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Crown-gall Tumor Stimulation or Inhibition: Correlation with DNA Strand Separation

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Abstract

The effects of the carcinogen dimethylbenz (a)anthracene, of antimitotic drugs (cyclophosphamide and daunorubicin), of the plant hormone (auxin IAA) and the antibiotic mitomycin C were investigated *in vitro* on cancer and healthy DNA from pea seedlings, inoculated or not, with oncogenic *Agrobacterium tumefaciens*. These substances stimulate *in vitro* both synthesis and strand separation of crown-gall DNA as well as oncogenic *A. tumefaciens* DNA, while they have little effect on normal plant DNA as is the case with *E. coli* and non-oncogenic *A. tumefaciens* DNA. This correlates with the substance-enhancing-power on *in vivo* crown-gall cell multiplication. Growth-stimulatory or inhibitory-effects are antagonized by the tumorless action of *E. coli* small size RNA-fragments. Plant ribonuclease is under control of all these compounds and the RNA-fragments compensate for increased or decreased ribonuclease activity induced by cyclophosphamide, daunorubicin, dimethylbenz(a) anthracene or auxin. There appears to be a correlation between ribonuclease activity and crown-gall cell development.

Introduction

A relatively close relationship exists between the carcinogenic processes in the animal and vegetable kingdoms. Chemical carcinogens induce mammalian (1) and plant (3, 15) tumors and when used at low doses they stimulate cancer cell multiplication in both cases (2, 24). Chemotherapeutic agents (cyclophosphamide (CP), daunorubicin) inhibit human cancers (16) but their carcinogenic potency established *in vitro* (5) and *in vivo* in mammals (26), has been extended to plants. We have shown (24) that once the tumor process was started the *in vivo* multiplication of pea cancer cells induced by *A. tumefaciens* B_6 could be either substantially accelerated or inhibited by CP, daunorubicin and DMBA [(IAA has exhibited a similar action (13)]. The effects obtained are dose-dependent. Carcinogens, antimitotics, which behave as carcinogens, and steroids preferentially stimulate mammalian cancer cell *in vitro* DNA synthesis and induce the strand separation of the DNA (12). We show here that CP,

daunorubicin, DMBA, mitomycin C and IAA also stimulate crown-gall cell and *A. tumefaciens in vitro* DNA synthesis through a destabilisation of these DNA but have little effect on DNA from healthy plant cells.

In addition, a correlation exists between CP, daunorubicin, DMBA, and IAA effects and RNase activity in plant tumor cells. Nucleases may produce a large range of biologically active RNA-fragments; primers for DNA replication (9), tumor-inducing RNA (active exclusively in the presence of IAA) (8, 10, 22), tumor-inhibiting RNA (whose effect may be overcome by IAA) (23), or RNA-fragments used here. These last have no direct effect on animal and plant tumor inhibition or evolution but are capable of binding to some sites on DNA which thereafter are no longer accessible to other substances (11).

The influence of endogenous or exogenous substances on RNase activity may lead to the liberation of ribooligomers in the cell which are either purin-rich, in which case they generally activate protein synthesis (19), or pyrimidin-rich, in which case they inhibit it (19). Furthermore, we show here that each of the drugs used (carcinogen, antimitotic, hormone, and RNA) acts on the plant cell RNase activity and that there appears to be a correlation between RNase activity and plant tumor development.

Materials and Methods

Reagents

Pancreatic RNase 4 x crystalized: I. C. N. Pharmaceuticals Inc., Cleveland, USA. Deoxyribonucleoside-5'-triphosphates (d-XTP) ^{12}C and ^{3}H-lithium salt (sp. act. 24-27 Ci/mmol): Schwarz Bioresearch, USA. Indolacetic acid (IAA): Prolabo, France. Mitomycin C: Sigma Co., St. Louis, USA. Daunorubicin- Rhône-Poulenc, France. Cyclophosphamide (CP): Lab. Lucien, France. 9, 10-dimethyl-1,2-benzanthracene (DMBA): N.B.C., Cleveland, USA. Phenol: Backer Chemicals, Holland. 8-hydroxychinolin: Merck, France.

Plant Material

Two-day-old etiolated decapitated epicotyls of *Pisum sativum* L. cv Annonay were used as already described (20). The oncogenic agent was *Agrobacterium tumefaciens* B_6 (about 10^8 cells per wound), aerobically grown overnight at 28°C (20). IAA, DMBA, daunorubicin, CP, and RNA-fragments tested here were dissolved in a buffered physiological saline solution, pH 7.0 and filtered through millipore prior to use.

In some instances, the wounds were infected (time 0) with a mixture of bacteria-drugs and in other experiments the drugs were introduced into bacteria-preinfected wounds (time 24 or 48 h) after gentle tissue scarification. The sterile saline solution was applied to control plants. The treated seedlings (30 plants) continued to grow in darkness for 12 days, then the fresh weight of the excised tumors was determined, and mean weights were compared by the Student's t-test. The tumors were used to prepare cancerous DNA and crude extracts for RNase activity detection. In

yet other experiments, the drugs were applied immediately to the cut subapical region and the segments were cut after 1 or 2 days and used for the preparation of crude extracts.

Isolation of DNA

Crown-gall cells from peas were placed in a pH 8 Lerman buffer (25) and DNA was extracted with water-saturated phenol containing 0.2% 8-hydroxychinolin as described previously (28). Light-grown pea shoots (about 2 weeks old) were used for healthy DNA extraction. DNA from *A. tumefaciens* was prepared in the same way except that lysis was performed in sterile distilled water. The integrity of DNA was controlled before use (12). The hyperchromic effect on DNA in the presence of the different substances was determined by UV absorbance in Tris-buffer 0.01 M pH 7.65 as described for mammalian DNA (12).

Isolation of DNA-dependent DNA Polymerase

The preparation of a partly purified enzyme from *E. coli* and incubation conditions for DNA synthesis have been described (12). All experiments were performed in the absence or presence of compounds to be tested.

Sources and Isolation of ^{3}H-labelled RNA and RNA-fragments

Bacteria *E. coli* T3000 aerobically grown at 37°C in a synthetic medium (6) supplemented with ^{3}H-adenine (500 µc/1) and ^{3}H-guanine (500 µc/1) were harvested during the exponential growth phase. Ribosomal RNA was isolated from washed cells as described (6). RNA-fragments were obtained by mild degradation of *E. coli* ribosomal RNA (r-RNA) using pancreatic RNase (11). Purin-rich RNA-fragments (about 25-50 nucleotides) are devoid of DNA. Their analysis and characteristics have been described (11).

Plant Ribonuclease

Healthy and cancerous tissues, drugtreated or not (from 50 plants), were homogenized in a cold mortar with sterile distilled water. The homogenate was twice centrifuged (5000 g for 10 min) and the dialysed supernatant (crude extract) was used as the enzyme source. Proteins were determined by Folin's method. Five to 10 µg of proteins were added to the assay solution (final vol. 0.1 ml) containing 100 µg of ^{3}H-r-RNA (15000-20000 CPM). After 10 min of incubation at 36°C the reaction was stopped by the addition of 5% TCA solution and the acid-precipitable product was filtered on a GF/C glass filter. It was then washed (TCA 5%) dried, and its radioactivity was measured in a Prias Liquid spectrometer.

Results

Crown-gall and Healthy Cell DNA *in Vitro* Synthesis

Fast development of crown-gall tumors implies an accelerated replication of DNA from these cells compared to that of healthy cells. DNA replication and cell division are interdependent. Using template DNA purified from the healthy or tumorous cells of pea seedlings and DNA-dependent DNA polymerase, we compared template activities of both

during DNA *in vitro* synthesis. Tumorous DNA exhibits a higher template activity compared with that of DNA from normal plant cells (Fig. 1, see origin of curves). From this first observation it appeared that cancer DNA contained a largernumber of single-stranded DNA regions required for DNA-dependent DNA polymerase activity. DNA synthesis, which requires all four d-XTP, is strongly inhibited in the presence of DNase (Table 1).

To parallel numerous observations on human cancer therapy and because of their effects on mammalian DNA synthesis, we investigated the *in vitro* synthesis of DNA from crown-gall and healthy pea cells in the presence of CP, daunorubicin, DMBA, mitomycin C or IAA. At low concentrations every one of these substances substantially stimulates the synthesis of tumorous DNA while they slightly enhance normal cell DNA synthesis (Fig. 1). High concentrations were inhibitory. It thus appears that crown-gall cell DNA is very susceptible to the extremely diversified molecules used. It is remarkable that *A. tumefaciens* DNA *in vitro* synthesis is greatly stimulated by the above compounds while that of DNA from *E. coli* or the non-oncogenic *A. tumefaciens* B_6-TrI (7) is not (Fig. 1). In this

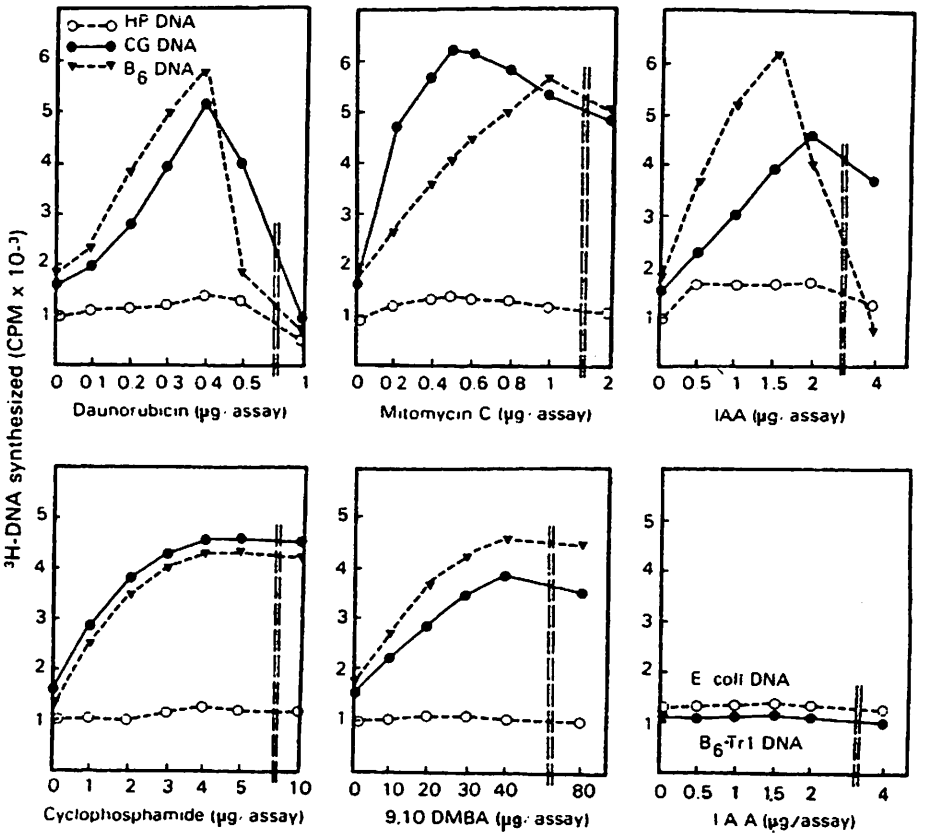

Fig. 1. Effects of various compounds on cancer and normal *in vitro* DNA synthesis of healthy pea cells DNA (HP DNA), crown gall DNA (CG DNA), and *A. tumefaciens* (B_6 DNA).

Table 1. Effect of DNase on crown-gall DNA *in vitro* synthesis.

Incubation medium (11)	^{3}H-TTP incorporated into DNA (CPM)	Inhibition %
Complete (0.5 µg of DNA)	6420	–
+ DNase 0.1 µg	643	90
+ DNase 1 µg	274	95
– d-ATP, d-CTP, d-GTP	447	93
heated enzyme at 100° for 5 min.	163	97

respect, crown-gall cell and *A. tumefaciens* DNA behave as do DNA purified from different mammalian cancerous tissues, i.e. carcinogens and several antimitotic drugs exhibit a considerable stimulatory effect on cancer DNA *in vitro* synthesis and a slight effect on that of DNA from healthy tissues (5).

Crown-gall Cells *in vivo* Multiplication

The particular *in vitro* stimulation of crown-gall DNA synthesis by the various substances used should be correlated by an *in vivo* acceleration of cancer cell multiplication. Thus, a range of concentrations of CP, daunorubicin or DMBA were introduced into preinfected wounds 24 or 48 h following bacterial infection (only a few hours are required for the transformation of normal pea cells into tumor cells). It is interesting that at low concentrations, these substances act as tumor-stimulants and increase tumor weight while at high concentrations they have a strong tumor-inhibiting action but do not disturb normal plant growth (Fig. 2). When drugs and bacteria are applied together on wounds (time 0), the same low concentrations have no effect on tumor development, whereas, high concentrations have a drastic inhibitory effect (Fig. 2). The non action of low concentrations at time 0 must be the consequence of the dilution or elimination of these substances unretained by healthy cells during the first hours following wounding. These results correlate the stimulation of *in vitro* crown-gall DNA synthesis and the absence of stimulation on DNA synthesis from healthy cells.

In Vitro **DNA Strand Separation**

The substantial and selective stimulation of *in vitro* crown-gall DNA synthesis and the significant increase of pea tumor cell multiplication under the influence of each of these compounds suggested that cancer DNA was "relaxed" compared to normal DNA and may undergo further destabilization in the presence of these substances. To verify this hypothesis, we studied the hyperchromic effect of these substances on healthy and cancer DNA. It is known that UV absorbance (260 nm) increases when DNA strands are separated. Maximum hyperchromicity obtained from both types of DNA on incubation with 0.1 M KOH ranges

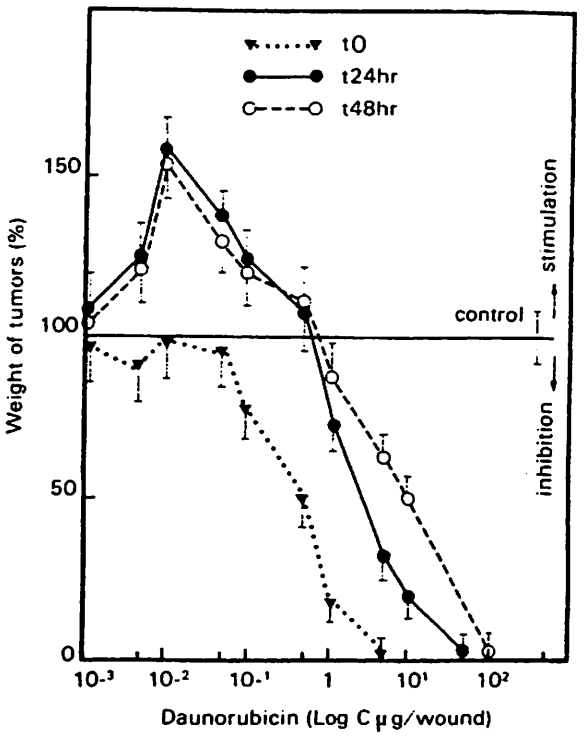

Fig. 2. Effect of daunorubicin on crown-gall tumor development. Drug was applied to the wounds either mixed with B_6 bacteria (t0) or separately 24 hours (t24) or 48 hours (t48) after bacterial infection. Each point represents mean tumor weight value for 30 plants and vertical bars indicate ± SE of the mean. Similar responses were obtained with CP and DMBA.

between 35 and 42%, which confirms that both DNA are well polymerized. Now, Fig. 3 shows that in the presence of each of the substances used, UV absorbance increases considerably for crown-gall DNA but not at all or only slightly for control cell DNA. There are optimal concentrations for DNA strand separation which vary according to the drug used (Fig. 3). They also locally separate the strands of DNA from *A. tumefaciens* B_6 but not from *E. coli* or non-oncogenic B_6-Trl. It is important to note that plant hormone IAA, daunorubicin, CP, and mitomycin C behave as carcinogens on cancer cell DNA but act very poorly on normal cell DNA. We should recall that tumorogenesis requires IAA for tumor induction with RNA (8, 10, 22). This hormone exhibits a small but detectable hyperchromic effect on plant DNA. DNA strand separation may explain both the stimulation of *in vitro* DNA synthesis and the *in vivo* acceleration of cancer cell multiplication.

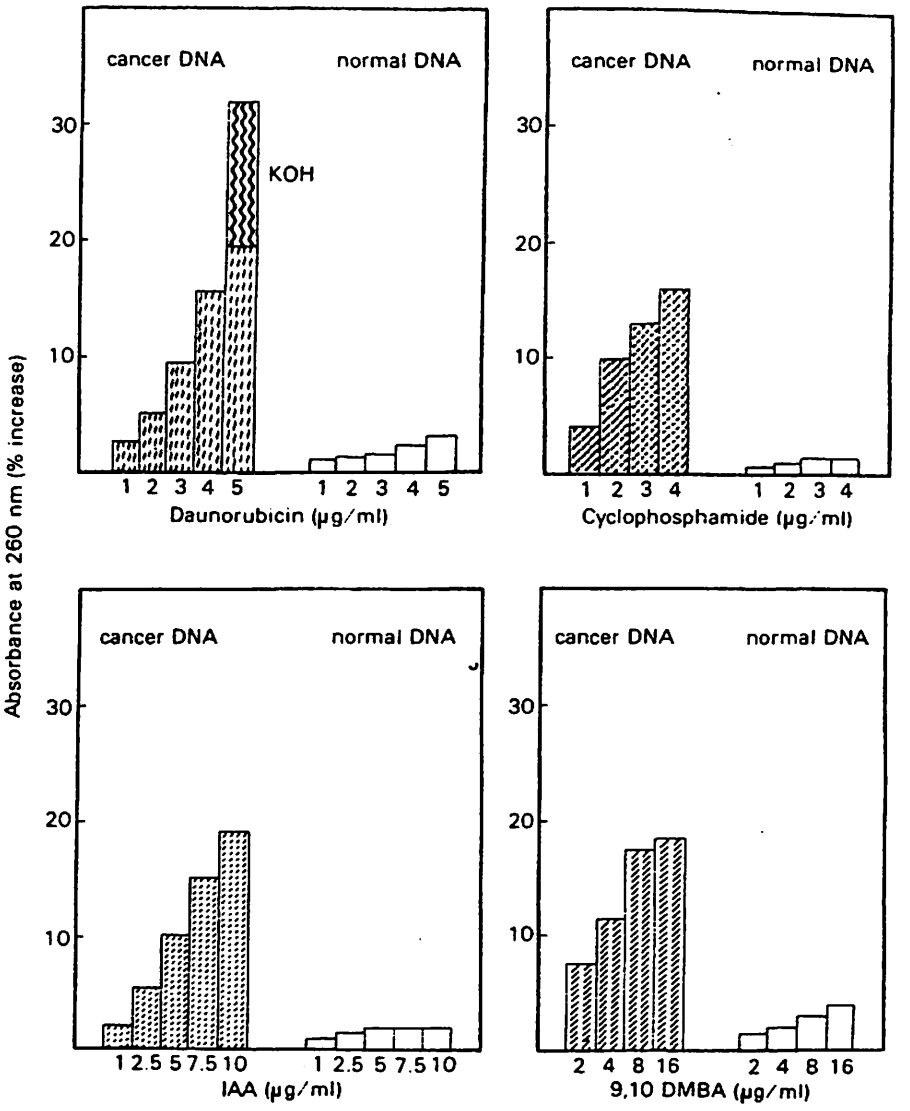

Fig. 3. Effects of drugs on crown-gall tumor and healthy pea cells DNA strand separation. UV absorbance (260 nm) of DNA was measured in the absence or presence of each compound tested at the indicated concentrations (see text).

and
Ribonuclease Activity in Healthy Crown-Gall DrugsTreated Cells

It is well known that *in vitro* replication of DNA by DNA-dependent DNA polymerase requires the presence of RNA primers (oligoribonucleotides) whose origin may be multiple (9, 27). RNase may provide these primers and we have shown that non-oncogenic long chain RNA may be degraded into

more or less purin-rich RNA-fragments, some of which inhibit preformed cancer cells (23). Others initiate DNA *in vitro* replication with a relatively high specificity towards DNA (4, 11). Plant cancer cells can be induced with RNA only in the presence of IAA (10). Thus, one should expect that, depending upon the nature of the cell and the speed of its division, RNA primers would differ and RNase activity would be modified. We therefore attempted to establish a possible correlation between *in vivo* increase or decrease in weight of tumors and RNase activity in plant and tumor extracts according to various treatments.

The crude pea extracts for the RNase activity detection were prepared either 24 h following application of the different drugs on healthy wounds (normal tissues) or at the 12th day following bacterial infection of wounds which have been post-treated 24 or 48 h after B_6 infection (tumorous tissues). Enzyme activity was measured by observing the degradation of ^{3}H-labelled r-RNA. Results are expressed as TCA-precipitated RNA (Table 2). Two important observations may be made: 1) CP, daunorubicin and DMBA after a 24 or 48 h aging period in the healthy wounded cells, decrease RNase activity, with low and high drugs doses. 2) Small doses of drugs present in the crown-gall cells lead to a considerable increase in RNase activity, while high doses greatly depress this activity.

An increase of RNase activity corresponds to an increase of tumor weight; both appear in the presence of small concentrations of drugs (Table 2). High doses decrease the tumor weight as well as RNase activity. Small

Table 2. RNase activity in the extracts from healthy pea cells and crown-gall tumors, both untreated or treated with different drugs.

	Per cent of degraded radioactive RNA		
Extracts from healthy plants		Extracts from tumor tissues	
Extract, no drug	32	Extract, no drug	42
" , CP 0.1 µg	32	" , CP 0.1 µg	55
" , CP 1 µg	10	" , CP 1 µg	70
" , CP 10 µg	8	" , CP 10 µg	21
" , CP 50 µg	1	" , CP 50 µg	1
" , daunorub. 0.005 µg	30	" , daunorub. 0.005 µg	76
" , daunorub. 1 µg	20	" , daunorub. 1 µg	16
" , daunorub. 2 µg	14	" , daunorub. 2 µg	8
" , daunorub. 5 µg	8	" , daunorub. 5 µg	11
" , DMBA 0.02 µg	33	" , DMBA 0.02 µg	49
" , DMBA 0.2 µg	32	" , DMBA 0.2 µg	63
" , DMBA 1 µg	30	" , DMBA 1 µg	35
" , DMBA 10 µg	28	" , DMBA 10 µg	28
No extract	0	No extract	0

doses of RNA-fragments introduced into infected wounds treated with small doses of drugs suppress the increase in tumor weight (Fig. 4) and decrease the enhanced RNase activity (Table 3). In the presence of high doses of drugs, large doses of RNA-fragments increase the RNase activity and practically suppress the tumor inhibitory effect of drugs (Fig. 4 and 5). Over a limit in concentration of drugs, RNA-fragments cannot reverse the effects observed. Thus, the amount of exogenous RNA-fragments may well modulate RNase activity in tumorous cells and thus bring the weight of tumors and RNase activity back to values found in untreated cancer cells. It should be stressed that RNA-fragments introduced into the wounds of decapitated epicotyls increase RNase activity (measured 24 h after administration), which is suppressed by low or large doses of drugs.

Table 3. RNase activity in the extract from crown-gall tumors treated with different drugs and RNA fragments.

Treatment	Per cent of degraded radioactive RNA
Extract without drug	42
Extract , CP 0.1 μg	55
" , CP 0.1 μg + RNA-fragts 0.005 μg	38
" , daunorub. 0.005 μg	76
" , daunorub. 0.005 μg + RNA-fragts 0.1 μg	40
" , DMBA 0.02 μg	63
" , DMBA 0.02 μg + RNA-fragts 0.4 μg	41
No extract	0

Conclusions

We used crown-gall tumors as a model to establish a correlation between *in vitro* DNA synthesis, DNA local strand separation, and multiplication of cancerous plant cells, with differential susceptibility to low and high doses of CP, daunorubicin, DMBA, or IAA. At low concentrations, these compounds (also mitomycin C) strongly stimulate crown-gall and *A. tumefaciens* B_6 DNA *in vitro* synthesis and DNA strand separation measured as a UV absorbance increase, and enhance tumorous cell development. Used at low or high concentrations, they have practically no effect on DNA from healthy pea cells, DNA from *E. coli* or from B_6-Tr-1 *A. tumefaciens* non-oncogenic strain. The action of IAA in these events is of particular interest. This plant hormone is required to increase the size of healthy cells (14) and is needed for tumor induction with tumor-inducing RNA (8, 10, 22). *In vitro,* it strongly stimulates the synthesis of crown-gall and B_6 DNA and induces DNA strand separation of both. In this

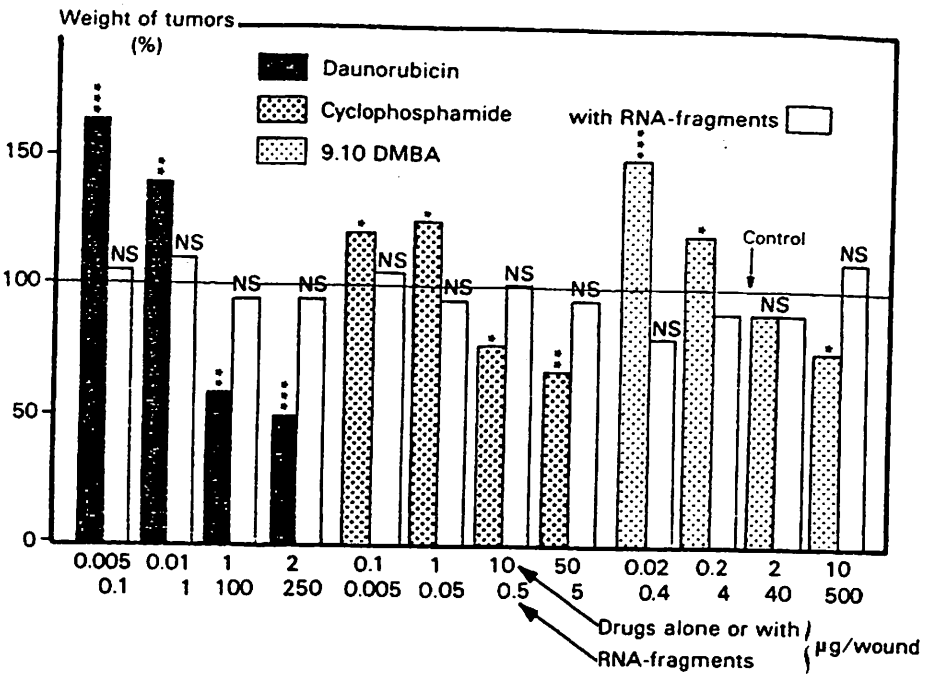

Fig. 4. Pea wound response following drugs or drugs + RNA-fragment application delayed by B_6 infection. The results based on 30 plants represent average of tumor weight expressed as percentage of control. NS; no significant difference. Significance of comparison to control (t-test), p values; 0.05*, 0.01**, 0.001***.

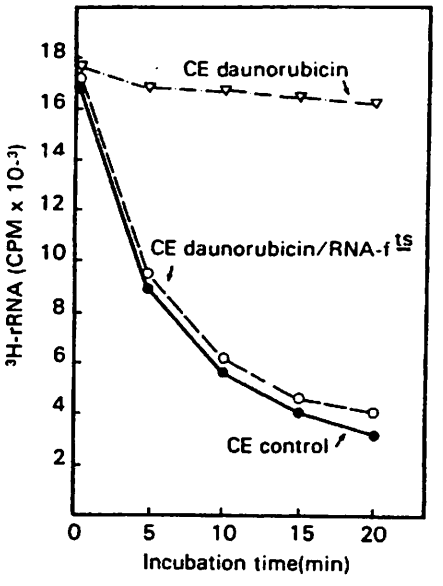

Fig. 5. RNase activity in the extracts from crown gall tumors treated with drug or drug + RNA fragments. The preinfected wounds were treated as described with distilled water (control), 2 µg daunorubicin or 2 µg daunorubicin + 100 µg RNA-fragments. Crude extracts (CE) were prepared (see text) and 10 µg proteins with 100 µg ^{3}H-r-RNA were used per assay. The results express the degraded radioactive r-RNA according to incubation time.

respect it has a detectable, although low, effect on DNA from healthy pea cells. Its contribution in tumor induction appears to be similar to that of carcinogenic compounds since its *in vitro* behavior is similar to theirs.

The interaction of hormones and ribonuclease was considered in plant cells (29). RNase activity is in our experiments of fundamental interest since this enzyme may provide RNA primers without which there is no possible DNA synthesis (9, 27) and, consequently, no cell division process. We have already demonstrated that, depending on the RNase, inert r-RNA may be used to provide either tumor-inducing RNA (8, 10, 22), tumor-necrosing RNA (23) or specific primers for this or that DNA template (4, 11). Thus, all modifications of RNase activity interfere with the endogeneous primers which this enzyme provides for cell multiplication.

It is worthwhile noting that a given cell DNA requires different RNA primers, according to its normal or cancerous origin (unpublished results). Here we saw that in healthy plant cells, low and high concentrations of these substances inhibit RNase activity while at low doses in crown-gall they strongly stimulate RNase activity. High doses are inhibitory and decrease tumor weight.

There appears to be a correlation between crown-gall tumor weight increment and the increased RNase activity of these cells. This correlation is observed only if small doses are introduced into wounded plants 24 h after infection with B_6. This indicates that these compounds act preferentially on tumorous cells whose formation requires at least 6 h following infection (18). Exogenously introduced RNA-fragments regulate RNase activity and suppress either the stimulating or inhibiting effect of the substances active in plant tumor development. It was reported that mitomycin C used at low concentration stimulated the multiplication of *A. tumefaciens* and consequently substantially increases tumor initiation (17). Moreover multiplication of *A. tumefaciens* inside tumorous cells does not appear to be essential for tumor weight increase (21). Here we have shown that mitomycin C at low doses induces crown-gall and B_6 DNA *in vitro* strand separation and strongly enhances DNA synthesis without affecting DNA from healthy pea cells. DNA local strand separation is a part of the gene activation process in healthy cells and particularly in tumorous cells; it provides single-stranded DNA regions required by DNA and RNA polymerases to accomplish their action necessary for cell growth and division.

Data presented here and other data obtained with mammalian cancer cells DNA (12) demonstrate the existence of a common denominator for cancer DNA i.e. that these DNA are destabilized and then become susceptible to the action of various compounds and may exhibit a high template activity in comparison to DNA from healthy cells.

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