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Development and characterization of a clinically useful animal model of epithelial ovarian cancer in the Fischer 344 rat

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OBJECTIVE: Our purpose was to develop and characterize a spontaneously arising, nonimmunogenic experimental animal model of epithelial ovarian cancer.

STUDY DESIGN: NuTu-19 is a cell line derived from a poorly differentiated adenocarcinoma formed in a female athymic mouse after subcutaneous injection of spontaneously transformed Fischer 344 rat ovarian surface epithelial cells. This cell line was injected intraperitoneally into naive, immunocompetent Fischer 344 rats to determine tumor growth and animal survival. Immunogenicity of this cell line was determined by repetitive vaccination of naive rats with either mitomycin C–treated or irradiated (5000 cGy) NuTu-19 cells, followed by intraperitoneal rechallenge with viable tumor cells. Kaplan-Meier survival analysis was used to analyze survival data. Major histocompatibility complex class I and class II and intercellular adhesion molecule-1 cell surface antigens were determined by fluorescence-activated cell sorting analysis.

RESULTS: NuTu-19 cells injected intraperitoneally grew progressively as numerous serosal nodules (peritoneum, omentum, diaphragm, liver, bowel), exhibited local tissue invasion and formed malignant ascites in a manner typical for human ovarian epithelial carcinomas. Animal survival was dosage dependent where as few as 10^4 cells were fatal when introduced intraperitoneally; mean animal survival was noted to be approximately 49 days when 10^6 cells were injected intraperitoneally. Repetitive immunizations of animals with large doses (10^7) of inactivated NuTu-19 cells did not confer immunity to the animals, which all died on subsequent challenge with viable parental tumor cells. NuTu-19 cells expressed high levels of major histocompatibility complex class I and intercellular adhesion molecule-1 cell surface antigens and very low levels of major histocompatibility complex class II antigens.

CONCLUSION: This is the first report of a reliable, spontaneously arising, nonimmunogenic epithelial ovarian cancer animal model. Because this model exists in an immunocompetent animal, it will be useful for studying the biologic and immunologic features of ovarian cancer. (Am J Obstet Gynecol 1996;175:593-9.)

Key words: NuTu-19, ovarian neoplasm, ovarian cancer, animal model, immunogenicity, Fischer 344

Ovarian carcinoma remains the fourth most frequent cause of cancer death in women in the United States and

Europe.[1] Although the ratio of incidence to survival has improved for most gynecologic malignancies, efforts to improve survival have been painfully slow with respect to ovarian cancer. Whereas improved patient awareness and accessibility of the cervix and endometrium to screening and diagnostic tests have resulted in detection of disease at an earlier stage, this has not been the case for epithelial ovarian cancers. In this latter disease there are no reliable signs, symptoms, or screening methods for its early detection. Evidence of this is indicated by the fact that 60% of patients with ovarian cancer are initially diagnosed with advanced disease (i.e., stage III or IV).[2] Hence the important goals of ovarian cancer research are to define approaches to prevent the disease, diagnose it at an earlier stage, and treat it more effectively. In efforts to achieve

these goals, one approach has been to develop model systems so that issues such as the behavior of early disease and response to experimental chemotherapy and immunotherapy can be examined. Currently available systems range from in vitro models such as ovarian cancer cell lines to several animal models. These models include (1) human ovarian cancer xenografts,[3] (2) an immunogenic chemically induced rat ovarian tumor,[4] (3) a germ cell tumor model,[5] and (4) models of nonovarian tumors injected intraperitoneally.[6] Although each of these models has applications in medical research, they are clearly not ideal for the study of the immunologic response to therapy and the biologic features of the most frequent form of ovarian cancer. In this report we describe a model where the tumor shows low immunogenicity in the presence of a syngeneic intact immune system, as is typical of clinical human ovarian cancer.

Material and methods

Cell line. The derivation and in vitro characterization of NuTu-19, a Fischer 344 rat-derived epithelial ovarian carcinoma cell line, is described in detail elsewhere.[7] Briefly, NuTu-19 was initiated from a poorly differentiated adenocarcinoma that arose in a female athymic mouse after injection of Fischer 344 ovarian surface epithelial cells that spontaneously underwent malignant transformation in vitro. Trypsinized Fischer 344 rat ovarian surface epithelial cells were cultured and allowed to spontaneously divide in vitro with serial passage. After in vitro malignant transformation and characterization, several cell lines were cloned and injected subcutaneously into nude mice. The Fischer 344 rat ovarian adenocarcinoma cell line NuTu-19 was derived from one of these nude mouse tumors.[7] NuTu-19 cells are growth factor independent, have been previously shown to be tumorigenic only in nude mice, and exhibit a doubling time of approximately 15 hours in vitro. The cells are maintained in complete media consisting of RPMI 1640 (Gibco Life Technologies, Grand Island, N.Y.) with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabassas, Calif.) at 37° C and 5% carbon dioxide. After initial passage through the Fischer 344 rat, the cell line was expanded and cryopreserved in liquid nitrogen (10^7 cells per vial) after testing negative for *Mycoplasma* contamination with a Mycotest kit (Gibco). Each experiment was performed by thawing a vial of cells and expanding them biweekly to generate the appropriate cell number.

Animals. Pathogen-free Fischer 344 female rats (100 to 125 gm) were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.) and housed in a pathogen-free animal facility at the University of California, Irvine. They were given commercial basal diet and water ad libitum. The experimental protocol for the use of these animals for these studies was approved by the Institutional Labora-tory Animal Care and Use Committee at the University of California, Irvine.

Determination of in vivo growth and tumorigenicity. NuTu-19 cells were harvested with 0.25% trypsin with ethylenediaminetetraacetic acid (EDTA) (Gibco), washed twice with Dulbecco's phosphate-buffered saline solution (Gibco), counted for cell number and viability with trypan blue exclusion, and injected intraperitoneally into groups of Fischer 344 rats at concentrations of 10^7, 10^6, 10^5, or 10^4 cells/ml of phosphate-buffered saline solution. Cell viability of at least 90% was required for experimental use. The animals were then observed twice daily and weighed weekly, and survival was monitored. An additional set of 12 animals were injected intraperitoneally (10^6 NuTu-19 cells) and then at different time intervals killed for necropsy and pathologic analysis. Two animals underwent pathologic analysis weekly for weeks 3 to 6. The remaining animals were killed and examined just before they died of intraperitoneal carcinomatosis and malignant ascites.

Determination of immunogenicity. To inactivate cell growth, NuTu-19 cells were harvested as above, treated with mitomycin C (0.05 mg/10^7 cells) for 1 hour at 37°, and washed twice in phosphate-buffered saline solution or irradiated with 5000 cGy (200 cGy/min) with a cesium 137–labeled source. Cells were then counted and resuspended in phosphate-buffered saline solution at a concentration of 10^7, 10^6, 10^5, and 10^4 cells per 100 µl. A cell viability count of ≥90% was required for experimental use. The mitomycin C–treated or irradiated cells were then injected subcutaneously (left flank) into groups of Fischer 344 rats on days 0 and 14. No tumor growth was noted at the subcutaneous injection site. This was followed by intraperitoneal challenge with 10^6 viable NuTu-19 parental cells (10^6 cells/ml of phosphate-buffered saline solution) on day 28. The animals were then followed up for survival.

Determination of cell surface antigen expression. Cells were harvested with 0.25% trypsin with EDTA, washed twice with phosphate-buffered saline solution, and resuspended in phosphate-buffered saline solution into 12×75 mm polystyrene tubes (Fisher Scientific, Pittsburgh) at 10^5 cells/100 µl per tube. The cells were then pelleted by centrifugation at 1000 revolutions/min for 5 minutes at 4° C. All phosphate-buffered saline solution was removed, and 10 µl of fluorescein isothiocyanate–labeled mouse antirat monoclonal antibodies (anti–major histocompatibility complex [MHC] class I [MCA 51F, Serotec, Washington, D.C.], anti-MHC class II [MCA 46F, Serotec]), and anti–intercellular adhesion molecule [ICAM-1] [MCA 773F, Serotec]) diluted in cold phosphate-buffered saline solution–0.5% sodium azide was added to the tubes in a total volume of 110 µl. Titration studies revealed that optimal cell surface staining was attained by diluting the anti-MHC class I and anti–
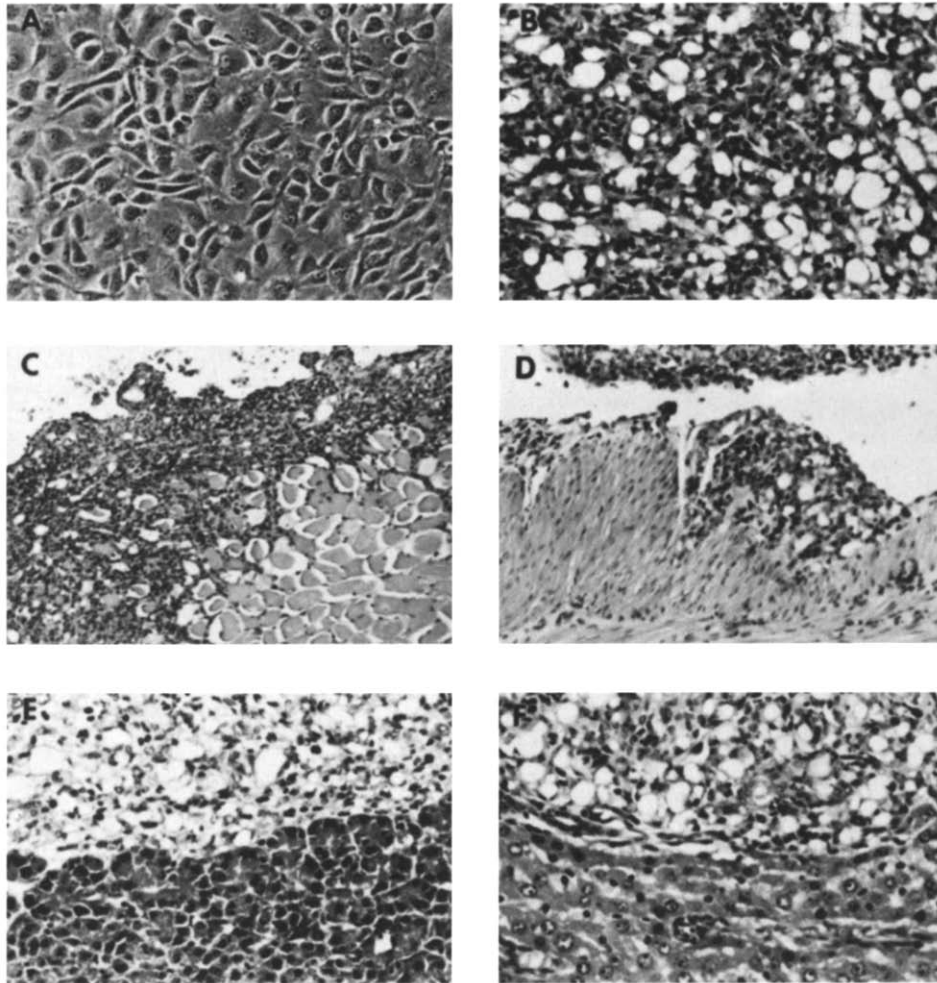
Fig. 1. **A,** Microscopic appearance of the NuTu-19 cell line in vitro. **B to F,** Microscopic appearance of NuTu-19 tumor implants on intraabdominal organs. **B,** Omentum. **C,** Diaphragmatic implant with papillary projections. **D,** Small bowel peritoneum. **E,** Pancreas. **F,** Liver serosa.

ICAM-1 antibodies 1:10 and the anti-MHC class II antibody 1:80. Fluorescein isothiocyanate–labeled mouse antihuman MHC class I (Becton-Dickinson, San Jose, Calif.) was used as an isotype control and was used diluted 1:10 with phosphate-buffered saline solution–0.5% sodium azide. The tubes were incubated for 45 minutes at 4° C, agitated every 15 minutes, washed twice with phosphate-buffered saline solution–2% fetal bovine serum, repelleted, and then resuspended in 200 µl of 1% paraformaldehyde. The tubes were sealed with parafilm, covered with foil, and maintained at 4° C until they were analyzed with a fluorescence-activated cell sorter (Becton-Dickinson) with a 15 mW argon laser with an excitation of 488 nm. Fluorescent signals were gated on the basis of forward and right-angle light scattering to eliminate dead cells and aggregates from analysis. Gated signals (10^4) were detected at 585 bandpass filter and analyzed with Lysis II software. Values were expressed as mean channel fluorescence intensity and percentage of positive cells.

Statistical analysis. Kaplan-Meier survival analysis was used to determine significance from survival data.

Results

Determination of in vivo growth and tumorigenicity (Figs. 1 to 3). NuTu-19 grows in vitro in a typical epithelial, cobblestone-like monolayer (Fig. 1, *A*). The cells are large and polygonal and exhibit atypical hyperchromatic nuclei with a high nucleus-to-cytoplasmic ratio. In our experience the doubling time under our in vitro growth conditions was approximately 15 hours, and we routinely obtained approximately 10^7 cells from a single confluent T150 tissue culture flask. Interestingly, NuTu-19 cells exhibit marked contact growth inhibition in vitro, and confluent flasks can be maintained at 37° C for several weeks without loss of cell viability.

In spite of the contact inhibition noted in vitro, when injected into the peritoneal cavity of the female Fischer 344 rats, the tumor cells grew progressively as numerous

Fig. 3. Survival of Fischer 344 female rats after intraperitoneal injection of viable NuTu-19 tumor cells. Animals were injected with 10^7, 10^6, 10^5, or 10^4 cells on day 0.

Fig. 2. Typical necropsy specimen from Fischer 344 rat after intraperitoneal injection of 10^6 viable NuTu-19 cells. Note large omental tumor masses (large arrow) and serosal implant on greater curvature of stomach (small arrow).

serosal nodules adherent to virtually all intraabdominal organs (peritoneum, omentum, diaphragm, bowel, liver, pancreas, spleen) and exhibited the capacity for local tissue invasion and formation of malignant ascites. Tumors first appeared grossly on the omentum by the third week as small nodules (1 mm) and continuously grew to form a confluent omental mass by the time the animals died (Fig. 2). Necropsies revealed massive hemorrhagic ascites, measuring up to 70 ml (containing 1 to 3×10^6 viable tumor cells/ml) and numerous tumor nodules, measuring 1 to 6 mm in diameter, studding the entire peritoneal surface and implanting the serosa of virtually all intraabdominal organs.

Microscopically, the intraperitoneal tumors grew as solid sheets and closely packed cords (with papillary projections of poorly differentiated adenocarcinoma cells that locally invaded underlying tissues (Fig. 1, B to F). Secretory products containing abundant hyaluronic acid (a characteristic of serous tumors) were evident in the cells and surrounding tissues. Desmoplastic reactions to locally invading tumor cells were minimal, as was histo-
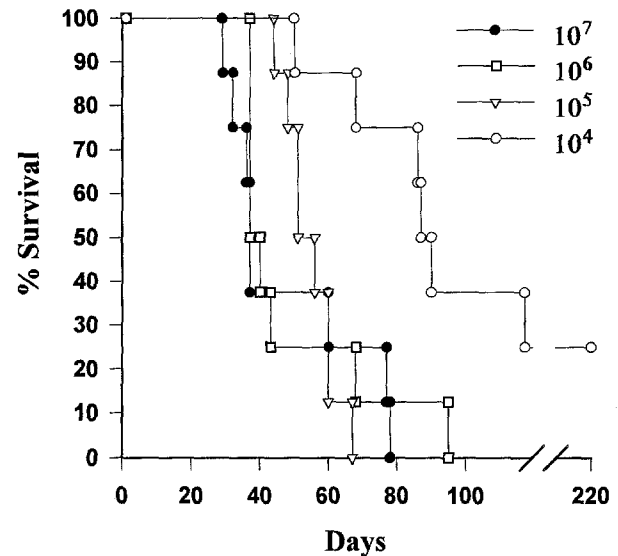
logic evidence of host inflammatory responses, which were virtually absent.

When injected intraperitoneally, as few as 10^4 NuTu-19 cells were fatal in 70% of the animals, whereas $\geq 10^5$ cells were fatal in 100% of the animals. Mean survival was found to be 54.6, 49.3, and 48.3 days for 10^5, 10^6, and 10^7 cells, respectively, and there was no statistically significant difference in the survival of these three groups (Fig. 3). At all dosages animals had gradual anemia with profound cachexia and ultimately died as a result of hemorrhagic shock from massive hemorrhagic ascites and intraperitoneal carcinomatosis. Although serosal implants were seen on the surface of the liver, no parenchymal metastases were found in the lung, liver, and brain.

Determination of immunogenicity (Fig. 4, A and B). Repeated immunizations of animals with various doses of mitomycin C–inactivated NuTu-19 cells (ranging from 10^4 to as high as 10^7 cells) did not confer immunity to the animals. Animals receiving the largest vaccine dose of 10^7 cells, which would be expected to be the strongest immunizing dose, all died of intraperitoneal carcinomatosis after intraperitoneal challenge with viable NuTu-19 cells, with a mean survival of approximately 40 days. There was no statistically significant difference in the survival of the animals injected with 10^5, 10^6, or 10^7 mitomycin C–inactivated cells because all died (after subsequent) challenge, with mean survival times of 42.2, 44.1, and 40.6 days, respectively (Fig. 4, A).

Repeated injections with 10^7 irradiated cells also did not have an impact on the survival of animals subsequently challenged with Nu-Tu-19 cells because all died of intraperitoneal carcinomatosis, with a mean survival of 45.5 days. There was no statistically significant difference in sur-
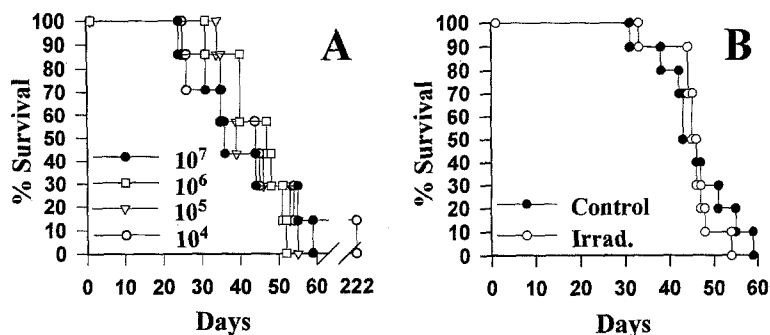
Fig. 4. A, Survival of Fischer 344 female rats challenged with 10^6 viable NuTu-19 tumor cells after repeated injections with graded doses of mitomycin C–treated NuTu-19 cells. Animals were injected twice (days 0 and 14) with graded doses of the inactivated tumor cells (10^7, 10^6, 10^5, 10^4) before challenge on day 28. B, Survival of Fischer 344 female rats injected on days 0 and 14 with 10^7 irradiated NuTu-19 tumor cells and challenged with 10^6 viable NuTu-19 tumor cells on day 28.

Table I. Cell surface antigen expression on NuTu-19 ovarian cancer cells

Cell line	Isotype control	MHC class I	MHC class II	ICAM-1
NuTu-19	94	425	151	547

Antigen expression was determined by flow cytometry; values represent mean channel fluorescence intensity.

vival between this subset of animals and the control animals, which received 10^6 viable NuTu-19 cells intraperitoneally and had a mean survival of 45 days (Fig. 4, B).

Determination of cell surface antigen expression (Table I). As can be seen in Table I, NuTu-19 cells expressed high levels of MHC class I and ICAM-1 cell surface antigens (mean channel fluorescence intensity of 425 and 547, respectively, vs isotype control of 94). MHC class II antigens were also expressed but to a much lesser degree (mean channel fluorescence intensity 151).

Comment

Ovarian carcinoma remains the fourth most frequent cause of cancer death in women in the United States and Europe and carries with it a disproportionately high mortality rate compared with other female pelvic cancers. Because patients with early-stage disease have no symptoms, in combination with inadequate screening methods, this disease is rarely detected at an early stage when it is consistently curable by standard surgical techniques. This is supported by the fact that up to 80% of patients with epithelial ovarian cancer are initially diagnosed with advanced-stage disease. We hypothesize that study of a model that closely mimics clinical ovarian cancer might provide insights into the diagnosis, treatment, or prevention of this disease more effectively.

We believe the model we present here has many advantages over existing ovarian cancer models. Approximately 1 in 70 women (14/1000) will have ovarian cancer at some time in her life. Many different species of domesticated animals and commonly used research animals[8-10]

have been reported to spontaneously form both benign and malignant ovarian tumors, but their incidence is generally very low, making their use for research purposes nearly impossible.[11] For example, the Fischer 344 rat has benign and malignant ovarian neoplasms at rates of 2 per 1000 and 3 per 1000,[9, 12, 13] whereas the rates in the Sprague/Dawley rat are reported as 23 per 1000 and 4 per 1000, respectively.[10] The exception to low incidence is the Osborne/Mendel rat in which benign granulosa cell ovarian tumors will spontaneously develop in one third of the animals by 18 months of age.[14] Stromal tumors (specifically granulosa cell tumors) are the most common histologic type of ovarian cancer that spontaneously occurs in laboratory animals.[8-10, 12] In contrast, 80% to 90% of all human ovarian cancers are histologically of epithelial origin, whereas only 3.3% of Fischer 344 rat ovarian carcinomas are derived from ovarian surface epithelial cells.[9, 12, 13] Furthermore, the most common form of human epithelial ovarian carcinoma, papillary serous adenocarcinoma, is found in only 0.13 of 1000 Fischer 344 rats.[9, 12] Given the rarity of the disease in general and this histologic cell type specifically in laboratory animals, it is easy to understand the difficulty in generating an adequate, reliable, reproducible animal model analogous to human epithelial ovarian cancer.

As an alternative to spontaneous tumor models, several animal models attempting to mimic human ovarian cancer have been developed. These include (1) human ovarian tumors xenografted into nude mice,[3] (2) chemically induced rat ovarian tumors,[4] (3) transplantable murine germ cell tumors,[5] and (4) models of nonovarian tumor

cells injected intraperitoneally so as to mimic ovarian cancer.[6] Although the human tumor–nude mouse xenograft model has shown utility for drug screening, the nude mouse lacks a competent immune system and hence is inappropriate for studies in which an intact immune system would be necessary. Moreover, the growth of human tumors in nude mice may obviate important physiologic interactions such as growth factor dependency or immune cell responses because of the xenogeneic-immunodeficient nature of the model. This may have a significant impact in the natural progression of the disease and the response to experimental therapy. One approach to circumvent some of these problems has been the development of a chemically transformed (with 7,12-dimethylbenzanthracene) ovarian carcinoma cell line. These cells, however, are highly immunogenic and do not grow in immunocompetent adult animals but rather in neonatal rats or in adult rats rendered partially immunodeficient by sublethal irradiation. The immunogenicity exhibited by these cells and of the mouse teratoma system also used in ovarian cancer research along with the germ cell histology of the latter make them less-ideal syngeneic models of ovarian cancer.[11]

In contrast to other models, the NuTu-19 in vivo ovarian cancer animal model we describe here resembles clinical ovarian cancer in many important ways. This tumor arises from the appropriate cell type and histologically is described as a poorly differentiated papillary serous ovarian adenocarcinoma. This is the most frequent histologic subtype of clinical ovarian cancer. The process of malignant transformation was achieved spontaneously apparently as the result of repetitious growth of the rat ovarian surface epithelial cells in vitro. This process in vivo (associated with ovulatory repair) has long been hypothesized to contribute to initiation of ovarian cancer in humans.[15] Furthermore, the transplantable tumor grows progressively in adult animals in a pattern consistent with clinical ovarian cancer and results in death from disease complications in a manner similar to the human disease.

Perhaps one of the most important features of this new syngeneic model of ovarian cancer is the weak immunogenicity of the tumor cells. The immunogenicity of human epithelial ovarian carcinomas has not been well studied. It is felt, however, that these spontaneously arising human tumors, which may or may not express tumor-associated antigens, resemble weakly or nonimmunogenic spontaneously arising animal cancers as opposed to highly immunogenic chemically or virally induced animal tumors that express oncofetal antigens.[11, 16] Hence, as opposed to the former, the latter highly immunogenic tumors can often be prevented or cured by various immunotherapy strategies.[11, 16] Some of the mechanisms that may render human ovarian cancers nonimmunogenic and capable of escaping recognition by the host immune

system involve down-regulation of the expression of tumor-associated cell surface antigens,[17] MHC class I and II molecules,[18] costimulatory and adhesion molecules such as B7 or ICAM-1,[19] or the production of immune-blocking factors.[20] Studies investigating the immunogenicity of animal tumors have involved attempted vaccination of animals with irradiated or mitomycin C growth-arrested cells, followed with parental cell challenge.[21] Investigators have immunized with as few as 10^5 cells to establish a cell line's immunogenicity. Here, we demonstrate that NuTu-19 is essentially nonimmunogenic because challenge with viable parental cells after attempted immunization with two injections of up to 10^7 cells failed to alter tumor progression.

In humans, MHC class I, class II, and ICAM-1 cell surface antigens are intricately involved in the host cell-mediated immune response. MHC class I antigen is expressed on all normal ovarian surface epithelium and on 80% of epithelial ovarian cancers, whereas MHC class II antigen is expressed on 16% to 40% of epithelial ovarian cancers and is not expressed on normal ovarian tissue.[22] In experimental animal models of tumors other than ovarian cancer, the expression or overexpression of MHC class I,[23] class II,[24] and ICAM-1[25] cell surface antigens enhanced the immunogenicity of tumor cells. NuTu-19 expresses high levels of MHC class I and ICAM-1 and low levels of MHC class II antigens. Because MHC class II may be linked to the inherent immunogenicity of a tumor, the low expression of MHC class II antigen on NuTu-19 cells may in part explain the poorly immunogenic nature of this tumor.

In summary, we believe this to be the first report of a syngeneic animal model of epithelial ovarian cancer that displays the low immunogenicity typical of clinical ovarian cancer. With this model it may now be possible to study various biologic features of and host therapeutic response to ovarian cancer in an immune-competent host.

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