JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1986, p. 527–531
0095-1137/86/100527-05$02.00/0
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Vol. 24, No. 4

In Vitro Effect of Ascorbic Acid on Infectivity of Herpesviruses and Paramyxoviruses

LENDELL A. WHITE,* CHARLOTTE Y. FREEMAN, BARBARA D. FORRESTER, AND W. ADRIAN CHAPPELL

Biological Products Program, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

Received 24 February 1986/Accepted 19 June 1986

Suspensions of herpes simplex virus types 1 and 2, cytomegalovirus, and parainfluenzavirus type 2 were inactivated within 24 h when treated at 37°C with 1 mg (5.05 mM) of copper-catalyzed sodium ascorbate per ml. The infectivity titer of respiratory syncytial virus was reduced substantially after 24 h but required 48 h for inactivation. Under these conditions, inactivation of these viruses was also successfully achieved with 5.68 mM catalyzed ascorbic acid. Copper (Cu^{2+}), when added with the ascorbate solution at 5 μg/ml (0.022 mM), exhibited a catalytic effect on the inactivation of these viruses. The rate of inactivation was affected by the incubation temperature, time of exposure, and the virus concentration. Ascorbate concentrations as high as 10 mg/ml (50.5 mM) demonstrated only a minimum increase in effect on viral inactivation. The loss of infectivity did not alter either the hemagglutination or complement fixation qualities of the antigens.

Procedures described by Chappell et al. (3) have established the use of beta-propiolactone and Tween 80-ethyl ether as standards for inactivating viral diagnostic reagents. Because of the hazards inherent in handling these chemicals, our laboratory was interested in evaluating safer and more efficient methods for inactivation of reagents.

During investigations of various chemical agents as preservatives and inactivators of viral diagnostic antigens, reference was found to the use of ascorbic acid as an antiviral product. Turner (12) reported the inactivation of vaccinia virus by ascorbic acid undergoing auto-oxidation catalyzed by copper. Similar reactions with bacterial, fungal, and viral agents were described by Ericsson and Ludbeck (4). While examining the survival of enteroviruses in food, Salo and Cliver (11) also investigated the effect of ascorbic acid on polio-, coxsackie-, and echoviruses. Inactivation based on loss of RNA infectivity was described. Bissell et al. (2) reported that ascorbic acid did not alter cell culture susceptibility to initial infection from Rous sarcoma virus but appeared to interfere with virus replication and infectivity.

We investigated by in vitro experiments the effects of ascorbic acid as an agent inactivating RNA and DNA viruses. The use and potency of these ascorbate-treated reagents in serologic assays were determined.

MATERIALS AND METHODS

Virus suspension. The following viruses were used in these experiments: herpes simplex virus type 1, strain MacIntyre (HSV-1); HSV-2, strain MS; cytomegalovirus, strain AD-169 (CMV); parainfluenzavirus type 2, strain Greer (Para-2); and respiratory syncytial virus, strain Long (RSV). CMV was grown in human embryonic lung fibroblast-like cells in monolayer cultures maintained with reinforced Eagle minimum essential medium (MEM) plus 2% fetal calf serum. The other four viruses were grown in monolayer cultures of Vero cells (African green monkey kidney) maintained with Improved MEM (no. 84-0017; GIBCO Laboratories, Grand Island, N.Y.) plus 1% fetal calf serum. Both media were used at pH 7.0 to 7.2. Virus passages and infectivity titrations were performed in the same host system used for virus growth. Cell cultures, virus seed stocks, and bovine serum products used in media were tested and determined to be free of bacterial, fungal, and mycoplasmal contamination.

Cell cultures grown in roller bottles were infected with virus and incubated at 37°C until a 3+ to 4+ cytopathic effect was observed. Cells were removed from the bottle surface by scraping, disrupted by a freeze-thaw cycle, and clarified of cell debris by centrifugation at 500 × *g* for 15 min. Portions were dispensed for future experiments and stored at −70°C. Infectivity titers were determined to range from $10^{4.5}$ with CMV to between $10^{7.8}$ and $10^{8.5}$ 50% tissue culture infective doses ($TCID_{50}$) per ml with the other viruses.

Chemicals. A stock solution of 0.1 M $CuSO_4$ (Cu^{2+}) was prepared in distilled water. It was added to the ascorbate-plus-virus mixtures to give a final Cu^{2+} concentration of 5 μg/ml (0.022 mM).

Stock solutions of ascorbic acid (Mallinckrodt, Inc., St. Louis, Mo.) at 568 mM and sodium ascorbate (Bronson Pharmaceuticals) at a 505 mM concentration were prepared in distilled water immediately before use. The stock solutions were diluted in the virus suspension to give the experimental concentrations of 0.1, 1.0, 5.0, and 10.0 mg/ml. The 1.0-mg/ml (5.05 mM) concentration was selected as the standard ascorbate concentration for the inactivation experiments.

The effect of ascorbate on the pH of the medium and the toxic effect of ascorbate on normal Vero cells were determined. The cell cultures were maintained with Improved MEM plus 1% fetal calf serum. The cultures were incubated at 37°C and observed daily for 7 to 10 days for viral cytopathic effect.

Effects of temperature on ascorbate treatment. HSV-1 grown in Vero cells to a titer of $10^{8.5}$ $TCID_{50}$/ml was treated with a mixture of 1 mg of sodium ascorbate per ml and 5 μg of Cu^{2+} per ml. The suspension was divided into three portions and incubated in water baths at 6, 27, and 37°C. Portions were removed at six different time intervals ranging from 10 to 48 h and frozen at −70°C until tested. Untreated HSV-1 control cultures were incubated under the same test conditions and time intervals as the ascorbate-treated suspensions. Viral infectivity was determined by serial titrations in Vero cells.

* Corresponding author.

TABLE 1. Effect of incubation temperatures on ascorbate inactivation of HSV-1

Additive (temp [°C])	Virus titer[a] after h of incubation:					
	0	10	18	24	36	48
Sodium ascorbate (1 mg/ml) + Cu^{2+} (5 μg/ml)						
6	7.3	ND[b]	6.5	≥6.5	5.5	5.3
27	7.3	ND	6.5	6.3	5.3	2.8
37	7.3	5.3	0.5	0	ND	0
None						
6	7.3	ND	≥7.5	7.3	6.5	7.3
27	7.3	ND	6.8	6.3	6.5	6.5
37	6.8	6.3	6.3	5.3	ND	3.8

[a] Log_{10}/Vero cells per $TCID_{50}$ per 0.1 ml.
[b] ND, Not determined.

Effect of ascorbate concentration on the inactivation rate of Para-2 virus. A suspension of Para-2 virus was prepared, and a portion was diluted with Improved MEM to yield two suspensions containing $10^{8.5}$ and $10^{5.5}$ $TCID_{50}$/ml. Each suspension was divided into three portions. Two portions of each suspension were treated with sodium ascorbate at concentrations of 1 and 5 mg/ml. Copper solution at a final concentration of 5 μg/ml was added immediately after the ascorbate. The third portion or untreated control suspension was incubated in a water bath at 37°C along with the ascorbate-treated suspensions. During incubation, aliquots from all six suspensions were removed at 6-, 10-, 14-, 18-, and 24-h intervals of incubation and stored at −70°C until tested. Infectivity titrations were performed in Vero cells. Hemagglutination (HA) titrations were performed with the portions removed from the treated and control portions of the high-titered suspension.

Test procedure. A measured volume of the frozen viral suspension was thawed, and either the ascorbic acid or sodium ascorbate stock solution was added to give the desired concentration of 1 mg/ml. The stock copper solution was added immediately to the virus-ascorbate mixture to give a final concentration of 5 μg/ml. The final mixture was vigorously stirred and incubated in a water bath at 37°C. Portions were removed from each sample at time intervals ranging from 6 to 24 h and stored at −70°C until tested. Serial 10-fold dilutions of the portions taken at each time interval were prepared in Hanks basic salt solution. Three monolayer cell cultures of either Vero or human lung fibroblast-like cells were inoculated for each dilution and incubated at 37°C. The maintenance medium was the same as the one used for the virus growth. Cell cultures were observed for 7 to 10 days for cytopathic effect. Infectivity titrations ($TCID_{50}$) were calculated by the method of Reed and Muench (10). Inactivation endpoints were confirmed by passage of the endpoint dilutions in the same cell culture host and observed for infectivity.

Untreated virus suspensions were incubated under the same test conditions, and samples were removed at the same time intervals as for the ascorbate-treated suspensions. Control suspensions were treated independently with the copper solution (5 μg/ml) and with the test dosage regimen of either ascorbic acid or sodium ascorbate.

Serologic assay. Diagnostic reagents were prepared by the procedures described by Chappell et al. (3) for use in the standardized complement fixation (CF) (9), HA inhibition (6), and the indirect HA (IHA) (1) tests. The bulk reagents were inactivated with the standard catalyzed ascorbate solution (1 mg/ml). The potency of these inactivated antigens was compared with the titer of the untreated antigens. The sensitivity and specificity of the antigens were compared by using positive control reference antiserum.

RESULTS

Effect of chemicals. No significant pH changes were observed with a concentration of ≤1 mg of ascorbic acid per ml and with ≤10 mg of sodium ascorbate per ml. Higher concentrations caused a rapid reduction in pH levels. Ascorbic acid concentrations of 5 and 10 mg/ml lowered the pH of the Improved MEM from 7.2 to between 3.8 and 4.7. When the medium was readjusted to pH 7.2, these ascorbic acid concentrations caused toxic or degenerative changes in the Vero cell monolayers, resulting in a sloughing of the tissue. The higher ascorbic acid concentrations also caused crystal deposits in the medium. No toxicity was observed with any concentrations of sodium ascorbate. The 1-mg/ml concentration of both products was free of all toxic effects and was used as the standard in all comparative studies. Vero cells pretreated with 1 and 5 mg of ascorbate per ml had no altered susceptibility to Para-2 infection.

Incubation temperatures. The effects of incubation temperatures on inactivation of HSV-1 by ascorbate are reported in Table 1. No significant decrease in infectivity titer was

TABLE 2. Inactivation of Para-2 by different ascorbate concentrations

Virus concn[a]	Concn (mg/ml) of sodium ascorbate + 5 μg of Cu^{2+}/ml	Virus titer[a] after h of incubation at 37°C:					
		0	6	10	14	18	24
4.5	None	4.5	≥5.0	4.0	4.3	4.0	4.3
	1	4.5	4.5	1.7	0	0	0
	5	4.5	4.3	2.7	0	0	0
7.5	None	7.5	6.5	7.5	6.7	7.0	7.3
	1	7.5	5.7	4.3	1.7	0	0
	5	7.5	6.5	4.5	3.3	0	0

[a] Log_{10}/Vero cells per $TCID_{50}$ per 0.1 ml.

TABLE 3. Ascorbate inactivation of HSV-1 and HSV-2

Additive	Virus titer[a] after h of incubation:							
	HSV-1				HSV-2			
	0	10	18	24	0	10	18	24
Sodium ascorbate (1 mg/ml) + Cu^{2+} (5 μg/ml)	6.8	5.3	0.5	0	6.5	3.5	<0.5	0
Controls								
No additive	6.8	6.3	6.5	5.3	6.5	7.3	5.5	4.8
Cu^{2+} (5 μg/ml)	6.8	5.8	5.3	3.8	6.5	5.3	4.3	3.8
Sodium ascorbate (1 mg/ml)	6.8	6.5	7.3	5.5	6.5	6.3	4.8	4.5

[a] Log_{10}/Vero cells per $TCID_{50}$ per 0.1 ml.

observed in ascorbate-treated suspensions incubated at either 6 or 27°C for 24 h. After 48 h, cultures incubated at 6°C had decreased by 2.0 logs in titer, and those incubated at 27°C had declined by 4.5 logs. Incubation at 37°C accelerated ascorbate inactivation. After 18 h there was a 6.0-log decline in infectivity, and cultures incubated for ≥24 h were inactivated. Untreated cultures showed a 3.0-log decrease in infectivity after 48 h of incubation at 37°C; however, cultures incubated at 6 and 27°C were unchanged by the incubation temperature.

Virus and ascorbate concentrations. The effect of ascorbate concentration on the rate of virus inactivation was determined with Para-2 (Table 2). Para-2 was inactivated more rapidly with the 1-mg/ml concentration than at the 5-mg/ml level. Little effect was observed at the 6-h interval, but gradual decline in infectivity was observed for both virus concentrations during the next 18 h. The time required for inactivation was proportional to the initial virus concentration. Portions of the suspension containing 8.5 logs of virus per ml were tested for HA reaction. The untreated control suspension gave an HA titer of 1:64 and remained stable at 1:32 through the 24-h incubation period at 37°C. No decline in HA titers occurred for the Para-2 suspensions treated with catalyzed ascorbate.

Inactivation experiments. Suspensions of each of the five viruses were treated with either sodium ascorbate or ascorbic acid at a concentration of 1 mg/ml catalyzed with 5 μg of Cu^{2+} per ml. The mixtures were incubated at 37°C in a water bath. Portions (1 ml) were removed from each suspension at various time intervals and stored at −70°C until tested. Controls were handled in an identical manner, and all specimens were titrated at the same time in the homologous host cell system.

HSV-1 and HSV-2 were inactivated by ascorbate catalyzed by Cu^{2+} (Table 3). A gradual decline in infectivity occurred between 10 and 18 h, with no infectious virus detected in the 24-h samples. Control suspensions treated with Cu^{2+} only demonstrated a less extensive reduction in titer. A decline in infectivity of 3.0 logs was observed with HSV-1, and with HSV-2 a decrease of 2.7 logs was observed. The ascorbate treatment alone showed no effect when compared with the medium controls. The infectivity titers of the untreated suspensions were stable through 18 h. However, a titer loss of 1.5 to 1.7 logs was observed after 24 h. Only the catalyzed ascorbate was totally effective in the inactivation of HSV-1 and HSV-2. Inclusion of Cu^{2+} increased both the rate and degree of inactivation occurring in the ascorbate-virus mixtures.

Experimental results comparing the effectiveness of sodium ascorbate with ascorbic acid on CMV, Para-2, and RSV are presented in Table 4. Both chemicals were catalyzed and were used at a concentration of 1 mg/ml. After incubation at 37°C for 6, 10, 18, and 24 h, the infectivity titers were compared with those of an untreated virus suspension. Incubation of the RSV suspension was extended to 48 h.

The CMV infectivity was reduced by 3.0 logs after a 6-h exposure to ascorbate-ascorbic acid and was inactivated by both chemicals after exposure to them for 10 h. Inactivation of Para-2 required between 10 and 18 h. While both chemicals were effective, catalyzed ascorbic acid inactivated the Para-2 virus more rapidly than did the catalyzed ascorbate. Inactivation of RSV occurred at a much slower rate than that observed with the other viruses. Inactivation was minimal during the first 10 h and increased by 18 h, but virus was still viable after 24 h. When treatment was extended to 48 h, inactivation was achieved. These results were replicated,

TABLE 4. Effects of ascorbate on viral infectivity titers

Virus	Additive	Virus titer[a] after h of incubation at 37°C:					
		0	6	10	18	24	48
CMV	Sodium ascorbate[b]	3.5	0.5	0	0	0	ND[c]
	Ascorbic acid[b]	3.5	0.5	0	0	0	ND
	None	3.5	3.5	3.5	3.5	2.3	ND
Para-2	Sodium ascorbate	4.5	4.5	1.7	0	0	ND
	Ascorbic acid	4.5	2.5	0	0	0	ND
	None	4.5	4.5	4.0	4.0	4.3	ND
RSV	Sodium ascorbate	6.5	5.5	5.5	4.5	2.3	0
	Ascorbic acid	6.5	5.3	5.0	3.8	2.5	0
	None	6.5	6.5	6.5	5.3	5.8	4.3

[a] Log_{10}/Vero cells per $TCID_{50}$ per 0.1 ml. For CMV, human lung fibroblast-like cells were used.
[b] 1 mg/ml plus 5 μg of Cu^{2+} per ml.
[c] ND, Not determined.

TABLE 5. Effects of ascorbate on diagnostic reagents

Viral antigen	Additive	Optimum antigen titer[a] after h of incubation:					
		CF		HA		IHA	
		0	24	0	24	0	24
Para-2	Ascorbate[b]	32	16	1,280	640		
	None	32	32	1,280	320		
RSV	Ascorbate	128	128				
	None	128	128				
CMV	Ascorbate	8	2			>16	16
	None	8	8			>16	4
HSV-1	Ascorbate	8	8				
	None	8	8				

[a] Reciprocal of dilution.
[b] 1 mg/ml plus 5 μg of Cu^{2+} per ml.

and no evidence of infectivity was observed after passage in Vero cells. This lack of infectivity by the passage material was accepted as confirmatory evidence of inactivation. The RSV medium control after 48 h of incubation demonstrated an infectivity titer in Vero cells of $10^{4.3}$ $TCID_{50}$/0.1 ml.

Untreated control suspensions of Para-2 and RSV gave a <1.0-log decrease in infectivity titer during the 24-h incubation period, while the untreated CMV material showed a decline of 1.2 logs in titer. Suspensions of CMV and Para-2 treated for 24 h, and RSV treated for 48 h with either Cu^{2+} or uncatalyzed ascorbate-ascorbic acid solutions demonstrated slightly reduced infectivity, as described with HSV (Table 3). This further confirmed the need for using catalyzed ascorbate for inactivation purposes.

Serologic assay. Bulk serologic reagents were prepared for Para-2, RSV, CMV, and HSV-1. The pools were split, and one-half of each reagent pool was inactivated with 1 mg of catalyzed ascorbate per ml at 37°C for 24 h. The infectivity and antigenic potency were compared with those of the untreated antigen. The Para-2, CMV, and HSV-1 antigens were inactivated by the treatment. An infectivity titer of <2.0 logs remained with the RSV antigen. Further treatment for 48 h was not attempted.

The serologic results are presented in Table 5. The potency and specificity of the Para-2 CF and HA antigens and the HSV-1 and RSV CF antigens remained stable after inactivation. The CMV CF antigen gave a fourfold decrease in titer after inactivation. The CMV IHA antigen was more stable after inactivation than was the untreated control antigen. However, the CMV IHA antigen demonstrated reduced sensitivity and was deemed unsatisfactory. Controls indicated that the reduced sensitivity was caused by the Cu^{2+} solution.

DISCUSSION

The importance of inactivating infectious viruses in diagnostic reagents cannot be minimized. Since various degrees of laboratory safety guidelines are practiced around the world, it is possible for a careless technician to become infected because of a laboratory accident involving an antigen that has not been inactivated.

For a number of years we have been content to use beta-propiolactone or gamma radiation for the inactivation of viral materials. In general, these methods are satisfactory. However, a gamma emission source is expensive and often not readily available. Also, the use of beta-propiolactone in production quantity places the producer at a risk in the use of this carcinogenic material.

The use of ascorbic acid (vitamin C) as an antiviral agent has been reported primarily as a prophylactic and therapeutic agent against the common cold. Our approach was to determine the in vitro effect of ascorbic acid on viruses in suspension, as measured by the infectivity of cell cultures. The results presented indicate that this procedure provides a method for inactivation of infectious viruses while utilizing a less hazardous chemical procedure. The procedure should be explored as a means of inactivation of other groups of viruses. The inactivated virus suspensions maintained their usefulness as diagnostic reagents. Four of the five viruses were successfully inactivated within 24 h. The infectious titer of the RSV preparation was reduced in 24 h and rendered innocuous after 48 h. The higher infectivity titer of the RSV suspension may have contributed to the additional time required for inactivation.

It has been reported that the stability of HSV and RSV infectivity is affected by changes in temperature, pH, and medium composition. Our experiments were conducted within a pH range of 7.0 to 7.2 at 37°C. Hambling (5) reported that the maximum infectivity of RSV was maintained at pH 7.5. Titrations showed that approximately only 10% of infectious RSV survived after 24 h of incubation at 37°C. The survival of our RSV controls (Table 4) surpassed this survival rate. We demonstrated a remaining infectivity of 88% after 24 h and 66% after 48 h of incubation at 37°C. The composition and pH of the suspending medium were reported by Lancz (8) and Lancz and Sample (7) to be interdependent factors of in vitro thermal inactivation of HSV. This thermal lability was an inconsistent observation, but as the investigators reported, it was related to pH values of ≤6.3 and ≥7.8. The addition of serum to the suspending medium was also reported to be beneficial in stabilizing viability. Our experiments with HSV-1 and HSV-2 controls (Tables 1 and 3) showed a survival rate of ≥74% after 24 h of incubation at 37°C.

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