Cytochemical Studies on the Mouse Mammary Tumor Virus

GILBERT H. SMITH
Viral Biology Branch, National Cancer Institute, Bethesda, Maryland 20014

SUMMARY

The chemical nature of the ultrastructure of the mouse mammary tumor virus has been investigated in situ. Small pieces of spontaneous mammary tumors from C3H mice were fixed in phosphate-buffered glutaraldehyde, infiltrated, and embedded in glycol methacrylate, a water-miscible plastic introduced in 1963 by Leduc and her collaborators as a medium suitable for cytochemical studies at the ultrastructural level. Electron microscopic examination of the tissue revealed the presence of numerous cytoplasmic A particles and extracellular B particles. Considerable evidence has accumulated demonstrating that the latter is the active, mature mammary tumor virus. The cytoplasmic A particle is considered to be formed in the cytoplasm as a precursor of the B particle; however, there is little evidence from immunologic, biochemical, and biophysical parameters to support this hypothesis. Ultrathin sections of the tumor containing both particles were subjected to digestion with RNase, DNase, pepsin, papain, and trypsin and combinations of these enzymes. Consecutive sections were incubated in the appropriate control solutions. The results reported provide evidence for the presence of RNA in the cytoplasmic A particle, indicate that the stereochemical nature of the A particle is synonymous with the nucleoid of the B particle, and present further support of Bernhard's hypothesis that the cytoplasmic A particle is the intracellular precursor of the mammary tumor virus.

INTRODUCTION

The discovery of the mammary tumor virus (MTV) in 1936 by Bittner (8) has led over the years to intensive studies concerning its biologic, morphologic, biophysical, and immunologic characteristics. Two types of particles visualized with the electron microscope and originally described by Bernhard are considered to represent MTV: (a) intracytoplasmic or type A particles, and (b) extracellular or type B particles. Bernhard (5) postulated that the A particles are formed in the cytoplasm as precursors of mature MTV and evolve into the latter by moving to the cell membrane to produce buds which are pinched off and subsequently develop into mature B particles.

Considerable evidence has accumulated since Bernhard's observations that support the hypothesis that the B particle represents the active MTV. Electron microscopic studies by Dmochowski (14, 15), Bang et al. (3, 4), Bernhard et al. (5), Suzuki (38), and Pitelka et al. (33, 34), Goldfeder et al. (18), Feldman (17), Haguenau et al. (19), and others have indicated the presence of B particles in spontaneous mammary tumors and in preneoplastic areolar nodules in a variety of inbred mouse strains infected with MTV. The biophysical studies of Moore et al. (30) and Lasfargues et al. (22) provided strong physical and biologic evidence that the B particle and its nucleoid represent the active MTV. Recent immunologic studies by Blair and Pitelka (9) have demonstrated that the specific antigenicity related to MTV, as measured by the immunodiffusion technic, is carried by the B particle. Despite these observations, there still exists a discrepancy between the biologic and morphologic results with reference to the presence of the B particle (34). Although at this time one cannot say that every B particle carried the classic MTV tumorigenicity, the fact that MTV activity is carried by a B particle is not in dispute.

The A particle has been observed in other murine neoplastic cells, e.g., Swiss lymphoma cells (13), spontaneous plasma cell tumors (10), and spontaneous leukemic cells (12). In these tumors they apparently are not transformed into B particles. It has also been suggested that the B particle may develop from buds at the cellular surface without the migration of the A particle to the membrane. (1, 18, 21, 30). These observations were challenged by Imai et al. (20), who examined serial sections of spontaneous C3H mammary cancers in three dimensions. He demonstrated that most of the buds were associated with complete A particles. He suggested that the earlier results were due to the observations made on thin nonserial sections. Still, no intermediate stages representing the alteration of the A particle to the eccentric nucleoid of the B particle following its release from the cell have been reported.

It is the purpose of the experiments to be reported here to establish the chemical nature of the ultrastructural morphology of the B particle, and to correlate the cytochemical nature of the A particle with the nucleoid of the B particle within the framework of Bernhard's hypothesis concerning the maturation of the mammary tumor virus.

MATERIALS AND METHODS

Tissue Preparation

Small pieces of a spontaneous mammary tumor taken from a C3H/AnWi female known to harbor the MTV were fixed in various concentrations of glutaraldehyde (2.0, 3.5, 5.0, 8.0%) in 0.1 M Sörensen phosphate buffer at pH 7.4. The blocks were processed according to the method of Leduc et al. (27). The samples were embedded in a mixture of 70% glycol methacrylate and 30% 85/15 butyl/methyl methacrylate which had been prepolymerized in an H2O bath at 90°C. Polymerization was completed in the cold under ultraviolet light.

1 NIH, USPHS, HEW.

Received May 8, 1967; accepted July 11, 1967.
**Enzymes**

The following enzymes and diluents were used: (a) bovine pancreatic ribonuclease (3 times crystallized, Worthington Biochemical Corp.), 0.1% in distilled H₂O at pH 37°C; (b) pepsin (5 times crystallized, Nutritional Biochemical Co.), 0.5% in 0.1 N HCl at 37°C; (c) papain (Worthington), 0.1% in 70 ml of H₂O + 20 ml of 0.01 M ethylenediaminetetraacetate + 0.5 ml of 0.03 M mercaptoethanol + 10 ml of 0.5 M cysteine (freshly prepared) adjusted to pH 6.2 at 37°C; (d) trypsin (Sigma Biochemical, type III), 0.25% or 0.3% in 0.046 M tris(hydroxymethyl)aminomethane (Tris)-HCl with 0.0115 M CaCl₂, pH 8.1, at 23°C; (e) deoxyribonuclease (once crystallized, Worthington Biochemical) in distilled H₂O with 0.005 M MgSO₄, pH 6.2, at 37°C.

**Enzyme Digestion Technic**

Ultrathin sections (silver-silver-gold) were cut with glass knives with an LKB Ultratome onto distilled water. The sections were transferred to the enzyme digestion baths with a loop. Simultaneously, consecutive sections were placed into the corresponding diluent of the enzyme being employed to provide a control for nonspecific extraction. The sections were then incubated at the recommended temperature for varying intervals to determine optimal digestion time. For digestions with two or more enzymes (Chart 1), the sections were transferred in the same manner to the digestion vessels, one with the enzyme, the other blank. Upon completion of the first digestion, the sections were transported through two changes of distilled water before being placed upon the succeeding enzyme solutions. The same was done with the control sections. The pH of the succeeding digestion mixture was monitored to insure near-optimum conditions for enzymatic activity. Additional control sections were incubated in alternating solutions, i.e., enzyme-blank-blank enzyme (Chart 1). Samples from these groups were compared to one another to determine the effect of nonspecific extractions on the resulting ultrastructural appearance. The same procedures were carried out during digestions involving three different enzymes. Samples were taken for electron microscopy at the end of each step to insure the effectiveness of that particular treatment. Because of the autocatalytic property of trypsin for incubations in this enzyme for longer than 2 hours, the sections were transferred to freshly prepared trypsin preparations at 2-hour intervals.

Following digestion the sections were mounted on Formvar-coated 300 mesh copper grids which had been shadowed with a thin layer of carbon and stained in 0.5% aqueous uranyl acetate at pH 4.9 and in Reynolds lead citrate (36). The specimens were examined in a Siemens Elmiskop I with a double condenser and a 50-µ objective at 80 kv accelerating voltage. Survey micrographs were taken at a magnification of 4,000-6,000 and structural details were studied at direct magnifications of 15,000-20,000. Calibration of the microscope was carried out with a diffraction grating with 28,800 lines per linear inch.
**RESULTS**

**Preliminary Observations**

Observations on thin sections from glutaraldehyde-fixed and postosmicated mammary tumor cells revealed the characteristic type A and B particles of Bernhard (5). The A particles were found singly or in large inclusions in the cytoplasm. In section they appeared as doughnuts consisting of two concentric rings the outer diameter of which measured 700-750 Å (Fig. 1). The innermost shell of the doughnut exhibited a strong affinity for electron staining with lead salts. This characteristic staining has been interpreted by several authors as an indication of the presence of nucleic acid, presumably RNA. The B particle, primarily extracellular, was characterized by possessing a lipoprotein sac measuring 1000-1400 Å, within which was contained an eccentrically located nucleoid measuring 400-500 Å (Fig. 2). The nucleoid possessed two distinct zones, an electron-dense core and an electron-lucent shell or cortex.

Following glutaraldehyde fixation, infiltration, and embedment in glycol methacrylate, the ultrastructural appearance of both the A and B particle is quite comparable to that observed after conventional treatment. The diameters of both the A particle and the nucleoid of the B particle are, however, greater; 900-1000 Å (Fig. 3) and 600-650 Å (Fig. 4), respectively. These measurements were consistent throughout all the blocks used during the course of the experiment and following all enzymatic treatments and their controls except those in which certain proteolytic enzymes were used.

The cellular ultrastructure closely paralleled the description given by Leduc et al. (25) in regard to the appearance of the nuclear chromatin, mitochondria, ribosomes, cellular membranes, and nucleolus for glutaraldehyde-fixed, glycol methacrylate-embedded tissues. No appreciable swelling or shrinkage was observed in any of the sections. In all instances, the contrast attained with double staining with aqueous 0.5% uranyl acetate and lead citrate was satisfactory.

**Effect of Single Enzyme Digestions**

**RNase.** After 20-30 minutes of incubation with 0.1% RNase, the densely staining ribosomes of the cytoplasm were lost (Fig. 5). The cytoplasm was homogeneous and moderately dense. The nuclear chromatin became more electron dense. With longer digestions, the granular component of the nucleolus was no longer present. No effect on the other components of the nucleus or cytoplasm was observed even with much longer digestions (5 hours). The A particle during these treatments reflected no consistent change, although in some cases the inner shell showed a decreased affinity for the lead stain (Fig. 6). In most sections, the inclusion bodies consisting of A particles were obscured by a diffuse electron-dense material. The B particle also remained unchanged in appearance. After 30 minutes of digestion with RNase, occasional B particles contained nucleoids with electron-lucent cores (Fig. 7). Although this phenomenon was not observed in the controls or in sections singly digested with other enzymes, no increase in the number of particles with electron-lucent cores was observed upon longer digestion with RNase.

**DNase.** The only cellular component that was affected during digestion with 0.1% DNase alone was the nuclear chromatin, which became more dense. Deoxyribonuclease digestion had no effect on either Particle A or B, even following incubation for periods up to seven hours.

**Pepsin.** An overall reduction of the density of the cell was effected with 1-hour digestion with 0.5% pepsin. The mitochondrial matrix, the agranular component of the nucleolus, and the nuclear chromatin were the organelles most obviously affected. The outer diameter of the A particles was reduced by 140-170 Å in comparison to those in control sections (Fig. 8), which had been incubated in 0.1 N HCl (Fig. 9). No change in the outer diameter of the B particle was observed. However, the outer coat of the eccentric nucleoid was degraded. The outer diameter of the nucleoid was reduced by 140 Å (Figs. 10, 11). The core of the nucleoid became more dense in appearance due to the loss of its outer coat. No further degradation of the outer coat of the nucleoid or the inner shell of the A particle could be obtained with longer digestion times (up to 5 hours).

**Trypsin.** The same overall reduction in the density of proteinaceous cellular components that was observed after pepsin digestion followed incubation in 0.3% trypsin. However, the effect was not as rapid. There was no alteration of the diameters of the A or B particle. The outer shell of the A particle was roughened in appearance after 4 hours incubation (Fig. 12) when compared with the controls incubated in 0.045 M Tris chloride buffer (Fig. 13). The nucleoid of the B particle was unaffected (Figs. 14, 15). No further change was observed for digestions up to seven hours.

**Papain.** The cellular components affected were the cytoplasm and the matrix of mitochondria. The nuclear chromatin remained dense. The ribosomes were found to clump into dense masses. The structure of the A particle and B particle was altered at the same manner as with pepsin digestion. Difficulty in keeping sections adherent to the Formvar-coated grids was noted following digestions in 0.1% papain of 60 minutes or more. Overt reduction in the density of the matrix of mitochondria and cytoplasm was observed abruptly after 90 minutes digestion. This phenomenon corresponded directly to the digestion period required for the alterations of the outer shell of the A particle and the outer coat of the B particle nucleoid.

**Effects of Double Enzyme Digestion**

**Pepsin-Nuclease.** Sections were incubated for two hours in 0.5% pepsin at 37°C to degrade the outer components of the A particle and the B particle nucleoid. The sections were then washed and transferred to solutions containing either 0.1% RNase, 0.1% DNase, or distilled water and incubated for 30 or 60 minutes at 37°C. Some sections were routinely sampled for electron microscopy after preliminary incubation in order to determine the effect of the proteolytic treatment. Additional controls were performed by incubating sections in 0.1 N HCl for two hours followed by 0.1% RNase, 0.1% DNase, and distilled H2O. Digestion with pepsin followed by RNase resulted in the decreased density of the agranular component of the nucleolus, matrix of mitochondria and nuclear chromatin, and the disappearance of the densely staining ribosomes and granular component of the nucleolus. The combination of pepsin and DNase hydrolysis brought about a striking loss of density in the nuclear chromatin, but ribosomal staining was unaffected. In sections...
Gilbert H. Smith

placed in distilled water after pepsin pretreatment, the ultrastructural changes were identical with those obtained with pepsin digestion alone. In the other controls, i.e., 0.1 N HCl pretreatment followed by RNase, DNase, or distilled H2O, the results were comparable to those observed when sections were incubated in the nuclease preparations alone or in 0.1 N HCl alone.

Some of the A particles were seen as open circles following digestion with pepsin and RNase (Fig. 16). When this occurred, the inner shell had little or no affinity for lead salt staining. However, the inner shell of the vast majority of the A particles remained unaffected by RNase hydrolysis. Deoxyribonuclease had no effect on any of the denuded A particles (Fig. 17). In all cases the inner shell was stained densely. Although the "cortex" of the nucleoid had been degraded by the action of pepsin in most of the B particles, the core remained densely stained and apparently resistant to the action of either nuclease (Figs. 18, 19).

Trypsin-Nuclease. The same ultrastructural alterations in cellular components that were observed for pepsin-nuclease digestion apply for sections incubated in 0.25% trypsin followed by either 0.1% RNase, 0.1% DNase, or distilled water for 30 or 60 minutes. The sections were exposed to tryptic proteolysis for four hours before transfer to succeeding solutions. The combination of trypsin and DNase had a more pronounced effect on the nuclear chromatin than did pepsin succeeded by DNase. Control sections were incubated in 0.045 M Tris chloride buffer for four hours and then transferred to RNase, DNase, or distilled water. None of the above procedures had any appreciable net effect on the ultrastructure of either the A or B particle when compared with the result observed in the controls or when either enzyme had been used alone. This was not true for cellular morphology. For example, trypsin followed by RNase produced no further changes in the A particle than had RNase digestion alone. Therefore, tryptic digestion alone provided no enhancement of the effect of ribonuclease on either the inner shell of the A particle or the core of the B particle nucleoid. On the other hand, trypsin pretreatment facilitated a much more rapid removal of ribosomal staining by ribonuclease action (≥5 minutes).

Pepsin-Trypsin. Two hours of digestion with 0.5% pepsin succeeded by two hours of incubation in 0.25% trypsin resulted in extreme overall decrease in the density of the cytoplasm, matrix of the mitochondria, the granular component of the nucleus, and the nuclear chromatin. The ribosomes were not stained intensely after this treatment and could not be readily observed. The same applied to the granular component of the nucleus in most sections. This situation was most likely due to the fact that commercial trypsin preparations are contaminated with low levels of ribonuclease activity. If the order was reversed, i.e., trypsin digestion preceding pepsin, the ribosomes remained intensely stained. Apparently, the proteolytic action of pepsin renders the ribonucleic acid component of the ribosomes more sensitive to the level of ribonuclease activity contaminating the trypsin preparation. The controls incubated in 0.1 N HCl and then trypsin or pepsin and then 0.045 M Tris chloride buffer gave results very similar to those observed in sections incubated with trypsin or pepsin singly. The A and B particles following double digestion with pepsin-trypsin or trypsin-pepsin were affected as with pepsin digestion alone, leaving the A particle with a much reduced outer diameter and an intact inner shell (Figs. 20, 21). The outer coat of the B particle nucleoid was completely removed, leaving only the dense core within the remnants of the lipoprotein sac (Figs. 22, 23).

Effect of Triple Enzyme Digestion

Pepsin-Trypsin-RNase. For each multiple digestion involving three different enzymes, there were seven controls (see Chart 1). The effect of this procedure on the cellular organelles and the MTV are represented in Table 1 in terms of the change in density in comparison with identical structures in unincubated control sections. Sections were incubated with 0.5% pepsin for 2 hours, 0.25% trypsin for 2 hours, and then placed on 0.1% ribonuclease for 30 minutes. The controls were done simultaneously. The ultrastructure changes noted for triple digestion with pepsin trypsin and RNase were similar to those observed when RNase followed protein hydrolysis. The reduction of the density in nuclear structure was so extreme that the nucleolus appeared in negative contrast to the nucleoplasm (Fig. 24). No trace of the A particles could be found except in the thicker portions of some sections and then only a faint outline of the inner shell was discernible. The juxtanuclear areas where remnants of inclusion bodies could be recognized were more electron lucent in appearance than the surrounding cytoplasm (Fig. 26). The B particles were detected in the extracellular lumina, but only by the presence of the lipoprotein sac. None of the nucleoid structure was present, in no instance were the dense cores of the nucleoids visible (Fig. 25).

The alterations observed in the fine structure of the A and B particles in the control sections which had been placed in (a) 0.1 N HCl followed by trypsin and RNase; (b) in pepsin followed by 0.045 M Tris chloride buffer and RNase; or (c) in 0.1 N HCl followed by 0.045 M Tris chloride and RNase were identical with those observed in the earlier experiments when RNase had been used separately or in combination with pepsin or trypsin. Thus, extinction of the structure of the A particle and the B particle nucleoid could only be attained if pepsin, trypsin, and RNase digestion was carried out consecutively. If trypsin hydrolysis was performed first for two hours and followed by pepsin and RNase, the A and B particles were degraded to the same extent as when pepsin and RNase were used together.

In order to be certain that the results obtained with consecutive hydrolysis with pepsin, trypsin, and RNase were due to the specific activity of ribonuclease, an identical experiment was carried out in which DNase digestion was substituted for RNase. In this experiment the sections were incubated for 40 minutes in 0.1% DNase after pepsin and trypsin proteolysis. In sections processed in this manner, the nucleus was rendered completely electron lucent and was seen in negative contrast surrounded by the more dense cytoplasm (Fig. 27). Deoxyribonuclease hydrolysis had no further effect on the A or B particle beyond that produced by the preceding digestions with pepsin and trypsin. The inner shell of the A particle and core of the B particle nucleoid were left intact and densely stained (Figs. 28, 29).

DISCUSSION

Extensive studies on the purification and analysis of MTV from milk and from mammary tumors of mouse strains with high indices of mammary cancer have been carried out by Moore and his collaborators (30, 37). As a result of these studies, the
**TABLE 1**

**Effects of Triple Digestion Procedures**

The effects of consecutive incubation of thin sections in three different enzymes and their controls are illustrated as a change in the relative electron densities of some of the cellular organelles and the A and B particle of mammary tumor virus. The maximum relative density is represented by the unincubated control and will be designated by 0. The degree of reduction from the density of the control by the various enzymatic extractions is indicated by the numerals 1, 2, and 3. The number 3 represents the maximum reduction achieved.

<table>
<thead>
<tr>
<th>Incubation media</th>
<th>Cell organelles</th>
<th>Mammary tumor virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nucleus</td>
<td>A particle</td>
</tr>
<tr>
<td></td>
<td>Nucleolus</td>
<td>B nucleoid</td>
</tr>
<tr>
<td></td>
<td>Granular</td>
<td>Shells</td>
</tr>
<tr>
<td></td>
<td>Agranular</td>
<td>Outer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inner</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Core</td>
</tr>
<tr>
<td>1. 0.5% pepsin-0.3% trypsin-0.1% RNase</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2. 0.5% pepsin-0.3% trypsin-distilled H$_2$O</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3. 0.5% pepsin-0.045 M Tris-0.1% RNase</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4. 0.5% pepsin-0.045 M Tris-distilled H$_2$O</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5. 0.1 N HCl-0.3% trypsin-0.1% RNase</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>6. 0.1 N HCl-0.045 M Tris-0.1% RNase</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>7. 0.1 N HCl-0.3% trypsin-distilled H$_2$O</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>8. 0.1 N HCl-0.45 M Tris-distilled H$_2$O</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>9. 0.3% trypsin-0.5% pepsin-0.1% RNase</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>10. 0.5% pepsin-0.3% trypsin-0.1% DNase</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>11. Unincubated control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Tris, tris(hydroxymethyl) aminomethane.

The biologic activity of MTV has been shown to be associated with the extracellular B particle. These observations have been confirmed by Sykes et al. (39) and Daniel et al. (11). Chemical determinations performed on B particle fractions purified by density gradient centrifugation or electrophoresis have demonstrated the presence of RNA and lipid (28, 29, 31). The amounts of RNA and lipid calculated as a fraction of the dry weight were 0.8% and 30%, respectively. The value estimated for the average mass of RNA per particle was $3.7 \times 10^6$ daltons (29). More recently, Duesberg and Blair (16) have isolated a single-stranded $70 S_{20,w}$ RNA from purified MTV derived from two different mouse strains. These studies have provided strong evidence that the B particle represents the active MTV and that MTV is an RNA virus.

Most RNA viruses carry out their replication in the cytoplasm of the infected cell. Although there is little information on the replicative cycle of MTV, Lasfargues et al. (21) have shown that the production of B particles in MTV-infected mammary gland organ cultures is related to two hormone-mediated phenomena, alveolarization of the primary explant and lactation by the induced glandular elements. Both of these events occur following a period during which there is a high rate of protein synthesis in the cytoplasm of the mammary epithelial cells. The A particle has been implicated as a cytoplasmic stage in the replicative cycle of MTV by numerous morphologic studies. It has been observed in normal, preneoplastic and neoplastic mammary tissue in a variety of MTV-infected mouse strains. Early studies by Bernhard and Guérin (6) indicated that an inverse relationship existed between the number of A particles and the number of B particles in spontaneous mammary tumors. They suggested that the A particles were rapidly eliminated and transformed into B particles. The latter may persist for long periods of time either at the cell surface or in the intercellular spaces. In spite of these numerous morphologic observations no evidence exists concerning the immunologic, biochemical, or biophysical nature of the A particle to support its proposed relationship as the precursor of the B particle.

Ultrastructural cytochemical technic employing embedment in water-miscible plastics followed by specific enzyme extraction or acid hydrolysis were first introduced by Leduc and Bernhard (23, 24, 27) and later as a specific technic for studying virus infection by Bernhard and Tournier (7). The procedure has
Gilbert H. Smith

advanced over recent years to provide good ultrastructural preservation combined with a minimum of denaturation of cellular proteins and nucleic acids so that specific enzyme hydrolysis is possible within sections of embedded tissues (25, 26, 40).

In the present study, the chemical nature of the intracellular A particle and the extracellular B particle, with special reference to the nucleoid of the latter, has been investigated with respect to alterations in their ultrastructural appearance and staining properties in thin sections subjected to a variety of specific enzymatic digestions. Adequate controls were carried out to document the specificity of enzyme hydrolysis and to provide information on the nature and extent of nonspecific extraction due to the chemical and physical character of the enzyme diluents. In all experiments high concentrations of enzymes were used to reduce the necessary reaction time and, therefore, nonspecific effects, and also because of the limited mobility of the enzyme molecules within the plastic polymer.

The results obtained with single enzyme digestions were indicative of good specificity with regard to target components within the sections. For example, RNase digestion resulted only in a loss of staining of the ribosomes and the granular component of the nucleolus. However, the structures within the A particle and B particle that were presumed to contain RNA were inconsistently altered. A point that bears directly on this observation is the consideration of the plane of section. In order for the nucleic acid presumably present within the inner shell of the A particle and core of the B particle nucleoid to be in contact with the enzyme solution, two requirements must be met: (a) the plane of section must pass through the outer shell exposing the inner surface; (b) the cut or open face must be oriented within the section toward the enzyme solution to facilitate the penetration of and contact with the enzyme. If the outer diameters of the A particle and B particle nucleoid, 1000 Å and 600–690 Å, respectively, are compared with the average thickness of section selected, 600 Å, it is readily apparent that all the A particles observed in any one thin section have been cut. On the other hand, in only a few B particles will the plane of section pass through the core of the nucleoid. If both requirements are satisfied, then one of the nucleases, DNase or RNase, should be effective in removing the density associated with one or both of these structures. The inner shells of some of the A particles and occasional B particle nucleoid cores were rendered electron lucent by ribonuclease action. This phenomenon was never observed following incubation with DNase or distilled water.

Because of the problem encountered due to orientation and plane of section, an attempt was made to remove the outer shell of the A particle and the B particle nucleoid within the thin sections with protease. Pepsin and papain hydrolysis were effective in this regard, pepsin reducing the outer diameter of the A particle and the nucleoid of the B particle by 140–170 Å. Trypsin had only a limited effect on these structures. This probably indicates that only a small number of lysine or arginine residues are associated with the protein composing the outer component of these structures. Pretreatment of thin sections with either pepsin or trypsin promoted no significant enhancement of the effect of ribonuclease on either particle. However, preliminary proteolysis with either enzyme brought about a more rapid and complete hydrolysis of other cell constituents with succeeding nuclease digestion, e.g., nuclear chromatin by protease-DNase or ribosomes by protease-RNase.

When pepsin and trypsin hydrolysis were combined, the inner shell of the A particle and the core of the B particle were unaffected. However, this treatment rendered those structures susceptible to RNase activity but not to DNase. The importance of tryptic digestion on the inner shell of the A and core of the nucleoid of the B particle was clarified by the fact that RNase was ineffective against either structure when trypsin incubation preceded pepsin digestion. The same was true if Tris chloride buffer was substituted for trypsin after pepsin incubation and before RNase. The activity of all the enzymes employed was well documented throughout the course of the experiment. The necessity of tryptic lysis prior to ribonuclease hydrolysis suggests that the inner shell of the A particle and core of the B particle nucleoid are basic protein-nucleic acid conjugates. Trypsin action is quite narrowly restricted to "basic" bonds, i.e., such bonds which link the carboxyl group of a basic amino acid (arginine and lysine) to the amino group of another. Basic proteins such as protamines and polyamines are often associated with the nucleic acid components of the bacterial viruses (2).

In conclusion, the results reported support the hypothesis that the A particle is the precursor of the nucleoid of the B particle, since the chemical organization at the ultrastructural level is the same in both. This is further evidence in support of Bernhard's postulate regarding the maturation of MTV. In terms of their relative susceptibility to enzymatic degradation, the outer shell of the intracellular A particle and the outer coat of the extracellular B particle nucleoid appear to have the same stereochemical structure. Both are attacked by pepsin and papain but not by trypsin. The diameter of both structures are reduced by approximately the same amount following pepsin digestion. The inner shell of the A particle and core of the B particle nucleoid are subject to RNase hydrolysis but not after pepsin and trypsin proteolysis. This observation is compatible with the concept that their chemical construction is similar, and furthermore that they are basic protein-ribonucleic acid conjugates.

The final proof that the A particle is truly an intracellular, immature form of MTV rests upon (a) the demonstration of MTV virion-associated antigencinity with the A particle and (b) the incorporation of isotopically labeled A particles into mature active MTV virions. The work presented here provides the first convincing evidence for the presence of RNA and protein in the ultrastructure of the A particle. The observations furnish a model that can be tested with the use of mammary tumor tissue isotopically labeled with protein and RNA precursors in conjunction with the water-miscible cytochemical technique. This is possible because the A and B particles apparently possess structures that are differentially susceptible to enzymatic hydrolysis in thin sections when compared to the cellular organelles. The new discovery of a soluble antigen MTV, by Ninowski et al. (32), which apparently is an internal component of the MTV virion, opens the possibility of correlating this antigen to the intracellular A particle in vivo cytochemically, with the use of specific ferritin-labeled antibody.

ACKNOWLEDGMENTS

The author sincerely appreciates the helpful criticisms and comments of Drs. A. J. Dalton and E. H. Leduc during the prep-
aration of the manuscript and the valuable technical assistance of Mr. David Longfellow.

REFERENCES


37. Stone, R. S., and Moore, D. H. Purification of Mouse Mam-
Gilbert H. Smith


FIG. 1. A small cytoplasmic inclusion body of type A particles. Their structure consists of two concentric shells; the inner shell characteristically stains more intensely (arrow). The center of the A particle appears variably electron-dense, this presumably is due to the plane of section. Specimen fixed in 4.5% phosphate-buffered glutaraldehyde, pH 7.4, for twenty minutes, post-fixed in 2% OsO4 in the same buffer for 1 hour and embedded in Epon. Section stained with Karnofsky’s lead hydroxide. X 70,000.

FIG. 2. A small extracellular lumen in a thin section from a spontaneous mammary tumor containing numerous type B particles. The B particles are characterized by a loose lipoprotein sac containing an eccentric-dense nucleoid. The nucleoid possesses two distinct components, an electron-lucent “cortex” or coat (arrow) and an electron-dense core. Specimen fixed according to the schedule given for Fig. 1. Uranyl acetate and lead citrate (Reynolds), X 70,000.

FIGS. 3-29. These micrographs were taken of thin sections from glutaraldehyde-fixed, glycol methacrylate-embedded mouse mammary tumor, doubly stained with 0.5% aqueous uranyl acetate and Reynolds’s lead citrate and subjected to various experimental procedures.

FIG. 3. An inclusion body of cytoplasmic A particles in an unincubated control section. X 69,500.

FIG. 4. Extracellular B particles lying in an extracellular lumen in an unincubated control section. Several budding A particles at the ends of microvillus-like projections can be seen (long arrows). The two components of the B particle are easily discerned (short arrow). The lipoprotein membranes appear in negative image because of the absence of OsO4 in the fixation procedure. X 69,500.
Mouse Mammary Tumor Virus

NOVEMBER 1967

Downloaded from cancerres.aacrjournals.org on January 2, 2018. © 1967 American Association for Cancer Research.
Fig. 5. Low-power micrograph showing the effect of incubation in 0.1% RNase for 20 minutes at 37°C. The dense-staining ribosomes are absent from the cytoplasm. The cytoplasm appears homogeneous and moderately dense. The nuclear chromatin exhibits a deeper contrast. × 21,600.

Fig. 6. An A particle inclusion body after incubation in 0.1% RNase for 60 minutes. The inner shell in some of the particles shows a decreased affinity for lead staining (arrows); others appear to be unaffected. × 69,500.

Fig. 7. B particle ultrastructure following digestion of the section for 60 minutes in 0.1% RNase. The nucleoids of some of the particles have become electron-lucent (arrows). × 69,500.

Fig. 8. The outer shell of the A particles were partially degraded and the outer diameter of the particle reduced after digestion in 0.5% pepsin for 60 minutes. × 69,500.

Fig. 9. Control section for pepsin hydrolysis which had been incubated for 90 minutes in 0.1 N HCl. This treatment had no effect on the ultrastructural morphology of the A particle. × 69,500.

Fig. 10. B particles after proteolysis in 0.5% pepsin for 60 minutes. The “cortex” or electron-lucent coat of the B particle nucleoid is degraded. The core of the nucleoid becomes more prominent in appearance due to the heightened contrast between it and the background. The lipoprotein sac is still in evidence (arrow). × 69,500.

Fig. 11. The structure of the B particle is unaltered after incubation in 0.1 N HCl for 60 minutes. × 69,500.

Fig. 12. No overt reduction in the outer diameter of the A particle is attained after 4 hours of incubation in 0.3% trypsin. The outer shell has a ragged appearance, suggesting some effect of the protease on that structure. The inner shell retains its affinity for lead salts. × 69,500.

Fig. 13. A particles in a trypsin control section incubated in 0.045 M tris(hydroxymethyl)aminomethane chloride buffer for 4 hours. × 69,500.

Fig. 14. No effect was observed on B particle structure following incubation in 0.3% trypsin for 4 hours. × 69,500.

Fig. 15. B particles in a section incubated for 4 hours in 0.045 M tris(hydroxymethyl)aminomethane chloride buffer as a control for fig. 14. × 69,500.

Fig. 16. A particles after treatment with 0.5% pepsin for 2 hours and 0.1% RNase for 30 minutes. The outer shell has been degraded by pepsin hydrolysis. The inner shell of some particles have a reduced density (arrow). × 69,500.

Fig. 17. A particles following 0.5% pepsin digestion for two hours and 0.1% DNase hydrolysis for 60 minutes. × 69,500.

Fig. 18. Budding A particles and extracellular B particles in a section treated for 2 hours with 0.5% pepsin and 30 minutes with 0.1% RNase. Most of the particles have lost the cortex of their nucleoids. The core, however, remains densely stained (short arrows). One of the B particles has an electron-lucent core which can be observed surrounded by the thin shell of the remnants of the nucleoid cortex (long arrow). × 69,500.

Fig. 19. B particles and cytoplasmic and budding A particles in a section consecutively treated with 0.5% pepsin for 2 hours and 0.1% DNase for 60 minutes. × 69,500.

Fig. 20. This micrograph portrays A particle morphology following treatment of the thin sections in 0.5% pepsin for two hours succeeded by two hours in 0.3% trypsin. The inner shell of the particle is intact. The outer shell has been completely removed. × 69,500.

Fig. 21. One of the control sections incubated in 0.1 N HCl for two hours then placed in 0.045 M tris(hydroxymethyl)aminomethane chloride buffer for two hours. Some nonspecific extraction of the outer shell of the A particles is apparent, but the overall diameter of the particle and the dense inner shell is unaffected. × 69,500.

Fig. 22. An extracellular lumen packed with B particles. This section was incubated for two hours in 0.5% pepsin followed by two hours in 0.25% trypsin. The core of the B particle nucleoid is unaffected. The cortex of the nucleoid in most of the particles is degraded. Part of the cortex is still present in some of the particles (arrow). × 69,500.

Fig. 23. B particles in a control section for pepsin and trypsin hydrolysis in which tris(hydroxymethyl)aminomethane chloride buffer was substituted for trypsin. The preliminary pepsin hydrolysis has clearly degraded the cortex of the B particle nucleoid to the extent that it is readily soluble in the tris(hydroxymethyl)aminomethane buffer. Remnants of the lipoprotein sac are present (arrow). × 69,500.

Fig. 24. The nucleolus (N) appears in negative contrast within the nucleus (Nu) in sections incubated consecutively in pepsin, trypsin, and RNase. The membranes of the Golgi apparatus (g) and mitochondria (m) are dense against the lighter cytoplasm. × 52,500.

Fig. 25. This micrograph illustrates a typical extracellular lumen (Lu) with microvilli (mv) and B particles in sections triply digested with 0.5% pepsin, 0.25% trypsin, and 0.1% RNase. The cores (co) of the B particle nucleoids are electron-lucent. An outline of the nucleoid cortex can be seen within the remnants of the lipoprotein sac. × 69,500.

Fig. 26. In the thicker portions of sections triply digested with pepsin, trypsin, and RNase, the faint outline of the degraded A particles can be discerned (arrows). The area immediately adjacent to the inclusion bodies is more electron lucent than the surrounding cytoplasm. × 69,500.

Figs. 27-29. These three micrographs were taken from the same section which had been incubated in 0.5% pepsin, 0.25% trypsin, and 0.1% DNase. The nucleus (Nu) in Fig. 27 (× 52,500) appears in negative contrast to the cytoplasm (Cy), indicating the specific hydrolysis of its contents by DNase. The dense core of the nucleoid of the B particle pictured in Fig. 28 and the inner shell of the cytoplasmic A particle in Fig. 29 are unaffected. × 69,500.
Mouse Mammary Tumor Virus
Gilbert H. Smith
# Cancer Research


## Cytochemical Studies on the Mouse Mammary Tumor Virus

Gilbert H. Smith

*Cancer Res* 1967;27:2179-2196.

| Updated version | Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/27/11_Part_1/2179 |

| E-mail alerts | Sign up to receive free email-alerts related to this article or journal. |
| Reprints and Subscriptions | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |
| Permissions | To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/27/11_Part_1/2179. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site. |