ANTIGENIC MODULATION OF FRIEND VIRUS ERYTHROLEUKEMIC CELLS IN VITRO BY SERUM FROM MICE WITH DORMANT ERYTHROLEUKEMIA*

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In the tumor dormant state, small numbers of tumor cells persist in the host without outgrowth to overt neoplasia. The cancer literature contains numerous reports describing recurrence of solid tumors and leukemias years after apparent successful treatment of the primary neoplasia (1). These reports suggest that residual tumor cells can persist in a dormant state during the prolonged clinical remission. Few experimental models have been developed to study the tumor dormant state. In this laboratory we have been investigating a murine model in which the rapidly fatal Friend virus erythroleukemia can be suppressed to a dormant state by treatment with statolon, an extract of mycoplasma-infected Penicillium stoloniferum cultures (2-4), or the double-stranded RNA extracted from statolon (5). This report describes our continuing analysis of host mechanisms involved in maintenance of Friend leukemia virus (FLV) in a dormant state.

Infection of DBA/2 mice with FLV causes a depression in humoral (6) and cellular immunity (7) and macrophage functions (8) followed by the development of a fatal erythroleukemia (2). Treatment of FLV-infected mice with statolon abrogates the depression of humoral immunity and macrophage functions (6, 8). Between 50 and 70% of these mice maintain immunocompetence and survive, suppressing the erythroleukemia to a dormant state, which lasts for the normal lifespan of most of these mice (2-4, 9). However, late in life, some of these mice develop erythroleukemia from which FLV can be isolated (2, 3). Mice with dormant FLV infections contain antibodies that complex FLV virion polypeptides and are cytotoxic for FLV-transformed cells, and these antibodies appear to be crucial for leukemosuppression and maintenance of the virus in a dormant state (9-11). Serum from mice with dormant FLV infections is referred to as dormant FLV-immune serum (FVIS).

The precise mechanism by which FLV antibodies suppress FLV erythroleukemia and maintain the virus in a dormant state is not known. DBA/2 mice are deficient in the fifth component of complement (C5) (12) which is required for antibody-mediated complement-dependent immune cytolysis (13). Carlson and Terres demonstrated antibody-dependent killing of syngeneic lymphomas by the DBA/2 mouse in vivo (14). The mechanism involved in this destruction of the lymphomas was most likely antibody-dependent cell-

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Abbreviations used in this paper: ADCC, antibody-dependent cell-mediated cytotoxicity; EBV, Epstein-Barr virus; FLC-745 cells, clone 745 of Friend erythroleukemia cells; fl-GAMg, fluorescein-conjugated goat anti-mouse 7S gamma globulin; FLV, Friend leukemia virus; FVIS, immune serum from mice with dormant Friend virus infection; GCSA, Gross virus cell surface antigen; MuMTV, murine mammary tumor virus; NMS, normal mouse serum; PBS, phosphate-buffered saline; TL, thymus-leukemia.
mediated cytotoxicity (ADCC); however, extensive attempts in our laboratory to demonstrate ADCC in vitro using FLV erythroleukemic cells as targets and FVIS have been negative (unpublished results). A third type of antibody-tumor cell interaction is antigenic modulation. We therefore explored the possibility that FVIS might maintain FLV in a dormant state through antigenic modulation.

Antigenic modulation is the phenotypic suppression of a cell surface antigen during exposure to specific antibody in the absence of complement (C); withdrawal of antibody from the environment of modulated cells results in the re-expression of cell surface antigen (15, 16). Boyse et al. (17) demonstrated that antigenic modulation was the mechanism involved in the escape and outgrowth of thymus-leukemia (TL) antigen-positive thymomas in TL antigen-immune mice. Modulation of TL antigen expression on the surface of leukemic cells was also observed after in vitro exposure to specific antibody (15, 18). In vitro antigenic modulation has also been described for the Gross virus cell surface antigen (GCSA) in mice (19), Gross leukemia virus expression in rats (20), fetal antigens on Rauscher leukemia virus-induced C57/BL6 lymphomas (21), and SV-40 virus antigen expression in transformed hamster fibroblasts (22). Antigenic modulation was also observed in vitro with human melanoma cell surface antigens (23), Epstein-Barr virus (EBV) antigen expression in Burkitt lymphoma cells (23, 24), murine H-2 antigens (16, 25), murine mammary tumor virus (MuMTV) antigens (26), membrane-associated immunoglobulins of lymphoid cells (16, 27-29), and measles virus antigens in persistently and acutely infected HeLa cells (30).

The interaction of antibody with cell surface antigen may result in antigen-antibody complexes which redistribute on the cell surface into patches and caps (31, 32), and this capping phenomenon has been associated with antigenic modulation (31). Antigen capping and antigenic modulation are dependent upon active cellular metabolism (15, 25, 32, 33), but each event can occur independently (34-36).

In order to determine whether modulation of FLV antigens is involved in maintenance of dormant FLV infection, an in vitro model was developed. This communication reports that FLV erythroleukemic cells cultured in FVIS, in the absence of C are markedly diminished in their susceptibility to FLV-specific immune cytolysis. Immunofluorescent studies demonstrate a temporal correlation between capping and modulation of FLV antigens. Finally, evidence for inhibition of the expression of the FLV genome in modulated Friend erythroleukemic cells is presented.

**Materials and Methods**

**Cells.** Clone 745 of Friend erythroleukemia cells (FLC-745) were obtained from the Genetic Mutant Cell Repository, Institute for Medical Research, Camden, N. J., which has designated this clone GM86. The cell line was derived from solid leukemic tumors passaged in DBA/2 mice (37).

The cells were maintained in suspension culture in 75 cm² plastic tissue culture flasks, and transferred twice weekly at a final concentration of 10⁶ cells/ml of culture medium, consisting of RPMI 1640 (pH 7.0) with 20% heat-inactivated fetal bovine serum and 100 µg/ml streptomycin, 100 U/ml penicillin, 100 µg/ml gentamycin, and 2 mM glutamine. All cultures were incubated at 37°C in a humidified chamber containing a 5% CO₂ atmosphere.

**Mouse Serum.** Mouse serum was obtained from ether anesthetized mice, bled from the right axilla. The blood was pooled and allowed to clot for 2-4 h at 4°C. Serum was collected after centrifugation at 300 g for 10 min, and stored at -70°C. Serum was heat inactivated by incubation in a 56°C water bath for 30 min. Heat-inactivated serum was diluted in culture medium to obtain a final 5% concentration for experimental cell culture medium, and filtered through a 0.45 µm Millipore filter (Millipore Filter Corp., Bedford, Mass.). Normal mouse serum (NMS) was obtained from 6- to 8-wk-old female DBA/2 mice (The Jackson Laboratory, Bar Harbor, Maine).

FVIS was obtained from mice with dormant FLV infections. Mice were infected with 2,000
leukemia-producing doses of FLV, treated with chlorite-oxidized oxyamylose and statolon as previously described (38). 38 days after FLV infection, those mice displaying no gross evidence of erythroleukemia were designated as having dormant FLV infections, and were bled for their sera. FVIS is cytotoxic for FLV-transformed cells in the presence of guinea pig C (9-11). Heat inactivation does not alter the C-dependent cytotoxic titer of FVIS (data not shown). Anti-DBA/2 C-dependent cytotoxic serum was obtained from C57BL/6 mice (The Jackson Laboratory), immunized with DBA/2 spleen cells as described by Davies (39).

**Experimental Cell Culture System.** For in vitro cell culture experiments, 1.5 × 10^6 cells were suspended in 3 ml of culture medium containing 5% heat-inactivated mouse serum in 16 × 125 mm plastic tissue culture tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). At 24-h intervals, a sample was removed and cell number determined by hemocytometer counts and viability determined by trypan blue dye exclusion. Except where indicated, cells were maintained in logarithmic growth phase by redilution every 24 h to 5 × 10^5 cells/ml in a final 3-ml culture volume, using fresh medium containing 5% heat-inactivated mouse serum. Under these culture conditions, FLC-745 cells have a population doubling time of 18–20 h.

**Assay for Cell Susceptibility to Immune Cytolysis.** Cell susceptibility to immune cytolysis was measured by a modified 51Cr-release method originally described by Wigzell (40). FLC-745 cells were centrifuged at 300 g for 10 min, and resuspended to a concentration of 3 × 10^6 viable cells/ml in the experimental culture medium in which they were grown. The use of the experimental culture medium assured that the cells were maintained in the appropriate experimental environments during the 51Cr-labeling period. The labeled cells were centrifuged through a 0.5 ml heat-inactivated fetal bovine serum underlay, then washed twice at 4°C in assay medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, and resuspended in fresh assay medium at a concentration of 2.5 × 10^5 cells/ml. This suspension was maintained at 4°C and used immediately for the assay.

The assay was performed in ISFB-96 Linbro plastic microcytotoxicity plates (Linbro Chemical Co., New Haven, Conn.) containing 2.5 × 10^4 51Cr-labeled cells in medium with 5% (vol/vol) guinea pig C (Baltimore Biological Laboratories, Cockeysville, Md.), and 5% FVIS cytotoxic serum in a final vol of 200 μl/well. This concentration of FVIS produces maximum lysis of FLC-745 target cells in the presence of C. The plates were incubated at 37°C for 90 min, then centrifuged for 10 min at 300 g in an IEC Model No. 442 flat-bottomed centrifuge rotor (Damon/IEC Div., Needham Heights, Mass.), and counted in a Searle gamma counter (Amersham/Searle Corp., Arlington Heights, Ill.). Supernatant control wells contained 2.5 × 10^5 target cells in 200 μl of assay medium. To test for cell susceptibility to lysis by C alone, target cells were mixed with assay medium containing a final 5% (vol/vol) guinea pig C. Maximum release was determined by hypotonic water lysis of 2.5 × 10^5 target cells diluted 1:20 in distilled water. All assays were done in duplicate. Percent specific release was calculated as follows:

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\text{Percent specific release} = \left( \frac{\text{Supernate cpm of experimental groups} - \text{Supernate cpm of spontaneous release}}{\text{Supernate cpm of maximal release} - \text{Supernate cpm of spontaneous release}} \right) \times 100\%.
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**Membrane Immunofluorescence Assay for FVIS Gamma Globulin and FLV Antigens.** In order to detect the presence of FVIS 7S gamma globulin bound to the cell surface, experimentally cultured FLC-745 cells were washed in phosphate-buffered saline (PBS), pH 7.4, at 4°C, centrifuged at 300 g for 10 min, and resuspended to a final concentration of 2.5 × 10^6 cells/ml in a 1:4 dilution of fluorescein-conjugated goat anti-mouse 7S gammaglobulin (FLGM; Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) in PBS. The cells were maintained in suspension at 4°C for 20 min with occasional agitation, then washed three times in PBS by centrifugation, resuspended, and examined under an American Optical Vertical Fluorescence Microscope (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). The labeled cell suspension was viewed with a tungsten light source or an ultraviolet light source, obtained from an Osram HBO 50W mercury vapor lamp, in combination with a BG-12 exciter filter and an OG-1 barrier filter. 100–125 viable cells per slide were counted and examined for fluorescence and fluorescence distribution. The criterion used to determine the viability of fluorescein-stained cells was described by Möller (41). Fluorescence distribution was categorized as rim, patch, and cap as previously described by others (31, 32). In order to detect the expression of FLV antigens at the cell surface, experimentally cultured FLC-745 cells were resuspended in a 1:4 dilution of FVIS for 20
rain at 4°C, then washed, centrifuged, stained with fl-GAMg, and observed for fluorescence as described above.

Results

Modulation of FLV Cell Surface Antigens during Culture of Friend Erythroleukemic Cells in Dormant FVIS. FLC-745 cells were cultured in medium containing FVIS to determine the effect of antibodies directed against FLV cell surface antigens on cell growth, viability, and susceptibility to FVIS-mediated C-dependent cytolysis. Two cultures of FLC-745 cells were initiated with medium containing either 5% heat-inactivated NMS or FVIS. After 24 h, each culture was divided into two subcultures. One subculture was diluted with fresh medium containing FVIS or NMS to maintain the cells in logarithmic growth phase. Cells in the other subculture were not diluted, but allowed to grow into the stationary phase. At 24 and 48 h, the total cell number and viability was determined, and the cells were labeled with $^{51}$Cr, and tested for their susceptibility to cytolysis by FVIS in the presence of C or by C alone.

Table I shows that during the 48-h incubation period, there was, in both the logarithmic and stationary growth phase cultures, no difference in growth and viability of cells cultured in FVIS or NMS. However, FLC-745 cells cultured in FVIS for 48 h in the logarithmic growth phase were significantly more resistant to cytolysis by FVIS than were cells cultured logarithmically in NMS. Cells cultured in FVIS in the stationary growth phase developed resistance to cytolysis by FVIS, but to a lesser degree than did cells cultured in the logarithmic growth phase. Of the four experimental cultures, cells in the logarithmic growth phase grown in NMS were most susceptible to cytolysis by FVIS and cells in the logarithmic phase grown in FVIS were least susceptible. The cells from the various cultures were not significantly lysed by the addition of C alone. The data indicate that FVIS modulates FLV antigen expression on erythroleukemic cells resulting in a loss of susceptibility to cytolysis by FVIS. The degree of this modulation is affected by the growth phase of the cells in culture. Since modulation of FLV antigen expression on FLC-745 cells cultured in FVIS was optimal when the cells were in the logarithmic growth phase, these culture conditions were employed for all subsequent experiments.

Kinetics of Modulation of FLV Cell Surface Antigens on FLC-745 Cells and Reversal of Modulation. To determine the kinetics of antigenic modulation, FLC-745 cells were cultured in the logarithmic growth phase for 96 h in media containing FVIS and examined at 24-h intervals for cell growth, viability, and susceptibility to cytolysis by FVIS in the presence of C or by C alone. To determine whether antigenic modulation was reversible, FLC-745 cells were cultured in FVIS for 48 h, washed free of FVIS, and cultured for an additional 48 h in fresh media with either NMS or no mouse serum. Unmodulated cells grown in media with either NMS or media without mouse serum were treated identically and served as controls.

Fig. 1 shows that FLC-745 cells cultured in medium containing FVIS for 24 h became relatively resistant to cytolysis by FVIS and continued to be resistant throughout the 96-h culture period compared to FLC-745 cells cultured in medium devoid of FVIS. FLC-745 cells that were modulated after culture in medium containing FVIS for 48 h, regained completely their susceptibility to
TABLE I

| Time in culture*  | % Increase in† cell no. | % Viable cells | % 51Cr release¶ |
|-------------------|------------------------|---------------|----------------|
|                   | NMS§ | FVIS| NMS | FVIS | NMS | FVIS|
| h                 |       |     |     |      |     |     |
| 24                | 148  | 136 | 92  | 91   | ND** | ND  |
| 48 (logarithmic)  | 160  | 158 | 95  | 93   | 45.3 ± 1.0 | 8.5 ± 0.1 |
| 48 (stationary)   | 52   | 58  | 87  | 89   | 24.4 ± 0.4 | 16.6 ± 0.6 |

* All cell cultures were initiated with 5 × 10⁵ cells/ml of culture volume. At 24 h, the cultures were divided into logarithmic and stationary growth phase subcultures as indicated. Subcultures were maintained in logarithmic growth phase by dilution in fresh medium at 24 h. Stationary growth phase subcultures were achieved by not diluting cells at 24 h. At 48 h the cells were assessed for cytotoxic susceptibility to FVIS.
† % Increase in cell no. is determined by the ratio of the increase in number of cells during each 24-h culture period, divided by the number of cells present at the beginning of the 24-h period, multiplied by 100%.
§ Cells cultured in the presence of 5% heat-inactivated NMS.
| Cells cultured in the presence of 5% heat-inactivated FVIS.
¶ % 51Cr release from experimental FLC-745 target cells by a final 1:20 dilution of FVIS and C. The % 51Cr release from cells of the four cultures reacted with C alone was less than 5%.
** ND, not done.

Fig. 1. Kinetics of FLV antigenic modulation and reversal of modulation. Cells cultured in NMS (●—●); cells cultured in medium containing fetal bovine serum but devoid of mouse serum (△—△); cells cultured in FVIS (×—×); cells cultured in FVIS for 48 h then transferred into medium with NMS (○—○); cells cultured in FVIS for 48 h then transferred into medium devoid of mouse serum (▲—▲).
cytolysis within 24 h after transfer to medium containing NMS or no mouse serum, indicating that antigenic modulation was reversible. There was no significant cytolysis of the cells by C alone. Cell growth and viability was the same in each of the experimental cultures (data not shown). These results indicate that FLC-745 cells cultured in FVIS undergo a reversible phenotypic loss in susceptibility to cytolysis by FVIS.

Medium from cell cultures containing FVIS were titrated for residual FLV-specific cytotoxic antibody after 24, 48, and 72 h of incubation. For each 24-h interval there was a twofold decrease in cytotoxic antibody titer as compared to medium with FVIS incubated in the absence of cells. It was not possible to quantitate the amount of residual modulating antibody in the cell cultures at the end of each 24-h period, because of associated nutrient depletions in the media. Therefore, the relationship between the concentration of FVIS in the culture medium and the induction of FLV antigenic modulation was also examined. FLC-745 cells were cultured in media containing serial twofold dilutions of FVIS, ranging from 1:10 to 1:80. FLV antigenic modulation was achieved at dilutions of FVIS up to 1:40. The 5% FVIS concentration in the growth medium used in the antigenic modulation cultures is thus a twofold excess concentration required for FLV antigenic modulation. Furthermore if cytotoxic antibody and modulating antibody decline at the same rate in FLC-745 cell cultures, then sufficient antibody remained in the cultures at the end of each 24-h incubation period to achieve antigenic modulation.

**Susceptibility of FVIS-Modulated FLC-745 Cells to Immune Cytolysis by Anti-DBA/2 Serum.** The specificity of FVIS-induced FLV antigenic modulation of FLC-745 cells was tested by comparing the susceptibility of modulated and unmodulated cells to immune lysis by both FVIS and anti-DBA/2 serum in the presence of C. Table II shows that modulated FLC-745 cells were relatively resistant to immune lysis by FVIS, but were as susceptible as unmodulated cells to lysis by anti-DBA/2 serum. Modulated FLC-745 cells and unmodulated cells were also equally susceptible to lysis by serial twofold dilutions of anti-DBA/2 serum ranging from 1:80 to 1:640 (data not shown). Thus, FVIS modulates the susceptibility of FLC-745 cells to FLV-specific immune cytolysis without affecting susceptibility to cytolysis by anti-DBA/2 serum. This observation indicates that in the presence of FVIS, FLV cell surface antigens do not co-modulate with H-2<sup>d</sup> antigens. The possibility that loss of susceptibility to cytolysis by FVIS is due to an anti-complementary factor is also excluded.

**Correlation of Modulation of FLV Antigens with Capping of FLV-Specific Antigen-Antibody Complexes.** The distribution of FLV-specific antigen-antibody complexes on the surfaces of FLC-745 cells undergoing antigenic modulation was determined by membrane immunofluorescence. Samples of cells cultured in medium containing FVIS or NMS were reacted with fi-GAMg every 6 h to detect the presence and position of FLV antigen-antibody complexes during a 24-h incubation period. Samples of cells were also tested at 0, 16, and 24 h for their susceptibility to cytolysis by FVIS.

At the inception of culture, 91% of the 100% immunofluorescent-positive FLC-745 cells displayed FLV antigen-antibody complexes distributed evenly over the cell surface (Fig. 2 A) and are denoted as rimmed cells in Table III, and 9% displayed a patched distribution. By 6 h of culture, the percentage of patched
TABLE II

Susceptibility of Modulated FLC-745 Cells to Immune Cytolysis
By Anti-DBA/2 Serum

| Mouse serum in assay medium* | % 51Cr release |
|-----------------------------|----------------|
|                             | Control cells† | Modulated cells§ |
| FVIS 81.8 ± 1.2             | 35.7 ± 1.5     |
| Anti-DBA/2 serum 83.5 ± 6.9 | 87.9 ± 0.7     |
| None 0.9 ± 0.3              | 1.9 ± 0.1      |

* FVIS was used at a 1:20 final dilution with C and anti-DBA/2 serum was used at 1:80 final dilution with C in the serum cytotoxicity assay.
† Control FLC-745 cells were cultured for 48 h in medium with NMS before use in the serum cytotoxicity assay.
§ Modulated FLC-745 cells were cultured for 48 h in medium with FVIS before use in the serum cytotoxicity assay.
‖ None refers to the release of 51Cr from labeled target cells incubated with guinea pig C in the absence of cytotoxic serum.

Fig. 2. FLC-745 cells were cultured in FVIS, then stained at selected times, during a 24 h culture period with fl-GAMg, to assess the distribution of FLV-immune complexes. (A) Rimmed fluorescence pattern observed at 0 h. (B) Patched fluorescence pattern observed at 6 h. (C) Capped fluorescence pattern observed at 18 h. (D) A cell negative for fluorescence observed at 24 h. (× 5,000).
Table III

| Distribution of fluorescence on cell surface | % Cells with FLV antigen-antibody complexes in cultures containing dormant FVIS |
|---------------------------------------------|-------------------------------------------------------------------------------|
|                                             | Time in culture (h)* | 0  | 6  | 12 | 16 | 18 | 24 |
| Positive                                    | 100               | 99 | 87 | ND | 70 | 35 |
| Rimmed                                      | 91                | 30 | 10 | ND | 2  | 0  |
| Patched                                     | 9                 | 54 | 38 | ND | 9  | 4  |
| Capped                                      | 0                 | 15 | 39 | ND | 59 | 31 |
| Negative                                    | 0                 | 1  | 13 | ND | 30 | 65 |

Mouse serum in culture medium % 51Cr release by dormant FVIS

| FVIS             | 68.9 ± 1.0 | ND | ND | 51.9 ± 2.9 | ND | 35.7 ± 1.5 |
| NMS              | 67.3 ± 2.0 | ND | ND | 79.9 ± 0.7 | ND | 81.8 ± 1.2 |

* FLC-745 cells were cultured in medium with FVIS or NMS. At the indicated time points, the cells were washed and stained in suspension with fluorescein-conjugated goat anti-mouse gamma globulin.
† At the 0 h time point FLC-745 cells were reacted with FVIS, washed, then stained with fluorescein-conjugated goat anti-mouse gamma globulin.
‡ At 0, 16, and 24 h, samples of cells, from the same cultures used for fluorescence staining, were labeled with 51Cr and tested for their cytotoxic susceptibility to a 1:20 final dilution of FVIS and C. The percent 51Cr release from the cells from both cultures reacted with C alone was less than 5%.
§ ND, not done.

During the first 18 h of culture in FVIS, the FLC-745 cells became relatively resistant to cytolysis by FVIS, coincident with the appearance of a maximum percentage of cells displaying capping. At 24 h, when this experiment was terminated, 96% of the cells were either capped or immune complex negative and there was a further increase in resistance to cytolysis by FVIS; maximum levels of resistance usually occur between 24 and 48 h of culture (see Fig. 1). These results indicate a temporal relationship between redistribution and loss of FLV antigen-antibody complexes and decreased cell susceptibility to cytolysis.
by FVIS. The maximum resistance of cells to lysis by FVIS and C is preceded by
the appearance of a maximum percentage of cells with capped FLV antigen-
antibody complexes.

Expression of FLV Antigens on the Surface of Modulated FLC-745
Cells. Using the membrane immunofluorescence assay, control and modulated
FLC-745 cells were examined for FLV antigens, to determine whether antigenic
modulation was associated with suppressed expression of the FLV genome.
FLC-745 cells were cultured in medium containing FVIS for 72 h to achieve
modulation, and in medium containing NMS as a control. Samples of cells from
the cultures at 0, 24, 48, and 72 h were reacted with FVIS followed by fl-GAMg.
This procedure should result in the fluorescent labeling of all cells whose surface
membranes contain either uncomplexed or antibody-complexed FLV antigen
before the staining procedure.

As shown in Table IV, 26% of the modulated cells at 48 h were devoid of both
immune complexes and free FLV cell membrane antigens. This redistribution of
surface antigens also correlated with decreased susceptibility of FLC-745 cells to
lysis by FVIS and C. These results suggest that FVIS does suppress FLV
antigen expression at the cell surface. However, possible spatial and steric cell
surface alterations associated with capping may have interfered with the stain-
ing of immune complexes on modulated cells by fl-GAMg.

Discussion

In this report we have demonstrated that FLV cell surface antigens on FLV
erthroleukemic cells are modulated after in vitro cultivation in medium con-
taining serum from mice with dormant FLV infections. Modulated FLV-ery-
throleukemic cells were not altered in their rate of division and expressed less
FLV antigen on the cell surface as demonstrated by membrane immunofluores-
cent microscopy and their relative resistance to FLV-immune cytolysis. Modu-
lated cells transferred to medium containing NMS re-expressed their FLV cell
surface antigens within 24 h.

A decrease in the amount of FLV cell surface antigens could either be caused
by inhibition of genomic expression or be a manifestation of antigenic modula-
tion whose effects are confined to the cell membrane. Data from studies of
surface antigens on cells cultured in medium containing specific immune serum
must be viewed cautiously since the amount of surface antigen present has been
shown to be determined by the rates of antigen production, their complexing
with specific antibody, and shedding or internalization of the complexes (35, 36,
42). If FLV antigen is produced at a relatively slow rate, then removal of the
antigen from the cell surface during modulation may lead to a prolonged period
in which the surface is devoid of detectable antigen. An alternative mechanism
is that expression of the FLV genome is itself inhibited. The above mentioned
dynamics of appearance, accumulation, and shedding of FLV antigens on the
cell surface, in the presence of FVIS, prevents a clear interpretation of data
obtained from cell surface studies. Suppression of the FLV genome in modulated
cells can best be determined by evaluation of the synthesis of virion polypep-
tides. Such studies will be the subject of a future communication.

Identification of the modulating antibody in the FLV system is currently in
### Table IV

*Expression of FLV Antigens on the Surfaces of Modulated FLC-745 Cells*

| Mouse serum in culture medium | % Cells with FLV-specific immunofluorescence* | Time in culture (h) |
|-------------------------------|---------------------------------------------|---------------------|
|                               |                                              | 0                   | 24     | 48     | 72     |
| FVIS                          | 100 (0)‡                                     | 85 (15)             | 74 (26) | 77 (23) |
| NMS                           | 100 (0)‡                                     | 97 (3)              | 99 (1)  | 98 (2)  |

|                  | % $^{51}$Cr release by dormant FVIS‡         |                     |
|------------------|---------------------------------------------|---------------------|
| FVIS             | 80.6 ± 2.0                                   | 36.6 ± 0.0          | 23.3 ± 0.5 | 19.0 ± 0.4 |
| NMS              | 95.1 ± 4.5                                   | 78.5 ± 1.1          | 66.8 ± 0.1 | 58.7 ± 0.5 |

* FLC-745 cells were cultured in medium with FVIS or NMS. At the indicated time points, samples of cells were reacted with FVIS and stained with fluorescein-conjugated goat anti-mouse gamma globulin to determine the percentage of cells displaying FLV-specific immunofluorescence.

‡ The number in the parentheses indicates the percentage of cells in culture which were negative for FLV-specific immunofluorescence.

‡ At the time points indicated, samples of cells, from the same cultures used for fluorescence staining, were labeled with $^{51}$Cr and tested for their cytotoxic susceptibility to a 1:20 final dilution of FVIS and C. The % $^{51}$Cr release from the cells from both cultures reacted with C alone was less than 5%.

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progress. Analysis of dormant FLV-immune serum has revealed antibody to gp69/71, gp43, p15, and p12. Of special note is that serum from overtly leukemic mice contain the same antibody specificities except for an absence of antibody to p12. p12 may be the antigen which serves as the common target for antibody-mediated C-dependent lysis on Friend, Rauscher, and Moloney leukemic cells (R. Steeves, personal communication) and may have been the modulated cell surface antigen in the experiments described here. Although the precise role of antibody to p12 in dormant FLV infections remains to be determined, a correlation exists between FLV dormancy and the presence of p12 antibody, and production of this antibody may determine whether a statolon-treated FLV-infected mouse will develop dormant or overt FLV erythroleukemia.

Our experiments have revealed that FLC-745 cells in logarithmic growth phase cultures are more susceptible to FLV-immune cytolysis in the absence of FVIS and when cultured in the presence of FVIS, underwent antigenic modulation to a greater extent than cells in a resting state. FLV erythroleukemic cells have been shown to cease production of virus on entering the G0 or resting state (43). The decreased production of virus antigens in resting phase cells may be the cause of the decreased susceptibility to cytolysis. However, the reasons for the decreased ability of cells in G0 to undergo antigenic modulation are unknown and the problem should be further studied.

Modulation of FLV cell surface antigens was produced by medium containing a constant excess of cytotoxic antibody. Although between 70 and 87% of cells at 16 h in modulated cultures contained antigen-antibody complexes on their
surface (Table III), they were not lysed by C alone. The inability of C to lyse modulated FLC-745 cells despite the detection of immunoglobulin on these cells by immunofluorescence, suggests that the amount of antibody bound to the cells was below that required for C fixation and cell lysis (44). It is also possible that cells with capped antigen-antibody complexes were either unable to fix C or were not lysed due to cell membrane configurational changes associated with antigenic modulation.

FLV antigenic modulation, as measured by the increased resistance of modulated cells to FLV-specific antibody-mediated lysis, was temporally associated with cap formation on the surface of FLC-745 cells. The internalization or shedding of immune complexes (45, 46) from the cell surface may reduce the cell surface antigen density, and increase the resistance of modulated cells to antibody-mediated C-dependent cytolysis. Similar temporal correlations between modulation and capping were observed for MuMTV antigens (26). In contrast to the above systems, measles virus antigenic modulation was preceded by capping which was associated with a transient increase in susceptibility to immune cytolysis (30). However, studies of modulation of TL antigens have suggested that capping is not a requisite for antigenic modulation (34, 36). The mechanisms involved in antigenic modulation and any differences in association with capping may reflect the intrinsic metabolism and physiology of the various cell systems studied.

Antibody-induced capping and modulation of a specific antigen may affect the expression and distribution of other cell surface antigens (47). In this communication, evidence was presented showing that the modulation of FLV antigens on FLC-745 cells does not affect the cytotoxic susceptibility of these cells to allotype serum. No effect on the expression of cell surface allotype antigens and differentiation antigens was observed on GCSA and MuMTV antigen-modulated cells (19, 26). However, the modulation of TL antigens was accompanied by an increased expression of H-2D haplotype antigens (15). Antibody-induced capping of FLV cell surface antigens on BALB/c erythroblasts depressed the expression of H-2D antigens. This observation suggests that FLV cell surface antigens are physically associated with H-2D antigens (R. Steeves, personal communication). However, our studies were confined to the entire H-2 antigen complex which did not modulate with FLV cell surface antigens.

Antibody may play a role in persistent and latent viral diseases through antigenic modulation. Measles virus persistence in subacute sclerosing panencephalitis (30) and herpes simplex virus latency (48) may be achieved by antibody-induced modulation of viral antigens at the cell surface. The in vivo modulation of Gross leukemia virus antigens in rats (20), SV-40 virus antigens in hamsters (22), and the in vitro modulation of EBV antigens in Burkitt lymphoma cells by human convalescent EBV serum (23) has been associated with suppressed expression of viral genomes in transformed cells. In contrast, GCSA expression on murine lymphomas was suppressed after passage through immune mice without any effect on the expression of C-type particles and other associated virion antigens (19). These experiments are analogous to ours in one important respect: modulation was produced with serum obtained from the infected or tumor-bearing hosts. Such serum would be expected to contain a
complete range of antibodies directed against virion and virus-induced cell membrane antigens and, therefore, modulating and tumor-suppressive antibodies are more likely to be present than in a host immunized with single antigens. Prophylactic immunization of mice and passive transfer of antibodies against endogenous virus particles were reported to prevent spontaneous tumor emergence and endogenous virus expression (49, 50). The role of humoral immunity in these tumor models still needs to be delineated; however, antibody may act to regulate viro gene and oncogene expression at the cellular level by a mechanism similar to antigenic modulation. Thus, antigenic modulation of FLV erythro leukemic cells might inhibit FLV genome expression in vivo and be responsible for the tumor dormant state.

Since serum from mice containing dormant FLV-infected cells produced modulating effects on FLV-transformed cells in vitro, the role of antigenic modulation in tumor suppression and emergence in vivo must be re-evaluated, at least in the exogenous viral leukemias. Antigenic modulation has been previously demonstrated to be a mechanism by which tumor cells shed their cell surface tumor-associated antigens, and thereby escape from the immune response and grow out to overt neoplasia. However, in the dormant FLV-infected mouse, FLV-infected cells persist for many months without producing neoplasia in the presence of antibody that has antigenic-modulating properties in vitro. Our in vitro studies indicate that the rate of erythroleukemia cell growth is not altered during the modulation of FLV cell surface antigens. However, preliminary in vivo experiments have revealed that dormant FLV-infected mice suppress outgrowth of small numbers of FLC-745 cells, and the FLC-745 cells in Millipore diffusion chambers in dormant FLV-infected mice are inhibited in their rate of division as compared with FLC-745 cells in chambers in normal mice. Therefore the absence of C5 and ADCC reactions in the DBA/2 mouse, coupled with studies in progress and those reported here, suggest that FLV antibodies act in vivo in conjunction with other humoral factors to suppress division of FLC-745 cells, possibly through antigenic modulation.

Summary

Friend leukemia virus (FLV) erythro leukemic cells cultured in medium containing FLV-immune serum from dormant FLV-infected mice undergo modulation of FLV cell surface antigens. Modulation was determined by an increased resistance to FLV antibody-mediated complement-dependent lysis and was associated temporally with the capping of FLV-immune complexes at the cell surface. Modulated cells regained their susceptibility to FLV antibody-mediated complement-dependent lysis when transferred to medium containing normal mouse serum. After 48 h of culture in FLV-immune serum, 26% of the FLV erythro leukemic cells were devoid of FLV cell surface antigens as demonstrated by immunofluorescence. Antigenic modulation occurred to a greater extent in cells maintained in logarithmic growth than in cells in G0 or resting phase. FLV-antigenic modulation is discussed as a possible mechanism by which antibody induces and maintains FLV-transformed cells in a dormant state.
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