

Endogenous oncornaviral antigen in the bursa of Fabricius of 15B × 7₂ chickens

(bursa/RNA tumor viruses/immunofluorescence)

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ABSTRACT Oncornaviral antigen was detected in the bursal epithelium and in a subpopulation of bursal follicular cells of 15B × 7₂ chickens. This antigen is present in the bursal epithelium at 11 days of embryogenesis and persists there for at least 3 weeks after hatching. The absence of detectable antigen in the intestinal epithelium contiguous to the bursal epithelium indicates that the accumulation of viral antigen is a specific property of the bursal epithelium. The observation of C-type particles in the intraepithelial spaces suggests that the viral antigen is synthesized and assembled into virions by the bursal epithelial cells. In embryonic bursas, viral antigen-positive cells radiate from the surface epithelium toward the central region of the follicles. In bursas from post-hatch chickens, viral antigen-positive cells, including intrafollicular epithelial cells and cells resembling lymphocytes, are confined to the medullary region of the follicles.

At present it is not known what role, if any, endogenous tumor virus genes play in the normal physiology of animals. In the avian oncornavirus system, it has been determined (1, 2) that a close structural homology exists between the exogenous avian leukosis viruses, which are oncogenic for bursal-derived (B) lymphocytes (3, 4), and the endogenous virus, Rous-associated virus-0 (RAV-0), which has little or no oncogenic potential (5). Because lymphoma induction is associated with an aberrant pattern of B-cell differentiation (6), the structural homology of exogenous and endogenous viruses suggests the possibility that exogenous avian leukosis viruses exert their oncogenic effect by interfering with a physiological function mediated by the endogenous virus.

Because infection of chickens with exogenous leukosis viruses specifically induces a B-cell lymphoma that arises in the bursa of Fabricius (3), we set out to investigate the distribution of endogenous oncornaviral antigen in the bursas of 15B × 7₂ chickens. These chickens spontaneously produce high levels of RAV-0 (7, 8) and were chosen for analysis on the assumption that this phenotype would facilitate the detection of viral antigen.

MATERIALS AND METHODS

Chickens. Embryonated eggs from line 7₂ chickens and from the mating of line 15B with line 7₂ chickens (15B × 7₂) were obtained from the Regional Poultry Research Laboratory (East Lansing, MI). Both 7₂ and 15B × 7₂ embryos are positive for RAV-0 production [V(+)], as assayed with fibroblasts in tissue culture. Because cells of 15B × 7₂ chickens are susceptible to reinfection by RAV-0, the level of RAV-0 production by 15B × 7₂ cells is amplified to a level approximately 10³-fold greater than that for 7₂ cells (8). Spafas-MR49 embryos, which are negative for RAV-0 production [V(-)] as well as for expression of chicken helper factor, were obtained from Spafas Inc. (Norwich, CT).

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Preparation of Immune Sera. To prepare antisera to structural proteins of RAV-0 (anti-RAV-0 sera), rabbits were injected with Nonidet P-40 (NP40; 0.5%)-treated preparations of either RAV-0 or the RAV-0 pseudotype of the Bryan high-titer strain of Rous sarcoma virus [BH-RSV (RAV-0)]. RAV-0 was obtained from fibroblasts cultured from 10-day-old 15B × 7₂ embryos; BH-RSV(RAV-0) was obtained by the propagation of stock virus on fibroblasts derived from 10-day-old quail embryos, which do not express RAV-0-related antigens but are permissive for RAV-0 replication (7). The RAV-0 used to complement the BH-RSV of the stock preparation was produced by cells of a line 7 embryo (7). Virus preparations for immunization and competition studies were purified on sucrose gradients as described (9). The antisera were serially absorbed twice on phosphate-buffered saline-washed monolayers of fibroblasts derived from 10-day-old Spafas-MR49 embryos and then absorbed as described (10) with an equal volume of a lysate of quail embryo fibroblasts [5 × 10⁷ cells/ml in 1% NP40/1% deoxycholate/0.1 M NaCl/0.05 M Tris-HCl, pH 7.6/2 mM phenylmethylsulfonyl fluoride/0.05 M iodoacetamide].

Antiserum raised in rabbits against purified nucleoprotein cores of avian myeloblastosis virus (generously provided by Dani Bolognesi, Duke University School of Medicine, Durham, NC) was absorbed as described above. Antiserum raised in rabbits against chicken gamma globulin (Antibodies, Inc., Davis CA) was absorbed twice with lysates (prepared as described above) of 15B × 7₂ embryonic fibroblasts and of quail embryonic fibroblasts. Samples of normal (nonimmune) rabbit serum were absorbed in parallel with each immune serum as described above. Fluorescein-labeled goat anti-rabbit gamma globulin serum was purchased from Antibodies, Inc. and used unabsorbed. Purified N-tropic AKR murine leukemia virus (AKR-MuLV) was kindly provided by Nobuo Tsuchida, Wistar Institute (Philadelphia, PA).

Immunofluorescence Assays. The bursa of Fabricius and other organs were dissected from embryonic and post-hatch chickens and immediately frozen at -70°C. Frozen sections (6 μm) of tissue were cut and fixed with acetone for 10 min at -20°C.

The sections were hydrated in phosphate-buffered saline; exposed to an appropriate dilution (at least 1/40) in phosphate-buffered saline of anti-RAV-0, anti-chicken gamma globulin, or normal rabbit serum for 30 min at 37°C; washed thoroughly in phosphate-buffered saline; exposed to a 1/40 dilution in phosphate-buffered saline of fluorescein-labeled goat anti-rabbit gamma globulin serum; washed thoroughly in phosphate-buffered saline; and mounted in 25% glycerol in phosphate-buffered saline. The two anti-RAV-0 sera [elicited

Abbreviations: AKR-MuLV, N-tropic AKR murine leukemia virus; B-cells, bursal-derived lymphocytes; BH-RSV(RAV-0), RAV-0 pseudotype of the Bryan high-titer strain of Rous sarcoma virus; NP40, Nonidet P-40; RAV-0, Rous-associated virus-0; V(+) and V(-), phenotypes of chickens whose fibroblasts do and do not spontaneously produce endogenous virus, respectively.

to either RAV-0 or BH-RSV(RAV-0)] yielded identical results in the immunofluorescence assays and were used interchangeably for these assays. The stained sections were examined with a Leitz-Orthoplan microscope with epi-illumination and photographed on Kodak Tri-X film.

Electron Microscopy. Bursas from 18-day-old $15B \times 7_2$ and Spafas-MR49 embryos, which had been quick-frozen at -70°C , were bisected. One-half of each bursa was examined by immunofluorescence and the other half, still frozen, was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide, stained *en bloc* with uranyl acetate, and embedded in Epon resin according to standard procedures. Thin sections were stained with lead citrate and examined in a Zeiss electron microscope.

RESULTS

Distribution of Viral Antigen in the Bursa of $15B \times 7_2$ Chickens. Patches of cells in the bursal epithelium of 11- to 12-day-old $15B \times 7_2$ embryos reacted strongly with anti-RAV-0 serum in an indirect immunofluorescence assay (Fig. 1a). Only a low level of background fluorescence was detected with normal rabbit serum (Fig. 1b).

Fluorescence due to the reactivity of the anti-RAV-0 serum became uniformly distributed in the bursal epithelium by day 17 of embryogenesis, similar to the pattern shown here for the bursa of 19-day-old embryos (Fig. 1c). Only background fluorescence was detected in the bursal epithelium with normal rabbit serum (see Figs. 2d and 3b) and with anti-chicken gamma globulin serum, although the latter stained intrafollicular cells (Fig. 1d). The level of fluorescence mediated by anti-RAV-0 serum in the bursal epithelium remained significantly above the background level (defined as that obtained with normal rabbit serum) for at least 3 weeks after hatching (data not shown). In general, the cytoplasm of the epithelial cells was diffusely stained, and punctate accumulations of stain were observed at the periphery; the nuclei appeared unstained. In contrast, the nonfollicular cells subjacent to the epithelium exhibited only a low level of anti-RAV-0 serum-mediated fluorescence approximately equivalent to that detected with normal rabbit serum.

In addition to the bursal epithelium, a population of cells located in the central region of the bursal follicles of embryos 17 days old or older reacted with anti-RAV-0 serum (Fig. 1e) but not with normal rabbit serum (data not shown). In sections in which the junction between the follicle and epithelium was seen, the intrafollicular cells that reacted with anti-RAV-0 serum radiated out from the epithelium (Fig. 1e). In contrast, lymphocytes that reacted with anti-gamma globulin serum were distributed throughout the bursal follicles of 19-day-old embryos (Fig. 1d). Although the limited resolution of the immunofluorescence technique precludes an unambiguous determination of which intrafollicular cell types react with anti-RAV-0 serum (Fig. 1c) or anti-gamma globulin serum (Fig. 1d) indicate that, at most, only a subpopulation of lymphocytes in $15B \times 7_2$ embryos react with anti-RAV-0 serum.

In post-hatch chickens, the cortical and medullary follicular regions are demarcated by an epithelium; and, as shown here, cells in the medullary region of the follicles exhibited a high level of anti-RAV-0 serum-mediated fluorescence, whereas cells in the cortical region exhibited only a background level of staining. The epithelium that demarcates the cortex and medulla reacted with anti-RAV-0 serum, as did the great majority of the medullary cells, including many that morphologically resemble lymphocytes. The intensity of the anti-RAV-0-mediated fluorescence was not uniform among the medullary cells (Fig. 1f). Bursal lymphocytes that reacted to anti-chicken

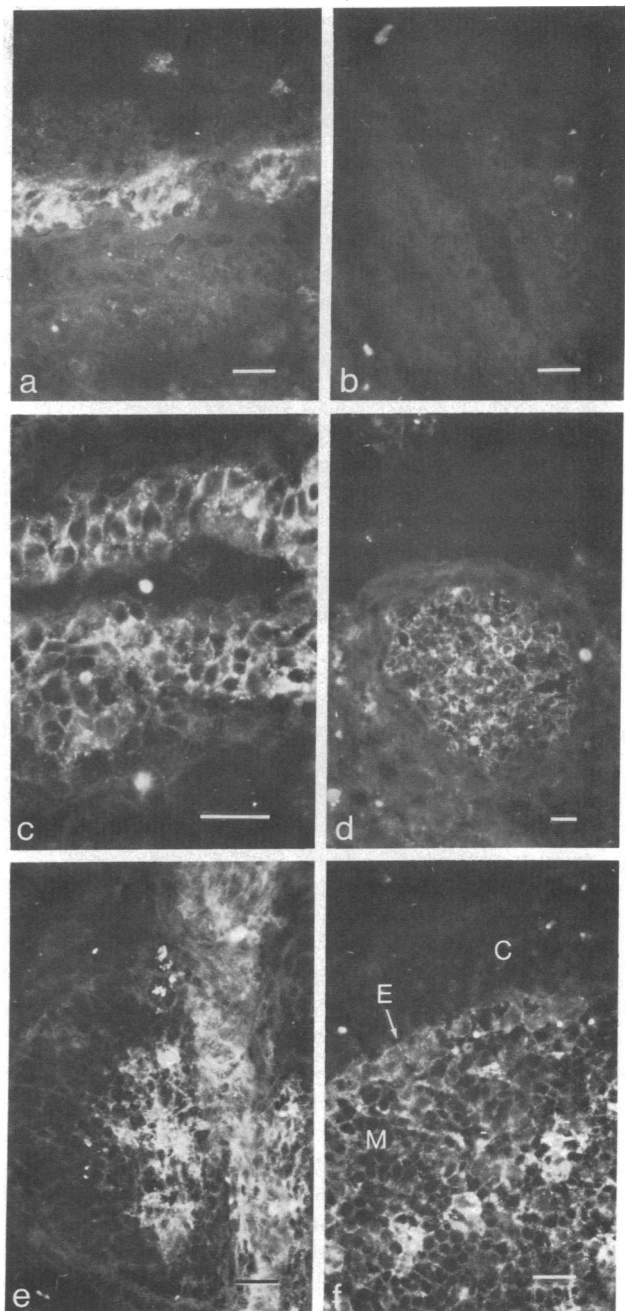


FIG. 1. Immunofluorescent staining of bursas from $15B \times 7_2$ chickens. (a) Eleven- to 12-day-old embryo, anti-RAV-0 serum; (b) 11- to 12-day-old embryo, normal rabbit serum; (c) 19-day old embryo, anti-RAV-0 serum; (d) 19-day-old embryo, anti-chicken gamma globulin serum; (e) 19-day-old embryo, anti-RAV-0 serum; (f) 11-day post-hatch chicken, anti-RAV-0 serum. C = cortex, M = medulla, E = intrafollicular epithelium. As resolved in Fig. 1e, the bursal epithelium appears as a vertical band traversing the right side of the figure; the follicle appears as a hemisphere to the left of the epithelium; the oncornaviral antigen-positive intrafollicular cells appear localized directly to the left of the epithelium. Bars indicate $30 \mu\text{m}$.

gamma globulin serum were confined to the medullary region of the follicle; the level of this reactivity was also not uniform among the medullary lymphocytes (refs. 11 and 12 and data not shown).

Characterization of the Reactivity to Anti-RAV-0 Sera. In competition experiments, lysates of BH-RSV(RAV-0) (Fig. 2b; compare Fig. 2a) but not of an unrelated oncornavirus, AKR-MuLV (Fig. 2c), decreased the level of staining mediated by anti-RAV-0 serum in the bursas of $15B \times 7_2$ embryos to the

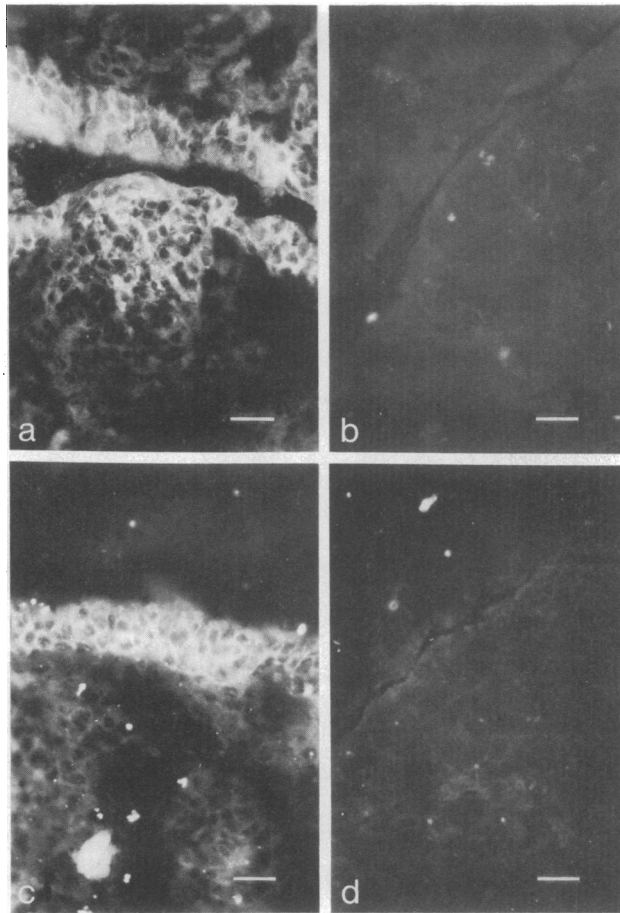


FIG. 2. Immunofluorescent staining of bursas from 18-day-old $15B \times 7_2$ chicken embryos. For competition experiments, purified preparations of BH-RSV(RAV-0) or AKR-MuLV were lysed in a buffer containing 1% NP40, 0.13 M NaCl, and 0.05 M Tris-HCl (pH 7.4); lysate at 100 μ g of protein per ml was mixed with an appropriate dilution of anti-RAV-0 serum prior to staining. (a) No competition; (b) competition with BH-RSV(RAV-0); (c) competition with AKR-MuLV; (d) normal rabbit serum control. Bars indicate 30 μ m.

background level obtained with normal rabbit serum (Fig. 2d). To minimize the possibility that in the competition experiments antigenically related nonviral components might be present in the immunizing and competing viral preparations, the antiserum had been raised against RAV-0 propagated in $15B \times 7_2$ fibroblasts whereas the competing BH-RSV-(RAV-0) was propagated in quail fibroblasts.

Identical results in competition experiments were obtained with antisera raised against BH-RSV(RAV-0) propagated in quail fibroblasts and antisera raised against purified nucleoprotein cores of avian myeloblastosis virus (data not shown). In additional competition experiments, purified chicken gamma globulin did not diminish the staining mediated by anti-RAV-0 serum but eliminated the staining mediated by anti-chicken gamma globulin serum (data not shown). These data, taken together, indicate that the anti-RAV-0 serum-mediated reactivity in the bursas of $15B \times 7_2$ embryos is due to avian tumor viral antigen.

A similar analysis applied to the bursas or cultured fibroblasts from Spafas-MR49 and line 7_2 chicken embryos, whose fibroblasts score as V(-) and low V(+), respectively, failed to demonstrate detectable levels of avian tumor viral antigen (data not shown).

Distribution of Viral Antigen in Intestinal Epithelia. In order to determine whether the presence of viral antigen in the

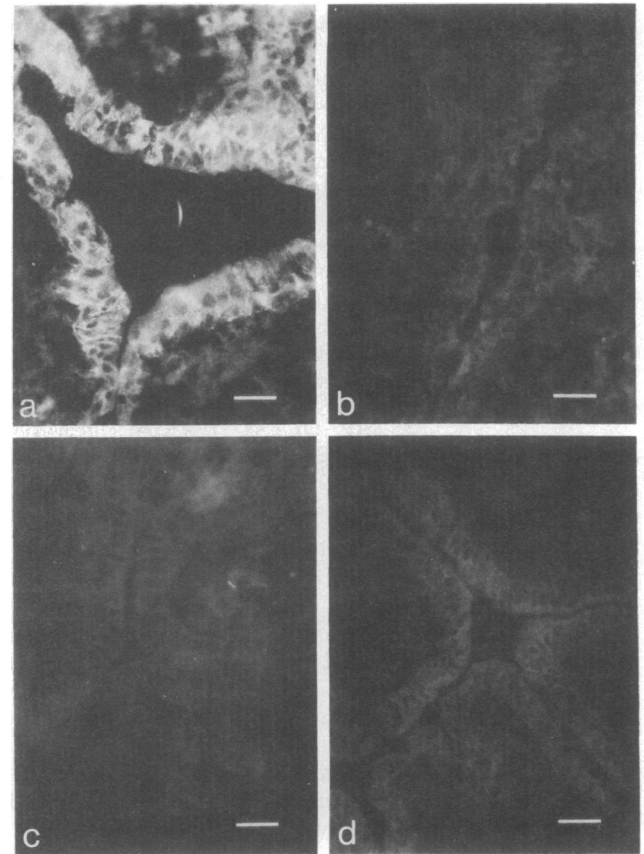


FIG. 3. Immunofluorescent staining of bursal and intestinal epithelia from 18-day-old $15B \times 7_2$ chicken embryos. (a) Bursa, anti-RAV-0 serum; (b) bursa, normal rabbit serum; (c) intestine, anti-RAV-0 serum; (d) intestine, normal rabbit serum. Bars indicate 30 μ m.

bursal epithelium is a general property of the intestinal epithelia of $15B \times 7_2$ chickens, we examined histological sections that contained both the bursa and a portion of the large intestine that is contiguous with the bursa. In a comparison of bursal (Fig. 3a and b) and intestinal (Fig. 3c and d) epithelia, each of which was exposed to both anti-RAV-0 serum and normal rabbit serum, significant reactivity was detected only with the combination of bursal epithelium and anti-RAV-0 serum (Fig. 3a).

Electron Microscopic Detection of Virions. The presence of oncornaviral antigen in the bursal epithelium of $15B \times 7_2$ chickens could represent accumulation due either to uptake or to synthesis by the epithelial cells themselves. An electron microscopic analysis was undertaken to test the latter possibility. In an examination of the bursal epithelium of $15B \times 7_2$ embryos, particles with a C-type morphology were found in the intraepithelial spaces (Fig. 4). Because the intraepithelial spaces are bounded on the luminal aspect by junctional complexes and on the basal aspect by a basement membrane, the most reasonable interpretation of the above results is that the bursal epithelial cells of $15B \times 7_2$ embryos produce endogenous viral particles and thus synthesize viral antigen. No particles were seen in bursas of Spafas-MR49 embryos (data not shown).

DISCUSSION

An analysis based on immunofluorescence has shown that anti-RAV-0 sera react specifically with tissue components localized in the bursal epithelium and in the central portion of the bursal follicles of $15B \times 7_2$ chickens. On the basis of the specificity of this reaction and the results of competition ex-

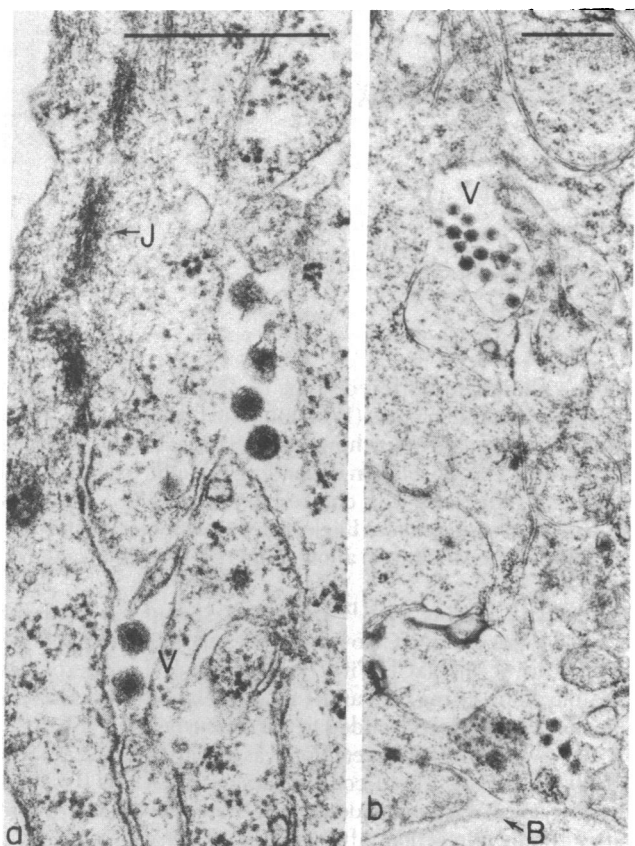


FIG. 4. Electron micrographs of the bursal epithelium of 18-day-old $15B \times 7_2$ chickens. (a) Luminal region and (b) basal region of intraepithelial cell boundaries. J = junctional complex; B = basement membrane; V = virus particles. Bars indicate 500 nm.

periments, we have concluded that this reactivity is due to the presence of avian tumor virus antigen. As analyzed with sections of large intestine, the confinement of viral antigen in the bursal epithelium indicates that the accumulation of viral antigen is not a general property of all the intestinal epithelia but rather a specific property of the bursal epithelium. The presence of C-type particles in the intraepithelial spaces, which are bounded by junctional complexes and a basement membrane, argues that viral antigen accumulates, at least in part, by synthesis in bursal epithelial cells and not exclusively as the result of uptake from the luminal contents (13, 14).

In addition to its expression in bursal epithelial cells of $15B \times 7_2$ chickens, viral antigen is detected within the central region of the lymphoid follicles. Because the follicle is comprised of lymphocytes and nonlymphocytes, higher resolution techniques are required before it can be determined with certainty which cell types contain the viral antigen. Nevertheless, given the presence of viral antigen in a large proportion of medullary cells, many of which resemble lymphocytes, our working hypothesis is that viral antigens are expressed by a population of $15B \times 7_2$ bursal lymphocytes.

An adjunct to this hypothesis is the possibility that the expression of endogenous viral genes is important in the physiology of development of bursal lymphocytes of all chickens; alternatively, the endogenous expression of viral antigen in the bursa may be restricted to $15B \times 7_2$ chickens and perhaps other V(+) chickens in which endogenous virus production is amplified by reinfection. Although no conclusive evidence exists to allow us to distinguish between these possibilities, the former may be related to recent findings showing that the expression of endogenous viral antigen in the murine system is a con-

comitant of hemopoiesis in general (15–18) and of antigen-driven B-lymphocyte differentiation in particular (19, 20). Because, in the present study, no reaction with anti-RAV-0 serum was detectable with bursas from V(-) chickens, the interpretation that endogenous virus expression is required for bursal development presupposes that the observed level of viral antigen in $15B \times 7_2$ chickens is an amplification of viral antigen expression that is functional in other chickens at a level below the limit of detection of our immunofluorescence assay. Assays based on more sensitive detection techniques may serve to resolve whether viral gene expression occurs in developing bursas of V(-) chickens.

Regardless of the possible role of endogenous virus in normal physiology, the observation that viral antigen is preferentially expressed in the bursal epithelium leads to questions about the nature of the interaction between the bursal epithelium and exogenous avian tumor virus, and the role, if any, this interaction plays in the induction of lymphoid leukemia. In the murine oncornavirus system, Waksal *et al.* (21) have presented evidence that murine leukemia virus expression in the thymic epithelium is involved in the induction of thymic lymphoma. Experiments are planned to ascertain whether the pattern of immunofluorescence detected in the bursal epithelium and intrafollicular cells of $15B \times 7_2$ chickens is recapitulated in the bursas of V(-) chickens infected with oncogenic leukemia viruses.

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