

BIOCHEMICAL EVIDENCE LINKING  
THE  $G_{IX}$  THYMOCYTE SURFACE ANTIGEN  
TO THE gp69/71 ENVELOPE GLYCOPROTEIN  
OF MURINE LEUKEMIA VIRUS\*

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The alloantigen  $G_{IX}$  is found on the surface of thymocytes of some strains of mice ( $G_{IX}^+$ ), where it is demonstrable by complement (C)-dependent lysis (cytotoxicity assay) with (W/Fu  $\times$  BN) $F_1$  anti-W/Fu(C58NT)D (anti-NTD)<sup>1</sup> serum, and not on the thymocytes of other strains ( $G_{IX}^-$ ). Its expression in mice of the 129 strain, where its genetics has been studied in most detail, is controlled by two unlinked chromosomal loci (1). (Control of its expression in other  $G_{IX}^+$  mouse strains may differ in detail from that of strain 129, and this is a subject of continuing study [2].) An important feature of  $G_{IX}$  antigen is its relation to leukemia virus and leukemogenesis. Thus, cells other than thymocytes, and from  $G_{IX}^-$  as well as  $G_{IX}^+$  mouse strains (and from rats), may express  $G_{IX}$  antigen if they become productively infected with murine leukemia virus (MuLV) (1).

In the accompanying paper, Obata et al. (3) give serological evidence that  $G_{IX}$  antigen is a constituent of the gp69/71 glycoprotein component (4, 5) of the MuLV envelope, implying that the mendelian inheritance of  $G_{IX}$  in certain mouse strains signifies the mendelian inheritance of a component of MuLV expressed selectively on cells following the T-lymphocyte differentiative pathway. We report here a related study in which a molecule resembling gp69/71 was isolated from intact thymocytes of 129 mice by enzymatic radioiodination of the thymocyte surface, followed by release of this glycoprotein with the nonionic detergent Nonidet P-40 (NP-40) and precipitation with anti-NTD serum; the product was then compared with envelope proteins isolated from purified MuLV. Our interpretation is that  $G_{IX}$  antigen is carried by a glycoprotein with a mol wt

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<sup>1</sup> Abbreviations used in this paper: anti-NTD, (W/Fu  $\times$  BN) $F_1$  anti-W/Fu (C58NT)D; 2-ME, 2-mercaptoethanol; MuLV, murine leukemia virus; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

of approximately 70,000 daltons which possesses other antigens identifying it as the gp69/71 glycoprotein component of MuLV.<sup>2</sup>

## Materials and Methods

*Antisera.* The anti-NTD serum referred to in the text is (W/Fu × BN)F<sub>1</sub> anti-W/Fu(C58NT)D (6). [W/Fu and BN are inbred rats and (C58NT)D is a transplanted W/Fu rat leukemia induced by wild-type MuLV from a C58 mouse.]

Goat anti-gp69/71 Rauscher antiserum: The goat antiserum to gp69/71 component of Rauscher-MuLV, isolated by phosphocellulose chromatography and Sephadex gel filtration, was kindly provided by Doctors M. Strand and J. T. August, Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, N. Y.

Goat antimouse immunoglobulin (Ig) has been described (7). Rabbit antirat Ig was provided by Dr. J. Phillips-Quagliata, Department of Pathology, New York University Medical Center, New York and by Dr. U. Hammerling, Memorial Sloan-Kettering Cancer Center, New York. Pig antigoat Ig was provided by Doctors Strand and August and by Dr. R. Wilsnack, Huntingdon Research Center, Brooklandville, Md.

*Preparation of Radioiodinated Cells.* Suspensions of washed mouse thymocytes (10<sup>8</sup>/ml) in phosphate-buffered saline (PBS) at pH 7.3 were radiiodinated with carrier-free <sup>125</sup>I (Amersham/Searle Corp., Arlington Heights, Ill.) by the lactoperoxidase method as described by Vitetta et al. (7). The cells were then lysed in 0.5% NP-40, and the nuclei and cell debris were removed by centrifugation at 10,000 *g* for 15 min. The supernatant fluids were dialyzed for 16 h at 4°C against PBS and centrifuged again at 10,000 *g*. Small aliquots of the supernatant fraction were then precipitated with 5% TCA containing 5 mM NaI, and radioactivity determined.

*Incorporation of Radioactive Metabolic Precursors.* Thymocyte suspensions were washed once with medium 199 (Grand Island Biological Co., Grand Island, N. Y.) and suspended in medium 199 containing 20% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at a concentration of 10<sup>7</sup> cells/ml. To this thymocyte suspension was added [<sup>3</sup>H]glucosamine (New England Nuclear, Boston, Mass.; 300–500 μCi/ml of cell suspension) and incubation was carried out at 37°C for 5 h in 5% CO<sub>2</sub> in air. Viability of the cells, according to trypan blue exclusion, was approximately 70% of the total population. The labeled cells were then washed once with PBS and lysed by NP-40 as for iodinated cells (see above).

*Preparation of [<sup>3</sup>H]Glucosamine-Proteins from MuLV.* Gross-MuLV was grown in NIH Swiss mouse embryo cells (8) in the presence of [<sup>3</sup>H]glucosamine, and purified by sucrose density gradient centrifugation according to the method of Nowinski et al. (9). [<sup>3</sup>H]glucosamine-labeled gp69/71 molecules were released from the virions by incubation in 0.1 M Tris buffer (pH 8.6) containing 8 M urea, 1% sodium dodecyl sulfate (SDS), and 2% 2-mercaptoethanol (2-ME) at 56°C for 30 min. Because gp69/71 is the major species labeled with [<sup>3</sup>H]glucosamine no steps were necessary to purify this species from the disrupted virus preparation for comparison with thymocyte-derived proteins.

*Preparation of <sup>125</sup>I-Labeled Proteins from MuLV.* Gross-MuLV (prepared from NIH Swiss mouse embryo cells as above and pelleted from a sucrose band) was radioiodinated by the lactoperoxidase method (see above). After iodination, the virus was twice centrifuged at 100,000 *g* for 1 h and the pellet resuspended in PBS. The resuspended radioiodinated virus was dialyzed with several changes against PBS for 3 days at 4°C and disrupted in the same way as [<sup>3</sup>H]glucosamine-MuLV.

*Immunoprecipitation.* The method of Vitetta et al. was used (7). Antigens in NP-40 lysates of thymocytes were precipitated by anti-NTD serum followed by rabbit antirat Ig. In controls, rat antiegg albumin or normal rat serum was substituted for anti-NTD serum. (These controls gave similar results, so normal rat serum was used in most experiments.) In later experiments goat antimouse Ig was used in place of rabbit antirat Ig because the former was found to react equally well

<sup>2</sup>In work related to that reported here, Del Villano and associates have demonstrated MuLV gp69/71 on the surfaces of normal and neoplastic mouse lymphoid cells. Del Villano, B. C., B. Nave, B. P. Croker, R. A. Lerner, and F. J. Dixon. 1975. The oncornavirus glycoprotein gp69/71: a constituent of the surface of normal and malignant thymocytes. *J. Exp. Med.* 141:172.

with rat Ig. With goat anti-gp69/71 antiserum, the control was normal goat serum (GIBCO), and the second precipitation was effected with pig antigoat Ig.

*Analyses of Immunoprecipitates by Polyacrylamide Gel Electrophoresis (PAGE).* Precipitates were dissolved at 56°C for 30 min in 0.1 M Tris buffer (pH 8.6) containing 8 M urea, 1% SDS, and 0.1 M 2-ME. Samples were electrophoresed on 7.5% SDS-polyacrylamide gels according to Weber and Osborn (10), and cut into 1-mm slices by a Gilson gel fractionator (model B-100/GAM-GCB, Gilson Medical Electronics, Inc., Middleton, Wis.). Gel slices containing radioiodinated polypeptides were counted in a Packard auto-gamma scintillation spectrometer (model 5358, Packard Instrument Co., Inc., Downers Grove, Ill.). Gel slices containing [<sup>3</sup>H]glucosamine-labeled polypeptides were counted in 10 ml of toluene-Triton X-100 (2:1) scintillation fluid in a Packard liquid scintillation spectrometer (model 3390, Packard Instrument Co., Inc.)

## Results and Discussion

The antiserum which defines G<sub>IX</sub> thymocyte surface antigen in the cytotoxicity assay is prepared in inbred rats by immunization with syngeneic MuLV-induced rat leukemia cells (6). For convenience, we shall refer to this as anti-NTD serum, as in the accompanying paper by Obata et al. (3). This serum identifies several MuLV-associated antigens (6) and precipitates at least four structural proteins of Gross-MuLV identified as gp69/71, gp45, p30, and one of the smaller proteins.<sup>3</sup> Since G<sub>IX</sub> antigen is specifically identified by the cytotoxicity assay with thymocytes, we used thymocytes from congenic mouse strains which differ for G<sub>IX</sub> antigen but share a common genetic background. In this study the congenic pair was 129, which is G<sub>IX</sub><sup>+</sup> (ie, thymocytes are lysed by G<sub>IX</sub> antibody and C), with its partner strain 129/G<sub>IX</sub><sup>-</sup> (thymocytes not lysed by G<sub>IX</sub> antibody and C) (1).

First we compared the radioactivities of crude precipitates obtained by reacting the NP-40 lysates of <sup>125</sup>I-labeled 129 thymocytes with (a) anti-NTD G<sub>IX</sub>-typing serum or (b) normal rat serum (control), followed by anti-Ig. In a series of such experiments the average ratio (a)/(b) was 2.6 ± 0.43 (nine experiments), as compared with 1.4 ± 0.55 (five experiments) for the average ratio (a)/(b) in duplicate tests with 129/G<sub>IX</sub><sup>-</sup> thymocytes. (An average of 3% of total counts in the dialyzed lysate were precipitated by anti-NTD serum.) This shows that a quantitative or qualitative difference between 129 and 129/G<sub>IX</sub><sup>-</sup> is identified in this system, but does not indicate whether this relates specifically to the G<sub>IX</sub> component recognized in the cytotoxicity assay, because the 129 mouse may be expressing other components of MuLV which the anti-NTD serum can identify in NP-40 lysates.

Next these immunoprecipitates were analyzed by SDS-PAGE. As shown in Fig. 1, under reducing conditions, immunoprecipitates from 129 thymocytes gave major peaks of mol wt of 70,000 (fraction 22) and 45,000 (fraction 36), and sometimes material in the region of the bromphenol blue marker (fractions 77-83). The 45,000 component appeared also in the control precipitates (derived from 129 thymocyte lysates reacted with normal rat serum); we shall not discuss this nonspecific component further, except to suggest that it may represent a superfluous reaction of anti-Ig with mouse antigen, or nonspecific coprecipitation of a very abundant cell surface protein.

Fig. 2 shows that the 70,000 peak is absent from the control precipitates of

<sup>3</sup> Tung, J-S., and E. S. Vitetta. Unpublished observations.

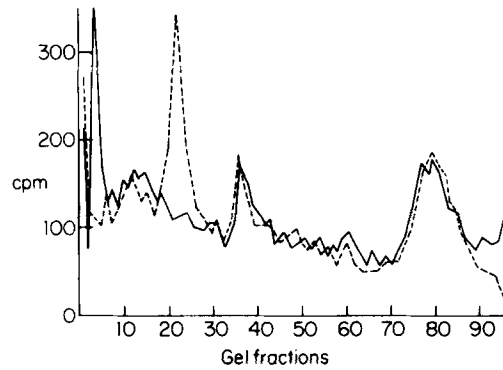


FIG. 1. PAGE in the presence of SDS of immunoprecipitates, from NP-40 lysates of surface-iodinated 129 thymocytes, formed by rat anti-NTD serum (----) and by normal rat serum (—). The  $^{125}\text{I}$ -labeled protein patterns are plotted together from parallel gels. The anode is on the right. Electrophoresis was carried out at 2.5 V/cm for 18 h at room temperature. The mol wt of species migrating at fractions 22 and 36 were determined to be 70,000 and 45,000 by comparison with mouse Ig subunits and MuLV virion proteins as markers for calibration.

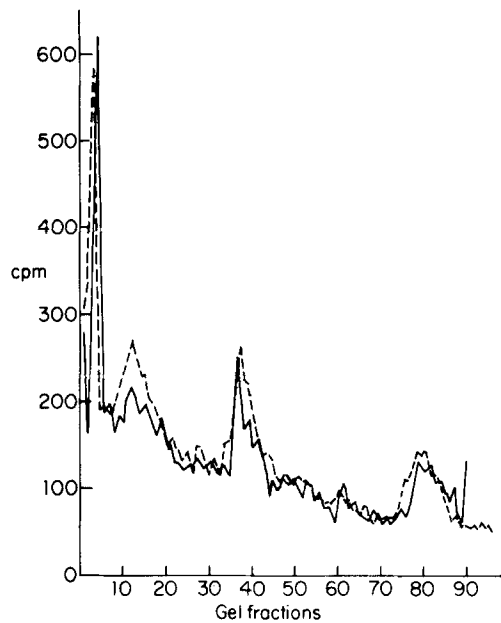


FIG. 2. SDS-PAGE patterns of iodinated cell surface proteins precipitated from NP-40 lysates of 129/ $G_{1X}^-$  thymocytes by rat anti-NTD serum (----) and by normal rat serum (—). The two patterns are plotted together from parallel gels. The electrophoresis conditions were the same as for Fig. 1.

129/ $G_{1X}^-$  thymocytes reacted with anti-NTD serum. Hence only one cell surface component consistently distinguishes 129 thymocytes from 129/ $G_{1X}^-$  thymocytes by this procedure, and that is the 70,000 component.

Fig. 3 shows that a 70,000 component is detected as well by SDS-PAGE of immunoprecipitates from [ $^3\text{H}$ ]glucosamine-labeled 129 (but not 129/ $G_{1X}^-$ )

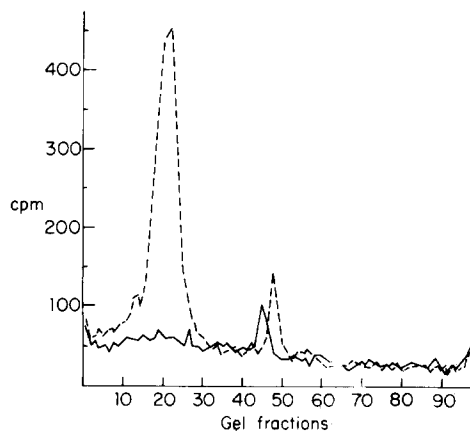


FIG. 3. SDS-PAGE patterns of immunoprecipitates, from NP-40 lysates of [<sup>3</sup>H]glucosamine-labeled 129 thymocytes (----) and 129/G<sub>IX</sub><sup>-</sup> thymocytes (—), formed by anti-NTD serum. The two patterns are plotted together from parallel gels. The electrophoresis conditions were the same as for Fig. 1.

thymocytes, and this component is thus presumably a glucosamine-containing glycoprotein. Thus, 129 thymocytes are specifically distinguishable from 129/G<sub>IX</sub><sup>-</sup> thymocytes not only (a) by susceptibility to lysis by anti-NTD serum in the cytotoxicity assay but also (b) by the possession of a glycoprotein of mol wt 70,000 revealed by SDS-PAGE analysis of immunoprecipitates with the same serum.

The gp69/71 envelope component of MuLV is also a glycoprotein of mol wt in the range of 70,000 and according to the serological data of Obata et al. (3) is the carrier of G<sub>IX</sub> antigen. Accordingly it seemed probable that the 70,000 iodinated protein peak detected by anti-NTD serum was in fact MuLV gp69/71 and would be detectable with anti-gp69/71 antiserum. The following experiments bear this out. Lysates of surface-iodinated 129 thymocytes were reacted with anti-gp69/71 antiserum (prepared by Strand and August [4, 5] against gp69/71 of Rauscher-MuLV) followed by pig antioat Ig. Fig. 4 shows that SDS-PAGE of the precipitate again demonstrated a 70,000 component, which is thereby identified as gp69/71. (In our SDS-PAGE system two glycoproteins of mol wt of 69,000 and 71,000 would not be resolved.) Next the supernate was further reacted with anti-NTD serum and rabbit antirat Ig. SDS-PAGE of this second precipitate demonstrated no additional 70,000 component, nor did it reveal any other 129-specific component that is absent from 129/G<sub>IX</sub><sup>-</sup> (data not shown).

Two explanations must be considered: (a) G<sub>IX</sub> antigen is a constituent of 129-gp69/71 and can therefore be precipitated by gp69/71 antiserum, or (b) 129-gp69/71 is exclusively precipitated by reaction with non-G<sub>IX</sub> antibody to gp69/71 which the anti-NTD serum most probably contains (see above), with G<sub>IX</sub> antigen being represented on some other protein species in the immunoprecipitate. The evidence thus far presented offers no support for the latter explanation—no other radioactive species specific to 129 thymocytes was detectable—and furthermore the serological data of Obata et al. (3) showing that G<sub>IX</sub> antigen

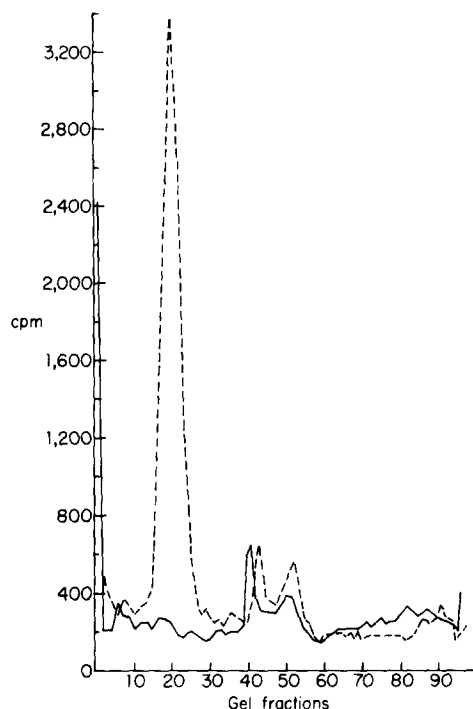


FIG. 4. SDS-PAGE patterns of immunoprecipitates, from NP-40 lysates of surface-iodinated 129 (----) and 129/G<sub>1X</sub><sup>-</sup> (—) thymocytes, formed by anti-gp69/71 antiserum. The two patterns are plotted together from parallel gels. The electrophoresis conditions were the same as for Fig. 1.

can be removed from solution by complexing with gp69/71 antiserum strongly imply that the former explanation is correct.

Next we compared gp69/71 isolated from Gross-MuLV with gp69/71 isolated from the thymocyte plasma membrane of the 129 mouse (129-gp69/71). Are they distinguishable? Fig. 5 shows in SDS-PAGE a small difference in mobility between Gross-MuLV-gp69/71 and 129-gp69/71 which has proved consistent in repeated tests. (A possible artifact produced in these tests by iodination of one but not the other is excluded by controls showing identical mobilities for [<sup>3</sup>H]gp69/71 of Gross-MuLV and [<sup>125</sup>I]gp69/71 of Gross-MuLV.) On the data given here we cannot rule out that the difference is due to the circumstances of isolation from thymocytes as opposed to virions; but as the procedures applied were identical, the most probable explanation is an intrinsic difference between the gp69/71 of an endogenous 129-MuLV and gp69/71 of Gross-MuLV. These points are being settled by current comparisons between gp69/71 components isolated from plasma membranes of different cells as well as from different MuLV virions and further discussion of it will therefore be deferred.

The fact that the 129-gp69/71 molecule occurs physiologically in both the membrane-bound state (thymocyte surface and virion envelope) and also free in the plasma (3) prompts consideration of which parts of the membrane-bound

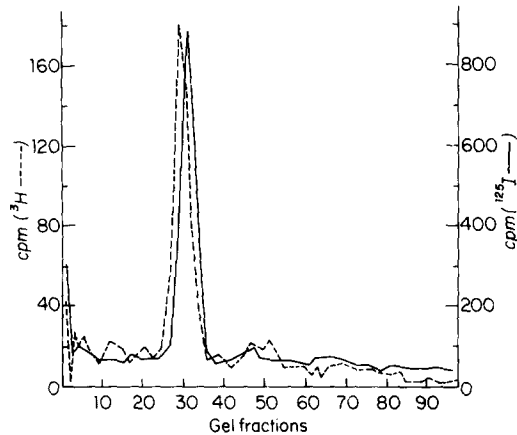


FIG. 5. Coelectrophoresis of [<sup>3</sup>H]gp69/71 of Gross-MuLV (----) and [<sup>125</sup>I]gp69/71 of 129 thymocytes (—), the latter precipitated from an NP-40 lysate by rat anti-NTD serum. The electrophoresis conditions were the same as for Fig. 1 except that the gel was electrophoresed for 24 h at room temperature.

molecule are accessible or inaccessible to antibody; i.e., which gp69/71 antigens of the intact thymocyte or virion are available for reaction with antibody and which are “buried?” G<sub>1X</sub> is the prime example of accessible antigen because it is demonstrable by the cytotoxicity assay and so is clearly an exposed segment of 129-gp69/71 in its membrane-bound state. But anti-NTD serum also evidently contains antibody to an inaccessible region of membrane-bound 129-gp69/71. This we infer from experiments (collaboration with Obata et al.) in which anti-G<sub>1X</sub> activity (in the cytotoxicity assay) could be removed from anti-NTD serum by absorption with intact 129 thymocytes without affecting its ability to precipitate 129-gp69/71 from NP-40 lysates of 129 thymocytes. Thus while the anti-G<sub>1X</sub> component of G<sub>1X</sub> antibody may be sufficient to precipitate 129-gp69/71 (of which there is no direct proof) it is not the only antibody in anti-NTD serum capable of doing so. This is not surprising, because anti-NTD serum is likely to contain antibody to more than one antigen of gp69/71 (cf. Geering et al. [6]) including the relatively conserved region conferring the group-specific antigenicity recognized by the goat anti-gp69/71 antiserum of Strand and August (4, 5). Thus, a useful hypothesis for future evaluation is that the group-specific region of membrane-bound 129-gp69/71 is relatively inaccessible, which as Obata et al. (3) point out would also account for the failure of group-specific antibody of goat anti-gp69/71 antiserum to give more than a weak reaction with intact 129 thymocytes in the cytotoxicity assay.

### Summary

It is known that the thymocyte surface antigen G<sub>1X</sub> is found in some strains of mice and not others, and that its expression in mice of strain 129, in which most extensive genetic studies have been made, is controlled by two unlinked cellular chromosomal loci. We have now isolated a protein with a mol wt of approximately

70,000 daltons from the surface of thymocytes from 129 mice, which have antigenic and biochemical properties characteristic of the gp69/71 envelope component of murine leukemia virus. Our evidence is compatible with the conclusion that it carries the G<sub>IX</sub> antigen.

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