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Serum amine oxidase activity contributes to crisis in mouse embryo cell lines

(senescence/spontaneous transformation/polyamine/free radical/cell culture)

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ABSTRACT **This paper reports the results of experiments to test the hypothesis that crisis of spontaneous transformation is caused by the hydrogen peroxide and/or aldehydes generated from endogenous polyamines by serum amine oxidase [amine:oxygen oxidoreductase (deaminating), EC 1.4.3.6]. After 4–5 weeks of culture, crisis occurred in 16 of 29 cell lines derived from limb buds of embryos from SJL/J, C3H, and CD-1 mice. In contrast, after the same time in culture but in medium supplemented with aminoguanidine, which inhibits serum amine oxidase, crisis occurred in only 1 of 41 cell lines. Protection against crisis was maximal in cell lines of SJL/J embryos, in which the incidence of crisis fell from 7 of 9 in untreated controls to 0 of 12 in the presence of 2 mM aminoguanidine. 2-Mercaptoethanol at 150–300 μM, which protects cells from serum amine oxidase-dependent polyamine toxicity, also protected the cell lines against crisis. These protected cell lines retained proliferative potential, diploid DNA content, and the mixture of cell types found in the primary cultures. These results indicate that cytotoxic catabolites generated by serum amine oxidase cause at least a large portion, but perhaps not all, of the cellular damage that leads to crisis in mouse embryo cell lines.**

Crisis in cell lines[§] derived from murine embryos is characterized by progressive deterioration of the cells, loss of their proliferative potential, and death of most or all of them (1–6). A few cells may survive crisis, remain dormant for a period of time, and then spontaneously transform into "immortalized" cells that can be passaged as continuous cell lines (1–5). These spontaneously transformed cells usually have abnormal karyotypes, DNA contents, and morphologies, although they may retain some characteristics of normal cells, such as anchorage-dependent growth and contact inhibition of proliferation and movement (1–3, 5, 6). Commonly used continuous cell lines like 3T3 have been derived from spontaneous transformants after crisis (1).

We found that mouse embryo cells derived from limb buds of day-14 embryos released spermine and spermidine into the bovine serum-supplemented medium during the time preceding crisis (7). It is well known that bovine sera contain high levels of an amine oxidase [amine:oxygen oxidoreductase (deaminating), EC 1.4.3.6] that catabolizes spermine and spermidine to hydrogen peroxide, aminoaldehydes, and lower order amines (8, 9). We hypothesized that these cytotoxic catabolites cause crisis in mouse embryo cell lines. To test this hypothesis, cell lines from limb buds of day-14 embryos from three strains of mice were cultivated in medium alone, in medium supplemented with aminoguanidine, an inhibitor of bovine serum amine oxidase (9), or in medium supplemented with relatively high concentrations of 2-mercaptoethanol, which protects cultured cells against serum amine oxidase-dependent polyamine toxicity (7, 10). The data show that these compounds prevent crisis without altering the original cellular composition, proliferative potential, or diploid DNA content of the cell lines.

METHODS

Pregnant CD-1 mice were obtained from Charles River Breeding Laboratories, and pregnant SJL/J and C3H mice were obtained from The Jackson Laboratory. At day-14 of gestation, the mice were killed by cervical dislocation, the embryos were removed, and their limb buds were amputated and stored briefly in 0.14 M NaCl/10 mM KH_2PO_4, pH 7.2, containing gentamicin sulfate at 20 mg/liter. Each limb bud was minced in the microwell of a 24-well plate containing medium. After 3 days, spent medium was replaced and clumps of tissue were removed, leaving a culture of adherent embryo cells. After 6–7 days, the cultures were confluent and were treated with trypsin/EDTA to obtain suspensions of single cells, which seeded new cultures. Thereafter the medium was replaced every 3–4 days, and the cell lines were passaged at confluency (usually 7 days after seeding). The medium of nonconfluent cultures was also replaced. The number of population doublings for each cell line was calculated as described (11). The incidence of crisis was scored on the day when signs of deterioration (either cytoplasmic granulation or cell death) were seen in 100% of control (untreated) cell lines. Cultures in crisis were identified by the presence of 50 or fewer cells.

Cultures were maintained in Ham's F-12 medium supplemented with 5% (vol/vol) fetal bovine serum and gentamicin sulfate at 20 mg/liter. For some cultures, the medium was supplemented with 1 or 2 mM aminoguanidine or 150 or 300 μM 2-mercaptoethanol. Aminoguanidine-supplemented medium was made immediately before use by mixing 10% fetal bovine serum/Ham's F-12 medium containing gentamicin sulfate at 20 mg/liter with an equal volume of Ham's F-12 medium/gentamicin sulfate at 20 mg/liter containing 2× concentrated aminoguanidine. 2-Mercaptoethanol-supplemented medium was made immediately before use by adding the 2-mercaptoethanol from a 1.0 M stock solution in water. Trypsin/EDTA was purchased from Irvine Scientific (Irvine, CA), fetal bovine serum was from HyClone (Logan, UT), Ham's F-12 medium was from GIBCO, and all other reagents were from Fluka.

For photography, cultures were fixed in 10% (vol/vol) formaldehyde in phosphate-buffered saline and then stained sequentially with Wright's stain and Giemsa stain. Flow cytometry was performed as described (12) on cells harvested after trypsinization.

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§The nomenclature for cell culture used in this paper was adopted from the recommendations of the Tissue Culture Association (39).

RESULTS

Primary cultures of mouse embryo cells established from day-14 limb buds of CD-1 mice contained a mixture of cell types, which was retained when the primary cultures were subcultured to initiate cell lines (Fig. 1*A*). Analysis by flow cytometry did not detect cells with abnormal DNA content at passage-2 (Fig. 1*B*). As expected, with subsequent passaging these cell lines began to show signs of deterioration, such as increased granularity of the cytoplasm and increased population doubling time. By day-25, after an average of 8.8 population doublings (an average of 3.8 passages), crisis was evident in four of six of the CD-1 cell lines (Fig. 1*C*, Table 1). After 5 more weeks of cultivation, one of the CD-1 cultures that had been in crisis contained two colonies of rapidly proliferating cells. Each colony was propagated separately as a continuous ("immortalized") cell line for eight passages. One colony had a fibroblastic morphology (Fig. 1*D*) and a highly aneuploid DNA content (Fig. 1*E*). The second line had a neural morphology and a hyperdiploid DNA content (data not shown).

Embryo cell lines from two other strains evolved similarly, although the time to crisis varied. Cell lines from both SJL/J

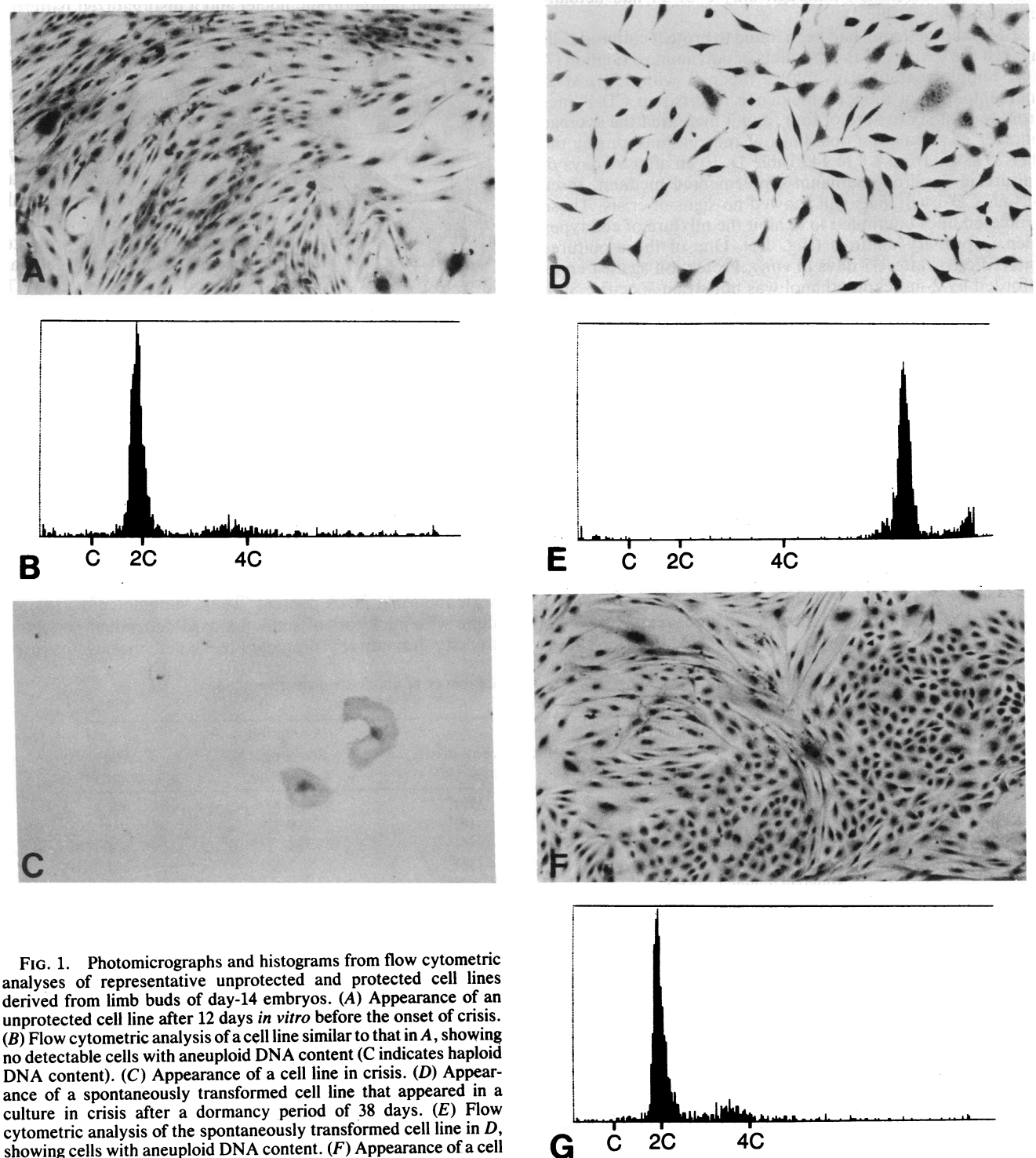

FIG. 1. Photomicrographs and histograms from flow cytometric analyses of representative unprotected and protected cell lines derived from limb buds of day-14 embryos. (*A*) Appearance of an unprotected cell line after 12 days *in vitro* before the onset of crisis. (*B*) Flow cytometric analysis of a cell line similar to that in *A*, showing no detectable cells with aneuploid DNA content (C indicates haploid DNA content). (*C*) Appearance of a cell line in crisis. (*D*) Appearance of a spontaneously transformed cell line that appeared in a culture in crisis after a dormancy period of 38 days. (*E*) Flow cytometric analysis of the spontaneously transformed cell line in *D*, showing cells with aneuploid DNA content. (*F*) Appearance of a cell line after 40 days *in vitro* in medium supplemented with 2 mM aminoguanidine. Note that crisis has been prevented and the mixture of cell types resembles that present at early passages (see *A*). (*G*) Flow cytometric analysis of a cell line similar to that in *F*, showing no detectable cells with aneuploid DNA content.

and C3H strains contained cells with morphologies similar to those in the CD-1 cell lines (Fig. 1*A*). Flow cytometric analysis of them at passage-2 did not detect any cells with abnormal DNA content (data not shown). The SJL/J cell lines seemed the most sensitive to the factors causing crisis. By day-35, after an average of only five population doublings (an average of 2.8 passages), 7 of 9 SJL/J cell lines had entered crisis (Table 1). The C3H cell lines deteriorated slowly and entered crisis later than cell lines from the other strains. By day-37, after an average of 7.5 population doublings (an average of four passages), all C3H lines showed signs of deterioration, although only 5 of 14 had actually entered crisis (Table 1).

2-Mercaptoethanol had been found to protect cultured cells against the toxicity of the products of polyamine oxidation (7, 10). Supplementation of culture medium with 150 μM 2-mercaptoethanol reduced incidence of crisis in CD-1 lines from four of six to zero of four, and it increased the average number of population doublings of the cell lines during the day-25 assay from 8.8 to 14 (Table 1). Even after 97 days of culture in 2-mercaptoethanol-supplemented medium, three of four CD-1 cell lines still showed no signs of crisis. These protected lines continued to exhibit the mixture of cell types seen in primary cultures (Fig. 1*A*). One of these cultures entered crisis after 37 days *in vitro*. Protection against crisis afforded by 2-mercaptoethanol was not strain-specific. Supplementation of culture medium with 300 μM 2-mercaptoethanol reduced the incidence of crisis in C3H cell lines from 5 of 14 to 1 of 14 but did not decrease the average number of population doublings during the assay period (Table 1).

Aminoguanidine is an inhibitor of bovine serum amine oxidase (9). Thus, cell lines from the three strains of mice were cultivated in medium supplemented with aminoguanidine at concentrations known to protect cultured cells against the serum-dependent toxicity of 0.5 mM spermine (7, 13). Including aminoguanidine in the medium afforded significant protection against crisis. Aminoguanidine at 1 mM reduced the incidence of crisis in CD-1 cell lines from four of six to zero of two and increased the average number of population doublings during the assay period from 8.8 to 9.3 (Table 1). The cell lines protected against crisis by aminoguanidine continued to exhibit that mixture of cell types seen in primary cultures (Fig. 1*F*). One of these two protected CD-1 cell lines was serially cultivated for 97 days without any evidence of crisis.

The protection against crisis afforded by aminoguanidine was also not strain-specific. Aminoguanidine at 2 mM reduced the incidence of crisis in C3H cell lines from 5 of 14 to 1 of 15 and increased the average number of population doublings during the assay period from 7.5 to 8.7 (Table 1). Furthermore, at the end of the experiment (day-40), cells in the protected cultures retained a mixed cellularity (Fig. 1*F*) as well as a proliferative potential similar to those of the original populations. Some cell lines did contain a few foci of cells with pleiomorphic nuclei and a disorganized pattern of growth (Fig. 2*A*). However, an analysis by flow cytometry did not detect cells with abnormal DNA content (Fig. 1*G*).

The protection by aminoguanidine was most striking in SJL/J cell lines. Supplementation of medium with 2 mM aminoguanidine had reduced the incidence of crisis from 7 of 9 to 0 of 12 by day-35, while raising the average number of population doublings during the assay period from 5 to 7.1 (Table 1). As with CD-1 and C3H cell lines, these protected SJL/J cell lines continued to exhibit proliferative potentials, diploid DNA contents, and mixed cellularities similar to those of the original populations. However, as in protected C3H lines, a few foci of apparently transformed cells appeared in some of these protected lines (Fig. 2*B*). The incidence of crisis in SJL/J cell lines remained 0 of 12 when the aminoguanidine concentration was lowered to 1 mM (Table 1). However, at day-40, cells in some of these cell lines exhibited reduced proliferative potentials as compared with those in 2 mM aminoguanidine.

When data from the three strains were combined, the incidence of crisis in cell lines cultivated without amine oxidase inhibitors was 16 of 29 (55%), but supplementation of the medium with 2-mercaptoethanol or aminoguanidine decreased the incidence of crisis to 1 of 18 (6%) or 1 of 41 (2%), respectively. The protected cell lines retained a capacity for regenerating a monolayer after subculture and a mixture of cell types similar to the primary cultures. Flow cytometry showed that these protected cell lines did not contain cells with abnormal DNA content. Thus, supplementing the medium with inhibitors of amine oxidase-dependent polyamine toxicity dramatically protected these cell lines against crisis

Table 1. Protection of mouse embryo cell lines from crisis by inhibitors of amine oxidase-dependent polyamine toxicity

Strain	Additive	Incidence of crisis, no./total (%)	Population doublings, average no.	Passages, average no.
CD-1	None	4/6 (66)*	8.8	3.8
	Aminoguanidine (1 mM)	0/2 (0)†	9.3	4.0
	2-Mercaptoethanol (150 μM)	0/4 (0)†	14	6.0
C3H	None	5/14 (38)	7.5	4.0
	Aminoguanidine (2 mM)	1/15 (7)	8.7	4.7
	2-Mercaptoethanol (300 μM)	1/14 (7)	7.5	4.0
SJL/J	None	7/9 (88)	5.0	2.8
	Aminoguanidine (1 mM)	0/12 (0)‡	6.2	3.3
	Aminoguanidine (2 mM)	0/12 (0)	7.1	3.7

Explant cultures from limb buds of day-14 embryos of CD-1, C3H, and SJL/J mice were initiated and subcultured as cell lines. The medium for some cell lines was supplemented either with aminoguanidine, an inhibitor of serum amine oxidase (9), or with 2-mercaptoethanol, which protects cells from amine oxidase-dependent polyamine toxicity (7, 10). The number of population doublings for each cell line was calculated as described (11). The incidence of crisis was scored on the day when signs of deterioration (either cytoplasmic granulation or cell death) were seen in 100% of control (untreated) cell lines (day-25 for CD-1 cell lines, day-37 for C3H lines, and day-35 for SJL/J lines). Cultures in crisis were identified by the presence of 50 or fewer cells.

*One culture in crisis contained two independent colonies of spontaneously transformed cells that were isolated and propagated as continuous cell lines, one of which is shown in Fig. 1 *D* and *E*.

†Only one of these cultures had entered crisis by day-97.

‡Although these cultures were not in crisis at the end of the assay, many cells exhibited granular cytoplasms and slower proliferation rates.

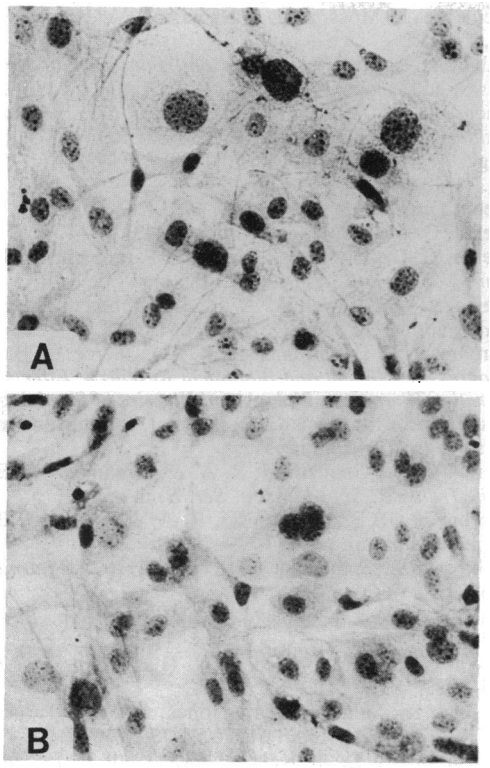

FIG. 2. Photomicrographs of areas of disorganization and nuclear pleomorphism seen in cell lines protected from crisis for 40 days by 2 mM aminoguanidine. Such morphologic abnormalities may be the first signs of spontaneous transformation, even though these cultures had no detectable cells with aneuploid DNA content (see Fig. 1*G*). (*A*) Culture from C3H embryos. (*B*) Culture from SJL/J embryos.

without apparently altering their basic biological characteristics.

DISCUSSION

Amine oxidases are a family of enzymes that include monoamine oxidases, diamine oxidases (histaminases), tissue polyamine oxidases, and serum amine oxidases (8, 14). These enzymes are thought important because they help regulate levels of monoamines and polyamines (15–17). Amine oxidases have in common the generation of hydrogen peroxide and aldehydes, reaction products usually considered to be waste products. However, recent studies on the mechanism of regulation of cancer by the embryo (18) have shown that these reaction products probably cause programmed cell death in the embryo and coincidentally kill cancer cells implanted into the areas where cell death occurs (7, 10, 19–23). These enzymes have been postulated to cause apoptosis in renewing tissues of the adult, thereby contributing to the regulation of tissue renewal (chalone-like activity) (7, 21, 22).

Our data lead to the conclusion that serum amine oxidase contributes to crisis in mouse embryo cell lines. Supplementation of the medium with either aminoguanidine or 2-mercaptoethanol protects the cell lines against crisis. Aminoguanidine is a known inhibitor of bovine serum amine oxidase (9), and high concentrations of 2-mercaptoethanol, such as used in these experiments, protect cultured cells against serum amine oxidase-dependent polyamine toxicity (7, 10). Although aminoguanidine and 2-mercaptoethanol could have several effects upon cells [e.g., aminoguanidine inhibits histidine decarboxylase (24) as well as some types of cation channels (25)], to our knowledge the only biological effect in common between these compounds is the protection they afford cultured cells against the cytotoxic products of polyamine oxidation.

Senescence of cell lines of human embryo fibroblasts occurs after a predictable number of population doublings (26). Thus, 2-mercaptoethanol and aminoguanidine might only appear to protect against crisis, when in reality they slow the population doubling time so that untreated cultures double more quickly and, therefore, reach maximum life-expectancy first. This idea is incompatible with the data in Table 1. Supplementation of medium with either 2-mercaptoethanol or aminoguanidine did not decrease the number of population doublings during the period of the assays. In fact, in many cases, these inhibitors of amine oxidase-dependent polyamine toxicity actually increased the number of population doublings over that of controls.

Thus, the most reasonable interpretation of our data is that crisis is caused by hydrogen peroxide and/or cytotoxic aldehydes generated by serum amine oxidase during its oxidation of endogenous amines. Polyamines added directly to the culture medium have been reported to be cytotoxic (9, 13). Our data show that the amount of endogenous amine substrate is sufficient to cause toxic effects. This mechanism of toxicity may contribute to the problems encountered in the long-term culture of other cell types.

Our conclusion is in accord with a previous report by Loo *et al.* (6) showing that crisis and its associated aneuploidy and spontaneous neoplastic transformation were caused by unknown factors in bovine serum. We show that bovine serum amine oxidase is, at least, one of these serum factors; however, it may not be the only one. The data in Table 1 demonstrate that supplementation of culture medium with aminoguanidine or 2-mercaptoethanol protects cell lines against crisis but does not completely prevent it. Two of 59 treated cell lines entered crisis at the same time as untreated controls, and two of six CD-1 cell lines entered crisis during long-term culture, even though the inhibitors of serum amine oxidase were present. Therefore, either these inhibitors cannot block all toxicity or other factors exist that contribute to crisis. Regardless, the results of our experiments bring the number of known damaging agents in the *in vitro* environment to two: bovine serum amine oxidase and overhead fluorescent light (27). Both of these damaging agents produce activated-oxygen species (13, 27).

Cell culture selects for rapidly proliferating cells and, therefore, probably for cells with high intracellular polyamine levels (16, 17). These intracellular polyamines may enter the extracellular environment by means of secretory or excretory pathways (28) and then come into contact with the serum amine oxidase in the medium. Alternatively, polyamines may enter the extracellular environment via leakage from cells damaged by the physical or chemical techniques used to dissociate them for passaging. We favor the latter explanation for several reasons. (*i*) Exposure of cultured cells to trypsin or EDTA causes leakage of small-molecular-weight cytoplasmic compounds (29). (*ii*) Diploid cells survive *in vitro* without crisis or senescence for much longer periods of time (i.e., 6 mo) when they are maintained as monolayers without passaging (30). (*iii*) Several studies have shown that both normal and abnormal cells exhibit a much higher plating efficiency in the presence of inhibitors of serum amine oxidase or under low oxygen tension (molecular oxygen is a required substrate for amine oxidases) (31, 32).

Many investigators have supported the hypothesis of an intrinsic mechanism of cellular aging to explain the finite life-span of cultured cells (11, 26). However, it is also admitted that currently available data do not exclude the alternative explanation of extrinsic factors that cause cumulative damage (26). It is interesting that free radicals, like those generated by serum amine oxidase and fluorescent light, produce cumulative cellular damage (27, 33). How a

very small number of cells survives during crisis is currently unknown. Perhaps different biological effects upon different cells are due to differences in the relative abilities of the cells to detoxify reactive oxygen species and aldehydes and/or to repair the damage produced by them.

Spontaneous transformation and even spontaneous neoplastic transformation can occur without crisis (34). Is it conceivable that at lower concentrations the products of polyamine oxidation act as carcinogens? Aldehydes and oxygen radicals can be carcinogenic, and activated oxygen species can produce DNA-strand breaks (35–38). If true, such a mechanism would explain how spontaneous neoplastic transformation occurs in the absence of crisis when cells are cultured in medium supplemented with horse serum (34). Horse sera contain much lower levels of serum amine oxidase than do bovine sera (13), and therefore levels of aldehydes and peroxides might be produced that are carcinogenic but not cytocidal. Similarly, this mechanism would explain why mouse embryo cells cultivated in serum-free medium did not transform (6). The foci of apparently transformed cells observed in our study in the presence of aminoguanidine or 2-mercaptoethanol (Fig. 2) may also result from exposure to subcytocidal levels of these reaction products.

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