  $G_{IX}$  representation on sperm has yet been made). By the usual methods of serial backcrossing, the  $G_{IX}$  phenotypes of 129 and B6 have been interchanged to produce two congenic lines of mice: 129/ $G_{IX}^-$  and B6/ $G_{IX}^+$  (1).

An indication that the  $G_{IX}^+$  character of 129 mouse thymocytes represents partial expression of murine leukemia virus (MuLV) genome comes from the fact that cells producing MuLV may express  $G_{IX}$  on their surfaces regardless of their tissue type and of their inherited  $G_{IX}$  genotype, which normally controls *expression vs. nonexpression* as outlined above. In other words, cells producing MuLV frequently express  $G_{IX}$  regardless of their differentiative history and hereditary, whereas in the absence of virus production  $G_{IX}$  behaves as a thymocyte differentiation alloantigen (4, 5).

It is understandable therefore that in mice that are lifelong overt producers of MuLV, like AKR, the tissue representation of  $G_{IX}$  is more widespread than in 129 mice, and that its expression is governed to a large extent by loci regulating the output of virus: *Akv-1*, *Akv-2* (6), and *Fv-1* (7), as is clear from another study (H. Ikeda, unpublished results). It is understandable also that on the cells of rats inoculated with MuLV, and on the leukemias thus induced in the rat,  $G_{IX}$  cell-surface antigen is expressed. In fact, the serological reaction used to

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<sup>1</sup> *Abbreviations used in this paper:* anti-NTD, (W/Fu × BN)F<sub>1</sub> rat anti-MuLV-induced W/Fu rat leukemia (C58NT)D; B6, C57BL/6; FMR, type antigen of the Friend-Moloney-Rauscher set of viruses; gs, group specific; IFA, immunofluorescence absorption; MuLV, murine leukemia virus.

demonstrate and analyze the  $G_{IX}$  system is the complement-dependent lysis of 129 thymocytes (cytotoxicity assay) by antibody produced in inbred rats against syngeneic MuLV-induced rat leukemia cells (1, 8). Antibodies directed to other MuLV-associated antigens, which this antiserum contains, do not compromise this  $G_{IX}$  cytotoxicity assay because no such known antigens are expressed on the 129 thymocyte surface (8).

The induction of  $G_{IX}$  antigen on infected rat cells in particular suggests that it might be coded by the MuLV genome, and might in fact represent a constituent of MuLV that is also a constituent of the thymocyte plasma membrane of certain mice, independent of complete virus production; in the latter circumstances its expression is seen to depend on the differentiative controls of the T-lymphocyte pathway. The following serological evidence now points to the correctness of this view. Supporting biochemical data are given in the accompanying paper (9).

## Materials and Methods

*Special Mouse Stocks.* In 1970, a shipment of C57BR/cdJ mice from the Jackson Laboratory, Bar Harbor, Maine, was found to include mice of both  $G_{IX}^-$  and  $G_{IX}^+$  phenotypes. Our present C57BR ( $G_{IX}^-$ ) and C57BR/ $G_{IX}^+$  substrains were derived by selective breeding of typed mice of this group. The two substrains are skin-graft compatible with each other, and identical for all cell-surface alloantigens tested.

In 1973, a mouse of our B6 ( $G_{IX}^-$ ) colony, used as a serological control, was found to be  $G_{IX}^+$ . The inheritance of this mutation (using "mutation" in its broadest connotation) was traced back through the pedigree, and our present B6 and B6/ $G_{IX}^+$ M substrains were established by selective breeding of typed  $G_{IX}^-$  and  $G_{IX}^+$  mice. These two substrains are skin-graft compatible and are identical for the following serological and biochemical markers: *Dip-1*, *Id-1* (chromosome 1), *Gpd-1*, *Mup-1* (chromosome 4), *Ly-2*, *Ly-3*, *Ldr-1* (chromosome 6), *Gpi-1*, *Hbb* (chromosome 7), *Es-1* (chromosome 8), *Thy-1*, *Mod-1* (chromosome 9), *Es-3* (chromosome 11), *H-2*, *Tla* (chromosome 17), *Ly-1*, *Pgm-1* (chromosome 19), *Pca* (chromosome ?), and kidney catalase (chromosome ?).

*Antisera.* (a) The antiserum (W/Fu  $\times$  BN) $F_1$  rat anti-MuLV-induced W/Fu rat leukemia (C58NT)D (8) (abbreviated "anti-NTD") contains antibodies to several MuLV-associated antigens in addition to  $G_{IX}$ . When referred to as " $G_{IX}$  antiserum" it is implied that the antiserum is being employed in the cytotoxicity assay with thymocytes of strain 129 mice (1), which express on their surfaces only this antigen ( $G_{IX}$ ) among the set of known MuLV antigens (for technical reasons, thymocytes of B6/ $G_{IX}^+$  congenic mice were often substituted for 129 thymocytes, with which they are precisely equivalent in the context of  $G_{IX}$  typing). The endpoint dilution of the anti-NTD serum used in this study (<50% thymocytes lysed) was 1/800.

(b) Anti-gp69/71 and anti-p30; the goat and rabbit antisera to the gp69/71 and p30 components of MuLV-Rauscher, isolated by phosphocellulose chromatography and Sephadex gel filtration (10) were kindly provided by Doctors M. Strand and J. T. August (Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, N. Y.).

*Complement (C)-dependent Cytotoxicity Assay for  $G_{IX}$  Antigen.* The method of Stockert et al. (1) was used. Thymocytes of 129 mice ( $5 \times 10^6$ /ml) were added in equal volumes to anti-NTD serum (serial dilutions) and rabbit serum (C source; required dilution predetermined). After incubating for 45 min at 37°C cell viability counts were made in the presence of trypan blue.

*Test for Free  $G_{IX}$  Antigen in Normal Mouse Serum by Inhibition of the  $G_{IX}$  Cytotoxicity Assay.* Sera from selected mouse strains were preabsorbed with BALB/c thymocytes to remove naturally occurring autoantibody to thymocytes (11) which would interfere with the cytotoxicity assay for  $G_{IX}$  antigen.

This preabsorbed serum (25  $\mu$ l; serial dilutions) was mixed with an equal volume of a predetermined dilution of anti-NTD serum (usually 1/200) and incubated for 30 min on ice. Then 50  $\mu$ l of thymocytes ( $5 \times 10^6$ /ml) were added and incubation continued for 30 min on ice. The cells were

washed once and resuspended in 100  $\mu$ l of C and incubated for 30 min at 37°C, followed by the usual viability counts.

*Immunofluorescence Absorption (IFA) Test for gp69/71 Antigen.* The method used was based on the method of Hilgers et al. (12). The standard test cells were E<sub>6</sub>G2 leukemia cells (transplanted B6 leukemia induced by passage A Gross virus) acetone fixed on slides. Goat and rabbit anti-gp69/71 serum give bright diffuse cytoplasmic staining in the indirect immunofluorescence test (titers in the region of 1/1,280 and 1/320, respectively). Soluble or particulate material is tested for the presence of group-specific (gs) antigen of gp69/71 by measuring its capacity to absorb this immunofluorescence reactivity of anti-gp69/71 serum, used at a predetermined critical dilution (in the region of 1/400) under standard conditions (13).

*Blocking Test for Proximity of Cell Surface Antigens (14).* In general terms, the test consists in the construction of absorption curves. For each curve, different numbers of cells (abscissa) are used to absorb standard volumes of an antiserum "anti-x" and plotted against the percentage of "x-positive" target cells lysed in the cytotoxicity assay by any remaining antibody (ordinate).

The cells used for absorption carry two antigens, "x" and "y," whose proximity is to be studied. Absorption curves are constructed for these cells after preliminary saturation with "anti-y," and for control purposes "anti-z," etc. In the case where x and y are adjacent, the absorption curve for x is selectively displaced by pre-exposure of the cells to anti-y. In the present study, anti-x = G<sub>1X</sub> antiserum, and anti-y = gp69/71 antiserum.

*Test for Absorption of G<sub>1X</sub> Antibody by MuLV.* MuLV "AKR-3T3" virus (lot number 346-59-12) was purchased from Electro-Nucleonics Laboratories, Inc., Bethesda, Md. This virus stock is described as derived from NIH/3T3 cells from NIH Swiss embryo cultures infected with AKR-MuLV, purified by double density gradient zonal centrifugation. Before use, the virus was pelleted and suspended in phosphate-buffered saline.

Equal volumes of virus suspensions (serial dilutions) and antibody diluted to the vicinity of the endpoint of the cytotoxicity assay were incubated for 15 min at 37°C, followed by 30 min on ice. After removal of the virus by centrifugation for 30 min at 110,000 g, 50  $\mu$ l of the supernate was mixed with 50  $\mu$ l of thymocytes ( $5 \times 10^6$ /ml). The absorption procedure was then completed as described above.

## Results and Discussion

One of the two major components of the MuLV envelope is gp69/71, comprising a pair of glycoproteins with mol wt of 69,000 and 71,000 and possessing common antigens, which can be isolated from disrupted virus (10). Antibody to purified gp69/71, made in goat or rabbit, identifies gp69/71 antigens in fixed virus-producing cells in the indirect immunofluorescence test (13); the presence of MuLV gp69/71 in any material can be assayed by absorption of this immunofluorescence reactivity from gp69/71 antiserum in the IFA test. As Table I shows: (a) Free gp69/71 antigen is demonstrable by the IFA test in the serum of mice of several strains (as reported by Yoshiki et al. [15] for the NZB strain), including not only overt life long virus producers like AKR and C58, but also 129 and several other mouse strains. (b) Mouse strains with gp69/71 antigen in their serum express G<sub>1X</sub> on their thymocytes in the cytotoxicity assay. (c) This concordance is not fortuitous, because it includes the congenic partners of B6, 129, and C57BR, which have similar genetic backgrounds but differ in their G<sub>1X</sub> thymocyte phenotype.

A new observation, illustrated in Fig. 1, is that G<sub>1X</sub> antigen also can be demonstrated free in the serum of 129 mice; this is shown by the capacity of 129 normal serum to inhibit the cytotoxicity of G<sub>1X</sub> antibody for 129 thymocytes. The concentration of G<sub>1X</sub> antigen in the serum increases substantially within the age range of 3-15 wk, as Fig. 1 also illustrates, although the concentration of G<sub>1X</sub>

TABLE I  
 Concordance of Tests for  $G_{IX}$  and gp69/71 Antigens in the Serum of Various  
 Mouse Strains

Mouse strain (age approximately 2 mo)	$G_{IX}$ on thymocytes*	Antigen in serum	
		$G_{IX}\ddagger$	gp69/71§
129, B6/ $G_{IX}^+$ , B6/ $G_{IX}^+M$ , C57BR/ $G_{IX}^+$ (see congenic partners below); and also CE, AKR, C58, I, DBA/2, A, SJJ/J, C3H/An	+	+	+
129/ $G_{IX}^-$ , B6, C57BR (see congenic partners above); and also C57L, BALB/c	-	-	-

\* C-dependent cytotoxicity assay.

‡ Inhibition test for  $G_{IX}$  antigen.

§ IFA test.

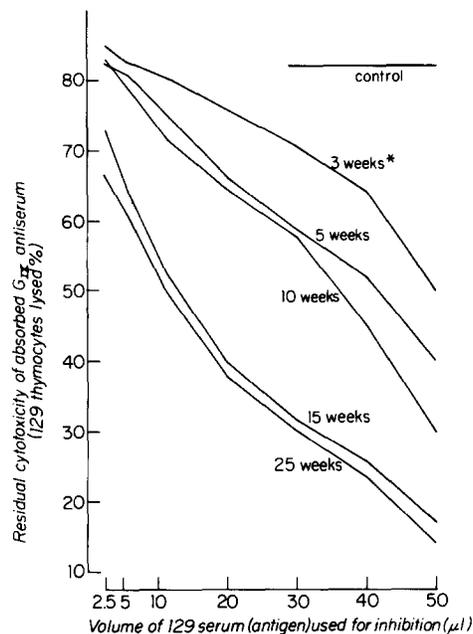


FIG. 1. Detection of free  $G_{IX}$  antigen in the serum of 129 mice of various ages by inhibition of the  $G_{IX}$  cytotoxicity assay. Control: serum of 129/ $G_{IX}^-$  mice (25 wk old). (\*), age of mice donating serum (pool from sets of three female mice).

antigen on thymocytes shows no similar change in 129 mice (Kohei Kawashima, personal communication). Table I shows that the presence of  $G_{IX}$  antigen in serum correlates with its presence on thymocytes, and with the presence of gp69/71 antigen in the serum; i.e. if the thymocytes of a mouse strain express  $G_{IX}$  on their surfaces, then both  $G_{IX}$  and gp69/71 antigens are demonstrable in the serum. This also held true in the genetic segregation test illustrated in Table II,

TABLE II  
*Concordance of Tests for (a) G<sub>IX</sub> Antigen on Thymocytes and (b) gp69/71 Antigen in the Serum of Backcross\* Segregant Mice*

G <sub>IX</sub> on thymocytes (cytotoxicity assay)	gp69/71 in serum (IFA test)	Number of segregants
++‡	++	34
++	+\$	0
+	++	0
+	+	38

\* C57BR/G<sub>IX</sub><sup>+</sup> × (C57BR/G<sub>IX</sub><sup>+</sup> × C57BR)F<sub>1</sub>.

‡ ++, same high reactivity as C57BR/G<sub>IX</sub><sup>+</sup>.

\$ +, same low reactivity as (C57BR/G<sub>IX</sub><sup>+</sup> × C57BR)F<sub>1</sub>.

in which the presence or absence of G<sub>IX</sub> on thymocytes was invariably correlated with presence or absence of gp69/71 antigen in the serum.

It should be emphasized that although soluble MuLV-related antigens (Friend-Moloney-Rauscher antigen [FMR] and Gross soluble antigen [GSA]) have previously been demonstrated free in serum (16, 17) this has been so only in mice producing large quantities of virus, whereas in the case of G<sub>IX</sub> the serum antigen is seen to be independent of viral output. The fact that the positive G<sub>IX</sub> phenotype (for thymocytes and serum) correlates with the presence of gp69/71 antigen in serum does not disclose whether G<sub>IX</sub> and gp69/71 antigens occur on a single molecule or as separate but codependent products.

To test this, we ascertained whether removal of the gp69/71 component from 129 mouse serum by complexing it with gp69/71 antibody would entail the removal of G<sub>IX</sub> antigen also. Once again, as shown in Table III, G<sub>IX</sub> antigen was not separable from gp69/71 antigen, i.e., G<sub>IX</sub> antigen was coprecipitated with gp69/71 by goat antiserum to gp69/71 MuLV-Rauscher. Among the control antisera included in Table III is goat antibody to MuLV-Rauscher p30, a major internal component of the virus (18), which did not coprecipitate G<sub>IX</sub>.

Although G<sub>IX</sub> antigen was thus genetically and physically inseparable from gp69/71 in the serum of all mice tested this does not imply that gp69/71 from all mouse sources necessarily carries the G<sub>IX</sub> specificity. The type specificity recognized on MuLV gp69/71 by radioimmunoassay (19), points to structural polymorphisms of this component of MuLV. In fact polymorphism of gp69/71 may well be responsible for most of the polymorphism of envelope antigens revealed by immunoelectron-microscopic studies of MuLV (20). This variation may extend to the G<sub>IX</sub>-determining region of the molecule, giving rise to gp69/71 molecules that are G<sub>IX</sub><sup>+</sup> and others that are G<sub>IX</sub><sup>-</sup>.

It seems somewhat less likely that G<sub>IX</sub> would be expressed on cell surfaces independently of the rest of the gp69/71 molecule bearing other antigens. But even this bears consideration, because gp69/71 is a composite molecule (glycoprotein) and it is not yet known which moieties account for any of its several antigens. Cell surface proteins are characteristically glycoproteins, although in only few instances is there evidence as to what part of the molecule is involved in antigenic variation. In the case of *H-2*, the alloantigenic differences are most probably determined by variation of the protein rather than the carbohydrate

TABLE III  
*Precipitation of  $G_{IX}$  Antigen from 129 Mouse Serum by Goat Antiserum to gp69/71\**

Antiserum (dilution)	Mouse serum from:	Residual antigen in supernate	
		$G_{IX}\ddagger$	gp69/71§
—	129	+	+
goat anti-gp69/71 (1/30)	129	—	—
normal goat serum (1/20)	129	+	+
goat anti-p30 (1/20)	129	+	+
—	129/ $G_{IX}^-$	—	—
goat anti-gp69/71 (1/30)	129/ $G_{IX}^-$	—	—

\* Antiserum (column 1), and undiluted mouse serum from 129 or 129/ $G_{IX}^-$  mice (column 2) were mixed in equal volumes (37°C/30 min), centrifuged to remove antigen-antibody complexes (110,000 g/30 min), and the supernate tested for residual  $G_{IX}$  and gp69/71 antigens.

‡ Inhibition of  $G_{IX}$  cytotoxic antibody.

§ IFA test with rabbit anti-gp69/71 serum.

moiety (21). The same question arises in connection with each antigen of gp69/71, the complete synthesis of which must involve more than one gene, in order to account for its composite structure. Clearly, future study of the chemical composition of this molecule in relation to  $G_{IX}$  and its other antigens will help in understanding the genetics of its specification, especially on the thymocytes of 129 and other mice where its expression is evidently independent of complete viral synthesis, and where the joint action of two unlinked chromosomal loci has already been implicated.

As yet, such detailed analysis is distant, but the following data support the view that the concordance of  $G_{IX}$  expression on thymocytes with presence of  $G_{IX}$ -gp69/71 in the serum is explicable in terms of a single glycoprotein that resides in the plasma membrane and can be released from it. Since the goat anti-gp69/71 serum identifies a particular segment or segments of the gp69/71 glycoprotein, and since  $G_{IX}$  is apparently a part of the same glycoprotein, goat anti-gp69/71 might be expected to react with thymocytes of 129 mice, provided that a relevant segment of gp69/71 is accessible in the plasma membrane. In fact this antiserum gave a weak but consistent reaction in the cytotoxicity assay with 129 thymocytes but not with 129/ $G_{IX}^-$  thymocytes.

A plausible reason for the weakness of this cytotoxic reaction on 129 thymocytes is that the gs antigen of gp69/71, to which the gp69/71 antiserum is directed in reactions with non-Rauscher-MuLV gp69/71, is relatively inaccessible on intact thymocytes. This interpretation is reinforced by tests we have made in which Nonidet P-40 lysates of 129 thymocytes, but not equivalent numbers of intact 129 thymocytes (nor lysates of 129/ $G_{IX}^-$  thymocytes), absorbed anti-gp69/71 antibody in the IFA assay.<sup>2</sup>

<sup>2</sup>The type-specific reactivity of the antiserum to gp69/71-Rauscher would not be involved in reaction with gp69/71 of 129 thymocytes nor with gp69/71-Gross of the standard test cells used in the IFA assay.

The physical association of the G<sub>IX</sub> and gp69/71 antigens implies that they should be situated close together on the thymocyte membrane. In investigating such questions of proximity we have previously shown that when a particular set of cell-surface antigens is saturated by the relevant antibody, subsequent absorption of antibody to a second set of antigens may be impeded, while at the same time antibody to a third set is not impeded; thus, the relative proximities of antigens on the cell surface have been estimated on the basis of steric interference by the competing antibodies (14).

Fig. 2 shows that saturation of 129 thymocytes with gp69/71 antibody, but not

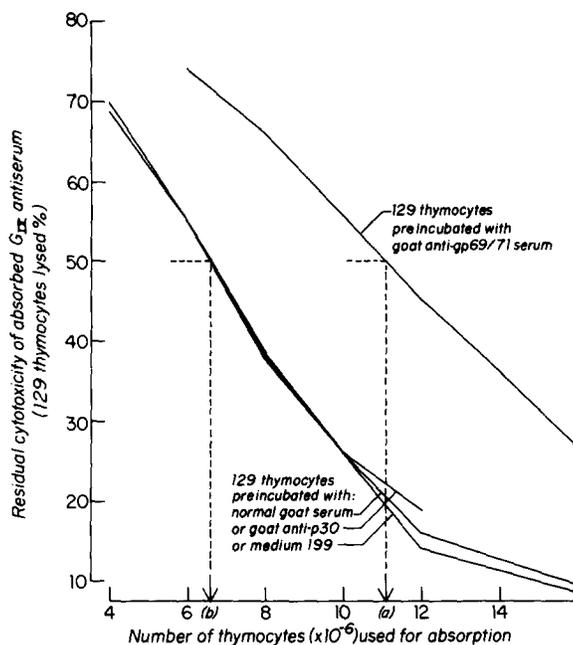


FIG. 2. Blocking of G<sub>IX</sub> antigen sites on 129 thymocytes by pre-exposure to gp69/71 antiserum. Procedure: Equal volumes of G<sub>IX</sub> antiserum were absorbed with different numbers of washed 129 thymocytes (abscissa) that had been preincubated with goat anti-gp69/71 or other (control) antisera as indicated; after removal of the cells by centrifugation, the absorbed antiserum was tested for residual G<sub>IX</sub> antibody in the cytotoxicity assay (ordinate). An index of the degree of interference between the two antibodies competing for the respective adjacent sites, G<sub>IX</sub> and gp69/71, is given by  $[(a-b)/a] \times 100 = [(11.2 - 6.6)/11.2] \times 100 = 41\%$  (14).

with p30 antibody, blocks the attachment of G<sub>IX</sub> antibody to the extent of approximately 41%, indicating that gp69/71 antiserum identifies a gp69/71 component present on 129 thymocytes, and that this is adjacent to G<sub>IX</sub> antigen. The fact that blocking was not complete can be interpreted most readily in terms of accessibility of membrane-associated gp69/71 antigen (as discussed above), or in terms of two gp69/71 antigenic sites that are sufficiently widely spaced on the cell surface to accommodate both the respective antibodies to some degree.

Finally the question arises: Is the G<sub>IX</sub> section of the gp69/71 molecule accessible to antibody when gp69/71 composes part of the virion coat rather than part of the plasma membrane? If so, then intact virus should be capable of

absorbing  $G_{IX}$  antibody. Table IV shows that intact MuLV does in fact absorb anti- $G_{IX}$  activity in the  $G_{IX}$  cytotoxicity assay, and the controls indicate that this is unlikely to be due to contamination of the virus preparation with  $G_{IX}$ -bearing fragments of plasma membrane or to the serological artifact of anticomplementarity.

In further tests, the absorption capacity of MuLV frozen once (for storage) was compared with that of the same MuLV after 10 cycles of freezing and thawing. The results indicate no increase in availability of viral  $G_{IX}$  antigen consequent upon disruption of virions.

We conclude that  $G_{IX}$  antigen is accessible on the intact virion, as it is on the cell surface. In this respect,  $G_{IX}$  antigen differs from FMR (MuLV associated) antigen. The latter is also a prominent surface antigen of infected cells (23),

TABLE IV  
*Absorption of  $G_{IX}$  Cytotoxic Antibody by Purified MuLV*

Antiserum (dilution)	Target cells	Residual cytotoxicity after absorption with the following concentrations of virus ( $\mu\text{g/ml}$ ):					
		400	200	100	50	25	None
		<i>cells lysed %</i>					
Anti- $G_{IX}$ (1/200)	B6/ $G_{IX}^+$ thymocytes	10	27	66	72	84	86
Rabbit antimouse cells (1/50)*	NIH Swiss lymphocytes	90					90
Anti-Ly-2.2 (1/200)‡	B6/ $G_{IX}^+$ thymocytes	78					79

Equal volumes of antiserum and virus suspension were mixed and incubated (absorption step), virus was removed by centrifugation, and the supernate (absorbed antiserum) tested for residual antibody (cytotoxicity assay); see Materials and Methods.

\* Control for contamination of virus preparation with plasma membranes of the cells that produced the virus (NIH/3T3).

‡ Control for anticomplementarity of the virus preparation; an anti-Ly system was selected because Ly-systems are high in demand for C (rabbit) and therefore sensitive indicators of anticomplementary effects (22).

recognized similarly by the cytotoxicity assay, but according to Lilly and Steeves (24) its demonstrability in virions is greatly increased by their disruption, suggesting that although it is accessible on the cell surface it is internally situated in the virion.

As discussed above, we do not assume that all variants of the murine gp69/71 molecule carry  $G_{IX}$  antigen. Further evidence on this point is now being sought.

## Summary

Expression of  $G_{IX}$  surface antigen on thymocytes is an inherited mendelian trait of certain strains of mice. We report here the following new findings: (a)  $G_{IX}$  antigen was found free in the serum of  $G_{IX}^+$  mouse strains. (b) Expression vs. nonexpression of  $G_{IX}$  antigen was invariably correlated with presence or absence of the group-specific antigen of Murine leukemia virus (MuLV) gp69/71 in the

serum of mice of inbred and segregating populations. (c) G<sub>IX</sub> antigen could be removed from normal G<sub>IX</sub><sup>+</sup> mouse serum by precipitation with antiserum to MuLV gp69/71. (d) Anti-gp69/71 serum was weakly cytotoxic for G<sub>IX</sub><sup>+</sup> thymocytes, and partially blocked the cytotoxic activity of G<sub>IX</sub> antibody for G<sub>IX</sub><sup>+</sup> thymocytes. (e) Purified AKR virus absorbed G<sub>IX</sub> activity, and disruption of the virions did not increase their absorbing capacity.

These serological data indicate that G<sub>IX</sub> antigen is a constituent of gp69/71, the glycoprotein which is the major component of the MuLV envelope. On present evidence, G<sub>IX</sub> antigen is represented in intact virions and is probably accessible to G<sub>IX</sub> antibody.

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