Multiplicity of Cell Response to the BAI strain A (Myeloblastosis) Avian Tumor Virus. VI. Ultrastructural Aspects of Adenosinetriphosphatase Activity of Nephroblastoma Cells and Virus 1,2

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SUMMARY

Renal tumors with characteristics of nephroblastomas were induced in the chicken by inoculation of the BAI strain A avian tumor virus. Ultracytochemical studies were made to determine the presence or absence of adenosinetriphosphatase in epithelial, sarcomatous, and chondromatous cellular components of the tumor, and of the enzyme associated with virus elaborated by the respective cells. Evidence of adenosinetriphosphatase was observed only at the brush border of cells corresponding to analogous elements of the proximal convoluted tubules of the normal nephron and at the membrane of cells resembling those of the distal convoluted tubule of the normal nephron. Virus budded from the membranes of the latter cells reacted positively for adenosinetriphosphatase. There was no reaction by virus associated with cells showing no enzyme at the cytoplasmic membrane. Virus in pellets sedimented from the blood plasma of chickens with myeloblastosis and treated with the same procedures gave a pronounced adenosinetriphosphatase reaction. The findings indicated that adenosinetriphosphatase in the virus in the nephroblastoma depended directly on incorporation of the enzyme into the particle as it budded from the membrane. The significance of the findings is discussed in relation to the influence of the respective host cells on the nature of the agent derived from them.—J Nat Cancer Inst 30: 1267–1301, 1963.

NUMEROUS STUDIES by a variety of techniques demonstrated (1–6) adenosinetriphosphatase activity associated with a particulate material having the properties of BAI strain A avian tumor virus (7). The enzyme is quantitatively proportional to the number of virus particles enumerated by electron microscopy in the circulating blood plasma of birds with myeloblastic leukemia induced by the agent, and in the medium of tissue cultures of the myeloblasts from diseased birds (8–10) or of the cells derived by treatment of normal bone marrow with the virus in vitro

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There is no unequivocal evidence to indicate significance of the enzyme with respect to the influence of the agent to induce or maintain neoplasia or other cell response. Cytochemical studies, however, suggested (12-15) a possible cell source of enzyme for incorporation in the virus as indicated by adenosinetriphosphatase in myeloblast cytoplasmic inclusions, presumably the site of virus synthesis in these cells. Results of recent work (16-18) revealed potentialities of ultrastructural cytochemistry for further advance in the knowledge of the enzyme relation to the virus particle and of possible mechanisms for inclusion of adenosinetriphosphatase in the agent. In the thymus of chickens with myeloblastosis, the normal cortical lymphoid elements are replaced by large blastlike cells exhibiting strong adenosinetriphosphatase activity at the cytoplasmic membrane (18-19). Though apparently not neoplastic, these cells are infected by the BAI strain A virus and, in contrast to the behavior of the myeloblasts, elaborate the agent by budding of the plasma membrane (18). In addition to the adenosinetriphosphatase activity of the cell membrane, individual virus particles in the intercellular spaces of the cortex showed (16-18) associated lead phosphate deposits at the particle's periphery after treatment with Wachstein-Meisel reagent (20). These findings not only revealed enzyme activity of the cell membrane and virus particle but suggested that the particle enzyme might have been derived by incorporation of the adenosinetriphosphatase-active cell membrane in the budding virus.

An opportunity for further clarification of these possible relationships was afforded by the characteristics of the nephroblastoma (21-26) induced by the BAI strain A virus. This tumor is a mixed growth consisting chiefly of epithelial components analogous to normal nephronic epithelial structures, sarcoma, and cartilage; virus is elaborated by all these cell types by budding of the cell membranes (24, 26). It was of interest to determine whether the membranes of the various cells exhibited adenosinetriphosphatase activity and whether there was a relationship between the cell enzyme and that of the virus apparently elaborated by these cells. Of equal interest were possible differences in enzyme activity between virus from the plasma of birds with myeloblastosis and virus synthesized by the kidney tumor cells. In the nephroblastoma, enzyme was in the brush border of cells resembling elements of the proximal convoluted tubule of the normal nephron (27). Enzyme was also in the cytoplasmic membranes of incompletely differentiated epithelial cells of components probably corresponding to the distal tubules of the normal nephron, and about virus particles nearby. All other cell types (except occasional capillary endothelial and red blood cells) and associated virus particles gave negative results. Control studies were made of nephronic elements and cartilage from normal birds. Similar studies were initiated to investigate the ultracytochemical aspects of BAI strain A virus in blood plasma of chickens with myeloblastosis. Some phases of the preliminary findings are presented as a basis for interpretations of the present results described in this report.

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MATERIALS AND METHODS

Induction of kidney tumors was effected, as described (25, 26), by intravenous inoculation of BAI strain A virus in White Leghorn chickens of line 15 (28) (Regional Poultry Research Laboratory, East Lansing, Mich.). For comparative studies with normal tissues, specimens were obtained from the kidneys of line 15 birds 3 days to 3 weeks old. Cartilage was taken from the distal end of the femur of 3-day-old normal birds.

Tissue specimens from the tumor and from normal birds were treated alike. Blocks of about 5 mm³ were fixed overnight in the cold. The efficacy of various fixatives was tested: 4 percent formaldehyde with calcium (29); buffered with phosphate (30) or Palade buffer (31); and 5 percent glutaraldehyde buffered with cacodylate (32) at pH 7.4. Glutaraldehyde preserved best and was used in most of the studies made on 16 kidney tumors.

After fixation the tissues were rinsed in 7.5 percent sucrose solution (33), frozen, and cut in 40 or 45 μ sections. The sections were rinsed in the cold sucrose solution for 20 to 30 minutes and incubated at 37° C in the Wachstein-Meisel medium with adenosine triphosphate as substrate (20) and containing 7.5 percent sucrose. Control sections were incubated in the same medium but without adenosine triphosphate substrate. The incubation period was varied from 15 to 60 or 70 minutes. The sections were transferred with wide-mouth pipettes through several changes of sucrose solution and sometimes rinsed briefly in 2 percent acetic acid to eliminate nonspecific deposits (34). The sections were washed again in sucrose solution, postfixed for 30 to 60 minutes in buffered (31) 1 percent OsO₄ containing 7.5 percent sucrose (35) and embedded in Epon 812 with the mixture B of Finck (36). Some sections were treated with ammonium sulfide solution and mounted for light microscopy.

Studies were made of the enzymatic behavior of the agent derived from the plasma of chickens with myeloblastic leukemia. Plasma exhibiting a high adenosinetriphosphatase activity, measured by photometric microdetermination (8), was centrifuged at 60,000 × g for 15 minutes. The resulting virus pellet was fixed in situ in the tube with 5 percent glutaraldehyde for 15 minutes. The pellet was disengaged from the wall of the tube, embedded in 5 percent agar, then frozen and sectioned, as were the tissues. Subsequent treatment to obtain thin sections was the same as that used with the tissues.

Thin sections were cut from the blocks (sections 40 μ thick) on a Porter-Blum ultramicrotome with Dupont diamond knives, stained with uranyl acetate (37) for 40 minutes, and for 5 minutes with lead hydroxide by method A of Karnovsky (38). Double staining with heavy metals was necessary for unequivocal identification of virus particles. Artifacts were not introduced by this technique as seen by examination of unstained sections.
RESULTS

Epithelial Structures

Figure 1 is an electron micrograph of a section of a glomerular corpuscle region of renal tumor B-524 at low magnification after treatment with Wachstein-Meisel reagent for 40 minutes. All structural and cellular elements of the normal corpuscle are represented, but in a state of incomplete cellular differentiation and organization (26). As in previous investigations on the normal rat kidney (27), there was no adenosinetriphosphatase activity of the respective epithelial cell membranes or intracellular structures of the avian glomerular tissue.

Podocytes of primitive differentiation in the nephroblastoma often showed intensive cell membrane budding in the elaboration of numerous virus particles sometimes seen (26) between or near the cells. Many virus particles were not found in the present material, but small groups (fig. 1) were scattered through the tissue. Figure 2 of the same tumor shows individual particles not only in spaces between the pedicels of the podocytes but also embedded in the basal membranes bounding the capillary wall. In other micrographs, virus particles were in podocyte vacuoles. Adenosinetriphosphatase activity was not evident in either the cell membranes or the virus particles.

These findings contrast with results of study of cells identified as incompletely differentiated elements corresponding to the normal proximal convoluted tubule. Figure 3 reveals evidence of a strong adenosinetriphosphatase reaction of the brush-border membrane in the same tumor, like that seen in the corresponding normal structure (27) but at no other part of the cell membrane (see also fig. 4). Figure 4 shows the continuation of the cytoplasmic membranes of cells adjacent to those of figure 3, toward the basal region, and a large portion of the membrane at the basal region of one cell. As in figure 3, there was no evidence of enzyme activity of the cell membranes.

An expanded intercellular space in figure 3 contains accumulated virus particles that show no associated lead phosphate deposit. Such particles without evidence of adenosinetriphosphatase were frequently in comparable situations. On the contrary, precipitate was characteristically layered about some particles (fig. 4) in the capillary space at the cell base. Particles with enzyme activity were often associated with capillary endothelial walls next to many tubular structures in this tumor, but not in the other renal growths examined. As noted with the virus in the thymus (16, 18), not all particles of this collection reacted positively. The particles were embedded in unidentified amorphous material that may have been fibrin deposit, endothelial cell cytoplasm, or badly differentiated basal membrane. The bird providing this tumor (B-524) was killed 115 days after inoculation with virus. Frequent examination of the blood smears revealed no evidence of myeloblastic leukemia, but the chicken had osteopetrosis.

The most striking results were obtained (fig. 5, tumor B-745) with
incompletely differentiated and organized cells corresponding to elements of distal convoluted tubules of the normal nephron. As in the corresponding normal structure (27), a heavy deposit of lead phosphate occurs at almost the entire extent of the cell membrane, including the interdigitating cell processes. There was no precipitate in the region of the terminal bars as observed in other micrographs. Figure 6 at higher magnification illustrates the character of the electron-dense material often deposited in small foci and in elongated masses at the cell membrane. The deposits were usually smoother and more dense toward the cell cytoplasm, and were mostly frayed and thinned on the opposite side.

Virus particles were distributed sparsely in the spaces between these epithelial cells, and some showed associated lead phosphate deposit (fig. 6, tumor F-914). These particles looked like the virus in the thymus (16-18).

**Sarcoma**

The ubiquitous supporting stroma in the nephroblastoma (25, 26) showed differentiation varying from epithelial or epithelioid elements to typical spindle-shaped mesenchymal cells as illustrated by tumor F-914 not treated with Wachstein-Meisel reagent (fig. 7). Such cells from the same tissue treated with this reagent (figs. 8A and B) never evidenced adenosinetriphosphatase activity at the cytoplasmic membrane. The cells elaborated virus particles by budding, but both buds and intercellular particles were rare. No precipitate was seen about the virus buds (inset, fig. 8A) or particles lying between the cells (inset, fig. 8B).

The principle of the findings with the sarcomatous elements is well illustrated, also, in the micrograph of the spindle-shaped cells in figure 5. Intercellular spaces contained much virus in close association with the cell membranes. There was no precipitate either at the cell membrane or about the virus particles, in contrast to the deposit at the epithelial cell membranes already described.

**Cartilage**

Normal chondrocytes (fig. 9) showed no deposit at the cell membrane after treatment with the Wachstein-Meisel reagent, nor did the neoplastic chondrocytes, as exemplified by tumor A-233 in figure 10. Virus particles budded from the tumor chondrocytes (fig. 11) were likewise free from significant deposit.

As shown earlier, nephroblastoma chondrocytes were derived by progressive differentiation of the stromal cells (fig. 7). Figure 12 (tumor B-745) shows a cell morphologically intermediate between the stromal elements (fig. 7) and fully differentiated chondrocytes (fig. 10). Nephroblastoma prechondrocytes (26) budded virus intensively, and many particles were close about the cells. This phenomenon is illustrated here in figure 12. Virus particles lie close to the cytoplasmic membrane.
of the prechondrocyte. Other particles are scattered in the tissue among other elements of spindle cell morphology. No deposit was associated either with the prechondrocyte, spindle cells, or virus particles.

**Virus From Blood Plasma of Chickens With Myeloblastosis**

Figure 13 shows the results of treatment with Wachstein-Meisel reagent of virus sedimented from leukemic blood plasma. Lead phosphate deposited densely between or about the virus particles obscures the relation of the precipitate to individual virus particles. At the pellet edge, however, there was precipitate at the surface of every distinguishable particle. The precipitate was usually in discontinuous masses at the particle surface as shown also in figure 6. The virus particle images showed well-defined nucleoids.

**DISCUSSION**

Earlier experiments (16–18) showed that lead phosphate precipitate indicating adenosinetriphosphatase activity was demonstrable in association with individual virus particles in the thymus of chickens with myeloblastosis after treatment with the Wachstein-Meisel reagent. This finding, obtained by direct examination of single particles, thus corroborated evidence (1–6) of the adenosinetriphosphatase activity of the BAI strain A virus determined by other methods. Results of the thymus study likewise suggested that the moiety of the virus displaying the enzyme activity was near the particle surface and was derived from the host-cell cytoplasmic membranes which were adenosinetriphosphatase-active and elaborated virus by budding.

The present results provide further evidence that virus enzyme is related to that of the host-cell membrane. Phosphate deposit occurred at the membrane of cells of epithelial structures corresponding probably to the distal convoluted tubules of the normal nephron, and virus intimately associated with these cells was positive (fig. 6). In contrast, sarcoma and cartilage cell membranes and those of the glomerular and proximal convoluted tubule cells showed no deposit, and there was none about virus particles associated with these respective membranes. Furthermore, although the brush border at the apexes of cells corresponding to proximal convoluted tubule elements was positive, virus in pockets between membranes of the same cells was negative, as was the cell membrane surrounding the particles away from the apex. There was no evidence that virus particles budded from the brush border.

Several factors might be expected to complicate interpretation of the results. These were particularly important with respect to the significance of absence of enzyme reaction. A negative result might be due to failure of the substrate to penetrate the tissue. This was partly controlled by routine preparation of thin blocks in sections 40 μ thick, cut on the freezing microtome. That the technical conditions were adequate was indicated.
by the positive results with just those neoplastic epithelial cells corresponding to analogous normal nephronic cells exhibiting adenosinetriphosphatase activity. In addition, both positive and negative cells were seen in the same field in the electron microscope (figs. 3, 4, and 5). Both positive and negative virus particles were also found in the same field (figs. 3 and 4). Thus the findings were in accord with the view that lack of reaction was not due to technical influences.

Much circumstantial evidence indicated that absence of reaction product about some virus particles was due to particle origin from cells without adenosinetriphosphatase in the cytoplasmic membrane. The close particle association with the prechondrocyte (fig. 12) left little doubt that the particles were derived from that cell, and neither the virus nor the cell exhibited the result of reaction. It seems significant in this respect that blood plasma particles of high activity measurable by other methods showed a massive phosphate deposit as seen in figure 13. This result and the mixture of positive and negative particles in the capillary of figure 4 increase the probability that the particles showing no response were devoid of enzyme. The source of the particles in the capillaries observed only in this tumor was not clear. Those with the phosphate deposit may have been derived from the distant osteopetrotic process, in which preliminary studies suggested the presence of adenosinetriphosphatase activity, or from distal convoluted tubule cells of the same growth. Similarly, the negative particles could have been elaborated by distant cells without adenosinetriphosphatase in the membrane and transported to the respective sites by the circulation. The enzyme of the virus is very stable (39, 40) so that inactivation of adenosinetriphosphatase was unlikely to be the cause of inactivity.

The validity of the interpretations that the virus properties are related to those of the cell is supported by other phenomena. The BAI strain A virus in the blood plasma of chickens with myeloblastosis may be derived from cytoplasmic structures of the myeloblasts of demonstrable adenosinetriphosphatase activity. Although budding does not appear to contribute to the major processes of virus elaboration by the myeloblast (14), preliminary studies showed, nevertheless, that the cytoplasmic membrane of the myeloblast gives a strong adenosinetriphosphatase reaction. There is evidence that segments of cartilage fibrils may be included in virus budded at the membrane of renal tumor chondrocytes, and that collagen formation by some nephroblastoma cells may complicate virus budding by the same cells (26). Host-tissue antigen and Forssman antigen are components of the BAI strain A virus (6). Host-tissue antigen is associated with influenza virus (41) budded from the cell membrane, and the character of the enclosing membrane of the herpes virus is like that of the host cell (42).

Of much fundamental interest has been the influence of host cells on the character of the particles synthesized by the respective elements of myeloblastic leukemia and nephroblastoma. Presumably myeloblastosis and the nephroblastoma are induced by the agent of blood plasma exhibit-
ing adenosinetriphosphatase activity such as that illustrated in figure 13. Yet the agent elaborated by some nephroblastoma cells exhibits changes not only in morphology (26) but in chemical constitution and behavior, as seen in the present work. With the mixture of differing virus particles in the nephroblastoma, it would be difficult to distinguish the extent of variations in biologic properties by inoculation of nephroblastoma extracts into test hosts. Morphologic differences and, perhaps, superficial chemical changes do not necessarily indicate true mutation concerned with presumable alteration of virus genetic constitution such as that which may occur (43) in the Rous sarcoma virus. Although the present experiments clarify some problems of virus enzyme origin, they do not contribute to the understanding of the activity of the agent in disease induction or maintenance.

The aggregate results strengthen the view that the cell membrane enzyme may be focally distributed (fig. 6). This would parallel a distribution of other activities and constituents of the cell, such as formation of dense bodies at the surface of chondrocytes (fig. 9) and elaboration of analogous structures designated as mucoastrosomes (44) by virus-induced neoplastic sarcoma cells. Such a distribution is suggested, also, by the appearance of the deposit on the virus particle.

Finally, neoplastic cells of the nephroblastoma exhibit not only the morphology of corresponding normal nephronic elements but some characteristics of cell membrane constitution and activity. Thus the influence of the virus on renal cells in the induction of the renal tumor effects no changes in the principles of the potentialities of nephrogenic cells for differentiation in morphology and function in these respects, but affects only minor aspects of differentiation.

REFERENCES


Figure 1.—Segment of nephroblastoma glomerular corpuscle (B-524) 115 days after virus inoculation. Cells of parietal layer (PAC) of Bowman’s capsule differentiated to flat epithelium, and hilum membranes (HS) enclose central mass cells (CM). Capillaries (CAP) lined by relatively well-differentiated endothelial cells (EC) and surrounded by basement membrane (BM) with attached pedicels (PC) of podocytes (PD) with well-formed major processes. Individual or small accumulations of virus particles (VP) are scattered in tissue. Virus bud (arrow) identifiable at higher magnification. Section incubated in Wachstein-Meisel medium for 40 minutes at 37°C. No evidence of adenosinetriphosphatase reaction products about cells or virus. $\times 6,500$
FIGURE 2.—Higher magnification of field like that of figure 1 from same tumor showing capillary with red blood cell (RBC) and lining endothelial cell cytoplasm (EC) surrounded by basement membrane (BM) and attached pedicel processes (PC) of podocytes (PD). Virus particles (VP) are scattered in basement membrane and in spaces between pedicel processes. Neither cell structures nor virus particles show precipitate indicative of adenosinetriphosphatase reaction after 40 minutes' incubation at 37° C in Wachstein-Meisel medium. × 46,000
PLATE 206

FIGURE 3.—Apical region of cells of same tumor as figure 1 identified as corresponding to elements of proximal convoluted tubule of normal nephron. Brush border (BB) shows result of adenosinetriphosphatase activity, but no deposit is at other parts of cell membrane including terminal bar (TB) region. Virus particles (VP) collected between cells show no evidence of adenosinetriphosphatase reaction. Incubation in Wachstein-Meisel medium 40 minutes at 37° C. × 38,000
Figure 4.—Basal portions of cells neighboring those of figure 3 show no precipitate at cytoplasmic or basement membrane (BM). Capillary space (CAP) contains amorphous material with embedded virus particles (VP) showing precipitate indicative of adenosinectriphosphatase activity. Some virus particles (arrows) show no precipitate. Incubation for 40 minutes at 37°C in Wachstein-Meisel medium. ×29,000
PLATE 208

FIGURE 5.—Region of tumor B-745 corresponding to incompletely differentiated and organized distal convoluted tubule. Plain and interdigitating cytoplasmic membranes of epithelial cells (arrows) show precipitate. Cell membranes in contact with primitively developed basement membrane (BM) are negative. Near pseudo-tubular structure (lower right) are spindle cells (SC) of neoplastic stroma with associated collagen (COL). None of the many virus particles (VP) scattered about shows associated precipitate. Incubation in Wachstein-Meisel medium 30 minutes at 37° C. × 6,500.
PLATE 209

FIGURE 6.—Virus particle (VP) adjacent to cytoplasmic membrane of cell in tumor F-914, like epithelial elements of figure 5 shows associated deposit of lead phosphate. Lead phosphate deposited in small focal or elongated masses at cell membranes. Incubation in Wachstein-Meisel medium 30 minutes at 37° C. × 160,000
Figure 7.— Stromal cells (tumor F-914) (not treated with Wachstein-Meisel reagent) of differentiation varying from epithelioid to spindle cell morphology. There is much associated collagen (COL). \( \times 5,700 \)
PLATE 211

Figures 8A and B.—Cells of same tissue as that of figure 7 treated with Wachstein-Meisel reagent for 30 minutes at 37° C showing absence of lead phosphate deposit at cell membranes. Dense, unidentified cytoplasmic bodies show precipitate at periphery.

Figure 8A.—Virus bud (arrow) shows no deposit. × 15,000. Inset of bud at higher magnification. × 150,000

Figure 8B.—Virus particle (arrow) shows no deposit. × 13,200. Inset of particle at higher magnification. × 105,000
PLATE 212

FIGURE 9.—Chondrocyte of cartilage from lower end of femur of normal chick 3 days old. No evidence of adenosinetriphosphatase reaction in cell membrane after incubation in Wachstein-Meisel reagent 60 minutes at 37° C. × 12,400

FIGURE 10.—Chondrocyte of nephroblastoma A-233 shows no precipitate with appearance of specific association with cell membrane. Incubation in Wachstein-Meisel medium 50 minutes at 37° C. × 13,500
Figure 11.—Portion of chondrocyte A-233 like that of figure 10 showing virus bud (B) in cytoplasmic membrane, immature virus particle (IVP) separated from cell, and free particles (VP) with appearance of mature virus. No precipitate associated with virus at any stage of elaboration. × 95,000
FIGURE 12.—Prechondrocyte (PCB) of nephroblastoma B-745 with virus particles aligned at surface. No evidence of adenosinetriphosphatase reaction at either cell membrane or in association with virus particles. Some virus particles (arrows) are of unusual morphology as previously reported (26). Spindle cell (SC) stromal elements are like those of figures 5 and 7. Incubation in Wachstein-Meisel medium 30 minutes at 37° C. × 15,000
FIGURE 13.—Micrograph of thin section of centrifuged pellet of BAI strain A virus obtained from circulating blood plasma of bird with myeloblastic leukemia. Treatment with Wachstein-Meisel reagent for 20 minutes at 37° C resulted in deposit of lead phosphate distributed between virus particles in pellet mass. Deposit at periphery of individual particles is illustrated by particles at edge of mass. × 36,000