Comparison of Indirect Hemagglutination and Immunodiffusion Tests for Detecting Type II Leukosis (Marek’s) Infection in S- and K-Line Chickens

CHOU C. HONG AND MARTIN SEVOIAN
Paige Laboratory, University of Massachusetts, Amherst, Massachusetts 01002

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The indirect hemagglutination and immunodiffusion tests were compared for detection of antigen and antibody to JM strain of leukosis virus infection between S- and K-line chickens. The indirect hemagglutination test was more sensitive than the immunodiffusion test for detecting the smallest amount of viral antigen and corresponding antibody in the plasma of infected chickens. The Cornell S-line had higher levels of antigen and antibody as compared with the Cornell K-line during the 20-week experimental period.

Type II lymphoid (Marek’s) leukosis (8) is common and widespread in poultry. Susceptible (S) and resistant (K) lines of White Leghorn chickens were developed over many years by F. B. Hutt and R. K. Cole of Cornell University by natural exposure to field infection followed by selection on the basis of highest and lowest incidence of leukosis, respectively, in various families (3, 6). The response of S- and K-line chickens to artificial challenge with JM virus has been previously established as susceptible and resistant, respectively (3, 9). Recently Zacharia and Sevoian (12) reported that birds infected with JM virus developed agglutinins. By sensitizing sheep red blood cells (SRBC) with JM viral antigen or antiserum, they have demonstrated quantitative differences of antigen and antibodies in the circulating blood.

The immunodiffusion (ID) technique has been used to detect type II leukosis infections. Kottaridis et al. (7) demonstrated that plasma and bone marrow from infected birds with JM or Connecticut A virus produced double lines against specific rabbit antisera. Antigen from noninfected birds produced a single precipitating line with the same antiserum, and the above workers suggested the appearance of an extra precipitin line due to the infection. In other strains of type II leukosis virus (HPRS-B-14 and GA strains), the precipitating antigen in infected kidney tissue culture has also been demonstrated against chicken immune serum by ID technique (2).

The present study used the ID and indirect hemagglutination (IHA) tests to determine (i) how soon after exposure each test may be used to detect immunogenic response and viremia in exposure to JM virus, (ii) the comparative relative sensitivity of the two tests in detecting JM infection, (iii) whether there was a corollary between IHA and ID responses to JM virus infections in chickens, and (iv) whether JM antigen and antibody responses were related to host resistance or susceptibility.

MATERIALS AND METHODS

Experimental design. The general design was experimentally to inoculate with or naturally expose to JM leukosis virus known S and K chickens at 1 day of age and, chronologically to measure in the plasma the sensitivity, rapidity, levels, and duration of antigen and antibody responses by using the ID and IHA tests.

One hundred S-line and 100 K-line day-old chicks were each allotted into three JM-infected groups and one control group. Group 1 chicks were infected via air-borne natural exposure, whereas group 2 and 3 chicks were inoculated intraabdominally with 0.25 ml of JM tumor suspension and 0.25 ml of JM-infected duck embryo fibroblasts; [DEF third passage tissue culture, $6 \times 10^3$ plaque-forming units (PFU)/ml], respectively. All chicks were kept in modified Horsfall units for maximal security against cross-infection. Control birds were isolated several miles away at South Deerfield Farm, University of Massa-

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chusetts. All groups of chicks were identified by wing-band numbers and were fed and managed similarly.

Samples of blood from each chicken were drawn via the alar vein into citrated tubes and were centrifuged. Samples were collected weekly from 1 day of age up to the termination of this experiment at 20 weeks postinfection and were tested as fresh plasma.

All birds which died during the experimental period were necropsied and recorded up to 20 weeks of age.

**Preparation of virus inocula.** Primary DEF were prepared by conventional trypsinization of decapitated 10- to 12-day-old embryo obtained from a flock of White Pekin ducks in Sterling, Mass. The cell culture (3.3 × 10^6 cells/ml) was grown in media 199 (Flow Laboratories, Rockville, Md.) supplemented with 10% tryptose phosphate broth, 5% bovine fetal serum, and penicillin, dihydrostreptomycin, and Fungizone with concentrations of 100 units, 100 mg, and 0.25 μg/ml, respectively. The pH of the medium was adjusted to 7.2 to 7.4 by the addition of sodium bicarbonate. The normal control cultures were incubated at 37 C with a 5% carbon dioxide atmosphere, and the cells were subcultured usually between 3 and 4 days, when monolayer growth became confluent.

JM virus (9) inocula were prepared from DEF tissue culture which had been overlaid with JM-infected chicken kidney cells, obtained from a 4-week-old S-line chicken experimentally infected with JM virus via natural air exposure. The same medium was used as described above. The third passage of infected DEF was allowed to grow until the cell sheets pealed and then was harvested in tissue culture fluid to give an approximate 20% cell suspension. The cells were treated with 10% dimethylsulfoxide (DMSO) and stored in sealed glass ampoules in a liquid nitrogen tank.

The JM-infected DEF tissue culture was frozen and thawed three times in a dry ice-alcohol solution, and the tissue culture fluid was collected and centrifuged at 1,000 × g for 10 min. The supernatant material was carefully removed, and the sedimented cell debris was discarded. The supernatant material was spun again at 15,000 × g for 1 hr, and the pellet was separated and set aside.

The antigens in the infected supernatant material were concentrated by the precipitation method with saturated ammonium sulfate. The fluid was cooled to 4 C and added to an equal volume of saturated ammonium sulfate ([NH₄]₂SO₄), and the mixture was homogenized on a magnetic stirrer for 1 hr at 4 C. The precipitate was taken up in the phosphate-buffered saline (PBS) at 4 C overnight to remove the remaining ammonium sulfate. Finally, the dialyzed viral antigen was later combined with pellet, dissolved in PBS (1:1, v/v), and stored in a freezer at −20 C until use.

**Preparation of JM tumor inocula.** The JM tumor suspension inoculum was prepared by using the procedure described by Sevoian et al. (10, 11). The volume of JM tumor suspension was 10^3 median infective doses (ID₅₀), as bioassayed in day-old S-line chicks.

**Preparation of antiserum.** JM antiserum was prepared in young adult New Zealand and Dutch Belted rabbits which received a minimum of six injections subcutaneously at weekly intervals. Each dose consisted of 1 ml of JM antigen in DEF mixed with equal volume of incomplete Freund's adjuvant.

The rabbits were bled via cardiac puncture for antiserum 10 days after the last injection. Antisera were absorbed with normal DEF and normal chicken tissue powder three times, respectively, and finally with dried fetal bovine sera, and then were centrifuged at 10,000 × g for 1 hr. The absorbed antisera were inactivated at 56 C for 30 min and were kept at −20 C until use.

Immunoglobulin was precipitated with 33% saturation of ammonium sulfate. The immunoglobulin precipitate fractions were dialyzed against PBS at 4 C overnight to remove ammonium sulfate. The immunoglobulin fractions were concentrated to 75% of the original volume by dialyzing against Carbowax (Union Carbide and Carbon Co., N.Y.) and stored at −20 C in a freezer. The immunoglobulin was used to sensitize the formalinized tumor sheep erythrocytes for detecting the JM virus levels in the infected chickens.

**Preparation of formalinized tanned sensitized sheep erythrocytes.** The process of formalization was carried out by dialysis method, suspending a dialysis bag filled with 10% sheep erythrocytes in a beaker containing 40% Formalin, pH 6.0. The beaker was gently shaken at 4 C for 2 hr, after which the dialysis bag was released and the Formalin floated over the cells. The mixture was then placed at 4 C for 6 to 8 hr with occasional swirling, after which the fixed cells were washed three times with two volumes of PBS and stored in the freezer at −20 C until use. The fixed-cell suspension (2.5%) was treated with 150,000 tannic acid at 37 C for 15 min and then washed twice in PBS (pH 6.4) solution by low-speed centrifugation (650 × g). The sediment was resuspended in PBS (pH 6.4) to 2.5% suspension. An equal volume of antigen or antibody was added in the suspension for sensitizing these cells in the water bath at 37 C for 30 min with occasional swirling. After sensitization, the cells were washed twice in four volumes of PBS (pH 6.4), and finally a 0.5% solution of fixed tanned sensitized cell suspension was made up with PBS (pH 6.4).

**IHA test.** Fixed tanned sheep erythrocytes (0.5%) sensitized with either JM virus antigen or rabbit anti-JM serum were used in these trials.

The Microtiter system, developed by Cooke Engineering Co., was used. A twofold dilution system was prepared in a Microtiter plate by adding 25 μlter of 1% bovine serum in PBS, pH 6.4, as a diluent to the appropriate wells of the plate. The 25-μlter microdiluter was placed and rotated in the testing plasma. Then 25 μlter was delivered to the first well, and further dilutions of testing plasma were prepared by transferring 25 μlter from one well to the next down the line. A 25-μlter amount was discarded from the last dilution. Finally, 25 μlter of 0.5% fixed tanned sheep erythrocytes sensitized with either JM viral antigen or rabbit anti-JM immunoglobulin was added to all wells for detecting
type II leukemia antibody and antigen response, respectively. The control samples were prepared in the same manner as above.

The wells were sealed by transparent tape sealer, incubated in the refrigerator (5°C), and then examined at the end of 12 hr by a test reading mirror. The IHA units were considered the highest dilution of either the antigen or the antibody showing complete agglutination.

**ID test.** The ID test was the petri dish (60 by 15 mm) method performed as described by Crowle (4). Supporting agar was 1.0% Difco agar prepared in 6% sodium chloride that contained 100 units of penicillin and 0.1 mg of streptomycin per ml. Five milliliters of the medium was dispensed into each plate. A central well and six circumferential wells were cut equal distances apart so that the distance from the edge of the center well to that of each surrounding well was 0.5 cm. The positive antiserum or known viral antigen was placed in the central well, and test plasma obtained from infected and control chickens was placed in the circumferential wells. The plates were kept at 37°C in a closed, moist chamber. Plates were observed every 8 hr, and the final readings were made until 72 hr of incubation.

**RESULTS**

**Leukosis mortality.** Mortality resulting from type II leukemia in both K- and S-line chickens during the 20-week experimental period is shown in Table 1. Similar to previous reports (3, 11), the K-line had significantly lower mortality (approximately 30%) than the S-line (approximately 80%). In addition, there was a delay of 3 weeks in the initial mortality in the K-line as compared with the S-line.

The routes of infection as well as the different inocula used had some influence on the incidence of mortality. Both K- and L-line chickens, when inoculated with the JM-infected DEF, had the lowest total mortality of 20 and 66.6%, respectively. Comparatively, S-line chickens inoculated with tumor suspension had higher mortality (100%) than the same line naturally exposed (82.6%). In contrast, no significant difference in mortality was found between groups of K-line chickens inoculated with tumor suspension or naturally exposed.

**Detection of antigen and antibody.** When JM-infected chicken plasma was tested against rabbit JM antiserum, a precipitin line was observed as early as 8 hr after incubation at 37°C, though more often the precipitin line was observed between 12 and 24 hr of incubation (Fig. 1). However, the final readings were not made until the test plates had been incubated for 72 hr to ensure completion of the reaction. Positive antigen reaction usually consisted of a single precipitin line, but some samples showed double lines when the IHA antigen titers were 6 log₂ or above. All attempts to demonstrate antigen in the plasma of control chickens by ID test failed.

When the immunodiffusion test was used to determine antibody response in the infected chickens, the precipitin line usually did not appear until after 24 hr of incubation at 37°C. A positive reaction always appeared as one precipitin line. (Fig. 2). No precipitin line was observed in the plasma of the control chickens.

**Relationship between IHA and ID tests.** Table 2 compares the results of IHA and ID tests. Antigen and antibody were detected in ID tests only when IHA titers were 3 to 4 log₂ levels or more. Thus, the IHA test was the more sensitive of the two tests.

| Table 1. Comparison of mortality between S- and K-line chickens during the 20-week experiment with JM virus infection |
|--------------------------------------------------|
| Treatment | Line | No. of chickens | Percent mortality |
|-----------------|--------|-----------------|-------------------|
| JM infected tumor | S    | 24              | 100.0%            |
| JM infected DEF* | K     | 23              | 82.6%             |
| Uninoculated control | S    | 19              | 0.0%              |

*The median infective dose of inoculum was bioassayed as approximately 10⁻⁸ PFU/ml in S-line chicks.
*The third passage of JM-infected duck embryo fibroblast (DEF) (6 x 10⁴ FFU/ml).
FIG. 2. Detection of JM antibody in testing plasma. Wells 1, 2, 3, 4, 5, and 6 were obtained from infected chickens. Center well was JM antigen (prepared from JM-infected DEF).

**Table 2. Comparison of immunodiffusion (ID) and indirect hemagglutination (IHA) tests for detecting JM virus and its antibody in plasma of susceptible (S) and resistant (K) lines of chickens**

| IHA Titer* | Per cent positive ID test | Testing for antigen in | Testing for antibody in |
|------------|---------------------------|------------------------|------------------------|
|            |                           | S line | K line | S line | K line |
| 1          |                           | 0      | 0      | 0      | 0      |
| 2          |                           | 9      | 0      | 0      | 0      |
| 3          |                           | 85     | 76     | 0      | 0      |
| 4          |                           | 89     | 89     | 9      | 6      |
| 5          |                           | 100    | 95     | 71     | 67     |
| 6          |                           | 100    | 100    | 100    | 100    |
| 7          |                           | 100    | 100    | 100    | 100    |
| 8          |                           | 100    | 100    | 100    | 100    |

* The infected chickens were tested weekly from 4 to 20 weeks postinfection.

**Comparison of type II leukosis infection between S- and K-line chickens by ID test.**

Within the three types of infection, positive antigen reaction was found in 98 to 100% of the S-line and 65 to 80% of the K-line chickens by ID test during the 20-week experimental period. Only 10 to 16% of the S-line and 6 to 7% of the K-line showed the positive antibody reaction during the same period of time. (Table 3).

The ID test results showed that the Cornell S-line had higher incidence of antigen or antibody response than the Cornell K-line from 4 to 20 weeks postinfection.

**Table 3. Distribution of positive reaction of immunodiffusion (ID) test in two lines of chickens with three treatments of JM virus infection from 4 to 20 weeks postinfection**

| Treatment                  | Testing | Line | Per cent positive ID test |
|----------------------------|---------|------|---------------------------|
| JM-infected tumor          | Antigen | S    | 99                        |
| suspension*                |         | K    | 65                        |
|                            | Antibody| S    | 10                        |
|                            |         | K    | 7                         |
| Natural exposure           | Antigen | S    | 100                       |
|                            |         | K    | 80                        |
|                            | Antibody| S    | 16                        |
|                            |         | K    | 6                         |
| JM-infected DEF*           | Antigen | S    | 98                        |
|                            |         | K    | 80                        |
|                            | Antibody| S    | 13                        |
|                            |         | K    | 6                         |

* The median infective dose of tumor suspension was bioassayed at approximately $10^{4.1}$ per ml.

**DISCUSSION**

The infected S-line chickens developed higher levels of antigen and antibody titers than did the infected K-line during the early stages of infection from 4 to 8 weeks of age. The line difference became smaller or nonsignificant after that period of time. These observations indicate that the genetically susceptible young S-line chicks may provide better cellular environment for growth or multiplication, or both, of type II leukosis virus as compared with the resistant K-line chicks. The levels of antibody response were proportionally and inversely correlated with antigen titers and the resistance to type II leukosis, respectively. The results of this study suggest that the IHA and ID antibodies do not possess a significant neutralizing or protective ability against type II leukosis.

The positive relationship between IHA and immunodiffusion tests for detecting viral antigen and corresponding antibody in infected chickens was observed consistently throughout the experiment. The IHA antigen and antibody titers had reached at least 3 log₂ and 4 log₉, respectively, before the positive precipitin band could be observed by immunodiffusion test. In this study, IHA test was shown to be more sensitive in detecting infection than was the ID test. This was expected, as Carpenter (1) stated that the indirect hemagglutination test can detect as little as 0.005 μg of antibody nitrogen per ml, whereas the immunodiffusion
test is able to detect no less than 5 μg per ml. When IHA antigen titers reached 6 log₂ or higher, a double precipitin line could be detected by immunodiffusion test, indicating the presence of at least two distinct antigenic components. One of the bands is undoubtedly of the JM-virus origin, while the other is postulated to be due to virus infection-associated antigen.

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