Lack of Correlation between Induction of Chemotactic Peptide Receptors and Stimulus-induced Actin Polymerization in HL-60 Cells Treated with Dibutyryl Cyclic Adenosine Monophosphate or Retinoic Acid

K. Murali Krishna Rao, Mark S. Currie, J. Cullen Ruff, and Harvey J. Cohen

Geriatric Research, Education and Clinical Center, Center for the Study of Aging and Human Development, and the Divisions of Geriatrics & Hematology/Oncology, Veterans Administration and Duke University Medical Centers, Durham, North Carolina 27705

ABSTRACT

We used the promyelocytic leukemic cell line HL-60 to explore the molecular mechanisms regulating stimulus-induced actin polymerization in myeloid cells. HL-60 cells express very few chemotactic peptide receptors in their undifferentiated state and fail to undergo actin polymerization when stimulated with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP). However, when the cells were induced to differentiate with dibutyryl cyclic AMP (dbcAMP) or retinoic acid, they acquired the ability to undergo actin polymerization on stimulation with FMLP or phorbol myristate acetate. Kinetic experiments revealed that in the first 48 h of retinoic acid treatment there was no increase in the chemotactic peptide receptors on HL-60 cells, but the cells were capable of undergoing actin polymerization on stimulation with FMLP. Similarly, treatment with dbcAMP showed no increase in chemotactic peptide receptors until 24 h but stimulus-induced actin polymerization was demonstrable as early as 4 h after the treatment. In addition, with dbcAMP-treated cells the magnitude of stimulus-induced actin polymerization showed large variation depending on the duration of exposure to the drug. Dual-label studies using propidium iodide to measure DNA content and NBD-phallacidin to measure the F-actin content revealed that these variations were not related to the stages of cell cycle. Cells in all stages of the cell cycle responded to stimulus-induced actin polymerization, but the magnitude of the response appeared to be more in cells in G2/M phase. The observations reported here indicate that the small number of chemotactic peptide receptors present on HL-60 cells are adequate to mount an actin polymerization response, provided the required intracellular mechanisms exist. Differentiation-inducing agents, therefore, must cause changes within the cell, such as induction of actin-binding proteins, to cause actin polymerization following FMLP stimulation. The HL-60 system serves as a useful model for studying the molecular mechanisms regulating stimulus-induced actin polymerization in human neutrophils.

INTRODUCTION

Stimulation of mature neutrophils with the chemotactic peptide FMLP causes rapid conversion of monomeric actin to polymeric actin (1–5). This conversion of monomeric actin to polymeric actin appears necessary for many functions of activated phagocytes such as chemotaxis (6), phagocytosis (7), antibody-dependent cellular cytotoxicity (8) and natural killer activity (9, 10). The signal(s) involved in the induction of this change is not known. Calcium flux induced by FMLP does not seem to be involved in the initiation of the actin polymerization (11–12). Activation of protein kinase was not found necessary for the change in FMLP-induced actin polymerization (13, 14). In macrophages, dbcAMP was reported to influence the state of actin in unstimulated cells and inhibit the FMLP-induced actin polymerization (15). However, the concentration of dbcAMP used (200 μM) does not preclude nonspecific effects at the membrane level. We have found that phenylmethylsulfonyl fluoride at high concentrations (2 mM) inhibits FMLP-induced actin polymerization by mechanisms unrelated to its anti-protease activity. Similarly, polymyxin B, which acts by perturbing the cell surface membrane, causes profound changes in the actin content in unstimulated cells and inhibits FMLP-induced actin polymerization (16).

In order to define the molecular mechanisms involved in actin polymerization, we studied this phenomenon in a human promyelocytic leukemic cell line, HL-60. This cell line was found useful in studying the differentiation events associated with the maturation of myeloid cells. HL-60 cells can be induced to differentiate into more mature cells by treatment with a variety of substances, such as phorbol esters, dimethyl sulfoxide, retinoic acid, dbcAMP and 1,2-dihydroxyvitamin D3 (17–20).

The differentiation process is accompanied by functional changes (17, 21, 22). In the present report we used HL-60 cells as a model system to study the stimulus-induced actin polymerization in neutrophils. We found that undifferentiated HL-60 cells do not undergo actin polymerization when stimulated with FMLP. However, when the cells were induced to differentiate into mature cells with either retinoic acid or dbcAMP they acquired the ability to undergo actin polymerization. HL-60 cells treated with tumor necrosis factor and N,N-dimethylformamide have also been shown to acquire the ability to undergo stimulus-induced actin polymerization (6, 23). In this report we show that there is no correlation between the acquisition of chemotactic peptide receptors and FMLP-stimulated actin polymerization in HL-60 cells induced to differentiate either with dbcAMP or retinoic acid.

MATERIALS AND METHODS

The fluoresceininated hexapeptide FNLPNTL and NBD-phallacidin were from Molecular Probes, Inc. (Eugene, OR). Retinoic acid, dbcAMP, unlabeled hexapeptide, L-α-lysophosphatidyleholine, type I (L-α-lyssolecithin), FMLP, BSA, propidium iodide, PMA and RNase were from Sigma (St. Louis, MO). The fluorescent hexapeptide was dissolved in dimethyl sulfoxide at a concentration of 3 mM and the unlabeled peptide to a concentration of 10 mM. Retinoic acid, FMLP, and PMA were also dissolved in dimethyl sulfoxide to a concentration of 1, 10, and 1 mM, respectively. All further dilutions were made in culture media or HBSS.

Medium and Serum. All cultures were done in RPMI-1640 (J. R. Scientific, Woodland, CA) supplemented with 1-glutamine and 10% fetal bovine serum (J. R. Scientific, Woodland, CA).

Culture Methods. The HL-60 cell line was from the American Tissue Culture Collection (Rockville, MD). Cells were used during passages 23 to 45. Maintenance cultures were passed two to three times each
ACTIN POLYMERIZATION IN HL-60 CELLS

week after initial seedings of 2.5–5 x 10^5/ml in 10% FBS. In experiments, the cells were cultured at 1 to 2 x 10^5/ml with the various additives at 37°C with 5% CO2 and 100% humidity in 25- or 75-cm² surface area plastic culture flasks (Becton Dickinson, Oxnard, CA). The cells were cultured for 3 to 6 days. In 6-day cultures the medium was changed on the third day, the replacement medium also contained the inducing agent. The cells were washed three times and were suspended in HBSS or PBS for further experiments. Cell viability was determined by trypsin blue exclusion.

Cytochemistry. After appropriate cultures, the cells were collected onto glass slides using a Shandon cytoseptorifice (Shandon Southern, Astmoor, England). Nonspecific esterase (a-naphthylbutyrate esterase) stain was done as described before (24). Morphology was ascertained by Wright's stain.

Quantitation of the Filamentous Actin (F-actin) Content. The amount of F-actin in resting and stimulated cells was measured by NBD-phallacidin labeling followed by methanol extraction as described by Howard and Oresajo (25). Briefly, 100 μl of the cell suspension (20 million cells/ml) was incubated with FMLP (100 nm) or PMA (1 μM) for one min and then the cells were fixed in a single step by adding 100 μl of a freshly prepared staining cocktail consisting of 6.4% paraformaldehyde, 200 μg/ml of lysophosphatidyl choline (lysolecithin) and 0.6 μM NBD-phallacidin in PBS for 30 min at room temperature. The cells were spun at 12,000 x g for one min in a microfuge and the cell pellet was washed twice with one ml PBS. The dye in the cell pellet was extracted into one ml of methanol for 10 min and the procedure repeated. The RFI of the pooled methanol extract (2 ml) was measured in a SPF-125 spectrofluorometer (American Instrument Co., Silver Spring, MD) set at 465 nm for excitation and 535 nm for emission. The percentage of change in F-actin was calculated as follows:

(RFI of stimulated cells – RFI of unstimulated cells) × 100
RFI of unstimulated cells

In some experiments the amount of F-actin content was measured by flow cytometry as described below.

Measurement of Chemotactic Peptide Receptor Expression. The expression of chemotactic peptide receptors on HL-60 cells was determined by binding studies using the fluorescent hexapeptide (FNLPNTL). 100 μl of the cell suspension (20 million cells/ml) was incubated with 100 nm hexapeptide (488 nm) for 1 min at 37°C, the cells were fixed by adding 100 μl of cold 6.4% paraformaldehyde. After 30 min in ice the cells were pelleted in a microfuge and washed twice with PBS containing 0.1% BSA and finally suspended in one ml of the same buffer. The cells were stored at 4°C until analysis by flow cytometer. The specificity of the binding was demonstrated by incubating the cells with fluoresceinated peptide for 1 min in the presence of a 500-fold excess of the unlabeled hexapeptide (23).

Dual Labeling of Cells for DNA and F-actin Content. The cells were stained for DNA and F-actin in a two step procedure. The first step was similar to that described for the quantitation of F-actin. For DNA staining the cells were washed twice with PBS after the 30 min incubation with the NBD-phallacidin staining cocktail and suspended in 0.5 ml of propidium iodide cocktail (6.25 mg propidium iodide and 2 mg RNase in 100 ml PBS, pH adjusted to 7.4 with NaOH) for 5 min. The cells were washed twice in Beckman Microfuge B with 0.1% BSA and finally suspended in one ml of the same buffer. The cells were stored at 4°C until analysis by flow cytometer. The RFI of the pooled methanol extract (2 ml) was measured in a SPF-125 spectrofluorometer (American Instrument Co., Silver Spring, MD) set at 465 nm for excitation and 535 nm for emission. The percentage of change in F-actin was calculated as follows:

(RFI of stimulated cells – RFI of unstimulated cells) × 100
RFI of unstimulated cells

In some experiments the amount of F-actin content was measured by flow cytometry as described below.

Flow Cytometric Analysis. Samples were analyzed for green fluorescence using an EPICS 753 fluorescent-activated cell sorter (Coulter Electronics, Hialeah, FL). The excitation source was an Argon-ion 5W laser (Coherent, Palo Alto, CA) emitting 400 nm of light at a wavelength of 488 nm. The emission wavelength to be analyzed was selected by placing both a 488/515-nm laser blocking filter and a 525-nm band pass filter in front of the fluorescence detector. Forward angle light scatter and perpendicular light scatter were used to gate preaggregates to exclude nonviable cells and cell debris from analysis. All other cells were analyzed. For each sample, 1 x 10⁶ cells were analyzed at an approximate flow rate of 2 x 10⁶ cells/s. Electronic pulses were sent from the EPICS unit to an MDADS computer system (Coulter Electronics) and digitized using analog to digital converters. For each sample, a 256-channel histogram (linear scale) was generated and analyzed for percentage of positive cells and mean channel fluorescence number. For two color analysis propidium iodide fluorescence was analyzed using a 575-nm band pass filter. NBD-phallacidin fluorescence was collected using a 525-nm band pass filter. Spectral overlap of NBD-phallacidin fluorescence into the propidium iodide fluorescence detector was eliminated using an electronic subtraction circuit.

Statistical Analysis. Student’s t test was used to estimate the differences between control and test group.

RESULTS

Effect of Retinoic Acid and dbcAMP on Cell Differentiation in HL-60 Cells. Culturing HL-60 cells with retinoic acid (1 μM) for 5 to 6 days caused granulocytic differentiation with the appearance of many mature polymorphonuclear leukocytes. The cells were nonspecific esterase negative. Differentiation induced by dbcAMP (750 μM) on the other hand led to maturation into monocyteic series as assessed by morphology of the Wright’s-stained cells and by nonspecific esterase positivity. Undifferentiated HL-60 cells showed only occasional nonspecific esterase-positive cells.

Effect of Retinoic Acid and dbcAMP on Chemotactic Peptide Receptor Expression. HL-60 cells were induced to differentiate into mature cells with either retinoic acid or dbcAMP for various time periods and the expression of receptors for the chemotactic peptide was determined using the fluoresceinated hexapeptide, FNLPNTL (Fig. 1). Undifferentiated HL-60 cells had very small numbers of receptors as assessed by flow cytometry. On stimulation with retinoic acid, the cells acquired chemotactic peptide receptors; however, the density of the receptors was very low as estimated by the intensity of the fluorescence of these cells read as mean channel fluorescence. DbcAMP stimulation resulted in the expression of receptors at a much higher density. The low level of expression of receptors observed with retinoic acid might have been interpreted as negative response in certain studies (26).

Effect of Differentiation on the Stimulus-Induced Actin Polymerization. Mature neutrophils demonstrate rapid conversion of monomeric actin to polymeric actin on stimulation with FMLP. In contrast, undifferentiated HL-60 cells do not undergo actin polymerization when stimulated with FMLP. However, when HL-60 cells were induced to differentiate with either retinoic acid or dbcAMP they acquired the ability to undergo actin polymerization on stimulation with FMLP as shown in Table 1. The tumor promoter, PMA was also capable of inducing actin polymerization in retinoic acid treated cells (66 ± 9%, mean ± SEM, N = 5, P < 0.001) but failed to induce significant change in F-actin in dbcAMP treated cells (9 ± 4%, mean ± SEM, N = 4, P > 0.05).

Time-Course of Chemotactic Peptide Receptor Expression and FMLP-induced Actin Polymerization (Fig. 2). HL-60 cells were incubated with either dbcAMP or retinoic acid for various time periods and the expression of chemotactic peptide receptors and response to chemotactic peptide in terms of actin polymerization was determined as described above. There was no increase in the chemotactic peptide receptor expression in retinoic acid treated cells in the first 48 h, but a significant increase in F-actin on stimulation with FMLP was noticeable at 48 h (P < 0.003). On the other hand a gradual increase in the receptor expression was seen with dbcAMP with a significant increase in receptor number by 24 h. The stimulus-induced increase in actin polymerization was demonstrable as early as 4 h after treatment with dbcAMP (with some batches of cells,

6722
ACTIN POLYMERIZATION IN HL-60 CELLS

HL-60 Cells

Dbc AMP 750 μM
(3 days)

Retinoic Acid 10^-6 M
(6 days)

Linear Fluorescence Intensity

Fig. 1. Chemotactic peptide receptor expression in HL-60 cells. The cells were treated with dbcAMP (750 μM) for 3 days or retinoic acid (1 μM) for 6 days, labeled with fluoresceinated hexapeptide and analyzed by flow cytometry.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>% Change in F-actin</th>
<th>P versus control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>0.4 ± 2*</td>
<td>&gt;0.001</td>
</tr>
<tr>
<td>Retinoic acid-treated cells</td>
<td>43 ± 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DbcAMP-treated cells</td>
<td>30 ± 6</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

* Mean ± SEM of five different experiments.

Fig. 2. Kinetics of chemotactic peptide receptor expression and acquisition of ability to undergo actin polymerization in undifferentiated, dbcAMP treated, and retinoic acid treated HL-60 cells. A, chemotactic peptide receptor expression; B, actin polymerization induced by FMLP. Bars, mean ± SD of three separate experiments.

the earliest time stimulus-induced actin polymerization noted was at 8 h. Thus, both in the case of dbcAMP and retinoic acid, a complete dissociation was noted with respect to the appearance of chemotactic peptide receptors and the acquisition of the ability to undergo actin polymerization to FMLP in HL-60 cells.

Cell Cycle-related Changes in Stimulus-induced Actin Polymerization. DbcAMP-induced cells showed a curious pattern of variation in actin polymerization response to FMLP (Fig. 2B). The peaks and valleys varied from experiment to experiment (accounting for the large standard deviation). We hypothesized that these variations might be related to the cells' position in the cell cycle. In order to explore this possibility, we determined the DNA content and F-actin content in FMLP-stimulated and resting cells in undifferentiated HL-60 cells, and in HL-60 cells induced to differentiate with either dbcAMP or retinoic acid. The cells were labeled with propidium iodide and NBD-Phallacdin in a two-step procedure and analyzed by flow cytometer for two color fluorescence. Fig. 3A shows a typical histogram of two color fluorescence. The X-axis shows red fluorescence (DNA content) and the Y-axis represents green fluorescence (F-actin content). The spectrum of red fluorescence was gated into three maps; map 1 representing G0/G1 phase, map 2 representing S phase, and map 3 representing G2/M phase. The green fluorescence in each area was measured in resting and FMLP-stimulated cells for each time point in control and dbcAMP-treated cells. Thus, in Fig. 3B, the three frames in panel 1 represent histograms of F-actin content (green fluorescence) in pairs (unstimulated and FMLP-stimulated) in the three regions gated on the basis of red fluorescence, in 8-h control cells. In each frame, A indicates the mean channel fluorescence of unstimulated cells and B represents the mean channel fluorescence of FMLP-stimulated cells. The mean channel fluorescence is directly proportional to the F-actin content.

In both 8-h control (panel 1) and 12 h control cells (panel 3) there was no change in the F-actin content on stimulation with FMLP in any of the three map areas (G0/G1, S, and G2/M). In contrast, in dbcAMP treated-cells both at 8 h (panel 2) and 12 h (panel 4) FMLP induces an increase in the F-actin content in all three map areas. Thus, treatment with dbcAMP confers the ability to undergo actin polymerization in cells at all phases of cell cycle. However, the magnitude of response was greater in cells in G2/M phase. Also it is important to note that in dbcAMP treated cells, the actin polymerization response at 12 h is less in each of the three map areas compared to the cells at 8 h. This may explain the variation of response in actin polymerization seen in Fig. 2B. Since the decreased response is seen in all three map areas the total decrease in response seen at 12 h cannot be attributed to mere differences in the number of cells in different phases of cell cycle. The reason(s) for the
variations noted in stimulus-induced actin polymerization at different time points remains unclear at the present time.

**DISCUSSION**

Stimulation of mature neutrophils with a variety of ligands induces actin polymerization (1–5). This conversion of monomeric actin to polymeric actin appears necessary for many functions of activated phagocytes including chemotaxis (6), phagocytosis (7), antibody-dependent cellular cytotoxicity (8) and natural killer activity (9, 10). Actin has been postulated to play an important role in metabolic regulation in cells (27, 28), RNA transcription (29, 30), and cellular activation in general. In addition, alterations in actin state have been noted in neoplastic diseases. The patterns of organization of actin and myosin are shown to be different in normal and neoplastic cells (31). Differences in the amount and behavior of actin have been demonstrated in melanoma cells of differing metastatic potential (32). We have shown that in several transformed cell lines of lymphocyte origin, two thirds of the actin is in polymeric form as opposed to one third in the normal lymphocytes (33). These observations suggest that the study of factors regulating actin state in cells might yield valuable insight into the abnormal behavior of cancer cells. At present very little is known about the molecular mechanisms involved in the conversion of monomeric to polymeric actin. Known secondary messengers such as calcium fluxes, protein kinase C, and cyclic nucleotides do not seem to play primary roles in this process (11–15). Some evidence exists for a role for polyphosphoinositides in this process (34, 35). Although differentiated HL-60 cells are shown to generate inositol phosphates following FMLP stimulation (36, 37), which may indeed have some role in FMLP-induced actin polymerization, the induction of actin polymerization by PMA (which bypasses the inositol phosphate pathway) in retinoic acid-treated cells suggests that additional processes are involved. Pertussis toxin-sensitive G-Proteins have been shown to be involved in stimulus-induced actin polymerization (38) and generation of inositol phosphates (36). However, much remains to be learned about the exact molecular mechanisms regulating conversion of monomeric to polymeric actin. In order to study these mechanisms in depth, we utilized HL-60 cells as a model for studying stimulus-induced actin polymerization.

HL-60, a promyelocytic leukemic cell line, can be induced to undergo differentiation when cultured with a variety of stimuli. These include several chemical substances (17–20) and cytokines such as tumor necrosis factor and IFN-γ (23, 24, 39, 40). We have previously shown that induction of differentiation with tumor necrosis factor and IFN-γ results in the acquisition of chemotactic peptide receptors and the ability to undergo actin polymerization following simulation with the chemotactic peptide FMLP. Further, these two cytokines seem to exert a cooperative effect (23). Induction with the two cytokines leads to differentiation into monocytoid cells showing nonspecific esterase positivity. Previous studies have shown that induction of HL-60 cells with retinoic acid leads to maturation into granulocytes and our observations were consistent with those reports. However, with dbcAMP we found that the cells matured into monocyctic cells which were nonspecific esterase positive; this was different from one study where granulocytic differentiation was reported (41).

Both dbcAMP- and retinoic acid-stimulated cells acquired the ability to undergo actin polymerization on stimulation with FMLP and also expressed chemotactic peptide receptors. However, the time-course experiments revealed that there was no relationship between the expression of the chemotactic peptide receptors and the ability to respond to the chemotactic peptide in terms of actin polymerization. We reported a similar lack of correlation between the expression of chemotactic peptide receptors and stimulus-induced actin polymerization in HL-60 cells treated with the cytokines tumor necrosis factor and IFN-γ. Although IFN-γ induces expression of chemotactic peptide

---

**Fig. 3.** Analysis of DNA content and F-actin content of HL-60 cells by double labeling of the cells with propidium iodide and NBD-phallacidin. HL-60 cells were treated with dbcAMP for 8 h and 12 h. Control cells were cultured for the same time periods without dbcAMP. The cells were harvested and stained with NBD-phallacidin and propidium iodide before and after FMLP-stimulation (1 min at 37°C). A, a typical histogram of red versus green fluorescence; The three maps shown are the regions gated to obtain the green fluorescence (F-actin content) histograms depicted in B. B, each panel shows the histograms of green fluorescence of unstimulated and FMLP-stimulated cells in the three map regions indicated in A.
receptors in HL-60 cells, actin polymerization does not occur when the cells are stimulated with FMLP (23). Thus, these observations seem to suggest that appearance of chemotactic peptide receptors is unrelated to the ability of differentiated HL-60 cells to undergo actin polymerization. A small number of chemotactic peptide receptors have been shown to be present in undifferentiated HL-60 cells (42, 43). Receptor-binding studies have shown that binding of as few as 100 receptors can lead to actin polymerization response in neutrophils (11). This suggests that the small number of chemotactic peptide receptors present on HL-60 cells are adequate to mount an actin polymerization response provided other components involved in the actin polymerization response are present and functional. It is becoming apparent that there are several actin-binding proteins in non-muscle cells, which might play a crucial role in regulating the actin state in the cells (44, 45). It appears that the inability to undergo actin polymerization noted in HL-60 cells is not related to the lack of receptors but to the deficiencies in the internal (post receptor) mechanisms required for inducing actin polymerization. Supporting such a conclusion is the recent observation that monocytoid differentiation of HL-60 cells leads to specific induction of gelsolin and a-actinin (46), proteins proposed to have a role in the regulation of actin state in cells.

We observed time-dependent variations in actin polymerization response to FMLP in cells treated with dbcAMP. We hypothesized that these variations may be related to the stages in cell cycle. However, in our dual label experiments (propidium iodide and NBD-phallacandin) we found that cells in all stages of cell cycle were capable of undergoing actin polymerization in response to FMLP in differentiated HL-60 cells. The cells in G2/M phase showed the maximum response. However, the variations in actin polymerization in dbcAMP treated cells depicted in Fig. 2 do not se12. em to be due to differences in the number of cells in various phases of cell cycle, because the magnitude of the response was less in all phases of the cell cycle at 12 h compared to the cells at 8 h. The variations in stimulus-induced actin polymerization might be related to quantitative changes in second messengers or molecules mediating the signals; these may be related to the time of incubation with dbcAMP. Since G-proteins are shown to be involved in stimulus-induced actin polymerization (38), studies on time related changes in G-proteins might prove useful.

In summary, we have shown that induction of maturation of HL-60 cells with dbcAMP or retinoic acid confers the ability to undergo actin polymerization on stimulation with FMLP. This is not related to the expression of chemotactic peptide receptors. Therefore, the ability to undergo actin polymerization might be due to the expression of new proteins which may have a role in regulating actin state. The findings reported here show that HL-60 cell line is a useful tool for delineating the factors regulating actin polymerization during maturation of myeloid cells.

ACKNOWLEDGMENTS

We thank David Leslie for assistance with the flow cytometric data and Dr. Bruce Weinberg for critically reviewing the manuscript.

REFERENCES


Lack of Correlation between Induction of Chemotactic Peptide Receptors and Stimulus-induced Actin Polymerization in HL-60 Cells Treated with Dibutyryl Cyclic Adenosine Monophosphate or Retinoic Acid


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/23/6721

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

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

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