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Characterization of Molecular Species Carrying Gross Cell Surface Antigen

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The Gross cell surface antigen (GCSA), associated with expression of endogenous Gross-type murine leukemia virus (G-MuLV) in tissues of mice, is defined by the cytotoxic reaction of a C57BL/6 antiserum, anti-AKR spontaneous leukemia K36, with cells of the Gross virus-induced C57BL/6 leukemia, E♂G2. Sequential lactoperoxidase-catalyzed radioiodination of E♂G2 cells, Nonidet P-40 lysis, precipitation with anti-K36 serum, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis identified molecules with properties of polyproteins encoded by the *gag* region of the viral genome. These cell surface species could also be labeled by in vitro culturing of E♂G2 with radioactive glucosamine. The viral specificity of these molecules and their participation in the GCSA typing system were established as follows. (i) Absorption of anti-K36 serum with GCSA$^+$, but not GCSA$^-$, leukemias led to a marked decrease in precipitation of these proteins. (ii) The same E♂G2 cell surface proteins were also precipitated by antisera against the MuLV virion proteins p30 and p15. (iii) Anti-K36 was shown to possess antibodies against Gross virus p30 and p15. (iv) "Clearing" the E♂G2 lysate of molecules reactive with anti-p30 or anti-p15 sera removed molecules reactive with anti-K36 serum. (v) Absorption of anti-K36 serum with disrupted G-MuLV virions or with Gross p30 or p15 removed GCSA cytotoxic antibodies; partial absorption was achieved with disrupted Rauscher-MuLV (R-MuLV) or with R-MuLV p30, and no absorption was found with R-MuLV p15. These data show that E♂G2 cells express, on their surfaces, MuLV core polyproteins that apparently can be glycosylated and on which the determinants of GCSA are located.

Since its discovery, the Gross cell surface antigen (GCSA, originally the G-antigen [44]) has played a pivotal role in investigations of the serology, physiology, and viral etiology of murine leukemias. This antigen system and its counterpart from Friend, Moloney, and Rauscher virus-induced leukemias (the FMR antigen) served initially to classify murine leukemias into two broad classes, the presence of GCSA signifying the expression of endogenous murine leukemia virus (MuLV). In subsequent work, the GCSA phenotype of mouse tissues and spontaneous leukemias has proved a valuable marker for genetic and immunological studies of leukemogenesis, most recently in defining stages in the development of disease in the classical AKR system (24, 25). To date, comparatively little information has been obtained concerning the molecular species bearing GCSA antigenic determinants. The purpose of this report is to identify the nature of the molecular structures carrying GCSA on mouse leukemia cells.

GCSA is defined by the cytotoxic activity of the antiserum C57BL/6 anti-AKR spontaneous leukemia K36 for cells of the syngeneic C57BL/6 leukemia E♂G2 (originally induced by Gross virus) (44). Other tissues can be typed for presence (GCSA$^+$) or absence (GCSA$^-$) of the antigen by assaying their capacity to absorb cytotoxic activity in this test system. By this method, GCSA was found on cells of all leukemias induced by Gross virus, on cells of spontaneous leukemias arising in high-leukemia-incidence strains of mice (e.g., AKR and C58), on normal lymphoid tissue of these high-incidence strains, and on cells of some leukemias induced in low-incidence strains (e.g., BALB/c and A) whose normal lymphoid tissues are negative for expression of this antigen (44). Recent work of O'Donnell and Stockert (40) has shown that expression of GCSA in cultured fibroblasts occurs upon infection by particular ecotropic and xenotropic MuLV isolates.

GCSA was demonstrable on the surface of E♂G2 cells by immune electron microscopy; however, neither budding virions nor free virions released from these cells were labeled (2).

This result suggested that the MuLV genome might specify nonvirion cell surface elements and that GCSA might represent such an element. A new facet of this problem was uncovered by Tung et al. (55), who showed that determinants of viral core proteins can be expressed on the exterior surfaces of lymphoid cells as parts of a glycosylated polyprotein molecule. The presence of this novel molecular species on the cell surface provided an explanation for data from a number of laboratories indicating that antigenic determinants of MuLV proteins that are not parts of the virion envelope, such as p30, p15, and p10, can be expressed on the surfaces of mouse tumor cells (7, 9, 12, 16–18, 22, 27, 31, 33, 37, 47, 58).

In the experiments that we present here, GCSA was characterized by enzymatic radioiodination of viable E♂G2 cells, precipitation of detergent-solubilized antigen with the anti-GCSA typing serum or anti-MuLV protein sera, and analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Since the protein species which were observed were precipitated by anti-GCSA serum and by antisera against MuLV p30 or p15, the possibility was pursued that GCSA is situated on MuLV polyprotein constituents similar to those found on the exterior of AKR lymphoid cells (30, 55). Data supporting this hypothesis have been obtained by absorption of the GCSA typing serum with GCSA-positive leukemias and with purified MuLV protein components.

MATERIALS AND METHODS

Leukemias. The E♂G2 leukemia was induced in C57BL/6 (B6) by passage A Gross-MuLV (G-MuLV) (44) and has been maintained by serial transplantation of leukemic spleen cells in the strain of origin. The origins of the leukemias of ERLD (B6), EL4 (B6), RADA1 (A strain), and RL♂1 (BALB/c) are recorded elsewhere (15, 42–44). The spontaneous AKR leukemia (AKR-SL) came from our own colony.

Viruses. Purified preparations of AKR-L1 (lot no. 408-52-6) and Rauscher MuLV (R-MuLV) (lot no. 442-3-4) were purchased from Electro-Nucleonics Laboratories, Inc., and were used for absorption of antiserum. R-MuLV (lot no. 237-49-6), from Litton Bionetics, and G-MuLV (lot no. 325-38-11), obtained from the Virus Cancer Program of the National Cancer Institute through the courtesy of Jack Gruber, were used for the preparation of individual viral proteins by gel filtration in 6 M guanidine-hydrochloride (10). The quality of virion and virion protein preparations was monitored by PAGE in slab gels and staining with Coomassie brilliant blue.

Preparation of radiolabeled virus. Radiolabeled G-MuLV and AKR-MuLV were prepared by incubating infected SC-1 mouse embryo cells for 18 h in the presence of medium containing radioactive amino acid precursors. The quantities of radioactive precursors used and the method of purifying the virus are described elsewhere (49).

Antisera. The GCSA typing serum B6 anti-AKR spontaneous leukemia K36 (abbreviated anti-K36) was prepared by immunization of B6 female mice with cells from a spontaneous leukemia of AKR (K36) according to the schedule previously described (44). As a rule, mice were bled between 5 and 9 months of age, and 40 to 50 sera with high anti-GCSA activity were pooled. Preparation of rabbit antiserum directed against R-MuLV p30 has been detailed elsewhere (21). A goat antiserum directed against AKR-MuLV p15 was similarly prepared. Antisera to viral structural proteins were monospecific, as shown by their reactivities with Nonidet P-40 (NP-40)-solubilized proteins of ^{3}H-amino acid-labeled G-MuLV (21). Other sera used were: rabbit anti-mouse immunoglobulin, provided by U. Hammerling, Memorial Sloan-Kettering Cancer Center, New York, N.Y.; goat anti-mouse immunoglobulin, purchased from Pocono Rabbit Farm and Laboratory, Canadensia, Pa.; goat anti-rabbit 7S globulins, purchased from Hyland Laboratories, Costa Mesa, Calif.; and bovine anti-goat immunoglobulin, provided by R. Wilsnack, Huntington Research Center, Brooklandville, Md.

Cytotoxic tests. The GCSA typing system is based on the standard complement-dependent cytotoxic test (44): equal volumes (50 μl) of serially diluted anti-K36 serum were mixed with E♂G2 cells (5×10^{6}/ml) and complement (pooled guinea pig serum diluted 1:2) and incubated at 37°C for 45 min. Viability counts were made after addition of trypan blue. The diluent in all cytotoxic tests was medium 199.

Absorption tests. (i) With leukemia cells. Anti-K36 serum (see Table 1) was diluted 1:2 (approximately five twofold dilutions below the 50% end point of the serum with E♂G2 cells) and mixed with an equal volume of washed, packed cells. After incubation at 4°C for 30 min, the serum was recovered by centrifugation at $900 \times g$ and tested for residual activity in the standard cytotoxic test (44).

(ii) With MuLV. Purified virus preparations (see Fig. 6) were pelleted by centrifugation for 60 min at $110{,}000 \times g$ and suspended in phosphate-buffered saline (PBS) to a final concentration of 800 μg of protein per ml, as determined by the method of Lowry et al. (32). Equal volumes (0.25 ml) of the virus suspensions (serially diluted in PBS) and anti-K36 serum (diluted 1:8) were mixed and incubated at 37°C for 15 min, followed by 30 min on ice. The virus was removed by centrifugation at $110{,}000 \times g$ for 30 min in cut off SW50 tubes, and 50 μl of the supernatant (i.e. absorbed antiserum) was used in the standard cytotoxic test (39). Controls were included in all experiments and consisted of the antiserum mixed with PBS alone. Control experiments were performed (data not shown) to ascertain that the virus preparations were not detectably contaminated with plasma membranes of the cells that produced the virus (absorption tests for H-2) and that

TABLE 1. *Activity of B6 anti-K36 serum after absorption with murine leukemia cells*

Serum	Leukemia used for absorption	No. of absorptions	Residual cytotoxicity		Residual precipitating activity with[b]:				
					E♂G2 cell surface proteins			G-MuLV proteins	
			% E♂G2 cells lysed at 1:2	Titer[a]	p(150)	p(85)	p(45)	p30	p15
B6 anti-K36	None	0	95	32	100	100	100	100	100
B6 anti-K36	RADA1	2	95	32	84	75	79	92	87
B6 anti-K36	ERLD	1	95	32	81	63	65	73	68
B6 anti-K36	EL4	1	95	32	81	75	72	75	71
B6 anti-K36	RL1	2	47	<2	74	44	74	48	53
B6 anti-K36	AKR-SL	1	<5	<2	49	38	57	21	46
B6 anti-K36	E♂G2	2	<5	<2	43	28	49	27	46
B6 normal mouse serum	None	0	ND[c]	ND	20	32	40	7	20
Normal goat serum	None	0	ND	ND	13	26	18	ND	ND

[a] Dilution of unabsorbed or absorbed B6 anti-K36 that lysed 50% of E♂G2 cells.

[b] Immunoprecipitates formed between absorbed and unabsorbed B6 anti-K36 or control sera and either E♂G2 cell surface proteins or G-MuLV proteins were analyzed by SDS-PAGE as detailed in Materials and Methods and the legends to Fig. 2 and 5. After determination of radioactivity corresponding to each of the peaks in the electropherograms, the recovery of each protein was expressed as a percentage of that recovered by unabsorbed B6 anti-K36 serum.

[c] ND, Not done.

the observed absorption capacity of some virus preparations was not due to anti-complementary effects (absorption tests for Ly2.2 and see reference 39).

(iii) With viral proteins. Individual viral proteins (see Fig. 7) obtained by agarose gel filtration in 6 M guanidine-hydrochloride were dialyzed free of the denaturant and lyophilized. Before use in absorption tests, the individual proteins were resuspended in 0.02 M Tris-hydrochloride, pH 7.6, to a final concentration of 200 μg of protein per ml. The viral proteins were then serially diluted in medium 199. Twenty-five microliters was mixed with 25 μl of anti-K36, diluted 1:10, and incubated on ice for 30 min. Fifty microliters of E♂G2 cells (5×10^6/ml) was added, and the mixture was incubated once again on ice for 30 min. After the cells were washed once in 1.5 ml of medium 199, they were resuspended in 100 μl of complement (guinea pig serum, diluted 1:2) and incubated at 37°C for 30 min. Viability counts were made as usual. This "two-step" cytotoxic test (39) was used for these experiments to avoid any anti-complementary effect from the high protein concentrations of the viral fractions.

Preparation of radioiodinated E♂G2 cells. Suspensions of E♂G2 cells were prepared from leukemic spleens by mincing in PBS; large fragments were removed by passing through a wire-mesh screen. The cells were washed three times by centrifugation in a refrigerated PRJ centrifuge at 800 rpm for 8 min and at 1,200 rpm for 2 min. This technique yielded preparations containing >90% leukemia cells. Suspensions of 10^8 washed E♂G2 cells in 1 ml of PBS were radioiodinated with carrier-free ^{125}I (Amersham/Searle Corp.) by the lactoperoxidase method (58). The incorporation of ^{125}I was 23×10^6 trichloroacetic acid-precipitable cpm per 10^8 cells. Cell viability was shown to be 95% before and after radioiodination by trypan blue exclusion. The iodination procedure has been shown to be specific for labeling proteins on the exterior surface of the plasma membrane; details of experiments leading to this interpretation have been published elsewhere (55, 58).

Incubation of E♂G2 cells with [^{3}H]glucosamine. This procedure was performed under conditions described by Tung et al. (55).

Immunoprecipitation of labeled proteins from cell lysates and lysed virions. Surface proteins of leukemia cells were radiolabeled with ^{125}I as described above. The cells were then lysed in 0.5% NP-40, and the nuclei and cell debris were removed (54). Immunoprecipitation of labeled antigens in lysates was performed after NP-40 lysis as described previously (46, 54, 57), except that overnight dialysis of lysates was not carried out. The latter step was omitted because labeled molecular species on E♂G2 cells were found in preliminary experiments to be sensitive to proteolytic degradation in NP-40 lysates. The amounts of sera sufficient for maximum precipitation of available antigen from cell lysates were determined by means of titration experiments.

Mouse splenic lymphocytes have immunoglobulin on the cell surface (58). Thus, care had to be taken that MuLV-specific cell surface antigens were not confused with labeled surface immunoglobulin, which would be precipitated by the anti-mouse immunoglobulin used as the second antibody for mouse antiserum (J.-S. Tung, personal communication). The amount of rabbit anti-mouse immunoglobulin needed to clear cell lysates of surface immunoglobulin was determined in titration experiments. A maximum of 2% of the available acid-precipitable radioactivity from a lysate of 10^8 cells could be precipitated with anti-mouse immunoglobulin. This recovery, as well as the SDS-PAGE pattern of the labeled

immunoglobulin components in the precipitate, was consistent with previous results detailed by Vitetta et al. (58).

The efficiency of the "immunoglobulin clearing" procedure was tested by titrating the supernatant from the first precipitation for reactivity with additional rabbit anti-mouse immunoglobulin serum as well as goat anti-mouse immunoglobulin. A maximum of only 0.3% residual radioactivity was precipitated from the supernatant with these sera. Normal rabbit serum and normal goat serum precipitated similar amounts of radioactivity. Immunoglobulin-cleared cell lysates were used in all experiments with E♂G2 lysates.

PAGE. Labeled polypeptides in immunoprecipitates were analyzed by PAGE in the presence of SDS and 2-mercaptoethanol for 3 h at 3 mA/gel in cylindrical gels (10-cm length, 7.5% acrylamide) or for 6 h at 4 mA/gel in 15-cm cylindrical gels containing 15% acrylamide according to the method of Laemmli (28).

RESULTS

PAGE of E♂G2 cell surface proteins precipitated with GCSA typing serum. An analysis by SDS-PAGE of proteins precipitated by anti-K36 from a lysate of surface iodinated E♂G2 cells is shown in Fig. 1. Three major protein peaks were observed; their molecular weights (in order of increasing electrophoretic mobility) were estimated to be 150,000, 85,000, and 45,000. These polypeptides will be provisionally referred to as p(150), p(85), and p(45). In some experiments [^{3}H]glucosamine-labeled AKR virus was subjected to coelectrophoresis to distinguish p(85) by its mobility from the glycoprotein gp70 (Fig. 2). As can be seen in Fig. 1, only small amounts of p(150) and p(85) were precipitated by normal B6 serum; these molecules were not precipitated by anti-K36 serum from ^{125}I-labeled B6 normal spleen cells or from cells of the (GCSA$^-$) B6 leukemia ERLD (not shown). A larger fraction of available counts per minute in the p(45) region from E♂G2 cell lysates was precipitated by anti-K36 serum than by normal mouse serum (or by normal goat and rabbit sera); this verified the presence of a polypeptide recognized by anti-K36 in this size range (see Discussion).

E♂G2 surface proteins precipitated with GCSA typing serum absorbed with murine leukemia cells. We sought next to determine whether antigens represented on p(150), p(85), p(45) have the same distribution as GCSA among various leukemias. Absorption of the E♂G2-directed cytotoxic activity from anti-K36 serum has been the means for determining the distribution of GCSA among leukemias and normal tissues of many mouse strains (13, 44). Leukemia cells known to be GCSA$^+$ or GCSA$^-$ were tested for their capacity to absorb precipitating activity in anti-K36 for E♂G2 surface proteins. Two absorptions of anti-K36 with RADA1 cells (GCSA$^-$) had a negligible effect on precipitation of p(150), p(85), and p(45) (Fig. 2B) as compared with control (unabsorbed) serum (Fig. 2A). On the other hand, one absorption of anti-K36 with AKR spontaneous leukemia cells (GCSA$^+$) led to a marked decrease in precipitation of all three proteins (Fig. 2C), such that the electropherogram resembled that obtained with normal B6 serum (Fig. 2D). Results obtained using serum absorbed with other GCSA$^+$ and GCSA$^-$ leukemias are summarized in Table 1 and discussed below.

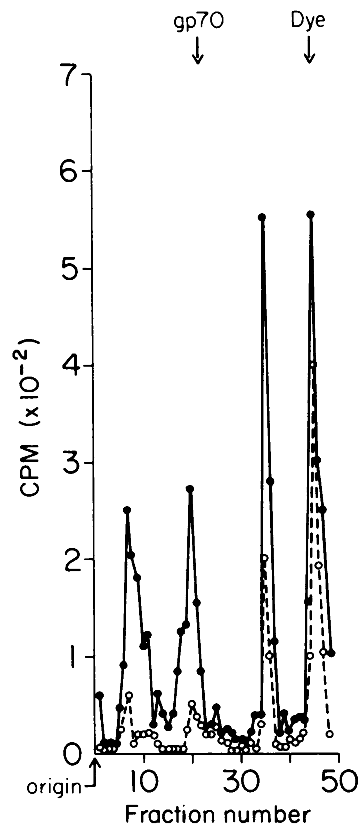

FIG. 1. *SDS-PAGE of ^{125}I-labeled surface proteins of E♂G2 precipitated from a cell lysate by B6 anti-K36 serum (●) and B6 normal mouse serum (○). Portions of immunoglobulin-cleared cell lysate from 10^7 cells (400-μl volume, 4.2×10^5 cpm of ^{125}I) were incubated with 20 μl of the indicated serum, and immune complexes were precipitated by subsequent addition of 200 μl of the appropriate antiglobulin. ^{125}I-labeled proteins in washed precipitates were analyzed in SDS-PAGE containing 7.5% acrylamide. [^{3}H]glucosamine-labeled AKR virus was subjected to coelectrophoresis with immunoprecipitates to serve as a marker for viral gp70 (arrow).*

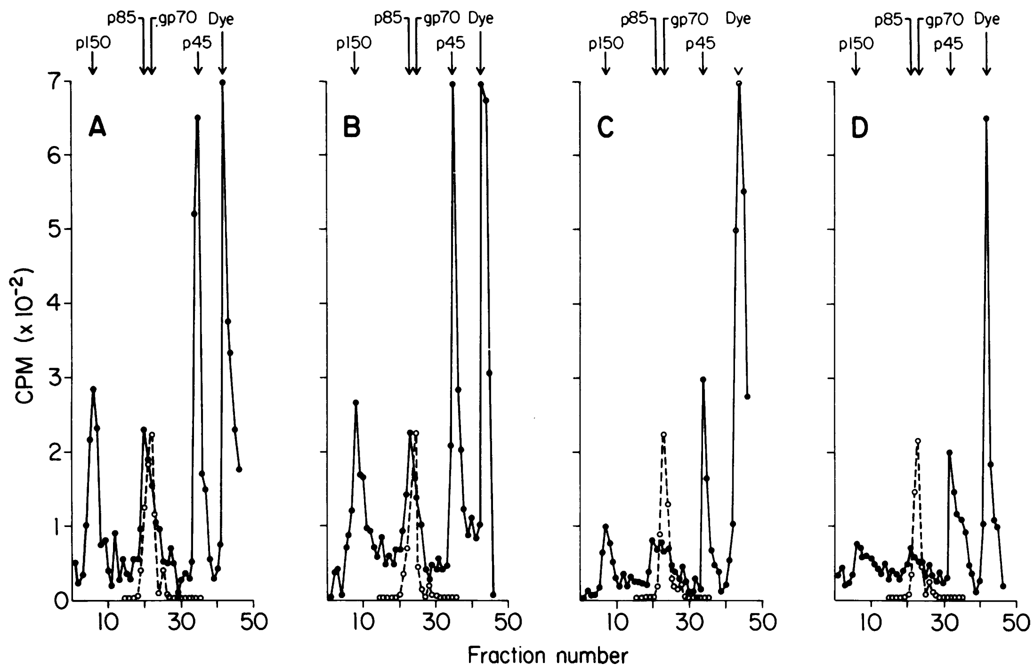

FIG. 2. *Coelectrophoresis of [³H]glucosamine-labeled AKR virus proteins* (○) *with ¹²⁵I-labeled surface proteins of E♂G2* (●) *precipitated from a cell lysate by unabsorbed B6 anti-K36 serum (A), B6 anti-K36 serum absorbed with RADA1 cells (B), B6 anti-K36 absorbed with AKR-SL cells (C), and B6 normal mouse serum (D). Portions of immunoglobulin-cleared lysate from 10^7 cells (400-μl volume, 4.9 × 10^5 cpm of ¹²⁵I), 20 μl of B6 anti-K36, and 200 μl of goat anti-mouse immunoglobulin serum were used. SDS-PAGE was performed in gels containing 7.5% acrylamide.*

Identification of MuLV-specific proteins on the surface of E♂G2 cells. Recent experiments by Tung et al. have identified molecules resembling p(85) and p(45), precipitable by antisera against MuLV p30, p15, or p10, on the surface of AKR spontaneous leukemia cells (55, 56). [The molecule provisionally referred to as p(75) in reference 55 and corresponding to p(85) in this study has been shown by more accurate criteria to consist of two components with molecular weights of 85,000 and 95,000 (50). Recent evidence indicates that both on AKR mouse leukemia cells (56) and on E♂G2 cells (29) the cell surface MuLV core polyprotein species in the 85,000-molecular-weight range actually consist of two components, one of 95,000 and the other of 85,000 molecular weight. These are designated $gP95^{gag}$ and $gP85^{gag}$, respectively (56).] Analysis by SDS-PAGE of labeled proteins precipitated from an immunoglobulin-cleared lysate of E♂G2 cells by rabbit anti-R-MuLV p30 serum and goat anti-AKR MuLV p15 serum (Fig. 3A) showed that p(150), p(85), and p(45) of these cells are also precipitated by these antisera.

It is noteworthy that neither MuLV p30 nor p15 was present on the cell surface in its monomeric form; no labeled proteins smaller than p(45) have been detected in immunoprecipitates with anti-K36, anti-MuLV p30, or anti-p15 serum. ³H-labeled MuLV p30 in AKR virus subjected to coelectrophoresis migrated as a sharp peak at fractions 40 to 42, where no ¹²⁵I-labeled material was evident. Electropherograms of immunoprecipitates from E♂G2 in a gel system affording better resolution of lower-molecular-weight polypeptides (19) also exhibited no ¹²⁵I-labeled proteins between p(45) and the dye front.

Preliminary experiments were performed to determine whether the cell surface species detected were glycosylated. NP-40 lysates were made from E♂G2 cells labeled with [³H]-glucosamine in culture, and proteins reactive with anti-MuLV p30 and anti-K36 were analyzed by the same procedures used for ¹²⁵I-labeled proteins. The results indicated a major peak of radioactivity migrating in SDS gels in the region of p(85) and a smaller amount of radioactivity migrating in the region of p(45) (Fig. 3B). These results are comparable to those obtained by Tung et al. (55, 56) for the cell surface polyproteins of AKR spontaneous leukemia cells. The p(150) molecule did not appear

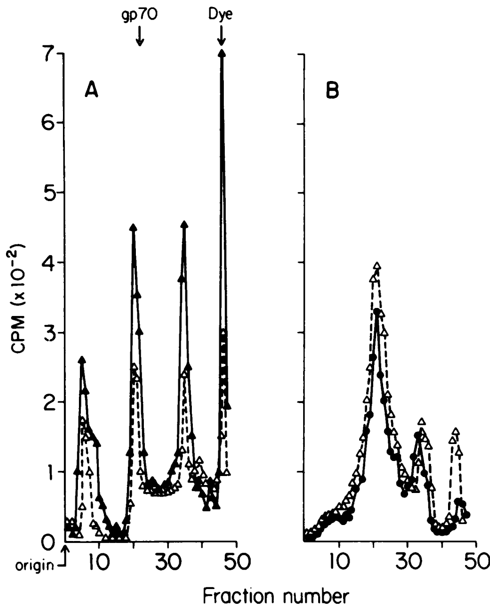

FIG. 3. *SDS-PAGE of (A) ^{125}I-labeled surface proteins of E♂G2 precipitated from a cell lysate by rabbit anti-R-MuLV p30 serum (▲) and goat anti-AKR MuLV p15 serum (△). (B) [^{3}H]glucosamine-labeled proteins of E♂G2 precipitated from a cell lysate by B6 anti-K36 serum (●) and goat anti-AKR p15 serum (△). Precipitations were performed with portions of immunoglobulin-cleared lysates from 10^7 cells as described in the legend to Fig. 1.*

to contain glucosamine. In view of this property and the physical characteristics (size) of p(150), further data will be needed to establish that this protein is MuLV coded and not co-precipitated with p(85) and p(45).

Identity of E♂G2 cell surface proteins precipitated by anti-K36 and by anti-MuLV p30 and anti-MuLV p15 sera. The experimental results shown in Fig. 1 and 3 suggested that anti-K36 and MuLV p30 and MuLV p15 antisera were recognizing identical molecular species on E♂G2 cells. To test this hypothesis, the following approach was used, modeled on a procedure for sequential precipitation of viral proteins in cell lysates developed by Tung et al. (53, 54). Lysates of radioiodinated E♂G2 cells, cleared of surface immunoglobulin, were reacted with either anti-MuLV p30 or anti-K36 sera (Fig. 4A and D). Supernatants from these precipitations were again reacted with both antisera. SDS-PAGE analyses of labeled proteins in these second precipitates are shown in Figure 4B, C, E, and F. The results show that when molecules in a cell lysate reactive with anti-MuLV p30 were removed, little remained that was reactive with anti-K36 serum (or with additional anti-p30 serum). However, antigens reactive with anti-MuLV gp70 were not removed (not shown). Removal of molecules precipitable by anti-K36 serum also sharply reduced the recovery of labeled cell surface polypeptides by subsequent reaction with anti-MuLV p30 (or more anti-K36) serum. These results establish that the determinants on E♂G2 recognized by anti-MuLV p30 and anti-K36 are on the same molecular species. In related experiments, lysates cleared of cell surface molecules reactive with either of the above antisera no longer possessed iodinated molecules reactive with anti-MuLV p15 serum, and vice versa.

Specificity of B6 anti-K36 serum for G-MuLV polypeptides. The above could have been obtained if anti-K36 possessed antibodies to any of the core proteins of Gross virus. To identify the particular antibody specificities present in anti-K36, ^{3}H-labeled proteins from NP-40-disrupted G-MuLV were precipitated with anti-K36 serum and goat anti-mouse immunoglobulin serum. Virion proteins in immunoprecipitates were analyzed by SDS-PAGE and identified by means of ^{14}C-amino acid-labeled G-MuLV proteins subjected to coelectrophoresis (Fig. 5). Anti-K36 displayed strong reactivity with viral p30 (26% recovery under these conditions) and p15 (35% recovery) (Fig. 5B) compared with normal B6 serum (1.8% of p30 and 7% of p15 [Fig. 5E]). The recovery of p15 with this normal B6 serum may reflect autogenous immunity to endogenous MuLV (19, 20, 36, 38) or an artifact due to the known aggregating properties of this molecule (46). Significantly, absorption of anti-K36 serum with the GCSA$^-$ leukemia RADA1 had little effect on reactivity with G-MuLV p30 and p15 (Fig. 5C), whereas absorption with AKR-SL (GCSA$^+$) cells had a marked effect, particularly on reactivity with p30 (Fig. 5D). Similar results were also obtained with serum absorbed with other GCSA$^+$ and GCSA$^-$ leukemias (Table 1). Negligible p12 and p10 were precipitated by anti-K36 serum. Some reactivity to p15(E) was detected in this serum (19, 29, 46), but it could not be GCSA related since it was not absorbed by AKR spontaneous leukemia cells (Fig. 5D).

Summary of results obtained using anti-K36 serum absorbed with leukemia cells. Three parameters were assayed in absorption tests with anti-K36 (Table 1; Fig. 2 and 5): (i) cytotoxicity for E♂G2 cells, (ii) precipitation of E♂G2 cell surface proteins, and (iii) precipitation of p30 and p15 from lysed G-MuLV virions by B6 anti-K36 serum. Absorption of the serum with RADA1, EL4, or ERLD cells, all GCSA$^-$, had little effect on the ability of the serum to precipitate either cell surface proteins or G-

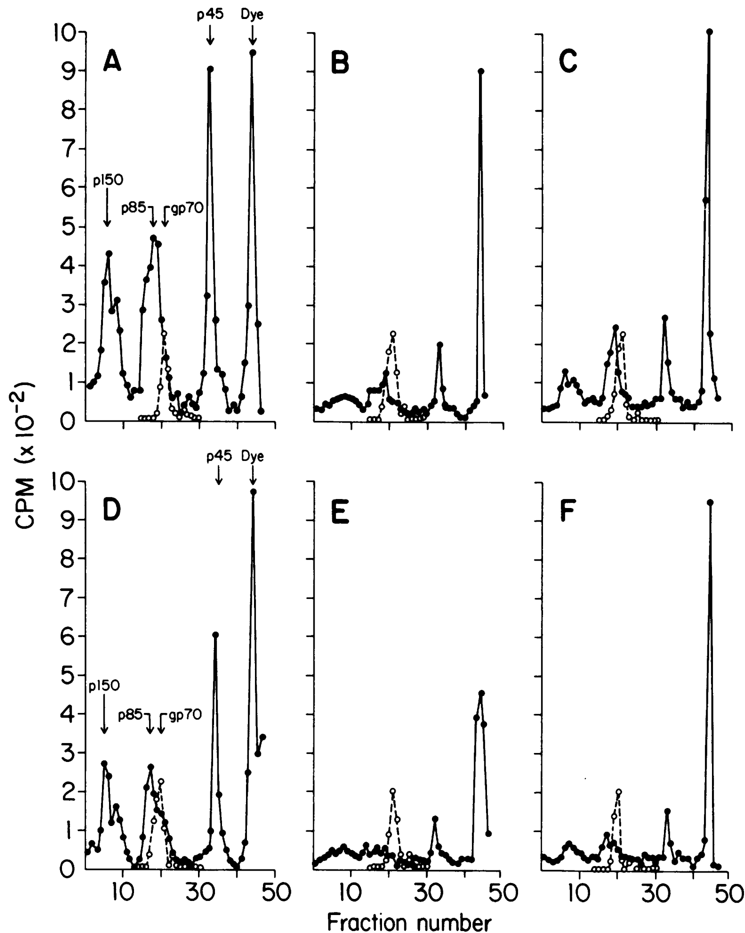

FIG. 4. *SDS-PAGE of (A) 125I-labeled surface proteins of E♂G2 precipitated from a cell lysate by rabbit anti-R-MuLV p30 serum, (B) residual labeled E♂G2 surface proteins in anti-p30-cleared lysate, precipitated with additional anti-MuLV p30 serum, (C) residual labeled E♂G2 surface proteins in a B6 anti-K36-cleared lysate, precipitated with additional anti-p30 serum, (D) 125I-labeled surface proteins of E♂G2, precipitated from a cell lysate with B6 anti-K36 serum, (E) residual labeled surface proteins in an anti-p30 cleared lysate, precipitated with B6 anti-K36 serum, and (F) residual labeled surface proteins in an anti-K36 serum-cleared lysate, precipitated with additional B6 anti-K36 serum. Initial precipitations were performed with immunoglobulin-cleared lysates from 10^7 cells (400-μl volume, 4.0 × 10^5 cpm of 125I) and 20 μl of antiserum. Symbols: (●) 125I-labeled protein; (○) [3H]glucosamine-labeled AKR virus subjected to coelectrophoresis as a marker for viral gp70. SDS-PAGE was performed in gels containing 7.5% acrylamide.*

MuLV-derived proteins. On the other hand, absorption of the serum with E♂G2 or AKR-SL cells, both GCSA+, lowered the same precipitation activities significantly.

Interestingly, two absorptions of anti-K36 serum with RL1 cells (GCSA+) did not remove all the cytotoxic activity. To obtain sufficient serum for immunoprecipitation experiments, these absorptions were done at a 1:2 serum dilution, instead of 1:8 or 1:16 as in the case of the standard GCSA typing test (40). Thus, the residual cytotoxicity in this serum is probably due to a low amount of antigen on RL1 cells and not to expression of a partially cross-reacting antigen on these cells. It is consistent with our other data that the incomplete absorption of cytotoxicity is directly correlated with intermediate precipitation of both cell surface and virion proteins compared with the other absorbed sera.

Even those sera completely devoid of cytotoxic antibodies apparently have small amounts of precipitating antibodies. This might be explained by the presence of large amounts of soluble GCSA in the plasma of mice bearing G+ leukemias (1). Thus, the mouse is exposed to determinants that may otherwise be buried in the tumor cell membrane and not

available for participation in the cytotoxic reaction. Antibodies to such determinants, which may be exemplified by the natural antibodies in many older B6 mice to MuLV p30 (50), would not be absorbed by intact leukemia cells and would therefore be available to bind to some NP-40-released virus-specific proteins from cells. An alternative explanation might be that absorption of cytotoxic activity may be achieved without complete removal of specific antibody (see Discussion).

Absorption of GCSA cytotoxic antibody by purified virus preparations and individual viral proteins. Experiments were performed to determine whether intact or disrupted virions could absorb GCSA specificity. Figure 6 shows quantitative absorption tests with purified preparations of AKR and Rauscher viruses frozen once for storage (intact) or subjected to 10 cycles of freezing and thawing (disrupted). Preparations of intact AKR virus partially absorbed GCSA activity but only under conditions in which identical amounts of disrupted virus showed a much greater absorption capacity (10-fold greater at 50% residual cytotoxicity). This marked increase in availability of GCSA-related antigen due to disruption of virions indicates that the determinants shared with GCSA are represented on internal virion components; the observed absorption with once-frozen virus may reflect the presence of a small amount of disrupted virus in the preparation.

Intact Rauscher viruses failed to absorb any cytotoxic activity of anti-K36 serum, although the activity of the antiserum was slightly reduced by absorption with high concentrations of disrupted virus. In a parallel study, intact and disrupted Moloney virus gave identical results. The reduced activity of the GCSA antiserum absorbed with these disrupted viruses may denote partial cross-reactivity between internal components of Rauscher and Moloney viruses and the GCSA-related internal components of AKR virus. Such a result would be consistent with the previously reported partial absorption of GCSA antiserum by FMR leukemia cells (44)

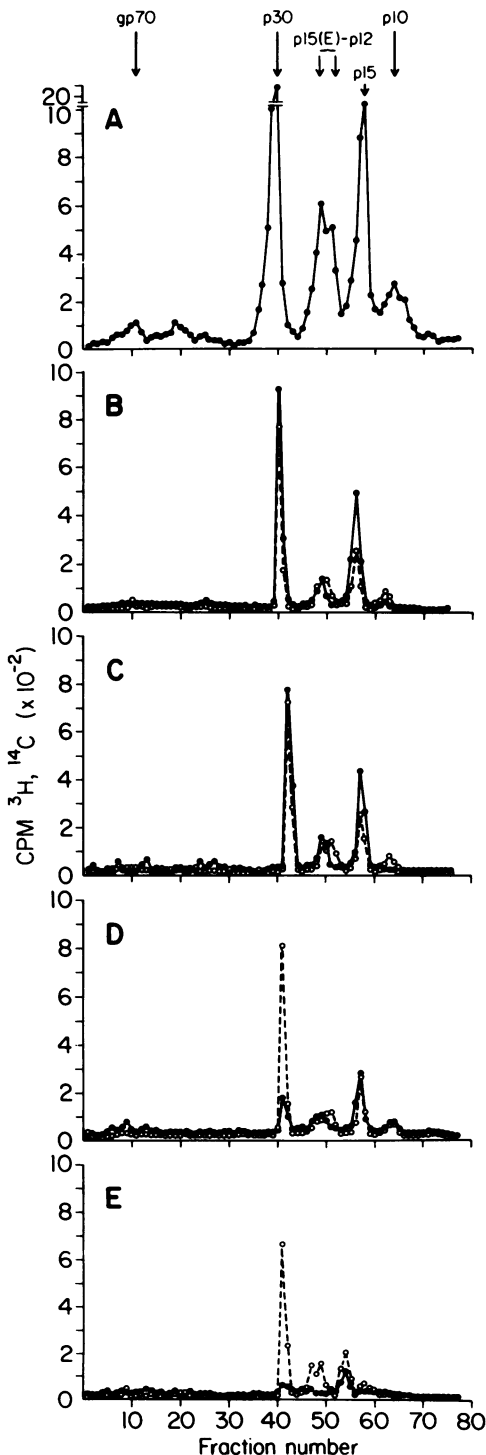

FIG. 5. *(A) SDS-PAGE of ^{3}H-amino acid-labeled G-MuLV proteins (6 μg of protein, 1.5 × 10^4 cpm). (B-D) Coelectrophoresis of ^{14}C-amino acid-labeled G-MuLV proteins with ^{3}H-amino acid-labeled G-MuLV proteins precipitated with B6 anti-K36 serum (B), B6 anti-K36 serum absorbed with RADA1 leukemia cells (C), B6 anti-K36 serum absorbed with AKR spontaneous leukemia cells (D), and normal B6 serum (E). Precipitations were performed as detailed previously (19) in 500 μl of NP-40 containing lysis buffer (6 μg of protein, 1.5 × 10^4 cpm) with 25 μl of the indicated antiserum and 250 μl of goat anti-mouse immunoglobulin. Labeled proteins in marker virus (○) and immunoprecipitates (●) were analyzed in SDS-PAGE containing 15% acrylamide (8). Viral proteins were identified and labeled according to criteria established in reference 4.*

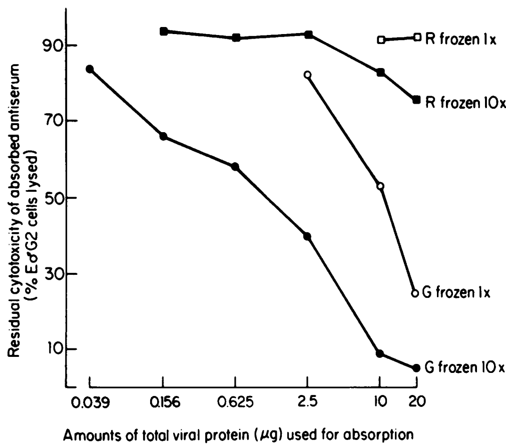

FIG. 6. *Absorption of GCSA cytotoxic antibody by purified virus preparations. Equal volumes of virus suspension (frozen once for storage or subjected to 10 cycles of freezing and thawing) and B6 anti-K36 serum diluted 1:8 were mixed and incubated. Virus was removed by centrifugation, and the absorbed antiserum (supernatant) was used in the standard cytotoxic test for residual antibody activity on E♂G2 cells (see Material and Methods). Unabsorbed anti-K36 serum (diluted 1:8 with PBS) lysed 85% of E♂G2 cells in the AKR-MuLV experiment and 92% of E♂G2 cells in the R-MuLV experiment. G, N-tropic virus from AKR; R, R-MuLV.*

as well as by FMR-infected tissue culture cells (40).

Control experiments were also performed to detect the possible presence of H-2 antigen in these virus preparations. Although the results were completely negative, the possibility could not be rigorously excluded that contamination of the AKR virus preparation with plasma membranes of the cells in which the virus was grown was in part responsible for the absorption of anti-GCSA activity. To exclude this possibility and to gain more insight into the nature of the GCSA cross-reactive internal component(s) of Rauscher virus, absorption of GCSA cytotoxic antibody by individual viral proteins was performed.

Figure 7 shows results of absorption experiments with purified viral proteins of G-MuLV and R-MuLV. As expected, only the internal virion components p15 and p30 of Gross virus were able to absorb the activity of anti-K36. A mixture of p15 and p30 was more effective in absorption than either protein alone (data not shown). (Some absorption was also detected with Gross p12; this latter effect requires further evaluation to exclude a minor component of p15 in the antigen preparation.) Rauscher p30 absorbed partially to a plateau level; Rauscher p15 was totally negative. The slight reduction in cytotoxicity by absorption with Gross gp70 was ascribable to the presence in this virion fraction of p65 core polyprotein (20, 51), which we have detected in stained protein gels of MuLV preparations used for these experiments. The results, thus, confirmed that the positive absorption of AKR virus shown in Fig. 6 is due to internal viral proteins and not due to contaminating plasma membrane.

DISCUSSION

The identity of the molecular species bearing GCSA has been an intriguing puzzle for tumor immunologists since the definition of this antigen system more than a decade ago (41, 44). The frequent association of GCSA with MuLV-positive leukemias, with lymphoid tissues of high-leukemic mouse strains, and, as recently demonstrated, with MuLV-producing cells in culture (36), illustrates the importance of this antigen system in typing for expression of endogenous MuLV genes. Earlier studies failed to resolve whether GCSA represented a virion structural component or a virus-coded, nonvirion protein, although the latter interpretation received some support from the failure of GCSA typing serum to label budding virions by immuno-electron microscopy (2). The evidence that we have presented here establishes that

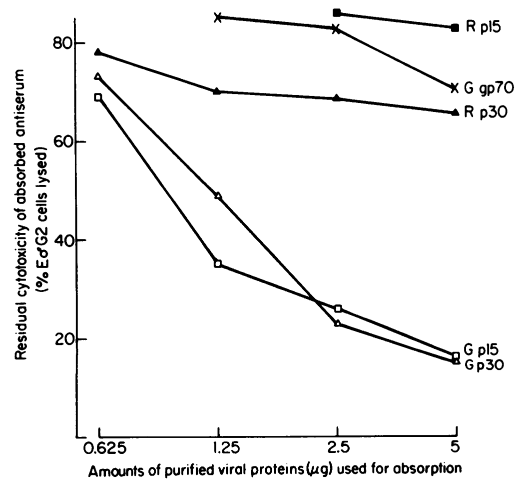

FIG. 7. *Absorption of GCSA cytotoxic antibody by purified viral protein preparations. Equal volumes of individual protein suspensions and B6 anti-K36 serum diluted 1:10 were mixed and incubated. The residual antibody activity on E♂G2 cells was tested in the "two-step" cytotoxic test (see Materials and Methods). Diluted anti-K36 serum incubated with buffer only lysed 84% of E♂G2 cells. G, G-MuLV; R, R-MuLV.*

GCSA does represent virion structural protein information, but in the form of glycosylated polyprotein species that do not themselves enter into virions. This finding derives from the work of Tung et al. (55), who originally demonstrated the presence of viral core polyprotein on the surface of $GCSA^+$ AKR spontaneous leukemia cells; in that system, precipitation of radioiodinated polyproteins was carried out with the GCSA-reactive rat serum (W/Fu × BN)F_1 anti-W/Fu (C58NT)D (often called anti-NTD serum) (13). The data presented here are consistent with those previous results. Recently, Ledbetter and co-workers (29, 30) also found glycosylated MuLV core polyproteins on the surface of $GCSA^+$ mouse leukemia cells.

To identify GCSA-bearing molecules we performed lactoperoxidase-catalyzed radioiodination on the standard GCSA typing cell, E♂G2, and precipitated an NP-40 lysate with the GCSA typing serum, anti-K36. SDS-PAGE analysis revealed three molecular species, designated p(150), p(85), and p(45), which are specifically precipitated by the typing serum (Fig. 1). [Other studies (29, 56) indicate that p(85) on AKR leukemia cells and on E♂G2 actually comprises two species, $gP85^{gag}$ and $gP95^{gag}$ (56), which can be resolved by slab gel electrophoresis and autoradiography.] Some material in the size range of p(45) and perhaps p(150) appeared to be cellular in origin (e.g., actin) and was either highly aggregatable or easily adsorbed to immunoglobulins or to viral proteins (11, 29, 55). Since GCSA is defined in terms of reactivity with cytotoxic antibody, it was important to show that absorption of such antibodies with $GCSA^+$, but not $GCSA^-$, leukemia cells would reduce the ability of anti-K36 serum to precipitate p(150), p(85), and p(45). The reactive antigens did co-type with GCSA, as shown in Fig. 2 and Table 1.

Surface-labeled molecules of E♂G2 cells similar to those precipitated by anti-K36 serum were precipitated by antisera against MuLV p30 and p15 (Fig. 3). Moreover, pretreatment of the lysate with anti-MuLV p30 and anti-immunoglobulin removed molecules reactive with either anti-K36 serum or additional anti-p30 serum (Fig. 4). Finally, antibodies to p30 and p15 in anti-K36 serum could be assayed directly by reaction with lysed MuLV (Fig. 5).

The evidence then in hand established the existence of polyproteins incorporating the p30 and p15 sequences on the surface of $GCSA^-$ cells and the presence of antibodies to p30 and p15 in anti-K36 serum capable of precipitating these polyproteins. It remained to be shown that the anti-p30 and anti-p15 antibodies were the same as those assayed in the GCSA cytotoxic test. In fact, absorption of anti-K36 serum with disrupted G-MuLV virions or with purified G-MuLV p30 or p15 significantly reduced GCSA cytotoxic antibodies (Fig. 6 and 7). The data indicated a relatively strong absorption of anti-K36 cytotoxic activity with individual p30 and p15 preparations. However, it should be noted that the percentage of cells killed is not strictly proportional to antibody titer in the cytotoxic test: in systems such as GCSA (or H-2, for example) a twofold dilution of a cytotoxic serum can result in considerably more than a twofold reduction in cell kill. The data in Fig. 7 would also be consistent with a model wherein p30 and p15 have GCSA sequences in common. This explanation is unlikely, however, since, in experiments like those detailed in the legend to Fig. 5, absorption of anti-K36 serum with p30 only removed precipitating activity for p30 of G-MuLV, and absorption with p15 only removed precipitating activity for p15.

The polypeptide sequences of the cell surface polyproteins are presumed to be comparable to those of intracellular MuLV core polyproteins found in infected tissue culture cells (3, 8, 34, 48, 57) and of products formed in cell-free protein-synthesizing systems in response to MuLV-specific RNA (14, 26, 35). Cell surface p(150) has not been described previously; a portion of this molecule may not derive from the *gag* region of the viral genome. The fact that the p(150) species has not been detected as readily in other AKR-spontaneous leukemia cell surface-labeling experiments may be a reflection of its relative instability in the presence of cellular proteases. Alternatively, p(150) may represent a cellular protein, more plentiful in E♂G2 cells, with a high affinity for viral *gag* polypeptide sequences in immunoprecipitates.

The roles of G-MuLV p30 and p15 in the GCSA system help to explain earlier findings that GCSA involves multiple antigenic determinants (40, 44). The presence of group-specific determinants is implied by the partial absorption of GCSA typing serum with FMR-type leukemias (44), with tissue culture cells infected by FMR or xenotropic viruses (40), or with R-MuLV p30 (Fig. 7). That these cross-reacting determinants are contained in p30 is consistent with the predominantly group-specific character of this protein (51). Type-specific determinants are revealed by the complete absorption of GCSA typing serum by Gross leukemias (44), by tissue culture cells infected with Gross-AKR-type viruses (40), and by G-MuLV p30 and p15. Such determinants would be expected in the type-specific region(s) of p30 (45) and espectially in p15, which is a strongly type-specific core protein (6, 45, 52). Absorption experiments

by Strand et al. (52) suggest that major determinants of the FMR cell surface antigen, an analog of GCSA, are also associated with determinants of the p15 of R-MuLV.

It is clear that the complex structure of the molecule bearing the GCSA antigens, its disposition on E♂G2, and the biological requirements of the cytotoxicity test permit certain distinctions to be made among different antigenic specificities in this system. The specificities recognized by anti-K36 on the core polyprotein probably represent a subset of the exposed determinants on this molecule as it is presented on viable E♂G2 cells. An additional group of determinants are presumably buried in the plasma membrane and are revealed by NP-40 solubilization. Clearly, hyperimmune sera against viral proteins made in species other than the mouse (e.g., "anti-NTD" serum [12]) may not detect the same determinants on a given protein as does anti-K36. Nor do precipitating antibodies against a given protein necessarily coincide with cytotoxic antibodies directed against that protein positioned in the membrane. The same considerations apply a fortiori to the additional antibodies in anti-K36 directed against p15(E), an activity also found in many sera from mice not subjected to hyperimmunization (19, 20, 36, 46). There is no evidence that anti-p15(E) antibodies in anti-K36 serum are cytotoxic for E♂G2.

One portion of the polyprotein that may be only partly exposed is the p15 region: although our type-specific anti-AKR-MuLV p15 serum is able to bind to E♂G2 cells and partially block the ability of these cells to absorb antibody from anti-K36 in a standard blocking test (5), the anti-p15 is only weakly cytotoxic (unpublished data). This suggests the possibility that the highly selective anti-p30 and anti-p15 specificities in anti-K36 may, to some extent, function cooperatively in cell killing. Thus, especially if p(85) molecules are relatively dispersed on the cell surface, simultaneous binding of immunoglobulin to adjacent p30 and p15 determinants could enhance effective binding of complement.

In conclusion, it appears that certain core-related MuLV polyprotein species need not be processed into virions, but instead may be directed to the cell surface via an alternative processing pathway that can include glycosylation. The molecular species that are involved derive from the *gag* gene of MuLV; in spite of the fact that these species may be encoded by endogenous MuLV loci, recognition by the immune apparatus of the host is possible, depending on the genetic constitution of the particular mouse strain (2, 38, 50). Further elucidation of these processes should help to clarify the events involved in the origin and establishment of leukemic cell clones.

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ADDENDUM IN PROOF

Peptide mapping of p(150) and p(85), radioiodinated on cells in situ, by J.-S. Tung indicates that p(150) does not contain exposed peptides characteristic of MuLV *gag* proteins, which are found in p(85) components. This strengthens the interpretation that p(150) is a cellular protein which complexes readily with p(85) and p(45) in immunoprecipitates.

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