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The Effect of 'Aging' of an Inhibitor of Spontaneous Tumors in Mice. II. Observations at Aging from 101–1346 Days

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In keeping with the policy established several years ago, the present article is considered to be a progress report. Much is already known on the control of spontaneous tumors of mammary gland origin in mice, but several final conclusions have not been reached. The most urgent present needs are (1) to identify, separate, and purify the ingredients of a liver emulsion known to contain an active inhibitor of spontaneous tumors (this program has been initiated at Roswell Park Memorial Institute), (2) to test out purified fractions on known malignant disease at the San Diego laboratory of Strong Foundation, and (3) to analyze genetically the nature of a transmissible entity already known to have an effect on spontaneous tumors in mice and possibly induced (?) by the injection of a liver emulsion containing a mixture which is at least partially known. Hence the conclusion is reached that the present problem for the control of spontaneous tumors in mice remains an immunogenetic problem. The present data verify a previous conclusion that the aging of a liver emulsion, at least up to 1346 days, increases significantly the biological effect of suppressing spontaneous cancerous growth in mice.

INTRODUCTION

In a recent communication, evidence was presented for the derivation of the following conclusions: '(1) The length of time that a liver preparation containing an inhibitor of spontaneous tumors in mice has been kept in a refrigerator (28 F) alters the effectiveness of inhibition on suppressing the growth capacity of these tumors; (2) these changes may be cyclic, since the maximal effectiveness of the

This experiment has been made possible by contributions from private individuals in the United States. Many of these contributions have been given as memorials for friends and relatives who have died of cancer.

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"inhibitor" is at the beginning and at the end of a dated series of experiments; and (3) this phenomenon of altered effectiveness may be associated with particle aggregation in an emulsion' (Strong, 1969*a*).

The above conclusions were based upon aging of the tumor 'inhibitor' alone and not upon whether or not the liver emulsion had been pasteurized before aging.

A verification of the effect of aging of the tumor 'inhibitor' on the suppression of tumor growth was obtained by using two series of mice bearing spontaneous tumors. One of the series received liver emulsion that had been pastuerized (56 C for 30 min); the second series of mice was given unpasteurized material. The liver material for both series of mice was from the same source, and therefore the only difference was the process of pasteurization of the material used in one of the series. The conclusions derived from this research were presented in a second paper as follows: '(1) Heating a liver extract to 56 C for 30 min during its preparation attenuated a tumor inhibitor contained therein; (2) kept under refrigeration following its preparation, including pasteurization, the tumor inhibitor recovered its effectiveness of suppressing spontaneous tumors at a fairly uniform rate; (3) the data suggest, but do not prove, that the "attenuated inhibitor" may completely recover its full effectiveness in suppressing tumor growth (i.e., as compared to unpasteurized inhibitor) if kept for a long enough period of time' (Strong, 1970).

A further analysis of the rhythms or cycles of activity associated with aging of the liver emulsion containing a tumor inhibitor in suppressing the growth capacity of spontaneous tumors was reported in a third publication. The conclusions were as follows: '(1) Significant fluctuations of the effect of an inhibitor are a common occurrence. These differ only in magnitude in separate experiments. (2) In the aging of the inhibitor under refrigeration, two phenomena appear, as follows: (a) the inhibition of the growth rate of spontaneous tumors is increased and (b) the cycles or rhythms of activity appear to diminish. (3) Treating three aliquots of the same liver emulsion containing a tumor inhibitor with different degrees of temperature and different schedules of changes of temperature produces different results on the suppression of tumor growth of spontaneous tumors in mice. (4) In one case, the data clearly show that in one moiety (that kept under refrigeration at −2.2 C when not in use), there was a progressive increase of suppression of tumor growth up to 350 days of age. (5) With the moiety aged at room temperature for 305 days, there were produced pronounced cycles of the effect on the suppression of tumor growth. The maximal degree of suppression of tumor growth was obtained when the liver emulsion was 90 days old. (6) The material aged at room temperature for 90 days (ART series) and then placed under refrigeration (−2.2 C) produced smaller variations of tumor growth than the original ART emulsion, and eventually suppressed the growth rate of tumors more than the part of the emulsion which was kept continuously at room temperature. (7) The material heated to 45 C for 14 days had a maximal effect on the suppression of tumor growth (with 100% complete regression of tumors) but gradually lost its capacity to suppress tumor growth and at the same time continued to produce rhythmic activity. (8) A radical or sudden

change of temperature increased the phenomenon of rhythmic activity of the inhibitor, but at the same time brought about the loss of inhibition of tumor growth and, in some cases, a *stimulation* of tumor growth. (9) With aging, two emulsions treated differently with temperature changes, even though derived originally from the same source, will eventually have a similar effect on tumor growth. This return to a common base of inhibitory action takes place even though, in the intermediate stages, a significant difference on tumor inhibition may have been evident. (10) The tumor inhibitor derived from liver extract is "heat sensitive" ' (Strong, in press).

The purposes of the present experiment are (1) to bring the conclusions of these three investigations mentioned above closer together by adding new data obtained on 16 new groups of tumor-bearing mice (four in each group) to the data already published in *Cytobios* (Strong, 1969*a*) and (2) to refer briefly to the program that has been initiated to ascertain the biochemical nature of the inhibitor.

It is intended to report that further changes in the effectiveness of the liver emulsions containing the tumor inhibitor have been ascertained with longer storage in the refrigerator than has been recorded (Strong, 1969*a*,*b*, 1970, in press).

These age changes, which are not being considered finally conclusive, should throw some light on the nature of the tumor inhibitor. The phenomena of aging, sensitivity, and rhythmic activity of the inhibitor must be correlated, as soon as possible, with the actual components of the liver emulsion.

Again, as in previous publications, this new report must be considered as a progress report.

MATERIALS AND METHODS

The same liver emulsions were used in this experiment as were used in previous experiments of this series dealing with tumor inhibition (Strong, 1969*a*,*b*, 1970, in press). The only difference is that now the liver emulsions are much older than they were in the earlier research. For example, in the experiment on the effect of pasteurization of the liver emulsion (Strong, 1970), the liver material was between 101 and 1087 days old; in the present experiment the same liver material was between 101 and 1346 days old. The new data on tumor inhibition between 1087 and 1346 days are added to the already published data for between 101 to 1087 days of age (Strong, 1969*b*) in order to present a clearer overall relationship between the old and the new data.

Nothing new has been added to the method of preparation of the liver emulsions used in previous experiments, and description of this procedure need not be repeated again (Strong, 1969*a*,*b*, 1970, in press).

A preliminary analysis of the rhythmic nature of the tumor inhibition process is attempted by presenting two charts obtained by the use of the same material divided into two moieties that differed only in that one part had been pasteurized before being placed in the refrigerator and the other remained unpasteurized. For this purpose, a series of tumor-bearing mice was injected periodically with

either pasteurized or unpasteurized liver extract beginning at 28 days of refrigerator storage to 302 days.

For the partial analysis of the nature of the rhythms of activity of the inhibitor on the suppression of growth capacity of spontaneous tumors, the curve obtained with pasteurized liver emulsion was moved 57 days to the right in order to compare the data with those obtained with the unpasteurized material at approximately the same degree of tumor inhibition.

RESULTS

The new data obtained in this experiment are presented in three figures.

Figure 1 contains the data on growth rates of spontaneous tumors in two series of mice, as measured by the averaged increments of growth at the fifth observation period. The data for the tumor-bearing mice receiving pasteurized liver emulsion containing the tumor inhibitor are on the solid line (labeled 'P'); data for the mice receiving the unpasteurized liver extract are on the dashed line (labeled 'UNP'). The averaged increments of tumor growth at the fifth period are on the ordinate. Successive series of four groups of mice each (16) are on the solid line at the 37 mm^2 level. The data from groups 1–16 (combined as 1–4, 5–8, 9–12, and 13–16) were published *in Cytobios* (Strong, 1970); new data are added between

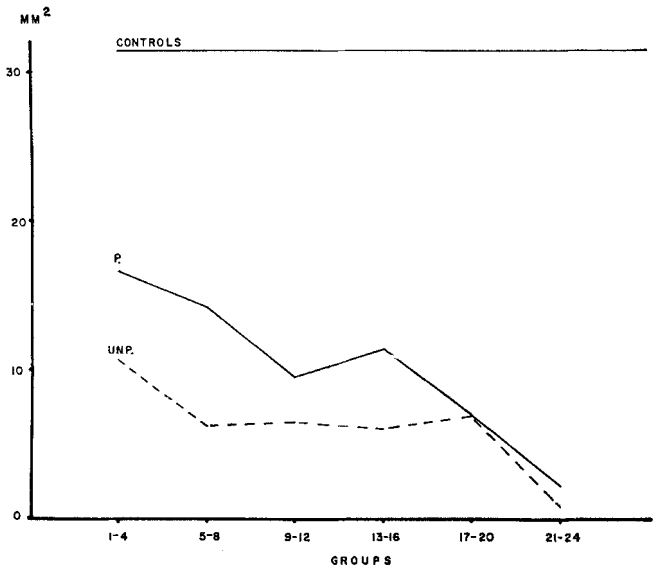

Fig. 1. Average increments of growth rates of spontaneous tumors at the fifth observation period for the controls (solid line) and the two series of experimental mice receiving the tumor inhibitor. Data for the pasteurized material at 56 C for 30 min are on the solid line (labeled 'P'). The new data are at series 17–20 and 21–24, joined to the old data of series 1–16. Ordinate: increments of tumor growth. Abscissa: successive groups of 16 liver-treated mice with tumors.

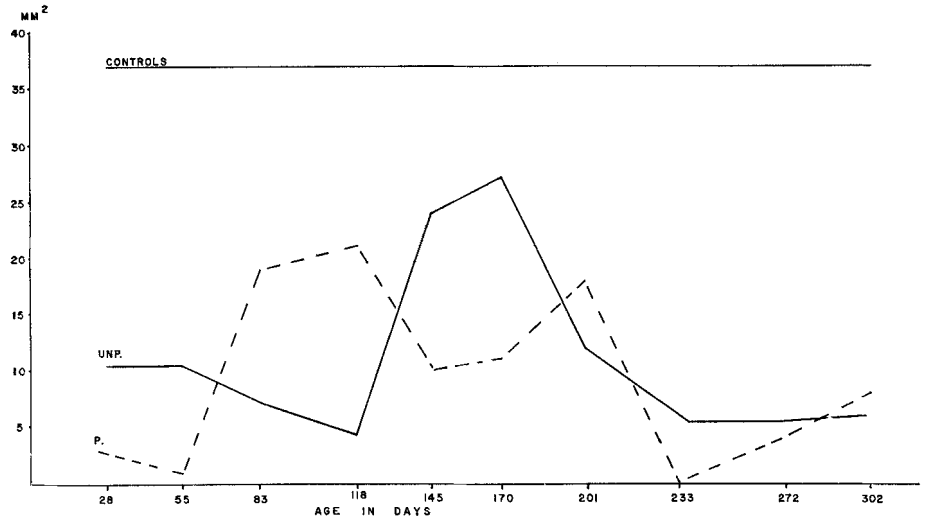

Fig. 2. Average increments of growth rates of spontaneous tumors at successive 5-day intervals. Data for those mice receiving pasteurized liver material are on the dashed line; data for mice given the unpasteurized material are on the solid line; data for the controls are all indicated at the 37 mm² level since there is no indication of a change or trend with time. Ordinate: increments of tumor growth. Abscissa: time of 'aging' of the liver material. The upper series is for the pasteurized material, and the lower is for the unpasteurized material.

groups 17 to 24 (combined as 17–21 and 21–24). The number of tumor-bearing mice is therefore 192 (24 groups of 4 mice each = 96 times 2 series or 192 mice). The maximal amount of suppression of tumor growth rate was therefore obtained in groups 21–24 of mice (in both pasteurized and unpasteurized groups) at which

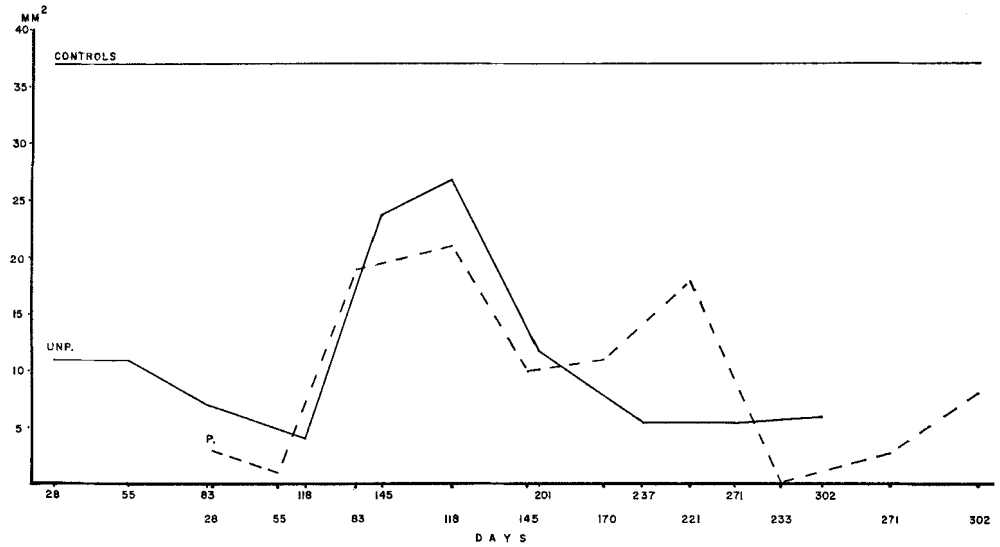

Fig. 3. The same data presented in Fig. 2; the only difference from Fig. 2 is that the data on growth increments of tumors are moved to the right 57 days for the mice of the pasteurized series. Ordinate: increments of tumor growth. Abscissa: time of storage of liver emulsions in days.

time the liver emulsion had been aged for 1346 days in the refrigerator (3.7 years).

Figure 2 presents the data on growth rate of tumors in another series in which a liver extract from beef was used. The time of storage under refrigeration was between 28 and 302 days. A series of mice receiving pasteurized material is compared to another series receiving an unpasteurized moiety of the same material. The data indicate a cyclic phenomenon which has been the rule for many separate experiments dealing with liver extracts from several species of animals. It is obvious that the two curves are similar (pasteurized and unpasteurized) but appear to differ in time only.

Figure 3 shows an attempt to compare the two curves of Fig. 2 by moving the curve based upon observation of data with the pasteurized liver emulsion to the right, or to the point in time where the two peaks of tumor suppression seem to coincide.

It is obvious that the two curves in Fig. 3 appear to be more like each other than the same two curves in Fig. 2.

DISCUSSION

It can be seen by an examination of Fig. 1 that the greatest amount of inhibition of the growth rate of spontaneous tumors in mice has been brought about by a liver preparation that has been aged for 1346 days in the refrigerator (−2.2 C). These data are in series 21–24. In this experiment, there was practically no growth of the tumors at the fifth observation period in both the pasteurized and the unpasteurized series.

The two curves in Fig. 1, one from the use of pasteurized material (solid line) and the other of unpasteurized material (dashed line) converge to a point predicted in a previous publication as follows: 'The data suggest, but do not prove, that the attentuated inhibitor (i.e., the pasteurized) may completely recover its full effectiveness in suppressing tumor growth (i.e., as compared to unpasteurized inhibitor) if kept for a long enough time' (Strong, 1970). Thus another conclusion on the phenomenon of tumor inhibition by a specially prepared liver emulsion has been verified. The first conclusion, that the 'original observation of the suppression of mammary cancer in the C_3H/ST mouse has been substantiated,' was first indicated by Saunders (personal communication).

The data for Figs. 2 and 3 indicate, perhaps, a partial analysis of the cyclic or rhythmic phenomenon associated with the inhibitory activity of the liver preparation.

It is obvious that the two curves of Fig. 3 are very similar. In fact, they seem to be more similar than the two curves presented in Fig. 2, even though the curves are the same and the only difference is the moving of the curve based upon the data obtained with pasteurized material to the right by 57 days.

It appears that the age changes associated with the tumor inhibitor may have a time sequence, that is, a pattern, and that the cyclic periods of activity may be

correlated not only with time but also with heat. It is known that the inhibitor is heat sensitive as indicated by comparing the activity of the inhibitor kept at room temperature to the activity of the inhibitor kept under refrigeration (Strong, 1969*b*).

It may be considered, therefore, that pasteurization (heating to 56 C for 30 min) affects the time sequence or pattern of activity of the inhibitor, which may have a temporary effect rather than a permanent one.

The original suggestion that 'the altered effectiveness may be associated with particle aggregation in an emulsion' is still being considered as a factor in time changes of the activity of the inhibitor but may eventually have to be given up—perhaps after more is known of the biochemical nature of the tumor inhibitor.

Since it has been necessary to cooperate with biochemists to analyze the biochemical nature of the inhibitor, the following discussion is based upon reports to the senior author rather than to actual publications of these collaborators.

By 1960, four events made possible the first attempt to analyze the biochemical nature of a liver-derived tumor inhibitor. These were as follows: (1) the obtaining of evidence on the presence of a spontaneous tumor inhibitor from liver preparation, (2) the purchase of a Cary instrument for the Biological Station at Springville, N.Y., (3) the appointment of Dr. Fred Bock, a biochemist from the staff of Roswell Park Memorial Institute of Buffalo, N.Y., to the Springville group, and (4) numerous studies on world literature and laboratory research which made possible the entertainment of the idea that a source of an inhibitor for spontaneous tumor in mice might be obtained from the liver (at that time only from mouse liver).

Part of the data bearing upon the evidence of a liver-derived tumor inhibitor in mice were the basis for writing *Biological Aspects of Cancer and Aging: Studies in Pure Line Mice*, which appeared in 1968. Unfortunately, part of the data bearing upon the evidence for the presence of a tumor inhibitor and Dr. Bock's preliminary analysis on the Cary instrument were lost in transit to California in 1964 and were never found. The data that were available for the writing of the book I referred to above are as follows. There appear to be several significant facts concerning the presence of an inhibitor of spontaneous tumors in mice: (1) There was obtained a positive effect in preventing or delaying the onset of spontaneous cancer of mammary gland origin in mice (a preliminary or pilot experiment) by the periodic injection of a specially prepared liver emulsion. (2) There was also obtained a complete regression of $15.9 \pm 1.9\%$ of all spontaneous tumors of mammary gland origin used. (3) There was a reduction of multiple tumors from $73.3 \pm 4.5\%$ in the controls to $29.0 \pm 1.4\%$ in the group receiving the tumor inhibitor (that is, a difference of $9.43 \times$ PE between the controls and the experimentals). (4) There was a significant reduction of growth capacities of spontaneous tumors as compared to the controls (Strong, 1968).

The development of the concept that a tumor inhibitor may be expected in the liver was also partly considered in the same book.

The methods that were used in the preparation of liver-derived emulsions containing a hypothetical tumor inhibitor were based not on well-known recognized techniques to obtain a relatively pure specific entity already known but on a survey of elimination or concentration of a possible inhibitor, and several compounds or entities could be tested at one time—perhaps the identification of an active inhibitory entity could also be hastened.

Hence it was known that the original liver emulsion containing a definitely demonstrated inhibitor of tumor growth in mice was made up of several components—and it still remains a mixture primarily because the research budget for the present series of experiments bearing upon the control of cancer in mice has never been adequate.

However, because the possibilities of a limited appreach to the task at hand were fully realized, the liver material known to contain a tumor inhibitor was given to Dr. Fred Bock for analysis. The technical part of the assay was done by Mr. Fred Johnson.

Dr. Bock reported as follows: 'There is evidence of conjugated double bonds in the liver emulsion, possibly an aromatic system. The nature of absorbance is so nonspecific that I don't think you can be more precise than above' (Bock, personal communication). However, before any further work could be done on the identification of specific entities in the liver emulsion, the experiment had to be terminated at Springville due to a forced retirement of the senior author, and plans were formalized to move the research program to California.

By 1970, enough progress had been obtained in determining the biological characteristics of the liver-derived tumor inhibitor that another attempt was made to determine at least some of its biochemical features. These studies for the interpretation of the biological nature of the tumor inhibitor have been the source of 12 publications since the appearance of *Biological Aspects of Cancer and Aging: Studies in Pure Line Mice* (Strong, 1968).

Consequently, contact was made on February 23, 1970, with Dr. James T. Grace, Jr., Director of Roswell Park Memorial Institute of Buffalo, N.Y., in order to ascertain whether or not any staff member of his institution would be interested in collaborating with the author on the problem at hand. He expressed an interest. Unfortunately, a serious auto accident on March 9, 1970, prevented his continued employment. Fortunately, he had contacted Dr. Arnold Mittelman about the problem. Further contacts with Dr. Gerald P. Murphy, Dr. Grace's successor as director, Dr. Edwin Mirand, and Dr. Mittelman have produced some results, although of a preliminary nature.

Dr. Mittelman first reported as follows: the liver emulsion submitted for bioassay 'is a mixture of (at least) three nucleosides (1–2 two derivatives of adenosine and one 3 of uridosine), four lipid fatty acids and five cholesterol.' On December 3 1970, Dr. Mittleman reported further that 'in your extract numbered Bull II 456, we have clearly identified a small quantity of adenosine, a larger quantity of N^6 methyladenosine, and 5-methylcytidine' (Mittelman, personal communication).

RÉSUMÉ

A transmissible entity has been indicated in mice by the injection of a liver emulsion containing an inhibitor of spontaneous tumors of mammary gland origin (Strong and Matsunaga, 1970). The test for the presence of a transmissible entity was measured by the effects upon the biological characteristics of cancer, such as (1) growth rate of tumors, (2) percentage of regressions of tumors, and (3) the survival time of the cancer proband. It is known that when once introduced into a mouse the effect of the inhibitor of cancer in mice increases in potency through successive generations of the untreated descent. It is also known that the tumor inhibitor is heat sensitive and will increase in potency to affect spontaneous tumors with aging under both room temperature and refrigeration.

The present evidence demonstrated that the best inhibitor was 1346 days old (3.7 years) at the time it was used. Pasteurization once used in the preparation of the inhibitor will 'attenuate' it, but there was obtained a return to its ability to suppress cancer with aging.

There has been a preliminary attempt to ascertain the materials present in the liver emulsion containing the active inhibitor. This work has been handicapped by a restricted budget but does indicate some progress. But this time-consuming problem has just begun.

CONCLUSIONS

1. There is a timed pattern of aging in a liver emulsion containing an inhibitor of the growth capacity of spontaneous tumors in mice.

2. The timed pattern of changes consists of at least two characteristics.

3. These characteristics are (1) the presence of rhythms of activity of the apparent inhibitor and (2) the increased effectiveness of the inhibitor in suppressing the growth capacity of a spontaneous tumor with time.

4. Due to the fact that the inhibitor is heat sensitive, the application of heat (such as pasteurization at 56 C for 30 min) in its preparation alters the time pattern (hastens?) which is always found during the 'aging' process of the liver emulsion.

5. The liver emulsion contains adenosine, N^6 methyladenosine, and 5-methylcytidine (Mittelman, personal communication).

6. With the determination of a 'transmissible entity' associated with the injection of a liver emulsion containing a tumor inhibitor, the present problem is definitely in the field of immunogenetics.

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