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ASCORBIC ACID INHIBITS RNA-TUMOR VIRUS REPLICATION AND INFECTIVITY

ABSTRACT. Ascorbic acid, at non-toxic concentrations, causes a substantial reduction in the ability of avian tumor viruses to replicate in both primary avian tendon cells (PAT) and chick embryo fibroblasts (CEF). The vitamin does not act by altering the susceptibility of the cells to initial infection and transformation, but it interferes with the spread of infection through a reduction in virus replication and virus infectivity. The effect is dependent on the continuous presence of the vitamin in the culture medium and is reversible.

The mechanism of action of ascorbic acid at a cellular and molecular level remains remarkably unknown despite the widespread popular belief in its various beneficial roles (1). While studying the effect of this vitamin on collagen synthesis in primary avian tendon (PAT) cells (2) we observed that transformation of these cells by RNA tumor viruses would proceed at variable and unpredicted rates. We discovered in due course that the observed variability was a function of whether or not ascorbic acid was present in the daily change of culture medium. Furthermore, pretreatment of cells with ascorbic acid before viral infection made the cultures more resistant to spread of virus infection. We therefore set out to examine in detail the effect of this vitamin on RNA tumor virus infection, replication and transformation.

Ascorbic acid is very unstable under the usual culture conditions (3). Using as an assay the reduction of ferric ion to ferrous by ascorbate and the ability of α-α' dipyridyl to complex the ferrous (4), we determined that the half life of ascorbate in our culture medium at 37° (with or without cells) was only one hour (data not shown). Therefore, in order to observe the effect of ascorbic acid on either collagen synthesis (2) or virus production, freshly prepared ascorbic acid at 50 µg/ml (.28 mM) was added to the medium which was changed daily. The morphology of Rous sarcoma virus (RSV) infected PAT cells was distinctly different depending on whether or not the cells were pretreated and grown in 50 µg/ml ascorbic acid per ml of medium (Fig. 1). While the untreated cultures became entirely transformed after 5-6 days as shown by their characteristics morphology (Fig. 1, upper right), the ascorbic acid treated cells showed discreet foci of transformation which did not spread to the rest of the culture (lower right). Similar results were obtained with other subgroups of the virus.

To determine whether other criteria of transfomration--in addition to morphology-- were affected, 2-deoxy-D-glucose (2-dg) uptake was measured in normal and virus-transformed cells grown in the presence or absence of ascorbic acid. An increase in glucose uptake after RNA-tumor virus infection is an accepted criteria of malignant transformation. While the rate of sugar uptake varies with the population density, the rate of growth and the cell shape (5), it is still higher in virus-transformed cells when these factors are controlled (5). A comparison of 2-dg uptake in the presence and the absence of ascorbate (Fig. 2) indicated that cells pretreated with and grown in the presence of ascorbate had a rate of sugar transport which almost remained at the low level of normal cultures. There was also a small but consistent decrease in the rate of sugar transport in both normal and cells infected with a transformation defective virus after ascorbate treatment (Fig. 2B). This may be due to the slightly altered morphology of ascorbate-treated untransformed cells since it is known that alteration in cell shape changes the rate of sugar transport (5). (Compare the two figures on the left of Fig. 1). Nevertheless, the magnitude of this change was far less than that observed in ascorbate-treated, transformed cells. Thus, in addition to relatively normal morphology, the rate of sugar uptake in cells infected with transforming viruses of RSV and grown in ascorbate was only slightly above that of normal, uninfected cells.

Additionally, viral replication and infectivity (or transforming potential) as measured by focus forming units was severely reduced (Table I). While total virus particle production (as measured by reverse transcriptase assay) was also reduced, the inhibition was less than that of focus forming units. However, transformation was not necessary for a decrease in virus number since transformation-defective viruses also showed a substantial and comparable decrease in virus replication (Table 1).

There was no toxicity to either normal or transformed cells at 50 μg/ml ascorbic acid when cell number was measured within a 5-day period (not shown). Therefore, preferential toxicity to transformed cells could not explain the above findings.

Three possibilities for the mechanism of action of ascorbate were considered: 1) Ascorbate could directly inactivate the virus (both the initial inoculum and the replicated virus) to reduce primary and secondary infection, 2) pretreatment with ascorbate could render the cells resistant to viral infection by an as yet unknown mechanism (e.g. by increasing collagen synthesis which in turn could form a protective coat and prevent infection, and 3) ascorbate could interfere with viral replication and infectivity by altering the metabolic make-up of the cell and by interfering with some of the steps in viral assembly and release.

Ascorbic acid has been shown to inactivate double and single stranded RNA and DNA bacteriophages (8). However, pretreatment of the different subgroups of RNA tumor viruses with ascorbic acid (50-500 μg for 1-4 hrs) did not reduce their infectivity in subsequent focus assays. Indeed, there was a small but reproducible increase in focus forming units when virus was preincubated with ascorbic acid (data not shown). The first possibility was therefore ruled out. While the distinction between the second and third possibilities was not simple, we decided in favor of the third based on the following three lines of evidence:

a) If the only effect of ascorbate were to prevent virus infection, then the subsequent addition of ascorbate to cultures which were fully infected by the virus through a high multiplicity of infection (m.o.i) would show little or no influence on further virus production. However, in experiments where the initial innoculum of virus was varied by a factor of 100 (Table 2), and ascorbate was added after infection, comparable percent decreases in virus titre were observed.

b) When cells were infected with a temperature-sensitive mutant of the virus (LA24) at the non-permissive temperature and were shifted to the permissive temperature in the presence or the absence of ascorbate (Table 3), virus titre was reduced in the presence of ascorbate and inhibition was reduced when ascorbate was removed. This was also true for PAT and chick embryo fibroblast cells infected with the wild-type virus (data not shown). These results indicated that addition of ascorbate even after full infection would lead to a reduction in virus titre and that the effect is reversible.

c) Most importantly, when the monolayer of normal cells prepared for focus assay was pretreated with ascorbate, no reduction in the number of foci were observed, although the average size of a focus was considerably smaller in ascorbate treated cultures (data not shown).

The following picture has emerged from the above data: ascorbic acid at concentrations that are not toxic to either normal or virus-infected cells (.28 mM) causes a substantial reduction in virus replication and infectivity in both PAT and CEF cultures infected with RNA tumor viruses. The effect is brought about after a 10-15 hr treatment of the culture with the vitamin and is dependent on its continuous presence. The effect is not due to interference with virus attachment, penetration, and transformation, but due to interference with the production of virus particles. The virus produced is further not as infectious (or transforming) as the virus produced in the absence of ascorbate.

The mechanism by which ascorbate brings about the reduction in virus titre and infectivity is not clear at the moment. Despite the high level of collagen produced in the presence of ascorbate in PAT cells (2), collagen is not involved directly. The inhibition of virus replication occurs in both PAT and CEF cultures despite the fact that the former produces a 10-15 fold

higher level of collagen than the latter. Additionally, as we have discussed above, the initial infection is not affected by pretreatment of cultured cells with ascorbate. Isoascorbate, an epimer of ascorbate which is reported to have only 1/20th of the biological activity of ascorbate, and is not metabolized, is almost as effective as ascorbate in reducing the spread of virus infection in culture (data not shown). Thus, metabolism of ascorbate is not necessary for its anti-viral action which in turn may be directly related to ascorbate's reducing potential. This possibility is under investigation.

Two additional points merit discussion. Despite the fact that virus titre and infectivity is substantially lower in ascorbate-treated cultures, the cells still produce an appreciable amount of infectious virus (Table 1). Yet, regardless of how long the virus-infected, ascorbate-treated cultures are maintained, some cells always look flat and appear normal (Fig. 1). The reason for this observation may be two-fold. The first possibility is induction of interferon. Rous sarcoma viruses have been shown to induce interferon in chick cells (9). Additionally, ascorbic acid is shown to increase interferon production in L cells and mouse embryo fibroblasts by poly(rI), poly(rC) and in human embryo lung fibroblasts infected with New Castle disease virus (10). The latter results, however, were not well documented and have not been repeated by other laboratories. Additionally, WI-38 cells treated with ascorbic acid and glutathione and infected with rhinovirus did not show much interferon production (11). Nevertheless, it is conceivable that ascorbic acid under our culture conditions could increase interferon production by RSV-infected cultures and hence further retard virus spread. The other likely possibility is that some of the cells that appear normal, may indeed be infected. Since ascorbic acid causes the production of some defective virus (as shown by the fact that focus forming units are inhibited more severely than reverse transcriptase

activity), cells could be infected by the transformation-defective subpopulation, hence becoming resistent to super infection by the transforming virus. These two possibilities require further investigation.

Schwerdt and Schwerdt have shown (11) that ascorbic acid reduces rhinovirus replication in cultured human cells after the first cycle of virus replication. The "delayed" action of ascorbic acid on rhinovirus replication is reminiscent of its action on RSV replication in chick cells. The authors, however, interpret their data solely on the basis of interferon production--a possibility which is almost ruled out by their own data. It is more likely that in their system also the presence of ascorbic acid causes defective or reduced virus replication by a mechanism other than elaboration of interferon. Their data differs from ours in that the amount of ascorbic acid used was 250 µg/ml of medium and had to exceed 100 µg/ml before any effect on virus replication could be observed. The results reported here were obtained with 50 µg/ml of ascorbic acid regardless of the density of the culture. Even at high densities ascorbic acid in excess of 100 µg/ml was toxic to PAT cells. Additionally, at a m.o.i. greater than 4 plaque forming units per cell, rhinovirus replication was not suppressed by ascorbic acid in WI-38 cells. We have shown here that in chick cells infected with RNA tumor viruses, regardless of the initial m.o.i., there is a substantial decrease in virus replication after addition of ascorbic acid. Whether these differences are species specific or whether they reflect differences between cell lines and primary cultures remain to be established.

The significance of our findings with regard to the action of ascorbic acid on viral infection in other species and specifically with regard to the common cold in man (1), remains obscure. If the results with chick cells could be extrapolated to man, it is clear that there is

no reason to expect a reduction in the incidence of colds with the intake of ascorbic acid. On the other hand, if the severity of colds were the result of secondary infection, the intake of ascorbic acid could substantially reduce the level of secondary infection by interfering with viral replication. Aside from a minimum amount of support for this idea from the large-scale clinical studies of Anderson (12), the extrapolation should be considered pure speculation.

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Table 1

Ascorbate Inhibition of Virus Replication

and Infectivity in PAT Cells

Culture	Day 3		Day 4		Day 5	
	RT[1)]	FFU[2)]	RT	FFU	RT	FFU
PC	28760	3.2×10^5	61330	6.2×10^5	127660	1.4×10^6
PC + Asc.	9380	2.5×10^3	22870	$.7 \times 10^5$	23790	1.8×10^5
% Inhibition	67%	>99%	63%	89%	81%	87%
TDPC	18580	-	14870	-	23850	-
TDPC + Asc.	5740	-	6640	-	1400	-
% Inhibition	69%	-	55%	-	84%	-

Legend as in Fig. 2. Overnight medium was removed and 1 ml of appropriate medium was added and collected 2 hrs later. The result is the average of duplicate plates of two experiments.

1) RT, Reverse transcriptase activity, measured as described previously (6).

2) FFU, focus forming unit per ml of of medium, measured as described previously (7).

Table 2

Lack of Effect of Initial Virus Inoculum

on Inhibition by Ascorbic Acid

Virus / Cell m.o.i.[1]	Culture	FFU/mg Protein		
		Day 2	Day 3	Day 4
0.5	PA[2]	5.6×10^5	1.3×10^6	3.2×10^6
	PA + Asc.	0.35×10^5	$.3 \times 10^6$	1.2×10^6
	% Inhibition	94%	77%	62%
5.0	PA	13.6×10^6	$24.0.10^6$	20.0×10^6
	PA + Asc.	0.8×10^6	1.3×10^6	7.0×10^6
	% Inhibition	94%	95%	62%

PAT cells were infected in the absence of ascorbic acid. Medium was changed 4 hrs post infection and ascorbic acid (50 µg/ml) was added at that time. The result is the average of duplicate plates of 3 experiments.

1) m.o.i., multiciplicity of infection.

2) PA, Prague A subgroup of Rous sarcoma virus.

Table 3

Effect of Temperature Shift in the Presence and Absence of Ascorbate on Temperature Sensitive Virus Production

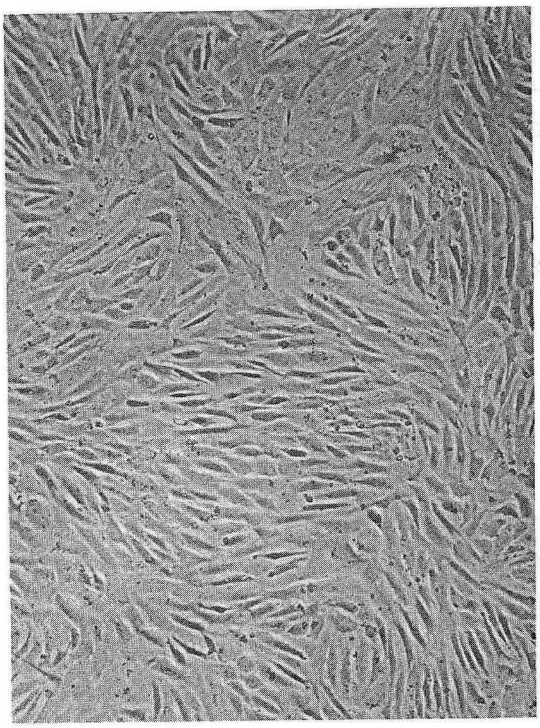
Culture Conditions		20 hr after shift		30 hr after shift	
Grown in:	Shifted in:	FFU/ml	% Inhibition	FFU/ml	% Inhibition
No Asc.	No Asc.	2.1×10^5	-	27×10^5	-
No Asc.	+ Asc.	$.9 \times 10^5$	57%	8×10^5	70%
+ Asc.	No Asc.	5.5×10^3	97%	1.8×10^5	94%
+ Asc.	+ Asc.	3.5×10^3	98%	1.5×10^5	94%

Cells were grown and infected with LA24 (a temperature-sensitive mutant virus defective in the src gene) at 41°. Medium was changed daily with or without ascorbate. The cells were shifted on day 5 post infection. Other legends as Fig. 2 and Table 1. The result is the average of duplicate plates of two experiments.

FIGURE LEGENDS

Fig. 1. Morphology of Ascorbate-treated PAT cells. PAT cells were prepared and infected with Rous sarcoma virus (Prague, Subgroup A) as described previously (2). Treated cultures received medium with 50 μg/ml ascorbic acid 24 hrs prior to infection and in subsequent changes of medium. Pictures were taken 6 days post infection.

Fig. 2. Sugar uptake in normal and virus-infected cells treated with ascorbic acid. Legend as in Fig. 1. Cells were infected with either Prague C (PC) strain of RSV or a transformation-defective Prague C (TdPC). The rate of [^{3}H]2-deoxy-D-glucose was measured in 5 min pulses as described previously (4).



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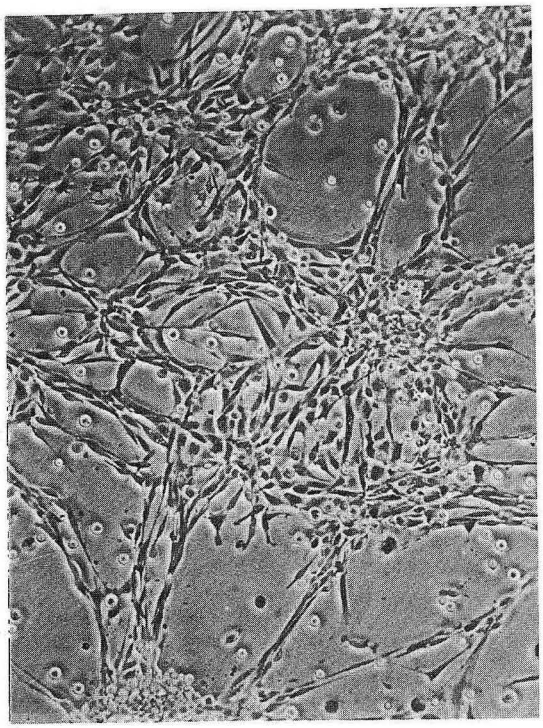

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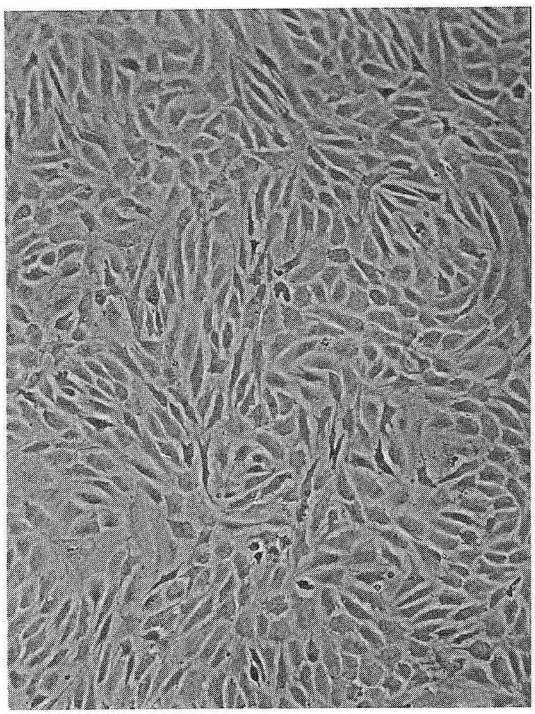

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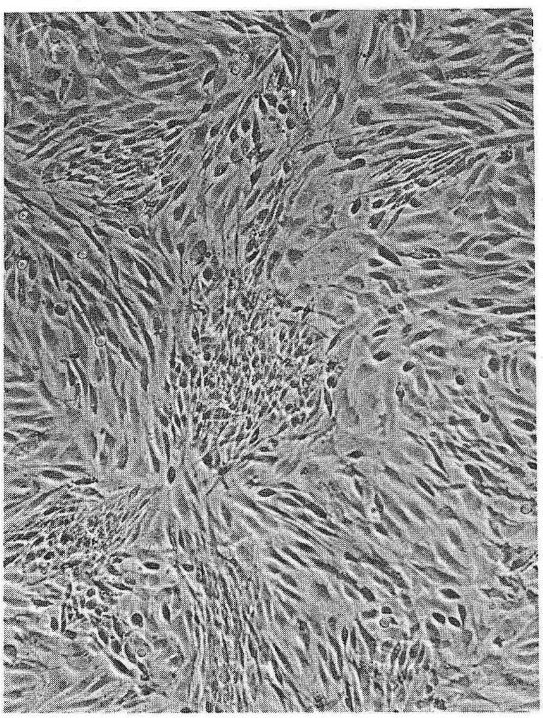

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+ ASCORBATE

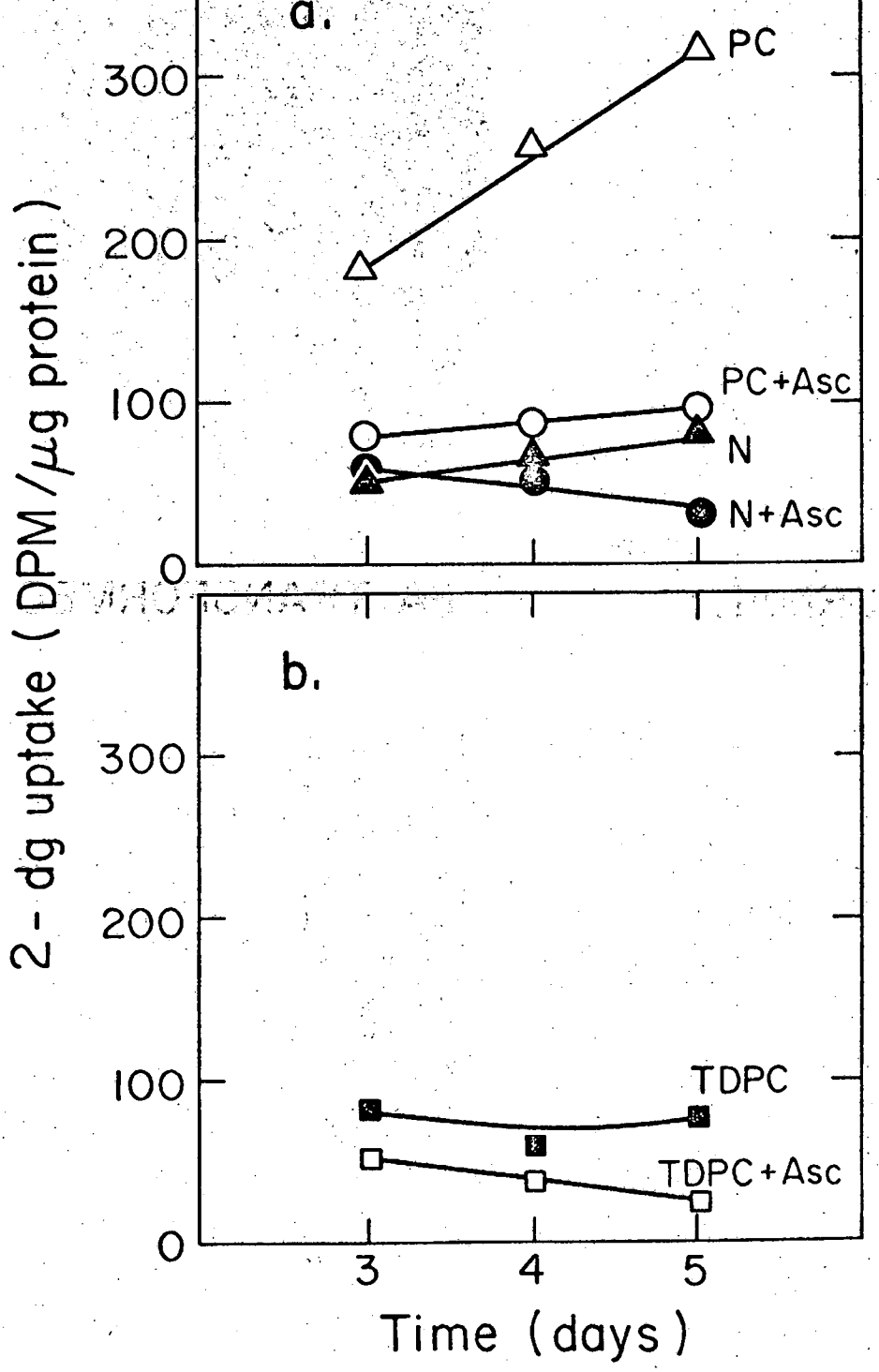

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Fig. 2

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