

The Effect of Vitamin C Deficiency on Complement Systems and Complement Components

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Summary The changes in the complement systems and complement hemolytic activities through classical and alternative pathways, and the individual components, C1, C4, C2 and C3 were demonstrated in the course of vitamin C deficiency in guinea pigs.

During the course of vitamin C deficiency during the first week after the start of the experiment, all components except C1 slightly decreased, gradually increasing in the following weeks. This indicates that this period is important in the formation of an immune defence system in the host.

At the time the symptoms of vitamin C deficiency appeared, C1, C2 and CH50 started to decrease. C3 increased when vitamin C deficiency became severe and showed a completely different pattern from those of the other components.

The lowered C1 will be due to a collagen-like region in the characteristic of C1q subcomponent, since insufficient vitamin C state produces impaired collagen formation.

The activity of the alternative pathway did not produce any change in this course, even in the severe stage. This indicates that the increase in C3 will contribute to maintaining the level of the alternative pathway and maintaining the body defence system in the vitamin C-deficient state, and that the complement system will be supported through the alternative pathway.

Key Words complement activity, vitamin C deficiency, alternative pathway, classical pathway, complement components, immune system

Various reviews (1, 2) have dealt with the effects of vitamin C (Vc) on the immune response, and studies have been made on this topic such as those on the effects of ascorbic acid on various immune systems including complement levels (3-5). Ascorbate appears not to influence complement titers (3, 4), but an investigator has claimed that complement titers in scorbutic guinea pigs were reduced (6). From

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these reports it would not be possible to obtain the definite evidence of the changes on abnormal complement activity during ascorbate deficiency.

In our last experiment (7), the complement titer was seen to be low in the severe stage of vitamin C deficiency. There was found a relationship between serum vitamin C levels and the complement titer in scorbutic guinea pigs.

In the present paper we report the effect of vitamin C deficiency on the complement systems and components in guinea pigs maintained for seven weeks on an ascorbate-free diet.

MATERIALS AND METHODS

Animals. Outbred, male, Hartley guinea pigs (Funabashi farm, Chiba, Japan), weighing about 250 g at the outset were fed on a commercial chow diet for 1 week after being received from the suppliers.

The animals were kept in a room with the temperature maintained at $24 \pm 2^\circ\text{C}$ and humidity at $50 \pm 10\%$. Experimental procedures were started immediately after the period of adaptation. The animals were observed for 8 weeks to confirm vitamin C deficiency clinically.

Diets. During the experimental period, the experimental group of guinea pigs was fed on an ascorbic acid (AsA)-deficient diet (containing AsA 2.3 mg/100 g in the diet), (Funabashi farm, Chiba, Japan) while the control group was fed on the same diet supplemented with AsA 1 g/100 g wt (8) twice weekly according to the body weight measurement. The guinea pigs were then maintained on 30 g/day of limited diets and water *ad libitum*. They were weighed twice weekly.

Blood collection. During the experiment, blood was collected from the heart twice weekly at the end of the experiment. The blood was allowed to clot at room temperature for 30 min and was then centrifuged at 3,000 rpm for 10 min at 4°C . After the centrifugation a part of the serum samples was used for the measurement of ascorbate in serum. The rest of the sera was stored at -80°C until used.

Observations

Body weight. Twice weekly.

AsA intake. AsA intake was calculated both through the dietary intake and AsA given orally at 1 g per 100 g body weight.

Clinical observations. The severity of the AsA-deficient state was classified into seven stages according to the daily clinical signs, as follows: 1) action laxity, 2) abnormality of joints, 3) difficulty in walking, 4) difficulty in walking (severe), 5) inability to walk, 6) bleeding from oral cavity, and 7) death.

AsA in serum. At the end of the experiment, the AsA in serum was measured by Bessy's method using hydrazine modified by Roe (9). In the cases of death before termination of the experiment, the AsA in serum was measured just prior to death. The AsA in the serum was measured immediately after the blood was taken.

Complement activity, CH50. CH50 was measured by Mayer's method (10). CH50 was expressed as a reciprocal dilution of serum samples which hemolysed

50% of sheep erythrocytes sensitized with an optimal amount of rabbit antibody at 37°C for 60 min.

ACH50. Complement hemolysis through the alternative pathway was followed by Amano (11). Reciprocal dilution of serum samples was carried out to determine the lysis 50% of rabbit erythrocytes in 0.03 M EGTA gelatin veronal buffer.

Complement components, C1, C4, C2 and C3. C1, C4 and C2 were measured by immune hemolysis reaction (12) and C3 was measured by immune adherence (13).

Statistical analysis. Group data were compared using Student's *t*-test.

RESULTS

Food intake and body weight

There were significant changes in body weight (Fig. 1) of guinea pigs fed on the AsA-deficient diet. The food intake was 11 g at the end of the 1st week and increased to 29 g at the end of the 2nd week. This level was maintained until the end of the 5th week but decreased remarkably to 15 g at the beginning of the 6th week and to 10 g in the 7th week, when marked clinical signs of vitamin C deficiency were observed. In the guinea pigs No. 9 and No. 10, the clinical signs and the decrease of the body weight appeared at the 4th week. They died in the 6th week. Guinea pig No. 1 showed the same tendency as the first in the 6th week and died in the 7th week.

The average body weight of the AsA-deficient group started to decrease at the

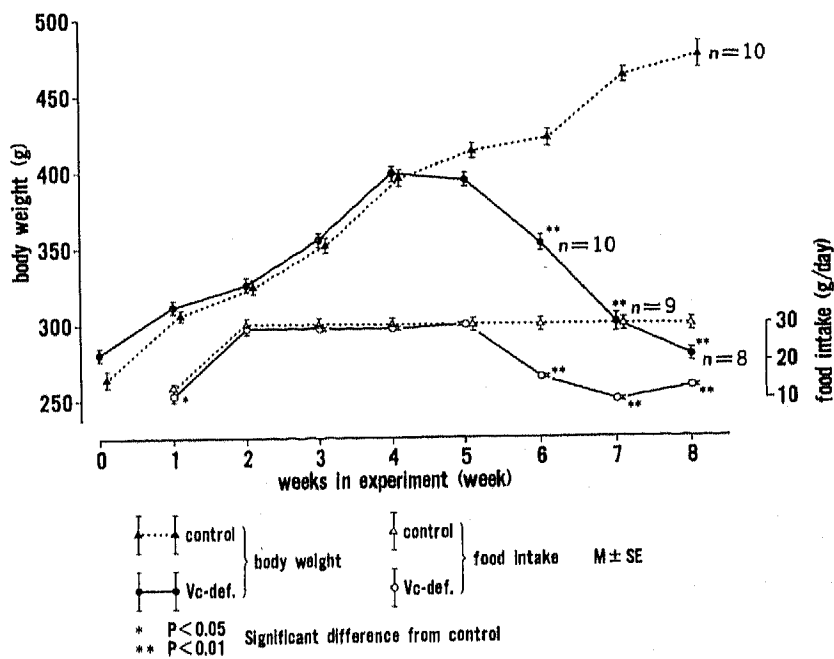


Fig. 1. Changes in body weight in vitamin C-deficient guinea pigs.

Table 1. Individual follow-up observations of the clinical signs of vitamin C-deficient guinea pigs.^a

No. of guinea pigs	Weeks in experiment (week)						
	1-3	4	5	5.5	6	7	8
1	0	1	3	5	6	7(†)	
2	0	0	5	5	—	6	7
3	0	0	2	4	6	—	7
4	0	0	1	4	6	—	7
5	0	1	2	5	5	—	7
6	0	1	2	2	5	—	7
7	0	0	1	2	5	—	7
8	0	1	3	4	6	—	7
9	0	1	4	6	7(†)	—	
10	0	1	4	6	7(†)	—	

^a The clinical signs were expressed by the following numbers: 0, no signs; 1, action laxity; 2, abnormality of joints; 3, difficulty in walking; 4, difficulty in walking (severe); 5, inability to walk; 6, bleeding from oral cavity; 7, death or killed at the end of the experiment. (†) Death before the termination of the experiment. —, no observation.

Table 2. AsA intake and concentration of AsA in the serum of vitamin C-deficient guinea pigs.

	AsA-deficient <i>n</i> = 10	Control <i>n</i> = 10
AsA intake (mg/day) ^a	0.5 ± 0.071*	4.6 ± 0.208
AsA in serum (μg/ml) ^b	0.696 ± 0.055*	1.07 ± 0.087

* *p* < 0.05 significant difference from control. ^a The average AsA intake was calculated both through the food intake of all the guinea pigs daily and AsA given orally 1 g/100 g body weight. ^b The AsA in serum was measured just before the guinea pigs died or were killed at the end of the experiment.

5th week, and at the 7th and 8th week, decreased to 304.2 ± 10.80 g (*p* < 0.01) and 278.8 ± 7.63 g (*p* < 0.01), respectively. The reduction in the average body weight of the experimental group was due to the food intake being reduced to 15.1 g/day, 10.0 g/day and 13.7 g/day, at weeks 6, 7 and 8, respectively. The body weight of the control group increased continuously throughout the experimental period (initial 253.3 ± 3.91 g, final 488.8 ± 9.70 g).

Individual follow up observations are described in Table 1.

AsA intake and AsA level in serum

Data on the effects of vitamin C deficiency of 7 weeks duration on the

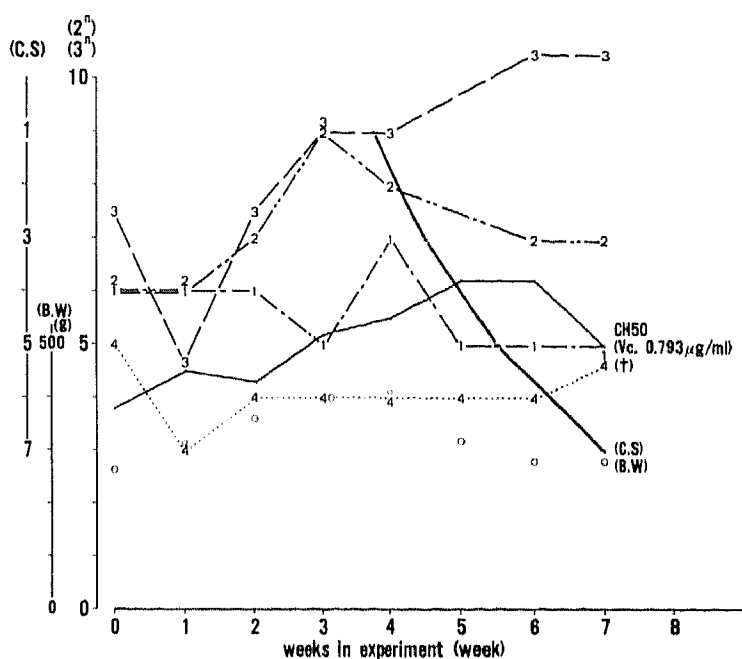


Fig. 2. Levels of component in follow up study of No. 1.

Fig. 2.-Fig. 6. Levels of CH50, ACH50, C1, C4, C2 and C3 in follow-up studies of guinea pigs No. 1 to No. 5.

○, body weight (B.W.).

●—●, CH50; vertical scale shows the numbers divided by 10 between maximum and minimum titers.

1—1, C1; } vertical scale shows dilution of the serum

4—4, C4; } 2ⁿ shows 2+ immune hemolytic reaction.

2—2, C2; } 3ⁿ shows 2+ immune adherence hemagglutination reaction.

3—3, C3; vertical scale shows dilution of the serum 3ⁿ shows 2+ immune adherence hemagglutination reaction.

●—●, (C.S.) clinical symptoms were classified in 7 stages according to the clinical signs and indicated by the stages numbers.

(), AsA in the serum.

concentration of AsA in serum and the AsA intake are shown in Table 2. The average of the AsA intake in the experimental group was low ($p < 0.05$). The AsA level in serum was measured at the end of the experiment. The AsA concentration in serum of the individual guinea pig is shown in the graphs of the individual follow up observations (Fig. 2-Fig. 6).

The average concentration of AsA in serum at the end of the experiment was $0.696 \pm 0.055 \mu\text{g/ml}$ in the AsA-deficient group, which was significantly lower ($p < 0.05$) than the level in the control, $1.07 \pm 0.08 \mu\text{g/ml}$.

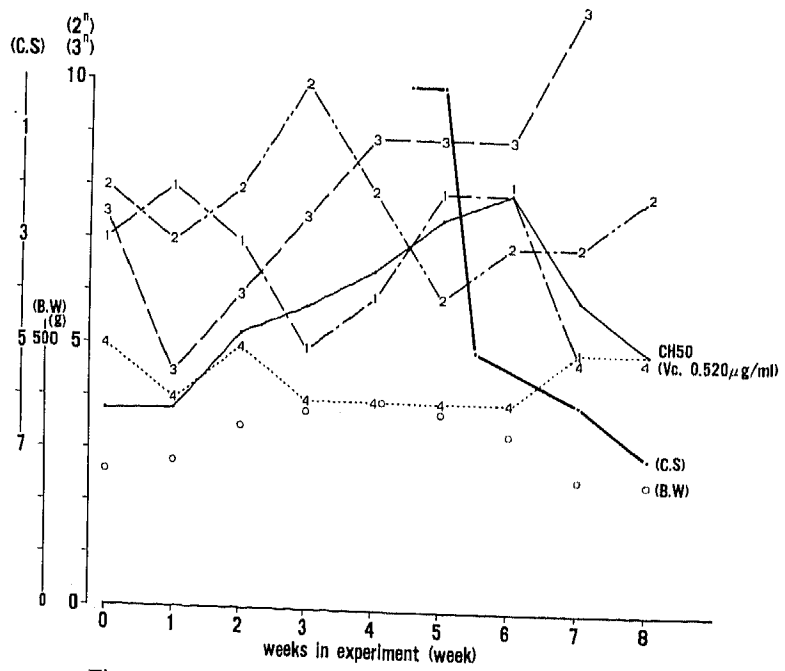


Fig. 3. Levels of components in follow up study of No. 2.

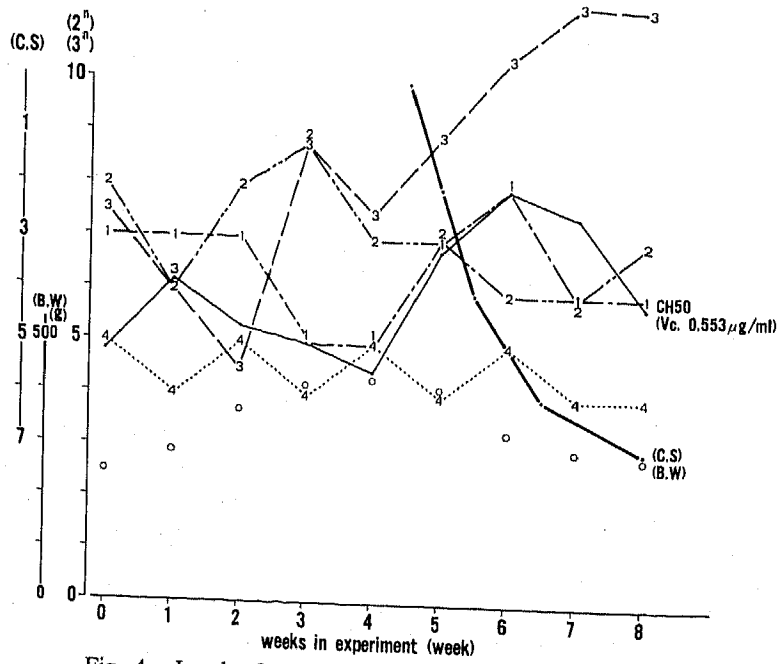


Fig. 4. Levels of components in follow up study of No. 3.

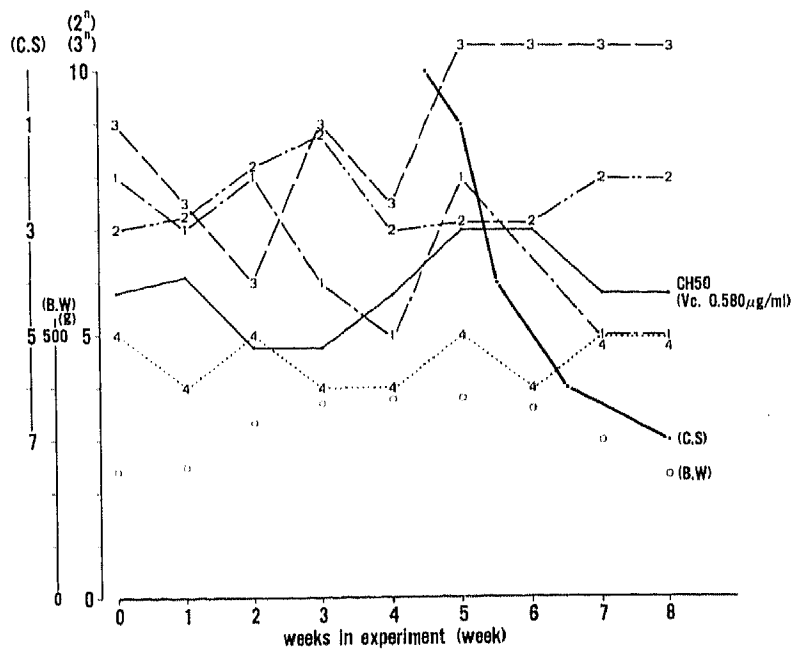


Fig. 5. Levels of components in follow up study of No. 4.

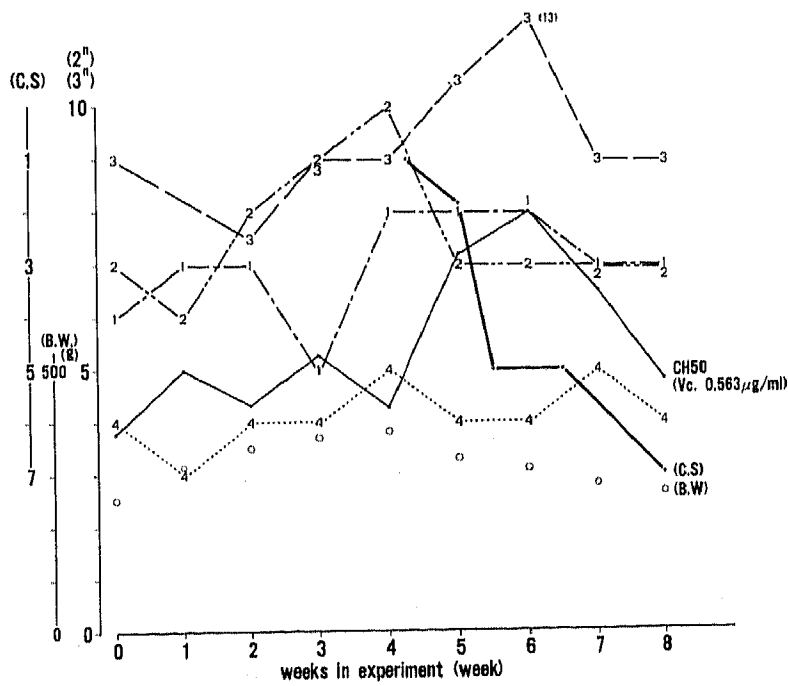


Fig. 6. Levels of components in follow up study of No. 5.

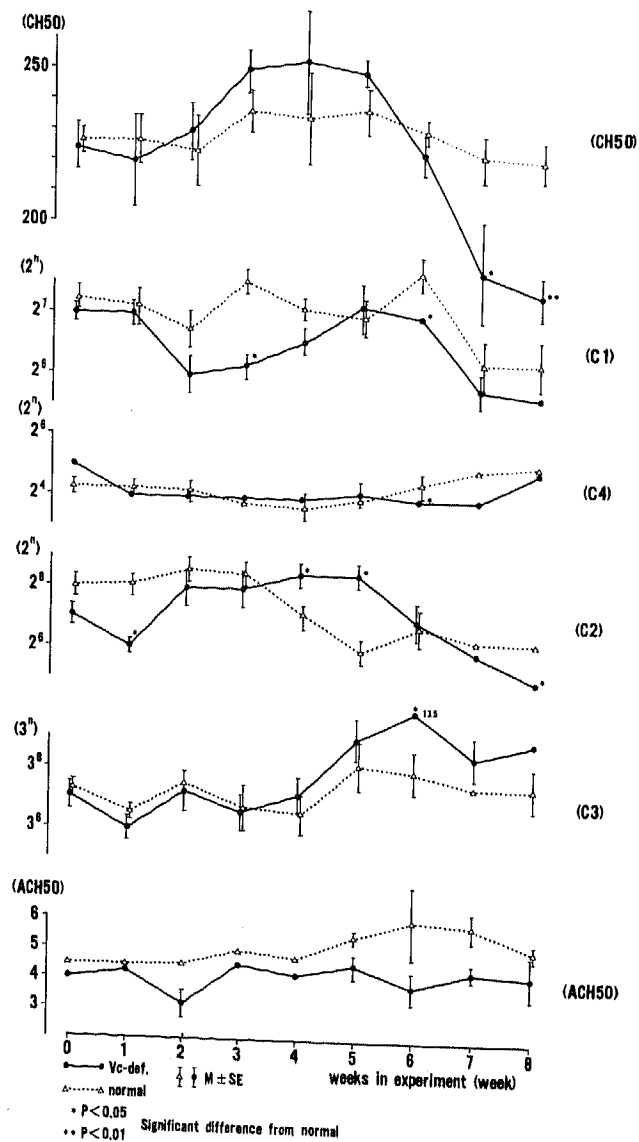


Fig. 7. Changes in CH50, ACH50 and individual components, C1, C4, C2 and C3 in the course of vitamin C deficiency in guinea pigs. Vertical scale showing dilution of the serum 2ⁿ in C1, C4, C2 and C3.

Clinical observations on the deficient group

From the 5th week all the animals started to lose body weight. Three of them showed abnormalities in walking and action laxity. At the 7th week, all the animals had edema in their feet and could not move. At the end of the 7th week, bleeding

under the skin of their legs and in their mouths and gums was observed. All the animals were in a stage of severe Vc deficiency clinically.

Levels of CH50, ACH50, C1, C4, C2 and C3 in the follow up study

The individual levels of CH50, components C1, C4, C2 and C3 were followed up together with the clinical symptoms and the body weight in the course of Vc deficiency as shown in Fig. 2–Fig. 6. They are summarized in Fig. 7.

CH50. Complement hemolysis titer for the classical pathway gradually increased until week 4, the time of the appearance of the clinical signs of AsA deficiency, and then started to decrease. On week 6, at the time of the clinical signs becoming severe, CH50 was still close to the normal level. On week 7, CH50 became low ($p < 0.05$) in the severe stage of AsA deficiency and lower ($p < 0.01$) at the end of the experiment. CH50 was maintained until a more severe stage of AsA deficiency occurred, and in the severe stage, the complement activity was also depressed.

C1. C1 was low ($p < 0.05$) during the 3rd week of the experiment and gradually increased up to the 5th week when the clinical symptoms appeared, then decreased, showing the same tendency as the CH50 titer.

C4. C4 did not show any particular change and maintained the same level throughout the experiment.

C2. From the beginning of the experiment, C2 gradually increased until the clinical symptoms appeared and was significantly high ($p < 0.05$) during the 4th and the 5th weeks. It then decreased along with the clinical signs.

C3. At the beginning of the experiment, C3 in the experimental group was at the same level as in the control group until the 5th week when Vc deficiency symptoms appeared. On the appearance of these symptoms, C3 was increased remarkably ($p < 0.05$) and remained at a higher level until the completion of the experiment. This pattern is completely opposite to that of C2, and when the other components were depressed, only C3 increased.

Complement hemolysis through the alternative pathway (ACH50)

ACH50 in the experimental group showed a tendency to be lower than the control group, but throughout the experiment there were no significant differences between the two groups. This indicates that in Vc deficiency the complement system is maintained through the alternative pathway. The classical pathway activated from antigen-antibody and components C1, C4 and C2 were depressed in the severe Vc-deficient stage. The alternative pathway was maintained for the body defence system in the Vc-deficient state. C3 in particular will potentiate the alternative pathway system of the complement.

DISCUSSION

The changes in the complement systems, CH50, ACH50 and the individual components, C1, C4, C2 and C3 in the course of vitamin C deficiency in guinea pigs

were demonstrated.

The state of vitamin C deficiency was confirmed by body weight change, the concentration of AsA in serum and the clinical observations. During the experiment, body weight decreased when the clinical signs of vitamin C deficiency appeared. When the body weight started to decrease on week 5, food intake was still normal. At the time when the body weight decreased remarkably, the food intake was also significantly low. In the last stage of the experiment, however, the food intakes were between 10 g/day and 15 g/day of diet containing 24% of protein. Enwonwu (14) reported that to produce protein-calorie malnutrition in the guinea pigs, it takes 3 to 4 weeks feeding with 5–11 g/day of diet containing 3% of protein. In our experiment, the guinea pigs were given 10 times more protein than were the protein-deficient guinea pigs and moreover, the daily food intake was more than that with those guinea pigs. Therefore the reduction in the body weight of the experimental group was not due entirely to inanition or not due, at least, to protein-calorie malnutrition. We consider the reduction in the body weight is mostly due to vitamin C deficiency.

There were several changes in the complement systems during the experimental periods.

The first period was the first week after the experiment started. The activities of the complement systems, excepting for the alternative pathway and C1, showed a slight decrease. C2 showed a significant decrease ($p < 0.05$) but the changes of other components were not significant. This indicates that this stage is an important period in the formation of immune systems in the host, because this phenomenon *i.e.*, the complement activity, was lowered at the very earliest stage in tumor-bearing rats and was frequently observed in the experiments on tumor-induced rats (Sakamoto *et al.*, unpublished data).

The second period was the time of the appearance of the Vc deficiency symptoms. C1 decreased ($p < 0.05$) in this period. On the other hand C2 and C3 gradually increased. This indicates that in the early stage of Vc deficiency the first component C1 was consumed. After that, C1 increased gradually until the clinical symptoms appeared and then decreased ($p < 0.05$) again.

The third period was the time when C2 increased gradually and reached the peak, and then decreased along with the clinical manifestation of AsA deficiency. At the same time, C3 also increased up to the same peak as C2 and remained at a high level even when the clinical symptoms became severe.

C4 did not show any notable change throughout the experiment.

C3 showed a completely different pattern from the other components in the course of Vc deficiency. Of the individual components, C3 increased significantly when the host was in a severe Vc-deficient state. Component C3 had shown a strong effect in increasing the complement activity by enhancing activation of both the classical and the alternative pathways, since C3 is known to be the junction of both the complement pathways. The response of C3 was more pronounced than that of C1, C4 or C2 when the host was in a severe stage of Vc deficiency. This is in line with

the observation that phylogenetically and ontogenetically the C3 system remains up to the time of the severe stage of the Vc deficiency (15) and plays an important role in the body defence mechanism, combining the alternative and the classical pathways.

On the other hand, C1 showed a lower level throughout the experiment and was significantly low ($p < 0.05$) at weeks 3 and 6. The lowered C1 component will be due to the characteristic of the C1q subcomponent. There are several investigators mentioned about the characteristic of C1q that there is a collagen-like region in the A chain of the C1q subcomponent which constitutes most of the N-terminal half of the chain and that similar collagen-like regions is found in the B and C chains (1, 16-18). Vitamin C is required for the proper hydroxylation of collagen lysin and proline; without a sufficiency of vitamin C, collagen formation is impaired. From these investigations the lowered C1 component might be closely related to the impaired collagen formation due to the vitamin C deficiency.

The lowered C1 will also be triggered the depression of the classical pathway, since a mega dosis of AsA administration contributes to increasing C1s-subunits (personal communication with Murata).

The activity of the alternative pathway did not show any change in the course of vitamin C deficiency, even in the severe stage. The complement system will be supported through the alternative pathway in the vitamin C-deficient state.

There were no investigations as to how or which part of the complement systems vitamin C exerts its effects for the activation of the individual components or both pathways. The cause of such effects of vitamin C deficiency on the complement systems and the complement components should also be investigated further. Detailed experiments would be needed to clarify the biochemical and physiological mechanisms of these phenomena.

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REFERENCES

- 1) Thomas, W. R., and Holt, P. G. (1978): Vitamin C and Immunity: an assessment of evidence. *Clin. Exp. Immunol.*, **32**, 370-379.
- 2) Worthington, B. S. (1974): Effect of nutritional status on immune phenomena. *J. Am. Dietet. Assoc.*, **65**, 123-126.
- 3) Spink, W. W., Michelsen, O., and Agnew, S. (1941): The relation of ascorbic acid to human complement. *J. Clin. Invest.*, **20**, 434-438.
- 4) Crandon, J. H., Lund, C. C., and Dill, D. B. (1940): Experimental human scurvy. *New Engl. J. Med.*, **223**, 353-356.
- 5) Anderson, R., Oesthuizen, R., Maritz, R., Theron, A., and Van Rosburg, A. J. (1980): The effects of increasing weekly doses of ascorbate on certain cellular and humoral immune functions in normal volunteers. *Am. J. Clin. Nutr.*, **33**, 71-76.
- 6) Mursh, F. (1936): Ascorbic acid and a precursor of serum complement. *Nature (London)*, **137**, 618.

- 7) Sakamoto, M., Kobayashi, S., Ishii, S., Katoo, K., and Shimazono, N. (1981): Changes of complement systems in vitamin C deficient and vitamin C excess on guinea pigs. *Eiyō To Shokuryō (J. Jpn. Soc. Food Nutr.)*, **34**, 325-334.
- 8) National Academy of Sciences, National Research Council (1978): Nutrient Requirements of Laboratory Animals, 3rd ed.
- 9) Fujita, A. (1955): The Measurement of Vitamins, Nankodo, Inc., Tokyo, pp. 605-607.
- 10) Mayer, M. M. (1961): Experimental Immunochemistry, 2nd ed., ed. by Kabat, E. A. and Mayer, M. M., Charles C. Thomas, Illinois, pp. 133-240.
- 11) Amano, T., Yoshinouchi, T., Miyashima, K., Mitsuhashi, Y., and Oofuji, M. (1976): Alternate pathway in SLE. *Jpn. Clin. Immunol.*, **8**, 289-297.
- 12) Nelson, R. A., Jr., Jensen, J., Gigli, I., and Tamura, N. (1966): Methods for the separation, purification and measurement of nine components of hemolytic complement in guinea pig serum. *Immunochemistry*, **3**, 111-135.
- 13) Nishioka, K. (1963): Measurements of complement by agglutination of human erythrocytes reacting in immune-adherence. *J. Immunol.*, **90**, 86-97.
- 14) Enwonwu, C. O. (1973): Experimental protein-calorie malnutrition in the guinea pig and evaluation of the role of ascorbic acid status. *Lab. Invest.*, **29**, 17-26.
- 15) Nishioka, K., Kawamura, K., Hirayama, T., Kawashima, T., Shimada, K., and Kogure, M. (1976): The complement system in tumor immunity: Significance of elevated levels of complement in tumor bearing hosts. *Ann. N. Y. Acad. Sci.*, **276**, 303-315.
- 16) Reid, K. B. M. (1974): A collagen-like amino acid sequence in a polypeptide chain of human Clq (a subcomponent of the first component of complement). *Biochem. J.*, **141**, 189-203.
- 17) Muller-Everhard, H. J. (1975): Complement Clq: A collagen-like glycoprotein. *Am. Rev. Biochem.*, **44**, 700-701.
- 18) Hughs, R. E. (1977): Nonscorbutic effects of vitamin C: Biochemical aspects. *Proc. Roy. Soc. Med.*, **70**, 86-89.