Detection of Avian Leukosis Virus Antigens by the ELISA and Its Use for Detecting Infectious Virus after Cultivation of Samples and Partial Characterization of Specific Pathogen-Free Chicken Lines Maintained in This Laboratory

Kenji TSUKAMOTO, Hiroshi HIHARA, and Yuji KONO

Poultry Disease Laboratory, National Institute of Animal Health, 4909-58 Kurachi, Seki, Gifu 501-32 and National Institute of Animal Health, 3-1-1 Kannondai, Tsukuba, Ibaraki 305, Japan

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ABSTRACT. An enzyme-linked immunosorbent assay (ELISA) for detecting avian leukosis virus (ALV) antigens was developed with rabbit anti-ALV serum. The ELISA detected purified ALV of subgroups A and B at a concentration of 0.4 ng/well and about 10^3 infectious units/well estimated by a resistance-inducing factor (RIF) test, and antigens in culture fluids from chicken embryo fibroblasts infected with subgroups A, B or E of ALV. These results showed that common antigens among the subgroups were detected by the ELISA. When virus titration was performed, virus infectivity could be determined by the ELISA within 7 days after cultivation. The titer was similar to that obtained by the RIF test on 19 days after 3 subcultures. These results indicate that the ALV-isolation test by the ELISA was superior to the RIF test in rapidity and applicability to large-scale field trials. Four specific pathogen-free (SPF) chicken lines maintained in this laboratory were examined for endogenous ALV antigens by the ELISA. Sera from laying hens had considerably high absorbance (A) values, whereas albumen samples showed low A values except for some samples (7/40 hens). Although most of sera from 1-day-old SPF chicks showed lower A values than those from laying hens, some sera showed A values as high as those from viremic chicks in 2 lines. Endogenous ALV was isolated from sera from laying hens (6/40) and their albumens (4/7) with high A values. Two SPF chicken lines were found to produce endogenous virus at a high frequency.—KEY WORDS: ALV, ELISA, endogenous ALV, SPF chicken.

Avian leukemia virus (ALV) infection is an important disease of chickens since it induces not only lymphoid leukemia (LL) [25] but also a decrease in egg production and hatching ability [10–12, 17].

A program to reduce the rate of ALV infection in flocks by detection and elimination of congenitally transmitting hens has been initiated in recent years [18, 26, 35]. Previous studies had shown a strong correlation between the congenitally transmitting hens and the presence of ALV antigens in egg albumen [8, 21, 22, 36]. Smith et al. [33] and Clark and Dougherty [2] independently had developed an enzyme-linked immunosorbent assay (ELISA) for detecting group-specific (gs) antigens of ALV by using anti-gs antiserum and showed that it was useful for the program [1]. Although the ability of the ELISA to reduce the rate of ALV infection in the field was confirmed by other scientists [5–7, 15, 16, 19, 21, 24], the ELISA gave a false-positive reaction due to endogenous virus antigens [3, 5, 7, 8, 33] and could not detect all transmitters [5, 6, 16, 21, 24, 35], probably because some of them produce eggs which contained lesser amounts of virus than the detection limit of the ELISA. Therefore, a specific and more sensitive assay should be required to identify all of the congenitally transmitting hens. It is one way to develop a simple virus-isolation test which detect exogenous virus by testing ALV antigens in the cultured samples with the ELISA. For this assay, C/E phenotype (resistant to the infection with endogenous ALV) of cells which express no endogenous ALV antigens is essential.

The purposes of this investigation were to establish a rapid and specific assay for detecting infectious exogenous ALV by the ELISA after cultivation of samples and to characterize 4 specific pathogen-free (SPF) chicken lines maintained in this laboratory for the presence of endogenous ALV.

MATERIALS AND METHODS

VIRUSES: Rous-associated virus (RAV)-1 (subgroup A), RAV-2 (subgroup B) and RAV-60 (subgroup E) were used as reference strains for each subgroup of ALV. Rous sarcoma virus (RSV) pseudotypes, RSV (RAV-1) and RSV (RAV-2), were used for the resistance-inducing factor (RIF) test as the challenge viruses. Marek's disease virus (MDV, strain GA), herpesvirus of turkey (HVT, strain FC-126) and reticuloendotheliosis virus

(REV, strain T) were used to determine the specificity of the ELISA established. Both MDV and HVT from Dr. K. Imai and REV from Dr. N. Yuasa in our laboratory were kindly provided, respectively. Each strain except for RAV-60 was propagated in chicken embryo fibroblasts (CEF) cultures of line 151, and strain RAV-60 was propagated in quail embryo fibroblasts (QEF) cultures.

Cell cultures: CEF cells were prepared as described previously [37] from 10-day-old SPF chicken embryos of line 151 (C/CE or C/E) and line M (C/O), which was purchased from Nippon Institute for Biological Science (Ome-shi, Tokyo). QEF cells were prepared from 6-day-old Japanese quail embryos (Q/B) received from the Research Foundation for Microbial Diseases of Osaka University (Suita-shi, Osaka). The CEF and QEF cells were grown in growth medium [F10 medium containing 5% bovine serum (BS), 0.5% chicken serum (CS), 10% tryptose phosphate broth (TPB) and antibiotics] until the cells became confluent, and then the cells were maintained in maintenance medium (F10 medium containing 2% BS, 0.5% CS, 10% TPB and antibiotics).

Resistance-inducing factor (RIF) test: The RIF test was essentially the same as that described by Rubin [29]. A total of $2.5 \times 10^6$ primary CEF cells (line 151) in growth medium was plated in 60-mm plastic petri dishes (No. 3002, Falcon, Becton Dickinson Labware, Oxnard, CA) and inoculated with 0.1 ml of serial 10-fold dilutions of ALV. After 3 days, these CEF cultures were transferred to 2 dishes for each sample at the rate of $1 \times 10^6$ cells per dish. One dish was challenged immediately with 1,000 focus-forming units (FFU) of the same subgroup of RSV and the other was subcultured for another 3 days. These processes (subculture and challenge) were repeated 3 times. On the next day after the challenge with RSV, the dish was overlaid with growth medium containing 0.9% Bacto agar and examined for RSV-induced foci 8 days after the challenge. A culture was considered positive for ALV if there was a 10-fold or greater reduction in FFU of RSV as compared with the uninfected control culture.

Rabbit antisera to ALV: ALV was purified as described previously [37] with slight modification. LSCC-BK3 cells, of a B lymphoid cell line producing subgroup A of ALV [13], were cultured in a micro-carrier spinner flask (Bellco Glass Inc., Vineland, NJ) in suspension culture medium (F10 medium containing 5% BS, 10% TPB and antibiotics). Culture fluids were harvested every 4 days and centrifuged at 4,200 g for 30 minutes. After another centrifugation at 49,300 g (RP19 rotor, Hitachi Inc., Hitachi, Ibaraki) for 120 minutes at 4°C the pelleted virus was resuspended in TNE buffer (0.01 M Tris-HCl at pH 7.5, 0.1 M NaCl, 1 mM EDTA). Subsequent steps of the purification were described previously [37]. Two New Zealand White rabbits were injected subcutaneously and intramuscularly with 1-ml quantity of the purified ALV with an equal volume of Freund's complete adjuvant. They received 2 booster injections of the same dose of the virus with incomplete adjuvant 2 and 4 weeks after the first injections. The serum anti-ALV antibody titers of these rabbits were more than $10^5$ both to subgroups A and B of ALV antigens by the ELISA [37] 4 weeks after the final administration. Anti-ALV immunoglobulins were prepared by 3 repeated precipitations with 33% ammonium sulfate and dialyzed against phosphate-buffered saline (PBS) at pH 7.4. This solution was centrifuged at 13,000 g for 10 minutes and the supernatant was stored at -80°C before use.

Protein concentration: Protein concentrations of immunoglobulin and purified ALV were determined with a commercial kit (Protein assay, Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin used as a reference standard.

Conjugation of immunoglobulin with horseradish peroxidase (HRPO): The rabbit anti-ALV immunoglobulin was labeled with HRPO with NaIO₄ as described by Wilson and Nakane [38]. HRPO-conjugated IgG was purified on a Sephadex G-200 column and adsorbed with chicken liver powder and normal CEF cells (line 151) to minimize reactivity against host cell or media components that may be adhered to the virus preparations.

ELISA for detecting antibodies to ALV in chicken sera: An ELISA was performed as described previously [37]. A Nunc module plate coated with purified and Triton X-100 treated ALV of subgroup A, which was produced from LSCC-BK3 [13], and HRPO-conjugated rabbit anti-chicken IgG (No. 3204-0082, Cappel Laboratories, Cochranville, PA) were used. Absorbance (A) values more than 0.5 were considered as positive.

ELISA for detecting ALV antigens: One hundred μl of the rabbit anti-ALV immunoglobulins diluted 1:5,000 (1.6 μg/ml) with carbonate-bicarbonate buffer was added to each well of a Nunc module plate
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(No. 4–69957, A/S Nunc, Roskilde). After incubation at room temperature for 24 hours, the antibody-coated wells were washed 5 times with washing solution (PBS containing 0.1% Tween 20). The same volume of samples diluted with a diluent (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% Tween 20 and 2% skim milk powder) was added to the antibody-coated wells. After incubation at 37°C for 2 hours the wells were washed as before and 100 μl of HRPO-conjugated rabbit anti-ALV immunoglobulin diluted 1:100 was added. The plate was incubated for 1 hour at 37°C. After the plate was washed, 100 μl of freshly prepared solution of HRPO substrate (8 mg of O-phenylenediamine in 20 ml of 0.1 M citrate and 0.2 M phosphate buffer, 0.005% H₂O₂) was added and incubation was continued for 20 minutes at 37°C. Then, 100 μl of 3 N H₂SO₄ was added to each well and A value was measured at 492 nm with an automatic microplate reader (MTP-22, Corona Electric Inc., Katsuta, Ibaraki). Cell extracts (1:4) from RAV-1-infected and uninfected CEF cells (line 151) were used as positive and negative controls, respectively, throughout the experiment.

Detection of infectious ALV by the ELISA after cultivation of samples: Each well of collagen-coated 24-well plates (MS-0024, Sumitomo Bakelite, Co., Ltd. Tiyoda-ku, Tokyo) which contained about 2 × 10⁵ primary CEF cells of line 151 (C/CE or C/E) or line M (C/O) was inoculated with 0.1 ml of samples and cultured for total 8 days. After 2 days-cultivation, growth medium was changed with maintenance medium and followed by another change after cultivation for subsequent 3 days. In this assay, both media did not contain chicken serum. Culture fluids and/or cell extracts were examined for the presence of ALV antigens by the ELISA.

Cell extracts: Cell extracts were prepared by adding 0.25 ml or 2.5 ml of 0.25% Triton X-100 in PBS to each well of the 24-well or 6-well plate after removing the culture fluids, followed by freezing and thawing. The 1:4 dilutions of the cell extracts with the ELISA diluent were examined for the presence of ALV antigens by the ELISA.

Detection of infectious ALV in field sera by the ELISA and the RIF test: Field serum samples collected from layers affected with lymphoid leukaemia or haemangiomata were examined for the presence of infectious exogenous ALV by both tests after inoculating them on primary CEF cells of line 151 (C/CE or C/E). A 100 μl quantity of each sample was inoculated both into a well of a 6-well plate (No. 152795, A/S Nunc, Roskilde) which contained 1.2 × 10⁶ cells and into a tissue culture dish (60 mm, No. 3002, Becton Dickinson Labware Company, Oxnard, CA, U.S.A.) which contained 3 × 10⁶ cells. The culture supernatants and cell extracts harvested from 6-well plates on the days designated in RESULTS were diluted 1:4 and examined for the presence of ALV antigens by the ELISA. Tissue culture dishes were examined for the presence of infectious ALV by the RIF test. The samples were tested for anti-ALV antibodies by the ELISA [37].

SPF chickens and their samples: Chickens of 4 White Leghorn SPF lines, 151 (C/CE or C/E) [31], BK (C/BED or C/E) [14], PDL-1 [9] and P2 [30], which were reared in this laboratory, were used throughout the experiment. Line P2 was kindly provided by Dr. B. W. Calnek (New York State Veterinary College, Cornell University, Ithaca, NY) in 1985. Serum and 3 albumen samples were collected from each of 10 laying hens of 4 SPF chicken lines. Sera collected from laying hens of a White Leghorn SPF line M which has gs (−) and chicken helper factor (chf) (−) phenotypes were kindly provided by Drs. Y. Otaki and Y. Nomura (Nippon Institute for Biological Science) and used as control. These sera and albumens were stored at −80°C before use.

Detection of endogenous ALV antigens and infectious endogenous ALV in samples from SPF laying hens: Sera and albumens from laying hens of SPF lines maintained in this laboratory (lines PDL-1, BK, P2 and 151) diluted 1:10 were examined for the presence of endogenous ALV antigens by the ELISA. A total of 20 sera from laying hens of line M was used as negative control for gs (−) chf (−) phenotypes. All of 40 sera from 4 SPF chicken lines and 7 albumen samples which showed high A values were also tested for the presence of infectious endogenous virus by the ELISA as described above. ALV that grew in CEF cells of line M (C/O) but not in those of line 151 (C/CE or C/E) were regarded as endogenous ALV.

Comparison of A values between SPF and viremic chick sera: RAV-1 strain of subgroup A of ALV (10⁶ IU/0.2 ml) was inoculated into a york sac of 4- to 6-day-old embryos of 4 SPF chicken lines (PDL-1, BK, P2 and 151). After hatching, all the virus-inoculated chicks were tested for viremic by the virus-isolation test and only positive sera were used.
as congenitally infected chick sera. Sera from 1-day-old SPF chicks of same lines were used as negative control. All samples were diluted 1:10 and examined by the ELISA.

RESULTS

Sensitivity of the ELISA for detecting ALV antigens: Purified ALVs of subgroups A and B were sequentially diluted and the dilutions were tested by the ELISA. As a result, viral proteins as little as 0.4 ng per well were detected, as shown in Fig. 1. Dilutions of ALV-infected CEF culture fluids as high as 1:64 or 1:256 were also positive by the ELISA (Fig. 2). These dilutions of culture fluids had virus titers of about $10^3$ infectious units (IU) of ALV per well by the RIF test.

Specificity of the ELISA: Culture supernatants from CEF (line 151, C/CE) cells infected with strains RAV-1 or RAV-2 and from QEF cells infected with strain RAV-60 were tested by the ELISA. Those from CEF cells infected with MDV and HVT were harvested when 80% of the cells showed a cytopathic effect and those from REV-infected CEF cells were harvested after subcultured, and both of them were used as controls of the test. As shown in Fig. 2, culture supernatants from CEF and QEF cells infected with RAV-1, RAV-2 or RAV-60 strains reacted positively in the ELISA, but those from uninfected control and MDV-, HVT- and REV-infected cultures did not.

Detection of virus propagation in CEF cultures by the ELISA: Serially diluted stock solutions of the RAV-1 and RAV-2 strains were inoculated into wells of a 24-well plate (MS-0024, Sumitomo Bakelite Co., Ltd.) which contained about $2 \times 10^5$ primary CEF cells of line 151. Culture medium was changed with maintenance medium on 3 or 4 days before each harvest. As shown in Fig. 3, ALV antigens increased in both cell and fluid phases of CEF cultures in parallel with the increase in concentration of inoculated RAV-1 or RAV-2 within 7 days after inoculation. The highest dilutions of seed viruses that were ELISA positive were $10^{-6}$ and $10^{-5}$ IU/0.1 ml for strains RAV-1 and RAV-2, respectively, which were similar to the titers obtained by the RIF test.

Comparison of the ELISA and RIF test for the detection of infectious ALV: As shown in Table 1, viral antigens or infectivity appeared successively in subcultures in parallel with the increase in concentration of the inocula and finally the highest infectivity obtained both by the ELISA and RIF tests was identical. However, when the ELISA was used, the highest infectivity was detected as early as the first subculture (6 days after virus inoculation) of RAV-1-inoculated cultures and the 2nd subculture (10 days after inoculation) of RAV-2-inoculated cultures. In contrast, when the RIF test was used, it was not found until the 3rd subcultures (19 days
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Antibodies. As shown in Table 2, subgroup A of ALV was detected in 9 of the 12 samples by the RIF test. These samples were inoculated into CEF cultures and 1:4 dilutions of culture fluids collected on days 6, 8 and 10 after inoculation were examined for the presence of ALV antigens by the ELISA. All the RIF (+) samples showed A values of more than 0.5 on day 6 after inoculation and the values increased thereafter except for T4 and T6 (Table 2), whereas reaming RIF (-) samples showed A values as low as those of negative control thorough the experiment. None of the RIF(+) samples had antibody to ALV when tested by the ELISA, whereas all RIF(-) had anti-ALV antibodies except one sample (K8).

Effect of egg albumen on the detection of ALV antigens: Four fold dilutions of the stock solutions of strains RAV-1 and RAV-2 were mixed with an equal volume of a 1:5 dilution of egg albumen from a SPF chicken of line 15I and the mixtures were examined by the ELISA. The ELISA was capable of detecting ALV gs antigens in the egg albumen with no decrease in sensitivity.

Values of sera and egg albumen from 4 SPF chicken lines: Sera from each of 10 hens in each of 4 SPF chicken lines and from 20 hens of line M were examined for the presence of endogenous ALV antigens by the ELISA. All the sera collected from the 4 lines showed A values and there was a marked difference in the values among individual samples (Fig. 4). Also, a difference in the rate of appearance of the prominently highest A values was found among the chicken lines. In contrast, sera collected

Table 1. Comparison of the sensitivity of the ELISA and the RIF test in the detection of infectious ALV

<table>
<thead>
<tr>
<th></th>
<th>ELISA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RIF test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>RAV-1</td>
<td>(3) &lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>+</td>
</tr>
<tr>
<td>T2</td>
<td>(10)</td>
<td>+</td>
</tr>
<tr>
<td>T3</td>
<td>(15)</td>
<td>+</td>
</tr>
<tr>
<td>P</td>
<td>(3)</td>
<td>+</td>
</tr>
<tr>
<td>RAV-2</td>
<td>(6)</td>
<td>+</td>
</tr>
<tr>
<td>T2</td>
<td>(10)</td>
<td>+</td>
</tr>
<tr>
<td>T3</td>
<td>(15)</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>T3</td>
<td>(15)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each 10-fold dilution of RAV-1 and RAV-2 was inoculated onto CEF cultures of line 15I and the CEF cultures were subcultured to 2 new plates (60 mm) at intervals of 3 or 4 days of cultivation. The cell extracts were tested for the presence of viral antigens by the ELISA.

<sup>b</sup> The numbers indicate the reciprocal of the virus dilution.

<sup>c</sup> P means virus-inoculated primary CEF cultures, and T1, T2 and T3 indicate the number of subcultures. The numbers in parentheses are the days when the results were obtained in each test.

<sup>d</sup> A values of more than 0.5 were considered as positive.
Table 2. Detection of infectious ALV and antibodies to ALV in sera from commercial layers affected with lymphoid leukosis or haemangiomia by the ELISA and the RIF test

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Absorbance values of culture fluid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>cell extract&lt;sup&gt;b&lt;/sup&gt; ALV&lt;sup&gt;ab&lt;/sup&gt; Antibody to ALV&lt;sup&gt;c&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>6PID</td>
<td>8PID</td>
</tr>
<tr>
<td>N- 1</td>
<td>0.598</td>
<td>0.866</td>
</tr>
<tr>
<td>N- 2</td>
<td>1.264</td>
<td>≥2.0</td>
</tr>
<tr>
<td>T- 4</td>
<td>1.690</td>
<td>1.804</td>
</tr>
<tr>
<td>T- 5</td>
<td>1.062</td>
<td>1.324</td>
</tr>
<tr>
<td>T- 6</td>
<td>≥2.0</td>
<td>≥2.0</td>
</tr>
<tr>
<td>K- 3</td>
<td>0.872</td>
<td>1.199</td>
</tr>
<tr>
<td>K- 5</td>
<td>0.623</td>
<td>0.844</td>
</tr>
<tr>
<td>K- 7</td>
<td>0.839</td>
<td>1.002</td>
</tr>
<tr>
<td>K- 8</td>
<td>0.364</td>
<td>0.384</td>
</tr>
<tr>
<td>K- 9</td>
<td>0.303</td>
<td>0.396</td>
</tr>
<tr>
<td>K-10</td>
<td>0.853</td>
<td>1.226</td>
</tr>
<tr>
<td>K-17</td>
<td>0.314</td>
<td>0.339</td>
</tr>
<tr>
<td>Control</td>
<td>0.291</td>
<td>0.346</td>
</tr>
</tbody>
</table>

a) Culture fluids and cell extracts were tested by the ELISA for the presence of ALV antigens on days 6, 8 and 10 after inoculation of serum samples.
b) ALV was detected by the RIF test.
c) ELISA antibody titers to ALV are expressed as the reciprocal of the serum dilution showing positive.

from line M showed relatively low A values and there was little difference in the values among individuals. Endogenous ALV was isolated from 6 of the 7 sera showing high A values among 40 sera tested, and their titers were 10<sup>2</sup>-10<sup>6</sup> IU/ml (Fig. 4).

When the A value of egg albumen was determined, the hens could be roughly divided into 3 groups: hens that produced eggs with albumen of high A value (higher than 0.5), relatively low A values (about 0.2) and low A value (lower than 0.1) (Fig. 5). The number of hens in each group differed among the chicken lines. High A values were found in the albumen of 21 eggs collected from the 7 hens. Among them, 4, 2 and 1 samples were derived from lines P2, BK and 15I, respectively. Antigen titers of the highly reactive albumens from the 7 hens were 1:64 (3/7) and ≥ 1:256 (4/7). Endogenous ALV was isolated from 4 of the 7 highly reactive albumen, and virus titers were 10–10<sup>2</sup> IU/ml (Fig. 5).

The A values among 3 albumen samples collected from the same hen were generally similar, except for the samples that had extremely high A values. However, as shown in Fig. 4, an egg that had a higher A value than the other 2 was found in 2 hens of line P2 (*)

Identification of congenitally infected chicks: The RAV-1 strain was inoculated into the yolk sac of 4- to 6-day-old embryos of the 4 SPF chicken lines at a concentration of 10<sup>6</sup> IU/0.2 ml. After hatching, all the inoculated chicks were confirmed as viremic by testing the cell extracts for the presence of ALV antigens by the ELISA after cultivation of their sera. Although most of the viremic sera reacted more strongly than the SPF sera in each line, 3 and 6 SPF sera from lines BK and P2 were reactive as strongly as those from the viremic chicks of the lines, respectively (Fig. 6).

DISCUSSION

An ELISA for detection of ALV antigen using rabbit anti-ALV antibodies was developed and found to be specific for ALV. Its sensitivity was almost the same as that of ELISA using anti-gs antibodies [2, 33]. However, this ELISA could detect ALV antigens in the culture fluid (Fig. 3) as well as in the cell extracts, while it was hard to detect ALV antigens in the culture fluids by the ELISA using anti-gs antibodies [23]. Since the anti-ALV antiserum used for the present study contained the antibodies against gp85 as envelope glycoprotein in addition to the gs proteins by a western blotting technique (data not shown), the anti-gp85 antibodies should be necessary for the detection of virus particles or envelope antigens in the culture fluids.

The titer of infectious ALV has been usually
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Fig. 4. Absorbance values of serum samples collected from 5 SPF lines. Isolation of infectious endogenous ALV from sera except line M (*) was demonstrated by detecting ALV antigens in cultured CEF cell extracts of line M on day 8 after inoculation. The symbols @the numbers mean the virus titers expressed as log (IU/ml) and • indicate positive and negative for isolation of endogenous ALV, respectively.

determined by the RIF test [29] or a phenotypic mixing test [20]. In the RIF test, CEF cells inoculated with viral samples are subcultured at least 3 times and it takes 18 days for the titration [29]. However, by using the ELISA, the titers of infectious ALVs were determined within a week after inoculation of CEF cells with RAV-1 or RAV-2 (Table 1, Fig. 3). The result of the ELISA for the detection of infectious ALV from field sera was consistent with that of the RIF test (Table 2). Thus, isolation of infectious ALV by detecting ALV antigens in cultured samples with the ELISA on 8 days after inoculation is superior to the RIF test in rapidity, simplicity and applicability to the large-scale field trials. Since the C/E phenotype of CEF cells which express no endogenous ALV antigens is needed for this assay, we used CEF cells of line 15I described below. There was a tendency that subgroup A of ALV appeared in the cell extracts a little earlier than in the culture fluids (Fig. 3).

In this study, 4 SPF chicken lines maintained in this laboratory were examined for the presence of endogenous ALV. Lines P2 and BK were found to produce endogenous ALV at a high frequency (Figs. 4 and 5). Line PDL-1 should express endogenous ALV antigens since some sera showed higher A values than those of line M (Fig. 4) and some albumen samples showed higher A values than those of line 15I (Fig. 5). In contrast to the 3 lines, the incidence of endogenous virus expression was low in line 15I except for few samples when tested by the ELISA (Figs. 4, 5 and 6). Since the expression level of endogenous ALV or its viral antigens were different among individual chickens and chicken lines [28, 32], large numbers of chickens should be examined in each line to confirm the presence of endogenous ALV and its viral antigens.

Although the 3 albumen samples from most of the hens (38/40) showed consistent A values, those of 2 hens of line P2 were not consistent (Fig. 5). Endogenous viral gene 7 (ev7) occasionally induces complete endogenous ALV at a low frequency in line 15R [4, 27]. These phenomena lead to the conclusion that such hens having ev7 will be difficult to identify by testing a single albumen for the presence of ALV antigens by the ELISA. Since such sporadic transmitters are suspected to exist in the field, it is important to study the mechanism of the sporadic transmission for the progress of ALV eradication program.

There was a strong correlation between the presence of ALV antigens in albumens and the congenitally transmitting hens [8, 16, 21, 36]. However, ELISA gave a false-positive reaction due to endogenous viral antigens [3, 5, 7, 8]. Smith et al. [33, 34] also reported that endogenous ALV antigens detectable by the ELISA were found in the egg albumens from viremic hens for endogenous ALV. We also confirmed that some albumens from 2 of the 4 SPF lines were strongly reactive in the ELISA (Fig. 5). Furthermore, Okaizaki et al. [19] reported the presence of “hard lines” which was difficult to reduce the rate of ALV infection by selecting dams.
Fig. 5. Absorbance (A) values of albumen samples collected from 10 hens from each of 4 SPF lines. A values of 3 albumen samples collected from the same hen are shown in a vertical line. ©: Positive for isolation of endogenous ALV, the numbers indicate the virus titers expressed as log (IU/ml). Ø: Negative for isolation of endogenous ALV. •: Not tested for endogenous ALV. *: Egg albumen having a higher A value than the other 2 in the same hen.

Fig. 6. Absorbance values of the sera from embryonally inoculated and uninoculated SPF chicks of 1-day-old age. Closed symbols indicate chicks which were congenitally infected with ALV and open symbols indicate uninfected SPF chicks.
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Such "hard lines" might be caused by the successive appearance of the chickens expressing endogenous ALV. These facts indicate that direct tests for ALV gs antigens in albumens by the ELISA mistakenly identify hens expressing endogenous ALV antigens as congenitally transmitting hens. This false-positive reaction is a problem especially in chicken lines which produce endogenous ALV antigens at a high frequency such as lines BK and P2 when the eradication is performed.

Possibility of identifying viremic chicks by detecting ALV antigens in sera was partially validated in this study (Fig. 6). However, some sera from day-old SPF chicks (Fig. 6) were reactive as strongly as those of the viremic chicks for exogenous ALV in the ELISA. Since the highly reactive SPF samples were derived from 2 SPF chicken lines which express endogenous ALV (Figs. 4 and 5), the reaction was probably due to endogenous ALV antigens. These results suggest that chicks infected congenitally with exogenous virus could not be detected specifically by testing the sera for the presence of ALV antigens by the ELISA. There were some differences in the values among individual viremic chickens of line PDL-1 (Fig. 6). This fact also confirmed the previous report that for complete eradication day-old chicks should be selected on the basis of the biological assay of blood [8].

However, sera from laying hens showed background A value in the ELISA as described previously [33]. The A values were higher than those of day-old chicks (Figs. 4 and 6). Also, the average A value of sera from 20 laying hens of line M which was gs (−) chf (−) phenotype is as high as 0.530±0.051 (Fig. 4). Therefore, the high A value of laying hens is not always due to endogenous ALV, and the direct test for ALV antigens in serum by the ELISA is not suitable for identification of the viremic hens. The reason for this high reactivity of hen's sera is not known.

The ELISA for detecting ALV antigens was shown to induce a false-positive reaction due to endogenous ALV antigens in 2 of the 4 SPF lines at a high frequency, as described previously [3, 5, 7, 8, 15]. The false-positive reaction due to endogenous ALV antigens can be excluded by cultivation of samples on C/E phenotype of cells, and at the same time, cultivation of samples leads to the increase in the amount of exogenous ALV. Accordingly, we developed the ALV-isolation test, which was sensitive and specific for exogenous ALV, with 24-well plates and the ELISA for the application to large numbers of samples. This test will be useful to identify the hens transmitting exogenous ALV to their chicks. Because some transmitters which shed no detectable ALV antigens but a small amount of infectious exogenous ALV in albumen can not be detected unless samples were culture [36]. Detection of such hens may be the key to reduce the rate of ALV infection further in the field.

REFERENCES


