A Simple Technique for the Detection and Classification of Latent Avian RNA Tumor Viruses

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Dedicated to Prof. WERNER SCHÄFER on the occasion of his 60th birthday

A rapid and simple technique for the detection of latent RNA tumor viruses has been described. It is based on phenotypic mixing between the latent agent(s) and a nondefective strain of RSV used as indicator virus. Most of the embryos from two different chicken lines tested so far contain such agents. According to their host range and serological properties they belong to subgroup E. The phenotypic mixing assay may also be applied for the titration of nontransforming viruses in general.

Two methods have been mainly applied for the detection of nontransforming avian tumor viruses in chick embryo cells. The resistance inducing factor (RIF) test is based on the property of these viruses to interfere with the superinfection of another avian tumor virus possessing a similar coat antigenicity. By means of this technique, as well as others, avian tumor viruses were classified into the subgroups A, B, C and D. The second method, the COFAL test, is based on the detection of viral group specific (gs) antigen produced by virus infected cells. Despite the availability of both techniques, widespread latent avian tumor viruses remained undetected until very recently. These agents, called chicken helper factor, RAV-0 or RAV-60, were shown to belong to a new subgroup, E. In addition they seem to differ from common “RIF” viruses in that they are frequently contained in chicken cells in a repressed form. From this state they may be released after superinfection with another chicken tumor virus or after exposure of the cells to various physical or chemical treatments.

Conventional assay techniques are not adequate for the detection of such repressed agents. The RIF test is not useful because a latent virus infection would only be detectable if virus particles or viral coat antigen is produced by the cell. And even then the assay might be inappropriate since RAV-0 producing cells have been found to interfere only slightly with the superinfection of a subgroup E pseudotype of RSV. Similarly, the COFAL test is not adequate since not only gs+ but also gs− cells may posses active helper factor. The assay methods which have been used to date instead, have been based on the capacity of latent viruses to provide defective RSV (RSVβ(0)) or RSV(−) with coat properties necessary for the infection of susceptible host cells. These tests are complicated by the fact that detectiveness of the indicator virus has to be overcome by the use of pseudotypes of RSV(−), obtained after double infection of RSV(−) with a nontransforming virus of a subgroup other than E. This paper describes a simple technique which is suitable for the detection of latent viruses in avian embryo cells. It is also based on phenotypic mixing of virus coat antigens, in this case, however, a nondefective RSV strain is used as an indicator virus.

C/E and C/BE cells were derived from the Spafas chicken line, and C/O, C/A, C/E and C/AE cells from the L-15 line. They were typed for their susceptibility to focus formation with viruses of all 5 subgroups. (C/BE cells, for example, are defined as being resistant to B and E viruses.)

A cloned, nondefective Schmidt Ruppin RSV strain was used as a source of indicator virus. It belongs to subgroup A and will be abbreviated as SRV-A. The origin of the chicken lines and of the viruses, and the procedures for tissue culture and virus assay have been described elsewhere. In order to obtain an indicator virus free of contaminating subgroup E virus, SRV-A was grown on different embryos and then assayed on C/A cells. This type of cell, resistant to infection with A virus, should reveal in the samples, the presence of a virus belonging to another subgroup.

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Table 1. Plating efficiency of SRV-A obtained from embryo cells of two chicken lines on C/A, C/E and C/AE cells. * This figure (X) was calculated by multiplying the FFU/ml obtained on C/A cells (E) by $1 \times 10^8$ divided by the FFU/ml on C/E cells (A): $X = \frac{E \times 1 \times 10^8}{A}$. ** SRV-A grown on this embryo was used as a source of indicator virus.

if, due to phenotypic mixing, SRV-A had been provided with the envelope antigenicity of the latter. From the samples tested, SRV-A grown on cells of Spafas embryo 10/1 (SRV-A 10/1) was chosen as a stock with which all subsequent experiments were performed. Envelope properties of this virus material were determined by infecting different types of chick cells. As shown in Table 1, $1.5 \times 10^6$ focus forming units (FFU) were obtained on C/E cells and $3.5 \times 10^4$ FFU von C/A cells. This is consistent with the idea that this stock of SRV-A contains rather low amounts of subgroup E virus.

The assay for latent viruses in embryo cells consisted of infecting primary cultures derived from separate 11-day old chick embryos with 0.2 ml of indicator virus (SRV-A 10/1 diluted 1000 fold in order to eliminate subgroup E virus) and transferring them two times at 3 and 5 day intervals. Three days after the second transfer, when most of the cells were transformed, the supernatants were harvested and frozen at $-70^\circ$C until tested on the appropriate types of chicken cells. Repeated freezing and thawing did not reduce the titers of the samples to any significant degree. As shown in Table 1, there is a wide variation in the titers of the samples tested on C/A cells ranging from $6.5 \times 10^4$ FFU/ml to nondetectable amounts, whereas, as expected, rather constant titers were obtained on C/E cells. For a better comparison, titers obtained on C/A cells were standardized to $1 \times 10^6$ FFU/ml obtained on C/E cells (last column in Table 1). That in no case any foci were obtained on C/AE cells demonstrates that genetic resistance to certain avian tumor viruses is rather absolute.

In order to determine whether the described assay may be used for a quantitative estimation of latent viruses, experiments were performed in a model system using myeloblastosis associated virus of subgroup B (MAV-B). The obvious experiment would consist in infecting chick embryo cells with various MAV-B dilutions and superinfecting these cells with constant amounts of SRV-A. Titration of the supernatants several days later should then reveal whether or not there is a linear relationship between the concentration of nontransforming virus and the number of foci obtained on C/A cells. However, the experiments were performed the other way around: C/O cells fully infected with SRV-A were
superinfected with different MAV-B dilutions and the supernatants tested on C/AE cells 4 and 5 days later. (Medium was changed at daily intervals and contained 1% DMSO.) This experimental device yields essentially the same information and in addition it might provide a new method for the titration of non-transforming viruses. (C/AE cells were used rather than C/A cells in order to eliminate "background" titers of SRV-A due to possible latent E virus in the SRV-A infected cells.) The results plotted in Fig. 1 show that there is indeed a linear relationship between the amount of MAV-B inoculum and the titer of focus forming units of the harvest, with a plateau at higher MAV-B concentrations. The titer of the same samples assayed on C/O cells ranged around $1 \times 10^7$ FFU ml. This method appears therefore appropriate for the determination of the relative titers of nontransforming viruses. An endpoint dilution technique for the titration of chicken leukemia viruses based on a similar principle has been described by other authors.  

The host range classification of the latent virus(es) contained in Spajas and L-15 cells as members of subgroup E is based on the assumption that the RSV (RAV-O) used for the cell typing represents indeed a subgroup E pseudotype. Since this virus was passaged several times in Spajas and L-15 cells after it was obtained from Dr. P. K. Vogt, the question arose as to whether or not latent viruses were picked up which might have led to a change in its host range. In order to clarify this, a neutralization assay was performed with RSV (RAV-O) and SRV-A obtained from both Spajas and L-15 cell lines and a rabbit serum prepared against RAV-O in Dr. Vogt's laboratory. In addition, the specificity of the reaction was controlled with chicken immune sera prepared against viruses of subgroups A to D and by using a known pseudotype of SRV-A, SRV-A (MAV-B), obtained in the experiment described above. The sera were diluted 1:25 and the virus incubation was performed following standard procedures. The samples were then assayed on C/A cells. As shown in Table 2, there is a strong neutralization of SRV (MAV-B) with anti-B serum and a weak reaction with anti-D serum. Such a cross reaction between subgroup B and D has been observed earlier. In contrast, RSV (RAV-O), SRV-A (Spajas), and SRV-A (L-15) are neutralized to a significant extent only with anti-E serum. This result indicates that most of the embryos from Spajas and L-15 lines tested so far contain an activable latent virus and that this or these agents belong to subgroup E.

The advantages of the described technique for the detection of latent viruses are its wide range of sensitivity and its relative simplicity. It is enough to infect one plate per embryo with a stock of

### Table 2. Serological characterization of MAV-B and 'chicken helper factors' in phenotypic mixtures with SRV-A.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Serum prepared against virus of subgroup</th>
<th>Control chicken serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>RSV (RAV-O)</td>
<td>0.5*</td>
<td>0.8</td>
</tr>
<tr>
<td>SRV-A (Spajas)**</td>
<td>NT</td>
<td>0.8</td>
</tr>
<tr>
<td>SRV-A (L-15)**</td>
<td>NT</td>
<td>1.8</td>
</tr>
<tr>
<td>SRV-A (MAV-B)</td>
<td>0.8</td>
<td>0.015</td>
</tr>
</tbody>
</table>

* Fraction of surviving foci. Figures in italics represent significant neutralization. NT: not tested. ** These virus stocks were derived from Spajas embryo 37/4 and L-15 embryo 46/2 respectively, after infection with SRV-A indicator virus.
SRV-A free of E virus and harvest the supernatants 4 to 5 days later when most of the cells are transformed. These samples may then be stored at –70 °C until tested. As shown in this report, high titered stocks of SRV-A free of detectable E virus can be obtained from cells of certain *Spafas* embryos. SRV-A grown on quail cells should also be free of contaminating E virus. Our technique may be varied by using nondefective B, C or D viruses as indicator viruses. In this case, other test cells have to be used, such as quail cells for an assay with nondefective sarcoma virus of subgroup B. Neutralization experiments on appropriate chicken cell types then provide an additional method for the classification of the activated viruses. Under the assumption that latent viruses specify their own coat antigen after superinfection with an indicator virus, our technique allows the detection of avian RNA tumor viruses from all known subgroups, regardless of whether or not these antigens are expressed in the carrier cell.

More experiments have to be performed in order to support our findings that there are chicken embryos which cannot be activated by superinfection to produce a “chicken helper factor”. In addition, it would be interesting to determine whether such cells remain negative even after treatment with ionizing radiation or chemical carcinogens.

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Abbreviations: RSV: Rous sarcoma virus; RAV: Rous associated virus; DMSO: dimethylsulfoxide.