Complement Fixation Reaction for the Diagnosis of Type II Avian (Marek’s) Leukosis

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The present study was designed to find a complement fixation (CF) reaction for the diagnosis of type II lymphoid leukosis, to learn some of the characteristics of the CF antigen, and to investigate the development of CF antibody response to this infection. JM virus-specific antigen was demonstrated in tumors of chicken tissue, in JM virus-infected chick embryo material, in JM virus-infected chicken kidney, and in duck embryo fibroblast tissue culture by using JM virus-immune rabbit serum. This CF antigen did not show cross-reactivity with Rous sarcoma virus or with RIF-type viruses. It was partially heat-labile. The CF activity was restored at −70 C for 10 months and was resistant to intermittent freeze-thaw treatment. The CF antigen may be denatured by ethyl alcohol, but no significant deleterious effects were noted after ether or chloroform treatment. JM virus-specific CF antibody could not be demonstrated by the direct complement dilution method or by the indirect or inhibition form of the CF test in infected or immunized chicken sera.

Type II lymphoid leukosis (10) is the most prevalent form of the avian leukosis complex. To date, the most reliable method for detecting the virus is the inoculation of susceptible 1-day-old chicks. Of foremost interest is an in vitro test to detect the etiological viruses, specific antigens, or antibodies associated with this infection.

The present investigation is involved with a complement fixation (CF) study for the diagnosis of type II lymphoid leukosis. Attempts were made to detect JM virus-specific CF antigens in different JM virus-host systems, to study the thermal and certain chemical effects on these CF antigens and to investigate the development of CF antibody response in chicken sera to JM virus exposure.

MATERIALS AND METHODS

Viruses. The JM virus strain (11) of type II avian leukemia complex (10) was propagated either in COFAL (9)-negative White Leghorn stock (SpAFAS) of Connecticut or S-line White Leghorn stock developed by Hutt and Cole of Cornell University and previously established as susceptible to infection by the JM virus (11). RIF-type viruses (RPL-12 and RAV-2) and Rous sarcoma viruses (BH-RSV (RAV-1) and BH-RSV (RAV-2)) were used in the COFAL test (9) and in the study of the antigenic relationship between type I and type II leukemia viruses.

Chicken kidney tissue culture antigen. Kidney tissue cells derived from 3-week-old JM-infected and non-infected control chicks were grown in medium 199 supplemented with 10% tryptose phosphate broth and 5% bovine fetal serum. Penicillin, dihydrostreptomycin, and fungizone were incorporated into the media at concentrations of 100 units, 100 μg, and 25 μg per ml, respectively. The pH of the medium was adjusted to 7.2 to 7.4 by the addition of 7.5% sodium bicarbonate. The bovine fetal serum was inactivated at 56 C for 30 min before use.

Trypsin (0.25%) in phosphate-buffered saline was employed for the preparation of primary cell culture. The kidney tissue suspension was incubated at 37 C in a 5% CO2 atmosphere for 12 days. The media were changed when necessary. At the end of the incubation period, the cells were harvested (with rubber policeman) in one-tenth volume of the tissue culture maintenance medium. The resulting suspension was subjected to three successive cycles of freezing (in a mixture of carbon dioxide ice and 95% ethanol) and thawing (in a 37 C water bath) after which it was clarified by light centrifugation. The supernatant fluids from control and JM virus-infected kidney tissue cultures serve as the negative control and JM-infected antigen, respectively.

Antigen from duck embryo fibroblast tissue culture. Preparation of both normal control and JM virus-infected duck embryo fibroblast tissue culture antigens was conducted in the same manner as was the preparation of chicken kidney tissue culture antigens.

Preparation of normal control and tumor ovarian and kidney chicken antigens. Tissues were homogenized in Veronal-buffered saline (12), subjected to three successive cycles of freezing and thawing (explained above), and then clarified at 1,500 × g for 30 min. The supernatant fluid was centrifuged at 10,000 × g for 30 min. The supernatant fluid was frozen at −70 C until needed and served as antigen.
Preparation of control and JM virus-infected antigens in chick embryos. Five-day-old fertile eggs were inoculated with 0.1 ml of JM-infected blood via the yolk sac. The eggs were kept in an incubator at 38 °C until the 20th day. The eggs were opened, and the live embryos were examined for pathologic lesions of JM virus (M. Sevoian, Abstr., Proc. 38th Annu. Meeting Northeastern Conf., Avian Disease, Univ. of Delaware, 27–29 June 1966).

The embryonic liver, spleen, and kidneys were harvested and emulsified into Veronal-buffered saline to make a 1:10 (w/v) suspension; the rest of the procedure was carried out in the same way as with tumor ovarian and kidney chicken antigen. Corresponding normal control antigen was prepared in the same way from chick embryos inoculated with normal blood.

JM hyperimmune rabbit serum. Antisera were obtained by inoculating young adult New Zealand and Dutch Belted rabbits. Each rabbit received intramuscularly four to six weekly injections of JM chicken kidney tissue culture antigen. Each dose consisted of 1.0 ml of antigen, which contained approximately 10³ TCID₅₀ before subjecting it to the freeze-thaw treatment, mixed with an equal volume of incomplete Freund's adjuvant. Blood was withdrawn from the heart 1 week after the last injection. Sera were separated and frozen until needed. The sera used for JM antibody in the CF test were prepared by adsorption with normal chicken kidney tissue powder three times and then with normal chicken kidney cells and powdered fetal bovine sera and were finally centrifuged at 10,000 × g for 1 hr.

Chicken sera. Blood samples were collected from the wing vein of sixty 12-week-old chickens representing three different groups: (i) paralytic chickens which had been naturally exposed to JM virus, (ii) chickens intramuscularly inoculated with JM virus at 4 weeks of age which did not show signs of disease at time of bleeding, and (iii) nonexposed control chickens. The blood samples were allowed to clot, and sera were separated and used in testing for CF antibody.

CF test. Veronal buffer diluent (VBD) was employed at pH 7.2. For the blood cell suspension, sheep erythrocytes (2.8%) were standardized by centrifugation or spectrophotometrically, or by both methods. The plateau method was used to determine the amount of hemolysis used in the test. Five H₉₀ of complement was used in the titrations of antigens and rabbit immune sera and in the diagnostic test for detecting antigens.

Technique. The LBCF technique (12) was essentially used in the titrations and standardization of reagents and in the demonstration of antigens. In detection of antigens in the diagnostic test, three antigen control tubes for each sample were used. These control tubes received normal control rabbit serum corresponding to the JM hyperimmune rabbit serum in the test proper.

In trying to demonstrate antibodies in chicken sera, the complement dilution method (6) was considered to be superior because of the anticomplementary (AC) characteristic of the avian sera. The principle involved is the measurement of the CF capacity of a definite amount of assumed serum antibody and JM virus-specific antigen. Serum and antigen were therefore used in a fixed amount and complement was diluted in series. The complement was initially added in a relatively high concentration, usually reaching nine serial dilutions with a 20% decrease of complement in each dilution. The test was done in pairs with normal control antigen and standardized positive antigen which were previously balanced with respect to their AC activity. The rest of the procedure was essentially the same as in the LBCF test. The determination of results was based on the following formula: fixation titer (FT) = (H₀ – Hₚ/H₀) × 10₀, H₀ = total percentage of hemolysis in four to six tubes around 80% of the normal series and Hₚ = total percentage of hemolysis in the corresponding four to six tubes of the positive series.

The interpretation of the reaction was as follows: FT < 20, negative; FT > 20, positive.

Inhibition form of the CF test (7) was used to detect antibodies in chicken sera. JM virus-specific CF antigen in its optimal reactive dilution (as concluded from block titration with JM virus-specific hyperimmune rabbit serum) was incubated with serial dilutions of chicken serum and 5 H₉₀ of complement for 2 hr before 4 units of rabbit antiserum was added. The rest of the procedure is as in the direct CF test. The test is considered positive if specific fixation is inhibited (hemolysis occurs) and vice versa.

RESULTS

Detection of CF antigens in JM virus-infected tissues. CF antigens were demonstrated with the use of JM immune rabbit serum in JM virus-infected chick embryo, in JM virus leukotic tumors of ovaries and kidney of chickens, and in chicken kidney and duck embryo fibroblast tissue cultures infected with JM virus. Corresponding control antigens did not fix complement with immune rabbit serum nor did the normal control rabbit serum when tested with JM-infected antigens. An example of JM virus-infected chicken embryo antigen titration versus immune rabbit serum is shown in Fig. 1. The optimal antigen dilution (giving the highest antibody titer with specific antiserum) was 1:8, even though a slight amount of CF activity was noted at a 1:48 dilution of antigen.

Chicken tumor tissue resulted in similar CF activity (Fig. 2). The optimal antigen dilution was 1:8. RSV, SR tumor antigen prepared in the same way as the JM-specific antigen, did not show fixation of complement when incubated with JM immune rabbit serum.

Chicken kidney tissue culture infected with JM virus also showed CF activity when tested with JM immune rabbit serum (Fig. 3). The optimal antigen dilution was 1:8. The COFAL-
Utilization of JM-specific CF test in diagnosis of type II lymphoid leukemia. Antigens were prepared from chick embryos inoculated with blood samples from 20 birds (4 weeks of age) exposed to natural infection at 1 day of age. The prepared antigens were tested for JM virus specificity by using JM immune rabbit serum (Table 1). The control tubes received normal control serum corresponding to JM-specific immune serum in the positive antigen did not fix complement when tested with JM immune rabbit serum.

Duck embryo fibroblast tissue culture infected with JM virus also exhibited CF antigen which reacted with rabbit immune serum (Fig. 4). The optimal antigen dilution was 1:8.

In all of the antigen titrations, the complement controls indicated that the antigens, the serum and the VBD were not anticomplementary.
culture antigens utilizing the complement dilution technique with normal and positive antigen pair. The difference in hemolysis between the normal and positive antigen series in each sample was calculated in 4 to 6 tubes which showed about 80% hemolysis in the normal series. No significant differences (FT < 20) in reaction have been observed in sera from normal control, infected, or immunized birds.

No avian serum antibody was detected by using the indirect or inhibition form of the CF test. The avian serum when incubated with standardized JM antigen did not inhibit the fixation of complement when rabbit antiserum was added and subjected to the usual incubation period before the addition of the incubator system. The degree of fixation was the same (complete fixation or 0% hemolysis) in the test for inhibition of fixation as in the control tubes that did not receive avian serum.

**DISCUSSION**

Demonstration of JM-specific CF antigen offers a laboratory technique to determine whether a chicken has developed a JM type-specific neoplasm of the avian leukosis complex. Therefore it offers a diagnostic tool that could be utilized in the study of epidemiology and immunology of the type II lymphoid leukemia by tracing CF antigens in infected tissues. The specificity of the antigens is strongly supported not only by the use of the adsorption technique but also by its reactivity with the JM viral immune rabbit sera irrespective of the species or tissue of origin of the cells infected and in the absence of CF antigen in noninfected cells from several organs of chickens, chick embryos, chicken kidney, and duck embryo fibroblast tissue culture. No CF reactions were observed between JM virus-specific antigens and normal rabbit serum.
TABLE 1a. Per cent hemolysis in the detection of CF antigen in chicken embryos inoculated with chicken blood (continued from Table 1)

| Serial no. | Control antigen | Per cent hemolysis | Interpretation |
|------------|----------------|--------------------|----------------|
| 1 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | Negative |
| 2 | 90 | 95 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | Negative |
| 3 | 0 | 0 | 40 | 80 | 100 | 100 | 100 | 100 | 100 | Positive |
| 4 | 0 | 10 | 80 | 100 | 100 | 100 | 100 | 100 | 100 | Positive |
| 5 | 80 | 90 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | Positive |
| 6 | 0 | 0 | 30 | 50 | 80 | 100 | 100 | 100 | 100 | Positive |
| 7 | 0 | 0 | 20 | 70 | 100 | 100 | 100 | 100 | 100 | Positive |
| 8 | 0 | 0 | 30 | 50 | 70 | 100 | 100 | 100 | 100 | Positive |
| 9 | 0 | 0 | 0 | 0 | 50 | 70 | 100 | 100 | 100 | Positive |
| 10 | 0 | 0 | 0 | 10 | 40 | 90 | 100 | 100 | 100 | Positive |
| 11 | 0 | 0 | 70 | 100 | 100 | 100 | 100 | 100 | 100 | Positive |
| 12 | 0 | 0 | 50 | 95 | 100 | 100 | 100 | 100 | 100 | Positive |
| 13 | 30 | 30 | 60 | 90 | 100 | 100 | 100 | 100 | 100 | Positive |
| 14 | 0 | 0 | 10 | 30 | 70 | 100 | 100 | 100 | 100 | Positive |
| 15 | 30 | 50 | 70 | 90 | 100 | 100 | 100 | 100 | 100 | Positive |
| 16 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | Positive |
| 17 | 10 | 10 | 0 | 30 | 70 | 100 | 100 | 100 | 100 | Positive |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | Positive |
| 19 | 70 | 30 | 0 | 0 | 50 | 100 | 100 | 100 | 100 | Positive |
| 20 | 30 | 60 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | Positive |

TABLE 2. Effect of temperature (56°C) on CF antigen

| Antigen | Initial CF titer | CF titer after heat treatment at 56°C for |
|---------|----------------|----------------------------------------|
|         |                | 15 min | 30 min | 45 min | 1 hr |
| Kidney tissue culture | 1:32 | 1:16 | 1:16 | 1:16 | 1:16 |
| Duck fibroblast | 1:16 | 1:8 | 1:8 | 1:8 | 1:8 |
| Chick embryo | 1:16 | 1:8 | 1:8 | 1:8 | 1:8 |

TABLE 3. Effect of chemicals on CF antigen

| Antigen | Initial CF titer | CF titer after treatment for 1 hr with |
|---------|----------------|----------------------------------------|
|         |                | Chloroform | Ethyl alcohol |
| Kidney tissue culture | 1:32 | 1:32 | 1:32 |
| Duck fibroblast | 1:16 | 1:16 | 1:16 |
| Chick embryo | 1:16 | 1:16 | 1:16 |

This antigen is probably not viral in nature, since tumor cells induced by viruses do not usually release much infectious virus; however this phenomenon may be more apparent than real. It is possible, nevertheless, that this antigen is a "new" cellular component which is coded by the viral genome and only depends on the viral infection and the integrity of the viral nucleic acid. This speculation is in accordance with the conclusions on the induced CF antigen detected in other tumor viruses.

Studies in growth cycles of simian virus 40 and adenovirus 12 have been analyzed for CF and infectivity (3-5, 8). By the use of metabolic inhibitors, it has been found that development of induced CF antigen continues at a rate similar to that of untreated cells, whereas the synthesis of infectious virus and viral proteins is completely inhibited.

It has not been established in this work whether the CF antigens in different host systems are identical or whether they share one or the same antigen. It may be that there is a spectrum of different antigens produced in the various cells which share some antigenic determinants without necessarily sharing the same composition or function.

Interpretation of positive CF tests must be done with caution as the existence of normal isoantigens could complicate results. However, in demonstrating the CF antigen, adsorption of the rabbit immune serum with pooled "normal" chicken cells eliminates this factor.

The absence of cross-reactivity between RIF-type viruses and JM, which has already been reported (3; M. Sevoian, Abstr., Proc. 38th Annu. Meeting Northeastern Conf., Avian Disease, Univ. of Delaware, 27-29 June 1966), was confirmed in this work by negative CF reactions of...
COFAL-positive antigens (RPL-12 and RAV-2) with JM immune rabbit sera.

The low titer of JM CF antibody in immune rabbit sera agrees with Sevoian (Abstr., Proc. 38th Annu. Meeting Northeastern Conf., Avian Disease, Univ. of Delaware, 27–29 June 1966) that JM virus is a weak immunogen.

The finding that these CF antigens are partially heat-labile agrees with the results reported by Cook (1) that one antigen of Marek's disease agent (type II lymphoid leukemia) is heat-stable.

The negative effect of ether and chloroform on the CF antigen does not necessarily contradict the result reported by Cook (1) that ether and chloroform inactivate the infectious virus of Marek's disease agent, since antigenicity rather than infectivity was studied in this work.

A search for specific CF antibodies has been unsuccessful. Sera from a variety of "normal," JM virus-infected, and immunized chickens reacted equally when tested against antigens derived from virus-containing and virus-free tissues. The apparent failure of chickens to develop CF antibodies in their sera could not be fully explained, but agrees with the results of similar experiments on other oncogenic viruses; this leads to the conclusion that CF antibodies are not formed in the original host carrying the neoplasma.

CF antibodies also have not been demonstrated in RIF-type virus infections in chicken sera (2). Possibly, this is because antisera in chickens do not have a high level of antibodies to be demonstrable by the CF test.

The negative results of the indirect CF test could be explained as being due to the presence of an anticomplementary and inhibitory substance in high concentrations.

It is possible that the failure to demonstrate JM virus specific CF antibody in chicken sera is due to limitations in present techniques. If elimination of the inhibitory substances from chicken sera is possible, the inhibition form of the CF test may prove useful in detecting specific antibodies.

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