Localization of Intracytoplasmic A Particles in Mouse Tumors by Light Microscopy

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SUMMARY

Two methods for identifying aggregates of intracytoplasmic A particles (referred to as A particles) at the light microscopic level are described in this paper. The first is an immunofluorescent method using antisera against these particles. In mammary tumors examined both in sections and in smears, specific fluorescence was found in the form of discrete granules, confined to the cytoplasmic hemisphere proximal to the lumen. Neither nuclear nor diffuse cytoplasmic fluorescence was confirmed. All features of these fluorescing granules seem to justify their identification with A and B particles in various clusters. It has also been shown that transplanted tumors contain more A particles at the sacrifice of B particles than do spontaneous tumors. Specifically fluorescing granules were also observed in the cytoplasm of smears of mouse leukemia cells that were known to carry A particles. These granules were stained red by the second method described, with fuchsin acid and methylene blue as stains, and were referred to as inclusion bodies. Specificity of fuchsin acid staining was confirmed by a direct identification of inclusion bodies with specifically fluorescing granules in the same area of a smear. Because the staining is so simple and provides permanent preparations, it can be effectively used for studies on A particles in mouse leukemias.

INTRODUCTION

Mouse mammary tumors of viral origin usually contain aggregates of hollow spheres (doughnut-shaped in profiles of thin sections) in the cytoplasm. These were first designated as type A particles by Bernhard et al. (3, 4) and later re-designated as intracytoplasmic A particles (1) to distinguish them from the similar, intracellular virus-like, intracisternal A particles. Intracytoplasmic A particles (referred to as A particles hereafter) were also observed in other mouse tumors (5, 7, 10, 25, 28–30, 34) and leukemias (6, 11, 13, 15, 21, 22, 28, 35, 36).

A particles have long been assumed to be precursors of extracellular B particles of MTV (3, 9, 19, 31). This was substantiated by Tanaka et al. (35), who demonstrated common antigen(s) in purified A and B particles. More recently, Smith and Wivel (32) reported different polypeptide patterns of these 2 particles using SDS-polyacrylamide gel electrophoresis. It appears, therefore, that further investigations are required to establish the final relationship between A and B particles (see also Footnote 5).

To promote studies on A particles, it seems important that their occurrence in various mouse tissues under natural, as well as experimental, conditions be reported. However, electron microscopy, a method now widely used for identifying these particles, is laborious and unsuitable for accurate quantitative studies; simpler, more reliable methods have therefore been sought.

The present paper describes 2 methods that allow rapid identification of A-particle aggregates at the light microscopic level with well-defined specificity. The first is an immunofluorescent antibody method, using antibodies against A particles: the second is staining with fuchsin acid. The second method is so simple that it can be applied to leukemia cell smears in any laboratory with no specialized materials and equipment, yet the method proves to be comparable in specificity and reliability to the immunofluorescent method and, in fact, is superior to the latter in that it provides permanent preparations. A part of this work was reported in preliminary form elsewhere. See Refs. 27 and 37.

MATERIALS AND METHODS

Mouse Tumors. Mammary tumors and leukemias, both spontaneous and transplanted, of DBA/2 strain mice were mainly used. These mice were transferred to this laboratory in 1966 by Dr. Dan H. Moore of the Institute for Medical Research, Camden, N. J. and have been described in detail elsewhere (14, 35). Of these mice 17.4% of the breeding females developed mammary tumors at an average age of 11.5 months, and 38.2% of the females and 44.5% of the males developed lymphoid leukemias at 7.1 and 7.8 months.

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The abbreviations used are: MTV, mouse mammary tumor virus; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.
respectively. Numerous A particles and a small concentration of C particles were contained in some of these leukemias, but no B particles were present (35).

Mammary tumors of other mouse strains used are listed in Table 1, together with their sources and the results of the immunofluorescent studies.

Leukemias of other mouse strains were also used. Those of spontaneous origin were leukemias in SL/Ms mice (Dr. Yasuo Ichikawa of this Institute); in C57BL/10J × DBA/2 F1, or B10D2 mice (Dr. Tatsuya Tanaka of Aichi Cancer Center Research Institute, Nagoya, Japan); and in BALB/c mice from our colony. Rauscher virus-induced leukemias in C57BL/6 mice (Dr. Michiyuki Maeda of this Institute) and 2 sublines of L1210 leukemias maintained in C57BL/6J × DBA/2 F1 mice (Dr. Akira Hoschino of Aichi Cancer Center Research Institute and Dr. Kenji Yamaguchi of Shionogi Research Laboratory, Shionogi and Co., Ltd., Osaka, Japan) were also examined. Transplantable myelomas in BALB/c mice that contained an abundance of intracisternal A particles (35) were provided by Dr. Yujiro Namba of this Institute. Cells of lymph nodes and peripheral blood from young BALB/c and DBA/2 mice were checked as nonleukemic controls.

Preparation of Antisera. Rabbit antiserum against A particles purified from DBA/2 mouse leukemias and mammary tumors were those used in the previous study (35). These antisera reacted commonly with A and B particles of MTV (35). In some experiments, a rabbit antiserum against leukemia A particles treated with SDS was also used: purified A particles were treated with SDS (final concentration, 0.1%), and excess SDS was removed by dialysis. A rabbit was immunized with this material according to the method previously reported (35).

Preparation of Labeled Antibodies. The direct immuno

fluorescent method was used. γ-Globulin fractions prepared from the above antiserum were directly conjugated with fluorescein isothiocyanate and were sequentially purified by Sephadex G-50 and DEAE-cellulose column chromatography by the method of Kawamura (20). The conjugates were used for staining. For blocking tests, preparations were treated with unlabeled antisera at 37° for 1 hr, washed with PBS, and then subjected to the usual staining. Preparations were also stained with labeled, nonantibody rabbit globulin.

Fuchsin Acid Staining. Smears of leukemic lymph nodes were fixed with methanol for 10 min at room temperature and air-dried. These were stained for 10 min with 3% fuchsin acid (rubin S) [(Merck AG, dissolved in methanol:0.5% acetic acid, pH 4.5 (1:1)]. Stained smears were washed with 0.5% acetic acid, pH 3.0, and were counterstained for 5 min with a dilute methylene blue solution [methanol saturated with methylene blue:0.5% acetic acid, pH 3.0 (1:50)]. Smears were again washed with 0.5% acetic acid, air-dried, and examined with a light microscope. The whole procedure was completed within 30 min. Stained smears could be stored in a dark place for more than 1 year without noticeable fading.

Enzyme Digestion Tests. Smears were fixed with 10% formalin in 0.1 M phosphate buffer, pH 7.2, at 4° for 15 min. After a washing with distilled water, these were incubated in enzyme solution at 37° for 15 to 30 min. Enzymes used were 0.1% bovine pancreatic RNAse (5 times crystallized, Sigma Chemical Co., St. Louis, Mo.) in distilled water, pH 5.5; 0.5% peptic (twice crystallized, Sigma) in 0.1 N HCl, pH 1.2; and 0.3% trypsin (twice crystallized, Sigma) in distilled water, pH 8.0. Control preparations were similarly incubated in the above-mentioned solution, lacking the enzyme. Smears were then washed with distilled water and stained with fuchsin acid as indicated above.

Identification of Fuchsin Acid-stained Structures with A-Particle Aggregates. A set of smears was made from the same lymph nodes. One was stained with fuchsin acid and the other was examined by the immunofluorescent method. The percentages of cells containing positively stained structures were counted in both smears and compared. A direct identification was attempted from the same area of a smear.
that had been subjected to the immunofluorescent examination first and then subjected to fuchsin acid staining. In this case, smears examined by the immunofluorescent method were washed with xylene to remove the immersion oil and paraffin used for mounting. Smears were further washed with descending ethanol and finally with tap water for 30 min. These were refixed with methanol:0.5% acetic acid (1:1) for 15 min and stained with fuchsin acid as indicated above. Identical areas were photographed for each examination.

Electron Microscopy. Tissues were examined by electron microscopy in sections prepared by conventional methods.

RESULTS

General Comments on Immunofluorescent Microscopy. Because labeled antibodies against intact A particles of DBA/2 mouse leukemias and mammary tumors gave identical results, no distinctions between them have been made in the following description. Observations using an antiserum against SDS-treated A particles did not differ from those using the previously mentioned antisera.

Specific fluorescence was not observed when preparations were stained with labeled, nonantibody globulin or with specific conjugates absorbed with purified A particles. Blocking with unlabeled antibodies reduced remarkably the intensity of specific fluorescence.

Immunofluorescent Microscopy of DBA/2 Mouse Mammary Tumors. In the cytoplasm (Figs. 1 and 2) smeared cells of mammary tumors contained specifically fluorescing granules. The granules were more variable in size and more numerous per cell than they were in leukemia cells (see Fig. 9). No diffuse cytoplasmic staining was confirmed. The nucleus itself lacked fluorescence, although some cytoplasmic granules appeared to be in the nuclear region due to superimposition.

Intracellular sites of these granules were more accurately determined in histological detail in sectioned mammary tumors. Histologically, unit constituents of mammary tumors are "nests," which consist of the lumen and its lining epithelia surrounded by a common base-membrane. In some areas, the nests resemble alveoli or acini of normal mammary glands (Fig. 8). In other areas, they are cellular cords of varying widths and lengths (Fig. 6) or groups of epithelia aggregated in an apparently random fashion (Figs. 3, 5, and 7). In spontaneous mammary tumors (Fig. 3), fine granules with specific fluorescence were found concentrated at the cell surface toward the lumen and in the thin layer of the cytoplasm adjacent to it, thus making the contours of the ramifying lumen of various sizes, which otherwise would be easily overlooked, clearly visible. No diffuse cytoplasmic fluorescence was observed and the nucleus was completely without fluorescence. Very similar observations were obtained with a labeled antibody against SDS-treated A particles (Fig. 4).

Appearance and distribution of these fluorescing granules in sectioned mammary tumors are identical to those of variously clustered A and B particles, observed by electron microscopy (12, 14, 35). Fine granules at the cell surface represent the free and budding B particles, and cytoplasmic granules represent A-particle aggregates of various sizes.

Considerably different findings were obtained with transplanted DBA/2 mouse mammary tumors, however. Larger granules appeared in the supranuclear region of the cytoplasm, while fine granules at the cell surface were more or less reduced in concentration (Figs. 5 and 6). This difference will be discussed later.

Immunofluorescent Microscopy of Mammary Tumors of Other Mouse Strains. Results of other strains of tumors are summarized in Table 1. All tumors from MTV-positive strains contained specifically fluorescing granules that were indistinguishable from those in DBA/2 tumors in morphology and intracellular distribution (Figs. 7 and 8). Different observations between spontaneous and transplanted tumors in DBA/2 mouse strain were reproduced and confirmed in other strain tumors (compare Figs. 7 and 8 with Figs. 3 to 6). Only 1 negative result was recorded with transplanted tumors of the AKR mouse strain, which are presumably MTV free.

Immunofluorescent Microscopy of Mouse Leukemias. Fig. 9 shows a lymph node smear from a spontaneous DBA/2 mouse leukemia in which specific fluorescence was found in the form of cytoplasmic granules in almost every leukemia cell. These granules varied in size, going up to 3 or 4 µm in diameter. Most cells contained a single granule, but some had several. In other cases of spontaneous DBA/2 mouse leukemias, however, cells with specifically fluorescing granules were found less frequently or were not observed at all. Nuclear fluorescence was not confirmed in all cases. Occurrence, intracellular site, and morphology of these fluorescing granules coincided ex-

Table 1

<table>
<thead>
<tr>
<th>Strains</th>
<th>Sources*</th>
<th>MTV contained*</th>
<th>Spon-</th>
<th>Trans-</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2</td>
<td>1</td>
<td>S and L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C3H/He</td>
<td>2</td>
<td>S and L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DDD</td>
<td>2</td>
<td>P</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DD</td>
<td>3</td>
<td>P</td>
<td>n.t.</td>
<td>+</td>
</tr>
<tr>
<td>FM</td>
<td>2</td>
<td>n.c.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>n.c.</td>
<td>+</td>
<td>n.t.</td>
</tr>
<tr>
<td>BALB/cDBA/2</td>
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<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BALB/cC3H/He</td>
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<td>S</td>
<td>n.t.</td>
<td>+</td>
</tr>
<tr>
<td>BALB/cDDDD</td>
<td>2</td>
<td>P</td>
<td>n.t.</td>
<td>+</td>
</tr>
<tr>
<td>AKR</td>
<td>5</td>
<td>None</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

* These tumors were obtained from: Source 1, our colony; Source 2, Dr. Tadashi Yamamoto and Dr. Akio Matsuzawa of the Institute of Medical Science, University of Tokyo; Source 3, Dr. Yoshikiko Tsubura of Nara Prefectural Medical College; Source 4, Dr. Michiyuki Maeda of this Institute; and Source 5, Dr. Toshio Takeda of the Chest Disease Research Institute, Kyoto University.

* S, L, and P are MTV-S, -L, and -P according to the systems of Bentvelzen and Daams (2) and Mühlbock and Bentvelzen (26).

* n.t., not tested; n.c., not classified.
Observations of DBA/2 Mouse Leukemias by Fuchsin Acid Staining. In smears of leukemic lymph nodes from DBA/2 mice, the nuclei were stained pale blue and the cytoplasm stained blue or violet. Structures supposedly identical to A-particle aggregates appeared as sharply outlined, deeply red-stained granules (Fig. 10). These were 1 to 3 μm in diameter and were usually located within, or near, the pale reddish-stained cell center of leukemia cells. These structures are hereafter referred to as inclusion bodies according to the suggestion of Guérin (16). A cell usually contained a single inclusion body, but sometimes there were 2 or more.

Size, morphology, intracellular site, and occurrence of inclusion bodies were very similar to those of A-particle aggregates, as revealed by electron microscopy (35) and by immunofluorescent microscopy (see above).

Contents of phagocytic vacuoles of lymph node macrophages were also stained dark red. In smears of peripheral blood, red blood cells and eosinophil granules were stained bright red and basophil granules were stained blue, while neutrophil granules showed no affinity to the dyes. However, no difficulties arose in distinguishing these structures from inclusion bodies. Lymphocytes of peripheral blood and lymph nodes from nonleukemic BALB/c mice were completely free of inclusion bodies.

Application of fuchsin acid staining to paraffin-embedded sections proved unsuccessful.

Observations of Other Mouse Leukemias by Fuchsin Acid Staining. Inclusion body-carrying cells were found in an average of 10.8% of B10D2 leukemia cells, and in 2.6 and 1.1%, respectively, of the Aichi and the Shionogi sublines of L1210 leukemia cells. However, no inclusion bodies were observed in spontaneous leukemias in SL/MS mice and BALB/c mice or in Rauscher virus-induced leukemias in C57BL/6 mice. In BALB/c mouse myeloma cells carrying intracisternal A particles, no structures like inclusion bodies were confirmed in the cytoplasm.

Inclusion Bodies after Enzyme Digestion. Pretreatment of smears with trypsin or pepsin completely eliminated red staining of inclusion bodies, whereas RNase did not have any effect. This indicated that protein was responsible for the staining.

Identification of Inclusion Bodies with A-Particle Aggregates. Similarities of these 2 structures in morphology and some other properties have already been mentioned. Chart 1 shows results of the immunofluorescent antibody method and fuchsin acid staining obtained from paired smears of the same leukemic lymph nodes. The percentages of cells with inclusion bodies are identical to those with specifically fluorescing granules. Furthermore, a direct identification of these 2 structures was shown in the same area of a lymph node smear, sequentially stained with labeled antibody and fuchsin acid (Figs. 11 and 12). All inclusion bodies (Fig. 12) appear at the same sites as do fluorescing granules (Fig. 11).

DISCUSSION

Attempts to detect aggregates of A particles at the light microscopic level are not new. Guérin (16) was the first to describe inclusion bodies in paraffin-embedded sections of mouse mammary tumors. These were immediately correlated with A-particle aggregates by Bernhard et al. (4), and this was later supported by Smoller et al. (33). Inclusion bodies were also observed by Bloom (5) in mouse mastocytoma that carried A particles. These authors used such reagents as hematoxylin-eosin, periodic acid-Schiff reagent, and Giemsa's and Mann's stains to paraffin-embedded sections. However, specificity of these staining methods remained for investigation, and, as it turned out, they all gave negative or inconsistent results when applied to smears. Experiments with Macchavello's stain and Ehrlich-Biondi's triacid stain were unsuccessful.

By contrast, the present paper shows that the immunofluorescent antibody method can be satisfactorily applied to both smears and sections with well-known immunological specificity. In mammary tumors and leukemias, specific fluorescence appeared in the form of discrete granules, which were readily identified with A-particle aggregates (and B-particle clusters at the cell surface, and in the lumen in the case of mammary tumors). Neither nuclear nor diffuse cytoplasmic fluorescence was observed in any of the cases. This is in agreement with our earlier description (35) that MTV-a antigen, an A-particle antigen shared by B particles, against which antisera used in this paper were prepared, is particle-associated. On this point, however, the present observations are inconsistent with many other reports which have demonstrated a somewhat diffuse cytoplasmic distribution of MTV antigens (8, 17, 18, 24, 28). Only Yagi (39) reported a granular fluorescence similar...
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to ours. The reason for this discrepancy is being investigated.6

Nevertheless, observations described in the present paper contain several interesting points to be discussed. First, fluorescing granules in mammary tumors were confined to the cytoplasmic hemisphere proximal to the lumen and no fluorescence was observed in the basal part of epithelia. This indicates that formation of A particles, like that of B particles, occurs in very intimate relationship with the functional polarity of mammary gland epithelium [It is well known that B particles are produced around the lumen. By contrast, C particles can bud at any part of the cell surface when they infect mammary tumors (H. Tanaka, unpublished data)].

Different immunofluorescent patterns observed regularly in spontaneous and transplanted mammary tumors of all MTV-carrying mouse strains tested are also worth mentioning. As described in "Results," transplanted tumors always contained more A particles than spontaneous tumors. This is consistent with the electron microscopic observations of Feldman (12), who shows that repeated transplantation of mammary tumors results in the interruption of MTV maturation at the budding stage, along with cytoplasmic accumulation of A particles. It appears that transplanted tumors provide an unfavorable condition for completion of MTV maturation; that is probably due to disturbed functional polarity, accompanied by the initial phases of histological dedifferentiation and/or evoked immunological defense mechanism of the hosts.

Essentially identical immunofluorescent observations were made in mammary tumors of all mouse strains known to carry MTV, although MTV that was contained was different in biological activity (Refs. 2 and 26; Table 1). This confirms our previous report (35) that MTV-a antigen is group specific for MTV.

In DBA/2 mouse leukemia cells, specific fluorescence usually appeared as a single granule located in or near the cell center. No fluorescence was observed at the cell surface because B particles are not formed by these cells. Another difference between leukemias and mammary tumors is that occurrence of fluorescing granules in leukemias is not constant, but varies greatly from case to case (38).

Inclusion bodies stained with fuchsin acid were successfully identified with A-particle aggregates, but there are still several points to be discussed with regard to the specificity of the staining. As shown by enzyme digestion tests, protein components are essential for the staining. However, intracisternal A particles in mouse myelomas could not be detected by the staining in spite of the overall chemical similarity to intracytoplasmic A particles (23, 35); this is probably because intracisternal A particles are not found in packed aggregates. This point suggests that fuchsin acid stains proteins distinctly only when accumulations occur at concentrations above a certain critical level. In addition, the isoelectric point of the protein was also critical, since washing of stained smears with solutions of pH 5.0 or higher resulted in complete dissociation of fuchsin acid, leaving inclusion bodies as colorless, empty images surrounded by blue cytoplasm (unpublished data). On the other hand, fuchsin acid stained erythrocytes, phagocytic vacuoles, and eosinophil granules in varying intensities, showing that the staining is no way specific for A-particle aggregates. Nevertheless, the staining can be considered "practically" specific because no other structures comparable to A-particle aggregates in the above-mentioned properties are found in DBA/2 mouse leukemias. Care should be taken, however, when the staining is applied to other mouse tissues, including mammary tumors, because the cellular architecture is usually much more complicated in these instances. It does seem possible, however, to apply the staining to inclusion bodies caused by other viruses when host cells are found to contain no confusing structures.

Notwithstanding the reservations discussed above the technical simplicity of using fuchsin acid staining and its advantage of allowing permanent preparations establishes its superiority over the immunofluorescent method. Using this stain, relatively few inclusion bodies were found in L1210 and B10D2 leukemias, and none were found in SL/Ms leukemias. However, this may not reflect the entire nature of A particle formation in these leukemias because of the limited number of cases examined. Furthermore, repeated transplantation of leukemias exerts a suppressive effect upon A particle formation (38).

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Figs. 1 and 2. Smears of transplanted DBA/2 mouse mammary tumor stained with labeled antibody against A particles of mammary tumors. × 1050.

Fig. 3. Section of spontaneous DBA/2 mouse mammary tumor stained with labeled antibody against A particles of leukemias. × 800.

Fig. 4. Same as Fig. 5 but stained with labeled antibody against SDS-treated A particles of leukemias. × 800.

Fig. 5. Section of transplanted DBA/2 mouse mammary tumor stained with labeled antibody against A particles of leukemias. × 800.

Fig. 6. Same as Fig. 7 but stained with labeled antibody against A particles of mammary tumors. × 800.

Fig. 7. Section of spontaneous C3H/He mouse mammary tumor stained with labeled antibody against A particles of mammary tumors. × 920.

Fig. 8. Section of transplanted C3H/He mouse mammary tumor stained with labeled antibody against A particles of mammary tumors. × 920.

Fig. 9. Smear of lymph node cells from spontaneous DBA/2 mouse leukemia stained with labeled antibody against A particles of mammary tumors. × 1200.

Fig. 10. Lymph node smear from spontaneous DBA/2 mouse leukemia stained with fuchsin acid. All leukemia cells except for one (arrow) contain a single inclusion body. × 1700.

Figs. 11 and 12. Identical area of lymph node smear from spontaneous DBA/2 mouse leukemia sequentially examined by immunofluorescent method (Fig. 11) and by fuchsin acid staining (Fig. 12). Fuchsin acid-stained inclusion bodies appear at the same sites of specifically fluorescing granules. An inclusion body in a cell (arrow) is slightly out of focus. Sizes of fluorescing granules in Fig. 11 are exaggerated by haloes due to overexposure to enhance background images. A red blood cell (R) is seen. × 1300.

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