Modulation of Nonspecific Mechanisms of Defense by Lactic Acid Bacteria: Effective Dose

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ABSTRACT

The purpose of this study was to determine the effect of a fermented milk product containing *Lactobacillus johnsonii* La1 (formerly known as *Lactobacillus acidophilus* La1) on the phagocytic activity of peripheral blood leukocytes in healthy adult volunteers. Furthermore, we sought to define the effective doses of the bacteria, examine the effect on respiratory burst activity, and, finally, examine the contribution made by the starter culture to the biological effects. Volunteers were randomly distributed among three groups; each subject received one pot (150 ml) of fermented milk each day for 3 wk. The first two groups received a freshly prepared product fermented by *Streptococcus thermophilus* (group A) alone or *S. thermophilus* and 10^7 cfu/ml *L. johnsonii* La1 (group B). Group C received a product stored for a period of 21 to 28 d and that contained *S. thermophilus* and 10^6 cfu/ml of *L. johnsonii* La1.

Ingestion of *L. johnsonii* La1 did not significantly increase fecal lactobacilli counts. However, *L. johnsonii* La1 was able to survive intestinal transit and was only recovered from the feces of the volunteers of groups B and C. The fermented base alone showed a weak effect on respiratory burst but not on phagocytic activity. However, the product containing 10^7 cfu/ml *L. johnsonii* La1 significantly enhanced both functions. The product containing 10^6 cfu/ml of *L. johnsonii* La1 had no significant effect on either function. These results suggest that fecal persistence may not necessarily reflect in vivo colonization and may not be a prerequisite for all forms of immune reactivity.
(**Key words**: lactic acid bacteria, dose response, phagocytosis, respiratory burst)

Abbreviation key: **LAB** = lactic acid bacteria.

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INTRODUCTION

Antagonism against intestinal pathogens, the barrier effect of the microflora, and modulation of the host's immune function are among some of the beneficial effects that probiotic bacteria provide to the host (1, 10). Our initial hypothesis proposed that bacterial strains adherent to the intestinal surface would perform such functions more effectively than would nonadherent organisms. For this reason, the avidity of microorganisms for the apical membrane of the intestinal epithelium has been a major criterion for strain selection.

Lactobacillus johnsonii La1 (formerly known as *Lactobacillus acidophilus* La1) is highly adherent to the microvillus membrane of human enterocytes in vitro and inhibits adhesion and invasion of Caco-2 human epithelial cells by a variety of diarrhoeaggenic bacteria (1). We have previously shown (10) that administration of a milk product fermented by *L. johnsonii* La1 enhanced the phagocytic activity of peripheral blood leukocytes in healthy volunteers. This physiological change took place without any detectable modification in the white cell differential count or lymphocyte subsets. Another study (7) has shown that *L. johnsonii* La1 administration during a program of oral vaccination with attenuated *Salmonella typhi* Ty21a enhances the specific humoral immune response against the vaccine. To our knowledge, these studies are among the first to show a direct relationship between ingestion of lactic acid bacteria (**LAB**) and modulation of the immune response in the human host.

The biological effects in our previous studies were attained with a daily consumption of 10^{10} cfu of *L. johnsonii* La1. The aims of the present study were (1) to determine the precise doses required to modulate phagocytosis, (2) to examine whether respiratory burst activity is similarly modulated, and (3) to discriminate between the specific effect of *L. johnsonii* La1 plus starter and that of the starter culture alone.

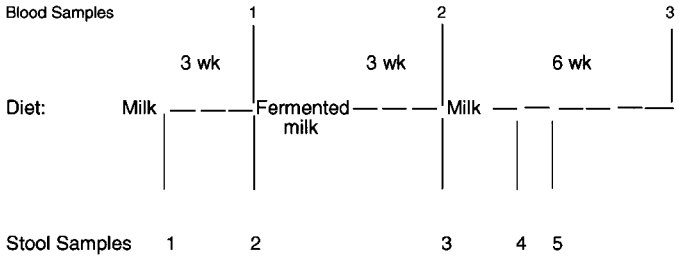

Figure 1. Experimental design.

MATERIALS AND METHODS

Volunteers and Experimental Design

Healthy adult volunteers (27 females and 15 males) from 21 to 57 yr of age were randomly distributed according to age, sex, and fecal lactobacilli counts into 3 groups, which, in addition to their normal diets, received different types of fermented products. Volunteers receiving antibiotics or suffering from metabolic or intestinal disorders were excluded from the study. Participants abstained from eating any fermented dairy product beyond those administered during the study.

Three products were used: a product fermented by the starter culture (*S. thermophilus*), a product fermented by the starter culture and 10^7 cfu/ml of *L. johnsonii* La1, and, finally, a product fermented by the starter culture and *L. johnsonii* La1 but containing a final count of 10^6 cfu/ml of *L. johnsonii* La1. All products were given orally, and the subsequent effects on phagocytic and respiratory burst activity were measured.

For the first 3 wk of the study, all groups received 150 ml of milk. Thereafter, they received one of the following for a second 3 wk: 150 ml of fermented milk base (control, no La1) (group A); 150 ml of milk freshly fermented with the starter culture and La1 (10^7 cfu/ml) (group B), or 150 ml of milk fermented with the starter culture and La1 but stored for 21 to 28 d to yield 10^6 cfu/ml La1 (group C).

In the final 6 wk of the study, all groups again received milk but no fermented products (Figure 1). This study was double blind and placebo-controlled, for which each volunteer served as his or her own control (10).

Preparation of Fermented Milks

The products used in this study were obtained through fermentation of pasteurized skimmed milk with *S. thermophilus* with or without *L. johnsonii* La1 at an incubation temperature typical for thermophilic bacteria (37 to 42°C) (Nestlé R&D Centre, Orbe, Switzerland and Vallette Factory, France). New batches consisting of seven pots per volunteer were produced on a weekly basis, and each batch was analyzed for viable bacterial counts according to the methodology described in Fecal Colonization by LAB. The final pH of the product was 4.29 (^{0}Dornic 106.74). Comparison of two fresh products containing different concentrations of *L. johnsonii* La1 would have been preferable. However, to minimize adaption of technological procedures at the factory level, the two products containing *L. johnsonii* La1 were inoculated with the same dose of bacteria, and the dose effect of viable organisms was ascertained through a comparison between a fresh and an aged product (21 to 28 d after manufacture) obtained during factory trials. The fermented products were distributed in 150-ml pots, and a code was attributed to each product.

Phagocytic Activity of Peripheral Blood Cells

Leukocyte phagocytosis in human whole blood was performed using opsonized, fluorescein isothiocyanate-labeled *Escherichia coli* (PHAGO-TEST™; Becton Dickinson, Basel, Switzerland) and flow cytometry. Briefly, 100 μl of fresh heparinized blood were mixed with 20 μl of the bacterial suspension (1×10^9/ml) such that the ratio of bacteria to leukocytes was approximately 20:1 (vol/vol). This mixture was then incubated in a water bath with controlled shaking for 10 min at 37°C. After quenching of extracellular bacteria, the blood was lysed and fixed, and the DNA of the leukocytes was stained using a propidium iodide solution. Measurements of phagocytic activity were with a FACScan™ flow cytometer using a blue-green excitation light (488 nm) and LYSYS II™ software (Becton-Dickinson). A live gate was set in the red fluorescence histogram such that the only events examined were those positive for propidium iodide staining and, thus, human diploid cells. In analysis of the data, the overall phagocytic activity and that of individual cell populations (granulocytes and monocytes) were considered. The median fluorescence intensity, which correlates to the number of bacteria per single leukocyte, was also examined.

Free Radical Generation by Peripheral Blood Cells

Leukocyte oxidative burst in heparinized whole blood was quantitatively determined using flow

cytometry and unlabeled, opsonized *E. coli* (Bursttest™; Becton Dickinson) Dihydrorhodamine 1-2-3, a nonfluorescent substrate, which becomes oxidized by cellular enzymes and superoxide ion to a fluorescent product, binds to the mitochondria of the phagocyte. The fluorescence emitted gives a measure of respiratory burst activity. Acquisition and analysis of data were performed as described previously for phagocytosis.

Fecal Colonization by LAB

Fecal specimens were taken at various time points during the study: at the beginning of the study, one day before and after the period of fermented milk intake, and d 7 and d 14 after product consumption (Figure 1). Freshly passed fecal samples were analyzed within 30 min for the total lactobacilli and for *L. johnsonii* La1.

The lactobacilli were counted on MRS agar (Difco, Detroit, MI) with antibiotics (0.8 mg/ml of phosphomycine, 0.93 mg/ml of sulfamethoxazole, and 50 μg/ml of trimethoprim). The plates were incubated anaerobically (Anaerocult A; Merck, Darmstadt, Germany) for 48 h at 37°C, and the different types of colonies were counted and isolated. The lactobacilli were identified by cell morphology, and the carbohydrate profile was determined by API 50CHL (Bio Mériuex, Marcy L'Etoile, France). The total lactobacilli and, more specifically, the *L. johnsonii* La1 colonies were assessed. Lactobacilli initially identified as *L. johnsonii* La1 were then determined to be *L. johnsonii* by dot blot hybridization with the 23S RNA probe ATAATATATGCATCCACAG (8).

Statistical Analysis

The data were analyzed using ANOVA on differences (end-beginning of treatment), followed by least significant difference multicomparison procedures. Within groups, these differences were tested by paired *t* tests.

RESULTS

The global phagocytic capacity of peripheral blood cells is due almost entirely to the granulocyte population. These cells constitute the most important cellular fraction (50 to 60%) of blood leukocytes (50 to 55% in the present study), and their individual activity is higher than that of monocytes, which represent between 2 to 10% of the leukocytes present (5 to 6% in the present study). Lymphocytes are nonphagocytic cells and were considered as negative internal standards in the system.

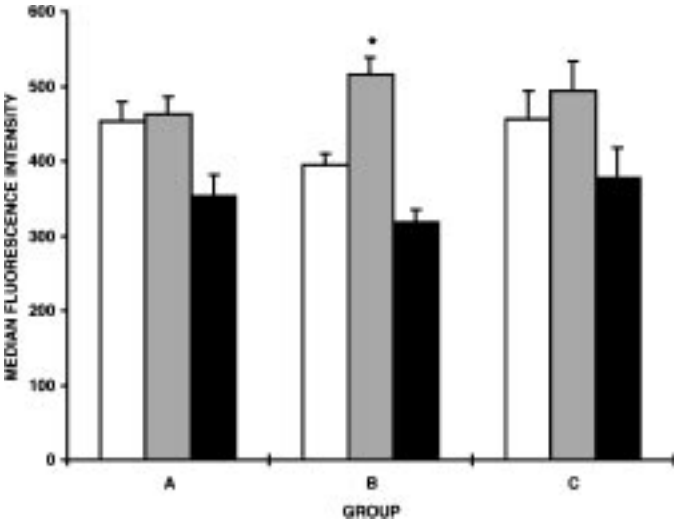

Figure 2. Median fluorescence values for phagocytic activity in the peripheral blood of volunteers receiving 0 (A), 10^9 (B), or 10^8 cfu (C) of *Lactobacillus johnsonii* La1/d. Median fluorescence values (± SEM) are shown for blood sample 1, at end of the equilibration period (□); blood sample 2 (grey shading), immediately after ingestion of the different products; and blood sample 3 (■) 6 wk after the end of fermented milk consumption. *$P < 0.001$ for the increment in activity between blood sample 1 and blood sample 2.

Although the volunteers were randomly distributed into the three groups, the basal phagocytic activity differed among groups. Nevertheless, after ingestion of the various products, there was an increase in overall phagocytic activity in group B, which was mainly due to the changes in the granulocyte activity because monocytes were less affected by the various diets (data not shown).

The group receiving the fresh product containing *L. johnsonii* La1 showed the most significant increase in the median fluorescence intensity of phagocytes (Figure 2) between time point 1, the end of the equilibration period, and time point 2, immediately after the ingestion of the fermented products ($P < 0.001$) compared with the control. The increment in activity for the control group A (*S. thermophilus* alone) and for group C receiving 10^6 cfu/ml of *L. johnsonii* La1 was not statistically significant. Furthermore, the ANOVA revealed that the increment in phagocytosis observed in group B was significantly higher than that for groups A and C ($P = 0.019$, LSD = 78). Six weeks after consumption of the fermented products had ended, the median fluorescence intensity decreased below pretreatment values for all groups.

Expressing data as the percentage increase in fluorescence intensity between time points 1 and 2

TABLE 1. The effect of different doses of viable *Lactobacillus johnsonii* La1 (La1) on fecal lactobacillus counts and La1 counts [colony-forming units per gram (log_{10}) of feces].[1]

Group	Daily intake of La1	Fecal lactobacilli: Before product intake (Stool sample 2)		Fecal lactobacilli: After product intake (Stool sample 3)		Fecal La1 in positive volunteers: Before product intake (Stool sample 2)	Fecal La1 in positive volunteers: After product intake (Stool sample 3)	
	[cfu (log_{10})]	(cfu/g)						
		$\bar{X}$	SD	$\bar{X}$	SD		$\bar{X}$	SD
A	0	4.60	1.06	4.27	1.15	<3.30	<3.30	
B	9.26	4.55	0.87	5.13	1.52	<3.30	4.82	1.18
C	8.10	4.46	1.00	4.43	1.06	<3.30	4.27	0.91

[1]Volunteers received 0 (A), 10^9 (B), or 10^8 cfu (C) of La1 daily. Counts are expressed as the mean ± standard deviation. The detection limit in the feces was 3.30 log_{10}.

ignores the disparity between groups at the beginning of the study (Figure 3). All groups showed an increase in phagocytosis, which only reached statistical significance for group B. Furthermore, this change was significantly higher than that observed in group A. Although the lower dose of *L. johnsonii* La1 also increased phagocytic activity, statistical significance was not reached.

To study respiratory burst activity of blood leukocytes, cells were preactivated with opsonized *E. coli* in a similar manner to that of the PHAGOTEST™. Differences in free radical production were thus expected to reflect differences seen in phagocytic activity, which was indeed the case. The group receiving 10^7 cfu/ml of *L. johnsonii* La1 showed a significant increment at time point 2 for the median fluorescence intensity of phagocytes (Figure 4, $P < 0.001$), and groups A and C showed a similar trend, which did not reach statistical significance. As seen for phagocytic activity, respiratory burst activity did not return to pretest values by time point 3 but were inferior to the original values.

When viable counts of total lactobacilli were assessed in the fecal samples of volunteers, group B showed a slight increment in total lactobacilli (0.58 log_{10}), which was not significant (Table 1). When the presence of *L. johnsonii* was investigated in the stool samples immediately after the period of fermented milk consumption (Table 1), only volunteers in groups B and C had detectable levels (zero for the control group A). Seven days after consumption had ended, *L. johnsonii* was no longer detected in the feces of these volunteers. Using our probe, none of the volunteers were positive for *L. johnsonii* in their feces before product consumption (<3.30 log_{10}). Only volunteers receiving *L. johnsonii* La1 had detectable levels after product consumption (Table 1).

DISCUSSION

As criteria for strain selection become more rigorous, certain strains of LAB are gaining credence as probiotic, and as such, functional components of food. Reported effects include immunomodulation and adjuvant activity after oral administration (7, 10) and adherence to human intestinal cells and exclusion of pathogens (1), as well as nonspecific resistance of the host to infections and tumors (2, 5). We have previously reported that a fermented product containing *L. johnsonii* La1 and a starter

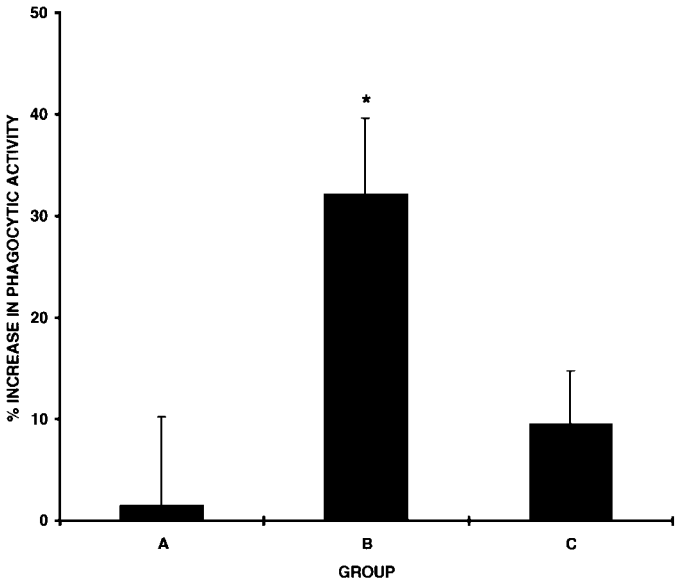

Figure 3. Percentage increase in phagocyte median fluorescence intensity between blood sample 1, at the end of the equilibration period, and blood sample 2 after 3 wk of fermented milk consumption. Volunteers received 0 (A), 10^9 (B), or 10^8 cfu (C) of *Lactobacillus johnsonii* La1 daily.

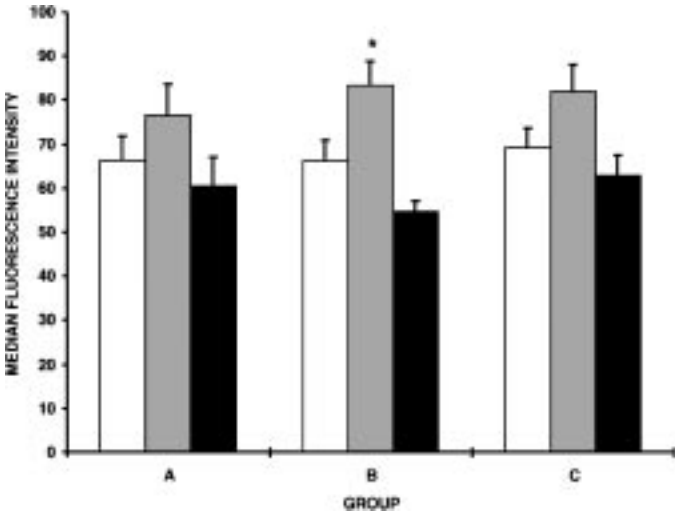

Figure 4. Median fluorescence values for the respiratory burst activity of phagocytes in the peripheral blood of volunteers receiving 0 (A), 10^9 (B), or 10^8 cfu (C) of *Lactobacillus johnsonii* La1 daily. Median fluorescence values (± SEM) are shown for blood sample 1 (□), at the end of the equilibration period; blood sample 2 (grey box), immediately after ingestion of the different products; and blood sample 3 (■), 6 wk after the end of fermented milk consumption. $*P < 0.001$.

culture was able to increase the phagocytic capacity of peripheral blood human leukocytes (10). A single daily dose (10^{10} cfu) of *L. johnsonii* La1 was tested in these experiments. However, the use of such bacteria as probiotics warrants an examination of effective doses. We are particularly interested in the effect of probiotics in fermented milk products. Because *L. johnsonii* La1 is used with *S. thermophilus* in the manufacture of such products, effective doses of *L. johnsonii* La1 were examined in milks fermented by the two bacterial species. The present study also considered the possiblity that the starter culture containing *S. thermophilus* alone, irrespective of its properties of adherence and survival, may contribute to the biological effects.

We observed that the increment in phagocytic activity immediately after the 3 wk ingestion period was statistically significant only for the group receiving 10^9 cfu of *L. johnsonii* La1/d. Respiratory burst activity was similarly affected. The group receiving 10^8 cfu *L. johnsonii* La1/d showed a similar trend, which was not, however, significant. Interestingly, the control group fermented with starter culture alone also showed a weak trend but only for burst activity. Nevertheless, this observation is in agreement with previous reports that showed that *Streptococcus* spp. were able to induce cytokine production by human blood cells (11, 12). In the present study, the *L. johnsonii* La1 clearly intensified the effect of the starter in a dose-dependent manner.

It has been postulated that the ability to adhere to intestinal cells in vitro may constitute better colonization in vivo. On this basis, our initial hypothesis proposed that *L. johnsonii* La1 could trigger stronger responses in the host than in less adherent strains (1). Certainly, many authors have suggested that survival in the feces following oral administration reflects large bowel colonization and is required for biological effects. It has also been suggested that the target for immunological modulation does not necessarily reside in the colon but may be a structure of the proximal small bowel such as the Peyer's patches of the gut-associated lymphoid tissue (GALT) (13, 14). It is also possible that there are other relevant sites. We have previously reported that colonization of germ-free mice with LAB induced an expansion of the intraepithelial lymphocyte population only in the proximal small bowel (6). Upon activation, this cellular compartment is able to produce interferon-γ and interleukin-2 (3, 4). If the target for immunomodulation does indeed reside in the small bowel, it follows that colonic colonization and the consequent persistence in the feces may not be strictly necessary to modulate immune reactivity.

Viable *L. johnsonii* La1 in the feces of our volunteers confirms the ability of *L. johnsonii* La1 to survive intestinal transit. Only volunteers in groups B and C had detectable levels of *L. johnsonii* La1 in their feces immediately after consumption of the test products had ended. In our previous study, feeding a daily dose of 10^{10} cfu resulted in a 1.22 $\log_{10}$ increase in total lactobacilli in the feces. Here, the fresh product fermented with the *L. johnsonii* La1 contained one log less of *L. johnsonii* La1. In this case, the increment in lactobacilli counts did not reach statistical significance. It is possible that there are different threshold doses for fecal colonization effects and immune reactivity. Probiotics, which are recovered at high levels in the feces, have been considered to provide a good barrier against pathogens in vivo. Passage of viable bacteria into the feces seems to be a reasonable criterion when selecting strains against intestinal infectious agents. However, our results show that for at least certain forms of immune reactivity, a high recovery in the feces is not a prerequisite. Indeed, in our previous study, levels of phagocytic acitivity were still significantly greater than were pretreatment values 4 wk after viable bacteria were no longer detected in the feces.

It is possible that the metabolites of *L. johnsonii* La1 may also contribute to the biological effects ob-

served, but we have not specifically addressed this point at the present time. However, our results suggest that the minimum daily dose of viable *L. johnsonii* La1, which will significantly modulate phagocytosis and respiratory burst activity, is 10^9 bacteria.

With consideration of the already high percentage of cells participating in both phagocytosis and free radical production at the beginning of the study, it is not suprising perhaps that the differences obtained in the present study were more subtle than were those previously reported (10). This difference may also explain our inability to sustain an effect on individual phagocytes after the test period. Unlike our previous study, the majority of phagocytes were already active. The number of cells participating in both phagocytosis and free radical production was high throughout the study and was unaffected by diet, the reason for which is unclear but may simply reflect the activity of individual batches of bacteria in the test kits or perhaps different sensitivities of the flow cytometer between studies. However, products containing the *L. johnsonii* La1 strain are now commercially available. Because the majority of our volunteers admit previous consumption of such products, the initially high levels of phagocytic activity we witnessed in this study might have reflected recent stimulation by the same bacterial strain. It may also explain why the levels of biological activity are lower at time point 3 than at time point 1, because the former reflects activity after a 6 wk abstention from fermented products, and the latter is after only a 3 wk abstention period.

In summary, these results confirm our previous study and show that oral daily administration of LAB in one pot of fermented milk over a period of 3 wk, modulates nonspecific mechanisms of defense. Furthermore, the threshold dose for immunomodulatory activity was determined. Since persistence in the feces was not a prerequisite for changes in immune reactivity, we speculate that different probiotic activities of LAB are taking place in different intestinal microenvironments but that the microflora barrier effect is probably active throughout the gastrointestinal tract. Certainly, pathogens find their niches in the stomach, the small bowel and the colon and in vitro and in vivo studies have shown that *L. johnsonii* La1 is capable of antagonizing pathogens at each of these intestinal environments. Finally, our results suggest that the minimum effective dose for certain forms of immune reactivity, is 10^9 cfu daily.

CONCLUSIONS

Ingestion of *L. johnsonii* La1 did not cause a signficant increase in fecal counts of lactobacilli even at a daily dose of 10^9 cfu. However, the strain survived intestinal transit and was recovered in the feces of volunteers who had ingested it. The milk fermented with *S. thermophilus* alone showed a weak effect on respiratory burst activity but not on phagocytic activity. However, both activities were significantly enhanced by the product fermented by *S. thermophilus* and 10^9 cfu La1. The product fermented by *S. thermophilus* and the lower daily dose of 10^8 cfu of *L. johnsonii* La1 had no significant effect on either function. In summary, a minimum daily dose of 10^9 *L. johnsonii* La1 is required to modulate certain forms of nonspecific, anti-infective mechanisms of defence. Fecal colonization may not be a prerequisite for this form of immune reactivity.

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