ASCORBIC ACID AND IMMUNITY

III. The Effect of Ascorbic Acid upon the Bactericidal Action of Human Blood[1, 2, 3]

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The function of vitamin C in the human body with relation to several infectious diseases has been the subject of many investigations. The voluminous literature has been summarized in part by Perla and Marmorstan (1941). Some investigators have maintained that the antibacterial activity of the blood was reduced in animals deficient in ascorbic acid, and the administration of ascorbic acid greatly increased the bactericidal titer (Jusatz, 1936, Ardy, 1939). On the other hand, Hamburger and Goldschmidt (1922–23) found no difference in the antibacterial action of blood from scorbutic animals and children, and normal controls. More recently, Pfannenstiel and Dotzer (1940) investigated the relationship in human blood between the vitamin C content and the bactericidal action against staphylococci. They concluded that the vitamin C level and bactericidal power were two independent variables. Further interest in this controversial problem was stimulated by the announcement of Ecker and his associates (1938, 1939) that the activity of complement in the serum of both guinea pigs and human beings was directly related to the presence of optimal concentrations of reduced ascorbic acid. Sera having little or no ascorbic acid possessed a low titer of complement. When the deficient sera were corrected by either the *in vitro* or *in vivo* addition of ascorbic acid an immediate rise in complement titer was demonstrated. We were unable to confirm these observations (Spink, Agnew and Mickelsen, 1942, Agnew, Spink and Mickelsen, 1942). Since it was impossible to present corroborative evidence that the hemolytic activity of complement in deficient human sera could be increased by either the *in vitro* or *in vivo* addition of ascorbic acid, the present study was undertaken. The purpose was to determine if the presence or absence of ascorbic acid influenced the bactericidal action of human blood. As Dingle, Fothergill and Chandler (1938) have pointed out, the bactericidal action of all sera involves antibody and complement. As to whether the same complement in a given serum is involved in bactericidal activity as in a hemolytic system, Dingle and his associates concluded that fresh human serum will complement the bactericidal action of immune guinea pig serum to an extent that roughly parallels its titer in complementing the hemolytic system.

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METHODS

Throughout this study, venous blood was obtained from individuals free from infections. The blood was permitted to clot at room-temperature, centrifuged, and the serum withdrawn. The macroscopic method of Mindlin and Butler (1938) was used for making the serum ascorbic acid determinations.

The first group of studies were carried out with the bloods of twelve individuals, of whom all except two were found to have a deficient serum ascorbic titer. The purpose was to compare the bactericidal action of whole blood for staphylococci before and after the intravenous injection of ascorbic acid. Whole defibrinated blood was used for the bactericidal tests. This was obtained by collecting and gently agitating venous blood in small Erlenmeyer flasks containing glass beads. Ten strains of coagulase-negative *Staphylococcus albus* were employed. The bactericidal tests were carried out as follows: 0.5 ml of whole defibrinated blood was placed into each of 7 small, pyrex glass tubes. Ten-fold dilutions of an 18-hour broth culture of *S. albus* ranging from 10^{-1} to 10^{-7} were prepared. Then 0.1 ml of each dilution was added to the series of tubes containing the defibrinated blood. The approximate number of organisms added was determined by making an agar pour plate with 0.1 ml of the 10^{-7} dilution. The tubes were sealed in a gas-oxygen flame and then rotated for 24 hours in a machine in an incubator set at 37C. At the end of this time, the tubes were opened and the contents cultured for viable organisms.

The amount of ascorbic acid in blood serum is not an absolute expression of the quantity found in a sample of whole blood since the erythrocytes contain appreciable amounts of ascorbic acid. Therefore, comparative antibacterial studies were made with serum alone. The second and third groups of observations were made in a similar manner with the blood sera of 13 individuals. The object was to compare the bactericidal action of serum for *E. coli communis* and *E. typhosa* (smooth H strain) before and after the intravenous injection of ascorbic acid. A strain of each of these organisms was obtained from Professor W. P. Larson of the Department of Bacteriology. Specimens of whole blood obtained before and shortly after the administration of ascorbic acid were allowed to clot and the serum removed after centrifugation. The bactericidal tests were performed in the following way: 0.5 ml of the fresh serum was placed in each of 7 tubes. Ten-fold broth dilutions of *E. coli* and *E. typhosa* were prepared from cultures grown on agar slopes for 20 hours. Then 0.1 ml. of each of the dilutions was added to each of the tubes containing the serum. A control series of 7 tubes for each of the strains was prepared by adding 0.1 ml of each dilution to 0.5 ml of serum heated at 56C for 30 minutes. All the tubes were shaken and then placed in the incubator set at 37C for 24 hours. At the end of this time, the contents of each of the tubes were cultured for viable organisms.

The next step was to compare the bactericidal power of normal, fresh, human serum with and without the presence of reduced ascorbic acid. A series of 25 observations were made with sera containing at least 0.5 mg of reduced ascorbic acid per 100 ml. Three strains of gram-negative organisms were employed. These included *E. typhosa*, *E. coli* and *Shigella paradysenteriae*

Flexner. Since traces of copper will act as a catalyst and quickly oxidize ascorbic acid, $CuCl_2$ in physiological sodium chloride solution was utilized to oxidize the ascorbic acid present in normal serum. One-tenth milliliter of $CuCl_2$ solution (0.22 mg) was sufficient to oxidize the ascorbic acid present in 5 ml of normal serum. Bactericidal tests were then performed with fresh serum and serum to which $CuCl_2$ had been added, observations being made with each of the 3 strains mentioned. The tests were performed as described above. Control observations were also made with each of the cultures using 0.5 ml of heated serum in a series of 7 tubes.

TABLE 1

Bactericidal action of whole defibrinated blood for Staphylococcus albus before and after intravenous injection of ascorbic acid

	PATIENT	AMOUNT OF ASCORBIC ACID INJECTED	NUMBER OF COLONIES	10^7 DILUTION AND STRAIN S. ALBUS	BEFORE INTRAVENOUS ASCORBIC ACID								AFTER INTRAVENOUS ASCORBIC ACID							
					Serum ascorbic acid	Killing power							Serum ascorbic acid	Killing power						
						10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
		mg			*mg*								*mg*							
1	38 F.	1,000	12	G	0.0176	+	+	0	0	0	0	0	6.72	+	+	+	+	0	0	0
2	60 F.	1,000	17	6476	0.06	+	+	+	+	+	0	0	7.15	+	+	+	+	+	0	0
3	59 F.	1,000	10	6482	0.00	+	+	+	+	+	0	0	5.20	+	+	+	+	+	0	0
4	66 F.	1,000	10	6482	0.10	+	+	+	0	0	0	0	9.76	+	+	0	0	0	0	0
5	21 F.	500	24	6773	0.13	+	+	0	0	0	0	0	2.85	+	+	+	+	0	0	0
6	54 F.	500	26	6492	0.08	+	+	+	+	+	+	0	5.6	+	+	+	+	+	+	+
7	28 F.	500	59	C32	0.03	+	+	+	0	0	0	0	4.36	+	+	+	0	0	0	0
8	37 F.	250	50	C32	1.31	+	+	+	0	0	0	0	3.2	+	+	+	0	0	0	0
9	43 F.	250	15	Niel	0.19	+	+	0	0	0	0	0	2.74	+	+	0	0	0	0	0
10	39 F.	200	34	#3	0.06	+	+	+	+	+	+	+	1.64	+	+	+	+	+	+	0
11	32 F.	200	11	6508	0.20	+	+	+	+	+	+	0	1.76	+	+	+	+	+	+	0
12	65 F.	200	2	6733	1.10	+	+	0	0	0	0	0	1.3	+	+	0	0	0	0	0

0 = No growth.
+ = Growth.

RESULTS

The intravenous injection of varying amounts of ascorbic acid into 12 human subjects was not followed by any increase in the bactericidal action of whole blood for coagulase-negative staphylococci. The results are tabulated in table 1. Four individuals received 1000 mg of ascorbic acid, which resulted in serum levels well above those found under normal, physiological conditions. This was also the case with persons receiving either 250 or 500 mg. High normal values resulted from the injection of 200 mg. All of the values recorded were obtained with bloods removed within 10 minutes after the injection of the ascorbic acid.

No significant change in the bactericidal action was noted with serum obtained before and after the intravenous injection of ascorbic acid. The results with *E. coli* and the sera of 8 persons are presented in table 2; observations made with *E. typhosa* and the sera of 5 individuals are shown in table 3.

A more crucial type of experiment involved a comparison of the bactericidal titer of sera having normal amounts of ascorbic acid with sera in which all of the ascorbic acid had been completely oxidized with the aid of small quantities of copper. Under these circumstances, the results with *E. coli* are given in table 4. In a series of 12 observations, it is to be noted that there was no significant difference in the bactericidal action of serum with a normal ascorbic

TABLE 2

Bactericidal power of human serum for E. coli before and after intravenous injection of ascorbic acid

	PATIENT	AMOUNT OF ASCORBIC ACID INJECTED	BEFORE INTRAVENOUS ASCORBIC ACID								AFTER INTRAVENOUS ASCORBIC ACID							
			Serum ascorbic acid	Bactericidal power							Serum ascorbic acid	Bactericidal power						
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
		mg	*mg*								*mg*							
1	28 F.	500	0.03	+	+	+	+	0	0	0	4.36	+	+	+	+	0	0	0
2	37 F.	250	1.31	+	0	0	0	0	0	0	3.20	0	0	0	0	0	0	0
3	43 F.	250	0.19	+	0	0	0	0	0	0	2.74	+	0	0	0	0	0	0
4	39 F.	200	0.06	+	0	0	0	0	0	0	1.64	0	0	0	0	0	0	0
5	32 F.	200	0.20	+	0	0	0	0	0	0	1.76	0	0	0	0	0	0	0
6	65 F.	200	0.10	+	0	0	0	0	0	0	1.30	0	0	0	0	0	0	0
7	58 F.	200	0.98	+	+	+	0	0	0	0	2.2	+	+	+	+	0	0	0
8	23 F.	200	0.07	+	+	+	0	0	0	0	1.426	+	+	+	+	+	0	0

0 = No growth.
+ = Growth.

TABLE 3

Bactericidal power of human serum for E. typhosa before and after intravenous injection of ascorbic acid

	PATIENT	AMOUNT OF ASCORBIC ACID INJECTED	BEFORE INTRAVENOUS ASCORBIC ACID								AFTER INTRAVENOUS ASCORBIC ACID							
			Serum ascorbic acid	Bactericidal power							Serum ascorbic acid	Bactericidal power						
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
		mg	*mg*								*mg*							
1	66 F.	1,000	0.1	+	0	0	0	0	0	0	9.76	+	0	0	0	0	0	0
2	21 F.	500	0.13	+	+	+	+	0	0	0	2.85	+	0	0	0	0	0	0
3	54 F.	500	0.08	+	0	0	0	0	0	0	5.6	0	0	0	0	0	0	0
4	58 F.	200	0.98	+	0	0	0	0	0	0	2.2	+	0	0	0	0	0	0
5	23 F.	200	0.07	+	+	+	+	0	0	0	1.426	+	+	+	+	0	0	0

0 = No growth.
+ = Growth.

acid content and a sample of the same serum in which all of the ascorbic acid had been completely oxidized. Similar results were obtained when strains of *E. typhosa* and *Shigella paradysenteriae Flexner* were employed, as shown in tables 5 and 6. It is of interest that the amount of $CuCl_2$ used to oxidize the ascorbic acid did not interfere with the bactericidal function of complement. It was also observed that this same amount of copper did not influence the action of complement in a hemolytic system.

TABLE 4

Bactericidal power of human serum for E. coli with ascorbic acid oxidized with $CuCl_2$
(0.022 mg $CuCl_2$ per 0.5 ml serum)

OBSERVATION	BACTERICIDAL POWER OF NORMAL SERUM							BACTERICIDAL POWER OF SERUM PLUS $CuCl_2$						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	+	+	+	0	0	0	0	+	0	0	0	0	0	0
2	+	+	0	0	0	0	0	+	0	0	0	0	0	0
3	0	0	0	0	0	0	0	+	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	+	0	0	0	0	0	0
6	+	0	0	0	0	0	0	0	0	0	0	0	0	0
7	+	+	+	0	0	0	0	+	0	0	0	0	0	0
8	+	+	0	0	0	0	0	+	0	0	0	0	0	0
9	0	0	0	0	0	0	0	+	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	+	0	0	0	0	0	0
12	+	0	0	0	0	0	0	0	0	0	0	0	0	0

0 = No growth.
+ = Growth.

TABLE 5

Bactericidal power of human serum for E. typhosa with ascorbic acid oxidized with $CuCl_2$
(0.022 mg $CuCl_2$ per 0.5 ml serum)

OBSERVATION	BACTERICIDAL POWER OF NORMAL SERUM							BACTERICIDAL POWER OF SERUM PLUS $CuCl_2$						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	+	+	+	0	0	0	0	+	+	+	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0 = No growth.
+ = Growth.

TABLE 6

Bactericidal power of human serum for Shigella paradysenteriae (Flexner) with ascorbic acid oxidized with $CuCl_2$
(0.022 mg $CuCl_2$ per 0.5 ml serum)

OBSERVATION	BACTERICIDAL POWER OF NORMAL SERUM							BACTERICIDAL POWER OF SERUM PLUS $CuCl_2$						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	+	+	+	+	+	0	0	+	+	+	+	+	0	0
2	+	+	+	0	0	0	0	+	+	+	+	+	0	0
3	+	+	0	0	0	0	0	+	+	0	0	0	0	0
4	+	+	+	+	+	0	0	+	+	+	+	+	0	0
5	+	+	+	+	0	0	0	+	+	+	+	0	0	0
6	+	+	0	0	0	0	0	+	+	+	0	0	0	0
7	+	+	0	0	0	0	0	+	0	0	0	0	0	0
8	+	0	0	0	0	0	0	+	+	+	0	0	0	0
9	+	+	+	0	0	0	0	+	+	0	0	0	0	0

0 = No growth.
+ = Growth.

DISCUSSION

The foregoing data show that ascorbic acid does not appear to influence the antibacterial activity of human blood. The limitations of the experimental procedures employed do not permit us to draw any final conclusions concerning the antibacterial role of ascorbic acid in the human body or even in the blood. The fixed tissues play an important part in the defense mechanism of the host. What function ascorbic acid has in this activity remains unknown. Another question that we have anticipated in citing the foregoing experimental data is, what effect would repeated injections of large doses of ascorbic acid have upon the antibacterial action of blood? A few observations directed along this line revealed no significant effects. However, such a type of investigation is beset with many obstacles. When different specimens of blood are obtained and studied several days apart, the experimental conditions are difficult to control. For this reason, the immediate effects of ascorbic acid were investigated.

SUMMARY

1. Synthetic ascorbic acid injected intravenously into human subjects having a deficiency of ascorbic acid in the serum did not result in an *in vitro* increase in the bactericidal action of whole blood for coagulase-negative staphylococci.

2. Under similar circumstances, the intravenous administration of ascorbic acid was not followed by a rise in the bactericidal titer of serum for *E. coli* and *E. typhosa*.

3. The bactericidal action of normal, human serum is not decreased for *E. coli*, *E. typhosa* and *Shigella paradysenteriae Flexner* when the ascorbic acid is completely oxidized in the presence of small amounts of copper.

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