Intake of nutrient supplements affects multiplication of *Leishmania donovani* in hamsters

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SUMMARY

The role of the essential nutrients, vitamins A, B (complex), C and E and iron, as prophylactic as well as supportive therapy in experimental visceral leishmaniasis (VL), was studied in hamsters. Prophylactic administration of vitamin C (50, 100 and 250 mg/kg) from day15 to day 0 (15 doses) significantly reduced the intake of *Leishmania donovani* in hamsters but had no therapeutic effect. In contrast, vitamins A, B complex and E and iron, whether used prophylactically or therapeutically, promoted parasite multiplication. The efficacy of sodium stibogluconate, a reference antileishmanial drug, was appreciably improved in animals administered prophylactically with vitamin C. However, supplementation of vitamin C during established infections resulted in reduced drug action. The results show that the prophylactic use of vitamin C may prevent the onset of leishmania infection and cautions against the indiscriminate use of nutrient supplements such as vitamin A, B complex, and E and iron in VL endemic areas.

Key words: *Leishmania donovani*, hamster, vitamin A, vitamin B complex, vitamin C, vitamin E, iron, sodium stibogluconate.

INTRODUCTION

Leishmania donovani is an obligate intracellular protozoan that colonizes macrophages in its vertebrate host, causing visceral leishmaniasis (VL) in humans. The most striking feature of visceral leishmaniasis is the profound impairment of the host's immune system. In drug-treated animals, therapeutic interventions that led to the resolution of the disease require a cell-mediated immune response (Wright & El Amin, 1989). Given the role of macrophages in both the initiation and resolution of infection, the survival of this parasite within these cells appears to be assisted by factors that inhibit or reduce the impact of macrophage microbicidal mechanisms (Moore, Labrecque & Marlashewski, 1993). *L. donovani*-infected macrophages are impaired in their ability to produce IL-1 (or express) class I or II MHC gene products, both of which are required for the induction of T cell-dependent immune responses (Reiner, 1987; Reiner *et al*. 1990). Infected macrophages also demonstrate an impaired oxidative burst, which is a primary defence of the cell during *L. donovani* invasion (Buchmuller-Rouiller & Mauel, 1987).

Enhanced nutrition reduces the risk of infections and improves recovery from infections of various types both in experimental animals and humans (Lesourd, Mazari & Ferry, 1998). More recently, it has been shown that non-specific immune enhancement can significantly increase resistance to bacterial, viral, rickettsial and fungal infections (Girodon, Lobard & Galan, 1997).

In the case of VL, high incidence was found to be associated with malnutrition, and undernourished children are highly prone to severe disease due to poor immune status (Cerf *et al*. 1987; Anstead *et al*. 2001). However, there are no reports regarding the relationship of immune status and malnutrition in individuals developing subclinical infection. Little is known regarding the effect of several nutritional supplements, which are vital for life and are known to enhance the non-specific immune status of the host (Leibovitz & Seigel, 1978; Thomas & Holt, 1978; Anderson, 1981; Bendich & Cohen, 1988; Bhaskaran, 1988) on the profile of several pathogens causing tropical diseases. Here we have employed the nutritional supplements vitamins A, B complex, C and E as well as iron, which are known not only to support healthy blood formation but also to enhance the natural resistance of the host, to see whether there is any positive effect when they are combined with anti-parasitic chemotherapy.

The present communication is the first report of the impact of the vitamins A, B complex, C and E as well as iron on the course of *Leishmania* infection. For this purpose, we used hamsters, which closely mimic the clinicopathological features of VL in humans. Systemic infection of the hamster with *L. donovani* results in a relentless increase in visceral

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parasite burden, progressive cachexia, hepatosplenomegaly, pancytopenia, hypergammaglobunemia and ultimately death (Melby *et al*. 1998). Using this model we studied the prophylactic as well as the therapeutic effect of the above stated nutrient supplements on the outcome of *Leishmania* infection. Their role as an adjunct to anti-leishmanial chemotherapy was also investigated.

MATERIALS AND METHODS

Animals

Laboratory bred, male golden hamsters (*Mesocricetus auratus*) of approximately 50 g weight were used as experimental hosts. They were housed in climatically controlled rooms in plastic cages and fed with standard rodent food pellet (Lipton India Ltd: Table 1) supplemented with seasonal fruits and vegetables and water *ad libitum*.

Parasites

L. donovani (MHOM/IN/80/DD8), a WHO reference strain, was obtained from Imperial College, London and maintained through *in vivo* serial passsge (amastigote to amastigote) in hamsters (Dube *et al*. 1998).

Isolation of parasite and infection to host

The isolation of parasites and infection of naïve hamsters were carried out as described by Pal *et al*. (1989). Briefly, heavily infected animals (carrying 40 to 60-day-old infections) were autopsied, their spleens removed, homogenized in Locke's solution and centrifuged (Model Hereaus, Germany) at 30 ***g*** for 5 min to sieve out tissue debris. The supernatant fraction containing parasites was aspirated and recentrifuged at 450 ***g*** for 30 min. The pellets of parasites formed at the bottom of the tubes were pooled and diluted to obtain a concentration of 1×10^7 amastigotes in 0·1 ml and this inoculum was given to each hamster intracardially.

Nutrient supplements and anti-leishmanial drug

The nutrient supplements used were: vitamin A (1 mg/kg, USV, India), vitamin B complex: (20 mg/kg, Glaxo India); vitamin C (Celin®, Glaxo India) at 250, 100, 50 and 25 mg/kg; vitamin E (30 mg/kg, Merck India); and iron (20 mg/kg, Fersolate®, Glaxo India). The constituents of the vitamins and iron are listed in Table 2. The doses of nutrient supplements used were decided on the basis of a conversion factor between rat and human (i.e. 1/7th dose of human) as described by Freireich *et al*. (1966).

Table 1. Composition of basal diet*

Constituents	Amount (G/100 g dry weight)
Protein	21·0
Fat	5·0
Crude fiber	4·0
Ash	8·0
Calcium	1·0
Phosphorus	0·6
Nitrogen free extract	53
Metabolically energy	3600 K cal/kg
Supplementation with vegetables and fruits	

* Gold Mohur Laboratory animal feed – Lipton India Limited.

Experimental protocol

(*1*) *Prophylactic and continued effects of nutrient supplements*. For each individual experiment a batch of 30 hamsters was equally divided into 3 groups. Animals of group 1 (prophylactic) were orally administered vitamin C (at a dose of 250 mg or 100 mg or 50 mg or 25 mg/kg) daily for 15 days [day 15 to day 0 post-infection (p.i.)] via a feeding cannula into the mouth. On day 0 the animals were challenged intracardially with 1×10^7 amastigotes. Hamsters of group 2 (continued) were similarly given vitamin C and challenge, but in these, the administration of vitamin C was continued for another 30 days (day 15 to day + 30 p.i.). Group 3, which received no vitamin C but only the infection, served as a control. Splenic biopsy was performed on day 31 p.i. Through a small incision in the upper left quarter of the abdomen, a small piece of splenic tissue was cut and dab smears were made on slides. The incision was stitched with nylon suturing thread. Following biopsy, an adequate amount of antibiotic powder (Neosporin; Burroughs Wellcome Ltd, India) was applied on the stitched region and finally sealed with Tincture of benzoin. In addition, Neosporin sulphate (100 mg/kg of body weight) was also given orally the day before and the day after the biopsy to assist healing. The smears were fixed in methanol and stained with Giemsa stain and the parasite burden expressed as number of amastigotes/1000 cell nuclei (Dube *et al*. 1998). The results were interpreted by comparison to the control group. The animals were kept for the assessment of efficacy of sodium stibogluconate and for their survival time.

The protocol as described for vitamin C was followed for assessing the effects of vitamin A, vitamin B complex, vitamin E and iron. The experimental animals were observed every week for gain or loss in body weight. Two to three replicates for each dose schedule were done with each nutrient supplement with their respective controls.

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Table 2. Composition of nutrient supplements

Nutrients	Source	Constituents per tablet/capsule
Iron	Fersolate®, Glaxo Smithkline Welcome, India	Ferrous iron (200 mg)
Vitamin C	Celin®, Glaxo Smithkline Welcome, India	Ascorbic acid (500 mg)
Vitamin A	Vitamin A Capsules®, USV limited, India	25 000 IU palmitate = Retinoic acetate (7·5 mg)
Vitamin E	EvionR, Merck, India	(α-Tocopherol (400 mg))
Vitamin B complex	Vitamin B complex®, Glaxo Smithkline Welcome, India	Vit.B1-IP-2 mg, B2-IP-2 mg, B6-IP-1 mg, B12-IP-1 mg, Nicotinamide- IP-20 mg, Panthothenate-IP-2·5 mg

(*2*) *Chemotherapeutic response in vitamin C-treated animals*. The grouping of animals, administration of vitamin C (50 mg/kg) and infection of animals were as described above. Half of the above-mentioned animals that received the vitamin prophylactically and were challenged on day 0, were administered sodium stibogluconate (SSG; 10 mg/kg × 5 days) intraperitoneally 2–3 days after the splenic biopsy. Simultaneously, drug treatment of non-vitamin C recipients (infected control) was made to allow comparison on day 7 post-treatment on autopsy (day 45 p.i.). Two replicates were done.

(*3*) *Combination therapy of nutrient supplements and SSG against established infection*. Hamsters were infected with 1×10^7 amastigotes and checked for infection on days 24–30 p.i. by spleen biopsy. Animals with confirmed infections were randomly divided into 4 groups each containing 10 animals. On day 30–35 p.i. the animals of group 1 were administered with SSG 10 mg/kg for 5 days; those of group 2 were given vitamin C (50 mg/kg) for 12 consecutive days; and the animals of group 3 received both SSG and vitamin C simultaneously in the dose schedule stated above. The animals of group 4 served as an infected untreated control. On day 42 p.i. the animals were autopsied to assess the effect of the therapeutic schedules.

A similar protocol was followed for vitamin A, B complex and E and iron. Comparisons were made between all the experimental groups.

Statistical analysis

Results were expressed as mean (S.D.). Parasite burdens for different nutrients were considered separately. Comparisons among the experimental groups were done with a One-Way Anova test using Sigma stat 2.0 software programs. The upper level of significance was chosen as $P < 0{\cdot}001$.

RESULTS

Prophylactic and continued effects of nutrient supplements

The prophylactic administration of vitamin C at 250, 100 and 50 mg/kg for 15 days made the animals relatively resistant to subsequent *Leishmania* infection, since there was significantly less parasite establishment compared to untreated controls (Fig. 1A–D). When compared to the untreated control, vitamin C-treated animals (prophylactic) showed an 85·54% ± 7·8 reduction in parasitic burden at a dose level of 250 mg/kg (Fig. 1A), 80·7% ± 4·6 at 100 mg/kg (Fig. 1B) and 80·43% ± 2·69 at 50 mg/kg (Fig. 1C). The lower dose of 25 mg/kg of vitamin C had no impact on the level of parasite burden (Fig. 1D). However, when the administration of vitamin C was continued after the challenge infection, the parasite burdens in such animals were comparable to untreated infected controls.

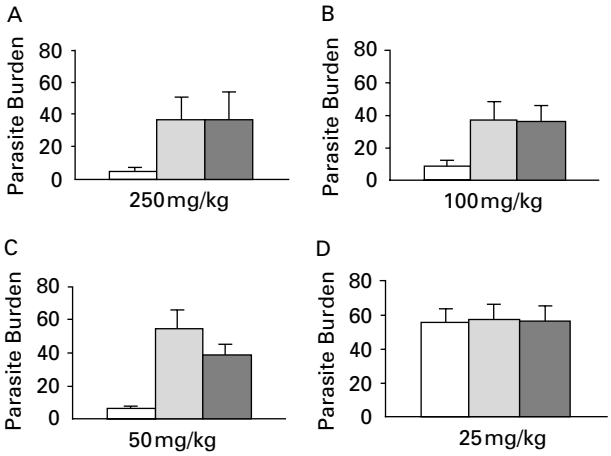

Fig. 1. Prophylactic and continued effect of vitamin C on *Leishmania donovani* infection in hamsters. □ Prophylactic (day 15 to day 0 p.i.). ▨ Continued (day 15 to day + 30 p.i.). ■ Control. Each bar in Fig. 1 A–D represents the pooled data with S.D. value of 2–3 replicates for each dose schedule. (A) 250 mg/kg (2 replicates); (B) 100 mg/kg (3 replicates); (C) 50 mg/kg (3 replicates); (D) 25 mg/kg (2 replicates).

In contrast, vitamins A, B complex and E and iron, whether given prophylactically or along with the infection, significantly assisted parasite multiplication. The prophylactic use of vitamins A and E enhanced the parasite burden to 254% and 314% of control infections, respectively, which was stastically significant ($P < 0{\cdot}05$), and when administration of vitamin E was continued there was significant difference between the prophylactic and continued groups ($P < 0{\cdot}05$) (Fig. 2A, B) as compared to

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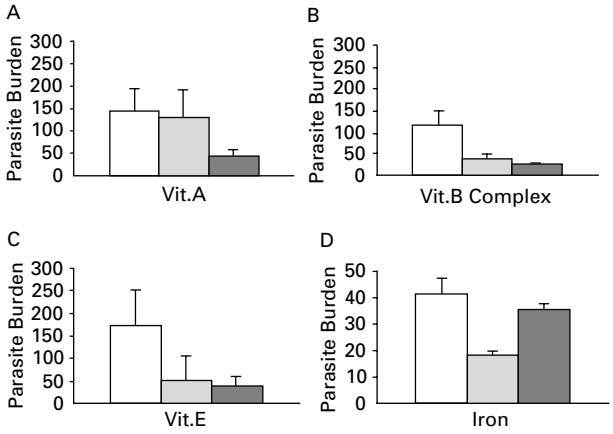

Fig. 2. Prophylactic and continued effect of vitamins A, B complex and E and iron on *Leishmania donovani* infection in hamsters. □ Prophylactic (day 15 to day 0 p.i.). ▨ Continued (day 15 to day +30 p.i.). ■ Control. Each bar in Fig. 2A–D represents the pooled data with S.D. value of 2–3 replicates for each vitamin. (A) Vitamin A (2 replicates); (B) vitamin B complex (3 replicates); (C) vitamin E (2 replicates); (D) iron (3 replicates).

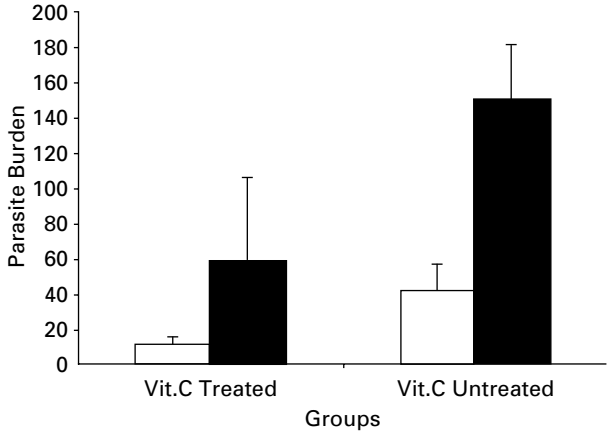

Fig. 3. Chemotherapeutic response of sodium stibogluconate (SSG) in vitamin C-treated hamsters infected with *Leishmania donovani*. □ SSG treated, ■ SSG untreated. Each bar represents the pooled data with S.D. value of 2 replicates.

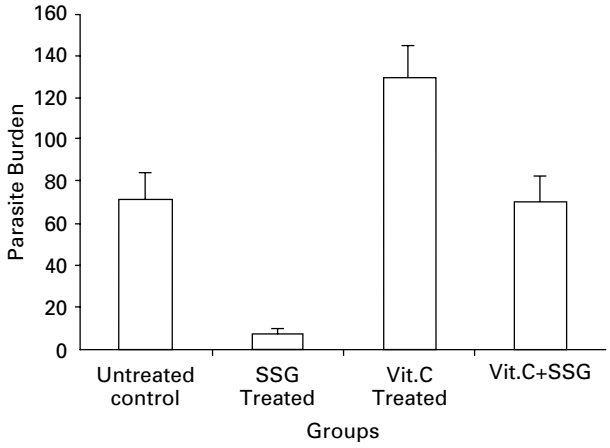

Fig. 4. Therapeutic efficacy of vitamin C + sodium stibogluconate (SSG) in established *Leishmania donovani* infection in hamsters. Each bar represents the pooled data with S.D. value of 2 replicates.

vitamin A-treated groups. Similarly, the prophylactic use of vitamin B complex and iron also significantly increased parasite multiplication ($P<0{\cdot}01$ and $P<0{\cdot}001$ respectively) (Fig. 2C, D). When the treatment with iron was continued a significant difference ($P<0{\cdot}01$) between the prophylactic and continued groups was observed.

A considerable increase in body weight was also observed in the animals given vitamin C prophylactically as compared to the groups treated with other nutrients. These animals also survived until the day the experiment was terminated i.e. on day 90 p.i., while the animals of the other experimental groups died between day 50 and day 60 p.i.

Chemotherapeutic response in vitamin C-treated animals

In vitamin C-treated animals the SSG therapy resulted in a $79{\cdot}9\% \pm 0{\cdot}8$ reduction in parasitic burden whereas in untreated animals the burden was reduced only by $64{\cdot}04\%$ (Fig. 3). Therefore, the chemotherapeutic effect of SSG in vitamin C-treated animals was improved by approximately 15%, which was a significant difference ($P<0{\cdot}05$).

Combination therapy of nutrient supplements and SSG against established infection

In hamsters with established *L. donovani* infections contrasting effects were observed when vitamin C was given at the same time as SSG, rather than prophylactically. The therapeutic efficacy of SSG given in combination with vitamin C was reduced by approximately 45% ($P<0{\cdot}05$) in comparison to animals, that were administered with SSG only (Fig. 4). In comparison to the untreated control group SSG-treated, vitamin C-treated and vitamin C + SSG-treated groups exhibited $81{\cdot}75\% \pm 0{\cdot}08$, $7{\cdot}46\% \pm 5{\cdot}34$ and $37{\cdot}11\% \pm 9{\cdot}91$ reduction in parasite burden respectively.

All the other nutrients (vitamins A, B complex and E, as well as iron) also resulted in suppressed efficacy of SSG (data not shown).

DISCUSSION

In visceral leishmaniasis, malnutrition contributes greatly to the risk of developing severe disease, adversely affecting the clinical picture and resulting in increased morbidity and mortality (Cerf *et al*. 1987). The symptoms include hepato-splenomegaly, anaemia, leucopoenia, and lymphadenopathy together with marked immunosuppression. Accordingly, in such cases the conventional therapeutic schedules fail

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to generate the desired results. It is well documented that the immune system can synergistically aid the therapeutic efficacy of drug treatment (Doenhoff *et al*. 1991). As such the efficacy of drugs could be considerably improved if they are used in combination with immunostimulants (Haidaris & Bonventre, 1983; Adinolfi *et al*. 1985; Pal *et al*. 1991; Zehra *et al*. 1995; Sharma *et al*. 1996) supplemented with treatment of symptoms. Disease manifestation and clinical success in visceral leishmaniasis depend greatly on the immunocompetence of the host (Doenhoff *et al*. 1991). Generally, infection with *L. donovani* is associated with defective cellular responses and a non-protective humoral response (Rezai *et al*. 1978; Halder *et al*. 1983; Ho *et al*. 1983; Ghose *et al*. 1979; Wyler, Weinbaum & Herrod, 1979; Carvalho *et al*. 1985*a*; Cillari *et al*. 1988, 1991). These altered immune responses are restored after successful chemotherapy (Halder *et al*. 1983; Cillari *et al*. 1988; Wyler, 1982; Carvalho *et al*. 1985*b*). Humoral immunity in leishmaniasis is non-protective, despite a significant increase of specific anti-leishmanial antibodies (Howard & Liew, 1984). It is, therefore, evident that protection against *L. donovani* infection, and response to treatment depend mainly on cellular immunity.

The relationship between nutrition and immune status in preventing infections is well established (Geber, Lefkowitz & Hung, 1975; Glasziou & Mackerras, 1993). Vitamins A, B complex (particularly B6, B12), C and E, and iron are essential nutrients that affect the immune response (Calder, 2001). Providing these nutrients to deficient individuals restores immune function and improves resistance to infection. Thus, appropriate nutrition is required in order for the host to maintain adequate immune defences towards bacteria, fungi, viruses, parasites and tumour cells (Calder, 2001).

In the present study it was observed that prophylactic administration of vitamin C caused significant suppression of parasite numbers, which is probably attributable to the enhancement of non-specific immune responses (Leibovitz & Siegel, 1978; Thomas & Holt, 1978; Anderson, 1981). A significant prophylactic activity of vitamin C was observed at 50 mg/kg for 15 days (day15 to day 0). However, enhancement of the dose failed to yield an additive effect. It has been reported that a high intake of vitamin C does not bring additional gains in combating infections (Walker, Bynoe & Tyrrell, 1967). In contrast, the continued administration of vitamin C after infection had no beneficial effect, rather it promoted parasite multiplication. *In vitro* studies have suggested that certain trypanosomatids, including *Leishmania* species, require ascorbic acid for their healthy growth and prolific multiplication (Chatterjee, 1967). The findings reported here endorse this claim.

Other nutrients used here also promoted parasite proliferation, whether given prophylactically or during the infection, endorsing the findings of Tait & Sacks (1988), Bhaskaran (1988) and Oppenheimer (1989). They observed that vitamin B and iron favours the faster multiplication of malaria. However, most research shows that while vitamin A supplementation helps people prevent or treat infections in developing countries where deficiencies are common (Glasziou & Mackerras, 1989), little or no positive effect, and even slight adverse effects, have resulted from giving vitamin A supplements to people in countries where most people consume adequate amounts of vitamin A (Pinnock, Douglas & Badcock, 1986; Murphy *et al*. 1992; Kjolhede, Chew & Gadomski, 1995; Bresee *et al*. 1996; Quinlan & Hayani, 1996; Stephensen, Franchi & Haernandez, 1998; Fawzi, Mbise & Spiegelman, 2000). Moreover, vitamin A supplementation during infections appears beneficial only in certain diseases (Ross, 1998). The effect of vitamin A on cell-mediated responses *in vitro* was studied in T cells derived from *Leishmania major* infections of mice (Frankenberg, Wang & Milner, 1998). Using lymph node cells and a T-cell line developed from infected susceptible and resistant mice it was observed that when added to cell cultures *in vitro*, vitamin A inhibits only the secretion of type 1 and not type 2 cytokines, possibly through an inhibitory effect on protein kinase C activity. This observation may explain our findings with vitamin A. Evidence from animal and human studies indicates that vitamin E plays an important role in the maintenance of the immune system. Even a marginal vitamin E deficiency impairs the immune response, while supplementation with higher than recommended dietary levels of vitamin E enhances humoral and cell-mediated immunity (Beharka *et al*. 1997). Supplementation with vitamin E during viral infections has been shown to increase T cell immune activity (Han, Wu & Ha, 2000) and reduce virus activity (Hayek, Taylor & Bender, 1997) in mice. This observation is in contrast to our findings.

The chemotherapeutic effect of a drug is inversely proportional to parasite numbers (Misra, Katiyar & Sen, 1980) and immunity of the host synergistically adds to the drug action (Katiyar, Misra & Visen, 1986; Pal *et al*. 1991). The reduced number of parasites and increased immune status in prophylactically vitamin C-treated animals would explain better the therapeutic efficacy of SSG. This would also explain the suppressed efficacy of SSG in vitamin A, B complex and C and iron-treated animals in which the parasite burden was either comparable or higher than the untreated infected controls. How these nutrients really interact with *Leishmania* infection remains is to be investigated at both biochemical and immunological levels.

The aim of supplementation was to increase the cell-mediated immune response, which is instrumental in combating pathogens. Nutritional immune enhancement will not only lessen the severity of

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infections, but it will buy valuable time until success can be achieved with an appropriate anti-leishmanial drug or vaccine. Our results suggest that prophylactic use of vitamin C may help in preventing *Leishmania* infection before progressing to an endemic area. However, the negative effects of the other nutrients cited above would certainly evoke a review of the practice of supplementing drug therapy for those resident in endemic areas. This would apply particularly to vitamin C, B complex and iron, especially in children and pregnant women, and suggests that modifications in therapeutic schedules should be investigated and considered.

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