Increased Tumorigenicity and Resistance to Antibody Lysis of Human Colon Tumor Cells Xenografted in Congenitally Athymic Mice

M. B. Tompkins, G. V. Rama Rao, and W. A. F. Tompkins

Center for Zoonoses and Comparative Medicine, College of Veterinary Medicine, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Congenital athymic mice are frequently used as in vivo culture systems for establishing human neoplastic cells for a number of different types of studies. This study reports that growth of human tumor cells in athymic mice may alter the biological and immunological properties of the cell line. Human colon tumor cell lines HCT-8R and HT-29 were grown in nude mice and explanted back into culture. These nude mouse-derived sublines were designated HCT-8R Nu1 and HT-29 Nu1. The nude mouse tumor-forming doses of the Nu1 sublines were 10- to 100-fold lower than the original cell lines. Using monoclonal antibodies to carcinoembryonic antigen in a complement-dependent 51Cr cytotoxicity assay, the Nu1 sublines were markedly more resistant to lysis than were the original cell lines. A quantitative 125I-anti carcinoembryonic antigen-binding assay revealed no significant difference in carcinoembryonic antigen on the surface of original cell lines and the Nu1 sublines. Nu1 sublines were also more resistant than the original cell lines to complement-dependent lysis by heterologous antisera against viable HCT-8R and HT-29 cells. Lack of correlation was observed between adsorption of anti-HCT-8R antibodies by HCT-8R and HCT-8R Nu1 and their respective susceptibility to antibody lysis. These results suggest that the nude mouse exerts some immunological and nonimmunological influences on xenografted tumor cells resulting in a cell population that is more tumorigenic and more resistant to antibody lysis. These modifications in the properties of Nu1 sublines do not appear to be related to changes in antigenic expression per se on the cell surface.

INTRODUCTION

The introduction of the athymic "nude" mouse as a host for xenografting tumors has provided a valuable model for the study of human neoplasms. Many investigators have demonstrated successful growth of tumor tissue fragments and established tumor cell lines in nude mice (7, 12, 13, 21, 22, 25). However, despite the absence of T-dependent immune responses in athymic mice, they appear to be able to influence the growth of grafted cells to some degree. Thus, different tumors show variable rates of growth, and certain malignant cells fail to grow in nude mice (6, 13, 14). Furthermore, tissue culture cell lines of normal origin capable of indefinite growth in vitro fail to grow in nude mice even when exceptionally large numbers of cells are injected (7, 21, 22). These observations indicate that nude mice are able to distinguish neoplastic cells from normal cells. While the mechanism(s) operative in tumor graft recognition in nude mice is not known at this time, there are data to suggest it is immunological. Prehn and Outzen (14) showed that syngeneic tumors of high immunogenicity grew better in irradiated adult or untreated newborn athymic mice than in control adult athymic mice. These authors suggested growth of immunogenic tumors in nude mice may be due to an overloading of a weak immune system. No evidence of immunological memory to the tumor antigen could be demonstrated in these mice. Campanile et al. (3) recently demonstrated a strong transplantation resistance in nude mice to allogeneic lymphomas. In contrast to the studies by Prehn and Outzen (14), this refractoriness to tumor growth was radioresistant.

The suggestions that athymic mice exert some immunological or nonimmunological restrictions on a variety of cell types are supported by recent observations in our laboratory that human colon tumor cells grown in nude mice were more tumorigenic on subsequent in vivo passages (18). It is possible that, even in the cases of tumors that grow readily in nude mice, some selective pressures are exerted by the host which may result in alterations in the phenotype and/or genotype of the cell population. The results of this study confirm our earlier observations that human colon tumor cells grown in nude mice are more tumorigenic on the second nude mouse passage and show that the nude mouse passage cells are more resistant to lysis by antibody and complement than the original cell lines.

MATERIALS AND METHODS

Cell Cultures. HCT-8R, a cloned line of human colon carcinoma cells, have been described previously (18). HT-29, a line of human colon carcinoma cells, were obtained from J. Fogh (Sloan Kettering Institute, N. Y.). HEI-407 is a line of human embryonic intestine cells (Grand Island Biological Co., Grand Island, N. Y.). The nude mouse-passaged tumor cells (Nu1 sublines) were produced as described previously (18). Briefly, young adult (8 to 12 weeks) female homozygous nu/nu mice were given s.c. injections of 1 x 10^6 viable cells. After 3 to 4 weeks of growth, tumors were surgically excised, minced, and dispersed with 0.25% trypsin. The cultures established from these tumors were designated HCT-8R Nu1, HT-29 Nu1, and HEI-407 Nu1. HCT-8R, HT-29, and HEI-407 cells at the same passage level used to inoculate nude mice were frozen and Nu1 counterparts at equivalent in vitro passage levels. The cultures established from these tumors were designated HCT-8R Nu1, HT-29 Nu1, and HEI-407 Nu1, HCT-8R, HT-29, and HEI-407 cells at the same passage level used to inoculate nude mice were frozen and stored in liquid nitrogen. The frozen cells were reestablished in culture at the time the tumors were excised from nude mice. This allowed us to compare the original tumor cells and their Nu1 counterparts at equivalent in vitro passage levels. In all cases, except where noted, the Nu1 sublines were established from tumors grown in a RNC strain of nude mice. Cytogenetic analysis was performed on all Nu1 sublines as described previously (18). All Nu1 cell lines possessed the characteristic human chromosomes of the parental line, and no mouse chromosomes were observed. All cultures were grown in Roswell Park Memorial Institute Tissue Culture Medium 1640 supple-
mented with 10% FBS, 3 penicillin (100 units/ml), streptomycin (100 µg/ml), and 0.03% L-glutamine and buffered with 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 0.75% NaHCO₃.

Congenital Athymic "Nude" Mice. The RNC strain of athymic mice was originally obtained from Dr. P. Mintz (Guelph University, Guelph, Ontario, Canada). Croy and Osoba (4) developed the RNC as a new inbred strain of mice by repeated inbreeding of mice heterozygous for the nude mutation (obtained from the Institute of Animal Genetics, Edinburgh). The resulting RNC strain is heterozygous for the nu gene and fixed at both the H-2 and M loci (H-2ª and M 1). The BALB/c genotype athymic mice were obtained from Sprague-Dawley. Homozygous nude males were raised by nu/nu male and nu/+ female mating in our laboratory. Mice were bred and maintained in a vertical flow HEPA filtered air room (Bionclean Laminar Air Flow Enclosure, Fieldstone Corp., Cincinnati, Ohio). During the course of this study there was no evidence of overt infections, and there were very few unaccountable deaths in our nude mouse colony.

Titration of Tumor-forming Dose in Nude Mice. The tumor-forming dose of HCT-8R, HT-29, and their Nu1 sublines was determined by s.c. inoculation of cells into 8- to 12-week-old female RNC nude mice. Tumor development was observed for 90 days after inoculation.

Antibody to CEA. CEA was extracted and purified from liver metastases of human intestinal carcinoma as described by Krupey et al. (8). Partially purified CEA was injected into goats (10 µg/injection) or horses (100 µg/injection) in incomplete Freund's adjuvant. Antiserum showing strong reactions with CEA by double diffusion in agar were adsorbed with glutaraldehyde-polymerized normal liver, colon, and plasma as described (2). Adsorbed antiserum which showed no reaction with normal tissue extracts were purified on a column of CEA coupled to CNBr-treated Sepharose as follows: 15 mg of purified CEA were coupled to CNBr-treated Sepharose and poured into a 1- x 10-cm column; 5 ml of CEA serum were loaded onto the column and incubated overnight at 25°C; the column was washed with 0.15 M borate buffer, pH 8.4, until no further protein was eluted as determined by absorbance at 280 nm; the anti-CEA antibodies were eluted by passage of 0.1 M glycine-HCl buffer at pH 2.8; the fractions were pooled, dialyzed 7 days against 0.15 M borate buffer and against distilled H₂O for 24 hr, and concentrated by lyophilization.

Labeling of Anti-CEA with 125I. Specifically purified goat anti-CEA and normal goat IgG were radiolabeled with 125I using the Bolton-Hunter reagent (New England Nuclear, Boston, Mass.). In this procedure, 0.125 mg of anti-CEA in 0.1 ml of 0.15 M borate buffer at pH 8.4 was reacted with dried Bolton-Hunter reagent containing 500 µCi of 125I. The reaction was allowed to proceed for 15 min at 0°C. The mixture was then reacted with 0.05 ml of 0.2 M glycine in 0.1 M borate buffer, pH 8.5, for 5 min at 0°C. The 125I-labeled protein was separated on a Sephadex G-50 (fine grade) column equilibrated with 0.15 M borate buffer, pH 8.4. One-ml fractions were collected and counted, and the data were plotted to determine the elution pattern. The 2 fractions of eluted immunoglobulin with the greatest number of counts were pooled. The total amount of protein was determined by absorbance at 280 nm, and the specific activity expressed as µCi 125I per µg of protein. Greater than 90% of the protein was recovered from the columns in each case. The specific activity of the recovered IgG ranged from 3 to 5 µCi/µg IgG.

Antisera to Whole Cells. Inbred LHC-Lak hamsters (Charles Rivers Lakeview Hamster Colony, Wilmington, Mass.) were immunized to HCT-8R or HT-29 cells by 3 i.p. injections at 10-day intervals of 1 x 10⁷ trypsin-EDTA dispersed cells. Antisera were collected 10 days after the 3rd injection, heat inactivated at 56°C for 30 min and stored at -20°C.

Adsorption of Anti-Whole-Cell Antisera. One ml of heat-inactivated hamster anti-HCT-8R serum diluted 1:10 in PBS was adsorbed with variable numbers of HCT-8R or HCT-8R Nu1 cells for 1 hr at room temperature. The suspensions were then centrifuged at 5000 x g for 15 min at 4°C, and the supernatants collected for antibody assay.

Complement-dependent Antibody Cytotoxicity Assay. Target cell damage due to antibody plus complement was assessed by release of 51Cr (sodium chromate) from labeled cells as described by Tompkins et al. (25). Briefly, monodispersed 51Cr labeled cells were washed 4 times in PBS plus 2% heat-inactivated FBS and adjusted to a concentration of 5 x 10⁵ viable cells/ml. One-tenth ml of cells was dispensed into 12- x 75-mm glass tubes and mixed with an equal volume of antisera at the dilutions to be tested. After 1 hr incubation at 37°C, 0.2 ml of guinea pig complement diluted 1:8 in PBS was added, and the tubes were incubated for an additional 90 min. At the end of this incubation, 2 ml of cold PBS with 2% FBS were added to each tube, and the cells were centrifuged at 400 x g for 3 min at 4°C. One ml of the supernatant was then counted for activity (Beckman Instruments, Inc., Fullerton, Calif.) for determining percentage specific 51Cr release according to the following:

% specific release =

Maximal 51Cr release (3 times freeze-thaw in distilled water) - spontaneous 51Cr release

The spontaneous release was taken as the highest of the values with antibody alone (serum toxicity control) or complement alone. As additional controls, cells were incubated with only PBS plus 2% FBS or with normal IgG (in the case of anti-CEA assay) or normal hamster serum (in the case of the assay with anti-HCT-8R antibodies) prior to addition of complement. These controls gave cytotoxicity values which were usually less than the spontaneous controls and were never significantly greater than the spontaneous controls. All test systems were done in triplicate for each experiment.

125I-Antibody Binding Assay for CEA. Near confluent cultures were dispensed with trypsin-EDTA, washed with PBS containing 2% FBS, and adjusted to a concentration of 1 x 10⁶ viable cells/ml. Cells were pelleted by centrifugation at 250 x g for 5 min and resuspended in 0.1 ml PBS and then 0.1 ml 125I-anti-CEA or 125I-normal goat IgG was added to tubes in triplicate. Controls consisted of labeled antibodies added to the tubes in the absence of cells. Cells were incubated for 60 min at 37°C and then washed 5 times in 2 ml of PBS containing 2% FBS. After the final wash, cells were resuspended in 2 ml of PBS plus 2% FBS, and 1 ml was transferred to a fresh tube for counting. Absolute numbers of counts bound to cells were

JUNE 1979 2161

Human Colon Tumor Cells Xenografted in Athymic Mice

The abbreviations used are: FBS, fetal bovine serum; CEA, carcinoembryonic antigen; PBS, phosphate-buffered saline (0.01 M, pH 7.2).

3 The abbreviations used are: FBS, fetal bovine serum; CEA, carcinoembryonic antigen; PBS, phosphate-buffered saline (0.01 M, pH 7.2).
calculated by subtracting the counts in tubes containing label alone from counts in tubes containing label and cells.

RESULTS

Tumor-forming Dose of Human Colon Tumor Cells in Nude Mice. HCT-8R, HT-29, and the Nu1 sublines were injected into RNC nude mice and observed for tumor formation. The results in Table 1 show that both HCT-8R Nu1 and HT-29 Nu1 were more tumorigenic in nude mice than their original counterparts. Thus, at a concentration of $10^6$ viable HCT-8R Nu1 cells, 100% of the mice developed tumors by 6 weeks. In contrast, $10^6$ HCT-8R cells were required to produce tumors by 6 weeks. Lower doses of HCT-8R failed to form tumors even after 12 weeks. HT-29 Nu1 showed a similar increase in tumorigenicity as compared to HT-29 cells. These results were obtained with Nu1 cells at in vitro passage 8, and similar results were observed at passage 20, suggesting that increased tumorigenicity is a stable phenotype of Nu1 sublines.

Complement-dependent Anti-CEA Lysis of Human Colon Tumor Cells. Previous studies have shown that HCT-8R, and to a lesser extent HT-29 cells, could be lysed by anti-CEA and complement. Therefore, a 51Cr release cytotoxicity assay was performed to determine if growth in nude mice altered the sensitivity of the cells to complement-dependent anti-CEA lysis. Lysis of the different target cells incubated with 0.5 µg amounts of monospecific anti-CEA antibodies is shown in Table 2. The Nu1 cells were completely resistant to lysis by both goat and horse anti-CEA. In contrast, horse anti-CEA caused significant lysis of both HCT-8R and HT-29 cells. Goat anti-CEA caused lysis of HCT-8R cells but not HT-29 as reported previously (25). These results were obtained from Nu1 sublines at the 8th in vitro subculture. Similarly, HCT-8R and HT-29 cells were passaged 8 times since they were used to produce the Nu1 sublines.

Quantitation of CEA on the Cell Surface by 125I-Anti-CEA Binding. Resistance of Nu1 cells to anti-CEA lysis could represent a reduction in CEA synthesis or expression on the cell surface. To examine this, 125I-anti-CEA was reacted with monodisperse tumor cells in a suspension-type radioimmunoassay (23). One $10^6$ target cells were reacted with 0.5 µg of specific anti-CEA labeled with 125I. This ratio of cells to µg of antibody was shown previously to be in the region of antibody excess (23). The results in Table 3 show that HCT-8R Nu1 and HT-29 Nu1 bind slightly more 125I-anti-CEA than their parental lines. Thus, HCT-8R and HT-29 bound 3921 ± 223 cpm, respectively, as compared to 4402 ± 439 and 7184 ± 443 cpm for HCT-8R Nu1 and HT-29 Nu1, respectively. Passage of HCT-8R Nu1 (CEA negative) cells through nude mice neither increased nor decreased the amount of 125I-anti-CEA bound to the surface, suggesting that the nonspecific binding properties of Nu1 sublines were not different from the original cell lines. Similarly, the nonspecific binding of 125I-normal IgG was similar for Nu1 sublines and the original cell lines.

Effect of In Vitro Culturing on Anti-CEA Reactions on the Surface of HCT-8R and HT-29 Nu1 Cells. This experiment was designed to determine if the expression of CEA as measured in vivo was altered by in vitro subculture.
ured by cytotoxicity and antibody binding was a stable characteristic of HCT-8R and the HCT-8R Nu1 subline grown in culture. Table 4 shows the results of anti-CEA cytotoxicity and 125I-anti-CEA binding when the 2 cell lines were tested at the 4th and 20th in vitro passage in the same assay. Thus, HCT-8R were equally sensitive to anti-CEA lysis when tested at passages 4 and 20. Similarly, the Nu1 subline was totally resistant to lysis at both passage levels. Normal goat IgG caused no significant lysis of any of the target cells. Examination of 125I-anti-CEA binding shows that there was no significant difference in CEA on the surface of HCT-8R and HCT-8R Nu1. Further, neither cell line showed any major shift in expression of CEA with increasing in vitro passage level. Thus, immunoresistance and cell surface CEA expression appear to be stable but independent phenotypes of HCT-8R and Nu1 cell lines.

**Lysis of Human Tumor Cells and Nu1 Sublines by Anti-Whole-Cell Antibody.** If immunoresistance of Nu1 cells to anti-CEA-complement lysis is not related to a change in expression of CEA per se, then nonspecific surface changes may be responsible. If this is the case, we would predict that the immunoresistance of Nu1 sublines is not restricted to CEA-anti-CEA reactions. To test this, antisera to viable HCT-8R and HT-29 was tested against the homologous cell line and the Nu1 counterpart. The results in Chart 1 show that anti-HCT-8R was strongly cytotoxic for HCT-8R cells but not for HCT-8R Nu1. Similarly, HT-29 Nu1 sublines were more resistant to lysis by anti-HT-29 than the homologous HT-29 cell. Table 5 compares the susceptibility of Nu1 sublines from an RNC nude mouse and a BALB/c nude mouse with the parental HCT-8R cell line to lysis by anti-HCT-8R serum. Both the RNC and BALB/c Nu1 sublines are more resistant to lysis than were the HCT-8R cells. A 1:32 dilution of antisera caused 68% lysis of HCT-8R cells as compared to 27% lysis of RNC Nu1 and 10.9% lysis of BALB/c Nu1. Similar differences were observed with other dilutions of antisera. Although data are not shown, normal hamster serum in the presence of complement caused no significant lysis of any of the target cells.

**Adsorption of Anti-HCT-8R Antibodies by HCT-8R and HCT-8R Nu1 Cells.** Anti-HCT-8R was adsorbed with equal dilutions of antiserum. Although data are not shown, normal as compared to 27% lysis of RNC Nu1 and 10.9% lysis of the susceptibility of Nu1 sublines from an RNC nude mouse and BALB/c nude mouse with the parental HCT-8R cell line to lysis by anti-HCT-8R serum. Both the RNC and BALB/c Nu1 sublines are more resistant to lysis than were the HCT-8R cells. A 1:32 dilution of antisera caused 68% lysis of HCT-8R cells as compared to 27% lysis of RNC Nu1 and 10.9% lysis of BALB/c Nu1. Similar differences were observed with other dilutions of antisera. Although data are not shown, normal hamster serum in the presence of complement caused no significant lysis of any of the target cells.

**Chart 1. Complement-dependent anti-HCT-8R and anti-HT-29 lysis of HCT-8R and HT-29 colon tumor cells and their Nu1 sublines.** $^{32}$Cr-labeled target cells were examined for antibody lysis in the presence of a 1:8 dilution of guinea pig complement. $\bigcirc$, anti-HCT-8R against HCT-8R; $\bullet$, anti-HCT-8R against HCT-8R Nu1; $\bigtriangledown$, anti-HT-29 against HT-29; $\blacksquare$, anti-HT-29 against HT-29 Nu1. Bars, S.E.

**DISCUSSION**

This study describes modifications of some biological properties of human colon tumor cells xenografted in congenital athymic mice. Human colon tumor cells, HCT-8R and HT-29, were inoculated into adult athymic mice, and the resulting tumors were explanted into culture. The explanted cells, designated Nu1 sublines, were compared to the original cell line for tumor-forming ability in nude mice. The Nu1 cells were found to be 10- to 100-fold more tumorigenic for nude mice than the original HCT-8R and HT-29 cells. Previous studies by Rosenthal et al. (18) also demonstrated that the tumor-forming dose of HCT-8R and HT-8 cells for nude mice decreased after a single passage in the mouse.

The colon tumor cell lines used in this study express CEA on their surface (18, 23, 25). Therefore, we were able to use CEA as a marker to determine if antigen expression was altered on Nu1 cells. Whereas the original HCT-8R and HT-29 cell lines were susceptible to anti-CEA-complement lysis, the Nu1 cells were totally resistant to lysis. Quantitation of CEA on the cell surface with an $^{125}$I-anti-CEA assay demonstrated that the Nu1-resistant cells and the more susceptible original cells possessed comparable amounts of CEA on the surface. Rosenthal et al. (18) used immunofluorescence and $^{125}$I-anti-CEA binding to show that some Nu1 sublines may express lower levels of surface CEA, whereas other Nu1 sublines may be unaltered from the original cells. This suggests that different clones of cells with varying levels of CEA expression can be selected by comparable amounts of anti-HCT-8R antibodies. This suggests that the concentration of major antigens participating in the cytotoxic reactions do not differ on the 2 cell types.
passage in nude mice. There was no apparent correlation between CEA concentration and tumorigenicity of the Nu sublines isolated by Rosenthal et al. (18).

In support of our results with anti-CEA, Nu cells were more resistant to lysis by antibodies produced against intact HCT-8R and HT-29 cells than were the original cell lines. No difference in adsorption of these antibodies by the 2 cell types was observed. Others have reported the failure of antibody-induced cytosis of tumor cells despite binding of antibodies and activation of the complement system (11, 16, 24). O'Hanian et al. (11) found a lack of correlation between surface antigen concentration and susceptibility to antibody lysis of guinea pig hepatoma cells. This antigen-independent resistance to antibody lysis has been attributed to a number of different factors (16) including the acquisition of nonspecific glycophorin on the cell surface which could block antibody or complement reactions (24). Studies are now in progress to examine these factors on the Nu sublines.

Möller (9, 10) showed that methylcholanthrene-induced sarcomas grown in H-2-incompatible newborn mice gained the ability to grow progressively in untreated H-2-incompatible adults. These nonspecific tumor sublines appeared to have a reduced concentration of H-2 alloantigens as measured by quantitative antibody adsorption (9) and antibody cytotoxicity (10) as compared to the strain dependent tumor of origin. Möller (10) suggested that the sublines appeared as a consequence of immunological selection of preexisting variants with reduced H-2 antigen from the inoculated tumor cell population. The fact that CEA expression and immunoresistance are unchanged with prolonged in vitro growth suggests that these phenotypes are genetically stable, and the Nu sublines have been selected from preexisting variants. This is supported by the observation that Nu sublines which express different levels of CEA can be selected (18). These high- and low-CEA-producer sublines are also stable in their CEA production when grown in culture. Our results are, however, at variance with Möller’s (10) contention that reduced surface antigen expression is a prerequisite for selection of more tumorigenic and immunoresistant sublines. It is evident from the data reported here that the observed increase in immunogenicity and tumorigenicity by passage through nude mice is independent of antigen expression per se. While there is a correlation between acquisition of immunoresistance and increased tumorigenicity for all Nu sublines tested, we do not know if these phenotypes are interdependent.

The selection pressures exerted on xenografted tumor cells by nude mice are not known at this time. Prehn and Outzen (14) suggested that athymic mice are able to exert weak immunological influences on tumor grafts, thus accounting for the variability in tumor growth by different tumor cell lines. Similarly, Campanile et al. (3) suggested that transplantation resistance of athymic mice to H-2-incompatible lymphomas was of immunological origin. While the results of Campanile et al. (3) did not support an involvement of T-dependent immunity, the possibility of B-cell dependent resistance or tumor inhibition by activated macrophages or naturally cytotoxic lymphocytes could not be ruled out. Studies are currently in progress to determine if antibody or other immune mechanisms are operative in our system. Preliminary studies not presented here have failed to demonstrate cytotoxic antibodies to either HCT-8R or HCT-8R Nu cells in tumor-bearing nude mice.

Finally, it has to be considered that the influences of the nude mouse host on human tumor cells are nonimmunological. One possibility is that the tumor cells are infected with host-derived viruses. A number of investigators have observed that nude mouse heterotransplanted tumors harbor murine xenotropic type C viruses (1, 5, 15, 20), and it has been suggested that these viruses might enhance the growth potential of tumor cells (5). Thus, it is possible that infection of the Nu sublines with persistent murine viruses could contribute to both the increased tumorigenicity and resistance to antibody-complement lysis described in this report.
REFERENCES


Increased Tumorigenicity and Resistance to Antibody Lysis of Human Colon Tumor Cells Xenografted in Congenitally Athymic Mice

M. B. Tompkins, G. V. Rama Rao and W. A. F. Tompkins


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/39/6_Part_1/2160

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.