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In Vitro and In Vivo Effects of Exogenous Nucleotides on the Proliferation and Maturation of Intestinal Epithelial Cells

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Summary To determine the nutritional role of nucleotides, the in vitro and in vivo effects of exogenous nucleotides on the development of intestine were investigated. First, the in vitro effects of nucleotides on the proliferation and maturation of enterocytes were studied by using a human colon tumor cell line (Caco-2) and a rat normal small intestinal crypt cell line (IEC-6). Second, the in vivo effects of nucleotides were also studied in early weaned rats fed nucleotide-unsupplemented or high-nucleotide-supplemented diet. Nucleotide composition resembled that of human milk (CMP : UMP : AMP : IMP : GMP = 10 : 1 : 1 : 1 : 1, in weight). Nucleotide supplement did not enhance Caco-2 cells proliferation; however, it significantly enhanced maltase and sucrase activities. In contrast, nucleotides supplement enhanced IEC-6 cells proliferation and maltase activity. CMP, predominantly contained in the mixture, enhanced most effectively the proliferation and maturation of cells. In the in vivo experiment, nucleotides significantly enhanced sucrase activity in the intestinal mucosa of early weaned rats. The results presented here suggest that a nucleotide supplement may enhance enterocyte proliferation and/or maturation in vivo and in vitro. Therefore exogenous nucleotides may play an important role in the development of the intestine.
Key Words Caco-2 cells, IEC-6 cells, nucleotides, intestinal development, early weaned rat

Human milk contains large amounts of nucleotide-monophosphates, in comparison with bovine milk (*1*). A previous analysis of nucleotide concentration showed that Japanese human milk contains five kinds of nucleotide-monophosphates (CMP, UMP, AMP, IMP, and GMP), in the proportion of 10 : 1 : 1 : 1 : 1 (CMP : UMP : AMP : IMP : GMP) (*1*). Furthermore, it is well known that nucleotides have various biological effects (*2, 3*). We have already reported that a supplement containing nucleotides in a proportion resembling that in human milk influenced

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the lipid metabolism of brain, and thereby it may contribute to the improvement of cerebral function in rats (*4*). Moreover, other investigators demonstrated that nucleotides and their metabolites stimulate polyunsaturated fatty acid and lipoprotein metabolism, modify the intestinal microflora, and enhance immune functions (*5–8*). Recently, therefore, interest has been increasing in the nutritional aspects of nucleotides, and infant formula has been supplemented with nucleotide-monophosphates.

Although intestinal epithelial cells can synthesize nucleotides from amino acid and other precursors, the synthesis may be limited and highly dependent on an exogenous supply of nucleotides for their proliferation and maturation (*9*). Nucleotides may therefore be conditionally essential nutrients for rapidly growing intestinal epithelium and lymphoid cells. The role of nucleotides and their metabolites on the proliferation and maturation of the intestine has been studied in previous investigations (*10–16*). Concerning the nutritional significance of nucleotides at cellular level, He et al demonstrated that nucleotide supplements may enhance the proliferation and differentiation of Caco-2 under conditions of nutritional stress, and of IEC-6 under nutritional stress conditions and also under normal culture conditions (*14*). Although their findings suggested that nucleotides may play a role in the proliferation and differentiation of enterocytes, their conclusions cannot be applied to the effects of human milk nucleotides because they used a nucleotide mixture with equal amounts of the five nucleotides (CMP, UMP, AMP, IMP, and GMP). Moreover, it is not known which of these nucleotides is most indispensable for cell proliferation and maturation. The first aim of this study was to investigate in vitro whether supplements containing nucleotides in a proportion resembling that in human milk would enhance the proliferation and maturation of epithelial cells and which nucleotides would be most effective. The second aim of this study was to investigate in vivo whether dietary nucleotide supplements would stimulate the development and maturation of the intestine in normal rats if administered during the weaning period. Some previous studies demonstrated that dietary nucleotides promote the repair of intestinal cells after injury, diarrhea or malnutrition in young rats, and after food deprivation in old rats (*9*, *12*, *13*, *15*, *16*). Uauy et al suggested that dietary nucleosides, added in equal amounts to the diet as stable precursors of nucleotides may improve the development and maturation of the intestine in weaning rats fed nucleotide-supplemented diet for 2 weeks (*11*). The effects of nucleotides on the development and maturation of the intestine immediately after weaning, however, have not yet been clarified.

Thus to investigate the effects of exogenous nucleotides on the development of the intestine, we undertook two experiments, one in vitro and the other in vivo. First, the effect of nucleotides mixed in the same proportion that they are found in human milk on the enhancement of cell density and enzyme activities, as the indices for enterocyte proliferation and maturation, were studied by using a human colon tumor cell line (Caco-2) and a normal rat small intestinal crypt cell line

(IEC-6). Second, the effects of these nucleotides on the intestinal mucosa weight, DNA, and protein contents and the mucosal enzyme activities of young rats as the indices of the development and maturation of the intestine were studied immediately after weaning.

MATERIALS AND METHODS

Chemicals and reagents. CMP, UMP, IMP, GMP, and AMP were purchased from Yamasa (Chiba, Japan). The nucleotide mixture was prepared in a proportion of 10 : 1 : 1 : 1 : 1 (= CMP : UMP : IMP : GMP : AMP) in weight. Dulbecco's modified Eagle medium (DMEM) and insulin were obtained from Sigma Chemicals (St. Louis, MO). Nonessential amino acids (NEAA) and fetal calf serum (FCS) were purchased from Dainihon Seiyaku (Osaka, Japan). HEPES, glutamine, and MITO were purchased from Cosmobio (Tokyo, Japan), Nissui (Tokyo, Japan), and Becton Dickinson Labware (Lincoln Park, NJ, USA), respectively. All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Culture of Caco-2 and IEC-6 cells. A Caco-2 cell line (ATCC No. HTB37), a human enterocyte carcinoma cell line, and IEC-6 (ATCC No. CRL1592), a rat normal intestinal epithelial cell line, were purchased from Dainihon Seiyaku (Osaka). Cells were routinely grown in DMEM containing 10 mM HEPES, 50 μg/mL transferrin, 10 μg/mL insulin, 0.6 mg/mL glutamine, 7% FCS, 10 μg/mL NEAA, and 3.7 mg/mL $NaHCO_3$, and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Determination of cell proliferation. Caco-2 and IEC-6 cells were cultured by using Matrigel™-precoated plates (Collaborative Research, Bedford, MA). The cells were seeded at a density of 1×10^4 cells/mL and cultured overnight in DMEM containing 10 mM HEPES, 50 μg/mL transferrin, 10 μg/mL insulin, 10 μg/mL NEAA, and 3.7 mg/mL $NaHCO_3$ with 0.6 mg/mL glutamine. Then, 1, 4, and 8 d after seeding, a fresh DMEM containing 10 mM HEPES, 50 μg/mL transferrin, 10 mg/mL NEAA, 1.0 mg/mL MITO, 0.6 mg/mL glutamine, 10 μg/mL insulin, and 3.7 mg/mL $NaHCO_3$ with and without nucleotides, was replaced.

The number of Caco-2 and IEC-6 cells was counted by using a hemocytometer 10 d after seeding. Cells were washed twice with 0.01 M phosphate buffer (pH 7.2, PBS) containing 0.15 M NaCl and trypsinized with 0.25% trypsin, 0.02% EDTA, and 0.9% NaCl in PBS. Fluorescence intensity was measured by using a fluorometer F-3000 (Hitachi, Tokyo) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm after incubation for 3 h at 37°C in medium containing 10% Aramar Blue™ reagents (BioSource International, USA).

Enzymatic assays. Caco-2 and IEC-6 cells were cultured as described above. Ten days after the seeding, the medium was removed by aspiration and the cells were washed three times with PBS. Then 0.1 mL maltose or sucrose (0.28 M), as the substrate, was directly added into the wells. The activities of maltase and sucrase were determined by measuring with a glucose test kit (Wako Pure Chemical

Table 1. Composition of the experimental diets.

Ingredient[a]	Diets (g/kg diet)	
	Control	1.0% Nucleotides
Casein	200	200
Cornstarch	585	575
Sucrose	30	30
Cellulose	80	80
Soybean oil	60	60
Mineral mixture[b]	35	35
Vitamin mixture[b]	10	10
Nucleotide mixture[c]	—	10

[a] Soybean oil was purchased from Ueda Oils and Fats Mfg. (Hyogo, Japan). Nucleotides were purchased from Yamasa (Chiba, Japan). All other ingredients were purchased from Oriental Yeast (Tokyo, Japan).
[b] AIN-76 mineral mixture and vitamin mixture contained 20% of choline bitartrate.
[c] The composition of the nucleotide mixture was shown in Materials and Methods.

Industries) the amount of glucose produced by the hydrolysis of maltose or sucrose. Enzyme activities were expressed as nmol/min/mg of protein. Protein content was measured by Lowry's method modified by Peterson (*17*) after the cells were harvested and solubilized by sodium dodecyl sulfate (SDS); bovine serum albumin (Sigma) was used as the standard.

Animals and diets. Male Sprague-Dawley rat pups (13 d old) were purchased from Japan Clea (Tokyo, Japan). They were reared in a litter and weaned when 17 d old. The rats were divided into two groups; nucleotide-unsupplemented (control) and nucleotide-supplemented. The composition of the experimental diets is shown in Table 1. The rats were housed in individual stainless steel cages at 23 ± 2°C in a temperature-controlled room with a 12 h light and dark cycle. Two or 3 d after being fed experimental diets, the rats were sacrificed and their organs rapidly weighed.

Preparation of intestinal mucosa. Intestinal mucosa was scraped with a glass slide on an ice-cold plate, then weighed and homogenized in 3 mL of cold saline. Its homogenates were filled up 10 mL, then frozen at -70°C until analysis. Protein content and sucrase and maltase activity were determined as described above. Similarly, lactase activity was determined by using lactose as the substrate and a glucose test-kit. Enzyme activity was expressed as nmol/min/mg of protein. DNA content was determined by the method of Schmidt and Thannhouser (*18*).

Statistical analysis. All results are presented as means$\pm$SD of three determinations. Statistical analysis was performed by Student's t test after analysis by ANOVA. Differences with a p value of <0.05 were considered statistically significant.

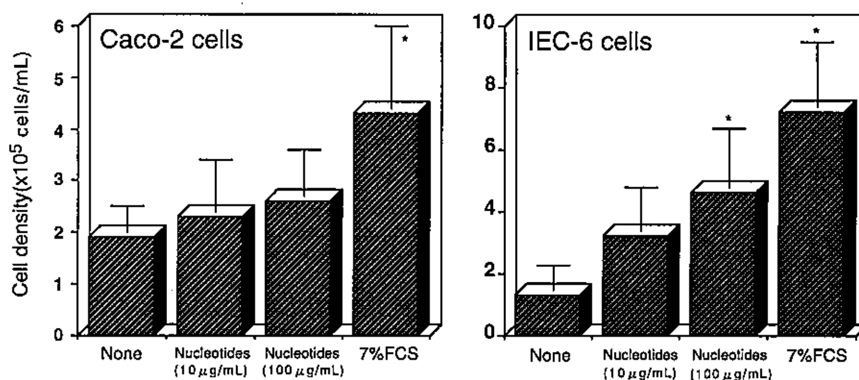

Fig. 1. Effect of exogenous nucleotides on the proliferation of Caco-2 and IEC-6 cells cultivated for 10 d. Values are means ± SD of three determinations. * Significantly different from the control culture medium without nucleotides, $p < 0.05$.

RESULTS

Effect of nucleotides on the proliferation of Caco-2 and IEC-6 cells

The effect of exogenous nucleotides on the proliferation of Caco-2 and IEC-6 cells is illustrated in Fig. 1. The supplement with nucleotides did not influence Caco-2 cell proliferation when cultured in medium supplemented with 0.6 mg/mL glutamine, but it resulted in a significant increase of IEC-6 cell proliferation. The proliferation of IEC-6 cells depended on the concentration of nucleotides in the culture medium.

Effect of nucleotides on the maturation of Caco-2 and IEC-6 cells

The maltase and sucrase activities of Caco-2 and IEC-6 cells cultured in media with different concentrations of nucleotides are presented in Table 2. In Caco-2 cells, the activities of maltase and sucrase were significantly higher when cultured in the presence of nucleotides. In IEC-6 cells, maltase activity was significantly higher when nucleotides were added to the medium. Sucrase activity in IEC-6 cells was not detected under the experimental conditions of this study. The two enzyme activities were much lower in IEC-6 cells than in Caco-2 cells.

Effect of each nucleotide on the proliferation and maturation of IEC-6 cells

The effect of each nucleotide on the proliferation and maturation of IEC-6 cells is shown in Figs. 2 and 3. The concentration of the nucleotide mixture in this study was 100 μg/mL and that of individual nucleotides was 70 μg/mL, because the concentration of CMP in the nucleotide mixture was about 70 μg/mL.

The nucleotide mixture and CMP promoted continuous proliferation (Fig. 2). Two days after addition of the nucleotide mixture, a significant increase of cell proliferation was observed compared with the nucleotide-unsupplement. IMP and

Table 2. Effect of exogenous nucleotides on enzyme activity of Caco-2 and IEC-6 cells cultivated for 10 d.

Supplement	Caco-2 cells		IEC-6 cells	
	Maltase (nmol/min/mg protein)	Sucrase (nmol/min/mg protein)	Maltase (nmol/min/mg protein)	Sucrase (nmol/min/mg protein)
None	48.8±10.1	1.6±1.4	0.3±0.1	<0.1
Nucleotides (10 μg/mL)	61.7±8.7*	6.4±1.3*	2.3±0.9*	<0.1
Nucleotides (100 μg/mL)	81.9±14.9*	9.8±1.8*	1.9±0.5*	<0.1
7% FCS	112.1±15.9*	18.5±4.7*	5.4±2.2*	1.8±0.4

Values are means±SD of three determinations. * Significantly different from the control culture medium without nucleotides, $p<0.05$.

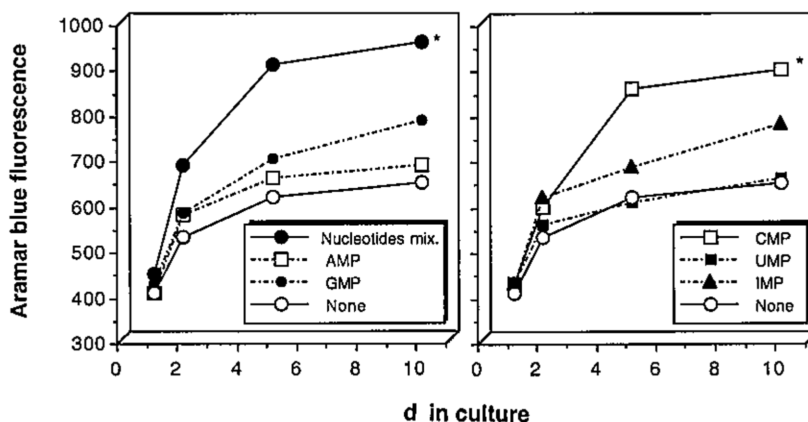

Fig. 2. Effect of individual nucleotides on the proliferation of IEC-6 cells. Values are means of three determinations. * Significantly different from the control culture medium without nucleotides, $p<0.05$. The nucleotide mixture was added at a concentration of 100 μg/mL of medium, and individual nucleotides were added at a concentration of 70 μg/mL of medium.

GMP also stimulated cell proliferation, but the increase was not significant. As illustrated in Fig. 3, the nucleotide mixture and CMP significantly stimulated cell maturation, as indicated by the increase in maltase activity; however, other nucleotides did not influence it.

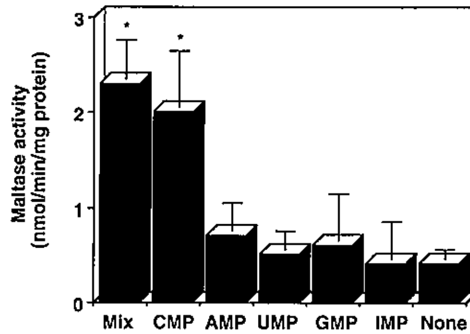

Fig. 3. Maltase activity in IEC-6 cells cultivated for 10 d. Each column represents means ± SD of three determinations. * Significantly different from the control culture medium without nucleotides, $p<0.05$. The nucleotides mixture was added at a concentration of 100 μg/mL of medium, and individual nucleotides were added at a concentration of 70 μg/mL of medium.

Table 3. Effects of nucleotides on body weight, organ weight, mucosal weight, protein, and DNA of early weaned rats.

		Control	1.0% Nucleotides
Initial body	2 d	40.5±1.2	40.8±3.2
weight (g)	3 d	41.1±3.1	41.3±3.6
Final body	2 d	42.7±1.2	42.1±1.7
weight (g)	3 d	48.9±2.8	48.6±3.5
Organ weight (g)			
Liver	2 d	1.86±0.22	1.74±0.09
	3 d	2.17±0.21	2.00±0.17
Small intestine	2 d	1.61±0.20	1.51±0.07
	3 d	2.01±0.33	1.79±0.17
Intestinal mucosa	2 d	0.81±0.08	0.72±0.13
(g)	3 d	0.86±0.15	0.88±0.09
Protein contents	2 d	11.94±1.19	13.46±1.48
(×10 mg/g mucosa)	3 d	13.34±3.09	12.46±1.71
DNA contents	2 d	5.96±0.78	7.03±0.91
(mg/g mucosa)	3 d	7.32±3.18	5.48±1.29

Values are means±SD, $n=6$.

ffects of dietary nucleotides on the development and maturation of intestine in early eaned rats

The effects of dietary nucleotides on the growth of early weaned rats are :esented in Table 3. No significant differences were evident with regard to body eight, nor in liver and small intestine weights between rats fed a 1% nucleotide diet

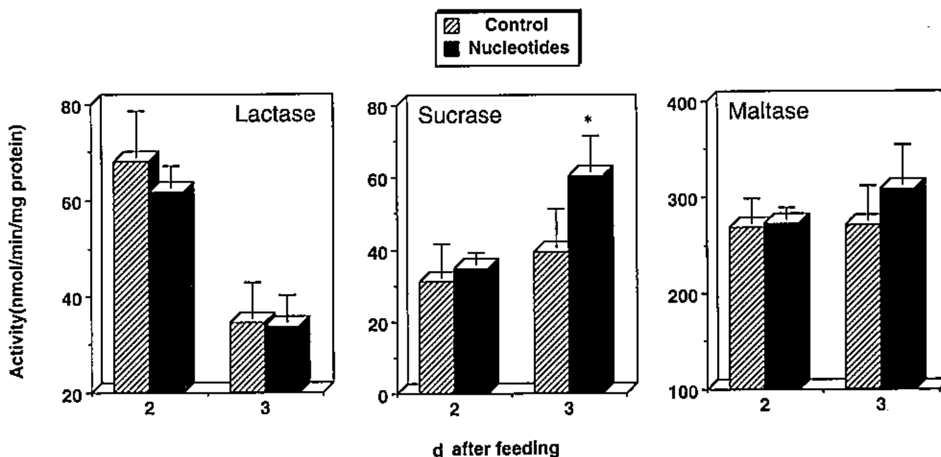

Fig. 4. Effect of dietary nucleotides on maturation of the intestine in early-weaned rats. Each column represents means ± SD, $n=6$. * Significantly different from the control, $p<0.05$.

and rats fed a control diet. Mucosal weight, protein, and DNA contents were also similar in the two groups. Sucrase activity in the nucleotide-fed group, however, was significantly higher after 3 d (Fig. 4). Maltase activity tended to be higher in the nucleotide-fed group, and lactase activity tended to be lower.

DISCUSSION

The small intestine has a limited capacity for de novo synthesis of nucleotides because of their rapid turnover rate, and it also requires a supply of exogenous nucleotides for DNA and RNA synthesis (*9*). Therefore nucleotides and their metabolites seem to be nutritionally essential for the intestine. So we utilized a normal rat small intestine crypt cell line (IEC-6) and a human carcinoma Caco-2 cell line to investigate the effects of nucleotides in vitro because these cells are frequently used as the model system to study proliferation and differentiation (*14*), physiology and pharmacology (*19*), and enteric bacterial pathogenesis (*20, 21*).

Our present results indicate that nucleotide supplement contributed to the proliferation of IEC-6 cells, but not of Caco-2 cells. Under glutamine-restricted conditions (0.1 mg/mL glutamine), nucleotides promoted Caco-2 cell proliferation (data not shown). Regarding cell maturation evaluated by measuring membrane enzyme markers, nucleotides supplement promoted cell maturation of both cells. He et al (*14*) have also shown that when glutamine is removed from the medium, exogenous nucleotides enhance the proliferation and differentiation of Caco-2 and IEC-6 cells. Furthermore, they have shown that nucleotide supplements under normal glutamine level do not enhance the proliferation and differentiation of

Caco-2 cells. Our results—that nucleotides did not influence Caco-2 cells proliferation under normal culture conditions and that they did promote cell proliferation under glutamine-depleted conditions—were consistent with their finding. Glutamine depletion from culture medium markedly inhibits DNA synthesis and cell proliferation because of the retardation in ATP production (*14, 22*). Therefore the level of glutamine in the medium seems to be important for cell proliferation and differentiation. Moreover, it has already been found that cancer cells characteristically have a greater capacity of de novo synthesis of nucleotides than normal cells do. Thus it is assumed that the level of glutamine used in this study was sufficient to activate a de novo pathway of Caco-2 cells as a source of purines and pyrimidine bases. In contrast to the results of He et al (*14*), we found that nucleotide supplement under normal culture conditions significantly enhanced membrane sucrase and maltase activities of Caco-2 cells. These reasons may reflect the differences of nucleotide proportion.

Japanese human milk contains five kinds of nucleotides and is characterized by CMP being the major nucleotide with a content of about 10-fold that of other nucleotides (*1*). Kubota (*23*) suggested the significance of human milk nucleotides on the basis of evidence that nucleotides orally administered are kept at a high concentration in newborn rats compared with mature rats. It was especially shown that CMP in comparison with others is kept longer in rats. However, the nutritional role of individual nucleotides has not yet been clarified. So we investigated the effects of individual nucleotides on proliferation and maturation by using IEC-6 cells because nucleotide mixture promoted cell proliferation and maturation alike. Our results proved that CMP had the strongest effect on cell proliferation and maturation, unlike the others, which exerted only a minimal effect. In this study, nucleotide concentration in medium was 10–100 μg/mL to provide the nutritional role of human milk nucleotides. The concentration of nucleotides in human milk ranges from 5 to 11 μg/mL (*1*), and that of free and enzymatically hydrolyzed nucleotides from RNA ranges from 21 to 104 μg/mL (82–402 nmol/mL) (*24*). Therefore the concentration of nucleotides in the present study seems to be physiological levels. In consideration that CMP is the most abundant in the nucleotide mixture and was found to have strongest effects in this study, it may be considered necessary for the development and maturation of intestine.

As mentioned above, our results showed that nucleotides enhanced cell proliferation and maturation in vitro. As illustrated in Fig. 2, the addition of 5 kinds of nucleotides promoted cell proliferation after culture for 2–5 d. In fact, it is known that the turnover of intestinal cells is rapid, with duration time of 2–3 d in most mammals and 3–5 d in humans (*25, 26*). It is possible that short feeding of nucleotides influences enterocyte proliferation and maturation in vivo. Some studies demonstrated that dietary nucleotides were beneficial for the development of the gastrointestinal tract during periods of recovery from diarrhea (*12, 15*), repair after intestinal injury, and sustenance under total parenteral nutrition (*13*). Furthermore, although dietary nucleotide supplements administered for 5 d had no effect on

intestinal brush-border enzymatic activities of old rats, refeeding them nucleotides for 3 d after food deprivation accelerated the physiological response of intestine (*16*). Regarding normal young rats (21 d old), Uauy et al (*11*) reported that the feeding of nucleosides, as a stable precursor of nucleotides, for 2 weeks may increase DNA and protein contents and the activity of maltase. However, the effect of nucleotides on intestinal development and maturation in rats immediately after weaning had not been investigated. Our results indicated that short feeding of dietary nucleotides also contributed to the intestinal maturation of normal young rats weaned at 17 d. These results supported that a supply of nucleotides may be beneficial to an optimal function during the period of weaning and to repair after injury or diarrhea, though nucleotides did not improve mucosal growth in rats. Furthermore, intestinal epithelial cells show a rapid turnover, and the mucosal enzymes drastically change in the weaning period. Therefore nucleotides may help to regulate the intestinal adaptation to intensive changes when switching from milk to a solid diet.

This study thus demonstrated that exogenous nucleotides enhanced the proliferation and/or maturation of rat and human epithelial cells. Simultaneously, our results showed that dietary nucleotides influenced the maturation of intestine in early weaned rats. Although these mechanisms are unclear, there may be possibilities that the stimulations of energy production, DNA/RNA synthesis, mRNA expression, and protein synthesis by an exogenous supply of nucleotides may induce cell proliferation or maturation, or both, if the depletion of nutritive elements, such as glutamine, occurs or if the salvage pathway works more efficiently than the de novo pathway does. Further studies are required to clarify these mechanisms and their significances to infants, but nucleotides in human milk may play an important role in the development of intestine. Our results support the idea that exogenous nucleotides may be considered as semiessential or conditionally essential nutrients for the development of intestine in infancy (*19, 27*).

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