Formation of a transformed follicle is necessary but not sufficient for development of an avian leukemia virus-induced lymphoma

(proeneoplasia/tumor progression/genetic resistance/c-myc/methyl green pyronin)

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ABSTRACT Avian leukemia virus (ALV) infection of susceptible chickens induces bursal lymphomas after a latent period of several months. The clonal development of these B-cell tumors is believed to be a multistep process. Histopathological changes, referred to as transformed follicles, occur within the target organ soon after virus infection and may represent a proximal stage of lymphogenesis. To establish further the significance of this lesion and its relationship to the subsequent development of lymphomas, we have compared the incidence of transformed follicles observed in animals susceptible or resistant to ALV-induced tumor development. During the 8 weeks following ALV infection, transformed follicles were detected in 82% of the susceptible animals and in 11% of the resistant animals. These results indicate that the incidence of transformed follicles in these animals correlates with their susceptibility to lymphoma development. Furthermore, each transformed follicle does not develop into a tumor. These observations suggest that the formation of a transformed follicle is necessary but not sufficient for lymphoma development.

The bursa of Fabricius is the site of primary tumor development in chickens susceptible to avian leukemia virus (ALV)-induced B-cell lymphomas (1). These lymphomas first appear 4–6 months after virus infection. Examination of DNA obtained from end-stage tumors has revealed that the cellular proto-oncogene c-myc has been interrupted by the insertion of ALV DNA sequences (2, 3). This insertion positions a viral long terminal repeat within the c-myc genetic locus and results in increased expression of c-myc RNA (2, 3). DNA transfer studies have identified an additional genetic locus believed to be important in lymphoma development. This locus, designated B-lym, produces morphological transformation of NIH 3T3 fibroblasts after transfection of tumor cell DNA (4–6). The relationship between the activation of c-myc, the role of B-lym, and the development of the lymphoma is currently not known.

Considerable data suggest that tumor development proceeds as a series of discontinuous events that are stochastic in nature (7, 8). ALV-induced B-cell lymphomas are thought to begin with the appearance, soon after infection, of a limited number of preneoplastic lesions within the bursa (9, 10). This histopathological feature, referred to as a transformed follicle (9), is characterized by an accumulation of large pyroninophilic lymphoblasts within individual bursal follicles. The hypothesis that transformed follicles are involved in the subsequent development of bursal lymphomas is supported currently by three observations. (i) Transformed follicles are absent from uninfected control animals. (ii) Transformed follicles occur within the target organ where primary lymphomas develop. (iii) Cells within these follicles are morphologically similar to those seen in end-stage tumors (9, 10). The data presented in this report demonstrate that, during the first 8 wk after infection with ALV, 82% of the susceptible animals and 11% of the resistant animals contained transformed follicles. The incidence of transformed follicles, therefore, correlates with the frequency of lymphomas observed in animals that are susceptible or resistant to tumor development. This observation strengthens the hypothesis that the transformed follicle represents a preneoplastic stage and further emphasizes the need for additional events to complete the tumorigenic process.

MATERIALS AND METHODS

Cells and Viruses. Methods for culturing chicken embryo fibroblasts and for virus production have been described (11). Rous-associated virus-1 (RAV-1) (kindly provided by L. Crittenden) was cloned by end-point dilution and used throughout this study. Interference analysis demonstrated the virus was characterized by subgroup A envelope glycoproteins (12).

Virus Infection, Animal Care, and Histopathology. Fertile eggs obtained from Hylane International (Dallas Center, IA) were incubated in a humidified incubator at 39°C. One day after hatching, Hylane SC (C/O, gs+ , chf+, V+) and Hylane FP (C/E, gs-, chf-, V-) chicks were infected intravenously with 2 x 105 infectious units (IU) of RAV-1 obtained from a single pool of biologically cloned virus. The animals were housed in isolation facilities in the Animal Resource Center at this institution as described (13). Bursas removed from chickens sacrificed 2, 4, 6, and 8 wk after infection were fixed in 10% neutral buffered formalin and were prepared for histological examination. For each bursa, serial sections (6 µm thick), prepared at 400-µm intervals throughout the entire organ, were stained with methyl green pyronin and examined microscopically as described (10).

RESULTS

Appearance of Transformed Follicles in Hylane SC and FP Chickens. We have previously characterized two lines of chickens obtained from Hylane International that respond differently to ALV infection. Hylane SC and FP chickens were shown to be, respectively, susceptible and resistant to the development of ALV-induced lymphomas (13). After ALV infection, 27 of 50 Hylane SC animals (54%) developed B-cell lymphomas. In contrast, none of the 36 FP animals examined developed tumors. Southern analysis of DNA prepared from tumor-bearing Hylane SC animals indicated that the tumors were clonal, with only one tumor present per animal.

Abbreviations: ALV, avian leukemia virus; RAV, Rous-associated virus; IU, infectious units.

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To determine whether the presence of transformed follicles is correlated with the frequency of primary bursal lymphoma, we have compared the incidence of these follicles occurring in these two lines of chickens (Hyline FP and SC), which differ in their susceptibility to ALV-induced lymphomagenesis. The appearance of transformed follicles

Table 1. Analysis of B-cell lymphoma development in Hyline SC and FP chickens

<table>
<thead>
<tr>
<th>Event analyzed</th>
<th>Hyline SC</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Hyline FP</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animals positive/animals tested</td>
<td>%</td>
<td>Expected positive</td>
<td>% reduction</td>
<td>%</td>
<td>Animals positive/animals tested</td>
<td>%</td>
<td>Expected positive</td>
<td>% reduction</td>
</tr>
<tr>
<td>Viremia (cumulative over 5 wk)</td>
<td>19/21</td>
<td>90</td>
<td>21/21</td>
<td>10</td>
<td>17/32</td>
<td>53</td>
<td>32/32</td>
<td>47*</td>
<td></td>
</tr>
<tr>
<td>Viral integration in bursal DNA (at 5 wk)</td>
<td>13/15</td>
<td>87</td>
<td>14/15</td>
<td>7</td>
<td>3/13</td>
<td>23</td>
<td>7/13</td>
<td>57*</td>
<td></td>
</tr>
<tr>
<td>Transformed follicles (at 4 wk)</td>
<td>19/20</td>
<td>95</td>
<td>17/20</td>
<td>—</td>
<td>4/15</td>
<td>27</td>
<td>3/15</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Tumor development (24 wk)</td>
<td>27/50</td>
<td>54</td>
<td>48/50</td>
<td>44*</td>
<td>0/36</td>
<td>&lt;3</td>
<td>10/36</td>
<td>&gt;90*</td>
<td></td>
</tr>
<tr>
<td>Metastasis (24 wk)</td>
<td>24/27</td>
<td>89</td>
<td>27/27</td>
<td>11</td>
<td>NA</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Data from analysis of viremia and viral DNA integration are from ref. 14, and data for tumor incidence and metastasis are from ref. 13. Animals were infected i.v. 1 day after hatching with $2 \times 10^7$ IU of RAV-1. They were examined over a 5-wk period for the presence of viremia and 5 wk after infection for integrated viral DNA. Plasma samples collected at different times were assayed for the presence of infectious virus. The sensitivity of the assay allows detection of $>400$ IU/ml. Integrated viral sequences were detected by digestion of DNA samples with the restriction enzyme EcoRI and by analysis by Southern DNA transfer and hybridization. Detection of integrated sequences required that 10% or more of the cells contain integrated viral DNA. Analysis of transformed follicles 4 wk after infection is from Fig. 2. Animals were observed for a 24-wk period for the development of primary and metastatic lymphoma. No significant quantitative differences were observed among any of the animals positive for viremia or viral DNA integration. Expected positive = (% positive observed at previous step) $\times$ (number tested). % reduction = $100 \times (1 - (number positive)/(number expected positive))$. NA, not applicable.

*P < 0.025.
in ALV-infected chickens is a relatively rare event occurring in 0.01%-0.1% of the bursal follicles (10). It is characterized as an accumulation of large pyroninophilic lymphoblastoid cells, which are located in individual follicles (Fig. 1). Microscopic analysis of bursal tissue serially sectioned at 400-μm intervals and stained with methyl green pyronin permits the detection of these rare follicles by directly examining each of the ~10² follicles within each bursa, on average, only once. One-day-old FP and SC chicks were infected with RAV-1. At different times after infection, animals were sacrificed and the bursas were prepared for histological analysis. Microscopic examination of bursal tissue from these chickens, sacrificed 2, 4, 6, and 8 wk after infection, revealed that significantly fewer resistant (FP) animals developed transformed follicles compared to the susceptible (SC) animals (Fig. 2). Seven of 61 FP animals

(11%) developed transformed follicles compared with 54 of 66 SC animals (82%). Furthermore, among those animals that developed transformed follicles, animals from the susceptible line contained approximately twice as many of these follicles as animals from the resistant line. The results from this analysis further demonstrate that the presence of transformed follicles in the susceptible line, detected as early as 2 wk after infection, reached a maximum value by 4 wk after infection. Moreover, no decrease in the incidence of these follicles was observed during the course of this study.

DISCUSSION

We have examined the progress of ALV infection and the subsequent development of bursal lymphomas in the Hyline SC and FP chickens. In addition to the analysis of transformed follicles presented in this report, we have previously examined the appearance of viremia and the establishment of integrated viral DNA sequences in bursal tissue (13, 14). If the development of a bursal lymphoma were a multistage process, then a reduction in the incidence of any of the requisite stages in this process would result in a decrease in the number of animals that would remain at risk for tumor development. Comparison of the incidence of these events in the SC and FP chickens identifies several stages where a significant reduction in the number of animals at risk for tumor development occurs. These data, summarized in Table 1, indicate that resistance to ALV-induced lymphomagenesis occurs at several steps. After ALV infection of the susceptible line (SC), 90% of the animals were viremic and 87% had integrated viral sequences in bursal DNA. In contrast, only 53% of the animals of the resistant line (FP) developed viremia, and only 23% contained integrated viral sequences. Both the appearance of viremia and the integration of viral DNA are indicators of the spread of virus infection. As such, they contribute to the development of bursal lymphomas. Thus, at each of two distinct stages early in lymphomagenesis, a significant number of FP chickens are prevented from progressing toward tumor development. In contrast, a block or reduction in the incidence of viremia or DNA integration does not occur in the SC line.

The analysis presented in Fig. 2 demonstrated that, at 4 wk after infection, 95% of the susceptible animals and 27% of the resistant animals developed transformed follicles (Table 1). Since 87% of the susceptible animals and 23% of the resistant animals contained integrated viral sequences, these data provide evidence for an association between integration and the presence of transformed follicles. Moreover, as virtually all of the viral integration in the SC animals occurred within the first 4 wk of infection (14), integration is associated temporally with both the appearance and the maximal incidence of these follicles. Although the incidence of transformed follicles observed in the FP animals is reduced when compared with that observed in susceptible animals, a similar association between viral integration and the presence of these follicles was observed in these resistant animals.

Finally, if the presence of transformed follicles were sufficient for lymphoma development, we would expect to see 95% of the SC animals and 27% of the FP animals develop lymphomas. However, only 54% of the SC animals and none of the FP animals developed tumors. Therefore, in addition to the events that occur prior to the development of transformed follicles, events occurring after the development of these follicles, in both susceptible and resistant chicken lines, must also participate in lymphomagenesis.

The temporal analysis of viral DNA integration and of the development of transformed follicles has provided data that suggest that viral integration within the first 4 wk of infection is associated with the development of these follicles. This
suggestion is consistent with the hypothesis that viral integration adjacent to c-myc, which modulates the expression of this proto-oncogene, is directly responsible for the development of the transformed follicles observed in susceptible and resistant animals. Accordingly, expression of c-myc, although essential for the development of transformed follicles, is insufficient for tumor development. The progression of a transformed follicle to a lymphoma requires additional events including, perhaps, the involvement of other genetic loci such as B-lym. Our observations (Table 1) suggest that a significant number of both SC and FP animals exhibit resistance to lymphoma development after the appearance of the transformed follicle.

The data presented above support the following two conclusions. First, the frequency of lymphoma development correlates with the incidence of transformed bursal follicles. Animals susceptible to ALV-induced lymphomas exhibited significantly more of these follicles than resistant animals. Second, the development of a transformed follicle is not sufficient for tumorigenesis, as revealed both by the presence of transformed follicles in resistant animals and by the identification of several transformed follicles (as many as 17) within a single susceptible animal that develops only one bursal tumor (13).

Our results are consistent with the multistep nature of tumor development and provide support for the hypothesis that the cells within the transformed follicle represent a preneoplastic population of cells from which a lymphoma can develop. Therefore, within an individual, the greater the number of transformed follicles, the greater the probability that this preneoplastic stage of the disease will progress to overt neoplasia.

The present model of preneoplasia is reminiscent of hyperplastic diseases in humans, which are considered to be premalignant states (7, 8). Multicentric lesions such as cervical hyperplasia, leukoplakia of the buccal mucosa, and colonic polyps are known to predispose the individual to the subsequent development of cancer. It will be of obvious significance to analyze this avian model in depth, with particular emphasis on the underlying molecular events responsible for the appearance of transformed follicles and the critical additional changes that cause a minute subset, probably a single cell within the transformed follicle, to become malignant.

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