Studies on Analogues of L-Cysteine and L-Cystine

II. The Effect of Selenium Cystine on Murphy Lymphosarcoma Tumor Cells in the Rat

By AUSTIN S. WEISBERGER AND LEIF G. SUHRLAND

It has frequently been suggested that sulfhydryl compounds may have an important role in cellular metabolism and in cell growth and division. Thus sulfhydryl (SH) compounds have been shown to stimulate mitosis in a wide variety of cells and tissues. Substances which oxidize SH compounds (SH → S—S) or combine with SH groups to inactivate them have been found to inhibit cell division. The literature pertaining to this subject has recently been reviewed extensively by Brachet,1 Contopolous and Anderson2 and by Barron.3 Sulfhydryl compounds have also been implicated in the growth of malignant tumors in general.1, 6 Thus tumor tissues have been found to contain a higher SH concentration than normal tissues. Low plasma SH levels have been demonstrated during the growth phase of malignant tumors and this has been attributed to an increased utilization of SH by proliferating tissue.1, 6, 7 Decreased availability of SH substances has been correlated with inhibition of tumor growth. Thus diets deficient in the SH amino acid, L-cysteine,8 suppress the growth of malignant tumors in animals8-10 whereas addition of either cysteine or glutathione to the diet results in increased tumor growth.9 Substances which inhibit the cellular incorporation of L-cysteine may have an effect similar to dietary deficiency of cysteine in suppressing tumor growth.

Selenium cystine (diseleno-dialanine), which has a structure identical with that of cystine except that selenium replaces sulfur in the molecule, is effective in low concentrations in decreasing the incorporation of radioactive L-cystine by leukemic leukocytes in vitro.11 It is not known whether selenium cystine also competitively inhibits cystine incorporation in the intact animal. It is possible that selenium cystine may function as a blocking analogue of cystine in vivo and that it may therefore affect cell growth and division, especially of malignant cells. The following studies were undertaken to determine whether selenium cystine decreases the influx of radioactive L-cystine into rat lymphosarcoma cells both in vitro and in vivo as well as to determine whether such changes can be correlated with alterations in tumor growth in the intact animal.

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* Since the reversible interconversion of cysteine and cystine is readily accomplished, these two compounds are considered as a single amino acid in metabolism.12 The terms are used interchangeably in this article.
ANALOGUES OF L-CYSTEINE AND L-CYSTEINE. II

MATERIAL AND METHODS

Murphy lymphosarcoma cells were transplanted into Wistar male albino rats, weighing 175 to 200 Gm. Tumors weighing 25 to 30 Gm. were used for transplanting. The donor animals were sacrificed and the tumor immediately dissected free. All necrotic tissue was removed and the tumor was homogenized in a Waring blender with cold physiologic saline so as to make a 20 to 25 per cent suspension. This was then filtered through gauze and 1.0 ml. of this suspension was injected subcutaneously into each animal in the region of the axilla. Transplants were successful in over 90 per cent of the rats by this method.

1. The Effect of Selenium Cystine on the Incorporation of Sulfur-35 (S35) Labeled L-Cystine* by Murphy Lymphosarcoma Cells In Vitro:

Tumors weighing 25 to 30 Gm. were removed from animals and homogenized with cold physiologic saline in a glass tissue grinder. The homogenate was then filtered through gauze, centrifuged and washed with physiologic saline. The cells were resuspended in rat serum so as to make approximately a 10 per cent suspension. 4.0 ml. of this tumor cell suspension were incubated with 8.0 micromoles (jAM) of selenium cystine† (2.0 μM per ml.) for 20 minutes at 37 C. Twenty to 25 micrograms of S35 L-cystine (150,000 counts per minute) were then added per ml. tumor suspension and incubated for 45 minutes at 37 C. The cells were then washed and the amount of radioactivity present per ml packed tumor cells was determined as counts per minute (c/min) per ml. packed tumor cells by methods previously described.‡ The radioactivity present in these cells was compared with that present in tumor cells which were not exposed to selenium cystine prior to adding S35 L-cystine. All experiments were performed in duplicate and the mean values are reported.

Similar experiments were performed with benzyl selenium cysteine (benzyl selenoalanine). This compound is structurally similar to selenium cysteine and is effective in decreasing the influx of L-cystine into leukemic leukocytes.

2. The Effect of Selenium Cystine on the Incorporation of S35 L-Cystine by Tumor Tissue as Compared with Liver Tissue In Vivo:

The pattern of S35 L-cystine incorporation by rat liver, spleen, kidney and muscle was determined as c/min per gram of tissue and was compared with the c/min incorporated per gram of tumor tissue. The acute effects of orally administered selenium cystine on the incorporation of S35 L-cystine by tumor and liver tissue were then determined.

Six hundred to 700 μg. (4,000,000 c/min) of S35 L-cystine were injected intraperitoneally into rats weighing 200 Gm. Two animals were sacrificed every 15 minutes for 4 hours and then hourly for 4 hours. Approximately 2.0 Gm. of tissue were ground with 20 ml. of physiologic saline in a glass tissue homogenizer. An aliquot of this material was placed in a planchette, dried and counted in a flow counter. The c/min per gram of tissue were calculated and the mean values reported.

The acute effects of selenium cystine on the incorporation of S35 L-cystine in vivo were then determined in tumor and in liver tissue. One hundred mg. of selenium cystine per Kg. body weight were administered orally to each of 4 rats by stomach tube. One hour after oral intubation of the selenium cystine, 600 to 700 micrograms (4,000,000 c/min) of S35 L-cystine were injected intraperitoneally. The animals were sacrificed 1 hour after injecting S35 L-cystine and the c/min per gram of tissue determined as previously described. Similar experiments were performed using 750 mg. of selenium cystine per Kg. body weight.

3. The Effect of Selenium Cystine on Tumor Growth:

The effect of selenium cystine on tumor growth was determined in 36 rats. Only those animals in which tumor transplants were successful are included in this group as well as in the other groups used for comparison.

Tumors were transplanted as described above. Selenium cystine was dissolved in dis-

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* S35 L-cystine was obtained from Abbott Laboratories, Chicago, Illinois.
† D, L-selemony cystine used in these experiments was furnished through the courtesy of Dr. Joseph Seifter of Wyeth Institute for Medical Research, Philadelphia, Pennsylvania.
‡ C/min per ml. packed tumor cells = 1 × c/min per ml. sample}

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tilled water in a concentration of 0.5 mg. per ml. Slight acidification was required to dissolve the selenium cystine. One mg. of selenium cystine per Kg. body weight was injected intraperitoneally daily for 14 days following tumor transplant. The tumor area was measured daily by taking the 2 largest diameters of the tumor. Observations on the weights of various tumors, compared to areas determined in this manner, indicate that this is a reliable index of tumor growth.

Since animals receiving selenium cystine failed to gain weight, the tumor growth in these animals was compared with that occurring in 15 rats which were pair fed with this group. Twenty-six rats on a normal diet served as an additional control group.

The effect of benzyl selenium cysteine on tumor growth was also determined in 19 rats. Two mgs. of benzyl selenium cysteine per Kg. body weight were administered intraperitoneally daily for 14 days following tumor transplant and the area of tumor growth was compared with that occurring in the control group and selenium cystine treated rats.

Results

1. The Effect of Selenium Cystine on the Incorporation of S\textsuperscript{35} L-Cystine by Tumor Cells In Vitro:

Murphy lymphosarcoma tumor cells readily incorporate S\textsuperscript{35} L-cystine in vitro. Incubation of the tumor cells with selenium cystine prior to adding S\textsuperscript{35} L-cystine results in a decreased influx of S\textsuperscript{35} L-cystine. Benzyl selenium cysteine, however, is ineffective in decreasing the influx of S\textsuperscript{35} L-cystine. Thus in a typical study, using the same cell population, the uptake of S\textsuperscript{35} was 29,700 c/m per ml packed tumor cells when incubated with S\textsuperscript{35} L-cystine alone (table 1). When another sample of the same tumor cell preparation was incubated with 2 \(\mu\)M selenium cystine per ml prior to adding S\textsuperscript{35} L-cystine, the amount of S\textsuperscript{35} incorporated was only 8,700 c/m per ml. packed tumor cells (29 per cent of the control value). When the same tumor cell population was incubated with 2 \(\mu\)M of benzyl selenium cysteine per ml the amount of S\textsuperscript{35} incorporated was increased to 37,500 c/m per ml. packed tumor cells (126 per cent of the control value).

2. The Effect of Selenium Cystine on the Incorporation of S\textsuperscript{35} L-Cystine by Tumor Tissue as Compared with Liver Tissue In Vivo:

S\textsuperscript{35} was rapidly incorporated into liver, spleen, kidney and tumor tissue following intraperitoneal injection of S\textsuperscript{35} L-cystine. There was a progressive increase in the S\textsuperscript{35} content of these tissues for the first 45 minutes following which there was a gradual fall in radioactivity over the next 4-hour period. The highest values were present in the liver, spleen and kidney (fig. 1).

The amount of S\textsuperscript{35} L-cystine incorporated in the liver and tumor tissue one hour after intraperitoneal injection of the labelled amino acid was determined in 10 additional rats (table 2). The mean values at the end of one hour were 18,500 c/m per Gm. of tumor tissue (range 14,000 to 27,000 c/m per Gm.) and 31,000 c/m per Gm. of liver tissue (range 23,000 to 40,000 c/m per Gm.). When

| Table 1.—Effect of Selenium Cystine and Benzyl Selenium Cysteine on the Incorporation of S\textsuperscript{35} L-Cystine by Murphy Lymphosarcoma Tumor Cells in Vitro |
|---------------------------------|-----------------|----------------|
|                                  | Counts Per Minute Per ML Packed Tumor Cells | Per cent of Control |
| Control                         | 29,700          | 100            |
| Selenium Cystine               | 8,700           | 29             |
| Benzyl Selenium Cysteine       | 37,500          | 126            |
Fig. 1.—Distribution of radioactivity in the tissues of rats following the intraperitoneal injection of $^{35}$S L-cystine. There is a rapid increase in the amount of radioactivity present in liver, kidney, spleen and tumor tissue during the first 45 minutes and a gradual decrease thereafter.

Table 2.—The Effect of Selenium Cystine on the Incorporation of $^{35}$S L-Cystine by Murphy Lymphosarcoma Cells and Liver Cells in the Rat in Vito

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<tr>
<th>Amount of $^{35}$S Incorporated</th>
<th>Lymphosarcoma Cells</th>
<th>Liver Cells</th>
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<tr>
<td></td>
<td>c/m Gm.</td>
<td>% of Control</td>
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</tr>
<tr>
<td>Control (12 rats)</td>
<td>18,500</td>
<td>100</td>
</tr>
<tr>
<td>Selenium Cystine 100 mg/Kg (4 rats)</td>
<td>10,100</td>
<td>55</td>
</tr>
<tr>
<td>Selenium Cystine 750 mg/Kg (4 rats)</td>
<td>8,600</td>
<td>47</td>
</tr>
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100 mg. of selenium cystine per Kg. body weight were administered orally one hour prior to intraperitoneal injection of S\textsuperscript{35} L-cystine to each of 4 rats, the mean value in the tumor tissue decreased from 18,500 c/m per Gm. to 10,100 c/m per Gm. (range 4,000 to 14,000 c/m per Gm.). In the liver, however, the S\textsuperscript{35} L-cystine uptake rose from 31,000 c/m per Gm. to 42,400 c/m per Gm. (range 38,000 to 49,500 c/m per Gm.).

When the amount of selenium cystine administered orally was increased to 750 mg. per Kg. body weight prior to injecting S\textsuperscript{35} L-cystine, the c/m per Gm. tumor tissue fell from 18,500 to 8,600 whereas in the liver the c/m per Gm. of tissue rose from 31,000 to 57,000.

3. The Effect of Selenium Cystine on Tumor Growth:

Animals receiving selenium cystine exhibited a significant reduction in tumor growth as well as a higher percentage of tumor regression as compared with the control group (fig. 2, table 3). The mean value for the tumor area in the control group on the 18th day following tumor transplant was 29 sq. cms. and 27 per cent of these animals exhibited spontaneous regression of tumor growth. In the group receiving selenium cystine the tumor area on the 18th day was 9 sq. cm. and 71 per cent of the animals exhibited regression of tumor growth. Animals which were pair fed with those receiving selenium cystine had tumor growths comparable to those of the control group. Animals receiving benzyl selenium cysteine exhibited no significant decrease in tumor growth (fig. 3).
Table 3.—The Effect of Selenium Cystine and Benzyl Selenium Cysteine on the Growth of Murphy Lymphosarcoma in the Rat

<table>
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<th>Average Area* (Sq. Cm.)</th>
<th>Percent Showing Regression of Tumor Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>27</td>
</tr>
<tr>
<td>Starvation</td>
<td>28.5</td>
<td>40</td>
</tr>
<tr>
<td>Selenium Cystine</td>
<td>9</td>
<td>71</td>
</tr>
<tr>
<td>Benzyl Selenium Cysteine</td>
<td>20</td>
<td>42</td>
</tr>
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</table>

* Areas taken 18 days after tumor transplant.

Fig. 3.—Animals receiving selenium cystine exhibit a reduction in tumor growth. Animals receiving benzyl selenium cysteine (benzyl selenium alanine) exhibit no significant reduction in tumor growth as compared with the control group.

Many of the animals receiving selenium cystine developed alopecia. Histologic examination of the liver, spleen, kidneys and bone marrow of animals receiving selenium cystine revealed no abnormalities.

Discussion

Selenium cystine decreases the influx of $^{35}$S-L-cystine into Murphy lymphosarcoma cells of the rat both in vitro and in vivo. The decreased incorporation of L-cystine by these tumor cells is associated with a decrease in the growth of the tumor in the intact animal. Since low concentrations of selenium cystine also decrease the influx of $^{35}$S-L-cystine into human leukemic leukocytes, it is possible that selenium cystine may likewise affect leukemic leukocytes in vivo.
Although selenium cysteine decreases the incorporation of $^{35}$S L-cystine into tumor cells in vivo, no such effect is apparent in liver cells. On the contrary, the administration of selenium cystine is associated with an increased incorporation of $^{35}$S L-cystine by liver cells. The increased turnover of L-cystine by liver cells under these conditions may be associated with a detoxifying action of the liver. The selective effect of selenium cystine in decreasing the incorporation of L-cystine into tumor cells as compared to liver cells may decrease the toxicity of selenium cystine in the intact animal. Thus the administration of 1 mg. of selenium cystine per Kg. body weight over a 2 week period resulted in anorexia, loss of weight and alopecia in the rats without histologic changes in the liver, spleen or bone marrow. The alopecia developing in these animals is of interest because of the high cystine content of hair. Inhibition of the rapidly proliferating cells of hair follicles may also account for the alopecia.

Although the mean value for tumor size in the selenium cystine treated animals was significantly smaller than that of the control animals (9 sq. cm. compared to 29 sq. cm.) some animals escaped the effect of selenium cystine and attained tumor growths comparable to those of the untreated animals. However, the number of regressions in tumor size (table 3) and the number manifesting decreased growth, as well as the marked differences in the mean areas, indicates that the differences observed with selenium cystine are significant.

Benzyl selenium cysteine is effective in decreasing the influx of $^{35}$S L-cystine into human leukemic leukocytes in vitro. This compound, however, is ineffective in decreasing the incorporation of $^{35}$S L-cystine by Murphy lymphosarcoma tumor cells in vitro. It is apparent that the failure of benzyl selenium cysteine to inhibit $^{35}$S L-cystine incorporation by lymphosarcoma cells in vitro is reflected in the failure of this compound to alter the growth of tumor in the intact animal. This would indicate that the potential effectiveness of a blocking analogue of cysteine (cystine) against malignant cells in vivo may be screened by its ability to decrease the incorporation of $^{35}$S L-cystine by these cells in vitro. The failure of benzyl selenium cysteine to affect tumor growth also indicates that the effects of selenium cystine are not due solely to a non-specific effect of selenium.

The mechanism of action of selenium cystine in decreasing tumor growth is not known. Since the effectiveness of analogues of cystine (cysteine) in the intact animal appears to be correlated with its effectiveness in vitro, it may be presumed that the mechanism of action is the same in both instances. Possible mechanisms of action in vitro have been discussed in the preceding article. The decrease in tumor growth associated with the administration of an analogue of cystine (cysteine) is further suggestive of the importance of the SH amino acids and related SH compounds in the metabolism of malignant cells.

**SUMMARY AND CONCLUSIONS**

Selenium cystine decreases the incorporation of $^{35}$S L-cystine by rat Murphy lymphosarcoma tumor cells both in vitro and in vivo. Selenium cystine also decreases the growth of the tumor in the intact animal.

Benzyl selenium cysteine does not inhibit the incorporation of $^{35}$S L-cystine by Murphy lymphosarcoma tumor cells in vitro nor does it affect tumor growth in the intact animal.
Thus the ability of selenium cystine to decrease the influx of $^{35}S$ L-cystine into tumor cells in vitro is associated with the ability of this compound to inhibit tumor growth in the intact animal. Furthermore, the inhibitory effect of selenium cystine is not due solely to the presence of selenium within the molecule but is related to its structural identity with cystine.

The data are further suggestive of the importance of SH compounds in the growth of malignant cells.

**SUMMARIO E CONCLUSIONES IN INTERLINGUA**

Selenio-cystina reduce le incorporation de L-cystina a $^{35}S$ per cellulas del lymphosarcoma de Murphy in rattos tanto in vitro como etiam in vivo. Selenio-cystina etiam reduce le crescentia del tumor in animales intacte.

Benzyl-selenio-cysteina non inhibi le incorporation de L-cystina a $^{35}S$ per cellulas del lymphosarcoma de Murphy in vitro; illo etiam non affice le crescentia del tumor in animales intacte.

Assi le capacitate de selenio-cystina a reducer le influxo de L-cystina a $^{35}S$ in le cellulas del tumor in vitro es associate con su capacitate a inhibir le crescentia del tumor in animales intacte. In plus, le effecto inhibitori de selenio-cystina non resulta simplemente del presentia intramolecular de selenium sed es connectite con le identitate structural del molecula de selenio-cystina con illo de cystina.

Le datos representa un indication additional del importantia de compositos sulfhydrylic in le crescentia de cellulas maligne.

**REFERENCES**

Studies on Analogues of L-Cysteine and L-Cystine: II. The Effect of Selenium Cystine on Murphy Lymphosarcoma Tumor Cells in the Rat

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