Detection and Evaluation of Feline Oncornavirus-induced Cell Surface Antigen(s) Shed from Cells in Vitro

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SUMMARY

A method for preparation of soluble feline oncornavirus-induced cell surface antigens was described. This technique relied upon the natural release of antigen(s) from FL-74 feline lymphoblastoid cells during their maintenance at 37°C in serum-deficient medium. When concentrated and clarified spent medium from 4-day cultures was tested for its antigen content by inhibition of humoral cytotoxicity, it was found that this natural production of soluble antigen provided more feline oncornavirus-associated cell membrane antigen per cell than did a solubilization procedure in which papain was used. The shed antigen preparation was immunogenic in cats, eliciting humoral antibody that was reactive with the surface of FL-74 cells and feline sarcoma virus-transformed nonproducer mink cells but was not reactive with feline leukemia virus in a virus neutralization assay.

INTRODUCTION

Neoantigens have been demonstrated on the surface of RNA tumor virus-transformed cells (16). One (or more) of these antigens is quite immunogenic and has received attention because it elicits an immune response in cats that is capable of protecting them against tumor development (16, 18-20, 26).

In a previous paper, we reported that surface antigen(s) including a nonvirion FOCMA could be recovered in soluble form following papain digestion of tumor cell membranes (38). This technique was not adequate for production of quantities of antigen needed for biochemical characterization and for vaccine studies. In addition, this type of antigen preparation was unstable upon storage.

We describe in this paper a method for preparation of surface antigens that are naturally released in culture and demonstrate that the soluble antigen prepared by this technique is immunogenic in cats.

MATERIALS AND METHODS

Cells and Virus. The FL-74 feline lymphoblastic cell line, derived from a KT-FeLV-induced cat lymphoma (36) was cultured as previously described (27). Feline sarcoma virus-transformed nonproducer mink cells and normal mink cells, designated 64F3Ci7 and Mvl-Lu, respectively, were kindly provided by Myron Essex, Harvard University, School of Public Health, Boston, Mass. These cells were maintained in Dulbecco's modified Eagle medium (Grand Island Biological Co., Grand Island, N. Y.) with 10% fetal calf serum. KT-FeLV was harvested and purified from FL-74 cells as described in an earlier report (25).

Antisera. Reference antisera for cytotoxicity inhibition assay was obtained from a cat that had regressed a Snyder-Theilen feline sarcoma virus-induced fibrosarcoma. The serum was adsorbed with 10¹¹ particles each of intact and ether-disrupted KT-FeLV. The incubation was carried out at 4°C for 15 hr, and virus was removed from the sera by centrifugation at 100,000 x g for 1 hr.

Antibody Assays. The indirect membrane fluorescence (18) and microcytotoxicity (24) assays were used to quantitate antibody specific for the cell surface of feline leukemia virus-transformed cells as described previously. In this test, 64F3Ci7 and Mvl-Lu cells were used at a concentration of 4 x 10⁶ cells/ml and FL-74 cells were used at a concentration of 8 x 10⁶ cells/ml. A virus neutralization assay was used to test for feline oncornavirus-specific antibody (31).

Recovery of Soluble Tumor Antigens from Culture Fluid. FL-74 cells, grown in roller bottles to a saturation density of 4 to 5 x 10⁶ cells/ml, were washed 3 times in serum-free McCoy's 5A growth medium (Grand Island Biological) containing gentamicin sulfate, 50 μg/ml (Gentocin; Schering Corporation, Kenilworth, N. J.), and mystatin, 10 units/ml (Mycostatin; E. R. Squibb and Sons, Inc., New York, N. Y.). Cells were resuspended to the original concentration in serum-free medium and maintained at 37°C for 4 days. For harvesting of the released antigen, the culture medium was clarified by centrifugation at 400 x g for 10 min and at 16,000 x g for 15 min. The supernatant was then concentrated 40-fold by ultrafiltration and dialysis against Aquacide III (Calbiochem, Los Angeles, Calif.). This was followed by a final clarification at 100,000 x g for 1 hr.

Quantitation of Soluble Tumor Antigens. To quantitate soluble tumor cell surface antigen, we used a cytotoxicity inhibition assay as reported previously (38).

Papain Solubilization of Cell-associated FOCMA. Cell membrane antigen solubilization was carried out according to the method of Law and Appella (22).

Protein Determinations. Protein was quantitated by the method of Lowry et al. (23) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard.
Evaluation of the Immunogenicity of Soluble Shed Antigen. Two specific-pathogen-free cats, 6.5 and 7.0 weeks old, received 4 weekly inoculations with 0.5 ml of the antigen preparation (20 mg of protein) mixed with either 0.5 ml of Freund's complete adjuvant (1st 3 inoculations) or 0.5 ml of incomplete Freund's adjuvant (last inoculation).

Adsorption of Hyperimmune Antisera with Normal Cat Thymocytes. An equal number of normal thymocytes from three 2-month-old specific-pathogen-free cats were washed in Hanks' balanced saline solution and resuspended in the hyperimmune antiserum at a concentration of \(1.5 \times 10^8\) cells/ml and incubated for 30 min at 37°. The serum was collected following centrifugation of the cell suspension at 400 x g for 10 min. It was then adsorbed 2 more times with the same number of cells.

RESULTS

Shed Tumor Surface Antigens from Cell Cultures. The release of soluble tumor surface antigen from FL-74 cells into the supernatant fluid occurred during the normal exponential growth of cells in culture and during maintenance of cells at 37° in fetal calf serum-free medium. Examination of cells in deficient medium showed that the viable cell count did not diminish more than 50%, and the concentration of antigens on the cell surface remained high during a 4-day maintenance period (Table 1).

Concentrated culture supernatants from cells maintained in serum-free medium for 4 days were capable of inhibiting the cytotoxic reactivity of an antiserum from a cat that had regressed a fibrosarcoma. This reference antiserum was specific for a nonviroin FOCMA component since it was adsorbed prior to use with both ether-disrupted and intact virus. In Chart 1, the degree of inhibition is shown for varying concentrations of antigen; a straight line was obtained when the antigen content was presented on a log scale.

A study was carried out to determine whether the antigen recovery in culture fluid was superior to that in papain digests. It was found that the overall yield of soluble FOCMA in either serum-containing or serum-free medium was 30 to 95 times that in papain preparations from an equivalent number of cells (Table 2). In addition, the specific activity of the serum-free medium preparation was 24 times that of medium with serum.

Immunogenicity of Soluble Antigen Preparation. The immunogenic potential of soluble antigen was tested in 6 to 7-month-old cats, 648B and 655B, who received 4 weekly inoculations of soluble antigen (from FL-74 cell serum-free spent culture medium) emulsified with adjuvant. Cat 648B also received an equivalent dose of antigen as a booster 6 weeks after the last inoculation. Antisera from serially bled cats were tested for indirect membrane fluorescence and microcytotoxicity activity on the KT-FeLV-infected and -transformed FL-74 cells and, as shown in Table 3, antibody activity was demonstrated in the sera of both cats by the 2nd or 3rd week following the initial inoculation. This activity increased to high levels from 2 to 5 weeks postinoculation. Antiserum from 648B (Postinoculation Week 12) and 655B (Postinoculation Week 5) did not produce fluorescence on thymus cells from 3 different specific-pathogen-free cats, and when the antisera were exhaustively adsorbed with normal thymocytes titer on FL-74 cells were not decreased. This demonstrated that the antiseras was not reacting with a normal feline surface antigen on the
FL-74 cells. Table 3 also presents the immunofluorescence titers of antisera from both animals when reacted with feline sarcoma virus-transformed nonproducer mink cells (64F3C17). Antisera that were positive in this assay did not produce membrane fluorescence on normal mink cells (MvI-Lu). Further testing showed that 64BB and 655B sera had no virus neutralization activity against feline leukemia virus pseudotypes of murine leukemia virus.

**DISCUSSION**

This study demonstrates that feline oncornavirus-induced cell surface antigens are shed from tumor cells in culture. When the phenomenon was exploited for the production of soluble antigen, we found that, from a given number of cells, we were able to obtain a greater amount of FOClMA than by a method in which papain solubilization was used (Table 2). This is significant because the use of harsh extraction procedures (e.g., treatment with high-molarity salt solutions, proteolytic enzymes, or detergents) in preparing any of the components can be avoided.

The release of soluble oncornavirus-induced antigen is most probably due to the turnover of membrane proteins, a normal process that occurs in metabolically active cells. Previously, we showed that production of FOClMA in FL-74 cells was cell cycle dependent, and it was proposed that the density of the antigen(s) on the cell surface is increased when cells pass through the cell cycle at a slow rate (27). This was in accordance with the observations of other investigators (6, 11, 12) who have shown maximum expression of cell surface antigens in the G1 phase of the cell cycle. It appears then that tumor antigen expression is enhanced rather than blocked in live cells that are unable to move on to mitosis. For this reason, we were not surprised that serum-deprived cells, most probably inhibited in their ability to synthesize DNA (35), were able to produce antigen abundantly; it was demonstrated both on the surface of cells and in the culture medium. The results are also consistent with those obtained for guinea pig and mouse tumor cell antigens, which indicate that the primary source of soluble antigen in culture is viable cells as opposed to autolyzing cells (7, 28).

The natural release of antigen from oncornavirus-transformed cells has interesting implications regarding the in vivo dynamics of tumor cell growth and resistance to immunological attack. One might suspect that in the cat, as in other experimental animals and man, soluble tumor antigen is released from progressively growing neoplasms into the body fluids (2, 8, 14, 15, 37) and that soluble antigen and antigen-antibody complexes block the destruction of tumor cells by lymphocytes (3, 5, 21, 32). Some investigators (1, 13) have observed a correlation between the amount of antigen shed from sarcoma cells in vitro and the extent of metastasis of the cells in vivo.

One objective of this study was to determine whether the immunogenic tumor surface component(s), when free from its normal association with the cell surface and in soluble form, was still capable of eliciting a response in cats. It was shown that cats could respond to the cell-free antigen(s) by producing high titers of tumor cell-specific antibody. In addition, results demonstrated that there were antibodies present in the serum that were reactive to a nonviral FOClMA surface component. The demonstration of a neoantigen that is unrelated to antigens of the oncornavirus is in accordance with previous work in our laboratory (38) and serological studies of Essex and others (10, 17, 31, 33, 34).

The immunogenic potential of these antigens is consistent with the capacity of other similar soluble antigens from oncornavirus-related tumors to elicit antitumor antibodies (4, 9). Sensitization of mice with soluble antigens has resulted in both protective immunity to tumors (9, 22) and blocking of cell-mediated immunity to tumors through the induction of enhancing antibody (30). Further investigations will be necessary to show whether or not soluble feline oncornavirus-induced antigens can elicit a positive protective response in cats.

Studies are continuing in our laboratory to deal with the purification and characterization of tumor antigens described in this study.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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