Significant Endogenous Synthesis of Nitrate Does Not Appear to Be a Feature of Influenza A Virus Infection

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Abstract

There is much concern about the role of nitrate in the formation of carcinogenic *N*-nitroso compounds. There has been renewed interest in the endogenous formation of nitrate arising as a host response to infection. This study was designed to investigate whether the large increases in nitrate excretion rate reported (6–15-fold) for certain infectious diseases is also a feature of systemic influenza infections. Volunteers were challenged either with an attenuated strain of influenza A virus or with saline; and excreted nitrate was measured in subsequent 24-h urine samples. Both with and without adjustment for potential confounding by dietary and other factors, it was clear that neither mild nor moderate influenza A virus infection resulted in substantial endogenous nitrate biosynthesis since all the variation in urinary nitrate excretion observed was within the range of normal daily fluctuations. It remains possible that a stronger and more consistent nitrate excretion response might be observed in other infectious illnesses with greater systemic disturbance.

Introduction

The hypothesis that humans are capable of the endogenous synthesis of nitrate has attracted considerable controversy (1–3). Since the work of Mitchell *et al.* (4) in 1916, a number of nitrate balance studies (5–8) have shown a consistent excess of nitrate in the urine (equivalent to about 1 mmol/24 h of synthesized nitrate) over that ingested from low-nitrate diets, suggesting that nitrate arising from endogenous synthesis in healthy individuals represents a significant proportion of normal daily nitrate exposure (9). Other studies have failed to confirm these findings (3, 10–13), and recently Packer *et al.* (14) demonstrated that endogenous nitrate biosynthesis was substantially less than 1 mmol/24 h and in the range of 0.15–0.43 mmol/24 h. Thus the extent to which nitrate synthesis in healthy human subjects relates to overall nitrate exposure remains open to question.

In 1982, Wagner and Tannenbaum (15) reported more than a 6-fold increase in urinary nitrate excretion in a human volunteer, maintained on a low-nitrate diet, 3 to 4 days after they had contracted a nonspecific intestinal infection. At the same time, Hegesh and Shiloah (16) observed a 15-fold increase in urinary nitrate excretion among 58 infants, hospitalized with acute diarrhea, in comparison with 30 control infants, both groups having been given identical food preparations. Similar increases in nitrate excretion have been reported in patients with giardiasis and ulcerative colitis but not viral meningitis (17, 18). It was suggested that nitrate biosynthesis might represent a host response to infection.

Furthermore, it has been demonstrated that immunostimulation of the reticuloendothelial system results in the oxidation of reduced nitrogen compounds (in particular arginine) to nitrate. Animal studies, in which rodents were challenged with a variety of immunostimulatory agents, have supported this hypothesis, and *in vitro* experiments have shown that murine macrophages are capable of synthesizing nitrate, with nitric oxide and nitrite as intermediates, when activated. Similarly, other mammalian cell types including endothelial cells, neural tissue, neutrophils and possibly hepatocytes and Kupffer cells have also been demonstrated to produce nitric oxide when appropriately stimulated (19–29).

At the present time, it is difficult to evaluate the relevance to humans of nitrate exposure arising from endogenous synthesis as a result of immunostimulation. It has been considered that the major source of nitrate exposure for humans is the diet, especially certain vegetables, preserved meats, and drinking water (30). Currently there is much discussion about environmental nitrate and its role, after reduction to nitrite, in the formation of carcinogenic *N*-nitroso compounds in the stomach (30, 31). Clearly, if it could be demonstrated that, in response to infections, the body burden of nitrate is periodically increased by as much as an order of magnitude, it would be necessary to assess the importance of this in relation to endogenous *N*-nitroso compound formation, particularly since it has been demonstrated that macrophages produce *N*-nitroso compounds concomitantly with nitrate and nitrite (32).

In order to investigate this problem more fully, it would be of interest to look at nitrate excretion patterns, under controlled conditions, in individuals before and after a challenge with an infectious agent. It is rarely possible for such studies to be organized around naturally occurring infections, and there are obvious ethical problems in experimentally exposing people to infectious agents. We conducted a study among human volunteers

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Table 1 Urinary nitrate excretion (mg/day) in volunteers before (days 1-2) and after (days 5-7) challenge with influenza A virus

Category	*n*	Mean clinical score	Mean days 1-2 ± SE	Mean days 5-7 ± SE	Mean difference (*P*)[a]	Adjusted mean difference[b] (*P*)[a]
Virus challenge	25	7.9	116.2 ± 7.8	133.4 ± 10.9	17.2 (0.034)	16.6 (0.056)
Saline challenge	4	2.0	113.6 ± 19.4	167.1 ± 46.0	53.5 (>0.1)	57.8 (0.016)
Virus challenge						
No infection	13	1.5	120.9 ± 8.8	132.1 ± 14.5	11.3 (>0.1)	9.1 (>0.1)
Infection	12[c]	14.8	111.0 ± 13.6	134.8 ± 17.0	23.7 (0.063)	26.1 (0.039)
Subclinical infection	3	2.7	89.4 ± 6.0	117.0 ± 8.9	27.6 (>0.1)	5.6 (>0.1)
Mild illness	7	14.1	128.2 ± 20.1	142.2 ± 25.1	14.0 (>0.1)	14.3 (>0.1)
Moderate illness	2	35.3	83.5 ± 32.6	135.5 ± 70.0	52.0 (>0.1)	95.1 (0.005)
Drug treatment	14	9.4	117.5 ± 11.6	134.4 ± 15.3	16.9 (>0.1)	17.1 (>0.1)
Placebo treatment	11	6.0	114.4 ± 10.6	132.1 ± 16.0	17.7 (>0.1)	17.5 (>0.1)

[a] Significance of comparison between mean difference and zero.
[b] Adjusted for difference between the two time periods in intake of vegetables, preserved meat, drinking water, number of cigarettes smoked, and alcohol, and whether or not subject consumed alcohol over study period.
[c] Six volunteers with clinical illness and laboratory confirmation, three with clinical illness only, and three with laboratory confirmation only (*i.e.*, subclinical infection).

participating in clinical trials of influenza at the Medical Research Council CCU.[2]

Materials and Methods

Twenty-nine healthy male volunteers, aged 19-49 years, took part in the study. Eight consecutive 24-h urine specimens were collected from each volunteer, commencing 2 days after the volunteers arrived at the CCU. In these initial 2 days, those volunteers with community-acquired infections were excluded. After two complete urine collections had been made, 25 volunteers were challenged intranasally with diluted allantoic fluid (EG4460) containing attenuated influenza virus (A/Eng/40/83) diluted $10^{-3.5}$ in Hanks' saline containing 0.2% bovine plasma albumen. This is a dose of $1-3 \times 10^4$ egg infectious doses/volunteer. Four volunteers received saline instead of virus. One day prior to virus (or saline) challenge, volunteers received intranasally either an experimental antiinfluenza drug or a placebo. Neither the volunteers nor the clinical observers were aware of the nature of the challenge (virus/saline) or the experimental treatment (drug/placebo). A full report of the drug trial and related procedures has been published elsewhere (33).

Throughout collection volunteers remained in isolation, were provided with similar meals by the CCU, and were examined clinically every day. A daily clinical score, derived from an aggregate of criteria such as temperature, number of tissues used, headaches, malaise, myalgia, and observed clinical signs, as used routinely at the CCU, was assigned to each volunteer by the clinical observer. At the end of the study period, the clinical observer also gave each volunteer an overall assessment as suffering either no illness or illness of mild, moderate, or severe nature. To confirm infection, the presence or absence of virus in daily nasal washings was assessed, and viral presence was taken as a specific indicator of infection. Also measured were titers of serum antibodies to influenza, prechallenge and 2-3 weeks postchallenge. A four-fold rise in antibody titer was regarded as an indication of infection. Volunteers with either of these laboratory indications but without clinical illness were categorized as having a subclinical infection.

Each volunteer also completed a daily questionnaire to ascertain the extent to which the meals provided by the CCU were consumed and to record additional intake of food, drink, and cigarettes. This information, together with reports of the composition of CCU meals, was used to derive semiquantitative measures of exogenous nitrate intake (from vegetables, preserved meat, and drinking water). Smoking and alcohol consumption were recorded as possible modifiers of nitrate metabolism.

Urine was collected over 10 g sodium hydroxide, the volume of each 24-h sample was recorded, and aliquots were stored frozen (−20°C) prior to nitrate analysis, within 1 month, using a standard automated procedure (14). Each sample at each time point was analyzed in duplicate. The coefficient of variation for the nitrate analysis method was 2.7%.[3]

Clinical trials at the CCU are approved by the Harrow District Ethical Committee at Northwich Park Hospital.

Results

Twenty-five of the 29 volunteers were challenged with virus, and, of these, nine were assessed as suffering a clinical illness (two were categorized as moderate and seven as mild). Six of the nine volunteers who were clinically ill, including both of those with moderate illness, had laboratory confirmation of these infections (*i.e.*, virus culture or significant antibody increase). A further three volunteers were subclinically infected, while 13 showed neither illness nor laboratory evidence of infection.

Summary urinary nitrate excretion results for the study are provided in Table 1. This shows the mean daily nitrate excretion before virus (or saline) challenge (days

[2] The abbreviations used are: CCU, Common Cold Unit; CS, clinical score; DT, drug treatment; PT, placebo treatment.

[3] P. Packer, unpublished observation.

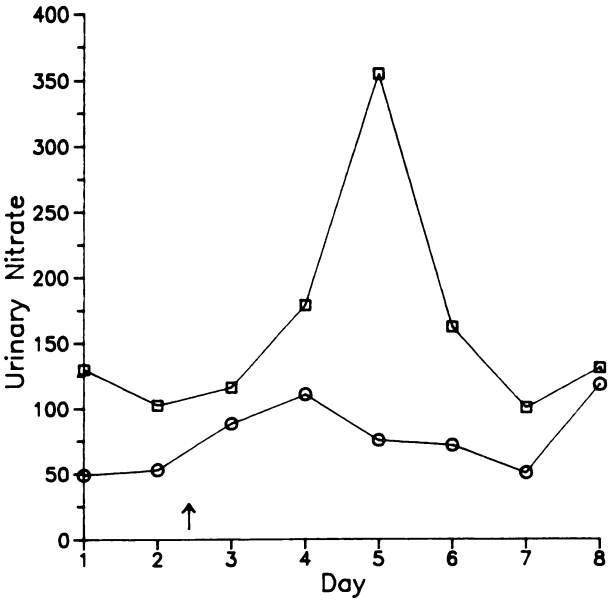

Fig. 1. Urinary nitrate excretion (mg/24 h) for each of the 8 study days for two volunteers, with moderately severe illness, following influenza virus challenge. □, CS 32 and PT; ○, CS 38.5 and DT. *Arrow*, virus challenge administered on day 2.

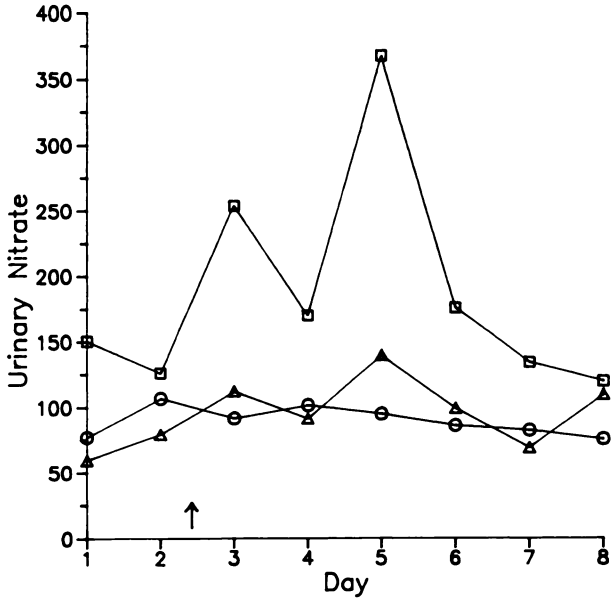

Fig. 2. Urinary nitrate excretion (mg/24 h) for each of the 8 study days for three volunteers, with mild illness, following influenza virus challenge. □, CS 19 and DT (one similar profile for volunteer with CS 14.5 and PT not shown); ○, CS 12 and DT (one similar profile for volunteer with CS 6 and DT not shown); △, CS 9 and PT (two similar profiles for volunteers with CS 9 and DT, and CS 29.5 and DT not shown). *Arrow*, virus challenge administered on day 2.

1 and 2) and 3 to 5 days after challenge (days 5 to 7, the peak period for illness) for the volunteers according to whether they received virus or saline and according to the extent of their illness. Results are also presented comparing volunteers who received the experimental drug with those who received placebo.

The results in Table 1 also show the mean difference in urinary nitrate excretion between the two time periods before and after adjustment for a number of covariates. These covariates were the mean change (between days 1 and 2 and days 5 to 7) in intake of vegetables, preserved meat, drinking water, alcohol, numbers of cigarettes smoked, and whether or not the volunteer ever consumed alcoholic drinks over the study period.

Although a significant but small increase in urinary nitrate excretion was observed following viral challenge (and specifically in volunteers with positive evidence of infection), a larger and more significant (after covariate adjustment) increase was observed in those individuals to whom saline was administered. Division of the infected group by severity of illness demonstrated that the major influence on the results was a doubling of urinary nitrate excretion in the two individuals with moderate illness and that all other changes in urinary nitrate excretion were within the normal range of individual day-to-day variation.

Table 1 also shows that drug treatment had no effect on urinary nitrate excretion, with drug and placebo groups showing similar levels of increase.

Representative individual time courses of nitrate excretion over all eight study days are shown in Figs. 1–5. Of the two men with moderate illness, only one showed an appropriately timed increase in daily nitrate excretion, consistent with a response to infection, whereas the other did not (Fig. 1). Similarly, not all men with mild disease or subclinical illness showed increases in nitrate excretion (Figs. 2 and 3), and, in contrast, several of the men without any evidence of infection, clinical or subclinical, showed a rise in nitrate excretion after challenge (Fig. 4).

Finally, Fig. 5 shows the time courses of nitrate excretion for the four volunteers who received the saline control instead of virus, two of whom received the drug and two the placebo. One of these excreted the highest daily amount of nitrate recorded in the study. Fig. 5 also indicates the considerable inter- and intraindividual variation that occurs in daily nitrate excretion rate even with broadly similar dietary intakes and in the absence of infection.

Discussion

The results presented provide little evidence to suggest that urinary nitrate excretion levels are increased following infection with an attenuated influenza A virus. Although there was a significant increase in excretion in the 12 volunteers with a definite infection (Table 1), the increase was small in magnitude, less than that observed in the four volunteers challenged with saline and largely attributable to one of the two volunteers in the moderate illness group. The extremely large increases in nitrate excretion after infection (6–15-fold) observed in other studies (15, 16, 18) were not observed in any individual. In these previous studies, endogenous synthesis of nitrate would seem to be the most reasonable explanation for the increase because it is unlikely that changes in nitrate intake or natural individual variation could explain such a large effect. In this study, however, it is not possible to conclude whether endogenous synthesis makes any contribution to overall postinfection nitrate excretion. Those changes which were observed could be explained by

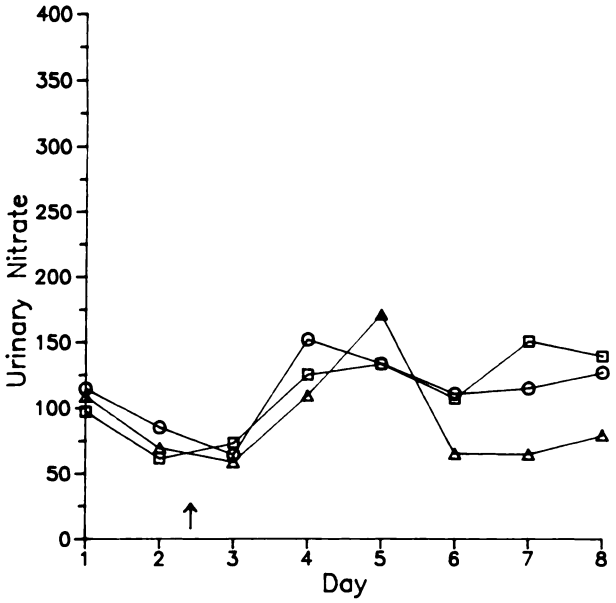

Fig. 3. Urinary nitrate excretion (mg/24 h) for each of the 8 study days for three volunteers, with subclinical infection, following influenza virus challenge. □, CS 1 and PT; ○, CS 5.5 and DT; △, CS 1.5 and DT. *Arrow*, virus challenge administered on day 2.

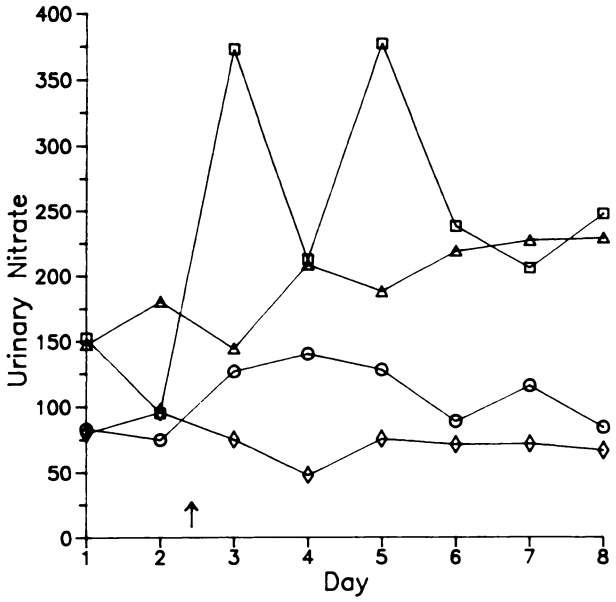

Fig. 5. Urinary nitrate excretion (mg/24 h) for each of the 8 study days for four volunteers with saline challenge. □, CS 0 and DT; ○, CS 0.5 and PT; △, CS 7.5 and DT; ◇, CS 0 and PT. *Arrow*, saline challenge administered on day 2.

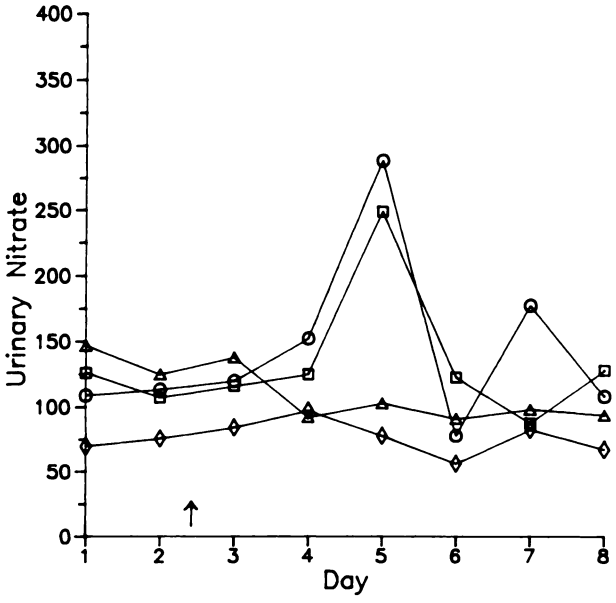

Fig. 4. Urinary nitrate excretion (mg/24 h) for each of the 8 study days for four volunteers, with no evidence of infection or illness, following influenza virus challenge. □, CS 0 and DT (three similar profiles for volunteers with CS 1 and DT, CS 5 and PT and CS 0 and PT not shown); ○, CS 0 and DT (one similar profile for volunteer with CS 0 and PT not shown); △, CS 0.5 and PT (three similar profiles for volunteers with CS 0 and PT, CS 1.5 and PT, and CS 7.5 and DT not shown); ◇, CS 0.5 and PT (two similar profiles for volunteers with CS 2.5 and PT, and 0.5 and DT not shown). *Arrow*, virus challenge administered on day 2.

variation in dietary intake or minor fluctuations in the metabolism of ingested nitrate, which were not completely controlled for in our statistical analysis.

In this study the virus strain used was deliberately attenuated in order to avoid extremely severe reactions. During the study, it also became apparent that the clinical response to infection with this strain was much milder than had been observed previously (34), probably because a closely related virus had caused infections in the general population and raised the level of host resistance. Nevertheless, the strain used was sufficiently virulent to produce systemic febrile symptoms in some individuals. Also the time period for the study was such that by the last urine collection (day 8) all subjects would be past the peak of any illness, which would normally be between days 5 and 7. The results exclude, therefore, the hypothesis that all systemic infections produce a dramatic increase in nitrate excretion to the extent observed in the earlier studies. It remains possible that a larger and more consistent nitrate excretion response might be observed subsequent to other infections which produce a more severe systemic illness. Those studies in which large changes in nitrate excretion were reported all involved illness with severe symptoms and, most frequently, gastrointestinal tract infections. Schulz *et al.* (17) found no such increases in individuals with viral meningitis. Thus the nitrate excretion response might be dependent on the type of infection, *i.e.*, bacterial, protozoan, etc., as well as the severity of illness.

Although over one-half of the volunteers with a virus challenge also received drug therapy, it is unlikely that this had any significant interaction with the pattern of nitrate excretion. Not only were mean urinary nitrate excretion levels similar in drug and placebo treated volunteers (Table 1), but also the individual profiles do not show any overall divergence between the two groups, *i.e.*, there was no evidence that drug therapy consistently suppresses (or enhances) nitrate excretion.

In conclusion, mild and moderate infections caused

by an attenuated influenza virus did not cause the substantial increase in host nitrate excretion that have been taken as indicative of increased endogenous nitrate synthesis in studies of certain other infections. The results presented show only changes in nitrate excretion which could be attributed to the smaller and naturally occurring inter- and intraindividual variations that are ordinarily observed. The results cannot be generalized to other conditions when the dynamics of nitrate excretion might be different.

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