Correction. In the article "Urinary acidification in turtle bladder is due to a reversible proton-translocating ATPase" by Troy E. Dixon and Quis Al-Awqati, which appeared in the July 1979 issue of Proc. Natl. Acad. Sci. USA (76, 3135–3138), the authors request that the following correction be noted. The title to Table 1 should read “ATP synthesis in poisoned cells.”

Correction. In the article "Developmental and mutational changes of glycoproteins in the mouse neuronal retina: Studies with bovine galactosyltransferase" by Barbara Wallenfels, which appeared in the July 1979 issue of Proc. Natl. Acad. Sci. USA (76, 3223–3227), the author requests that the following corrections be noted. On p. 3223, column 2, in line 23 the thickness should be 1 mm, in line 25 it should be 10% Ampholine mixture, and in line 27 “8–10” should be added after “7–9” and the ratio term should be corrected to “1:2:2:3:3:3:6.”

Correction. In the article "Interaction site of Escherichia coli cyclic AMP receptor protein on DNA of galactose operon promoters" by Taketoshi Taniguchi, Michael O'Neill, and Benoit de Crombrugghe, which appeared in the October 1979 issue of Proc. Natl. Acad. Sci. USA (76, 5090–5094), the authors request that the following corrections be noted. On p. 5094, the first line should end “and three (or more).” In the sequences that follow, the last two (ara -148 to -138 and -158 to -148) should be deleted.

Correction. In the article "Conditions for inhibiting and enhancing effects of the protease inhibitor antipain on x-ray-induced neoplastic transformation in hamster and mouse cells" by Carmia Borek, Richard Miller, Cynthia Pain, and Walter Troll, which appeared in the April 1979 issue of Proc. Natl. Acad. Sci. USA (76, 1800–1803), some of the numbers should be corrected. In Table 1 on p. 1802, in column A the numbers in the last four lines should be 2,200, 3,200, 3,900, and 4,200. In Table 2 on p. 1802, the heading of the fifth column should read B/A x 10^3. In column A, line 4 should be 6,500; lines 7 and 8 should be 17,000 and 11,000; and line 12 should be 10,000. The yield ratios in the last columns of these tables are correct as printed.

Correction. In the article "On the evolution of accuracy and cost of proofreading RNA aminoacylation" by Michael A. Savageau and Rolf R. Freier, which appeared in the September 1979 issue of Proc. Natl. Acad. Sci. USA (76, 4507–4510), an error occurred in the Proceedings editorial office. On page 4507, in the right-hand column, Eq. 1 should read:

\[
E = \frac{1}{2} \frac{1}{(P-1)(I+1)} \times (|(P-1) - (1+C) - I(1+PC)| \nonumber \\
+ \sqrt{((P-1) - (1+C) - I(1+PC))^2 + 4(P-1)(I+1)(1+C)}} \quad [1]
\]

Correction. In the article "Monoclonal antibody directed to a B-cell antigen present in rats, mice, and humans" by David L. Gasser, B. Alleen Winters, Jean B. Haas, Thomas J. McKearn, and Roger H. Kennett, which appeared in the September issue of Proc. Natl. Acad. Sci. USA (76, 4636–4640). Fig. 2 was printed upside down. The correct arrangement is:

FIG. 2. Autoradiography of polyacrylamide gel comparing ^35S-labeled H76 antibody (Left) with ^35S-labeled myeloma protein produced by the mouse P3/X63Ag8 cells (Right).
Conditions for inhibiting and enhancing effects of the protease inhibitor antipain on x-ray-induced neoplastic transformation in hamster and mouse cells

(proteases/DNA repair/x-ray damage/expression of carcinogenesis/fixation of transformation)

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Communicated by Richard B. Setlow, December 29, 1978

ABSTRACT Using cultured normal hamster embryo cells and the heteroploid mouse C3H cell line 10T½ clone 8, we have studied the effect of the protease inhibitor antipain on x-ray-induced neoplastic transformation. We found in both cell systems that, while there was no effect on cell survival as compared to irradiated controls, the addition of antipain at a concentration of 6 μg/ml to the cultures 24 hr prior to irradiation resulted in enhanced transformation as compared to the frequency in cultures exposed to radiation alone. Yet the addition of antipain to cultures 10 min after irradiation resulted in a decreased transformation rate. This decrease was not found when antipain was added to the mouse cells 48 hr after irradiation or to the hamster cells 48 hr after irradiation. These results suggest that the protease inhibitor antipain has more than one mechanism of action in modulating the fixation and expression of transformation by x-irradiation, possibly by the modification of DNA repair.

Protease inhibitors (1) have been shown to inhibit the expression of mutations in bacteria (2) and to inhibit the tumor-promoting effect of phorbol esters in mice (3).

We have investigated the effect of the protease inhibitor antipain on cell transformation by x-irradiation in two in vitro systems; in short-term cultures of freshly explanted hamster embryo cells (4–12) and in the 10T½ cell line derived and cloned from C3H mouse embryo (13, 14).

We found an enhancement of cell transformation when antipain was added to the cultures prior to irradiation and was present during the course of exposure to x-rays. On the other hand, we found an inhibition of cell transformation by x-rays when antipain was added 10 min after irradiation. No significant effects were observed in the mouse cells when antipain was added 24 hr after their exposure to x-rays or in the hamster cells when antipain was added 48 hr after x-irradiation.

MATERIALS AND METHODS

Cell Cultures. Hamster embryo cells. Minced midterm whole embryos from golden hamsters (Lakeview, Wilmington, MA) were used as the source of normal cells. Primary cultures were established by progressive dissociation of the minced fresh tissue (4–8). For transformation experiments, the cells in 3-day-old primary cultures were suspended by trypsinization and 10⁴ cells were cloned into 100-mm petri dishes (Falcon) on x-irradiated (4000 rad) (1 rad = 0.01 gray) feeder cells (15) of the same type in Dulbecco’s modified Eagle’s medium supple-mented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) (6–8). C3H 10T½ clone 8 cell line. This line was kindly supplied by C. Heidelberger (13, 14). Cell cultures were maintained in Eagle’s basal medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). For transformation experiments, 6000 cells were plated onto 100-mm petri dishes, allowing for the survival of 400–500 cells after radiation.

X-Irradiation. Cells were irradiated at room temperature, 24 hr after plating, with 300 rad of x-rays at a dose rate of 32.2 rad/min and incubated at 37°C with 5% CO₂ in air, with weekly medium changes. The source of x-rays was a Siemens 300 kVp constant potential generator with an added filter of 0.2 mm of copper. Controls consisting of unirradiated cultures with or without added antipain were maintained under the same conditions as the experimental cultures.

After the incubation period, 2 weeks for the hamster cells to allow for colony growth (6–8) and 6 weeks for the 10T½ cells to allow for focus formation (14), cells were fixed and stained with Giemsa and assayed for transformation.

Antipain. The water-soluble protease inhibitor antipain, [(S)-1-carboxy-2-phenylethyl] carbamoyl-L-arginyl-L-valyl-argininal (1), was obtained from the U.S.-Japan Cancer program. Antipain was added to the cell cultures in complete medium at a final concentration of 6 μg/ml. Fig. 1 describes the protocol employed. Either antipain was added at plating

![Diagram](https://example.com/antipain_diagram.png)
time and kept on during irradiation and throughout the period of incubation, or, alternately, antipain was added at 10 min, 24 hr, or 48 hr after irradiation. After the initial addition, antipain was maintained in the medium throughout the period of incubation.

**Transformation Assay.** *Hamster embryo cells.* After the appropriate period of incubation, medium was removed and cells were fixed and stained with Giemsa (4–8). A differential count was made of normal and transformed colonies, the latter being identified by their morphological appearance from among the surviving colonies (4–8). The number of normal and transformed colonies was scored so that both transformation frequency and cell survival could be assessed within the same experiment. Transformed colonies were identified by their irregular growth pattern and their tendency to form multilayers as compared to controls (4–8) (Fig. 2). The relationship between this morphology and the malignant nature of the cells has been documented (6–8).

**10T½ Cells.** After the 6-week incubation period, dense foci of transformed cells growing as a multilayer were easily distinguishable morphologically from the flat confluent sheets of untransformed cells (Fig. 2). The guidelines described by Borek et al. (18, 14) for assessing cell survival and evaluating cell transformation were strictly followed. Both type II and type III transformed foci (14, 16–18) were scored, type III being the more dense and irregular of the two.

**RESULTS**

A summary of the experimental protocol employed and the overall observations obtained are presented in Fig. 1.

Cell survival and transformation data after treatment with 300 rad of x-irradiation and antipain are presented in Table 1 for the hamster embryo cells and in Table 2 for the mouse 10T½ cells. While antipain treatment before or after exposure to irradiation did not alter plating efficiency or cell survival in any significant way as compared to cells treated with x-rays alone, the effects of antipain in modifying x-ray-induced neoplastic transformation are most striking. These effects are similar qualitatively in the two cell systems. When antipain is added to the culture medium prior to radiation treatment, the result is enhanced transformation frequency, yet the addition of antipain within 10 min after radiation results in decreased transformation frequency. The protective effect of antipain is diminished in the hamster cells when the protease inhibitor is

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**Fig. 2.** (A) A 14-day-old normal colony of hamster embryo cells. (Giemsa, X25.) (B) A 14-day-old colony of hamster cells transformed by 300 rad of x-irradiation. Note the dense multilayering within the colony compared to the normal and the criss-cross irregular pattern at the periphery of the colony. (Giemsa, X25.) (C) A monolayer of untransformed 10T½ mouse cells 6 weeks in culture. Note the flat sheet of cells. (Giemsa, X25.) (D) A type III focus of 10T½ cells transformed by 300 rad of x-irradiation and 6 weeks in culture. Note the dense multilayering within the focus. (Giemsa, X25.)
Antipain added before x-irradiation with 300 rad

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Surviving fraction</th>
<th>Total colonies (A)</th>
<th>No. colonies transformed (B)</th>
<th>Transformation incidence (B/A × 10^3)</th>
<th>Yield with antipain + x-ray</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>3,500</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>Antipain</td>
<td>0.94</td>
<td>3,500</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>X-irradiation</td>
<td>0.60</td>
<td>6,500</td>
<td>43</td>
<td>6.6 ± 1.0</td>
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</tr>
<tr>
<td>X-irradiation and antipain</td>
<td>0.55</td>
<td>8,700</td>
<td>106</td>
<td>12.3 ± 1.2</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>

Antipain added after x-irradiation with 300 rad

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Surviving fraction</th>
<th>Total colonies (A)</th>
<th>No. colonies transformed (B)</th>
<th>Transformation incidence (B/A × 10^3)</th>
<th>Yield with antipain + x-ray</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>20,000</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Antipain</td>
<td>0.96</td>
<td>16,000</td>
<td>0</td>
<td>0</td>
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<tr>
<td>X-irradiation</td>
<td>0.53</td>
<td>15,000</td>
<td>129</td>
<td>8.4 ± 0.7</td>
<td>0.4 ± 0.1</td>
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<tr>
<td>X-irradiation and antipain</td>
<td>0.44</td>
<td>17,000</td>
<td>62</td>
<td>3.7 ± 0.5</td>
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<tr>
<td>24 hr</td>
<td>1.00</td>
<td>14,000</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Antipain</td>
<td>0.92</td>
<td>14,000</td>
<td>0</td>
<td>0</td>
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<tr>
<td>X-irradiation</td>
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<td>13,000</td>
<td>85</td>
<td>6.5 ± 0.7</td>
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<tr>
<td>X-irradiation and antipain</td>
<td>0.59</td>
<td>15,000</td>
<td>75</td>
<td>5.0 ± 0.6</td>
<td>0.8 ± 0.1</td>
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<tr>
<td>48 hr</td>
<td>1.00</td>
<td>22,000</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Antipain</td>
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<td>32,000</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>X-irradiation</td>
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<td>39,000</td>
<td>25</td>
<td>6.4 ± 1.3</td>
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<tr>
<td>X-irradiation and antipain</td>
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<td>42,000</td>
<td>25</td>
<td>5.9 ± 1.2</td>
<td>0.9 ± 0.3</td>
</tr>
</tbody>
</table>

Surviving fraction = (No. of colonies)/[(No. of cells plated) (plating efficiency)].

... added 24 hr after irradiation and is virtually eliminated when added at 48 hr after radiation (Table 1). In the 10T½ mouse cells this loss of protection is observed when antipain is added 24 hr after irradiation (Table 2).

**DISCUSSION**

We have shown that the protease inhibitor antipain has two opposing modes of action in modulating the frequency of cell transformation in vitro by x-rays. In both diploid short term hamster embryo cell cultures and the heteroploid 10T½ mouse cell line the addition of antipain to the cultures prior to irradiation resulted in enhanced cell transformation. In sharp contrast, when added soon after irradiation, antipain had a protective effect, resulting in decreased cell transformation. Under both sets of conditions in which transformation rate was modified the presence of antipain did not influence cell survival in any significant way, suggesting, as in earlier work (7), that x-ray-induced cellular damage responsible for cell transformation may differ from that responsible for cell survival.

The two diametrically opposed effects of antipain strongly suggest that the protease inhibitor antipain has more than one mechanism of action in regulating cellular or molecular events involved in the fixation and expression of cell transformation by x-irradiation (5). Some of these may be involved in the modification of DNA repair (19-26).

The protective effect of antipain added after exposure of the cells to x-rays is a temporal phenomenon and is most efficient...
when antipain is added within minutes of irradiation. Its addition, to the mouse cells 24 hr after exposure to x-rays and to the hamster cells 48 hr after irradiation, had no significant effect on the radiation-induced frequency of transformation.

While the molecular mechanisms of this protective effect are not understood and are yet to be explored, it is of interest to speculate on the possibilities. It is at once clear that the effect is mediated by the inhibition of early events required for the fixation of transformation by x-rays [the fixation has been shown to be a temporal event (5) and a subsequent phenotypic expression of this fixation (5, 16)]. Antipain has been shown to inhibit the induction of postreplication error-prone DNA repair (SOS systems) in bacteria exposed to UV irradiation (2, 25, 26); thus the decreased yield of transformation found when antipain is added after irradiation could be interpreted as a result of the inhibition of postreplication DNA repair mechanisms.

While there is no direct molecular evidence for these DNA repair processes in mammalian cells exposed to x-irradiation, an induction of this type of repair is suggested by our transformation studies with fractionated doses of x-rays. We have reported (7, 17) that both hamster and 10T½ cells exposed to low doses of x-rays split into two fractions separated by 5 hr have enhanced cell transformation frequency as compared to the frequency observed after exposure to a single acute dose.

An alternate mechanism for the protective effect of antipain, or perhaps one that acts in addition or as part of the above mode of action, may be mediated by the inhibition of cellular proteases associated with the expression of neoplastic transformation (27-31).

The enhancement of transformation frequency of antipain when the protease inhibitor is added prior to irradiation could be due to a direct modification of DNA by x-irradiation and an inhibition by antipain of some rapid error-free DNA repair (22, 23), yet to be defined, involved in removing x-ray-damaged regions from DNA. This could result in a selective disturbance of DNA repair (32), different from that responsible for cell survival (32), and result in an enhanced rate of transformation.

This investigation was supported by Grant CA 12536 to the Radiological Research Laboratory, Grant CA 13696 to the Cancer Center/Institute of Cancer Research, and Grant CA 16060 to New York University, all awarded by the National Cancer Institute, U.S. Department of Health, Education and Welfare.