Correlation between the Loss of the Transformed Phenotype and an Increase in Superoxide Dismutase Activity in a Revertant Subclone of Sarcoma Virus-infected Mammalian Cells

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ABSTRACT

We have studied the effects of paraquat (methyl viologen), a herbicide that increases intracellular production of superoxide radical, on the viability of virus-transformed and nontransformed normal rat kidney (NRK) cells in culture. We have shown that a low concentration of paraquat (12.5 μM) is cytotoxic toward virus-transformed cell lines, including Kirsten sarcoma virus- and SV40-transformed NRK cells. The corresponding untransformed NRK cells were resistant to the same and a 4-fold higher concentration of paraquat. There was good correlation between the susceptibility of transformed and untransformed cells to paraquat cytotoxicity and their ability to increase the superoxide dismutase (SOD) enzymatic activity. We found that paraquat is cytotoxic toward Kirsten sarcoma virus-transformed and SV40-transformed NRK cells which showed low intracellular SOD activity. The relationship between SOD activity and paraquat cytotoxicity was strengthened by the finding that the tolerance of NRK cells to the drug was associated with high intracellular SOD activity.

This report also describes the isolation of a revertant (revertant RE8G3) cell line derived from Kirsten sarcoma virus-transformed NRK cells after paraquat treatment which contains SOD activity at levels much higher than those found in NRK cells. This revertant is undistinguishable from NRK cells with respect to its lack of transformed cell properties. Not only are these cells normal morphologically but also they do not grow in soft agar, an in vitro property that closely correlates with in vivo tumorigenicity. Several biological and biochemical properties of RE8G3 cells, including growth characteristics, surface receptors for both transferrin and epidermal growth factor (EGF), and the EGF-dependent 32P phosphorylation of specific membrane polypeptides have been studied. The most interesting conclusion that can be drawn from these studies is that there is a correlation between loss of the transformed phenotype and an increase in both EGF receptors and EGF-dependent 32P phosphorylation of a m.w. 170,000 membrane-associated protein.

INTRODUCTION

Paraquat (methyl viologen), a broad-spectrum herbicide, is toxic not only to vegetable organisms but also to mammalian tissues (5, 15, 26, 30). The major factor in paraquat toxicity appears to be increased production of superoxide anion arising from the reaction of reduced paraquat with molecular oxygen (13, 15, 16). Paraquat undergoes repeated cycles of oxidation and reduction; thus, it serves as a continuing source of O$_2^\cdot$ (16). This has been demonstrated in several biological systems including chloroplasts (14, 22), lung microsomes (23), and homogenates of kidney, liver, and lung (2).

SOD, an enzyme which catalyzes the dismutation of the potentially damaging reactivity of the superoxide anion to the less toxic hydrogen peroxide and molecular oxygen, is believed to be present in all oxygen-metabolizing cells (12, 13). The physiological function of SOD is to protect cells against the harmful activities of the superoxide radical generated by aerobic metabolic reactions (12, 13). It has been reported that the SOD activity in numerous transformed cell lines is abnormal (4, 24, 31). Reduced amounts of manganese-containing mitochondrial SOD have been found in all tumors examined (24). Diminished amounts of the copper- and zinc-containing cytosol SOD have been found in numerous, but not all, neoplasms (24). These findings suggest that diminished enzyme activities along with radical production by tumor cells may be responsible for many of the observed properties of cancer cells (24). Other studies have suggested that the differences between normal and cancer cell SOD activity may be exploited in the treatment of cancer (1, 24).

Because superoxide anion has been implicated in the toxicity of paraquat (13) and diminished SOD levels have been found in a number of transformed cells (24), it was of interest to examine the cytotoxicity of paraquat toward normal and virus-transformed cells in culture to determine whether it is cytotoxic only to virus-transformed cells. In addition, experiments were performed to determine whether these cells will increase their SOD specific activities in response to paraquat, which has been reported to increase intracellular levels of specific SOD isoenzymes in biological systems (13, 15). In this report, we document the selective cytotoxicity of paraquat toward virus-transformed cells as well as the relationship between SOD activity and paraquat cytotoxicity in cultured cells. This report also describes the isolation and characterization of an interesting revertant (revertant EB8G3) cell line from K-NRK cells after paraquat treatment which expresses a marked increase in its SOD activity when compared with the specific activity of SOD from NRK cells.

MATERIALS AND METHODS

Chemicals. Methyl viologen (1,1'-dimethyl-4'-bipyridinium dichloride), NBT, and xanthine were purchased from Sigma Chemical Co.

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pany (St. Louis, Mo.). Xanthine oxidase (0.4 unit/mg) was obtained from Boehringer Mannheim (New York, N. Y.); [γ-32P]ATP (1000 Ci/mmol) and Na125I (carrier free) were obtained from New England Nuclear (Boston, Mass.). Sources of other materials were as described previously (9–11).

Cells. Cells were grown in DME containing 10% (v/v) calf serum as indicated elsewhere (10). Stock cultures were started from frozen cells at approximately 2-month intervals. NRK-B clone 18, K-NRK clone 32, and SV-NRK-B clone 2 were kindly provided by Dr. R. Ting (Biotech Research, Inc., Rockville, Md.).

Viability was determined (a) by the capacity of the cells to resume normal growth following paraquat treatment; (b) by their plating efficiency on plastic, determined 10 days after seeding the cells; and (c) by their capacity to exclude trypan blue (7). Apparently, cells cannot be stained with trypan blue unless they are irreversibly damaged (7, 17).

Several revertant cell lines were isolated by cloning. Cells were diluted with culture medium to a final concentration of less than 1 cell/ml. The cell suspension (0.1 ml) was added to each well of a Falcon Microtest II plate. Twenty-four hr later, wells were scored for single cells.

 Colony formation in agar was measured according to the procedure of Macpherson and Montagnier (21). The number of colonies (>50 cells/colony) formed was determined on a portion of each 60-mm plate 14 days after plating 106 cells.

SOD Assay. Cultures were assayed for SOD activity after they had become confluent to minimize the fluctuations in activity that occur with changes in cell density. A cell grown in 100-mm dishes were placed on a bed of ice, the culture medium was decanted, and the monolayers were washed 4 times each with Dulbecco's phosphate-buffered saline at 0–4°C. The cells corresponding to 5 pooled dishes were scraped into 10 ml of this solution with a Teflon spatula and centrifuged at 3000 x g for 10 min at 0–4°C, and the pellet was resuspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.8). The cell suspensions were then sonicated at maximum output of a sonic dismembrator 300 (Artek Systems Co., Farmingdale, N. Y.); the cell suspensions were sonicated on ice in 1-min bursts for a total of 2 min. The samples were centrifuged at 30,000 x g for 60 min at 0–4°C, and the supernatant was used in the SOD assay. The samples were stored at −20°C until the assay was performed.

SOD activity was assayed according to the NBT method of Beauchamp and Fridovich (3). The initial rate of reduction of NBT was determined by monitoring the decrease in absorbance at 560 nm and 25°C. The reaction mixture contained 2.5 x 10−5 M NBT, 1 x 10−4 M xanthine, 10−3 M xanthine oxidase, 1 x 10−4 M EDTA, 50 mM sodium carbonate (pH 10.2), and varying sample protein concentrations. The initial rate of NBT reduction was measured by the difference in optical density at 560 nm and 25°C.

RESULTS

Effect of Paraquat on Growth and Viability of Normal and Transformed Cells. In initial experiments to examine the effects of paraquat on growth and viability of normal and transformed cells, cultures were incubated for 48 hr in medium with or without various doses of paraquat. Chart 1 shows that the growth of NRK cells was inhibited by paraquat in a dose-dependent manner. After 36 to 48 hr in 50 μM paraquat, NRK cells acquired a more flattened morphology, and no mitotic cells or further increase in cell number were observed. Following 48 hr incubation with paraquat (50 μM), normal growth rate and morphology were restored in 24 hr after removal of paraquat from the medium of NRK cells. Paraquat (50 μM) was not cytotoxic to NRK cells even after treatment for 72 hr (Table 1). Higher doses of paraquat (100 μM), however, caused a pronounced decrease in the rate of cell growth of NRK cells (Chart 1) and extensive cytotoxicity was noted by 36 to 48 hr (data not shown).

Paraquat also inhibited K-NRK and SV-NRK cell growth in a dose-dependent manner (Chart 1). After 24 hr of treatment with 50 or 100 μM paraquat, there was no further increase in cell number (Chart 1). When treated with 50 μM paraquat, most of the transformed cells acquired a rounded morphology by 24 hr and subsequently detached from the culture dish. The detached cells were not viable after 72 hr of treatment with 50 μM paraquat. The detached cells stained with trypan blue (Table 1) and did not reattach when subcultured in new medium without paraquat. The detached cells did not form colonies when plated at a density of 2000 cells/50 sq cm dish and cultured for 10 days. The majority of the few cells remaining attached to the dish after 72 hr of 50 μM paraquat treatment were also shown to be irreversibly damaged by trypan blue staining (Table 1). These cells did not form colonies when cultured in fresh medium for 10 days.

Effects of Prolonged Incubation with Paraquat and Isolation of Revertant Cells. The effects of prolonged incubation of NRK cells in medium containing a low dose of paraquat (12.5 μM) were tested. Paraquat (12.5 μM) caused a slight decrease in the rate of growth of NRK cells (15 to 25% inhibition) but caused no cell death in either sparse or confluent cultures after treatment for 4 days. With frequent media changes, NRK cells proliferated in the presence of paraquat and remained spread on the substratum in a normal manner for more than 5 weeks. Thus, NRK cells were remarkably tolerant to the cytotoxic action of 12.5 μM paraquat for prolonged periods of time.

In contrast, 12.5 μM paraquat was cytotoxic to K-NRK and SV-NRK cell lines. After 4 days in paraquat (12.5 μM), the growth rate of virus-transformed cells was markedly decreased, and many rounded cells detached from the culture dish and were lost in the medium changes. The majority of the detached cells (more than 90%) were not viable (data not shown). After 8 days, less than 0.01% of the transformed cells

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* Unpublished observations.
Selective Cytotoxicity of Paraquat and SOD Activity

Chart 1. Dose dependence of growth inhibition in NRK, K-NRK, and SV-NRK cells treated with paraquat. Cells were plated at 7.5 x 10^5 cells/dish; 24 hr later, the medium was removed, and new media containing paraquat at the indicated concentrations were added. The control cultures were treated identically but without the drug. Cell counts were determined at the indicated times; each point is the average of duplicate measurements from 2 cultures. ○, control; ●, 50 µM; ■, 100 µM.

Table 1

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Paraquat (µM)</th>
<th>% of survival ^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRK</td>
<td>12.5</td>
<td>Attached</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Detached</td>
</tr>
<tr>
<td>SV-NRK</td>
<td>12.5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>K-NRK</td>
<td>12.5</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>ND ^d</td>
</tr>
</tbody>
</table>

^a Cells were incubated in medium with or without the indicated concentrations of paraquat for 72 hr.
^b Fraction of total cells counted which did not stain with trypan blue. Cells attached to the dish or in suspension were exposed to trypan blue as indicated in Ref. 7 and counted. The percentage exclusion by untreated cultures was normalized to 100% for comparison with paraquat-treated cultures.
^c ND, not done.

The cells were incubated in medium with or without the indicated concentrations of paraquat for 72 hr. The percentage exclusion by untreated cultures was normalized to 100% for comparison with paraquat-treated cultures.

incubated with 12.5 µM paraquat remained attached to the substratum. These cells remained flat and attached to the substratum without any visible mitotic activity for an additional 6 days in paraquat. After this quiescent period, the cells began rapid proliferation and colonies developed. The majority of these cells appeared morphologically similar to normal, untransformed NRK cells. These transformed cells, which reverted morphologically to the normal phenotype, developed into monolayer populations of revertant cells resistant to 12.5 µM paraquat. Several revertant cell lines (clones C6, G5, and E8) derived from K-NRK cells were isolated by cloning in media containing 12.5 µM paraquat. After 16 passages in 12.5 µM paraquat, revertant line E8B4 was subcloned from revertant line E8. Subsequent recloning of line E8B4 resulted in revertant subclone E8G3. Although clones E8 and E8B4 and a clone (RP1) independently developed were utilized in these studies, the majority of the experiments described here were with clone E8G3. A total of 6 revertant clones have been isolated from SV-NRK cells grown in 12.5 µM paraquat. These clones have not been characterized further.

Biological Properties of Revertant Cells. By phase-contrast microscopy (Fig. 1A), the revertants resemble NRK cells. Revertant E8G3 cells selected from K-NRK cells had a flattened appearance quite different from the spindly and round shapes typical of K-NRK cells (Fig. 1). Unlike NRK cells, which were fibroblastic at low densities, the revertants tended to have the same epithelial morphology at all cell densities.

A comparison of various growth parameters of NRK, K-NRK, and revertant RE8G3 cells is given in Table 2. Revertant RE8G3 exhibited a population-doubling time slightly slower than that of NRK cells (15 to 20% increase in the generation time), whereas K-NRK demonstrated a slightly faster growth rate (10 to 10.5 hr). Upon reaching a single confluent layer of cells, both NRK and RE8G3 cells cease growing and have a characteristically low saturation density. The saturation density achieved by the revertant RE8G3 was somewhat lower than that of NRK cells and was significantly different from that of K-NRK cells. RE8G3 also resembles NRK cells in the concentration of serum required to sustain growth. In 1% serum, RE8G3 and NRK cells either fail to grow or grow with a greatly increased generation time (>100 hr). The K-NRK cells are capable of growing in medium supplemented with 1% serum with a somewhat increased generation time (<50% inhibition in growth rate). Another property by which the revertant RE8G3 resembles NRK cells is its inability to form colonies in soft agar, whereas K-NRK cells produce colonies with high efficiency. Thus, by this criterion, the revertants grown in paraquat retain the untransformed phenotype as stably as do NRK cells.

In the presence of 12.5 µM paraquat, the cellular morphology of clone RE8G3 has been maintained continuously for more than 200 generations, and no cultures have yet reverted to the transformed morphology. Chart 2 shows that morphological transition of RE8G3 cells occurred when cultures were deprived from the presence of paraquat. For RE8G3 cells adapted
caused a significant increase in SOD specific activity in both of paraquat, no significant changes were observed in SOD specific activity in NRK cells (Table 3). As shown in Table 3, specific activity in K-NRK cells exposed to the drug. Paraquat specific activity in NRK cells increased significantly in the presence of paraquat. The most important biological phenomenon correlated with these observations is that both normal and revertant cells are resistant to paraquat, whereas K-NRK is not.

**SOD Specific Activity of Normal, Transformed, and Revertant Cells.** Since it has been suggested that changes in SOD activity play a key role in the mechanism of adaptation to paraquat in both plant and animal systems (13), it was important to examine the SOD specific activity of normal, transformed, and revertant cells cultured in the absence and presence of paraquat. The results of such analysis with cells cultured in control media indicated that SOD specific activity present in NRK cells was higher than that of K-NRK (Table 3) and SV-NRK (data not shown) cells. Experiments in control media resulted in the most intriguing observation concerning the revertant RE8G3 (Table 3). This cell clone contained SOD activity with a drastically increased (by a factor of 6) SOD specific activity when compared with its direct parental cell line K-NRK. We have also found increased SOD specific activity in another revertant clone (RE8B4) cultured in control media when compared with NRK cells (Table 3). As shown in Table 3, the effects of addition of paraquat (100 μM) to cultures of NRK, K-NRK, and revertant cells were studied. While the SOD specific activity in NRK cells increased significantly in the presence of paraquat, no significant changes were observed in SOD specific activity in K-NRK cells exposed to the drug. Paraquat caused a significant increase in SOD specific activity in both RE8B4 and RE8G3 revertant clones (Table 3). Although in control experiments revertant RP1 contained SOD activity similar to that of K-NRK cells, we have also found that revertant RP1 exhibited the ability to increase significantly SOD activity in the presence of paraquat. The most important biological phenomenon correlated with these observations is that both normal and revertant cells are resistant to paraquat, whereas K-NRK is not.

**EGF and Transferrin Binding to NRK, K-NRK, and Revertant RE8G3 Cells.** In view of the previously reported loss of EGF-binding sites following transformation by RNA sarcoma viruses (28, 29), we examined the possibility that reversion of viral transformation might also result in restoration of EGF-binding sites. For this purpose, cultured NRK, K-NRK, and RE8G3 cells were analyzed for the ability to bind 125I-labeled EGF. Time-course studies showed that maximum binding to

### Table 2

**Growth properties of normal, transformed, and revertant RE8G3 cells**

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Growth pattern</th>
<th>Doubling time&lt;sup&gt;a&lt;/sup&gt; (hr)</th>
<th>Saturation density&lt;sup&gt;b&lt;/sup&gt; (x 10&lt;sup&gt;5&lt;/sup&gt;/sq cm)</th>
<th>Serum requirement&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Colony-forming efficiency in agar&lt;sup&gt;d&lt;/sup&gt; (%)</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>Flat</td>
<td>11–12</td>
<td>1.2</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Revertant RE8G3</td>
<td>Flat</td>
<td>13–14</td>
<td>1.1</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Transformed</td>
<td>Round, spindle-like</td>
<td>10–10.5</td>
<td>1.9</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>81.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> The doubling time was determined with cells grown in 10% serum while the cells were in log-phase growth.

<sup>b</sup> Due to decreased substratum adhesiveness of K-NRK cells, it was not possible to determine an accurate saturation density for these cells; the values are probably greater than those shown.

<sup>c</sup> +++, maximal growth rate; +, less than 50% inhibition in growth rate; –, no growth or greatly increased generation time.

<sup>d</sup> Cells (10<sup>5</sup>) were seeded per 60-mm dish. Colonies of >50 cells were counted at 2 weeks. Cloning efficiency = (number of colonies × 100)/10<sup>5</sup>.

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**Chart 2.** Growth and morphological transitions of revertant RE8G3 after removal of paraquat. Stock cultures were started simultaneously from frozen cells obtained at set time intervals after removal of paraquat. Cells were plated in normal medium at 1.5 x 10<sup>5</sup> cells/60-mm dish. After 48 hr, the medium was removed, and new medium was added. Forty-eight hr later, the cultures were photographed, the cells were counted, and the amount of protein per dish was determined. The appearances of the cells at the indicated times after removal of paraquat are shown. Each cell count is the average of duplicate measurements from 2 cultures. The amount of protein per dish is the average from 3 cultures. Note that the cellular densities at the time of harvest were similar except for Day 1. Similar morphology and growth patterns were observed in 4 separate experiments. For details, see the text.
NRK cells was reached between 60 and 90 min and indicated remarkable differences among NRK, K-NRK, and RE8G3 cells (Chart 3). We found that NRK cells exhibited a large $^{125\text{I}}$-EGF-binding capacity (Chart 3). In contrast, K-NRK cells showed little $^{125\text{I}}$-EGF binding, which was estimated to be about 10 to 15% of NRK cell binding (Chart 3). Revertant RE8G3 cells exhibited a greater $^{125\text{I}}$-EGF-binding capacity than that of K-NRK cells (Chart 3), which in cells grown in the presence of paraquat was estimated to be about 60% of NRK cell binding capacity. When RE8G3 cells were switched from paraquat-containing medium to control medium, $^{125\text{I}}$-EGF-binding capacity decreased gradually over 30 days (Chart 4A). At this time, RE8G3 cells exhibited 10 to 15% of NRK cell binding. Since viral transformation of certain cell lines is accompanied by changes in transferrin receptor activity (8), it was of interest to us to determine whether reversion of viral transformation might also lead to changes in $^{125\text{I}}$-transferrin-binding capacity analogous to those observed with EGF. Time-course studies demonstrated that maximum $^{125\text{I}}$-transferrin binding to NRK cells was reached between 45 and 60 min and indicated significant differences between NRK, K-NRK, and RE8G3 cells. We found that NRK cells consistently exhibited a large $^{125\text{I}}$-transferrin-binding capacity. In contrast, K-NRK cells showed a reduced $^{125\text{I}}$-transferrin-binding capacity, which was estimated to be about 30 to 35% of NRK cell binding. Revertant RE8G3 cultured in the presence of paraquat consistently bound a greater amount of $^{125\text{I}}$-transferrin which was determined to be about 70% of NRK cell binding. On transfer of the RE8G3 cells to medium without paraquat, $^{125\text{I}}$-transferrin-binding capacity declined gradually over 30 days (Chart 4B). At this time, the $^{125\text{I}}$-transferrin-binding capacity of RE8G3 cells was similar to that of K-NRK cells. From these experiments, we conclude that the reduction in both EGF and transferrin receptors in RE8G3 cells after removal of paraquat correlated with the transformed phenotype.

EGF-dependent Phosphorylation of NRK, K-NRK, and RE8G3 Membrane Components. We have previously shown that the incubation of both NRK and K-NRK membranes with $[\gamma^{32}\text{P}]$ATP resulted in the phosphorylation of numerous membrane components, the phosphorylation of which was enhanced by EGF (10, 11). We found that NRK membranes exhibited high levels of EGF-stimulated $^{32}\text{P}$ phosphorylation of a m.w. 170,000 protein (11). In contrast, K-NRK membranes exhibited a marked reduction in the EGF-dependent $^{32}\text{P}$ phosphorylation of the m.w. 170,000 protein (11). From a comparison of the results with NRK and K-NRK membranes (11) and the data presented in Fig. 2, Lanes a and b, it can be inferred that the levels of EGF-dependent phosphorylation of the m.w. 170,000 protein of RE8G3 cells were restored toward control NRK levels. Revertant RE8G3 cells cultured in paraquat-containing medium consistently exhibited high levels of EGF-dependent $^{32}\text{P}$ phosphorylation of the m.w. 170,000 protein which was estimated to be about 60 to 70% of the EGF-stimulated phosphorylation of the m.w. 170,000 protein of control NRK cells. When RE8G3 cells were switched from paraquat-containing medium to control media, the EGF-dependent phosphorylation of the m.w. 170,000 protein declined rapidly between 1 and 15 days (Chart 5A). The phosphorylation of the m.w. 170,000 protein reached a minimal level of approximately 20% of control levels (EGF-stimulated phosphorylation at t = 1 day) by 15 days. The patterns of radioactivity in Fig. 2 demonstrate that only the m.w. 170,000 band in RE8G3 cells switched from paraquat-containing media to control media has drastically reduced radioactivity while the radioactivity of other bands
Chart 4. Binding of EGF and transferrin (Tf) to revertant R-E8G3 cells as a function of time after removal of paraquat. The cells were grown and allowed to attain confluency as indicated in the legend to Chart 2. Cells were rinsed free from growth medium, and the binding of either 125I-EGF (A) or transferrin (B) was determined. Medium (1.5 ml) containing either 125I-EGF (2 ng/ml, 100,000 cpm/ng) or 125I-transferrin (0.5 ng/ml, 100,000 cpm/ng) was added to each confluent 60-mm culture dish for 90 or 60 min, respectively. Cells were then harvested, and specific binding was determined. These relationships were consistently observed in 3 separate binding experiments.

Chart 5. Relative phosphorylation of the m.w. 170,000 protein (A) and the m.w. 130,000 protein (B) are plotted as a function of time after removal of paraquat. The phosphorylation assays were performed in the absence (O, −) or presence (•, +) of 120 nM EGF.

either remained relatively constant or was reduced but apparently to a much lower extent. For example, Chart 5B shows that the radioactivity in one prominent m.w. 130,000 band was slightly reduced (15% decreased) after removal of the drug for 31 days. Furthermore, the decrease in the radioactivity of all the other protein bands, except the m.w. 170,000 and m.w. 130,000 bands, after removal of paraquat did not exceed 30% of control levels at all time points examined. For purposes of comparison the gel patterns of R-E8G3 membranes phosphorylated in the absence of EGF are also shown (Fig. 2). As expected from the much lower phosphorylation of membrane components in the absence of EGF, the m.w. 170,000 band was weakly phosphorylated in the basal state (Fig. 2, Lane a). As shown in Chart 5A, switching the R-E8G3 cells from paraquat-containing media to control media produced a detectable decrease in the basal phosphorylation of the m.w. 170,000 protein within 4 days. This was followed by relatively constant basal phosphorylation levels of the m.w. 170,000 protein. Thus, from this series of experiments, we conclude that in R-E8G3 cells there is a correlation between the transformed phenotype, the decrease in EGF receptors, and the reduction in EGF-dependent 32P phosphorylation of the m.w. 170,000 membrane protein.

DISCUSSION

The experiments presented here demonstrate that a low concentration of paraquat is cytotoxic toward SV40- and Kir-
sten sarcoma virus-transformed cell lines and is not toxic to nontransformed NRK cells under similar conditions. Analysis of SOD levels show that there is a correlation between that metabolic parameter and sensitivity to paraquat in virus-transformed cells; i.e., the levels of SOD activity are not significantly increased in the virus-transformed cells exposed to paraquat but are strongly enhanced in nontransformed NRK cells which are not killed by the paraquat treatment. We speculate that the selective cytotoxicity of this drug toward virus-transformed cells might reflect a general inability of tumor cells to survive an increased superoxide-producing state, while nontransformed cells might be relatively resistant because they are more effective in neutralizing the enhanced superoxide metabolism. However, we emphasize that the mechanism of paraquat toxicity remains unknown.

Revertant RE8G3 cells offer a number of interesting properties that contribute to their usefulness in the understanding of the nature of virus-induced transformation and adaptation to drugs. The isolated clone RE8G3 exhibits unique properties when compared with NRK cells. This clone of revertant cells exhibited a complete reversion not only in cellular morphology, saturation density, and serum requirements for growth but also in the ability to grow in agar suspension. Growth in soft agar has been found by others to be a reliable indicator of the transformed phenotype of a cell and tumorigenic potential (25). Thus, these cells are indistinguishable from NRK cells with regard to their growth properties and normal phenotype.

The most interesting aspect of the revertant subclone RE8G3 is the marked increase in its SOD activity in the presence and absence of paraquat when compared with the specific activity of SOD from NRK cells. The marked increase in SOD activity occasioned by aerobic paraquat may be an adaptive response which protected the revertant cells against superoxide lethality (13). The strong correlation between revertant RE8G3 inability to grow in soft agar and its ability to increase SOD levels in the presence of paraquat substantiates the earlier findings of an association between high SOD levels and the normal phenotype (24). The overall mechanism underlying the reversion of clone RE8G3 remains to be defined rigorously; however, it may be solely due to the increase in SOD levels, which may be sufficient to result in reversion of all the transformed cell properties examined.

The mechanism(s) leading to the reduced binding of both EGF and transferrin by RE8G3 cells after removal of paraquat is as yet a matter for speculation. Implicit in earlier findings (6, 28, 29) is the possibility that the reduction in EGF receptors in transformed cells may reflect the presence of a transformation-associated growth factor which may block EGF receptors in RE8G3 cells grown in the absence of paraquat. Of course, other factors, such as changes in receptor turnover rates or in receptor characteristics, may lead to reduced availability of EGF receptors in transformed cells. In approaching the second question, we note that certain SV40-transformed cells release into serum-free culture media a growth factor which facilitates iron transport into the transformed cells (9), implying that the transferrin requirements were reduced. Considering these observations, the possibility exists that the reduction in transferrin receptors in RE8G3 cells grown in the absence of paraquat may reflect the presence of a transformation-associated growth factor responsible for iron transport. However, a transformation-dependent defect in the processing of transferrin receptor in RE8G3 cells grown in the absence of paraquat is also possible and may account for the reduction in transferrin receptors.

The revertant subclone described here, although containing EGF-dependent 32P phosphorylation of the m.w. 170,000 protein at levels lower than that found in NRK cells (10, 11), exhibited a marked increase in the EGF-stimulated 32P phosphorylation of the m.w. 170,000 protein in comparison to K-NRK cells (11). Furthermore, we found that, after removal of paraquat, the EGF-dependent 32P phosphorylation of the m.w. 170,000 protein in the revertant cell membranes progressively declined to K-NRK levels. These results therefore provide further support for an association between the transformed phenotype and a reduction of EGF-dependent 32P phosphorylation of the m.w. 170,000 component (11). From a comparison of the results with [125I]-EGF (Chart 4A) and the data in Fig. 2 and Chart 5, it can be inferred that the loss of EGF-dependent 32P phosphorylation of the m.w. 170,000 protein is associated with a loss of EGF receptors. These findings suggest that both of these activities share some limiting step or alternatively that reduction of EGF receptors is itself the limiting transformation-induced step in the reduction of 32P phosphorylation of the m.w. 170,000 protein. It has been suggested that m.w. 170,000 and m.w. 150,000 phosphoproteins may be components of the EGF receptor of a variety of mammalian cells (18). It is possible, therefore, that the m.w. 170,000 protein that we have observed in RE8G3 membranes may represent a component of the EGF receptor of RE8G3 cells which is a substrate of the phosphorylation reaction.

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Fig. 2. Time-dependent effects of removal of paraquat on the extent of phosphorylation of the m.w. 170,000 membrane component incubated with [γ-32P]ATP in the absence (basal phosphorylation, −) or presence (+) of EGF. The cells were grown and allowed to attain confluency as indicated in the legend to Chart 2. The monolayers were washed, the cells were collected, and the membranes were isolated. Samples were prepared for phosphorylation, electrophoresis, Coomassie blue staining, autoradiography (24-hr exposure), and densitometry as described in “Materials and Methods.” The samples were analyzed in exponential 5 to 9% acrylamide gels; 50 µg of protein were applied to each lane. O, origin; F, front. Radioactive bands were quantitated with an electronic planimeter and expressed as peak area. Photographs of representative autoradiographs are shown. (a, b), (c, d), (e, f), (g, h), and (i, j), cells cultured in the absence of paraquat for 1, 4, 15, 25, and 31 days, respectively. M, molecular weight; K, thousands.
Correlation between the Loss of the Transformed Phenotype and an Increase in Superoxide Dismutase Activity in a Revertant Subclone of Sarcoma Virus-infected Mammalian Cells

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