Metabolism of Ascorbic Acid (Vitamin C) in Subjects Infected with Common Cold Viruses

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There are reports in the literature of 20 or more trials designed to assess the efficacy of vitamin C (L-xyloascorbic acid, ascorbic acid, AA) in the prevention and treatment of the common cold. Many of the trials failed to satisfy strict criteria of scientific experimentation and it is generally concluded that AA supplementation has at best a small effect on colds (1,2). The effect of suggestion may sometimes be relevant to the benefits reported (3).

Infectious processes, including colds, may nevertheless influence AA metabolism: The early reports of Harris and his colleagues (4-6) that less AA was excreted during infections were probably the historical origin of the use of AA supplements to treat common colds, but apart from its antiscorbutic effect the biological role of AA is still not clear. Little attention has been given to changes in AA metabolism during infections, although there are reports of decreases in plasma or leucocyte AA during common colds and other infective processes (7-10). However, any substantial involvement of AA in infection would presumably result in an increased tissue requirement for the vitamin, and it is not clear whether variations in leucocyte AA concentrations in humans provide a significant index of changes in tissue metabolism.

Few studies have attempted to assess the significance of the observed changes in blood AA by measuring urinary excretion—possibly because this assay is difficult in subjects on moderate intakes (11). Previous work (R. E. Hughes, unpublished) has indicated that subjects given a daily supplement of 100 mg AA achieve AA equilibrium in 3–6 days; at this stage a constant proportion of the AA consumed (usually about 75%)

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appears in the urine during the 6 hr immediately subsequent to the ingestion of the dose. Any increased tissue requirement for AA should therefore be reflected as a decrease in the total 6-hr AA excretion, with possibly an increase in the formation and excretion of its metabolites. We have therefore examined the influence of infection with common cold viruses (rhinoviruses) on the absorption of AA and on the excretion of AA, diketogulonic acid (DKG), and oxalic acid. DKG and oxalic acid are two possible metabolites of AA (12) and their determination in urine poses few problems. Substantial concentrations of DKG have been shown to occur in human tissue and there is presumptive evidence that it is formed from AA (13,14) although recent commentators have indicated that it is not a major metabolite of AA in man (15,16).

MATERIALS AND METHODS

Volunteers. Male and female volunteers at the Medical Research Council's Common Cold Unit (Harvard Hospital, Salisbury) took part in the study. All were healthy subjects aged 18–50, living in similar conditions; their food was supplied from a central kitchen and consisted of a standard normal diet except that the fresh fruit was restricted to apples. They lived in isolation singly or in groups of two or three for the duration of the experiments (10 days).

Experiment 1: AA metabolism. This was designed to examine the excretion of AA, diketogulonic acid (DKG), and oxalic acid in infected and noninfected subjects. Twelve subjects took part. All received 100 mg AA daily in addition to their diet. The AA was given at 2 PM on Day 1 and thereafter at 9 AM daily for a total of 9 days. On Days 2-9 inclusive the subjects emptied their bladders at 10 AM (because orally administered AA is known not to be excreted within the first hour) and the urine was collected between 10 AM and 1 PM and between 1 PM and 4 PM. In each individual, the totals of AA excretion for the 6-hr periods on Days 3+4 (preinoculation) and Days 7 + 8 (postinoculation) were measured; for DKG the totals for 3-hr periods on Days 3+4 and 7+8 were used, and for oxalic acid, the totals for 3-hr periods on Days 2+4 and 6+7. On Day 4 between 4:30 and 6 PM nine of the volunteers were inoculated with a preparation of rhinovirus and three with the diluent only. Blood samples for leucocyte AA and plasma AA were collected before inoculation (Day 4) and after it (Day 8). Blood for serum-antibody determination was collected on Days 4 and 21. Nasal washings for virus isolation were collected on Days 6, 7, 8, and 9 using Hanks' balanced salt solution and were stored at –70°C until tested.

Experiment 2: AA absorption. This was designed to determine the extent to which impairment of gastrointestinal absorption of AA could account for the reduction of AA and DKG excretion found in infected

subjects in Experiment 1. The rise in plasma AA following ingestion of 4 g AA was measured in a group of subjects, and the test was repeated 3 or 4 days later when about half of the subjects were in course of an experimental rhinovirus infection similar to that used in Experiment 1. Blood for AA estimation was collected from 11 volunteers on Day 0 at 9 AM (baseline) and at 1:00 PM , 4 g of AA being ingested at 9.05 AM. The comparatively large dose of AA (4 g) was used to ensure that measurable increase in plasma AA-occurred, and the rise in plasma AA during the 4 hr following the dose (4-hr value less baseline value) was taken as a measure of absorption. On the next day five of the subjects were infected with rhinovirus as in Experiment 1 and the AA-absorption test was repeated either 2 or 3 days after this (i.e., 3 or 4 days after the first test) when the infective process in each individual was judged to be at its maximum. Nasal washings for virus isolation and blood for serum-antibody responses were collected as before, to define the extent of the rhinovirus infection. In this experiment the 6 control (noninfected) subjects received no intranasal inoculation of either virus or saline, and were not in isolation but were tested under conditions exactly comparable to the infected subjects. The second absorption test in these controls was done 3 days after the first.

Analytical procedures. The volume of all urine samples was measured, and analyses for AA and DKG were done on the day of collection. AA was analyzed in all samples and DKG in the 1–4 PM samples. Oxalic acid determinations were done on frozen samples of the urine within 14 days of collection using the 1-4 PM samples collected on Days 2, 4. 6, and 7.

AA was determined in urine by the 2, 6-dichlorophenol indophenol dye technique after a preliminary treatment of the urine with a cation-exchange resin to remove interfering substances (11). Diketogulonic acid (DKG) and oxalic acid were determined as described by Roe and Kuether (17) and by Hodgkinson and Williams (18), respectively.

Plasma AA was determined by the dichlorophenol indophenol photometric method (19) using the filtrate obtained by treating 4 ml plasma with 4 ml of 20% metaphosphoric acid. Leucocyte AA was determined as described by Denson and Bowers (20) with minor modifications; leucocytes were counted with a Coulter Counter (Model 2F).

Rhinovirus infection. Volunteers were inoculated intranasally using drops containing rhinoviruses type 3 (100 $TCID_{50}$) and type 9 (50 $TCID_{50}$) prepared for administration to humans (21), or with diluent alone (0.2% bovine plasma albumin in Hanks' balanced salt solution). Infection was assessed clinically under double-blind conditions by daily examination (21) and virologically by isolation of virus from daily nasal washings and by assessment of fourfold or greater rises in serum antibodies against the challenge viruses 3 weeks after infection. Virus in nasal washings was

detected and titrated using rhinovirus-sensitive HeLa cells and neutralizing antibody was measured by a micromethod in the same cells (22).

RESULTS

In Experiment 1 five of the nine subjects given the rhinovirus inoculum developed mild coryzal symptoms and one moderate symptoms. Virus shedding was detected in six subjects, in two, three, or all four of the daily nasal washing specimens, with virus titers ranging from $10^{0.5}$ to $10^{3.5}$ TCD_{50} per milliliter. Two of these six subjects also developed a fourfold rise in antibody titer against one or both of the two challenge viruses. The six subjects (G–L) showing laboratory evidence of infection formed the "infected" group. The "uninfected" (control) group consisted of six subjects A–F. They comprised three saline-inoculated subjects A, D, and F who showed no laboratory evidence of infection and also three subjects B, C, and E who had received the virus inoculum but who also showed no virus in nasal washings collected 2, 3, 4, and 5 days after inoculation, nor any subsequent increase in specific antibody titer and who were, therefore, also judged not to have been infected.

The infected and uninfected groups were compared as regards their excretion of AA and DKG before inoculation. The mean value, with standard error, for excretion of AA on Days 3+4 for the uninfected group was 50.4 mg ± 13.2 and for the infected group 53.4 mg ± 16.7. Similarly mean DKG excretion on Days 3+4 in the uninfected group was 20.3 mg ± 3.4 and in the infected group 24.8 mg ± 4.8. These differences between the groups were not significant.

A change in excretion after infection was then sought: The excretion of AA in both infected and uninfected groups Days 3+4 (preinfection) was compared with their excretion on Days 7+8 (during infection), and DKG excretion before and during infection was compared in the same way (Fig. 1). In uninfected subjects the mean excretion of both AA and DKG was higher on Days 7+8 than on Days 3+4, AA being increased by 19.8% and DKG by 16.3%. This indicated that "AA equilibrium" had probably not been reached by all subjects on Days 3+4 despite the daily supplement of 100 mg. However, in the infected group mean excretion of both AA and DKG decreased markedly on Days 7+8 compared with Days 3+4, AA by 35.0% and DKG by 36.3%. Two infected subjects, K and L, and one uninfected, D, were clearly far from AA equilibrium on Days 3 and 4, when each of them excreted < 10 mg AA (Fig. 1): AA excretion in two of these subjects, K and L, subsequently remained virtually unchanged during their infection, whereas the noninfected subject's, D's, excretion of AA and DKG had increased considerably by Days 7 and 8. Evaluation of the falls in AA excretion by the paired *t* test showed $0.1 > P > 0.05$ for the six infected subjects, or $P < 0.05$ when K and L were excluded from

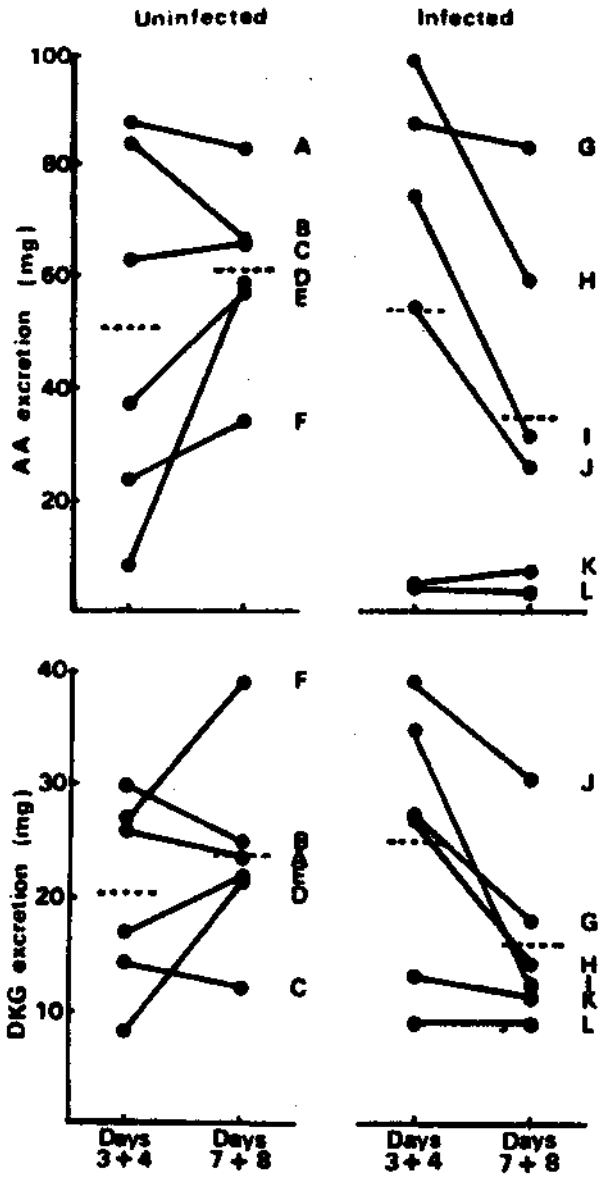

FIG. 1. Excretion of AA (total for 6-hr periods on 2 successive days) and DKG (total for 3-hr periods on 2 successive days) in uninfected controls (A–F) and in infected subjects (G–L) before (Days 3 + 4) and during (Days 7 + 8) rhinovirus infection. (----) Mean value for each group of observations.

the analysis. For DKG excretion the fall in infected subjects was significant ($P < 0.05$) whether subjects K and L were included in the assessment or not.

The oxalic acid excretion did not differ significantly in infected and uninfected subjects. The mean excretions (mg) were 10.3 ± 0.9 and 10.8 ± 1.9, before (Days 2 + 4) and after (Days 6 + 7) infection, respectively, for the infected group and 9.3 ± 0.7 and 8.8 ± 0.8 for the control group (mean values with standard errors). Neither the leucocyte-AA nor the plasma-AA concentration was significantly changed 4 days after infection. During infection (Day 8) the mean AA concentration in leucocytes was 31.96 ± 2.85 μg per 10^8 leucocytes, compared with 34.37 ± 3.46 in the same

subjects before infection (Day 4) (mean values with standard errors); the difference was not significant.

In Experiment 2 five subjects developed coryzal symptoms after virus inoculation and rhinovirus infection was proven by the recovery of virus from their serial nasal washings. The five infected subjects were all female, with a mean age of 30.2 years; the noninfected controls were five females and one male, with a mean age of 28.2 years. No clear difference in AA absorption after infection (Day +3) could be demonstrated compared with the preinfection values (Day 0). In the uninfected subjects the mean baseline concentrations of AA in plasma before and during infection were 0.54 ± 0.13 and 0.91 ± 0.08 mg per 100 ml plasma, respectively, and in the infected subjects they were (Day 0) 0.30 ± 0.12 and (Day +3) 0.78 ± 0.12 mg per 100 ml, respectively (means with standard errors). The rises in plasma AA after ingestion of the test dose on Day +3 (4-hr value less baseline value) (Fig. 2) were not significantly different in infected and control groups, nor did the rises in the infected subjects on Day + 3 differ significantly from the values in the same subjects before infection (Day 0).

DISCUSSION

The current study indicated that under controlled conditions of AA intake the urinary excretion of AA was significantly reduced in subjects experimentally infected with rhinoviruses. There was no concomitant

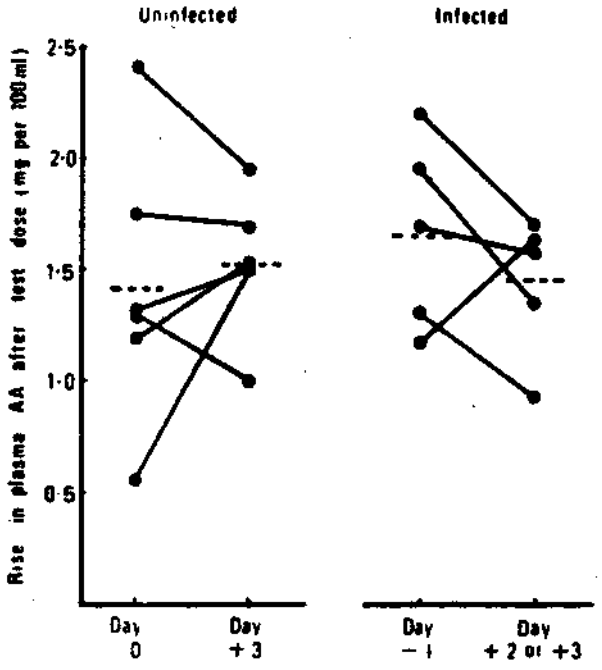

FIG. 2. Rise in plasma AA (4-hr concentration - baseline concentration) after a test dose of 4 g AA in uninfected controls (Days 0 and +3) and in subjects before (Day –1) and during (Days +2 or +3) rhinovinis infection. (----) Mean value for each group of observations.

increase in DKG and oxalic acid – two possible metabolites of AA – and this finding would tend to support the view that DKG is perhaps not a direct metabolite of AA in humans (15,16). The results did, however, show that excretion of DKG, like that of AA, falls during infection. It would seem interesting to investigate the fate of other metabolic products of AA in humans under similar experimental conditions, but apart from ascorbate-2-sulfate and oxalate these have not been defined (15). Our failure to show a consistent change in oxalic acid excretion during infection may be related to the fact that other endogenous and dietary sources make a significant contribution to urinary oxalic acid, and these other sources may mask any changes resulting from altered metabolism of AA.

A number of possible interpretations of these results present themselves. One is that during infection less AA is absorbed from the gastrointestinal tract. However, the second experiment produced no evidence that absorption of AA was reduced in the infected subjects. (It is however conceivable that AA absorption could occur more quickly or more slowly during infection, in which case our 4-hr determination of plasma AA would not necessarily be comparable with the 4-hr preinfection value.)

An alternative possibility is that during infection AA is retained by the tissues or perhaps diverted to organs or tissues which require more vitamin. Hume and Weyers reported a significant fall in leucocyte AA during a common cold infection (7); we were unable to confirm this but our second measurements of leucocyte and plasma AA were made on the 4th day after inoculation of rhinovirus, when the infectious process in some of the volunteers had probably passed its maximum. It is in any case difficult to assess the significance of changes in leucocyte AA: The total amount that could be "released" by the leucocytes would be too small to change significantly the concentration of AA in other organs and tissues.

Our results, obtained during a controlled experimental viral infection in man, confirm that AA metabolism is altered during the infection. However, the nature of the involvement of AA in the infectious process remains unclear.

SUMMARY

The influence of experimentally induced rhinovirus infection on the excretion of ascorbic acid (AA) and two of its possible metabolites, diketogulonic acid (DKG) and oxalic acid was determined in human volunteers receiving a controlled daily intake of AA. There was a significant fall in the excretion of both AA and DKG in the infected subjects but no change in the oxalic acid excretion. A simple absorption test provided no evidence of any impairment of gastrointestinal absorption of AA during infection. Possible mechanisms of this infection-induced change in AA metabolism are discussed.

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