Studies of Neoplastic Myelomonocytic Cells in BALB/c Mice Producing Infectious C-Type Viruses

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SUMMARY

C-type viruses were identified by electron microscopy in muramidase-producing transplantable leukemic cells of BALB/c mice. Neoplastic cells contained peroxidase-positive cytoplasmic granules and consisted of atypical monocytic and granulocytic precursors, undifferentiated stem cells, and histiocytes. Viral particles were present in all tumors studied and were principally of the intracisternal A and immature C types. Exceptionally, mature C-type virions were noted in large cytoplasmic vacuoles of tumor cells, in bone marrow megakaryocytes, and in extracellular spaces. Unusual cytoplasmic structures that may represent structural precursors of viral nucleoprotein assembly were also observed in some cells. Bone marrow cells from animals treated with cell-free filtrates contained numerous cylindrical inclusions, several of which demonstrated viral budding. Complement fixation and mouse embryo cytopathogenicity tests on tumor extracts identified the viral particles as members of the murine leukemia-sarcoma group and demonstrated high titers of infectivity.

INTRODUCTION

The development of transplantable myelomonocytic leukemia in a BALB/c mouse during experiments designed to produce plasma cell tumors was recently reported by Warner et al. (30). The tumor, designated WEHI-3, was composed of immature and atypical granulocytic and monocytic elements and contained substantial amounts of muramidase.

The present investigation was initiated in order to establish a base line at the electron microscope level for an animal model analog to our studies of the human disease (27, 28) since, in common with cases of human myelomonocytic leukemia, serum and urine from these animals contained elevated levels of the cationic enzyme muramidase (19).

This paper describes studies of 1 of the 4 sublines of myelomonocytic tumors derived from the original animal and reports the unexpected finding of viruses of the murine oncornavirus group (18). Preliminary findings were reported previously (24).

MATERIALS AND METHODS

Animals. Twenty-four of our inbred 6-week-old BALB/c mice (supplied by the Animal Procurement Branch, NIH, Bethesda, Md.) were vaccinated against ectromelia and sent to Dr. Donald Metcalf, Cancer Research Unit, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, who generously arranged for their i.p. implantation with passage 35 WEHI-3 subline B tumor cells.

In our facilities, the tumor has been maintained in 6-week-old sex-segregated animals from the same source as the original seeding colony; it is now in its 87th passage. Animals were cohorted with peer controls, 6 to 8 per Isosystem cage (Carworth Division of Baltimore Biological Laboratories, New City, N. Y.) on bedding of cedar shavings and were supplied with Purina laboratory chow and water ad libitum.

Test animals were given i.p. or s.c. injections of 0.5-ml aliquots of approximately 30% concentrations (v/v) of aseptically removed tumor, liver, and ascites cells or bone marrow suspensions in Hanks' base medium containing 10% calf serum and antibiotics (Becton, Dickinson and Co., Cockeysville, Md.). Most animals were moribund 10 to 18 days after grafting; in many instances, however, bone marrow-treated animals had latency periods up to 90 days. Littermate controls were always negative.

Newborn mice received 0.1 to 0.2 ml of bacteriologically negative cell-free filtrates prepared from tumor homogenates that had been clarified by centrifugation and seeded with viable Escherichia coli before being passed through 0.45-μm Millipore filters (Millipore Corp., Bedford, Mass.). In preliminary experiments, the shortest latency period in these animals was 125 days.

Phagocytic Function Test. In order to test phagocytic competence, cells harvested from ascitic fluid and single-cell tumor suspensions (prepared by serial passage of tumor minces through 18- to 25-gauge needles) were exposed to suspensions of thorium dioxide or Pseudomonas aeruginosa as previously described (27).

Sampling. Specimens were taken from each transplant generation. In order to check the constancy of characteristics of the WEHI-3 line as originally described (30), histological sections, blood, bone marrow smears, and tumor imprints were prepared by standard techniques and examined by light microscopy. Random tail blood counts and differentials were performed on 50 animals. Muramidase levels of plasma, urine, and tumor homogenates were determined by the Lysoplate method (19) on 122 samples from test animals during 35 transplant generations. Additionally, smears and tumor imprints were tested for peroxidase activity (17).

Virus Specificity Tests. Ten percent tumor extracts were examined for murine leukemia virus group-specific antigens by...
complement fixation (13) and the XC mixed-culture cytopathogenicity tests (23). These procedures were performed courtesy of Dr. J. Hartley and associates of the Laboratory of Viral Diseases, NIH, Bethesda, Md.

Electron Microscopy. Tissues were removed from test animals and controls under ether anesthesia and were prefixed in 3.5% cacodylate-buffered (pH 7.35) glutaraldehyde for 10 min at room temperature and then at 0℃ for 2 hr. Blocks were rinsed in buffer and postfixed in 2% OsO4, similarly buffered either with cacodylate or s-collidine in 10% sucrose. Staining en bloc with uranyl acetate was followed by dehydration in graded ethanol and embedding in Epon-Araldite by conventional methods. Thin sections were doubly stained with uranyl acetate and lead citrate and were viewed in a Siemens-Elmiskop 1A electron microscope equipped with double condenser and operated at 80 kV.

RESULTS

The gross pathology and histology of the WEHI-3 subline B tumor have been substantially constant throughout the transplant generations studied and did not deviate significantly from the original descriptions of early passages (30). In brief, in animals treated by i.p. injection, neoplastic cells characteristically infiltrated the pancreas, liver, spleen, mesenteric lymph nodes, perirenal tissues, and bone marrow. Implantation s.c. resulted in tumor formation at the injection site with subsequent systemic dissemination. Histological sections and imprints demonstrated the tumor to be composed of a mixed population of undifferentiated blasts, histiocytic cells, atypical monocytic and granulocytic elements, atypical mononuclear cells, and scattered mature neutrophils.

Peripheral blood white cell counts ranged between 25,000 and 50,000 with occasional levels to 150,000. Blood smears contained predominantly granulocytic and monocytic elements and several large, blast-like cells similar to those in tumor imprints. Bone marrows were hypercellular with numerous megakaryocytes, the gamut of granulocytic and monocytic cells, and many large neoplastic cells.

Enzymes

Muramidase. Of 110 specimens of urine, plasma, and ascitic fluid, only 13 from experimental animals contained levels below 25 µg/ml, with the highest concentration ranging up to 1900 µg/ml in urine. Excluding those with levels below 25 µg/ml, the mean value of 20 urine samples was 336 µg/ml, the mean of 47 plasma samples was 63 µg/ml, and the mean of 20 samples of ascitic fluid was 244 µg/ml. In 12 tumor homogenates tested, muramidase levels ranged from 75 to 600 µg/ml with a mean of 226. These values correlate fairly well with levels reported for earlier passages (30) and indicate that the neoplastic cells continue to produce the enzyme. Of the 10 control animals tested, urine muramidase was undetectable and plasma levels were below 18 µg/ml in 9 animals; in 1 presumed normal animal, in which no pathology was detected, urinary and plasma muramidase measured 30 and 100 µg/ml, respectively.

Peroxidase. Peroxidase activity was demonstrated in the majority of cells in tumor imprints.

Electron Microscopy

Tumor Cells. The fine structure of tumors harvested from i.p. and s.c. transfers demonstrates them to be composed principally of 2 major cell types. Cells with some characteristics of monocyte precursors (27—29, 32) have large, immature, irregularly shaped nuclei containing prominent pleomorphic nucleoli, hyperplastic Golgi elements, moderate numbers of dense granules, numerous vesicles, scattered lamellae of RER and microvillous surface extrusions (Figs. 1 and 3). Atypical neoplastic cells with some features of promyelocytes (2, 3, 6) represent the 2nd numerous population. In these cells (Fig. 1), round or oval nuclei contained finely dispersed chromatin clumped slightly at the nuclear membrane. In common with the monocytoid cells, the pars amorpha and pars fibrosa of the nucleoli (5) were frequently segregated. The cytoplasm was distinguished by markedly dilated cisternae of RER (Figs. 1 and 2) and many Golgi elements. Free ribosomes occurred singly or as polyribosomes, and electron-dense granules were randomly dispersed throughout the cytoplasmic matrix. Mitotubules were frequently noted.

The micromorphology of WEHI-3 tumor cells reveals considerable protein-synthesizing machinery (20) as indicated above. Still to be resolved is the localization of muramidase in these cells (4), since the presence of only moderate numbers of granules suggests that extragranular production and/or storage of muramidase may account for the considerable amounts of this enzyme in tumor homogenates.

Viruses. Virus-like particles of 3 types (22) were present in both cell populations. Doughnut-shaped intracisternal A-type particles measuring 70 to 80 nm in diameter were by far the most abundant (Figs. 1, 2, 4, 6, and 10), budding into the RER and within smooth-walled vesicles. Immature C particles 100 to 120 nm in diameter were contained within and budded from the walls of cytoplasmic vacuoles and from microvillous projections on plasma membranes of neoplastic cells (Figs. 1 and 3); these virions were also free in extracellular spaces (Figs. 4 and 11). Least numerous were mature C virions present in large vacuoles of monocytic cells, in dilated channels of bone marrow megakaryocytes, and in extracellular spaces (Figs. 1 and 4).

Unusual pentalaminar tubular structures (26) 25 nm wide and 100 to 900 nm long were noted in virus-producing tumor cells from several transplant generations (Figs. 5 and 6). These were often in continuity with membranes of the RER and in some instances were formed by constricted membranes of the same cistern that exhibited budding particles in dilated areas (Fig. 6). The similarity in dimension and homology of these structures to the outer lamellae of C particles, particularly striking in aberrant viral forms (Fig. 7), suggests the possibility that they may represent precursors of viral nucleoprotein assembly.

Megakaryocytes and mononuclear cells in bone marrow specimens from animals that had received cell-free filtrates*

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1The abbreviation used is: RER, ribosomal endoplasmic reticulum.
produced masses of immature C-type particles (Figs. 7 to 9) and exhibited atypical, varied structures, including virus-producing cylindrical bodies known to occur in virus-induced hematopoietic neoplasms (7, 11).

In the experiments designed to test phagocytic competence, very few neoplastic cells ingested particles; however, phagocytosis was demonstrable in some virus-producing cells (Figs. 10 and 11).

**Virus Specificity**

Tumor extracts contained complement-fixing antigen for the murine leukemia virus group (13) and significant titers of infectious viruses of the murine leukemia-sarcoma group as measured by the XC plaque test (23) (Table 1). Hartley additionally reported that virus was detected equally well in NIH and BALB/c mouse embryo cells, indicating that the virus recovered is an NB-trophic virus (14). Neutralization testing indicated that the virus is a member of the Friend-Moloney-Rauscher subgroup, being neutralized by high dilutions of murine sarcoma virus rat antisera but only poorly by anti-Gross passage A and AKR antiserum.

**DISCUSSION**

BALB/c mice respond to i.p. injection of mineral oil by the development of plasmacytomas that have been demonstrated to contain intracisternal A-type particles (8, 15). It was therefore not surprising to find such particles (Figs. 1, 2, 4 to 6, and 10) in the cells of the transplantable leukemia induced by the same agent.

The unexpected finding of C particles as well (Figs. 1, 3, 4, and 11) resulted in additional experiments that demonstrated them to be highly oncogenic virions of the murine leukemia-sarcoma group. Cell-free passages confirmed the in vitro tests (Figs. 7 to 9), and results of trans-species experiments (25) further demonstrated the oncogenic properties of these viruses.

Table 1

<table>
<thead>
<tr>
<th>Animal</th>
<th>Transplant generation</th>
<th>Material tested</th>
<th>Complement-fixing antigen</th>
<th>Infectivity titera,b,c</th>
<th>INFECTIVITY TITER</th>
<th>BALB/c</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NIH-ME</td>
<td>BALB/c</td>
</tr>
<tr>
<td>266-2</td>
<td>58</td>
<td>10% tumor extract Supernatant from NIH-ME culture</td>
<td>1:8</td>
<td>10^{3.1}</td>
<td>10^{3.6}</td>
<td></td>
</tr>
<tr>
<td>266-2</td>
<td>1st passage</td>
<td>Supernatant from NIH-ME culture</td>
<td></td>
<td>10^{5.1}</td>
<td>10^{5.2}</td>
<td></td>
</tr>
<tr>
<td>266-2</td>
<td>1st passage</td>
<td>Supernatant from BALB/c-ME culture</td>
<td></td>
<td>10^{5.9}</td>
<td>10^{5.1}</td>
<td></td>
</tr>
<tr>
<td>375-6</td>
<td>Cell-free passage</td>
<td>10% tumor extract</td>
<td>Not tested</td>
<td>10^{5.4}</td>
<td>10^{5.3}</td>
<td></td>
</tr>
</tbody>
</table>

a Tested against murine sarcoma virus rat antiserum and anti-murine leukemia virus group-specific (gt 1) guinea pig serum.

b Plaque-forming units/0.1 ml as measured by the XC plaque test.

c NIH mouse embryo tissue culture.

d BALB/c mouse embryo culture.

The significance of the finding of an NB-trophic, Friend-Moloney-Rauscher subgroup in the WEHI-3 subline B tumor is unclear. Murine leukemia virus recovered from naturally infected BALB/c mice has been either N or B trophic and falls into the Gross-AKR serological subgroup (Refs. 14 and 21; J. W. Hartley, personal communication).

To date, there is no conclusive evidence for biological activity of intracisternal A particles (12), although a promising finding, the localization of 60 to 70 S RNA in isolated intracisternal A particles, has been reported recently (31).

Of particular interest was the preponderance of immature C-type particles in WEHI-3 cells, extracts of which showed high titers of infectivity. The “immaturity” of such particles has been questioned by de Harven (9), who prefers to call the triple-layered particle with the lucent center “enveloped A” virions. Although attempts have been made to determine the comparative viability of both “enveloped A” and mature C types (10), these assays remain inconclusive.

Natural expression of the C-type RNA viral genome in BALB/c mice is nonexistent in newborns, very slight in animals under 12 months old, and quite high in older animals (16). Aaronson et al. (1) have shown that C-type viruses are present in unexpressed form in all BALB/c cells and can be elicited in vitro by transfers of virus-negative embryo cells.

The present studies have documented the phenotypic expression and biological activity of viruses in myelomonocytic leukemia induced in young BALB/c mice, a low-leukemia strain.

**ACKNOWLEDGMENTS**

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REFERENCES


Figs. 1 to 11. G followed by a number indicates transplant generation.

Fig. 1. G-42 tumor. Neoplastic monocytic (M) and promyelocytic (P) cells producing intracisternal particles (single arrows) and intravacuolar virions (double arrows); note hyperplastic Golgi (G), segregated fibrillar (f), and granular (g) components of the nucleoli and widely distended cisternae of RER (*). X 12,500.

Fig. 2. G-36 tumor. Detail of cell producing intracisternal A particles. Nuclear pore (arrows), mitochondria (m), RER (*). X 54,000.

Fig. 3. G-36 tumor. Immature C virions (arrows) bud from the cell membrane and from walls of cytoplasmic vacuoles. X 65,000.
Fig. 4. G-42 tumor. Maturing extracellular virions and intracisternal budding particles (arrow). X 72,000.

Fig. 5. G-62 tumor. Unusual linear structures (s) present in some tumor cells appear to encircle cytoplasmic vacuole; virus-like particles (arrows) are formed in the same cells. X 78,000.

Fig. 6. G-62 tumor. The tubular structure here appears to be formed by close approximation of cisternal membranes (double arrows) which produce budding particles in adjacent dilated areas (single arrows). X 65,000. Inset demonstrates unit membrane structure of outer surfaces and intermittent nature of the medial lamina. X 140,000.
Fig. 7. Cell-free studies. Bone marrow. Massive proliferation of immature C-type virions and aberrant forms in a mononuclear cell. Note points of junction (arrows) of replicating particles. X 108,000.

Fig. 8. Cell-free studies. Bone marrow. Detail of megakaryocyte containing cylindrical virus-producing structure (arrow) with inner and outer membranes corresponding to those of viruses. X 96,000.

Fig. 9. Cell-free studies. Bone marrow metamyelocyte with multiple nucleocapsids budding at the plasma membrane. X 21,600.
Fig. 10. G-36 tumor. A flagellated bacterium (B) in a phagocytic vacuole, in a neoplastic cell synthesizing intracisternal particles. X 61,500.

Fig. 11. G-37 ascites cells. Phagosome (ph) containing ingested Th02 particulates; a C-type virus (arrow) is present in the same cell. Note a dividing virion and marker particles in the extracellular space (es). X 50,400.
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