Ferritin-labeled Antibody Studies of Feline C-type Particles

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**SUMMARY**

The application of an indirect ferritin-labeled antibody method to tissue culture lines established from cats suffering from various malignant diseases revealed tagging of viruses and certain areas of the cell surface membranes. Dog antifeline sarcoma and cat antifeline sarcoma sera were used as the intermediate sera on feline C-type virus-positive lines established from 3 cases of malignant lymphoma, 1 erythroleukemia, and 1 idiopathic agranulocytosis. The tagging of viruses and membranes was similar in all the lines, indicating a similarity or sharing of antigenic components. Each instance of ferritin-labeled antibody tagging previously had been shown to be positive with fluorescent antibody staining, showing that there is correlation between fluorescent antibody staining of feline C-type virus-producing cells and ferritin tagging of the virus and infection-mediated membrane antigens.

**INTRODUCTION**

The application of the indirect fluorescent antibody method to tissue culture lines established from cats suffering from various malignant diseases revealed 2 distinct fluorescent staining patterns around the periphery of unfixed suspended cells. One pattern showed heavy staining on the cell surface indicative of a large accumulation of antigenic materials, and the other showed a sharp, thin layer of antigen on the cell surface. For determination of the morphology of the structures which were being stained, ferritin-labeled antibodies were applied to tissue culture cells established from several different feline tumors.

**MATERIALS AND METHODS**

**Antisera.** Serum XD25M was obtained from a 1-year-old female beagle which was inoculated with 1.5 ml of a 10% tissue homogenate from a tumor induced by inoculation of FSV into her litter of puppies 1 day postpartum. No tumor developed, and a 2nd inoculation of a similar preparation (4 ml in Freund’s complete adjuvant) was administered approximately 2 months later. The serum was obtained 6 weeks subsequent to the 2nd inoculation and absorbed with cat liver powder followed by a mixture of frozen and thawed BHK-21, BSC-1, and human fetal diploid lung tissue culture cells. Serum XC25M was obtained from a pregnant cat which had aborted her litter 2 days after her fetuses had been inoculated in utero with a concentrated FSV pool prepared by the method of Moloney (18). Two weeks later, the cat was inoculated (1 ml i.m.) with a similar Moloney preparation of FSV. A localized swelling occurred and subsequently regressed. The animal was exsanguinated 3 months after the i.m. inoculation, and serum was absorbed with cat liver powder followed by a mixture of frozen and thawed BHK-21, BSC-1, and human fetal diploid lung tissue culture cells. The antisera XD25M and XC25M were supplied by Dr. Murray B. Gardner (Department of Pathology, University of Southern California School of Medicine, Los Angeles, Calif.) and Dr. Paul Arnstein (National Cancer Institute, California State Department of Public Health, Berkeley, Calif.).

**Ferritin Conjugates.** Rabbit anti-dog γ globulin and rabbit anti-cat globulin were prepared by immunizing rabbits with either canine or feline γ-globulin. The immune rabbit serum was treated with 14% saturated sodium sulfate, and the resultant precipitate of crude γ-globulins was labeled with horse spleen ferritin (Pentex) by using xylylene diisocyanate (25, 27). The conjugate was absorbed with a pool of frozen and thawed human fetal diploid lung and BHK-21 and BSC-1 cells followed by frozen and thawed tissue culture cells established from normal, whole feline fetuses.

**Cell Culture.** The FL-74 cell line was obtained from Dr. G. H. Theilen and Dr. T. G. Kawakami, University of California, Davis, Calif. (30). The cells were derived from a cat with malignant lymphoma and were grown in suspension in 8-oz prescription bottles in L-15 medium (17) fortified with 20% FBS. The cells were subcultured at weekly intervals by dividing the cells equally to establish new cultures. The cultures were then fed once with an equal volume of medium approximately 3 to 4 days after being subcultured.

**F-1b and F-8 Cell Lines.** These 2 cell lines were established in this laboratory from trypsinized lymph nodes from cats with malignant lymphoma. The cells were grown as monolayers in 8-oz prescription bottles and were subcultured at 3- to 4-day intervals. Growth medium consisted of Eagle’s minimum essential medium in Earle’s balanced salt solution fortified with 10% FBS.

**F-25 Cell Line.** This cell line was established in this laboratory by trypsinization of a lymph node from a cat with...
erythroleukemia and was subcultured in the same manner as the F-1b and F-8 cell lines.

**F-31 Cell Line.** This cell line was established in this laboratory by trypsinization of a lymph node from a cat with an idiopathic agranulocytosis and was subcultured in the same manner as the F-1b, F-8, and F-25 cell lines. The interval between subcultures, however, was extended to 4 weeks because of a much slower growth rate.

**FFc-79 and NFF-35 Cell Line.** FFc-79 cell line was obtained from the Cell Culture Division, Naval Biological Laboratory, University of California, Oakland, Calif. NFF-35 cell line was established in this laboratory by Dr. John L. Riggs. The cells were established from a pool of several whole fetuses and maintained in culture as monolayers. Eagle’s minimum essential medium in Earle’s balanced salt solution fortified with 10% FBS was used as the outgrowth medium.

**In Vitro Studies.** FFc-79 cells were grown in a 2-oz prescription bottle and inoculated with 0.5 ml of virus from F-1b cell line containing 10⁴ tissue culture infectious doses/ml (as assayed by fluorescent microscopy). In order to increase the number of infected cells so that viruses can readily be observed by electron microscopy, the cells were subcultured 7 days after infection and again 4 days later. The cells from the 2nd passage were grown in Petri dishes containing coverglasses (22 mm sq). Three days later, the coverglasses containing infected cells were processed for ferritin-labeled antibody tagging.

**Staining Technique.** Cells were stained by the indirect ferritin-labeled antibody method. Five to 10 million cells from the suspension culture (FL-74) were recovered by centrifugation (500 X g for 5 min) in a siliconized conical centrifuge tube and then washed twice with PBS, pH 7.5, containing 0.05% gelatin. Two drops of a 1:4 dilution of the intermediate serum were added to the cell pellet, and cells were resuspended in the serum with a Vortex mixer. The cells were incubated at 37° for 1 hr and washed 4 times with PBS containing gelatin. Ferritin-labeled antibodies (3 drops) were added to the pellet from the final wash, resuspended with the Vortex mixer, and incubated for 30 min at 21°, followed by washing 4 times with PBS containing gelatin. The pellet was rinsed in phosphate buffer, pH 7.2, and postfixed in 1% osmium tetroxide. The pellet was rinsed in phosphate buffer, dehydrated in a graded series of ethanol concentrations, and embedded in Epon for thin sectioning. The sections were stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop I.

Cell lines F-1b, F-8, F-25, F-31, FFc-79, and NFF-35 were prepared on coverglasses (22 x 40 mm). The complete monolayer cultures were washed several times by dipping into beakers containing 0.1 M phosphate buffer, pH 7.2, and covered with 1:8 and 1:16 dilutions of XD25M serum. Similar dilutions of normal dog serum were used as controls. The coverglasses were incubated for 20 min at 37° and washed by initially flooding the coverglass with phosphate buffer, followed by immersion into 4 different containers of buffer. The washed cells were covered with ferritin-labeled rabbit anti-dog conjugate and incubated at 21° for 20 min. Unreacted ferritin was washed off by flooding the coverglass with buffer, followed by immersion and gentle agitation in 4 different containers of buffer over a 10-min period. The cells were then fixed by immersion in a 1% solution of glutaraldehyde, scraped, and pelleted. The pellet was washed in phosphate buffer and further processed for electron microscopy.

**RESULTS**

Canine anti-FSV (XD25M) and feline anti-FSV (XC25M) sera were found to have specific activity against feline C-type particles and infection-mediated cell surface antigens as determined by indirect ferritin-labeled antibody tagging. XD25M serum has also been shown to have neutralizing activity against the viruses from cell lines F-1b and F-8. Fig. 1 illustrates the distribution of particles on or near the surface of FL-74 cells prior to treatment with ferritin-labeled antibodies. When XD25M or XC25M serum was applied to FL-74 cells, the viral particles were tagged, in addition to certain areas of the cell surface membranes. Figs. 2 to 5 illustrate ferritin tagging of FL-74 cells with XD25M serum as the intermediate serum. Fig. 2 is a low-magnification view of the distribution of antigens on the surface of several cells which are in various stages of maturation. Cells labeled A, B, and C have a heavy accumulation of ferritin on large clusters of viral particles, while Cell D shows distinct membrane tagging (arrows). The distribution of viral particles around Cell B is shown in Fig. 3. Note the dense layer of material, presumably the complex formed by the dog anti-FSV and rabbit anti-dog γ-globulin, between the outer shell of the viral particles and ferritin granules.

The distribution of membrane antigens around a cross-section of the whole cell similar to that shown in Cell D (Fig. 2) is illustrated in another cell in Fig. 4. A higher magnification of the area depicted by arrows is illustrated in Fig. 5.

When the feline anti-FSV serum (XC25M) was used as the intermediate serum on FL-74 cells, the results were identical to those shown in Figs. 2 to 5. Both viral (V) and membrane (M) tagging (Fig. 6) were evident and were distributed in a similar manner. The budding surface of an immature particle is antigenically distinct from the host cell surface membrane, as is evident by ferritin tagging (Fig. 7). The ferritin on one side of the budding particle is presumably due to virus-induced membrane antigen. As shown in Fig. 8, the application of normal cat control serum resulted in much less viral or membrane tagging. Occasionally, ferritin granules are caught between the C-type particles which tend to aggregate in large clusters in this cell line.

For determination of whether other feline cell lines established from malignant lymphomas (F-1b and F-8), an erythroleukemia (F-25), a case of an idiopathic agranulocytosis (F-31), and normal whole embryos (FFc-79 and NFF-35) possessed similar antigens, XD25M serum was used as the intermediate serum on cells prepared on coverglasses (Table 1). Fig. 9 shows a budding particle from the first passage level of cell line F-1b. Like the particles in the FL-74 cell line, viral particles in cell line F-1b were tagged with ferritin (Fig. 10). The normal dog serum control was negative.
Cell line F-8, which was established from another case of malignant lymphoma, was similar to F-1b. Particles were observed in the lymph node (Fig. 11) and in the tissue culture cells established from it. The particles in the tissue culture cells were also readily tagged with ferritin-labeled antibodies (Fig. 12). Cell line F-25 was established from a case of erythroleukemia and contained particles in tissue culture cells. Viral (Fig. 13) and membrane (Fig. 14) tagging were also readily observed. The C-type particles present in cell line F-31 showed tagging similar to those observed in cell lines derived from neoplasms.

To exclude the possibility that the membrane tagging was due to altered surface antigens common to malignant cells having nothing to do with virus production, normal feline cells were infected in vitro and tested for ferritin-labeled antibody tagging. The viral and membrane tagging were similar to those observed in the lines established from affected host tissues.

All the lines were treated with a normal dog serum followed by ferritin-labeled rabbit antidog IgG antibody and were found to be negative. Two normal feline cell lines (FFc-79 and NFF-35) were treated with XD25M or normal dog serum and were also found to be negative.

**DISCUSSION**

Fluorescent antibody studies by Riggs et al. of the cell lines described in the present study suggested that ferritin tagging is closely correlated with fluorescent staining. The 2 distinct patterns of fluorescence which were observed correspond to the 2 types of structures which were tagged with ferritin-labeled antibodies. For example, the intense peripheral fluorescent staining representing large accumulations of antigenic material apparently correspond to the large clusters of ferritin-tagged virus which were distributed unevenly around the cell surface (Fig. 3).

The 2nd type of fluorescent pattern which was reported is the thin but intensely staining layer around the cell periphery and corresponded to the heavy layer of ferritin on certain areas of the cell surface membrane (Figs. 4 and 5). This type of virus-induced antigenic alteration of cell surface membranes is not unique with the feline leukemia virus, for it has also been shown by electron microscopy with influenza (3, 7, 19), herpes simplex (20), parainfluenza (12), mumps (6), and rubella (21) viruses. Similarly, dense layers of antibody have been observed by Dunkel and Zeigel (8) on Epstein-Barr virus and its infected cells when treated with antibody. Additional evidence for virus-induced membrane changes was shown by the observations of several investigators that hemadsorption can occur, between the infected cell plasma membrane and red blood cell, without the viral particle acting as the bridge (2, 4, 5, 6, 11, 21).

The possibility exists that the membrane antigens are virus-induced cell surface antigens which differ antigenically from the viral envelope. Inasmuch as the intermediate sera used in these studies were dilutions of whole serum, this method could not distinguish the existence of 2 or more antigens. In this regard, the use of ferritin coupled to pure antibody such as that which could be eluted from a complex of purified C-type particles and antibody to determine the antigenic differences or similarities between the viral envelope and the surface membrane antigen is contemplated. Alternatively, should antibody to only the infection-mediated cell surface antigen in the feline leukemia system, such as that described for the Gross leukemia cell system (1) or in the Epstein-Barr membrane-positive viral-negative serum system (23), become available, then the use of such an antibody could also distinguish the differences, if any, between the feline C-type viral envelope and its infection-mediated membrane antigen. Nevertheless, the observations indicate a similarity of these antigens, for there appears to be a direct correlation in the observation of the frequency of membrane tagging with the numbers of particles observed. That is, membrane tagging was readily observed when FL-74 cells were used, whereas cell line F-1b, which produces less virus than FL-74 cells, only rarely displayed membrane tagging. Similarly, F-25 cell line, which is intermediate in the numbers of viral particles observed, was also intermediate in the frequency in which
membrane tagging was observed. Furthermore, there was a good correlation between the presence of C-type particles and fluorescent staining. Pearson et al. (22) recently reported that Epstein-Barr virus-induced membrane antigens were also expressed on the enveloped virus particles.

The observation of viral and membrane tagging of in vitro-infected normal feline cells indicates that the membrane antigen was infection-mediated and also excludes the possibility that the membrane tagging observed in this study is a characteristic of transformed cells unrelated to viral infection. The observation of feline C-type virus from cats with various forms of tumors have now been reported in several instances (Refs. 9, 10, 13–16, 24, 26, 28–30 and Footnote 2). In the present study, each instance of ferritin-labeled antibody tagging had previously been shown to be positive with fluorescent antibody staining, and, therefore, there appears to be a good correlation between fluorescent antibody staining of feline C-type producing cells and ferritin-labeled antibody tagging of viruses and its infection-mediated membrane antigens. The cell surface membrane antigens were shown to be infection mediated, as indicated by ferritin tagging of viral particles and membranes of infected cells but not of normal cells. It must be emphasized, however, that the application of the ferritin-labeled antibody methods to unfixed whole cells would not label intracytoplasmic and intranuclear antigens. In our continuing studies of the possible intracellular location of viral envelope and group-specific antigens, the application of ferritin-labeled antibodies on frozen and thawed cells to permit entry of the ferritin-labeled antibody conjugates will be performed.

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REFERENCES

Fig. 1. Clusters of mature C-type particles are shown around an FL-74 cell. X 28,000.

Fig. 2. A low-magnification view of several cells showing viral particles tagged on the surface of Cells A, B, and C; Cell D shows surface membrane tagging (arrows). X 12,000.

Fig. 3. A higher magnification of Cell B from Fig. 2 showing numerous C-type particles tagged with ferritin-labeled antibodies. X 20,000.

Fig. 4. Whole cell, showing the distribution of membrane tagging. Arrows, area shown in Fig. 5. X 13,500.

Fig. 5. Part of cell showing surface membrane tagging. X 27,000.

Fig. 6. Parts of 3 FL-74 cells which have been treated with XC25M serum. Both viral particle tagging (V) and membrane tagging (M) are shown. X 22,000.

Fig. 7. A budding C-type particle tagged with ferritin-labeled antibody. X 60,000.

Fig. 8. Normal cat serum control on FL-74 cells in the indirect ferritin antibody method. Some ferritin granules are caught between viral particles since the C-type particles tend to aggregate in large clusters in this cell line. X 57,000.

Fig. 9. A budding C-type particle from cell line F-1b. X 60,000.

Fig. 10. C-type particles in cell line F-1b are tagged with ferritin-labeled antibodies. X 60,000.

Fig. 11. C-type particles within the cytoplasm of a macrophage from the lymph node of a cat (F-8) with malignant lymphoma. X 38,000.

Fig. 12. C-type particles in cell line F-8 are tagged with ferritin-labeled antibodies. X 60,000.

Fig. 13. C-type particles in cell line F-25 are tagged with ferritin-labeled antibodies. X 60,000.

Fig. 14. Part of cell line F-25 showing surface membrane tagging. X 60,000.
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