23 Dietary Nucleotides and Immunity

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23.1 INTRODUCTION

Nucleotides are ubiquitous, low molecular weight intracellular compounds with considerable structural diversity. They comprise three joined structures: a nitrogenous base, a pentose sugar, and at least one phosphate group. The most common nucleotides can be divided into two groups, purines and pyrimidines, based on the structure of the nitrogenous base. The pentose sugar that binds the base and phosphate within the compound is either ribose or deoxyribose. Ribonucleotides signify those purine or pyrimidine nucleotides linked by ribose, where the purine bases are adenine (A), guanine (G), or inosine (I), while the pyrimidine bases are cytosine (C), uracil (U), or thymine (T). Nucleotides, primarily as components of nucleoproteins, but also as free nucleotides and nucleic acids, are naturally present in all foods of animal and vegetable origin, although their concentration varies greatly between foods (Gil 2002). The nutritional requirement for nucleotides in humans has long been recognized, but nucleotides have not generally been regarded as essential given that *de novo* synthesis and salvage pathways exist in animals, including humans (Traut 2014). Accordingly, the requirement in humans is categorized as "conditionally essential" (van Buren and Rudolph 1997; Carver 1999). The essentiality of ribonucleotides is apparent during the following conditions: rapid growth, malnutrition, infection, or injury (Rudolph et al. 1990; Uauy et al. 1996; Carver 1999). This chapter

reviews what is known about nucleotides, immunity, and infection, and draws from studies in both experimental animal models and in human infants.

23.2 SOURCES OF DIETARY NUCLEOTIDES

Exogenous nucleotides are widely distributed in foods, especially those containing cellular elements and nucleoproteins (proteins conjugated with nucleic acids). Such foods include organ meats and seafood (Kojima 1974; Clifford and Story 1976; Barness 1994). Muscle protein is thought to be a relatively poor source of nucleotides as it is comprised mainly of actin-myosin protein (Devresse 2000). Human breast milk has been shown to contain significant concentrations of nucleotides, with profiles and concentrations substantially different from bovine milk (Gil and Sanchez-Medina 1982; Oddy 2002). Numerous studies on preterm and full-term neonates reliant on infant formulas containing supplemental nucleotides as the only alimentary source have demonstrated clear benefits in relation to improved immunity (see meta-analysis by Gutiérrez-Castrellón et al. 2007). Given that single-cell proteins (SCPs) have nucleic acid levels that are around seven times higher than meats (Ingledew 1999), yeasts provide a good source of nucleotides (Tibetts 1999; Li et al. 2007). Thus, industrially produced baker's yeast or brewer's yeast (*Saccharomyces cerevisiae*) has been shown to provide a particularly good source for commercial production of supplemental nucleotides. Commercially available 5'-nucleotides for supplemental use, or addition to infant formulae, include, in particular, AMP (adenosine 5'-monophosphate), GMP (guanine 5'-monophosphate), IMP (inosine 5'-monophosphate), UMP (uracil 5'-monophosphate), and CMP (cytosine 5'-monophosphate).

23.3 DIGESTION, ABSORPTION, AND METABOLIC FATE OF DIETARY NUCLEOTIDES

The absorption and degradation of nucleotides as well as their dephosphorylated forms, nucleosides, have been well established in a diverse range of species, including humans. Uric acid, obtained by oxidation of xanthine, is the final product of purine metabolism in humans, primates, birds, some reptiles, and the majority of insects—such species are referred to as *uricotelics*, since they all excrete uric acid into the urine. In other mammals, given the name *allantoinotelics*, uric acid is degraded by the enzyme uricase (urate oxygen oxidoreductase) to allantoin and carbon dioxide. Moreover, a number of teleostean fish excrete allantoic acid, as allantoin is hydrolyzed by allantoin amidohydrolase. Selacean fish, dipneusta, as well as some teleosteans and batracians, can degrade allantoic acid by allantoate ureohydrolase rendering two molecules of urea and one molecule of glyoxilic acid; (Gil 1984). As a uricotelic species, the products of pyrimidine nucleotide catabolism are harmless (e.g., beta-aminoisobutyrate) or indeed beneficial (e.g., carnosine, anserine, and beta-alanine). While urate (uric acid) is always produced in the body and excreted via the urine, very high intakes of purine nucleotides and purine-rich foods contribute to high serum levels, which can in turn lead to hyperuricemia (excess uric acid in the blood). This condition may cause the precipitation of urate crystals in the blood, tissue, and joints (gout). These mechanisms are described below.

23.3.1 Absorption of Dietary Nucleotides

The three principal features of nucleotide metabolism in humans are (a) *de novo* synthesis from metabolites such as glutamine, aspartate, and glycine, particularly in the liver; (b) salvage from RNA and DNA degradation; and (c) exogenous intake from dietary sources (Grimble and Westwood 2001). There are many factors that control the relative importance of each of these processes in maintaining the body's pool of nucleotides and nucleosides, and the relative contribution of *de novo*

and salvage pathways appears to vary both in different tissues and at different phases of the cell cycle (Fairbanks et al. 1999; Grimble et al. 2000; Grimble and Westwood 2001). The exogenous supply of nucleotides is thought to be particularly important in the case of high turnover tissues and cells, such as those growing rapidly or those associated with immunity.

Enterocytes, the terminally differentiated cells of the intestinal epithelium, may be particularly dependent on an exogenous supply of nucleotides in the diet, although hepatic *de novo* synthesis may provide some additional support (Grimble 1996).

Dietary nucleotides have a limited capacity for absorption in the intestinal tract (Sanderson and He 1994), possibly as a result of the lack of a nucleotide transport system and the presence of negatively charged phosphate groups, which hinder absorption (Mateo 2005). However, following dephosphorylation and conversion to nucleosides, they are well absorbed and metabolized. As a result, nucleosides are the major bioavailable form of purines and pyrimidines absorbed into gut epithelial cells. Both purine and pyrimidine nucleosides are actively absorbed through four concentrative gut Na^+-dependent transporters, and they are also absorbed passively through two equilibrative transporters, which exhibit different specificities for purine and pyrimidine derivatives (Ngo et al. 2001; Scharrer et al. 2002). Based on studies on rats, uptake appears to be dependent on diffusion and specific sodium ion–dependent, carrier-mediated mechanisms (Bronk and Hastewell 1987) and more than 90% of dietary and endogenous nucleosides and bases are taken up in the enterocyte (Salati et al. 1984; Uauy 1989). Metabolites are then available to the various salvage pathways that result in the resynthesis of nucleotides for the body's specific required pool (Rolfes 2006).

23.3.2 Nucleotide Degradation

The following subsection is based on material from Swanson et al. (2006) and Angstadt (1997).

23.3.2.1 Purine Nucleotide Catabolism

In the degradation of purine nucleotides, phosphate and ribose are removed first, then the nitrogenous bases are oxidized. The end product of purine catabolism in humans is uric acid, which is excreted in urine via the kidneys. Owing to the presence of the enzyme urate oxidase in most other mammals, the more soluble allantoin is the end product.

Guanine nucleotides are hydrolyzed to the nucleoside guanosine, which undergoes phosphorolysis to guanine and ribose 1-P. However, since intracellular nucleotidases are not very active against AMP in humans, AMP is deaminated by the enzyme adenylate (AMP) deaminase to IMP. In the catabolism of purine nucleotides, IMP is further degraded by hydrolysis with nucleotidase to inosine and then phosphorolysis to hypoxanthine.

Both adenine and guanine nucleotides converge at the common intermediate xanthine. Hypoxanthine, representing the original adenine, is oxidized to xanthine by the enzyme xanthine oxidase. Guanine is deaminated, with the amino group released as ammonia, to xanthine. If this process is occurring in tissues other than liver, most of the ammonia will be transported to the liver as glutamine for ultimate excretion as urea.

Xanthine, like hypoxanthine, is oxidized by oxygen and xanthine oxidase with the production of hydrogen peroxide. In humans, the urate is excreted and the hydrogen peroxide is degraded by catalase. Xanthine oxidase is present in significant concentration only in the liver and intestine. The pathway to the nucleosides, possibly to the free bases, is present in many tissues.

These catabolic pathways for purine nucleotides are shown in Figure 23.1.

23.3.2.2 Pyrimidine Nucleotide Catabolism

Pyrimidines, in contrast to purines, undergo ring cleavage and the usual end products of catabolism are beta-amino acids, ammonia, and carbon dioxide. Pyrimidines sourced either from nucleic acids or the body's energy pool are catabolized by nucleotidases and pyrimidine nucleoside phosphorylase

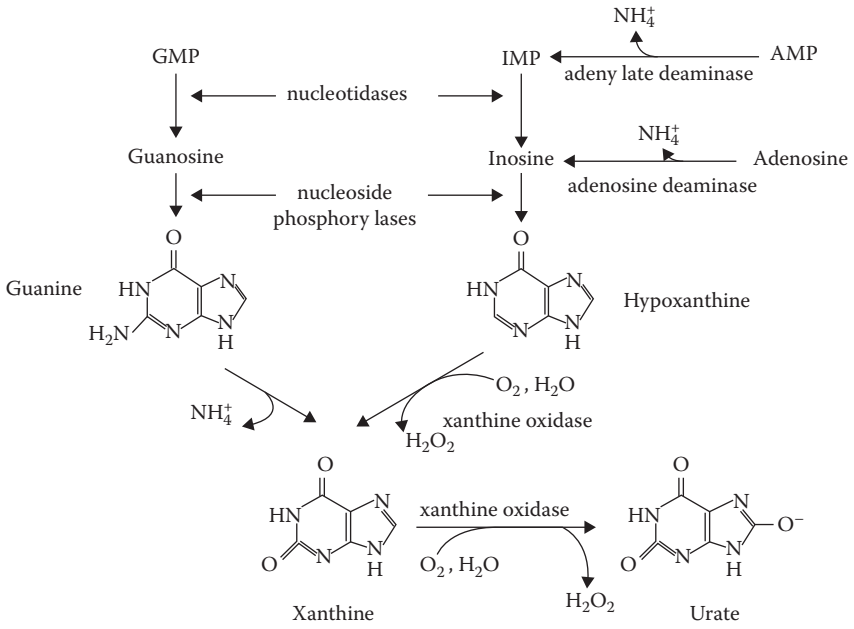

FIGURE 23.1 Typical catabolic pathways of purine nucleotides in humans.

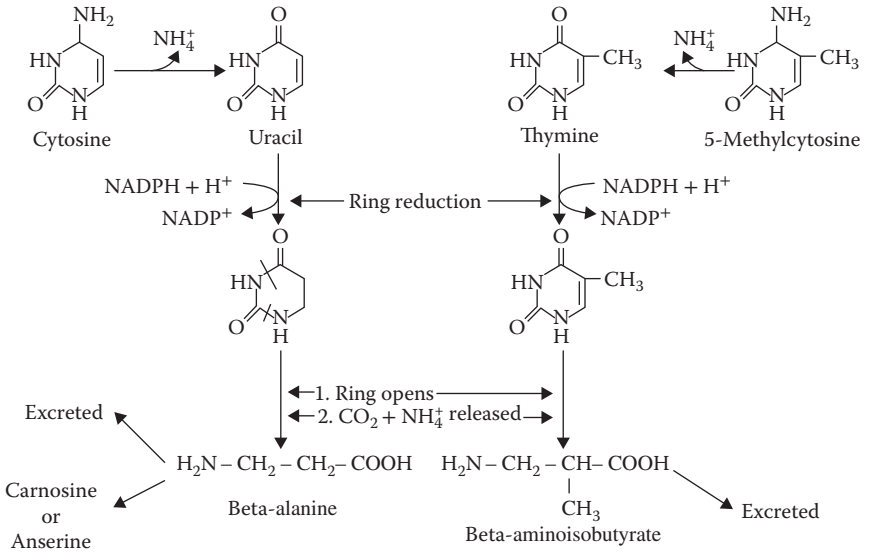

FIGURE 23.2 Typical catabolic pathways of pyrimidine nucleotides in humans.

to yield the free bases. The 4-amino group of both cytosine and 5-methyl cytosine is released as ammonia. These pathways of pyrimidine nucleotide catabolism are shown in Figure 23.2.

23.3.2.3 Undegraded Bases

Purine and pyrimidine bases that are not degraded are recycled through a range of salvage pathways and are so resynthesized as nucleotides (Rolfes 2006). This recycling, however, is not sufficient to meet total body requirements under all conditions and, accordingly, some *de novo* synthesis is

essential. *De novo* synthesis of both purine and pyrimidine nucleotides occurs from readily available components (Traut 2014).

23.4 ROLE OF DIETARY NUCLEOTIDES IN IMMUNITY

Although nucleotide deficiency has not been related to any particular disease, dietary nucleotides have been reported to be beneficial for infants, since they positively influence lipid metabolism and immunity, as well as tissue growth, development, and repair (Carver and Walker 1995; Sanchez-Pozo et al. 1999; Gil 2001). Taken together, the existing evidence indicates that dietary nucleotides may enhance the maturation of the immune and gastrointestinal tracts in infants (Carver and Stromquist 2006).

Rapidly proliferating tissues, such as the immune system or the intestine, are not able to fulfill the needs of cell nucleotides exclusively by *de novo* synthesis and they preferentially utilize the salvage pathway, recovering nucleosides and nucleobases from blood and diet. In accordance to this, the intestine is able to hydrolyze RNA and free nucleotides to nucleosides, which are efficiently absorbed by the enterocytes, with the exception of cytidine (Figures 23.3 and 23.4) (Gil et al. 2007). An exogenous supplement of these compounds through the diet may be essential to sustain intestinal growth and to maintain the cellular function in these tissues (Carver and Walker 1995; Uauy et al. 1996).

Nowadays it is well known that the gastrointestinal tract has not only the role of absorbing nutrients but also of protecting the body from potentially pathologic organisms while, at the same time, ensuring tolerance to commensal bacteria, food antigens, and self-antigens (Mason et al. 2008). The protective defenses of the gastrointestinal tract include physical barriers (glycocalyx and intestinal epithelium), antimicrobial compounds, and specialized immune responses (Mason et al. 2008). A significant proportion of the gastrointestinal tract comprises immune cells (mainly T and B lymphocytes, macrophages, and dendritic cells) (see Chapter 2), and even intestinal epithelial cells produce immunomodulatory molecules such as cytokines to regulate the immune function (Walker 1996).

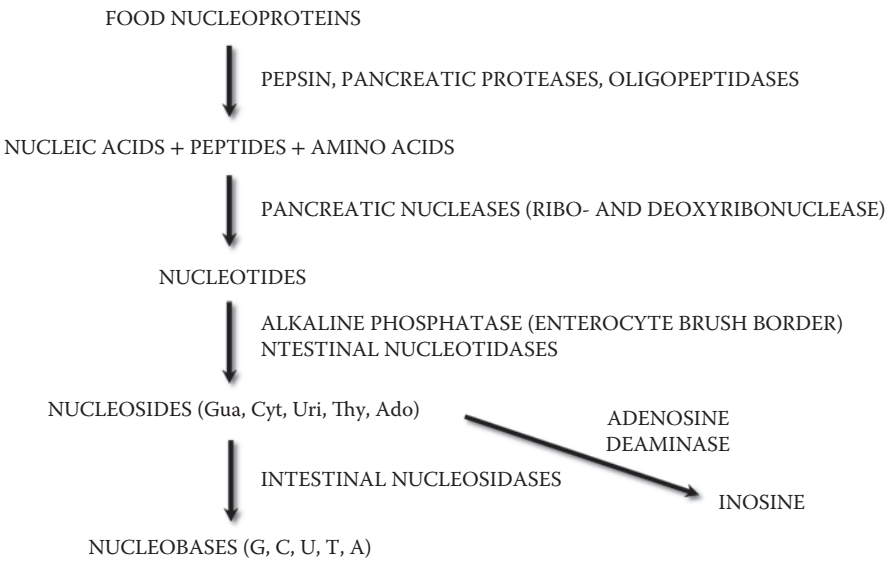

FIGURE 23.3 Digestion of dietary nucleotides. A, adenine; Ado, adenosine; C, cytidine; Cyt, cytosine; DNA, deoxyribonucleic acid; G, guanine; Gua, guanosine; RNA, ribonucleic acid; T, thymine; Thy, thymidine; U, uracil; Uri, uridine.

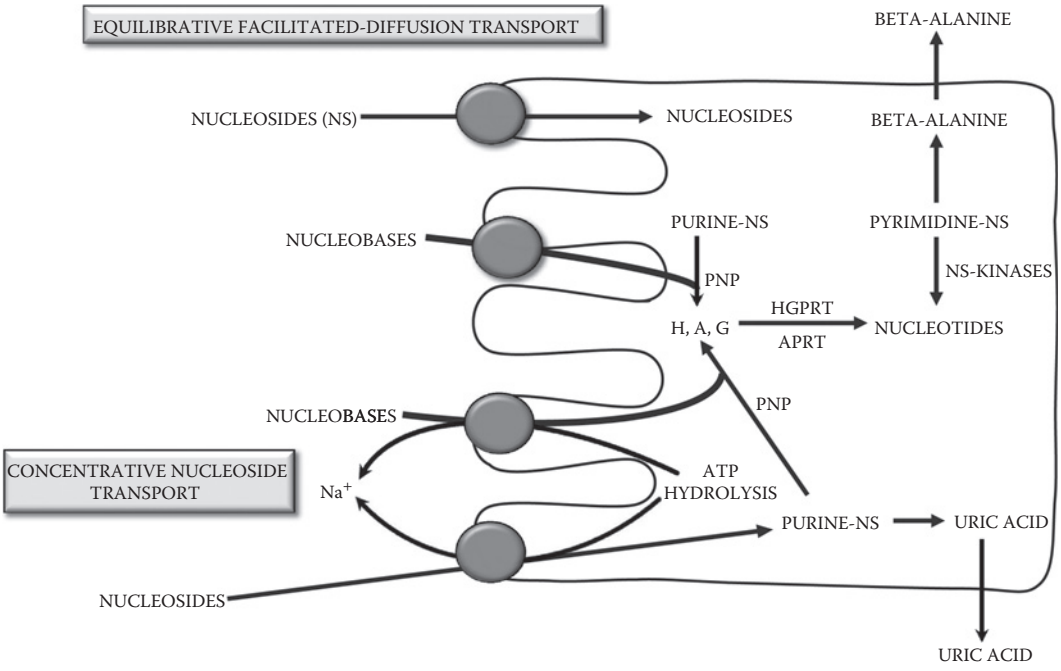

FIGURE 23.4 Absorption of dietary nucleotides. Equilibrative and concentrative nucleoside transporters are responsible for the absorption of nucleosides and, to a lesser extent, nucleobases. lnside the enterocyte, pyrimidine nucleotides are directly synthesized after phosphorylation of pyrimidine nucleosides, namely uridine. This reaction is catalyzed by nucleoside-kinases. In turn, purine nucleosides are first degraded to free bases by the purine phoshorylase (PNP), and then they enter the salvage pathway in which the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) catalyzes the synthesis of nucleotides. Purine nucleosides can also be degraded to uric acid in the enterocyte. A, adenine; ATP, adenosinetriphosphate; G, guanine; H, hipoxantine; NS, nucleosides.

The newborn immune system is relatively immature at least during the first year of life when the levels of IgA and IgG to viral or bacterial pathogens are reduced (Schaller et al. 2007). The delay in innate; acquired immune function in newborns is, however, compensated by in utero transfer of specific IgG and with specific factors present in human milk among which lipids, mucin, oligosaccharides, pathogen-specific antibodies, or nucleotides are included (Schaller et al. 2007). These nutrients influence the maturation of immune cells especially at weanling.

The development and maintenance of the immune tolerance starts very early in life, even prenatally. Tolerance is characterized by the polarization of T helper (Th) cells towards Th2 and T regulatory (Treg) phenotypes, together with a suppression of the Th1 response (Mason et al. 2008; Calder et al. 2006). Oral tolerance is characterized by the production of TGF-β and IL-10 by Treg cells, and IL-3, IL-4, IL-5, and IL-13 by Th2 lymphocytes. The production of TGF-β, IL-4, and IL-5 increases the production of secretory IgA (sIgA).

23.4.1 Nucleotide Effects on Lymphocyte Maturation, Activation, and Proliferation

The terminal deoxynucleotidyl transferase (TdT) enzyme has been referred as an index of lymphocyte immaturity (Drexler et al. 1993). Mice fed a nucleotide-free diet have shown a higher percentage of TdT-positive cells proceeding from the thymus and the spleen than those fed a diet supplemented with RNA, adenine, or uracil, suggesting that dietary nucleotides could stimulate the

maturation of lymphoid cells (Kulkarni et al.1989). The suggested mechanism proposes that dietary nucleotides exert a predominant effect upon the initial phase of antigen processing and lymphocyte proliferation suppressing the uncommitted T lymphocytes responses, as demonstrated by higher levels of TdT for undifferentiated lymphocytes in primary lymphoid organs in mice fed a nucleotide-free diet (Kulkarni et al. 1989). On the other hand, a regulatory role of dietary nucleotides in immunohematopoiesis has also been proposed (Jyonouchi et al. 1994).

There is considerable evidence demonstrating that exogenous nucleotides increase the proliferative response to T-cell dependent mitogens (PHA, ConA, and PWM), whereas no effects are seen when B-cell–dependent mitogens are used; this has been reviewed extensively in previous works (Carver et al. 1995; Gil 2001; Kulkarni et al. 1989; Jyonouchi et al. 1994; Gil et al. 1997; Rueda and Gil 2000).

In animal models stimulated with allogenic spleen cells, dietary nucleotides enhance the lymphoproliferative response, particularly during the recovery of protein-energy malnutrition, and it has also been reported that in nucleotide-starved rats the parenteral administration of a mixture of nucleotides and nucleosides (OG-VI) resulted in an increase in the proliferative response of spleen cells to the mitogen ConA. It has also been demonstrated that Balb/c and DBA/2 mice present an increase in the popliteal lymph node blastogenic response to antigens, allogens, and mitogens when they are fed with a diet supplemented with a mixture of nucleosides and nucleotides (Gil 2001; Kulkarni et al. 1989; Gil et al. 1997; Rueda and Gil 2000).

Holen et al. (2006) stimulated peripheral blood mononuclear cells (PBMCs) from healthy individuals with influenza virus antigen in the presence of DNA, RNA, dAMP, dCMP, dGMP, dUMP, or TMP. Specific nucleotide derivatives alone did not affect the growth of PBMCs. However, the nucleotide derivatives influenced immune cell growth and cytokine secretion when cocultured with specific antigen. DNA, RNA, dAMP, dCMP, and dUMP increased influenza virus antigen-induced immune cell proliferation. In contrast, dGMP and TMP inhibited the antigen-induced growth response (Holen et al. 2006). RNA and dAMP cocultured with virus antigen significantly increased PBMC secretion of IFN-γ, IL-10, and TNF-α. DNA increased virus antigen-induced immune cell secretion of IFN-γ only, whereas dUMP increased secretion of IL-10 only. Finally, dGMP completely inhibited virus-triggered IFN-γ secretion (Holen et al. 2006).

The effects of dietary nucleotides have been studied by Kulkarni et al. (2002) *in in vivo* and *in vitro* models of microgravity, which have adverse effects on the immune system. Popliteal lymph node response was significantly suppressed in mice subjected to experimental microgravity, and was restored by diets supplemented with either RNA or uracil. Splenocytes isolated from these mice had decreased PHA-stimulated proliferation and IL-2 and IFN-γ levels, and these effects were restored by RNA and uracil diets. Also, splenocytes cultured in microgravity conditions showed an inhibited PHA response, and uridine as well as a mixture of nucleosides and nucleotides restored the proliferative responses. Nucleotide supplementation, especially uridine, was also able to influence cell surface markers, indicating that the lymphocytes had acquired an activated phenotype (Kulkarni et al. 2002).

The effects of dietary nucleotides in newborn term infants was investigated in a double-blind study carried out by Buck et al. (2004), in which 477 subjects were enrolled. The children were divided into three different groups depending on the diet: infant formula with or without nucleotides, or human milk. In contrast with the studies mentioned above, these authors found that during the first year of age there were no changes in the total number of T-cells, B-cells, or NK cells in the bloodstream. Nevertheless T-cell subsets were affected (see below) (Buck et al. 2004). On the other hand, high levels of Tc1 and total IFN-γ-producing cells were found in nucleotide-fed infants. The effect of these cells could be beneficial, as increases in IFN-γ correlate with proliferative responses (Buck et al. 2004). These data suggest that nucleotides, as semiessential nutrients, could have a role in lymphocyte proliferation in several conditions like inflammation or immunosuppression but not in normal conditions.

23.4.2 Dietary Nucleotides and Lymphocyte Subpopulations

Differences in T and B lymphocyte subpopulations between mice fed diets containing nucleotides or not have been described (Buck et al. 2004; Manzano et al. 2003). Manzano et al. have reported that dietary nucleotides affect maturation and differentiation of intestinal lymphocytes that usually takes place at weanling (Manzano et al. 2003). These authors found that nucleotides exert selective effects on the different lymphocyte subsets (Peyer's patches, intraepithelial, and lamina propria). In general, nucleotides promote the development of T helper lymphocytes, and consequently the maturation and differentiation of B-cells. These effects would in part explain the positive modulation that nucleotides exert on immunoglobin production (see the "Modulation of Immunoglobin Production by Nucleotides" section below).

Jyonouchi et al. have described that dietary nucleotides modulate antigen-specific type 1 and type 2 T-cell responses in both C57BL/6 and BALB/cJ mice (Jyonouchi et al. 2000, 2001). The same authors challenged BALB/cJ mice with ovalbumin (OVA) plus incomplete Freund's adjuvant, a combination that predominantly induces Th2 response in these mice. Dietary ribonucleotides increased the OVA-specific Th1 cells after the primary antigen challenge and decreased OVA-specific Th2 cells after the secondary challenge. Costimulatory molecule (CD86 and CD154) expression and the activation state of total Th and cytotoxic cells were not affected in the regional draining lymph node. These results suggest that dietary ribonucleotides may modulate OVA-specific Th1/Th2 responses without nonspecific activation of T-cells (Jyonouchi et al. 2003).

The effects of dietary nucleotides on lymphocyte subset populations in preterm and full-term infants have been reported: there was a higher percentage of blood $CD4^+$ cells in preterm children fed a nucleotide-supplemented formula compared with those fed the standard formula at 10 days of life (Navarro et al. 1999). As mentioned above, Buck et al. investigated the effects of dietary ribonucleotides on the immune cell phenotype and function of infants in their first year of life (Buck et al. 2004). These authors found increases in the percentage of memory/effector T-cell populations and changes in NK cell subtypes in infants fed a nucleotide-supplemented formula compared with infants fed the same formula without nucleotides. The observed increase in memory T-cells correlated with higher vaccine antibody titers and improved cell-mediated immune responses. Furthermore, in this study, the increase in memory/effector T-cell populations was associated with an increase in Tc and Th2 cells, and with a decrease in the population of naive lymphocytes. The increase in the proportion of Th2 cells in the nucleotide-fed group could enhance the systemic and immune mucosal response. Decreases in naive T-cell populations and increases in memory T-cells characterize normal immune maturation/development and normal responses to vaccinations and infections. It is interesting to mention that the observed changes made the immune system of the nucleotide-fed children more similar to that of the breastfed children. Therefore, these results provide evidence that formula supplemented with levels of nucleotides similar to those found in human milk (72 mg/L) may facilitate maturation and immunoregulatory shifts in some lymphocyte populations. These shifts might support increased antibody responses and immune cell protection. The changes in NK cell subsets may also enhance innate immune responses against tumors and/or intracellular pathogens.

23.4.3 Modulation of the Macrophage Phagocytic Activity by Dietary Nucleotides

A number of reports have related dietary nucleotides and macrophage activity. In mice inoculated with *Staphylococcus aureus*, the phagocytosis of microorganisms was lower in those who were fed on a nucleotide-free diet than in those fed on a diet supplemented with RNA or adenine. Dietary nucleotides enhanced the interaction of macrophages and T-cells, explaining the higher susceptibility of mice fed a nucleotide-free diet to *Candida* infection (Kulkarni et al. 1989).

23.4.4 Nucleotide Modulation of the Delayed Hypersensitivity and Allograft and Tumor Responses

Early studies showed that mice fed a nucleotide-free diet and previously challenged with an intravenous stimulus of sheep red blood cells (SRBCs) exhibited a delayed cutaneous response when these cells were injected in the mice legs. An increase in the delayed hypersensitivity response to SRBCs and to DNFB in BALB/c and DBA/2 mice fed a diet supplemented with a mixture of nucleotides and nucleosides has been reported (Carver and Walker 1995; Kulkarni et al. 1989; Rueda and Gil 2000). One of the most studied models to ascertain the influence of dietary nucleotides on immunity is the evaluation of the response of the host against allografts. The duration of heart allografts implanted on the ear of mice was shown to increase when the diet was devoid of nucleotides, suggesting an impaired immune response; the addition of yeast RNA to the diet resulted in a reduced period of allograft survival. Likewise, the use of cyclosporine as an immunosuppressive agent in mice had a synergic effect when combined with a nucleotide-free diet leading to a higher period of allograft survival (Kulkarni et al. 1989). Also, administration of a nucleoside/nucleotide-free diet to rats subjected to transplantation of fetal small intestine without vascular anastomosis resulted in less graft rejection and lower plasma IL-2 levels (Ogita et al. 2004b). However, no differences were seen when mice were inoculated with a fibrosarcoma or the LSTRA syngeneic lymphoma, which is highly aggressive (Navarro et al. 1996).

Natural killer (NK) cells are one of the main populations involved in the immune response against transformed cells. The activity of NK cells is increased in mice fed a diet supplemented with nucleotides in respect to those fed a diet without nucleotides. Likewise, Carver has shown in human newborns that NK cell activity at the second month of life is increased in infants fed formula supplemented with nucleotides (Carver and Walker 1995).

23.4.5 Modulation of Immunoglobulin Production by Nucleotides

Experiments carried out in mice fed a nucleotide-free diet for 3 weeks have shown a profound decrease of specific antibody response to T-cell–dependent antigens and a retained response to T-cell–independent antigens and lipopolysaccharides (Jyonouchi et al. 1994). Likewise, a mononucleotide-nucleoside mixture used in experimental total parenteral nutrition restored the humoral immune responses to T-cell dependent antigens in mice fed a nucleotide-free diet. However, this solution did not show any effect on the *in vitro* specific antibody production in response to T-cell–dependent antigens. Our group reported in BALB/c mice that the addition of nucleotide mixtures to a nucleotide-free diet resulted in an increase in the response of hemolytic IgG-forming cells induced by previous immunization with sheep erythrocytes; when the diet was supplemented with single nucleotides, AMP, GMP, or UMP increased the IgG response whereas CMP and IMP had no effect. GMP was the only nucleotide that increased the hemolytic IgM-forming cell response. A study using ovalbumin-specific T-cell receptor transgenic mice indicated that dietary nucleotides increase mucosal IgA response against specific antigens by increasing the production of transforming growth factor beta by intestinal epithelial cells and the proportion of TCR$\gamma\delta$ IELs (Nagafuchi et al. 2002). Although previous studies have demonstrated the effect of dietary nucleotides on the differentiation of enterocytes, this study provides further evidence for the involvement of the enterocyte-mediated immune response in the effect of these compounds (Nagafuchi et al. 2002). In agreement with this, a study carried out in Caco-2 cells showed that exogenous nucleotides modify the expression and activity of transcription factors involved in immune response and inflammation (Ortega et al. 2011).

Our group has done a series of studies to determine the influence of dietary nucleotide supplementation to infant formulas on the levels of circulating antibodies in preterm infants. Total serum IgM and IgA levels increased significantly for the first 3 months of life, whereas no differences were detected for serum IgG; levels of IgE were undetectable (Gil et al. 1997; Navarro et al. 1996)

(Figure 23.5). The aforementioned increase in serum IgA has also been reported by Yau et al. in healthy full-term infants fed a nucleotide-supplemented formula (Yau et al. 2003).

The effect of dietary nucleotides on the antibody response to specific food antigens has been also studied in children. A study with preterm infants showed higher concentration of specific IgG against α-casein and β-lactoglobulin for the first month of life in newborns fed a low-birth-weight-infant nucleotide-supplemented formula (Martinez-Augustin et al. 1997). Nevertheless, no differences were observed in the serum levels of these specific immunoglobulins, nor in those of total immunoglobulins when malnourished children were fed with formulas supplemented with nucleotides (Martinez-Augustin et al. 1997).

Finally, several studies have demonstrated that dietary nucleotides enhance infants' responses to bacterial antigens and to vaccination (see Table 23.1). Thus, Pickering et al. (1998) showed that dietary nucleotides may modulate the immune response in normal infants, enhancing the production of specific IgG against low response antigens, namely *H. influenzae* type b, a bacterium responsible for meningitis episodes in early infancy (Pickering et al. 1998). On the other

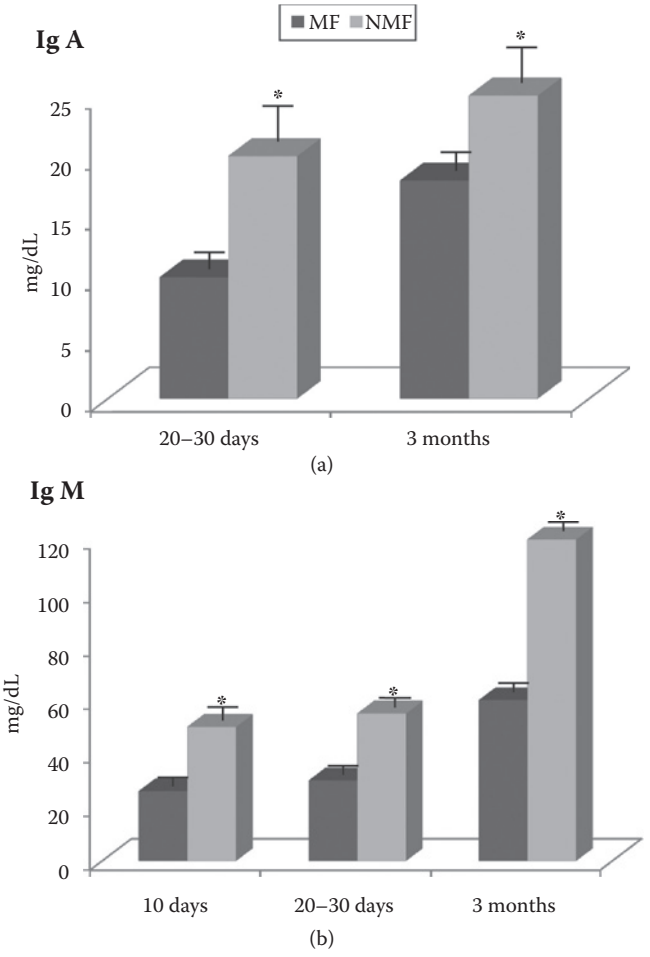

FIGURE 23.5 Nucleotides and humoral responses in preterm infants. Nucleotides increase the serum levels of IgA (a) and IgM (b) in preterm infants fed a nucleotide-supplemented milk formula (NMF) compared to those fed a control milk formula (MF). *$P < 0.05$ MF vs. NMF. (From *Neonatal Hematology and Immunology III,* Gil, A. et al., Role of dietary nucleotides in the modulation of the immune response, 139–144, 1997, with permission from Elsevier, and Navarro, J. et al., *Immunol Lett* 53, 141–145, 1996.)

TABLE 23.1
Effects of Dietary Nucleotides on Infant Immunity

Observed Effects of Nucleotides	Reference
***In vitro* analysis of specific blood cell populations for functional capacity:**	
Increase in the number of T memory/effector cells in the first year of life	Buck et al. (2004)
Lower levels of cytokine-producing T-cells (Tc1 and Th2 cells and total IFN-γ production) in the first year of life	Buck et al. (2004)
Decrease in naive T-cells in children in the first year of life	Buck et al. (2004)
Increased IL-2 production	Carver et al. (1991)
Increased NK cell activity	Carver et al. (1991)
Analysis of *in vivo* responses to antigenic challenge, measuring changes in serum or mucosal antibody concentrations:	
Antibody concentration or production	
Higher IgA and IgM production at 3 months of age	Navarro et al. (1999) Maldonado et al. (2001)
Higher serum IgA	Yau et al. (2003)
Higher antibodies against β-lactoglobulin at 1month of life	Martinez-Augustin et al. (1997)
Serum vaccine-specific antibodies	
Higher Hib antibodies at 7 and 12 months	Pickering et al. (1998) Ostrom et al. (2002)
Higher diphtheria antibodies at 7 months	Pickering et al. (1998) Hawkes et al. (2006)
Higher oral polio virusVN1 antibodies at 7 and 12 months	Schaller et al. (2007) Ostrom et al. (2002)
Higher tetanus antibodies at 7 months	Hawkes et al. (2006)
Analysis of the incidence or severity of infection following challenge with live or attenuated pathogens further supported by assessments *in vitro* or *in vivo*:	
Decreased diarrhea incidence	Yau et al. (2003) Pickering et al. (1998) Brunser et al. (1994) Merolla and Gruppo Pediatri Sperimentatori (2000) Lama More et al. (1998)
Fewer symptoms of upper respiratory disease after 3 and 7 months	Navarro et al. (1996) Hawkes et al. (2006)

Hib, Haemophilus influenzae; IFN, interferon; IL, interleukin; NK, natural killer.

hand, Schaller et al. (2004) explored the effects of ribonucleotide supplementation on antibody responses in children subjected to routine infant immunizations (Schaller et al. 2004). Infants fed a nucleotide-supplemented formula had significantly higher poliovirus type 1 neutralizing antibody responses than did infants fed a nucleotide-free formula (Schaller et al. 2004). Another study demonstrated higher responses to tetanus toxoid (Hawkes et al. 2006), and a thorough study by Buck et al. (2004) showed that ribonucleotide feeding increases antibodies responses to Hib and to poliovirus, making them more similar to those of breast-fed children (Buck et al. 2004).

More recently, the above-mentioned studies and others were systematically reviewed by Gutiérrez-Castrellón et al. (2007), concluding that there is sufficient evidence to support the

addition of nucleotides to infant formulas, the two main benefits being the improved maturation of the immune system and the decrease in the incidence of diarrhea (Gutiérrez-Castrellón et al. 2007).

23.4.6 Dietary Nucleotides and Defense against Infection

Animals injected intravenously with *Candida albicans* or *Staphylococcus aureus* and fed a nucleotide-free diet had a significantly lower survival rate than mice fed RNA, adenine, or uracil-supplemented diets. On the other hand, intraperitoneal administration of a nucleoside-nucleotide mixture for 14 days to mice was associated with reduced translocation of Gram-negative enterics to the mesenteric lymph nodes and spleen in comparison to control animals. The extent of the damaged mucosa was greater in controls, and these animals were more susceptible to the lethal effects of the lipopolysaccharide from *E. coli*, which suggests that dietary nucleotides may block bacterial translocation by preventing endotoxin-induced mucosal damage (Carver and Walker 1995; Gil 2001; Kulkarni et al. 1989).

One of the potential mechanisms by which nucleotides reduce the incidence of infection is the modulation of the intestinal microbiota. Our group reported for the first time that nucleotide supplementation to an infant formula reduced the counts of enterobacteria and increased the counts of bifidobacteria in the fecal microbiota (Gil and Uauy 1995; Uauy et al. 1996). These results suggested that nucleotides could act as prebiotics favoring the proliferation of the beneficial flora and inhibiting that of potential pathogens. A more recent study corroborates this, indicating that nucleotide supplementation of infant formulas decrease the ratio of *Bacteroides-Porphyromonas-Prevotella* group to *Bifidobacterium* species (Shingal et al. 2008). These results support clinical findings showing a low incidence of acute diarrhea in infants fed nucleotide-supplemented formula in developing (Brunser et al. 1994) and developed countries (Pickering et al. 1998).

Yau et al. (2003) investigated the effects of an infant formula fortified with nucleotides on the incidence of diarrhea, respiratory tract infections, and immune responses in healthy term infants (Yau et al. 2003). Compared with infants that received a nucleotide-free diet, those fed the supplemented formula had a significantly lower risk (25%) of diarrhea between 8 and 28 weeks. In contrast, the group of infants fed the nucleotide formula showed an increased risk of upper respiratory tract infections.

23.5 NUCLEOTIDES AND INTESTINAL INFLAMMATION

Intestinal inflammation is the hallmark of a range of diseases including inflammatory bowel disease. Because of the effect of nucleotides on immunity, intestinal healing, and proliferation, several studies have assessed their effect on intestinal inflammation in animal models. The first studies, in which nucleotide mixtures were administered to rats with DSS-induced colitis (Sukumar et al. 1998, 1999), described an exacerbation of the inflammatory reaction. In accordance with this, the administration of nucleoside-nucleotide-free diets was described to suppress cytokine production and to protect colonic mucosa in TNBS-induced colitis in rats (Adjei et al. 1996, 1997). These results are in agreement with the fact that the administration of nucleoside-nucleotide–free diets to Lewis rats reduced acute rejection of allogenic transplants. These rats received a 2-cm jejunum transplant from a donor Fischer rat into their abdominal wall (Ogita et al. 2004a,b). As a consequence, a proinflammatory effect of nucleotide mixtures is deduced, while nucleoside-nucleotide–free diets are considered to have immunosuppressive effects.

There is not a clear explanation for the proinflammatory role of dietary nucleotides, and specific studies are lacking, but nucleotides have been shown to be mediators of the purinergic system that plays an important role in maintaining gut homeostasis by regulating a variety of functions including secretion/absorption, immune/inflammatory, and nervous functions (Kolachala et al. 2008; Antonioli et al. 2013). In this sense, extracellular nucleotides liberated from different cell

types in stress conditions have been shown to alert the immune system to tissue injury or inflammation. In fact, released nucleotides and their derivatives, such as ATP, ADP, UTP, UDP-glucose, and adenosine, would act as ligands of purinergic receptors (P1 and P2). In general, these receptors work in an autocrine mode. Under normal conditions, low concentrations of nucleotides are present in body fluids and tissues, but during inflammation, large amounts of extracellular nucleotides are rapidly released into the extracellular environment at the site of inflammation, increasing nucleotide concentrations rapidly. For example, released ATP can stimulate P2 (for nucleoside tri-/diphosphate) receptors and is rapidly metabolized by ectonucleotidase into adenosine, that in turn acts on P1 (for adenosine and AMP) receptors and/or is recaptured by nucleoside transporters. Thus, the occurrence of purinergic signals depends on the integrated activity of enzymes and transporters deputed to finely modulate the magnitude and duration of purinergic responses, driving the shift from ATPergic (mainly proinflammatory) to adenosinergic responses, which predominantly ameliorate inflammation (Antonioli et al. 2013). The importance of the purinergic receptor system in the intestinal inflammatory response is illustrated by the fact that some of these receptors like PA(2A), P2X7, P2Y(2), and P2Y(6) are overexpressed in IBD patients or colitic animals and, for example, P2X7-deficient mice do not develop experimental colitis (Neves et al. 2011; Ochoa-Cortes et al. 2014). Furthermore, the release of ATP by damaged intestinal epithelial cells, commensal bacteria, macrophages, platelets, or neutrophils induces the generation of proinflammatory Th17 cells and the activation of mast cells, which in turn produce proinflammatory cytokines, chemokines, and other inflammatory mediators like leukotrienes and histamine (Kurashima et al. 2015). The consequence of the increased extracellular ATP levels is therefore a proinflammatory signal, but also a protective one, since it can help prevent infections by stimulating the immune system. In fact, ATP release has been shown to result from TLR stimulation by intestinal bacteria, and a protective role has been attributed to this phenomenon (Kurashima et al. 2015). After exonucleotidase metabolism ATP is degraded to AMP and adenosine, which interact with P1 receptors such as PA(2A) and PA(3), and which are involved in both the promotion and the resolution of inflammatory responses (Colgan and Eltzschig 2012; Ye and Rajendran 2009). The fact that PA(2A) knockout mice show an exacerbated colonic inflammatory response to infection, while PA(2A) and PA(3) selective agonists ameliorate intestinal inflammation, together with the need for PA(A2) and PA(3) receptor expression on T-cells and myeloid cells for the inhibition of intestinal inflammation (Ye and Rajendran 2009), indicates the importance of these receptors in the development and resolution of intestinal inflammation.

Dietary nucleotides are in direct contact with the intestinal epithelium and the lamina propria, where cells express purinergic receptors; therefore, although not directly demonstrated to our knowledge, it would be reasonable to think that the proinflammatory effects of nucleotide mixtures and the anti-inflammatory effects of nucleoside-nucleotide–free diets could be directly related to the stimulation of the purinergic receptor system.

It is interesting to point out that in models of ileitis induced by indomethacin, the intraperitoneal administration of nucleotides exerts anti-inflammatory effects (Veerabagu et al. 1996). Furthermore, the intraperitoneal administration of inosine (monophosphate disodium salt) has also been shown to attenuate TNBS-induced colitis in rats (Rahimian et al. 2010), an effect that is mediated by PA(2A) receptors. Therefore, it would be possible that the precise effect of nucleotides would depend on the luminal/apical or intraperitoneal/basolateral administration.

23.6 POTENTIAL MECHANISM OF ACTION OF DIETARY NUCLEOTIDES

It has been proposed that dietary nucleotides exert effects upon cellular immune function by acting on the T helper/inducer population with the predominant effect on the initial phase of antigen processing and lymphocyte proliferation. The suggested mechanism would be the suppression of uncommitted T lymphocyte responses as demonstrated by higher activities of deoxynucleotidyl

transferase, a marker of undifferentiated lymphocytes, in primary lymphoid organs of mice fed a nucleotide-free diet (Kulkarni et al. 1989).

Another hypothesis is that exogenous nucleotides may modulate T helper (Th) cell-mediated antibody production (Jyonouchi et al. 1994), favoring the balance of T-cell differentiation to Th-2 cells, which are mainly involved in the B-cell response and in the suppression of proinflammatory reactions induced by Th-1 cells.

The molecular mechanisms by which dietary nucleotides modulate the immune system are practically unknown. It has been suggested that the small intestine should play a key role in the regulatory effects of nucleotides upon the immune response. The gut-associated lymphoid tissue can initiate and regulate T-cell development and may act as a thymus analog (Walker 1996). Dietary nucleotides have been shown to enhance the production and the genetic expression of IL-6 and IL-8 by fetal small intestinal explants when challenged with IL-1β, the response being nucleotide concentration dependent. Furthermore, the addition of AMP to the culture medium resulted in the suppression of crypt cell proliferation followed by the restoration of differentiation and the induction of apoptosis across the human small intestinal epithelium (Sanchez-Pozo et al. 1999). Dietary nucleotides may influence the protein biosynthesis by regulating the intracellular nucleotide pool. In addition, signal transduction mediated by the interaction of exogenous nucleosides and their receptors may also contribute to modulate the expression of a number of genes, some of which can directly affect the levels of intestinal cytokines (Figure 23.6).

Nucleotides have been reported to modulate the gene expression of enzymes involved in their own metabolism, such as the purine salvage enzymes hypoxanthine-guanine phosphoribosyl transferase and adenine phosphoribosyl transferase in the small intestine and proximal colon (Leleiko et al. 1987; Leleiko and Walsh 1995). Recently, exogenous nucleosides have been shown to affect the expression and activity of several transcription factors involved in cell growth, differentiation, apoptosis, and in immune response and inflammation in Caco-2 cells. In fact, the addition of nucleosides to the medium increased the expression and activity of the general transcription factor CCAAT displacement protein (CUX1), and decreased the expression and activity of the general upstream stimulatory factor 1 (USF1), glucocorticoid receptor (NR3C1), and nuclear factor kappa B (NF-κB) (Ortega et al. 2011).

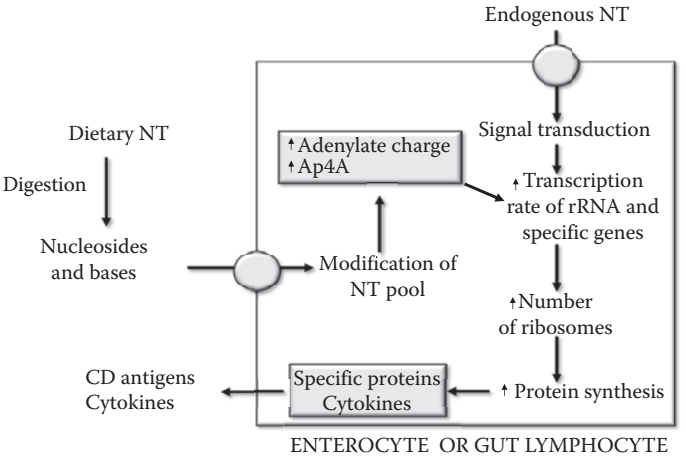

FIGURE 23.6 Potential mechanisms of action of dietary nucleotides. Dietary nucleotides, mainly absorbed as nucleosides in the gut, may influence enterocyte and Iymphocyte protein biosynthesis by regulating the intracellular nucleotide pool. In addition, signal membrane transduction mediated by the interaction of exogenous nucleosides and their receptors may also contribute to modulate the expression of a number of genes, some of which can directly affect the levels of intestinal cytokines. NT, = nucleotides; rRNA, = ribosomal ribonucleic acid.

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