Cell Culture of the Mucinous Variant of Human Colorectal Carcinoma

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ABSTRACT

Two cell lines, RW-2982 and RW-7213, have been established for the first time from the mucinous variant of human colorectal carcinoma, which is a distinctive and important subtype that has a worse prognosis than the more common nonmucinous large bowel carcinoma.

Methods of establishment and observations made during 7 and 3 years, respectively, of continuous culture are described. These cell lines required 4–9 months of adaptation to tissue culture conditions before noticeable growth occurred. Both cell lines have the following unique properties: (a) growth in vitro as delicate branching three-dimensional tumor particles within a wide gel of insoluble, often translucent mucus (proteoglycan); (b) production of large quantities of carcinobryonic antigen; (c) ability to survive or adapt to growth in media free of serum, hormones, growth factors, and all proteins; and (d) tumorigenicity in multiple sites in nude mice, including liver, with especially rapid growth in the peritoneal cavity as granular and noninvasive and thus resembles pseudomyxoma peritonei. Unlike other reported colorectal cell lines, these mucus-coated particulate cell lines will not readily grow as monolayers and grow much more slowly with a doubling time of 2 weeks or more.

A serially transplanted tumor from the RW-7213 surgical specimen has also been maintained in nude mice since August 8, 1984. This tumor retains properties of the original specimen.

Observations made on the tumor biology of mucogenic colorectal carcinoma using these cell lines are discussed.

INTRODUCTION

There will be an estimated 145,000 new cases of colorectal carcinoma in the United States in 1987, with 60,000 deaths (1). There is no chemotherapeutic treatment that prolongs survival once metastatic dissemination has taken place (2). For these reasons it is important to study tumor and cancer cell biology in this malignancy to understand the reasons for the lack of effective therapy and to enable development of future strategies for treatment. The availability of colorectal cell lines allows laboratory studies in cell biology, growth factors, and drug resistance. The use of the nude mouse expands these studies to include tumor biology, metastasis models, and chemotherapeutic index determination in vivo.

Many cell lines from human colorectal adenocarcinomas have been established (3–7). However, until very recently (8), there were no reports of cell lines derived from the mucus-producing variant of colorectal carcinoma. Classification of a colorectal tumor as mucinous (9) requires the presence of extracellular lakes and pools of mucus with acini or strips of epithelium which are often sparse and frequently dislodged from the periphery and floating in the mucus. Furthermore, for inclusion in this category, this distinctive histological pattern must be seen in the infiltrating or metastatic portions of the tumor and should comprise a minimum of 60% of the estimated tumor volume. The mucinous subtype, which comprises 15% of colorectal carcinomas (9), is important because it has a worse prognosis (9, 10) than the more common moderately differentiated nonmucus-producing tumor type. A distinctive form of i.p. mucinous malignancy known as pseudomyxoma peritonei is often derived from local spread of mucinous appendiceal carcinoma. Again, until very recently (8), there were no reports of human colorectal cell lines producing this distinctive appearance and growth pattern upon i.p. injection into nude mice.

The establishment, characterization, and biological properties of two new cell lines, RW-2982 and RW-7213, derived from human mucinous colorectal carcinoma are discussed in this paper.

MATERIALS AND METHODS

Surgical Specimen RW-2982. The RW-2982 cell line was cultured from metastatic omental tumor nodules submitted to the RWGH pathology department on May 12, 1980. The patient was a 69-year-old white male (blood type AB+) whose primary surgery was on June 12, 1979, for a mucin-producing, moderately well-differentiated adenocarcinoma of the rectosigmoid that extended through the entire thickness of the bowel wall with metastases in nine of 13 pericolic lymph nodes. Prior to surgery the serum CEA was 10.9 ng/ml. On December 4, 1979, the tumor had recurred at the anastamosis site resulting in mucus discharge from the rectum and later infiltration of the pelvis, and partial obstruction of the left ureter. A colostomy was performed and a hernia containing tumor repaired on January 11, 1980. Intestinal obstruction developed so a gastrostomy and jejunostomy were performed on May 12, 1980. At that time there was massive metastatic tumor involving the abdominal cavity with studdings on the peritoneal surface of the omentum. There was no evidence of liver metastases. The patient developed phlebitis of the left leg and died suddenly on May 22, 1980, presumably of pulmonary embolus. No autopsy was performed.

Historically, both the primary and metastatic tumors were of the mucus-producing subtype of colorectal carcinoma (Fig. 1).

Cell and Tissue Culture. RW-2982 tumor tissue was sterilely minced into 1-mm cubes using two sharp scalpels. Cubes were attached to the bottom of 25-cm² plastic tissue culture flasks, 10–12 per flask, using chicken plasma clots produced by mixing freshly reconstituted lyophilized chicken plasma and chick embryo extract. After the clots had formed for 15 min at 37°C, 5 ml of medium RPMI 1640, supplemented with 10% FCS, and containing Hapes Buffer (0.02 M), insulin (0.1 U/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and fungizone (5 µg/ml) was added. (All media and supplements were from Grand Island Biological Co., Grand Island, NY, except "Fungizone intravenous," a product of Squibb, Princeton, NJ that also contains 41 mg sodium desoxycholate per 50 mg amphotericin B.) Medium was changed weekly. After establishment, line RW-2982 was maintained in 75-cm² plastic flasks and fresh medium was added weekly by first pipetting off 80% of the old medium before replacing with 10 ml fresh medium. After more than a year in culture, insulin was withdrawn from the medium.

Surgical Specimen RW-7213. The RW-7213 tumor was both cultured
and xenografted into nude mice on August 8, 1984, from the invasive portion of a resected sigmoid colon containing a moderately differentiated mucus-producing adenocarcinoma extending through the entire thickness of the bowel wall and forming deposits in the serosal fat. One of 13 lymph nodes showed metastatic tumor. The patient was a 66-year-old white male (blood type A+). Preoperative CEA was 3.2 ng/ml; 10 months later there was diffuse abdominal recurrence (without liver metastasis) with obstruction requiring a laparotomy and colostomy with mucus fistula. Treatment was with local palliative radiation therapy and later chemotherapy with 5-fluorouracil and a nitrosourea. Serum CEA done at 24 months was 119 ng/ml. The patient was still alive with tumor at 30 months.

Microscopically the tumor was moderately well differentiated with both mucus-producing and nonmucus-producing areas. RW-7213, Cell and Tissue Culture. RW-7213 tumor tissue was washed in culture medium and finely minced using two scalpels. For xenografting, 0.2 ml of the minced tissue was injected s.c. into each of three nude mice. The remainder of the mince was placed into tissue culture flasks in RPMI 1640 medium with 10% FCS supplemented as above except without insulin. (In the 4 years since establishing RW-2982, the methods had been simplified by eliminating the plasma clot above except without insulin. (In the 4 years since establishing RW-2982, the methods had been simplified by eliminating the plasma clot above except without insulin.)

Growth Rate of RW-2982 Particles. Quadruplicate flasks were inoculated with approximately equal volumes of tumor particles. Each week the complete contents of each flask was transferred to sterile pipet to a sterile screw-cap 15-ml conical-graduated centrifuge tube (Kimax, Fisher 05-538-32B, Pittsburgh, PA). Tumor particles were centrifuged and the packed volume measured to the nearest 0.01 ml. The particles were then returned to their original flask in fresh medium, and reincubated. After five to six weekly determinations, average volume doubling time was determined using the slopes of graphing volume versus time.

Nude Mouse Injections and Tumor Weights. Nude mice (BALB/c background) were bred and maintained in the RWGH Cancer Center Animal Care Facility. All cages, bedding, and water bottles were autoclaved. In most experiments 6–12-week-old mice of either sex were utilized. Subcutaneous injections (usually on the lower abdominal flank) were performed using 0.15 ml of centrifuge-packed cell clumps. This was equivalent to 0.3-ml gravity sedimented clumps. The weight of 0.15-ml clumps was approximately 0.075 g after blotting on filter paper. Although this type of culture of colonie tumor in paniculate form cannot be readily enzymatically treated to obtain a single-cell suspension, a rough estimate done on particles of RW-2982 treated with trypsin suggests that 0.15 ml of packed clumps contains about 1.2 million cells. Injections were with a 1-cc tuberculin syringe using a 22-gauge needle. Intraperitoneal injections were usually 0.2-ml centrifuge-packed clumps (1.6 x 10⁶ cells). Subcutaneous tumors were weighed to the nearest 0.01 g after removal from mice sacrificed by cervical dislocation. The i.p. tumors were weighed by scraping all tumor from the peritoneal cavity onto absorbent filter paper (Whatman no. 1, 24 cm). The filter paper absorbed the majority of any contaminating blood (which was seen after several grams of tumor had accumulated). The larger cell clumps were loosely adherent to peritoneal structures but could be removed completely with gentle traction using curved forceps. (Tumor did not invade peritoneal organs or structures, nor was it firmly adherent.) The smaller free-floating clumps (having the appearance and consistency of tapica) were gently scraped or blotted from the peritoneal cavity using the same large piece of filter paper. The resulting tumor material was gently scraped together with a scalpel blade, trans-
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Splenic Injections. Nude mice were anesthetized with 100 mg/kg sodium amytal and the spleen was exposed after abdominal incision. Injections with a 1-cm³ syringe were made just under the capsule. The laparotomy incision was then closed in layers, first closing the peritoneum with suture material and the skin with stainless steel clips. The skin closure clips were removed at 1 week. Two methods were examined for preparing RW-2982 particles for injection into the nude mouse spleen. For the first, the particles were broken by vigorous shaking in a screw-cap sterile glass tube followed by centrifuge sedimentation. A volume of 0.02-ml packed particles was injected. For the second method, particles were again broken by vigorous shaking as before but the particles were repeatedly gravity sedimented for a short period with removal of the smaller particles and debris by pipeting off the supernatant. Finally, centrifuge packing was performed to obtain a concentrated pellet of particles for loading the syringe. In each case the number of splenic primaries and liver colonies was determined at necropsy on Day 31.

RESULTS

Establishment of Cell Line RW-2982. The attached explants of RW-2982 initially showed some slight monolayer outgrowth but no progressive growth. However, after 3–4 months of observation it was noted that the surfaces of two explants began to develop finger-like, fuzzy-surfaced projections. There was continuous growth of these three-dimensional structures and within an additional 1 or 2 months some clumps of tumor could be shaken loose. These free-floating, branching serpentine particles continued to grow and proliferate slowly in culture without any attachment to the flask or monolayer formation. When examined with a tissue culture microscope, a delicate halo of translucent mucus surrounding and evenly following the contour of each delicate particle was observed. The particles grew relatively slowly, compared to other (monolayer) colorectal cell lines, with a doubling time of about 2 weeks. During 7 years of continuous culture there has been a slow decline in the thickness of the external proteoglycan gel coating, which has become more opaque. Currently the cultured tumor particles have the appearance shown in Fig. 2.

Histology of RW-2982 Particles. Histological examination of RW-2982 particles showed cross-sections of a three-dimensional tumor coated with mucus (Fig. 3). The surrounding mucus gel stained intensely with Alcian blue indicating acid mucopolysaccharide content (not shown). The opaque nature of the external mucus gel in particles cultured continuously for several years is due to the presence of proteinaceous material and cellular debris (Fig. 3), which was not prominent in very early examinations. The decline in thickness of the mucus coat with time has been most dramatic with one RW-2982 subline which now consists of smaller particles due to a thinner coat of mucus (compare Fig. 4 with Fig. 3). The tumor formed s.c. in nude mice from injection of that subline of RW-2982 particles also demonstrated less mucus production than parental RW-2982 (not shown).

Histology of RW-2982 Nude Mouse Tumors. RW-2982 grown s.c., i.p., and in the spleen and liver of nude mice had the typical appearance of mucus-producing colorectal carcinoma. There were strips of malignant columnar epithelium surrounded by or adjacent to lakes of extracellular mucus. A typical pattern with clumps of serpentine tumor surrounded by mucus in a s.c. tumor is shown in Fig. 5. Goblet cells can be seen in some strips of epithelium. This appearance is very similar to the original patient tumor (compare Fig. 5 with Fig. 1). There was often little or no fibroblastic stroma in the nude mouse tumor. RW-2982 grown i.p. (not shown) appeared remarkably similar to cell blocks of the in vitro grown tumor particles (Fig. 3), except there was less proteinaceous debris within the mucus.

Mucus stains of nude mouse tumors showed intense Alcian blue staining of extracellular mucus with much less PAS staining (identical to the in vitro particles and original patient tumor).

Establishment of Transplantable RW-7213. The directly xen-
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Fig. 5. Tumor in nude mouse after injection of cultured RW-2982. Mucus-producing colonic adenocarcinoma (H & E, x 100).

Fig. 6. Transplantable RW-7213 in nude mouse (passage 6). Mucus-producing colonic adenocarcinoma with prominent fibroblastic stroma (H & E, x 100).

Fig. 7. Living particles of cultured RW-7213 showing translucent coat of mucus around delicate serpentine branching epithelial tumor (x 2.8).

Fig. 8. Living cultured particles of RW-7213 after 2 years in culture, with thick translucent coat of mucus. (Early RW-2982 had a similar appearance.) Background culture media contains dissolved Blue Dextran (Sigma) which is too large to diffuse into the mucus gel. The gel contains dead cells and refractile cellular debris (x 16).

This suspended mucus and cellular debris there were some small serpentine clumps of tumor tissue coated with a halo of mucus reminiscent of the particles of RW-2982. Consequently the suspended particulate material was retained and observed for growth. By about 7 months there appeared to be slight growth of a minority of the suspended tumor particles. Unquestionable substantial growth was noted by 9 months. By about 12 months from the date of original culture there was enough volume of tumor particles to begin to test the mucus-coated tumor particles, designated RW-7213, for histological appearance, ultrastructure, and tumorigenicity. The early growing RW-7213 particles had a relatively thick coat of proteoglycan (mucus) gel that was quite translucent (Fig. 7). Examination of these living particles with low magnification microscopy showed sharply outlined serpentine, branching solid tumor particles coated with insoluble pale mucus (Fig. 8). There was some variability in the thickness of the mucus halo among tumor particles and some flasks of continuously passaged particles have shown a decline in thickness, similar to the observed decline with the RW-2982 cell line.

Early Tumorigenicity Testing of Cultured RW-7213. In the first few weeks after placing RW-7213 in culture, when mucus-coated particles were first noted, some suspended material was injected i.p. into nude mice, since it had been noted that RW-2982 grew especially well in that location. However, these early ografted RW-7213 had grown well from the beginning s.c. in nude mice, and has been maintained as a serially transplantable tumor since August 8, 1984. New transplants are done about every 3 months, using groups of three to four mice. Histological sections of the transplantable RW-7213 tumor (Fig. 6) show typical mucinous colorectal carcinoma which is somewhat better differentiated than RW-2982 with more goblet cells. An interesting feature of the transplantable tumor is a prominent fibroblastic stroma (Fig. 6). This fibroblastic stroma was noted to be present in the RW-2982 and RW-7213 patient tumors, but is not observed as a component of tumors formed by injecting the cultured RW-2982 or RW-7213 tumor particles.

Establishment of Cell Line RW-7213. Although that portion of original tumor cultured without serum failed to survive beyond several months, success was achieved with the 10% FCS cultures. Within a week after tissue culture, small monolayer colonies of epithelial cells were noted. Over 1–2 months these colonies enlarged slightly but did not grow progressively. In the initial cultures of RW-7213 there was suspended, minced tumor with mucus and cellular debris present in the supernatant. The majority of the material was retained during weekly media changes by pipeting off the nonsedimenting material after brief gravity treatment. The rationale was that floating clumps of tumor cells might slowly attach to the plastic surface and form monolayer colonies. After a few weeks it was planned to discard this free-floating material. However, it was noted that within
nonproliferating clumps of RW-7213 failed to grow in the peritoneal cavity.

These same early RW-7213 suspended particles (tested at 6 months) were tumorigenic in nude mice s.c. but grew much more slowly than RW-7213 tested later (from about 14 months). Interestingly, the well-established RW-7213 tested first at 14 months now grows well i.p. in nude mice, similar to RW-2982, producing enlargement of abdominal girth due to extensive growth in the peritoneal cavity.

Selecting for High Mucus Variants of RW-7213. A simple method of maintaining RW-7213 particles with the thickest possible mucus gel is to periodically select for the high mucus variants in culture by separating them using a pipet. Our observation was that the high mucus particles grow more slowly and can be diluted out with time by the faster-growing particles with less mucus.

Comparison of Transplantable RW-7213 and Cultured RW-7213. Since with RW-7213, unlike RW-2982, we have maintained both a long term transplantable tumor in mice as well as a cell line, comparisons can be made between in vivo grown tumor and in vitro adapted tumor. One observation was that upon tissue culture of the transplantable RW-7213 tumor there is again a long adaptational period before noticeable growth in vitro. On two separate occasions it took 6 months for suspended tumor particles of RW-7213 from the transplantable mouse tumor to questionably begin to grow in vitro with definite growth by 9 months. This recapitulates the time span needed to adapt the patient RW-7213 tumor to definite in vitro growth. The transplantable RW-7213 might be useful as a model for studying the reasons for this long adaptation to tissue culture conditions. The use of the serum-free media supplements as reported by Murakami and Masui (14) were tried but did not facilitate adaptation to in vitro growth for the RW-7213 transplantable tumor.

Histology of RW-7213 Particles. Cell blocks of early RW-7213 particles showed an extracellular mucus gel occupying several times the volume of the enclosed solid tumor clumps (Fig. 9). Compared to late RW-2982, in particles of RW-7213 the mucus was more voluminous, more translucent, and less proteinaceous (compare Fig. 9 with Fig. 3). Examination of the solid tumor showed irregular serpentine tumor clumps only a few cells thick with generally columnar cells on the surface and containing numerous goblet cells (Figs. 10 and 11). Well-differentiated glandular structures were only rarely seen.

Histology of the RW-7213 Nude Mouse Tumors. Intrasplenic, s.c. or i.p. injections of RW-7213 particles into nude mice readily produced tumors. The histology was very similar to that of RW-2982, with clumps and strips of malignant colorectal epithelium embedded in or adjacent to a mucinous (proteoglycan) gel (Fig. 12). Sometimes there was more extracellular mucus than had been seen with RW-2982. Very little fibroblastic stroma was seen (Fig. 12). Stains for mucus showed intense staining with Alcian blue and minimal staining with PAS (not shown).

Electron Microscopy of Particles and Tumors. Ultrastructural examination of RW-2982 and RW-7213 particles and s.c. tumors from nude mice revealed similar appearances. Typical electron microscopic features of colonic carcinoma were seen with microvilli (Fig. 13) with dense cores of microfilaments extending into rootlets (Fig. 14) and abundant glycolcalceal bodies on the surface of cells (Fig. 14) and within the mucus gel. Well-formed goblet cells were seen within groups of columnar cells (Fig. 13) and junctional complexes between cells were noted (Fig. 14). Interestingly, groups of glycolcalceal bodies were seen within apparent secretory vesicles in the cytoplasm (Fig. 14). The extracellular mucus gel appeared to be a mixture of electron lucent goblet cell contents and glycolcalceal bodies (not shown).

Serum-Free Adaptation of RW-2982. RW-2982 was adapted over a period of about 1 year to grow in medium free of all serum, protein, hormones, and growth factors. The use of
bovine colostrum (12) as an initial serum replacement is probably not necessary and is cumbersome. An alternate method might be to gradually lower the amount of serum (15). The doubling time of RW-2982 was estimated to be 4 weeks, serum-free, and 2 weeks in 10% serum.

Assessment of Mucus Gel Coat. Histological sections of in vitro grown tumor particles demonstrated more mucus volume than epithelial cell volume (Figs. 3 and 9). The s.c. tumors from RW-2982 and RW-7213 grown in nude mice (Figs. 5 and 12) showed a ratio of mucus to epithelium of at least 3 or 4 to 1, with generally higher ratios for RW-7213 (Fig. 12).

Live-cultured particles of RW-7213 were examined microscopically and measured using an ocular micrometer. The solid central core of cohesive cells had an average width of 0.25–0.40 mm. The average thickness of the attached mucus gel coat was about 0.5 mm (with thinnest areas about 0.25 mm). The maximum width of the entire particles was 1.2–1.65 mm. This corresponds to the 1–2-mm size that tumors reach before they require a blood supply (16).

Micrometer measurements of histological sections of the particles revealed a maximum width of the total particles (solid tumor plus mucus) of 0.5–0.7 mm. The maximum width of the solid tumor within the particles was 0.15–0.20 mm. Thus, with fixation and histological processing there is up to 50% shrinkage of particles.

In attempting to darken the background of living particles for better photography, we found that trypan blue (M, 961) diffuses into the gel within minutes. However, blue dextran (M, 2,000,000) is too large to diffuse into the gel (Fig. 8).

CEA Determinations. Table 1 shows that high levels of CEA were found in the supernatant conditioned medium of both cell lines grown with or without serum. The highest level (5680 ng/ml) was found in conditioned medium from RW-7213.

Immunoperoxidase stains for CEA showed intense cytoplasmic staining in clumps of RW-2982 and RW-7213 cells of tumors grown in nude mice (not shown). The mucus was negative but the stroma around and within the tumor, and the surrounding fibroblasts, histiocytes, and neutrophils showed staining. This is probably due to both the heavy secretion of CEA causing staining of the stroma (17), and the cross-reaction to antigens in neutrophils and histiocytes (18).

We recently measured the CEA level in 7-day conditioned medium from high density cultures of RW-7213 that had continuously been in serum-free media for an entire year. These particles contained living cells, but no evidence of particle growth or cell division. Yet the 7-day conditioned media contained 8920 ng/ml CEA. This indicates that CEA is produced continuously in cultures without apparent cell division and does not require any serum components for synthesis.

Growth Rate of RW-2982 in Different Environments. RW-2982 was found to grow faster and attain a larger tumor mass after i.p. injection into nude mice as compared to s.c. injection.
A mean tumor weight of 7.25 g was attained i.p. compared with 0.83 g s.c. at 42 days (Table 2). This corresponds to an estimated doubling time of 3 days i.p. and 9 days s.c. In vitro, the volume doubling time for RW-2982 particles grown in 10% serum was estimated to be 2 weeks, with a 4-week doubling time under serum-free conditions.

Comparison of Properties of RW-2982 and RW-7213. RW-2982 and RW-7213 are very similar and have most properties in common. Some differences between the two cell lines may be a function of the different length of time each has been in continuous culture, which is since 1980 for RW-2982 and since 1984 for RW-7213. RW-7213 particles have a thicker, more translucent layer of mucus than RW-2982. The latter often has wider more opaque particles (Fig. 2) with more dead cells and cellular debris, often in a thinner layer of mucus (Fig. 3). But RW-2982 at a comparable age had an appearance similar to RW-7213. After injection into mice RW-7213 has a higher proportion of extracellular mucus (Fig. 12). To date RW-7213 has not shown long term adaptation to growth in serum-free medium. It will, however, readily survive for 2–3 months without serum, and high density cultures of RW-7213 particles have survived serum-free for as long as 16 months, but without visible proliferation. RW-7213 appears slightly better differentiated with more well-formed goblet cells and better differentiated strips of epithelium in vitro and in vivo. RW-7213 produces more CEA than RW-2982. Only RW-7213 secretes a factor that stimulates the growth of mouse stromal fibroblasts in culture (currently under investigation).

Growth in Spleen. In mice sacrificed shortly after splenic injection, histological sections of liver revealed clumps of mucus and particles of RW-2982 within portal vein branches, indicating immediate travel of some small particles into the liver via the splenic vein and portal vein branches (not shown). In a pilot study of growing RW-2982 in spleen, three of three primaries grew, but in each case one or more small nodules also developed in the liver at 1 month (Table 3). It was reasoned that by upward adjustment of the injected particle size by eliminating very small particles, fewer particles would enter the splenic vasculature and the result would be fewer immediate colonies growing in the liver. The identical experiment with particles washed to remove very small particles resulted in 60% fewer liver colonies at 1 month (Table 3). RW-7213 also grew readily in the nude mouse spleen and produced liver colonies (Fig. 15). Preliminary studies of RW-2982 particles injected into the tail vein of nude mice had not resulted in lung colonization.

DISCUSSION

The RW-2982 and RW-7213 cell lines, which grow in the form of delicate three-dimensional tumor particles coated with mucus, appear to be the first successful long term suspension cultures of the mucinous subtype of human colorectal carcinoma, which has a large amount of extracellular and extraglandular mucus. The reason other investigators have failed to establish a gross mucus-producing cell line from colorectal carcinoma is probably related to three unique features of this type of tumor. First, there is the previously unrecognized obligate nonadherent or suspended nature of the mucus-producing cells. Published classification systems for established colorectal cell lines have included only monolayer lines (3, 4). It is likely that cell culturists in the past have discarded the floating tumor particles along with the supernatant mucus and debris when monolayer colonies began to form in primary cultures of colorectal tumors. In fact, when initial cultures of the RW-7213 tumor resulted in some monolayer colony formation, supernatant mucus and debris was about to be discarded when particles similar to RW-2982 were noted. These particles were saved and observed and began to proliferate only after 6 months in culture to ultimately form the RW-7213 cell line. Second, methods that utilize enzymes to dissociate tumor minces to produce a single cell suspension prior to culture probably produce lethal damage to the mucus-producing cells. A third feature making mucinous colorectal carcinoma difficult to establish is the long time period needed for the tumor particles to adapt to tissue culture conditions and to begin to proliferate. The RW-2982 tumor (derived from a metastasis) had been in culture for 3 months before it was noted to be proliferating. An even longer adaptational period of 6 months was needed for noticeable proliferation of the RW-7213 line (derived from a primary tumor). Slow adaptation for cells capable of producing large amounts of mucus or CEA may be a general phenomenon since the LS174T monolayer line required 10 months for adaptation (7) and the SW-1116 line possibly a year in the same flask prior to successful passaging (3).

Although difficult and time consuming to establish in culture, the RW-lines are now very hardy and easy to grow and work with since they do not require frequent media change, are not

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Table 2 Comparative growth of RW-2982 in different sites in the nude mouse

<table>
<thead>
<tr>
<th>Treatment</th>
<th>S.c.</th>
<th>I.p.</th>
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<tbody>
<tr>
<td>Day 21</td>
<td>0.18 ± 0.02</td>
<td>0.45 ± 0.12</td>
</tr>
<tr>
<td>Day 28</td>
<td>0.33 ± 0.14</td>
<td>3.10 ± 1.02</td>
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<tr>
<td>Day 35</td>
<td>0.55 ± 0.25</td>
<td>6.46 ± 1.96</td>
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<tr>
<td>Day 42</td>
<td>0.83 ± 0.26</td>
<td>7.25 ± 2.02</td>
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* Mean ± SD.

Table 3 Effect of particle size on growth of RW-2982 in spleen and liver at 1 month

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breaking of clumps resulting in</td>
<td>3/3</td>
<td>3/3 (100%)</td>
</tr>
<tr>
<td>mixed particle size (no washing)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Removal of most small particles by washing</td>
<td>5/5</td>
<td>2/5 (40%)</td>
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**Fig. 15.** RW-7213 mucinous carcinoma growing in nude mouse liver 3 months after intrasplenic injection of cultured particles (H & E, x 25).
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adversely affected by acidic culture conditions, grow to high particle concentrations in each flask, and can be conveniently subdivided without trypsinization. These lines should prove useful to investigators who wish to study this subtype of colon cancer.

Until very recently, the only other published report of a cultured colorectal tumor physically and histologically resembling RW-2982 and RW-7213 was the Z516 organotypic culture (19). This tumor, grown for more than 10 years on chick embryo mesonephros before being discarded because of mycoplasma contamination, histologically appeared very similar to the RW lines (Fig. 17 of Ref. 19), with large amounts of extracellular mucus and production of detectable CEA. The recently described NCI-H498 mucinous colorectal tumor line appears to have properties similar to the RW lines (8). Another recent report (13) describes a new cell line (LIM1863) from colon carcinoma that grows as suspended organoids in culture (like the RW lines), but with no extracellular mucus gel.

The LS174T line has some properties in common with the RW lines, producing large amounts of CEA (7). Tumors produced in nude mice also show mucus production, but within large, well-differentiated glandular structures, without free lakes or pools of mucus as in the RW lines (20). Moreover LS174T is a monolayer line and does not produce much insoluble mucus in vitro, although recently high mucin variants of this line have been isolated and characterized (20). The mucus produced by LS174T stains intensely with both PAS and Alcian blue, unlike RW-2982 which principally stains with the latter stain, indicating mostly acid mucopolysaccharides.

The GW-39 tumor is a transplantable human CEA-producing colorectal mucinous tumor which has been maintained by Goldenberg in the cheek pouch of Syrian Golden hamsters (21). It has some features similar to the tumor produced by injecting RW-2982 and RW-7213 into nude mice. What is very interesting about the GW-39 tumor is that it will grow as a xenograft without conditioning (administering steroids or other immunosuppressives to the host). According to Goldenberg only two other human tumors grow in hamsters without conditioning and both also produce mucus (22). Apparently GW-39 has not been adapted to grow in vitro as a cell line except for relatively short term studies, where CEA production in vitro was reported to be 560 ng/ml after 26 days of culture without media change (23). The techniques used in establishing cell lines from the transplantable RW-7213 tumor (retaining the suspended particulate material and waiting up to 9 months for adaptation) may be useful in the future for establishment of a cell line from the interesting GW-39 transplantable tumor.

RW-2982 has been in continuous culture since 1980 and its organoid, suspended growth was initially regarded as a curiosity among colorectal cell lines. However, with the additional establishment of RW-7213 it is now apparent that suspended particulate growth is the obligate culture morphology for the gross-mucus-producing variant of colorectal cancer.

An important observation made with the RW cell lines is that the colorectal mucus secreted by this tumor type is insoluble and in the form of a gel with the inherent unique physical properties of gels (24). Because the mucus is a relatively transparent coat that stains with Alcian blue, and can be removed by trypsin digestion, it undoubtedly represents a three-dimensional gel structure of mucopolysaccharide covalently linked to protein, categorizing it as a proteoglycan as reviewed by Iozzo (25). The mucus gel completely surrounding the RW particles is apparently freely permeable to water and low molecular weight nutrients in solution. Colonic tumor mucus has the same basic proteoglycan gel structure (25) as does the proteoglycan gel making up the basement membrane of the glomerular filtration apparatus (26). The latter gel has been extensively studied and has been shown to have "pores" that exclude macromolecules above a certain molecular weight size and charge (26). The findings with trypan blue and blue dextran treatment indicate that the porous proteoglycan mucus gel surrounding the RW colorectal cells excludes macromolecules above a certain (presently undetermined) molecular weight size. The mucus gel surrounding mucinous colorectal carcinoma may possibly function as a physical barrier to immune effector cells such as macrophages and lymphocytes. The RW cell lines should be useful to investigators needing a source of relatively large quantities of colonic tumor mucus.

There are reports of the growth of human colon carcinoma cell lines in serum-free but hormone and growth factor supplemented culture medium (8, 14, 27). Besides providing its own three-dimensional tumor environment, the fact that RW-2982 will survive and slowly proliferate in medium devoid of all added protein, hormones, or growth factors suggests that the tumor particles have the ability to produce their own growth factors, if any are needed. It is possible that the RW-2982 cells growing without protein are a separate more primitive population, autonomous with respect to growth factor requirements. However, tumors formed from injecting the serum-free-adapted cells into nude mice show no difference in growth rate and have the same degree of histopathological and cytological differentiation and produce the same proportion of extracellular mucus as the parent line. This evidence along with the long adaptational period required and subsequent slow growth suggests that the RW-2982 cells have simply adapted to the protein-free environment. Since RW-7213 will not proliferate (but survives) in serum-free medium, it is perhaps a less primitive or better-differentiated cell type than RW-2982. This behavior correlates with the origin of RW-2982 from a metastasis and RW-7213 from a primary tumor.

Both RW-2982 and RW-7213 release large amounts of CEA into the culture medium. Both tumors stain intensely for CEA using immunoperoxidase methods. The serum-free RW-2982 subline is a potential source of relatively pure CEA for experimental and analytical purposes, since there is no exogenous protein present and the conditioned medium contains microgram quantities of CEA per milliliter. There appears to be a good correlation between mucus production and CEA production in colorectal cell lines or transplantable tumors since RW-2982, RW-7213, GW-39 (23), Z560 (19), LS174T (7), and NCI-H498 (8) all produce both mucus and CEA, often in large amounts.

Like most other human colorectal cell lines RW-2982 and RW-7213 readily produce tumors s.c. in nude mice. More unique for these mucinous lines compared to monolayer lines is the ability to grow rapidly in the peritoneal cavity of nude mice, producing several grams of only loosely adherent mucinous particulate material in several weeks. The physical appearance of this material closely approximates human pseudomyxoma peritonei in which there is also no invasion of the intraabdominal organs (28). Consequently RW-2982 and RW-7213 grown i.p. in nude mice may be a useful animal model for pseudomyxoma peritonei.

In addition to s.c. and i.p. growth of the mucinous cell lines, both RW lines also grow readily in the spleen of nude mice and produce liver colonies or metastases. The importance of models for studying liver colonization (one step in the multistep metastatic process) are obvious for colorectal cancer with its high
rate of and often exclusive metastasis to that organ. Although there are reports of the growth of human colorectal monolayer lines in nude mouse spleen (29) we were unable to induce HCT-8 or SW-480 to grow in that site despite multiple attempts. In this respect the RW lines are unique compared to some of the monolayer lines.

RW-2982 and RW-7213 both grow readily in the nude mouse liver, whereas neither of the patients from whom the tumors were derived developed liver metastases. A possible explanation is that these particulate lines have the ability to grow in the liver if placed directly with splenic injection via the splenic vein, but do not have the tendency to invade veins necessary to spontaneously reach the liver. This explanation is supported by the noninvasive character of the RW tumors grown i.p. and the lack of spontaneous hepatic metastasis under these latter conditions. An analogy can be made to pseudomyxoma peritonei, the most differentiated of mucinous carcinomas, where organs are not invaded and there are never any hepatic metastases (28).

Of all the human tumor cell lines in culture those of colorectal cells are among the hardiest or have some of the most aggressive properties experimentally. For example, as studied previously, colorectal cell lines will readily grow in the less severely immunosuppressed ATS-treated mice (30), and the GW-39 tumor will grow in hamsters without any immunosuppression (21). We have noted that the RW-2982 cell line will survive at room temperature for many days (11 days was the longest time tested). The ability of some colon cells to survive and proliferate in the hostile environment of the spleen with its population of immune and inflammatory cells is further evidence of the hardiness of malignant colorectal cells.

In summary, two cell lines have been established from the mucinous variant of human colorectal carcinoma. These lines have a unique physical appearance and secrete large amounts of CEA. It has been found that mucogenic colorectal carcinoma is tumorigenic in multiple sites in the nude mouse and appears to grow more readily and in more anatomic locations than some of the nonmucogenic cell lines. We are currently studying coinjected crude mucus preparations along with nonmucogenic colon carcinoma cells to see if the presence of mucus enhances tumorigenicity. Now that cell lines are available which can provide a convenient source of colorectal mucus, these and other studies can be designed to yield information about why mucus-producing colon tumors have a worse prognosis than the more common nonmucinous variant.

ACKNOWLEDGMENTS

We thank Debra Forde, Susan Ryan, Pamela Micheletti, Joseph Morrissette, Becky Melucci, and Karen Carreia for histological preparations and staining; Rocco Rosati for anatomical, technical, and photographic assistance; Craig Doremus for assistance in some of the nude mouse spleen injections; Jeanne Whitehead for cytology preparations; Janet Butmarch and Christine Ross for immunoperoxidase stains; Grant Jolly, Gary Hudson, and Audrey Manksy for technical assistance in electron microscopy and photography; Dr. George Coleman for follow-up information on his patient; and Janice Burnett and Marea Tumber of the RWGH Cancer Center Animal Care Facility. We are particularly grateful to Drs. Charles J. McDonald and Joseph C. Alper for providing space in their laboratory over the past decade.

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