[CANCER RESEARCH 35, 1064-1068, April 1975]

A Mixed-Immunoglobulin Rosette Technique for the Detection of Antibody to Feline Oncornavirus-associated Cell Membrane Antigen

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SUMMARY

A mixed-immunoglobulin rosette technique has been developed for the detection of antibodies to feline oncornavirus-associated cell membrane antigens. Lymphoblastoid cells infected with feline leukemia virus were incubated with test sera and then fixed in paraformaldehyde. They were then exposed to a rabbit anti-cat immunoglobulin G serum. Antigen-antibody reactions were detected by mixing the cells with sheep erythrocytes sensitized with cat anti-sheep red blood cell serum; the presence of cat antibody on the lymphoid cells was registered by the formation of sheep red blood cell rosettes around them. The method was shown to be at least 10 times more sensitive than indirect immunofluorescence. A high degree of correlation was shown between the mixed-immunoglobulin rosette and indirect immunofluorescence tests, using both feline leukemia virus-infected cat and dog cells as targets. The results indicate that the tests are likely to be measuring similar reactions. Using the indirect immunofluorescence test we found that 75% of cats with lymphoid neoplasia had no demonstrable antibodies. Twenty-four of such indirect immunofluorescence-negative sera were tested by the mixed-immunoglobulin rosette technique; antibody was shown in at least seven of these sera.

INTRODUCTION

The protective mechanisms in viral leukemia are of considerable practical and theoretical interest. In FeLV[2] infection, 3 systems of antigens have been studied, as reviewed in Ref. 8. The group-specific antigens are structural proteins of the virion and have not yet been shown to play a part in protective immunity. The type-specific antigens are glycoproteins which project as spikes or knobs from the outer membrane of the virion. Antibodies to these are virus neutralizing. A 3rd set of antigens distinct from the latter appears on the surface of virus-infected cells. It is not now known whether these are cell or virus coded; they have therefore been termed the feline oncornavirus-associated cell membrane antigens. They are known to be of major importance immunologically in preventing the establishment of chronic virus infection and in protecting against the pathogenic effects of the feline oncornaviruses (3, 9).

Antibodies to FOCMA have until now been detected by immunofluorescence (5, 10, 11). The test is sensitive since there are available leukemia virus-infected feline lymphoblastoid cell lines that grow easily in suspension culture and express on their surfaces large amounts of FOCMA. Cat sera are caused to react with these cells; in the presence of antibody, immunoglobulin becomes attached to the membrane antigens and this is demonstrated by fluorescein-labeled anti-cat Ig by the traditional indirect immunofluorescence method. In this paper we describe another technique for detecting immunoglobulin attached to cell membrane antigens. This is a MIR test which greatly increases the sensitivity of the system. Briefly, cultured FeLV-infected lymphoid cells are incubated with the test serum. They are then exposed to a rabbit anti-cat IgG serum. Antigen-antibody reaction is detected by mixing the lymphoid cells with SRBC that have been sensitized with cat anti-SRBC serum. The presence of cat immunoglobulin on the lymphoid cells is registered by the formation of SRBC rosettes around the lymphoid cells, the cell adhesion being mediated by the double immunoglobulin bridge. The technique has been used to examine sera of cats with naturally acquired lymphoid neoplasms, cats infected experimentally with FeLV, and sera from uninfected normal cats.

MATERIALS AND METHODS

Cells

Feline lymphoid cells were from the FL74 cell line of Theilen *et al.* (12) which are chronically infected with FeLV of Subgroups A, B, and C. These cells have been grown in continuous suspension culture in this laboratory for several years. No immunoglobulin can be detected on their surface by the mixed-antiglobulin reaction (L. Mackey and R. R. A. Coombs, unpublished results).

Dog lymphoid cells were from the CT45S line, infected with FeLV5 (7). As controls, uninfected dog cells were used. No FeLV-free feline lymphoblastoid cells are available.

SRBC were from Burroughs Wellcome Limited, London, England.

[1] This work was carried out with the help of a grant from the Cancer Research Campaign.

[2] The abbreviations used are: FeLV, feline leukemia virus; FOCMA, feline oncornavirus-associated cell membrane antigens; MIR, mixed immunoglobulin rosette; SRBC, sheep red blood cells; BPA, bovine plasma albumin; PRF, percentage rosette formation.

Received November 1, 1974; accepted January 10, 1975.

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Sera

Test Sera. Sera from cats with naturally occurring lymphosarcoma were taken by cardiac puncture under anesthesia immediately before euthanasia. Samples from normal cats and cats experimentally infected with FeLV were taken from the cephalic vein or by cardiac puncture in the case of cats undergoing euthanasia. Sera were inactivated at 56° for 1 hr before use.

Before reacting with dog lymphoblastoid cells, cat sera were absorbed with acetone-dried dog tissue powder and fresh dog liver homogenate. The tissue powder was used in a ratio of 100 mg powder per ml of serum; the powder was dampened with phosphate-buffered saline (NaCl, 0.8 g/liter; KCl, 0.2 g/liter; disodium orthophosphate, 1.15 g/liter; potassium dihydrogen phosphate, 0.2 g/liter; final pH 7.2 to 7.4) and the serum was mixed and incubated at 4° for 30 min. The liver homogenate was thoroughly washed in phosphate-buffered saline and mixed with the serum in equal volumes; incubation was at 37° for 30 min. The serum was then recovered and reinactivated at 56°.

Rabbit Antiserum to Cat Globulin. Chromatographically purified feline IgG (15 mg/ml) was emulsified with Freund's complete adjuvant in equal parts. One ml was injected i.m. into each hind leg. Two months later, a further 1 ml of purified IgG was injected i.v. and the rabbit was bled for serum 1 week later. The serum was absorbed with normal cat RBC and SRBC and used at a dilution of 1:5.

Cat Antiserum to SRBC. This was kindly provided by Professor R. R. A. Coombs and used at a dilution of 1:40. The dilutions of this and the antiglobulin were determined in indirect antiglobulin tests. The rabbit antiserum was used in a range of dilutions against SRBC sensitized with different dilutions of the cat serum. The dilutions used were the highest that caused agglutination of SRBC.

Sensitization of FeLV-infected Cells. Lymphoid cells, from a culture of viability over 75% were taken into 5-cm siliconed tubes. Into each tube were placed 10^6 cells. Test sera were added at appropriate dilutions, 0.25 ml per tube, and incubated at 37° for 30 min. The cells were then washed 3 times in phosphate-buffered saline, pH 7.2.

Paraformaldehyde Treatment. Coombs *et al.* (2) demonstrated immunoglobulin on the surface of B-lymphocyte using the mixed-antiglobulin reaction. This was subsequently improved in sensitivity by S. Kent using paraformaldehyde fixation of such lymphocytes (R. R. A. Coombs, personal communication). The paraformaldehyde solution was prepared as follows. Two g paraformaldehyde were suspended in 20 ml distilled water and heated to 60°. NaOH (0.1 N) was added drop by drop until the solution cleared. After the solution was removed from the water bath, 30 ml of 0.25 M phosphate buffer, pH 7.2, and then 425 mg NaCl were added. The solution was cooled to +4° for use. The sensitized lymphoid cells were washed 3 times in phosphate-buffered saline, added to the paraformaldehyde solution at a concentration of 5×10^6 cells/ml, and kept in the solution for 30 min at +4°. The cells were then washed twice in phosphate-buffered saline, resuspended in phosphate-buffered saline, and kept overnight at +4° to allow bound formaldehyde to elute. Before use in the test, the cells were washed in phosphate-buffered saline.

Mixed-Antiglobulin Reaction. Washed SRBC at a concentration of 2% in phosphate-buffered saline were incubated for 1 hr at 22° with an equal volume of cat anti-SRBC serum diluted 1:40. After 3 washes, a dilution of 0.8% SRBC was made for use in the tests. The sensitized lymphoid cells were washed twice at +4° with phosphate-buffered albumin containing 0.2% crystalline BPA. Two drops of cell suspension (concentration adjusted to the original 10^6/ml) were mixed with 2 drops of rabbit anti-cat Ig at 1:5 dilution. Incubation was carried out at +4°C for 1 hr. The cells were washed 3 times in phosphate-buffered saline with BPA. Two drops of sensitized SRBC were mixed with 2 drops of lymphoid cells in LP2 tubes (Luckham Ltd., Burgess Hill, Sussex, England). These were spun at 1800 rpm for 2 min. One-half of the supernatant was removed, and the cells were very gently resuspended in the remaining drop and then transferred to a siliconed slide in a drop of toluidine blue. The stain was prepared by depositing 0.5% toluidine blue in methanol onto siliconed slides, 4 drops at a time. The slides were then dried and the stain was reconstituted with phosphate-buffered saline and BPA. One drop was transferred to siliconed slides where the drop of cell suspension was added and mixed. The preparations were mounted and wax sealed. In these, 200 lymphocytes were counted and the percentage showing rosettes was estimated. A rosette was identified as a lymphocyte bearing 3 or more SRBC.

Indirect Immunofluorescence Test. Details of this technique have been published (5).

RESULTS

Experiment 1. This experiment was designed to determine whether antibodies to FOCMA, as shown by indirect immunofluorescence, could also be shown by the MIR method. The data obtained were used to establish the dose response by regressing percentage of cells showing rosette formation on serum dilution and to carry out a correlation analysis between the indirect immunofluorescence-FOCMA and MIR-FOCMA methods.

For this study, cat sera were first used in the indirect immunofluorescence-FOCMA test and the antibody titer was measured. The same sera were then used to sensitize FL74 cells in the MIR test, using the sera in a range of dilutions including dilutions beyond the indirect immunofluorescence end point.

The results indicated that the relationship between serum dilution and rosette formation followed a sigmoid distribution. The dose-response data are shown in Chart 1. The *x*-axis represents stepwise logarithmic dilutions of sera beyond the 95 to 100 PRF[8] dilution. It will be seen that the relation is linear over the 80 to 20 PRF points. Below 20, individual sera showed considerable variation in the rate of reduction in rosette formation through subsequent serum dilutions. To ensure that the test reading fell within the linear portion of the dose-response curve, a PRF of 25 was taken as the end point of the test.

Comparison of the indirect and immunofluorescence and

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MIR tests on sera covering a wide range of antibody titers showed that the tests were closely correlated and that the MIR method had a higher sensitivity. The correlation between the indirect immunofluorescence-FOCMA and MIR-FOCMA end points is shown in Chart 2. There was a high degree of correlation (r = 0.9624) between the methods, and the sensitivity of the MIR test was 3 to 4 $\log_2$ units greater than that of the indirect immunofluorescence test.

Experiment 2. Designating the end point as 25% depends on 2 factors: (*a*) the linear dose-response relation shown above; and (*b*) the degree of rosette formation resulting from nonspecific effect. It appeared that serum dilution might influence the latter. Sera of cat fetuses from definitely uninfected mothers, the ideal sera for this purpose, were not available. The nearest approximation we could obtain were sera from kittens from our isolated experimental cattery. These had no antibodies on the indirect immunofluorescence-FOCMA test and were not infected with conventional FeLV. A range of dilutions of sera from such kittens was tested, and the results are shown in Chart 3. Sera from 22 kittens were examined at a dilution of 1:4 and 8 of these were also used as 1:1 and 1:2 dilutions. The mean percentage of rosettes observed using the 22 cat sera at 1:4 dilution was ±2.7. At 1:1 and 1:2 dilutions, a much greater range was found. The result at the 1:4 serum dilution indicates that 25% rosette formation lies above the nonspecific range.

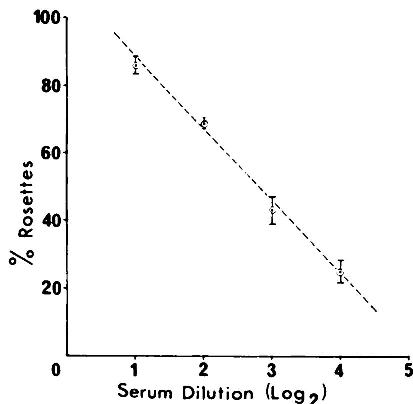

Chart 1. The regression of percentage of lymphoblasts forming rosettes on serial dilutions of sera from 10 cats with FOCMA antibodies. *Points*, group means; *bars*, S.E. The x-axis represents stepwise $\log_2$ dilutions of sera beyond the dilution at which approximately 95% of cells formed rosettes.

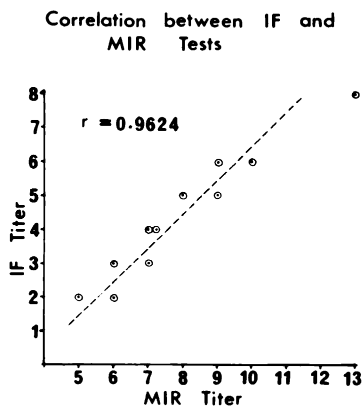

Chart 2. Correlation of the indirect immunofluorescence and MIR tests in sera from 11 cats with antibody to FOCMA. The line of regression is shown.

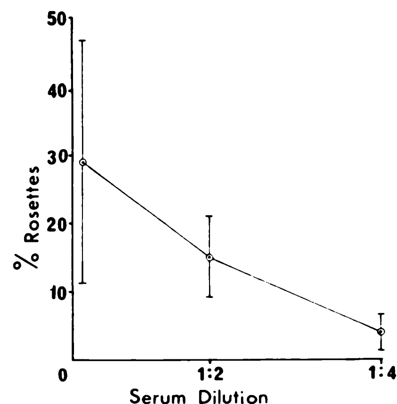

Chart 3. Results of the MIR test applied to sera from 22 kittens showing no evidence of exposure to FeLV; the sera were used at dilutions 1:1, 1:2, and 1:14. *Points*, mean; *bars*, S.D.

Experiment 3. The greater sensitivity of the MIR method shown by the above results is of obvious advantage in detecting low levels of antibodies. We therefore examined sera from 32 cats with naturally occurring lymphoid cancers. Only 8 of these had shown antibody by the indirect immunofluorescence-FOCMA test (10). FL74 cells were sensitized with sera from the 24 indirect immunofluorescence-negative cases at a dilution of 1:4. Of these 24 sera, 7 proved to contain antibody as detected by the MIR test. In the individual cases, the PRF varied from 25 to 80.

Experiment 4. It is known that FOCMA is expressed on dog cells that have been infected with FeLV (4). It was of interest to determine whether the quantitative relationship between MIR and indirect immunofluorescence, which was found using cat cells, also existed in the dog system. The use of dog cells has the further important advantage that uninfected lymphoblastoid cell lines are available for use as controls. No such feline lymphoblastoid cell lines have yet been established.

The test sera were cat sera containing FOCMA antibodies, as shown by indirect immunofluorescence testing on FL74 cells, and absorbed with dog tissue powder and liver homogenate. These absorptions were carried out because we have found in the indirect immunofluorescence test that some cat sera contain heterophile antibody that reacts with dog cells. Removal of such antibody does not reduce the titer of FOCMA antibody (W. Jarrett and L. Mackey, unpublished results). Absorbed sera were then tested by both immunofluorescence and MIR using uninfected dog cells. The results were negative.

The dog cells were infected with FeLV and the cat sera were caused to react with them. The indirect immunofluorescence and MIR tests were carried out using the same batch of dog cells on the same day for the indirect immunofluorescence tests and for the 1st-stage incubation in the MIR test. Normal cat sera absorbed with dog tissue

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were included as controls. The results are in Table 1. It can be seen that the indirect immunofluorescence titer obtained with sera using infected dog cells was approximately 3 $\log_2$ units less than the indirect immunofluorescence titer on cat cells, reflecting a lower amount of FOCMA on the dog cell membranes. The MIR titer using the dog cells was approximately 3 $\log_2$ units greater than the corresponding indirect immunofluorescence titer. There was good correlation between the 2 sets of results. Normal cat sera gave negative results on both infected cat and dog cells.

DISCUSSION

The measurement of antibodies to FOCMA has been of fundamental importance in studies on feline leukemia and sarcoma. Using the indirect immunofluorescence test, it has been shown that, when cats become infected with FeLV, they may, under certain circumstances, respond with the production of antibody to antigen(s) expressed on the cell membrane of cells infected with the virus. These antibodies play a significant role in protective immunity. They protect against the establishment of chronic FeLV infection and the development of leukemia (9), and they may prevent or cause the regression of sarcomas (3). The test has also been of great value in epidemiological studies. The incidence of FOCMA antibodies has been shown to be high in certain urban cat populations where there is considerable contact between cats (3, 10), and hence it is assumed that exposure to FeLV infection is common.

The nature and origin of the cell membrane antigens on the FeLV-infected cell are not yet known. The membrane may contain antigens of both cell and viral origin and such antigens may be coded by viral or cell genome, in the latter case virus induced. Until this system has been analyzed, it will be impossible to define the specificity of antisera. However, it is probable that the indirect immunofluorescence and MIR tests measure the same antigen-antibody reactions, since they are both simply techniques for detecting immunoglobulin on the surface of the same cells. Results obtained using the 2 methods in a series of animals and over a wide range of antibody titers were closely correlated. It is unlikely that different antigen-antibody reactions were being measured, as individual cats would be expected to respond unequally to a range of antigens. We have also shown that antibody to FOCMA can be found in cats that have previously had antibody measurable by indirect immunofluorescence. In an experiment in which 39 kittens were infected neonatally with FeLV, antibody to FOCMA was produced by all of the kittens within 3 months (our unpublished results). After several months the titers dropped, and in 12 cases antibody was no longer detectable by indirect immunofluorescence. In all of these, the MIR test gave positive results. The fact that the 2 methods also showed good correlation when dog cells were used in the tests increases the likelihood that the same antibodies were being registered.

Table 1

Comparison of the indirect immunofluorescence and MIR tests in the measurement of antibody to FOCMA on FeLV-infected dog cells

Serum	Indirect immunofluorescence titer (FL74 cells)	Indirect immunofluorescence titer (CT45S cells)	MIR titer (CT45S cells)	Logs greater sensitivity MIR/indirect immunofluorescence
1	256	32	256	3
2	256	16	128	3
3	128	16	128	3
4	128	32	128	2
5	128	16	256	4
6	64	4	32	3

The sensitivity of the test system depends on the demonstration of immunoglobulin on the cell surface in the 2nd stage of the reaction. An important practical point was the finding that nonspecific rosette formation often occurred when undiluted or 1:2 diluted sera were used. The reason for this is not known at present. However, it seems possible that small amounts of globulin could become attached to the cells during incubation with concentrated serum. Because of the high sensitivity of the immunoglobulin detection system, this would register in the test.

Our results show that MIR is approximately 10 times more sensitive than indirect immunofluorescence for the detection of antibody to FOCMA. Hence we were able to find antibody in leukemic cats where none had been shown by indirect immunofluorescence. The incidence of measurable antibody in our series of cats with naturally occurring lymphoid cancers was therefore approximately 50%. Based on indirect immunofluorescence results, our previous estimate was 25% (10). Since negative results were found using sera from young rural cats free from FeLV infection, it is unlikely that the MIR test gave false-positive results. Although no virus-free cat lymphoblastoid cells are available, we showed that uninfected dog cells did not form rosettes when caused to react with antibody-containing sera.

The MIR method is similar to the mixed-hemadsorption test of Fagreus and Espmark (6). It differs in the fact that the antigen-bearing cells are in suspension and the attached immunoglobulin is stabilized by paraformaldehyde, which increases the sensitivity of the system. A major advantage of the MIR method is the ability to quantitate the results accurately, since individual rosette-carrying cells are easily counted. The main disadvantage of the test is that it is more time consuming than indirect immunofluorescence, involving a 2-stage process that cannot be carried out on the same day. However, its sensitivity allows very small amounts of antibody to be detected, which is of great value in several aspects of virus leukemia work. Cats can be screened for the presence of antibody by simply testing the serum at the 1:4 dilution. The method is also valuable in confirming a doubtful result obtained in the indirect immunofluorescence test, where positive reactions in undiluted and 1:2 diluted sera can be difficult to assess. We found that homologous antigenic immunosuppression is common in neonatally infected kittens and may be an important primary event in leukemogenesis (1, 9). In both epidemiological and pathogenesis studies, therefore, it is important to have antibody test systems of high sensitivity. The test will also be applicable in vaccination experiments, where it is essential

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to determine whether animals have previously been exposed to virus infection. Conversely, when high-titered sera are available, it is possible to utilize the method for the detection of cell membrane antigens. At the moment, we are studying the possible expression of virion structural antigens on membranes of infected cells using antibodies against purified viral polypeptides. We are also investigating the possible relationship between FOCMA and fetal antigens by applying the technique to the interaction of fetal cells and high-titered feline FOCMA antisera.

It seems likely that this technique could be adapted for use in other systems for the measurement of antibodies to cell membrane antigens. Further advantages of the method are that no expensive equipment or labeled sera are required.

ACKNOWLEDGMENTS

We are grateful to R. R. A. Coombs and B. W. Gurner for help with the technique of demonstrating immunoglobulin on lymphocytes.

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