THE EFFECTS OF ASCORBIC ACID ON DIPHTHERIA TOXIN AND INTOXICATED HEŁA CELLS

Charles E. CLARK1 and Timothy J. SMITH

Department of Health Sciences, College of Health, East Tennessee
State University, Johnson City, Tennessee 37601

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Summary Ascorbic acid (vitamin C) prevented diphtheria toxin from inhibiting the incorporation of [U-14C]-alanine into trichloroacetic acid precipitable material in HeLa cells. Ascorbic acid did not exhibit an effect on the adenosine diphosphate-ribosylation of amino acyl transferase II nor did it separate fragment A from fragment B in "nicked" toxin. A non-specific reducing agent, para-methylaminophenol sulfate, exhibited an effect on HeLa cells very similar to the results of ascorbic acid. Citric acid, a tricarboxylic acid, had no effect on HeLa cells.

Widespread attention, both public and scientific, has been focused on ascorbic acid (vitamin C) by the book "Vitamin C and the Common Cold" written by Pauling (1). Since its publication, many double-blind studies concerning the effects of ascorbic acid on the common cold and other respiratory diseases have been reported (2–5). These studies involve a small segment of the population and the effects of ascorbic acid on individuals. Although receiving much attention at this time, ascorbic acid has been the subject of research and controversy since its isolation by Szent-Gyorgyi in 1928 (6). These studies include the action of ascorbic acid on diseases of viral, fungal, and bacterial etiology (7–10). One example is diphtheria, a disease resulting from the powerful exotoxin released from the bacterium Corynebacterium diphtheriae (8, 9).

During the latter part of the 1930's, Jungblut (8) concluded that ascorbic acid, when used in controlled dosages, was capable of eliciting a protective effect on diphtheria intoxicated guinea pigs as well as inactivating the toxin in vitro. Klenner (9) reported human case histories of diphtheria treated with massive doses (46 g in 48 hr) of ascorbic acid in conjunction with diphtheria antitoxin.

In 1959, Strauss and Hendee (17) reported the diphtheria toxin-dependent inhibition of protein synthesis in HeLa cells. Diphtheria toxin is protein in nature with two fragments that can be separated by the action of trypsin and

1 To whom requests for reprints should be addressed.
thiols such as 2-mercaptoethanol. DRAZIN et al. (11) have shown that fragment A must be linked to fragment B which binds to the cell membrane for fragment A to enter the cell and catalyze the ADP-ribosylation of transferase II. Once ADP-ribosylated, the transferase can not function to translocate the growing polypeptide chain from the amino acyl site to the peptidyl site on the ribosome. Thus, synthesis of proteins is decreased in susceptible cells.

The purpose of this experiment is to ascertain if ascorbic acid could prevent the action of diphtheria toxin on HeLa cells grown in lab culture as it does in the intact animal and could ascorbic acid prevent the *in vitro* cell-free ADP-ribosylation reaction. Since JUNGEBLUT (8) concluded that ascorbic acid could inactivate diphtheria toxin *in vitro*, we used disc-gel electrophoresis to see if ascorbic acid could reduce the disulfide bond of “nicked” toxin which would prevent its inhibition of protein synthesis. These data should show if ascorbic acid has a specific effect on the diphtheria toxin protein or a more general effect such as the cellular membrane.

**MATERIALS AND METHODS**

The diphtheria toxin in these experiments was supplied by Lederle Laboratories and used without further purification. The protein concentration of the toxin was approximated by the Biuret method. Dilutions were made in the appropriate buffers used in the different experiments.

*SDS-disc gel electrophoresis.* The reducing effects of ascorbic acid and 2-mercaptoethanol on diphtheria toxin previously “nicked” with trypsin were studied by the use of SDS-disc gel electrophoresis. Polyacrylamide gels were prepared as described previously by WEBER and O'SBORN (14). The reduction of “nicked” diphtheria toxin by 2-mercaptoethanol has been described by GILL and DINIUS (10) and COLLIER and KANDEL (12). For each gel 10 μg of diphtheria toxin in 0.1 ml of 0.01 M phosphate buffer, pH 7.0, containing 1% SDS, and 10% glycerol was heated for 1 min at 100°C. To reduce “nicked” toxin, 2-mercaptoethanol was added to give a concentration of 1% prior to heating. When ascorbic acid was tested for its ability to reduce “nicked” toxin, solid ascorbic acid (Sigma Chemical Company) replaced 2-mercaptoethanol. Several ascorbic acid concentrations ranging from 0.09 to 1.0 mg were used and exhibited identical results. Toxin samples were electrophoresed at a current of 10 milliamps/gel for 6 hr on a Buchler Polyanalyst disc gel electrophoresis apparatus. Following electrophoresis, the gels were removed from the tubes and stained with Coomassie brilliant blue (Bio-Rad Laboratories). Destaining was accomplished by diffusion.

*ADP-ribosylation of transferase II.* Isolation and purification of transferase II was performed by methods described by HONJO et al. (13). The purification procedures included ammonium sulfate fractionation, diethylaminoethane Sephadex and hydroxyapatite column chromatography. The protein concentra-
The purification of the purified transferase II preparation was determined by the method of Lowry et al. (15).

Diphtheria toxin was assayed for NAD: EF-2 ADPR transferase activity in a manner described previously by HCNJO et al. (13). This standard assay mixture contained diphtheria toxin (0.8 \( \mu \)g); 300 picomoles nicotinamide [U-\(^{14}\)C]-adenine dinucleotide in 0.01 ml of 20 \( \mu \)M Tris-Cl buffer, pH 7.6; 66.0 \( \mu \)g of purified transferase II in Tris-Cl buffer and 0.23 ml of 20 \( \mu \)M Tris-Cl buffer, pH 7.6 in a total volume of 0.35 ml. NAD (specific activity 167 mC/mmmole) obtained from Amersham Searle was diluted 5-fold in 20 \( \mu \)M Tris-Cl buffer, pH 7.6 prior to use. Trizma base was obtained from Sigma Chemical Company. When ascorbic acid was added to the system, the desired concentration (3, 10, 30 \( \mu \)g/ml) was added as part of the 0.23 ml of 20 \( \mu \)M Tris-Cl buffer, pH 7.6. The mixtures were incubated for 15 min at 37°C, and the reaction was stopped by the addition of 20% trichloroacetic acid. After chilling the tubes, the precipitate was collected on Millipore filters, washed, dried, and its radioactivity measured in a Toluene-PPO-POPOP cocktail in a Packard Tri-Carb Liquid Scintillation Spectrometer. Counting efficiency was 60% for \(^{14}\)C. Liquid Scintillation cocktail reagents were obtained from Fisher Chemical Company.

HeLa cell studies. HeLa cell-line 04012 obtained from Flow Laboratories was grown in Eagle's Minimal Essential Medium with Earle's salts and L-glutamine and without sodium bicarbonate. Also, the culture medium contained in final concentration penicillin (50 \( \mu \)g/ml), streptomycin (1 \( \mu \)g/ml), neomycin (100 \( \mu \)g/ml) fungizone (2.5 \( \mu \)g/ml), and calf serum (5%). All ingredients for HeLa cells culture were obtained from Grand Island Biological Company.

Growing cells were removed from Falcon flasks, washed with phosphate buffered saline, suspended in culture medium, and 2 ml plated in 60 x 100 mm plastic petri dishes. The cells were incubated in a 90% air-10% CO\(_2\) atmosphere overnight. The next morning culture medium was replaced with fresh medium to which ascorbic acid, citric acid, paramethylaminophenol sulfate (p-MAPS), or antitoxin had been added if required. The amount of each substance is specified in Table 1. After incubation for 30 min at 37°C, diphtheria toxin (1.5 \( \mu \)g/2 ml) was added to all petri dishes except controls. Following three hours of incubation, 7.6 nanomoles of [U-\(^{14}\)C] alanine (130 mC/mmmole) was added to all petri dishes and again incubated for three hours. Radioactive alanine was purchased from California Bionuclear. Cells were washed with phosphate buffered saline, removed with trypsin and treated with cold 20% trichloroacetic acid. The trichloroacetic acid insoluble material was collected on Millipore filters, washed with phosphate buffered saline and trichloroacetic acid, dried, and the radioactivity determined. The uptake of [U-\(^{14}\)C] alanine by control cells which contained no toxin was considered as 100%. All other cells were compared to controls. Trichloroacetic acid and salts used to prepare phosphate buffered saline were purchased from Fisher Chemical Company. Double distilled (in glass) water...
Table 1. Percent incorporation of $^{14}$C-alanine into TCA precipitable material.

The complete system contained HeLa cells, $[U-^{14}$C] alanine (specific activity 130 mCurie/m mole) and diphtheria toxin (1.5 µg/2 ml) in minimum essential medium. Cells incubated with ascorbic acid (0.5, 30, and 90 µg/ml) but without toxin were not different from complete minus toxin. Percent incorporation values are the mean ± standard error of the mean. B was different from A, C, D, and G, while A was different from B, D, E, F, and H, $P<0.01$ in all cases. All values were at least six determinations except H which was 4 determinations.

<table>
<thead>
<tr>
<th>Contents of petri dishes</th>
<th>% Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Complete minus toxin</td>
<td>100±0</td>
</tr>
<tr>
<td>B Complete</td>
<td>60.2±5.7</td>
</tr>
<tr>
<td>C Complete+ascorbic acid (90 µg)</td>
<td>98.3±7.4</td>
</tr>
<tr>
<td>D Complete+ascorbic acid (30 µg)</td>
<td>83.3±5.2</td>
</tr>
<tr>
<td>E Complete+ascorbic acid (0.5 µg)</td>
<td>61.2±4.5</td>
</tr>
<tr>
<td>F Complete+citric acid (30 µg)</td>
<td>60.0±7.1</td>
</tr>
<tr>
<td>G Complete+antitoxin (25 units)</td>
<td>101.6±4.9</td>
</tr>
<tr>
<td>H Complete+p-MAPS (30 µg)</td>
<td>78.0</td>
</tr>
</tbody>
</table>

was used throughout the experiments.

Statistical significance was determined by Student's $t$ test for nonpaired varieties.

RESULTS

Diphtheria toxin inhibited the incorporation of $[U-^{14}$C] alanine into trichloroacetic acid precipitable material in HeLa cell cultures (Table 1). The single toxin concentration (1.5 µg/2 ml of culture medium) used throughout these experiments prevented the incorporation of $[U-^{14}$C] alanine such that intoxicated cells incorporated only 60% of the labeled amino acid when compared to controls never exposed to toxin (Table 1).

HeLa cells pre-incubated with certain concentrations of ascorbic acid for 30 min prior to the addition of diphtheria toxin incorporated $[U-^{14}$C] alanine into trichloroacetic acid precipitable material to the same extent as non-intoxicated cells. An ascorbic acid concentrations of 90 µg/ml of culture medium completely blocked the ability of the toxin to inhibit the incorporation of $[U-^{14}$C] alanine (Table 1). When smaller concentrations of ascorbic acid were used, the protection decreased until a concentration of 0.5 µg/ml afforded no protection whatsoever.

Citric acid (30 µg/ml), a tricarboxylic acid, does not protect against the inhibition of $[U-^{14}$C] alanine incorporation (Table 1). At this same concentration, $p$-MAPS, a non-specific reducing agent, protects almost as well as ascorbic acid. Excess diphtheria antitoxin completely blocked the action of the toxin. Thus, ascorbic acid, a very strong reducing agent, does prevent the shutdown of protein synthesis by diphtheria toxin in HeLa cell cultures.
The effect of ascorbic acid on “nicked” toxin was studied by SDS disc gel electrophoresis. A preparation of “nicked” toxin treated with 2-mercaptoethanol exhibits three bands (Fig. 1, gel C). These three bands migrating from top to bottom represent the three species which can be found in a “nicked” toxin preparation treated with disulfide reducing reagents. The slowest migrating band represents intact toxin or toxin nicked with trypsin but not reduced by thiols. The middle band is fragment B while the fastest moving band is fragment A. Neither of the two bands representing fragments A and B is seen in gel B (Fig. 1) when ascorbic acid replaces 2-mercaptoethanol as the reducing agent. Toxin treated with trypsin, but not reduced, also exhibited only one band on SDS disc gel electrophoresis (Fig. 1, gel A).

Also, ascorbic acid had no effect on the in vitro ADP-ribosylation of transferase II (Table 2). The ADP-ribosylation of transferase II (NAD: EF-2-ADPR transferase) requires NAD, transferase II and the catalyst, diphtheria toxin. Three different concentrations of ascorbic acid exhibiting a 10-fold range neither stimulated nor inhibited the NAD: EF-2-ADPR transferase activity (Table 2). The highest concentration of ascorbic acid (30 µg/ml) used in these in vitro studies is the same concentration which inhibited the incorporation of [U-¹⁴C] alanine in the HeLa cell experiments by 17%. When NAD alone and NAD plus toxin, or
Table 2. CPM in TCA precipitable material in ADP-ribosylation studies.

The assay system is described in MATERIALS AND METHODS. The complete system includes NAD (specific activity 167 mCi/mmole), diphtheria toxin and transferase II in Tris-Cl buffer, pH 7.6. CPM values are the average of 3 determinations.

<table>
<thead>
<tr>
<th>Contents of the reaction system</th>
<th>CPM</th>
</tr>
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<tbody>
<tr>
<td>NAD*</td>
<td>30</td>
</tr>
<tr>
<td>NAD* + 10 μg ascorbic acid</td>
<td>33</td>
</tr>
<tr>
<td>NAD* + toxin + 10 μg ascorbic acid</td>
<td>31</td>
</tr>
<tr>
<td>NAD* + transferase II + 10 μg ascorbic acid</td>
<td>35</td>
</tr>
<tr>
<td>Complete</td>
<td>460</td>
</tr>
<tr>
<td>Complete + 3.0 μg ascorbic acid</td>
<td>420</td>
</tr>
<tr>
<td>Complete + 10 μg ascorbic acid</td>
<td>410</td>
</tr>
<tr>
<td>Complete + 30 μg ascorbic acid</td>
<td>440</td>
</tr>
</tbody>
</table>

* Indicates radioactive NAD.

NAD plus transferase II in the presence of ascorbic acid were the components of the reaction mixture, the radioactivity found in trichloroacetic acid precipitable material was only slightly above background (Table 2).

DISCUSSION

Ascorbic acid does prevent the inhibition of [U-14C] alanine incorporation into trichloroacetic acid precipitable material in diphtheria intoxicated HeLa cells. The in vitro experiments reported here suggest that this protective effect is not a result of ascorbic acid reducing the disulfide bond in "nicked" toxin which causes the separation of fragment A from B, nor does ascorbic acid affect the NAD: EF-2-ADPR transferase activity of diphtheria toxin. Since ascorbic acid and p-MAPS, two reducing agents, but not citric acid which is not a reducing agent, did prevent the action of diphtheria toxin, it seems likely that the mechanism of action of ascorbic acid involves reduction. It is possible that this reduction is taking place at the cell membrane. IGLEWSKI and RITTENBERG (16) have shown that HeLa and other cancerous cells are more susceptible to diphtheria toxin than normal cells. They suggest that the resistance to diphtheria toxin by normal cells is a property of the cell membrane. Thus, ascorbic acid may reduce or alter an important part of the membrane receptor specific for diphtheria toxin.

Since ascorbic acid is capable of eliciting a protective effect on diphtheria intoxicated HeLa cells, it seems highly probable that protection could be offered in the pharyngeal tissues where the diphtheria bacillus seats itself during the primary stage of infection. MAKILA and KIRVESKARI (18) noted that when subjects were administered 500 mg doses of ascorbic acid there was an increase from 0.6 to 1.2 μg/ml in parotid secretions of the vitamin within 2 and reaching a peak 6 hours after administration. PAULING (19) and STONE (20) feel that doses of ascorbic acid of 10 g or more a day have no deleterious effect on humans. No
research on parotid secretions of test subjects on massive doses of ascorbic acid has been reported, but KLENNER (9) has shown clinical evidence which indicates that ascorbic acid not only can be tolerated in massive doses but also is beneficial to an individual in the diseased state. It follows that a proportional increase in parotid ascorbic acid secretion would follow larger doses of ascorbic acid. This would bathe the pharyngeal tissues with high concentrations of ascorbic acid which would certainly diminish the localized infection of diphtheria. These experiments suggest a pharmacological effect of ascorbic acid as postulated by PAULING (19) and routinely performed by KLENNER (9) in his medical practice.

Currently we are attempting to ascertain if the effect is specific for diphtheria toxin toward HeLa cells or will other bacterial toxins and other cell lines be affected by ascorbic acid.

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REFERENCES