Role of Suppressor Cells in Feline Leukemia Virus-associated Immunosuppression

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ABSTRACT

Protein from detergent-disrupted feline leukemia virus was found to suppress lymphocyte blastogenic responsiveness of peripheral blood mononuclear cells from normal cats. When suppressive doses of this protein were substituted for mitogen in the pretreatment step of the concanavalin A-induced suppressor cell assay, significant suppression was observed in five of eleven cats tested. These results suggest that feline leukemia virus-associated immunosuppression may be mediated by a virus-induced suppressor cell.

INTRODUCTION

Oncogenic viruses have been shown to impair immune responsiveness, as first demonstrated in Friend leukemia virus-infected mice (22). Since this initial observation, many systems have been used to document and understand the effect of oncogenic viruses on lymphocyte function (for reviews, see Refs. 4 and 7). For example, a variety of cellular and humoral immune functions have been found to be impaired in mice infected with Friend leukemia virus (2), Rauscher virus (10), Moloney virus (14), and Gross virus (23) and in chickens infected with avian myeloblastosis virus (12) and avian leukemia virus (24).

A model which has been utilized to study tumor-associated immunosuppression is FeLV infection of cats. FeLV is an oncogenic retrovirus which has been shown to be immunosuppressive, either in association with tumor development or as a nononcogenic manifestation of viral infection (9). FeLV has been shown to impair immune reactivity in infected cats, manifested by decreased lymphocyte blastogenic responsiveness (5) and increased incidence of intercurrent disease (6). The mechanisms of FeLV-induced immunosuppression are poorly understood, but there is evidence for a direct viral effect on lymphocyte function. This is shown by the ability of whole killed FeLV to inhibit mitogen-induced blastogenic responsiveness of normal cat lymphocytes (11). More specifically, the p15 protein antigen of the virus envelope has been shown to be responsible for this inhibition (19).

Particular attention has been given to suppressor cell function in the immunosuppression associated with virus-induced tumors in animals. This activity has been identified in murine (3, 13, 14) and avian (1, 18) retrovirus-induced tumors. Recently, several assays for suppressor cell activity in cats have been developed; for example, a short-lived suppressor cell population, which loses its effect after 24 hr in culture, has been identified. Furthermore, FeLV-infected cats demonstrated a loss of this function following viral infection (25). Secondly, it has been shown that the cat has a Con A-induced suppressor cell population (15) similar to those reported in humans and mice (8).

The purpose of the present study was to determine whether FeLV protein is capable of generating suppressor cell activity in a manner similar to that induced by Con A, when substituted for Con A in the Con A-induced suppressor cell assay.

MATERIALS AND METHODS

Animals. Cats of varied ages were used as a source of PBMC. All cats were from the specific-pathogen-free colony of the Division of Laboratory Animal Services, New York State College of Veterinary Medicine, Cornell University, and were free of disease, including FeLV as determined by absence of detectable antigen as assayed by enzyme-linked immunosorbent assay (18).

Reagents. Cell cultures were established in Roswell Park Memorial Institute Medium 1640, containing 2 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, gentamicin (100 µg/ml; Schering Corp., Kenilworth, N. J.), and 10% heat-inactivated fetal calf serum. Cells were washed in Hank's balanced salt solution containing 100 units penicillin, 100 µg streptomycin, and 0.25 µg Fungizone per ml. Con A (Sigma Chemical Co., St. Louis, Mo.) was used at a final concentration of 5 µg/ml in Roswell Park Memorial Institute Medium 1640 for lymphocyte blastogenesis, suppressor cell preactivation, and coculture stimulation. Unless otherwise indicated, all reagents were from Grand Island Biological Co., Grand Island, N. Y.

Mononuclear Cell Preparations. PBMC were prepared as described previously (15). Briefly, blood was collected into heparinized tubes from cats under light anesthesia via i.m. injection of ketamine hydrochloride (Ketaset; Bristol Laboratories, Syracuse, N. Y.). PBMC were separated from heparinized blood, diluted 1:2 with Hanks' balanced salt solution using a Ficol-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) gradient (400 g; 30 min). Cells from the Ficol interface were washed once and resuspended to 10⁶ cells/ml in culture medium.

Lymphocyte Blastogenesis and Con A-induced Suppressor Cell Assay. Aliquots (0.1 ml) of the PBMC suspension were cultured in quadruplicate with 0.1 ml of culture medium, with or without Con A (5 µg/ml) in 96-well flat-bottomed microtiter plates (Costar; Rochester Scientific Co., Rochester, N. Y.), in a humidified, 5% CO₂ incubator at 37°C for 72 hr. Tritiated thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, Mass.), at 0.5 µCi/0.1 ml culture medium, was added for the last 18 hr of culture.

Con A-induced suppressor cell function was assayed according to a method described previously (15). Briefly, an aliquot of the above-mentioned mononuclear cell suspension was preactivated in the presence of Con A (5 µg/ml) in a humified 5% CO₂ atmosphere at 37°C for 18 hr (Con A-preactivated cells). Another aliquot of cells was cultured under the same conditions without Con A (media-preactivated cells). A third aliquot of cells was held at 4°C for use as responder cells. After 18 hr, all aliquots were washed 3 times and brought to 1 x 10⁶/ml in culture medium. Fifty µl of responder cells were cocultured with 50 µl each of responder cells. The purpose of the present study was to determine whether FeLV protein is capable of generating suppressor cell activity in a manner similar to that induced by Con A, when substituted for Con A in the Con A-induced suppressor cell assay.

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Con A-preactivated or media-preactivated cells in exactly the same filter discs using an automated multi-sample harvester (MASH II; M. A. Bioproducts, Walkersville, Md.) and tritiated thymidine incorporation was measured by liquid scintillation spectrophotometry (LS-230; Beckman Instruments, Fullerton, Calif.).

Lymphocyte blastogenesis results were expressed as net cpm, calculated by subtracting cpm of unstimulated from stimulated cultures. Con A-induced suppressor cell activity was expressed as percentage suppression, calculated using the equation

\[
\text{% suppression} = \left(1 - \frac{A - B}{C - D}\right) \times 100
\]

where A is Con A-stimulated, Con A-preactivated cpm, B is unstimulated Con A-preactivated cpm, C is Con A-stimulated, media-preactivated cpm, and D is unstimulated media-preactivated cpm.

Preparation of FeLV Protein. Cell-free viral extract was prepared from culture supernatants of FL-74 cells, a continuous feline lymphoblastoid cell line producing FeLV (26), by the standard Moloney (21) method of differential centrifugation. The resulting extract was inactivated by incubation with Dulbecco's phosphate-buffered saline containing 0.1% Triton X-100 (Sigma), with agitation at 37° for 30 min. The detergent was removed with successive ether extractions, and the ether was blown off with N2 gas. The resulting viral protein was concentrated and exhaustively dialyzed by negative-pressure dialysis against Dulbecco's phosphate-buffered saline. The final concentration of protein was 1 mg/ml as determined by the technique of Lowry et al. (17). This protein showed reactivity with a caprine anti-FeLV serum in immunodiffusion, and did not differ from the initial cell-free virus concentrate, as determined by polyacrylamide gel electrophoresis. BSA was similarly processed and brought to the same protein concentration and used as a control for a nonspecific protein effect.

Results

Effect of FeLV Protein on Lymphocyte Blastogenesis. Cultures were established with 0, 0.5, 5.0, or 50 μg of either FeLV or BSA protein per well of 10^6 cells in addition to normal stimulatory concentrations of Con A. No significant differences were seen between FeLV and BSA at 0, 0.5, or 5.0 μg of protein (Table 1). However, at 50 μg protein per well, there was a statistically significant depression of blastogenesis by FeLV compared to BSA in all 3 cats tested. Therefore, 50 μg FeLV protein per 10^6 peripheral blood lymphocytes were used as a preactivating dose in suppressor cell experiments.

Ability of FeLV to Generate Suppressor Cells. Cells from 11 cats were tested for their ability to generate suppressor cells in the presence of Con A or FeLV. Six of these 11 cats generated suppressor cell activity with Con A preactivation; however, statistically significant suppression was observed in only 3 of these 6 cats (Table 2). Eight of 11 cats generated suppressor cell activity with FeLV preactivation; however, statistically significant suppression was observed in only 5 of these 8 cats. None of the cats that generated significant suppression with Con A preactivation (Cats 5, 6, and 11) generated significant suppression with FeLV preactivation. Conversely, none of the cats that generated significant suppression with FeLV preactivation (Cats 1, 2, 3, 7, and 10) generated significant suppression with Con A preactivation.

Viability of Preactivated Cell Suspensions. Viability of preactivated cells after the preactivation step was determined by trypan blue exclusion, following washing, prior to coculture. Cells were 98 ± 1 (S.E.), 96 ± 2, and 96 ± 2% viable for media-preactivated, Con A-preactivated, and FeLV-preactivated cells, respectively. Viability was also determined following the 72-hr coculture period. Unstimulated cocultures were 77 ± 2, 72 ± 7,

### Table 2

<table>
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<tr>
<th>Cat</th>
<th>Media</th>
<th>Con A</th>
<th>FeLV</th>
<th>Con A</th>
<th>FeLV</th>
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*Net cpm (stimulated minus unstimulated cocultures).

* p < 0.005 between media-preactivated and Con A- or FeLV-preactivated cocultures (Student's t test).

* p < 0.05 between media-preactivated and Con A- or FeLV-preactivated cocultures.

### Table 1

<table>
<thead>
<tr>
<th>Cat</th>
<th>Protein</th>
<th>Con A</th>
<th>0 μg protein/well</th>
<th>5 μg protein/well</th>
<th>10 μg protein/well</th>
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<tbody>
<tr>
<td>1</td>
<td>FeLV</td>
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<td>15,712 ± 689a</td>
<td>19,041 ± 1,210</td>
<td>18,472 ± 1,205</td>
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<td></td>
<td>BSA</td>
<td></td>
<td>141 ± 37</td>
<td>99 ± 14</td>
<td>82 ± 8</td>
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<td></td>
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<td>70 ± 8</td>
<td>98 ± 8</td>
<td>77 ± 18</td>
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<tr>
<td>2</td>
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<td>11,194 ± 158</td>
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<tr>
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<td>88 ± 9</td>
<td>88 ± 16</td>
<td>103 ± 16</td>
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<td></td>
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<td>103 ± 5</td>
<td>80 ± 10</td>
<td>80 ± 10</td>
</tr>
<tr>
<td>3</td>
<td>FeLV</td>
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<td>11,170 ± 1,156</td>
<td>11,521 ± 259</td>
<td>9,733 ± 614</td>
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<tr>
<td></td>
<td>BSA</td>
<td></td>
<td>79 ± 3</td>
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<td>93 ± 7</td>
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<td></td>
<td></td>
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<td>76 ± 6</td>
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</table>

*Mean ± S.E.

* p < 0.001 between cultures containing equal concentrations of either FeLV or BSA protein (Student's t test).
and 80 ± 1% viable for media-preactivated, Con A-preactivated, and FeLV-preactivated cells, respectively. Stimulated cocultures were 61 ± 4, 60 ± 6, and 75 ± 2% viable for media-preactivated, Con A-preactivated, and FeLV-preactivated cells, respectively.

**DISCUSSION**

The results of this study have shown that protein derived from detergent-disrupted FeLV is capable of suppressing lymphocyte blastogenic responses of lymphoid cells from normal cats. Furthermore, when suppressive concentrations of this protein were substituted for Con A in the Con A-induced suppressor cell assay, suppressor cell activity was observed in cocultures containing FeLV-preactivated cells.

The finding that detergent-disrupted virus can inhibit lymphocyte blastogenic responsiveness is in agreement with previous studies (11) which demonstrated that UV-inactivated FeLV suppresses lymphocyte blastogenesis. It is noteworthy that both killed, intact virus and disrupted virus are capable of suppressing lymphocyte blastogenesis, since the nature of the virus preparation has been shown to be important in other systems of virus-mediated suppression of lymphocyte blastogenesis (12).

The suppression observed in the present study was not due to cytotoxicity against the responding lymphocytes since there was no difference in viability of cells exposed to FeLV protein compared to cells exposed to medium alone, or to mitogenic doses of Con A. Furthermore, there was no carryover of toxic substances from the disruption procedure since equal concentrations of BSA, treated with the same disruption procedure, failed to suppress blastogenesis. It should be noted, however, that the amount of viral protein used is based on a crude virus preparation, and the amount of specific virus protein is probably lower than the total protein present in the preparation.

A mechanism which may be relevant in virus-mediated suppression of lymphocyte blastogenesis is the induction of virus-induced suppressor cells. While this has been observed in vivo with several murine (3, 13, 14) and avian (1, 16) retroviruses, the in vitro induction of suppressor cells by these viruses has not been identified. Therefore, in the present study, FeLV protein was substituted for Con A in the preactivation step of the Con A-induced suppressor cell assay, in an attempt to detect FeLV-induced suppressor cells. When this was done, statistically significant suppression was seen in 5 of 11 cats tested. PBMC from the same 11 cats were also tested in the mitogen-induced suppressor cell assay, and only 3 of 11 cats generated significant suppressor cell activity. This is a much lower incidence than was seen in previous studies of feline Con A-induced suppressor cells (15) and is unexplained. It is interesting to note, however, that there was a mutual exclusion in the ability to generate suppressor cells, in that cells which generated suppressor activity following Con A preactivation did not generate FeLV-induced suppressor activity, and vice versa. The reasons for and the strictness of the mutual exclusion of the ability of cells from any given cat to generate either Con A- or FeLV-induced suppressor activity are unknown. However, the results are compatible with the hypothesis that diminished blastogenesis in FeLV-infected cats is due to a virus-induced suppressor cell which is not detectable in the Con A-induced suppressor cell assay.

Additional studies are necessary to determine the significance of virus-induced suppressor cells in FeLV-associated immunosuppression. (a) Experiments should be performed to detect the activity of in vivo-generated, virus-induced suppressor cells during FeLV infection and FeLV-induced neoplasia, as assayed by the ability of PBMC from infected animals to suppress proliferation of normal homologous cells. (b) It would be appropriate to attempt to identify an in vitro-generated, virus-induced suppressor cell in other examples of oncogenic virus infection where in vivo-generated suppressor cell activity has been identified (1, 3, 13, 14, 16). (c) Since the p15 viral envelope antigen has been shown to be the immunosuppressive moiety of FeLV (19, 20), if virus-induced suppressor cells are significant in FeLV-associated immunosuppression, it would be expected that p15 could induce suppressor cell activity similar to that induced by FeLV protein in the present study and, conversely, that removal of p15 from the FeLV protein mixture would abrogate this suppressive activity.

It is also necessary to determine the relationship between the Con A- and FeLV-induced suppressor cells. It is necessary to determine whether FeLV and Con A induce separate suppressor populations and whether the FeLV-induced cell can affect the activity of the Con A-induced cell. (a) The characteristics of the FeLV-induced suppressor cell need to be identified and compared to the characteristics of the Con A-induced cell. (b) The effect of anti-FeLV serum on either cell population needs to be investigated. It is expected that the induction of the FeLV-induced cell could be inhibited by this antiserum, but the induction of the Con A-induced cell would not be affected. (c) An appropriate way of examining the interaction between the Con A- and FeLV-induced suppressor cell would be to isolate the suppressor cell populations after preactivation with either Con A or FeLV and to examine what effect the alternate preactivator has on either cell population.

In conclusion, results presented in this study have added to the knowledge of feline immunoregulatory networks. Our results suggest that FeLV-associated immunosuppression may be mediated by a virus-induced suppressor cell, which is distinct from other nonspecific suppressor cells which are active in both healthy and FeLV-infected cats.

**ACKNOWLEDGMENTS**

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