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NUCLEOTIDES AND NUTRITION

Proceedings from a roundtable symposium held at the Hotel Inter-Continental New Orleans
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Guest Editor, W. Allan Walker

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Symposium: Nucleotides and Nutrition

Nucleotides and Nutrition: Role as Dietary Supplement[1,2]

W. ALLAN WALKER

Departments of Pediatrics and Nutrition, Harvard Medical School, Combined Program in Pediatric Gastroenterology and Nutrition, Children's Hospital and Massachusetts General Hospital, Charleston, MA 02129

It is apparent from the number of symposia published in the last few years that the role of nucleotides in the diet is an extremely important issue. This roundtable, entitled Nucleotides and Nutrition, helped update our knowledge on the use of nucleotides as a supplement in newborns and in older children and adults. We were very fortunate to have many of the world's authorities in the field available to discuss the salient issues surrounding this topic.

In session I Frederick B. Rudolph and Lewis A. Barness reviewed the current knowledge on the chemistry, physiology and composition of nucleotides in breast milk from parturition until weaning. Dr. Rudolph described the metabolism of nucleotides, underscoring their importance in cellular function and the consequences of dysfunction vis-à-vis various human disease states. He reviewed his studies with Charles T. Van Buren over the last several years examining the role of nucleotides in optimal host defense against infection and as a substrate for a normal immune response to noxious foreign stimuli. He pointed out, based on his research, that the absence of nucleotides tends to cause an arrest in the cell cycle between the G_0-G_1 and the S phases of T helper cells. It was apparent from this review that additional basic research is necessary to further define the importance of nucleotides within various metabolic pathways of specific cell types and under various conditions in which there may be higher requirements for them as substrates for normal host defense function.

Dr. Barness reviewed data on the composition of the nonprotein nitrogen pool in breast milk and the contribution made by nucleotides. He also indicated that the total nucleotide pool, including the nucleotides in the soluble fraction and those released by the breakdown of cells in the breast milk, may be grossly underestimated and that any consideration of nucleotide supplementation of infant formula must include a reexamination of these fluids using such modern quantitative techniques as HPLC. Very little is now known about the capacity of the enterocytes in premature and full-term human infants to use nucleotides through the salvage pathway or the de novo synthesis pathway. Again, this presentation underscored the need for further molecular and cellular studies in human development, as well as for a reassessment of our knowledge of the composition and quantity of nucleotides, before recommendations for meaningful supplementation in premature infants and in posttraumatic stress can be made.

In session II Ian R. Sanderson and Harumi Jyonouchi reviewed molecular studies involving nucleotide uptake and metabolism in human intestinal cell lines and organ cultures from human fetuses, as well as in vivo and in vitro studies of specific mechanisms of lymphocyte function. Dr. Sanderson reviewed the data obtained in his laboratory using Caco-2 cells, a human cancer cell line that differentiates in a manner similar to that of the crypt villus differentiation pathway, and IEC-6 cells, a noncancer rodent crypt cell line that requires growth factors from an extracellular matrix for maturation to occur. In the neoplastic cells (Caco-2) the de novo synthetic pathway for nucleotides was active and, under conditions of normal growth, exogenous nucleotides were not required. However, under conditions of nutritional stress, they became essential. With the nonneoplastic crypt cells (IEC-6) in the presence of an extracellular matrix (Matrigel), nucleotides provided an additive stimulus for the differentiation.

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[1]Presented at the symposium "Nucleotides and Nutrition" held in New Orleans, LA, March 28, 1993. The symposium was cosponsored by the American Institute of Nutrition and Wyeth-Ayerst International. Funding for the symposium and the publication of the supplement was provided through an educational grant from Wyeth-Ayerst International. Guest editor for this supplement was W. Allen Walker, Massachusetts General Hospital, 149 13th Street, Charleston, MA 02129.

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Dr. Jyonouchi reviewed the need for polynucleotides and RNA as demonstrated in both in vivo and in vitro experiments involving B cells in the presence of stimulus by T-cell–dependent and T-cell–independent antigens. These studies showed that polynucleotides were an effective stimulus for the increased production of antibodies in response to T-cell–dependent antigens, suggesting that the primary response was mediated by T cells, most likely T helper cells. Polynucleotides failed to increase the antibody responses to T-cell–independent antigens or polyclonal B-cell activators in vitro. Whole immune responsiveness was rapidly restored in vivo by parenteral administration of nucleotides to mice fed a nucleotide-free diet. There was a discrepancy, however, in the ability of RNA and mononucleotides to stimulate immune responsiveness in vitro, indicating a need for comprehensive studies to define the mechanism of nucleotide and RNA stimulus and suggesting that the process may be more complex or may require several steps before the ultimate stimulus to B cells occurs. These results demonstrate that polynucleotides are important to B-cell responsiveness, particularly under conditions of bacterial sepsis, but additional experiments to sort out the disparity between the in vitro and the in vivo observations are required. Using techniques such as in situ hybridization or RT-PCR of cytokine mRNA may be necessary to unravel the in vivo and in vitro response.

Jane Carver reviewed the literature and her own work examining the role of nucleotides in maintenance of cellular immunity. She provided data that suggested that exogenous nucleotides facilitate the clearing of organisms after parenteral exposure to bacteria and that their action is exerted at the post-phagocytosis step of bacterial destruction within the phagocytes. Dr. Carver described a small study that evaluated the clinical and immunological effects of human milk and nucleotide-supplemented (physiologic levels) formula compared with those of unsupplemented formula on infants in the first 6 mo of life. The results did not indicate significant differences in terms of weight gain or the incidence of infections; however, the natural killer cell activity in the infants given breast milk or supplemented formula was greater than that in the infants given unsupplemented formula. She also provided preliminary data that support the importance of nucleotides as essential nutrients in liver and gut function. These studies suggest that further observations in both in vitro and in vivo systems are necessary to sort out the specific mechanism of nucleotide stimulus to host defenses and gut and liver function. It was suggested that, in general, nucleotide supplements provide an optimum environment for an appropriate host defense response but do not necessarily stimulate host responsiveness beyond that which is normally required to counteract foreign antigens and microorganisms in the microenvironment.

In session III Sergio A. Bustamante reviewed his studies on the effects of dietary nucleotides on the gastrointestinal tract in newborns. Through a literature review, he presented data that nucleotides tend to stimulate an increase of DNA levels, enterocyte differentiation and an increased crypt-villus ratio. Dr. Bustamante also presented data demonstrating that there are increased gram-positive indigenous gut flora (bifidobacteria) in babies fed a nucleotide-supplemented diet, compared with the flora in babies given an unsupplemented diet. He concluded his presentation by reviewing the role of nucleotides in preventing severe injury in postradiation damage to the gut. In the presence of nucleotides, radiation injury was decreased but occurred more rapidly. Again, these studies, although provocative, suggest that the importance of nucleotides must be defined at the molecular level, particularly in controlling the positive and negative actions of cytokines in the inflammatory response.

Ricardo Uauy reviewed the nonimmune effects of nucleotides, discussing microfloral colonization and the role of nucleotides in liver regeneration. He also discussed the role of nucleotides on lipid metabolism, particularly as a stimulus for essential fatty acid metabolism. Dr. Uauy reviewed previous studies and discussed ongoing studies in his laboratory that demonstrate a profound positive effect of nucleotides on the functions of the gastrointestinal tract and the liver, and he indicated that modern cellular and molecular biology techniques will be necessary to delineate the specific process at the basic level.

In the final presentation, Charles Van Buren reviewed the need for nucleotides in adult nutrition. He discussed several excellent multicenter studies with burn and surgical posttrauma patients that demonstrate the importance of intravenous and enteric nucleotide supplementation in reducing postoperative complications and hospital stays. Dr. Van Buren stressed the need for ongoing comprehensive studies that can further define the optimum supplementation of these essential nutrients in stressed adult patients.

The roundtable helped clarify and expand our knowledge of dietary nucleotides as an important and possibly essential nutritional supplement. It was apparent that additional research to delineate the role of nucleotides in both immune and nonimmune processes is needed. Future studies should address the following areas:

1) The basic mechanisms of nucleotide function in host defense by lymphocyte elements and at both the enteric organs and the liver must be defined by using modern cellular techniques. Additional studies may help to define the importance of nucleotides at various stages of human development, more must be known about the development and function of enterocytes, thereby making possible an objective estimate of the exogenous needs at various stages of development. The same logic pertains in older children

and adults under condition of increased nutritional stress or general stress.

2) The specific role of nucleotides, as well as the appropriate composition of the nucleotides or RNA to use as supplementation, must be defined.

3) Multicenter studies are important in defining the approaches for nutritional management in patients in the 21st century and for reducing hospitalization.

4) Molecular studies in immunology should be conducted to determine the within- and between-lymphocyte communications at the B- and T-cell level and the contribution that nucleotides might make to normalizing this process in neonates and poststress patients.

This was an extremely productive roundtable. A great deal of new information was presented and clear indications were given for the future direction of studies that will help further our knowledge of the importance of supplemental nucleotides in the human diet.

Symposium: Nucleotides and Nutrition

The Biochemistry and Physiology of Nucleotides[1,2,3]

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ABSTRACT Nucleotides are phosphate esters of nucleosides that contain a sugar linked through a glycosidic linkage with purine and pyrimidine bases. Purine and pyrimidine nucleotides are major components of the cells that make up the monomeric units of DNA and RNA, and they function in all cellular processes. Biosynthesis, interconversion, catabolism and other aspects of nucleotide metabolism, along with various cellular roles of nucleotides, will be discussed, and the possible use of dietary sources of preformed purines and pyrimidines will be considered. J. Nutr. 124: 124S–127S, 1994.

INDEXING KEY WORDS:

• *nucleotide* • *purine* • *pyrimidine*
• *biosynthesis* • *catabolism*

Purine and pyrimidine nucleotides are involved in almost all cellular processes and play a major role in structural, metabolic, energetic and regulatory functions. They make up the monomeric units of RNA and DNA; RNA synthesis is required for protein synthesis and DNA synthesis is required for growth and cell division. Adenosine triphosphate, an adenine nucleotide, is the major source of the chemical energy used in metabolism, driving almost all cellular processes. Nucleotides are physiological mediators in a number of metabolic processes. Cyclic adenosine monophosphate and cGMP regulate a large number of cellular events, and adenosine is important in regulating blood flow and smooth-muscle activity. Guanosine triphosphate is involved in signal transduction, RNA structure and microtubule formation. Many other nucleotides are involved in regulating other cellular processes.

Nucleotides function as activated intermediates in the synthesis of glycogen and glycoproteins; they are also intermediates in the synthesis of phospholipids and serve as methyl and sulfate donors. They are structural components of a number of coenzymes that are crucial to many metabolic pathways, and they function as allosteric effectors controlling the regulatory steps of major metabolic pathways. For general references on nucleotides see Blakely (1993), Henderson and Paterson (1973), Jones (1980) and Uauy (1989).

STRUCTURES

Nucleotides consist of a nitrogenous base (either a purine or a pyrimidine), a sugar and one or more phosphate groups. The term nucleotide in the context of the title of this article refers to the several forms in which purines and pyrimidines occur and implies not a specific form of the compounds but all forms that contain purine and pyrimidine bases.

The major purine and pyrimidine bases are shown in **Figure 1**. Uric acid, which is derived from purines, is also found in significant levels. Other pyrimidines and purines are also present in smaller amounts, and they have significant roles, particularly in RNA structure and function.

The components of a nucleotide can be a base, either a purine or pyrimidine, a sugar, and a phosphate. A nucleoside, which does not have a phosphate group, is formed from a base and a pentose through a glycosidic bond between the N-1 nitrogen of a pyrimidine or the N-8 of a purine and the C-1′ carbon of the pentose. The pentose is ribose or 2′-deoxyribose. The major function of the 2′-deoxyribose

[1]Presented at the symposium "Nucleotides and Nutrition" held in New Orleans, LA, March 28, 1993. The symposium was cosponsored by the American Institute of Nutrition and Wyeth-Ayerst International. Funding for the symposium and the publication of the supplement was provided through an educational grant from Wyeth-Ayerst International. Guest editor for this supplement was W. Allen Walker, Massachusetts General Hospital, 149 13th Street, Charleston, MA 02129.

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[3]Supported in part by grants no. GM42436 from the National Institutes of Health and no. C-1041 from the Robert A. Welch Foundation.

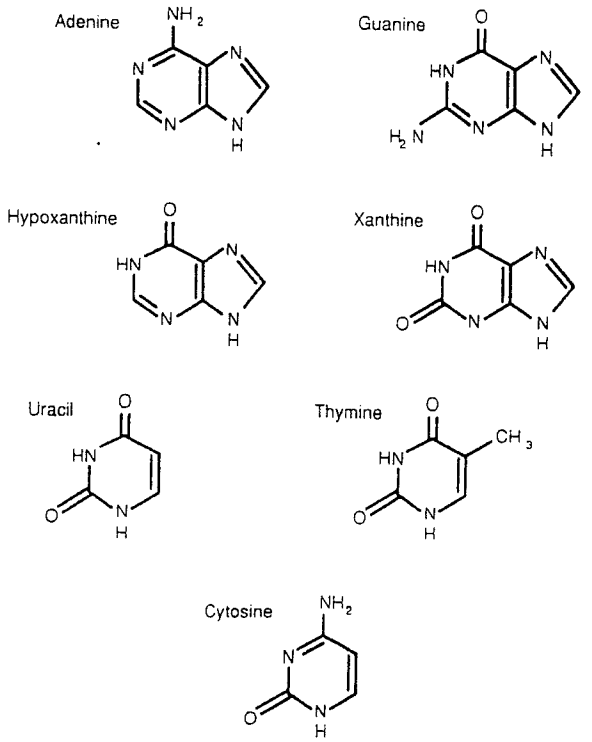

FIGURE 1 Structures of the major purine and pyrimidine bases.

nucleotides is as the structural components of DNA. The ribonucleotides are the monomeric units of RNA, but also serve in most other cellular and metabolic functions of nucleotides. The phosphoryl group of nucleotides is most commonly esterified to the C-5′ hydroxyl of the pentose. In cyclic nucleotides the phosphate is esterified to both the C-5′ and the C-3′ hydroxyl groups. The number of phosphate groups attached is indicated by the mono-, di- or tri- designation.

BIOSYNTHESIS, SALVAGE AND INTERCONVERSION OF NUCLEOTIDES

Purines and pyrimidines can be formed by de novo biosynthesis or by salvage of preformed bases and interconversion to the desired compound. The biosynthetic origin of the various atoms in purine and pyrimidine bases is shown in **Figure 2**. Almost all of the atoms in both bases are derived directly or indirectly from amino acids. Phosphoribosylpyrophosphate (PRPP) is the pentose source both for purine and pyrimidine biosynthesis and for salvage of bases. This compound is formed from ribose-5-phosphate. Deoxyribonucleotides are subsequently formed

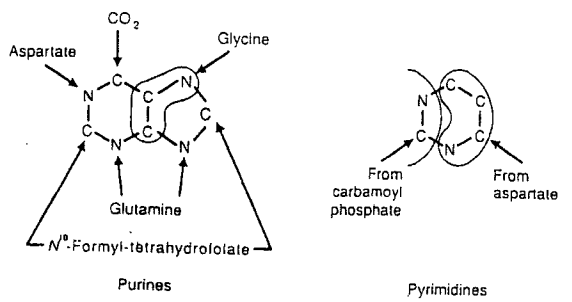

FIGURE 2 Biosynthetic origin of the atoms in purine and pyrimidine bases.

from the ribonucleotides. The pathway for purine biosynthesis consists of 10 steps, as illustrated schematically in **Figure 3**. The initial step involving PRPP and glutamine condensation that is catalyzed by PRPP aminotransferase is likely the rate-limiting step and is feedback-inhibited by AMP and GMP. Inosine monophosphate is the first purine formed, and it is converted to either AMP or GMP depending on the needs of the cells. Regulation occurs at these steps also. The monophosphates of both purines and pyrimidines are readily converted to diphosphates and triphosphates by various kinase enzymes that use ATP as a phosphate source.

Pyrimidine biosynthesis is illustrated in **Figure 4**. Phosphoribosylpyrophosphate is not added until the intact pyrimidine is formed as orotic acid. Orotidine-5′- monophosphate (OMP) is the first pyrimidine formed, but its function in the cell is only as a precursor of other pyrimidines. Uridine monophosphate is formed from OMP and then CTP and TTP are derived from UMP. In eukaryotes, regulation of pyrimidine synthesis occurs primarily at carbamoyl phosphate synthesis with inhibition by pyrimidine nucleotides and activation by purine nucleotides.

Deoxyribonucleotide synthesis is catalyzed by ribonucleotide reductase, an enzyme that converts both purines and pyrimidines to their deoxyribose forms. The reductase is controlled in a complex manner by both substrates and products to allow synthesis of equimolar levels of the various deoxyribonucleotides (Mathews et al. 1988). Because the deoxynucleotides are used only for DNA synthesis, the levels of the purines and the pyrimidines must be equal. Thymidine triphosphate is then formed as the monophosphate from dUMP. The levels of the deoxyribonucleotides are typically in the range of 2–60 μmol/L (Blakely 1993) while the levels of the ribonucleotides are typically much higher, with ATP concentrations in the range of 2–10 mmol/L and concentrations of other ribonucleotides from 0.05 to 2

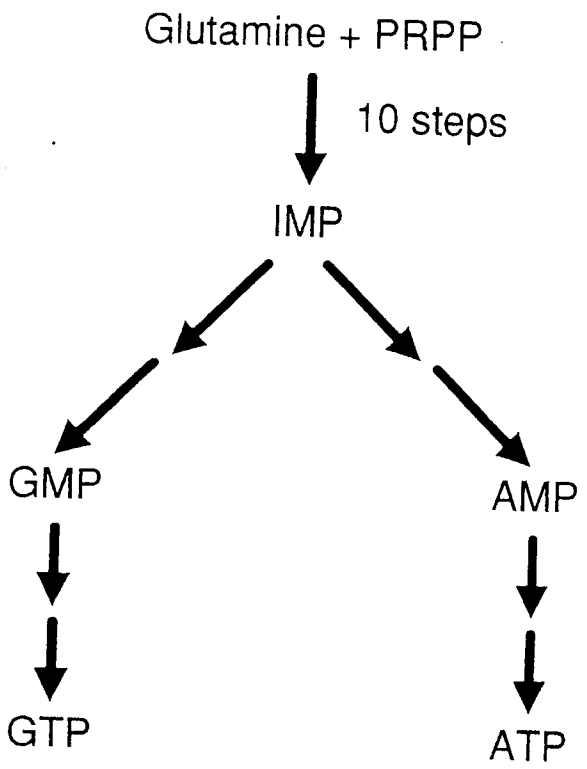

FIGURE 3 Schematic pathway for purine biosynthesis. PRPP = phosphoribosylpyrophosphate.

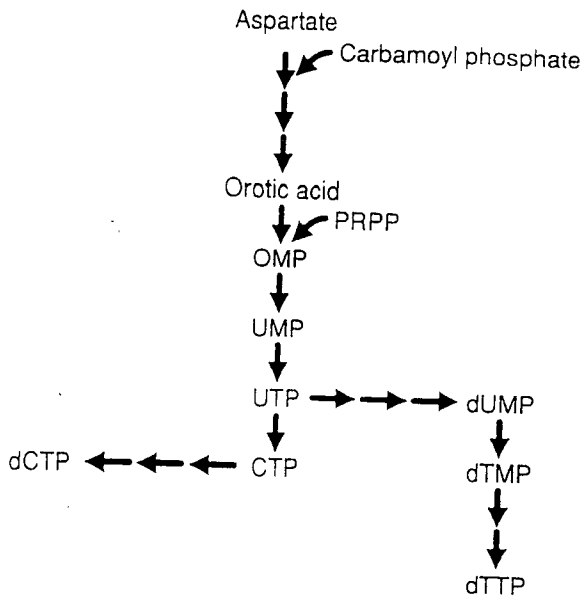

FIGURE 4 Schematic pathway for pyrimidine biosynthesis. PRPP = phosphoribosylpyrophosphate. OMP = orotidine-5'-monophosphate.

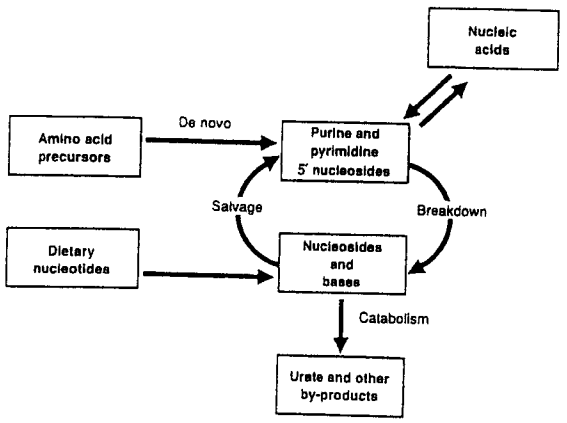

FIGURE 5 Pathways for nucleotide uptake, interconversion and degradation.

mmol/L. The levels of diphosphates and monophosphates are typically lower than those of the triphosphates. The levels of both ribonucleotides and deoxyribonucleotides will vary considerably, depending on the phase of the cell cycle and under various metabolic conditions.

Catabolism of purines and pyrimidines also occurs. In primates uric acid is the end product of purine catabolism, while other species can convert it to more-soluble forms. Gout results from elevated plasma levels of uric acid. The end products of pyrimidine catabolism are β-alanine and β-amino isobutyrate, both of which are soluble and easily excreted. Less is known about pyrimidine catabolism, because no clinical effects of the end products occur. The catabolic pathways operate in the digestive system, converting DNA and RNA and free nucleotides to nucleosides and free bases. Pyrimidine bases and nucleosides are taken up and readily incorporated into tissues (Sonoda and Tatibana 1978). Dietary nucleotides appear to be important in supporting cellular metabolism, particularly in rapidly dividing tissues such as lymphoid cells and the intestine (Rudolph et al. 1990, Uauy 1989).

The uptake of purines and pyrimidines from the intestine and from the cellular turnover of nucleotides particularly from mRNA provides preformed bases that avoid the metabolic cost of de novo biosynthesis. Synthesis of both purines and pyrimidines consumes a significant amount of energy.

The overall interactions involved in nucleotide uptake, synthesis, salvage and catabolism are illustrated in **Figure 5**. It is important to note the role of amino acids in nucleotide synthesis and the salvage of dietary and cellular sources of nucleotides. A balance exists between these different pathways, affording

proper levels of nucleotides in cells with minimal metabolic expense.

CLINICAL ASPECTS OF NUCLEOTIDE METABOLISM

A number of metabolic inhibitors for purine and pyrimidine pathways have important clinical uses (for a general discussion, see Blakely 1993). Such inhibitors are often toxic to cells, as they interfere with fundamental pathways. These include 6-mercaptopurine, tiazofurin, 5-fluorouracil, hydroxyurea, azaserine, sulfonamides, methotrexate and trimethoprim. A number of new drugs, including zidovudine, didanosine, acyclovir and cytarabine, that interfere specifically with viral DNA polymerases and thereby inhibit viral replication are being used to treat viral infections. Among the disease states that involve nucleotide metabolism are gout, severe combined immunodeficiency disease, Lesch-Nyhan syndrome and orotic aciduria.

CONCLUSIONS

Nucleotide metabolism is a crucial part of most cellular processes. Recent studies indicate requirements for dietary sources of nucleotides that were not previously recognized. These results suggest that future studies to further define the roles of nucleotides as well as the effects of various drugs on nucleotide metabolism are required. This should be an area of fruitful research in the future.

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Dietary Sources of Nucleotides—From Breast Milk to Weaning[1,2]

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ABSTRACT Human breast milk contains large quantities of nucleotides compared with those in cow's milk and infant formulas. Dietary nucleotides may have significant effects in infants. J. Nutr. 124: 128S–130S, 1994.

INDEXING KEY WORDS:

- humans • nucleotides
- breast milk • infant formulas

Purine and pyrimidine nucleotides are important metabolites that participate in many biological functions. They form the basis of genetic information (DNA, RNA), serve as energy stores (AMP, GMP, etc.), mediate hormonal action (cAMP), participate in immunity (adenosine deaminase) and are essential in other cellular actions. Nucleotides are one of the nonprotein nitrogen constituents of human milk (Table 1).

POTENTIAL NEED FOR DIETARY NUCLEOTIDES

Nonprotein nitrogen accounts for ~20% of the nitrogen in human milk and ~5% of the nitrogen in cow's milk. The largest nonprotein nitrogen constituent is urea, the nutritional function of which is not known. Many of the other nonprotein nitrogen constituents are important biologically and are usable when ingested. Free and cellular nucleotides (primarily CMP and AMP) account for ~2–5% of the nonprotein nitrogen in human milk. Nucleotides are present in human milk in large amounts compared with those in the milks of other species. The function of nucleotides in immunity and their presence in human milk has led to exploration of their possible dietary necessity for human infants.

De novo synthesis of nucleotides from amino acids, ribose, formate and CO_2 requires considerable energy. The salvage pathway to create new nucleotides from nucleotide fragments or from external sources requires less energy than from de novo synthesis. Inconsistency in measuring nucleotides in biological fluids, uncertainty regarding absorption of dietary nucleotides in infants and older children and lack of knowledge about the local effects of dietary nucleotides on intestinal lymphocytes are among the stimuli for further studies with human infants.

CONCENTRATION OF NUCLEOTIDES IN VARIOUS TISSUES

The concentration of nucleotides varies in different tissues. In RBC, for example, adenine compounds predominate. In the liver, uridine and other nucleotides are more prominent. In all cells, concentrations of RNA are higher than those of DNA, the former being ~1000 times more concentrated than DNA and relatively constant while DNA concentration varies with the stage of the cell cycle.

Sanguansermsri et al. (1974) reported DNA levels of 10–120 mg/L and RNA levels of 100–600 mg/L in human milk from d 5 to wk 8 of lactation. Ho et al. (1979) noted little change in the absolute cell counts or the proportion of leukocyte types from the

[1]Presented at the symposium "Nucleotides and Nutrition" held in New Orleans, LA, March 28, 1993. The symposium was cosponsored by the American Institute of Nutrition and Wyeth-Ayerst International. Funding for the symposium and the publication of the supplement was provided through an educational grant from Wyeth-Ayerst International. Guest editor for this supplement was W. Allen Walker, Massachusetts General Hospital, 149 13th Street, Charleston, MA 02129.

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TABLE 1

Compounds contributing to nonprotein nitrogen of human milk[1]

	Nitrogen	
	<30 d of lactation	>30 d of lactation
	μmol/L	
N-Acetylglucosamine	16,400	10,700
Urea	10,500	7900–12,800
Free amino acids	3100	2600–4300
N-Acetylneuraminic acid	4500	210–1900
Peptides	—	1200–4300
Choline and ethanolamine	500–1400	700–1400
Nucleic acids	—	1400
Creatinine	—	500–800
Creatine	—	800
Uric acid	—	300
Nucleotides	210	210
Ammonia	140	140
Carnitine	70	50
Polyamines	7	14
Cyclic nucleotides	2–14	2–14

[1]Adapted from Carlson (1985).

prepartum period to the immediately postpartum period. The total cell count dropped sharply as lactation became established, and the percentage of macrophages increased as that of polymorphonuclear leukocytes decreased. The lymphocyte percentage remained constant. Over the first 2 wk, however, although the cell count dropped, the volume of colostrum ingested increased; hence the total number of leukocytes ingested by the infant remained essentially constant. Janas and Picciano (1982) reported the nucleotide content of human milk over 3 mo and found relatively little variation in the concentrations of UMP, GMP, UDP, CDP, ADP and GDP over that period. The concentration of CMP and AMP decreased and that of IMP increased over the same period.

Gil and Sanchez-Medina (1981 and 1982) measured the nucleotide content of the milks of humans, cows, goats and sheep (Tables 2 and 3). Many nucleotides were in lower concentration than that of human milk, with the lowest being that of cow's milk. Orotate is the major nucleotide in cow's milk, and this nucleotide is apparently poorly salvageable by human infants.

As other foods are added to the diet of an infant, any that contains cellular elements provides dietary nucleotides. Organ meats, fish and poultry are especially rich sources of nucleoprotein. The increase in urinary uric acid following the consumption of such foods is presumptive evidence that dietary nucleotides are absorbed (Wilson et al. 1954). Fruits, vegetables and milk products are poor sources of nucleotides.

SUMMARY

Nucleotides are present in human milk in larger quantities than in cow's milk or infant formulas and the nucleotide profiles vary. Nucleotide-supplemented infant formulas are commercially available. Further study of the absorbability of exogenous nucleotides and their function as dietary supplements is being pursued.

TABLE 2

Nucleotides in pooled human colostrum at different stages of lactation[1]

	Nucleotide					
	48 h	72 h	6 d	15 d	1 mo	3 mo
			μmol/L			
CMP	55.1	34.5	31.0	26.4	18.7	18.3
AMP	33.4	24.1	22.4	26.0	20.2	15.1
GMP	3.3	3.6	5.0	ND[2]	3.2	ND
UMP	17.7	13.2	14.9	7.0	12.9	9.3
GDP-mannose	5.3	9.7	5.4	4.6	4.6	4.4
UDP-Ac-hexosamine	4.5	35.7	23.2	31.4[3]	19.6	22.0
UDP-hexose	ND	ND	13.1	ND	8.2	10.1
UDP	14.1	6.8	5.3	4.0	7.6	6.5

[1]Adapted from Gil and Sanchez-Medina (1982).
[2]ND = not detected.
[3]UDP-Ac-hexosamine + UDP-hexose.

TABLE 3

Nucleotide monophosphates in milk from cow, goat and sheep at four stages of lactation[1]

	Nucleotide monophosphate			
	0–1 d	1–2 d	5–10 d	1 mo
		μmol/L		
AMP				
Cow	39.7 ± 7.5	61.8 ± 4.9	41.9 ± 2.9	27.5 ± 1.4
Goat	37.8 ± 4.8	47.0 ± 3.1	62.7 ± 4.4	61.0 ± 6.5
Sheep	210.9 ± 32.3	297.3 ± 8.0	144.2 ± 13.2	93.6 ± 5.5
GMP				
Cow	ND	ND	ND	ND
Goat	ND	ND	ND	ND
Sheep	11.5 ± 4.3	34.6 ± 4.3	4.9 ± 3.1	ND
CMP				
Cow	31.9 ± 4.1	52.5 ± 5.1	47.4 ± 4.1	33.2 ± 2.4
Goat	40.6 ± 4.4	64.5 ± 6.8	53.3 ± 3.3	49.1 ± 3.0
Sheep	167.9 ± 18.8	327.5 ± 27.9	150.2 ± 1.6	74.3 ± 5.1
UMP				
Cow	186.3 ± 50.1	390.0 ± 54.3	31.2 ± 7.4	ND
Goat	59.9 ± 8.6	537.7 ± 7.6	120.1 ± 6.0	143.3 ± 6.2
Sheep	1022.8 ± 48.7	1133.0 ± 70.1	584.6 ± 27.4	250.9 ± 26.0

[1]Values are means ± SEM, n = 4–7. ND = not detected. Adapted from Gil and Sanchez-Medina (1981).

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Nucleotide Uptake and Metabolism by Intestinal Epithelial Cells[1,2,3]

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ABSTRACT The epithelial cells of the gastrointestinal tract are the first to encounter ingested nucleotides. Enterocytes metabolize or transport nucleotides (often partially metabolized) to other cell types. Nucleotides may also affect enterocyte gene expression. These interactions in intestinal cell lines (Caco-2 and IEC-6 cells) are described. Nucleotides and nucleosides are efficiently taken up by neoplastic cells (Caco-2) and substantially metabolized during absorption by epithelial monolayers. In nonmalignant cells (IEC-6), nucleotide pools are smaller than enterocytes of neoplastic origin (Caco-2). Consequently, cell proliferation in IEC-6 cells is more dependent on an external supply of nucleotides. Cell differentiation was examined by measuring brush border enzyme activities (sucrase, lactase and alkaline phosphatase). Nucleotides enhanced the expression of brush border enzymes in carcinoma cells only when stressed by glutamine deprivation. IEC-6 cells, which are poorly differentiated in optimal media, require basement membrane (Matrigel) for expression of brush border enzymes. Under these conditions, adding nucleotides to the culture medium enhanced enzyme activity. In addition to being substrates for intestinal absorption, nucleotides may affect enterocyte differentiation. J. Nutr. 124: 131S–137S, 1994.

INDEXING KEY WORDS:

- *small intestine* • *purine*
- *pyrimidine* • *nucleotides*

The importance of nucleotides in human nutrition is now an area of intensive research. Nutritional requirements are most crucial in the very young, and there has been much recent interest in the role of nucleotides in infant nutrition. Nucleotides are present in breast milk, and their uptake enhances a number of immunologic as well as nonimmunologic functions. Understanding how the nucleotides in breast milk exert their activity requires knowing their interaction with enterocytes. The gastrointestinal tract develops under the influence of nutritional and hormonal factors. In vivo studies have shown that nucleotides have a role in gastrointestinal development (Pita et al. 1988, Uauy 1989, Uauy and Stringel 1988, Uauy et al. 1990) and may be beneficial to the function of enterocytes after repair (Quan et al. 1990). Indeed, the nucleotide content may be an important factor by which dietary intake influences gene expression in the intestinal epithelium (Sanderson and Walker 1993).

In this article we will review work performed in our laboratory that examined how nucleotides affect the proliferation and differentiation of intestinal cell lines (He et al. 1993). We will also review work that examined the uptake of nucleosides by enterocyte cell lines (He et al. unpublished data) and will conclude by outlining how these studies could be developed into future research.

EFFECTS OF NUCLEOTIDE SUPPLEMENTATION ON THE PROLIFERATION AND DIFFERENTIATION OF CULTURED CACO-2 AND IEC-6 CELLS

[1]Presented at the symposium "Nucleotides and Nutrition" held in New Orleans, LA, March 28, 1993. The symposium was cosponsored by the American Institute of Nutrition and Wyeth-Ayerst International. Funding for the symposium and the publication of the supplement was provided through an educational grant from Wyeth-Ayerst International. Guest editor for this supplement was W. Allen Walker, Massachusetts General Hospital, 149 13th Street, Charlestown, MA 02129.

[2]The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

[3]This work was supported by grants from the National Institutes of Health (R01-HD-12437 and PO1-DK33506) and by grants from Wyeth-Ayerst Research Division of Clinical Nutrition and Critical Care of America. Dr. Sanderson is supported by the Crohn's and Colitis Foundation of America.

In our studies we used a human colon carcinoma cell line, Caco-2, that undergoes spontaneous differentiation in culture after having reached confluence, making it useful as an in vitro model for studying the intestinal differentiation and transport function (Hidalgo et al. 1989) and for morphological characterization, including studying polarity during enterocyte development (Traber et al. 1987). The capacity for de novo and salvage pathway synthesis of nucleic acids in cancer cells is increased with a concurrent decrease in the activity of key enzymes in nucleotide degradation, suggesting that nucleobases are reused more efficiently in neoplastic than in nonneoplastic cells (Holley 1972, Weber 1983). Therefore, although Caco-2 cells may be one of the best models available for studying various aspects of enterocyte differentiation, they may not be appropriate for defining the needs of normal enterocytes for exogenous nucleotides during development. Consequently, a normal rat small intestine epithelial cell line, IEC-6, (Quaroni et al. 1979) was used for studying the effects of nucleotide supplementation on the proliferation and differentiation of normal enterocytes. This cell line exists as an undifferentiated crypt cell in culture; however, cellular maturation is induced when these cells are grown on a collagen matrix (Matrigel, Collaborative Research, Bedford, MA) harvested from Engelbreth-Holm-Swarm sarcoma tissue (Carroll et al. 1988).

The Caco-2 and IEC-6 cell lines were used for studying the absorption of exogenous nucleotides by enterocytes and the effects of nucleotides on cell proliferation and differentiation. In particular, we examined how nucleotides could compensate for the stress induced by the removal of both glutamine and nonessential amino acids (He et al. 1993).

MATERIALS AND METHODS

The techniques used for studying these effects are shown in **Table 1**. The cellular uptake of nucleotides was determined by using radiolabeled AMP and the cellular pools of nucleotides and their metabolites were determined by using HPLC. Solubility in trichloroacetic acid was used for distinguishing nucleic acid from nucleotides, nucleosides and nucleobases. Cell proliferation was determined by counting the cells in a hemocytometer. Cell differentiation was determined by measuring the activity of brush border enzymes.

UPTAKE AND USE OF NUCLEOTIDES

The results (He et al. 1993) showed that Caco-2 cells take up nucleotides from the culture medium in a linear fashion until a nucleotide concentration of 1500 μmol/L. The process was rapid, with the rates of uptake remaining similar at 2 h (**Fig. 1**). The uptake of AMP by Caco-2 cells decreased at higher concentrations, indicating saturation.

TABLE 1

Methods for determining the effects of nucleotides on enterocyte proliferation and differentiation[1]

Parameter	Method
Nucleotide uptake	Cellular uptake of [^{14}C]AMP
Cell proliferation	Alternate-day cell counts
Levels of nucleotides and metabolites in cellular extract	HPLC
Enterocyte differentiation	Brush border enzyme activity

[1]Adapted from He et al. (1993).

The rate of uptake of AMP depended on the state of differentiation of the Caco-2 cells, and the uptake by undifferentiated cells was less efficient than that by differentiated cells (Fig. 1). On the basis of these experiments, it could be predicted that uptake would vary in different areas of the crypt-villus surface because the differentiation of epithelial cells in vivo proceeds as they pass along the crypt-villus axis. The difference in uptake between the differentiated and the undifferentiated cells was accounted for by the difference in uptake by the acid-soluble fraction. The incorporation of AMP into nucleic acids was similar in both states, but the differentiated cells expanded their soluble pool of nucleotides, nucleosides and nucleobases more rapidly than did the undifferentiated cells.

NUCLEOTIDE AND NUCLEOSIDE POOLS

Nucleotides are stored in enterocytes ready for use in many essential biochemical reactions, including the synthesis of nucleic acids and cellular metabolic functions. As shown in **Figure 2**, the pools of AMP, ADP and ATP are larger in differentiated cells than in undifferentiated cells and in cancerous cells (Caco-2) than in nonmalignant cells (IEC-6). Similar patterns can be seen for other nucleotides and nucleosides (He et al. unpublished data).

EFFECTS OF NUCLEOTIDE UPTAKE ON POOL SIZES

Because enterocytes contain soluble pools of nucleotides and their metabolites, it is relevant to ask how these pools might be affected by nucleotide

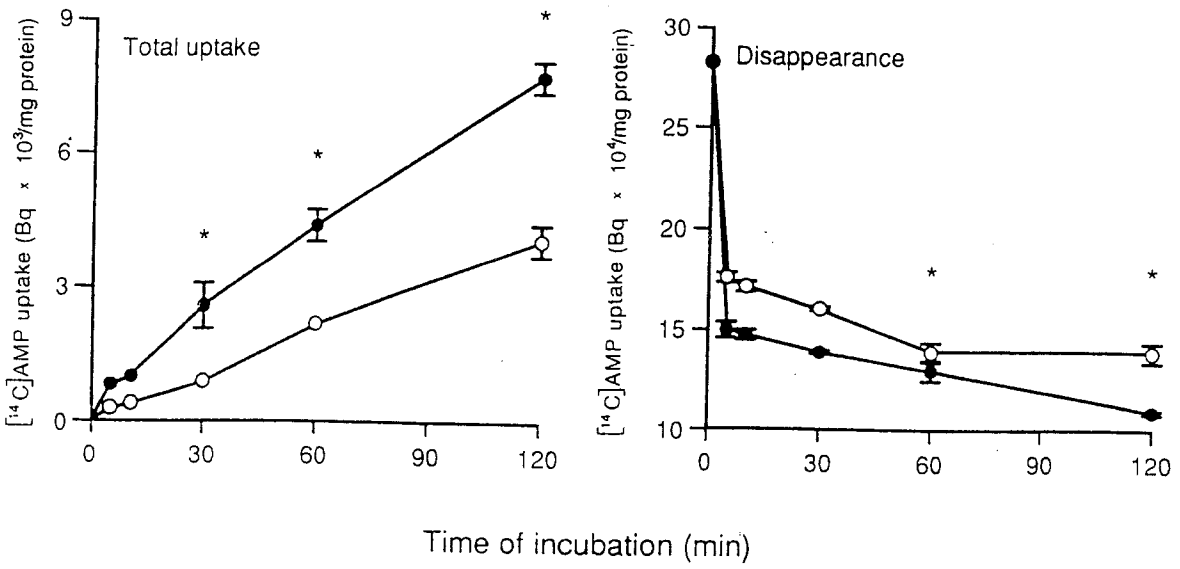

FIGURE 1 Changes in [8-^{14}C]AMP uptake by Caco-2 cell monolayers. [8-^{14}C]AMP was added to the medium at 30 μmol/L. The cells were incubated at 37°C and exposed to radiolabeled AMP for up to 3 h. Total, acid-soluble and acid-insoluble fractions of [8-^{14}C]AMP uptake were estimated at 30-min intervals. Values are means ± SD of three determinations. Solid circles (●), differentiated cells; open circles (○), undifferentiated cells. *P < 0.05 vs. undifferentiated cells.

uptake. We studied the effect of AMP uptake on the different fractions of the soluble pools and found increases in the levels of the metabolites of AMP in the pools. Different metabolites were increased in the acid-soluble pools of the IEC-6 and the differentiated and undifferentiated Caco-2 cells (**Table 2**). The products of AMP uptake were less degraded in the cancerous cells than in the IEC-6 cells, in which the levels of only xanthine and hypoxanthine were increased. Thus, the metabolites in the Caco-2 cell pools were in a form that could be more readily assimilated into essential biochemical pathways. These data suggest that, because they have smaller pools, nonmalignant (normal) enterocytes would be more dependent on an external supply of nucleotides. Normal enterocytes are less able to expand their pools in the face of AMP supplementation, and the pools themselves contain metabolites that are more catabolized than those in the pools of the malignant cells. We tested this hypothesis by subjecting cells to different levels of nutritional stress.

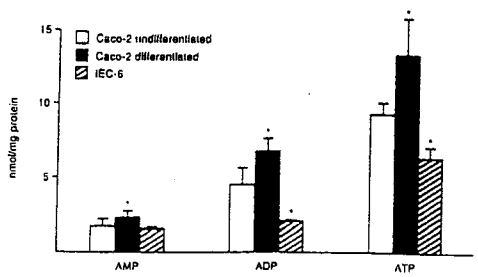

FIGURE 2 Pool sizes of AMP, ADP and ATP in Caco-2 and IEC-6 cells. *P < 0.05 vs. undifferentiated Caco-2 cells. Data from He et al. (1993).

TABLE 2

Effects of AMP supplementation on purine pools of Caco-2 cells and IEC-6 cells[1]

	Change in pool size		
	Undifferentiated Caco-2 cells	Differentiated Caco-2 cells	IEC-6 cells
		%	
AMP	150	162	
ATP	95	65	
β-NAD	209	52	
GDP	65		
Adenine		54	
Adenosine		49	
Hypoxanthine			70
Xanthine			114

[1]Data are shown only for significant increases (P < 0.05). Data from He et al. (1993).

EFFECTS OF NUCLEOTIDE SUPPLEMENTATION ON THE PROLIFERATION AND DIFFERENTIATION OF ENTEROCYTES

Nucleotides are salvaged into pools available for the synthesis of nucleic acids. We tested the hypothesis that, because of the differences in pool sizes discussed above, removing nucleotides from the incubation medium would have a greater effect on the nonmalignant cells than on the malignant cells. The dependency of cells on nucleotides was examined by subjecting them to nutritional stress by removing glutamine or nonessential amino acids or both. Equal amounts (10 mg/L each) of five nucleotides (AMP, CMP, GMP, IMP and UMP) were dissolved in standard culture medium that was constituted without fetal bovine serum. When glutamine and nonessential amino acids were present, adding nucleotides had no significant effect on the proliferation of Caco-2 cells (**Fig. 3***A*). However, nucleotide supplementation increased the proliferation of IEC-6 cells (Fig. 3*B*). Similarly, when cells were grown in the absence of nonessential amino acids, the Caco-2 cells showed no dependence on nucleotides (Fig. 3*C*) and the growth of the IEC-6 cells became more marked (Fig. 3*D*). Only when glutamine was removed from the culture medium did the nucleotides have an effect on the proliferation of the Caco-2 cells (Fig. 3*E*). When both nonessential amino acids and glutamine were removed, both cell lines required nucleotide supplementation to survive (Fig. 3*G* and 3*H*).

The effect of nucleotide supplementation on the differentiation of IEC-6 cells was evaluated by determining the changes in intestinal enzyme markers. Differentiation also depended on nucleotide supplementation (He et al. 1993). Caco-2 cells spontaneously differentiate in culture; therefore, the effects of nucleotides can be examined directly. Under conditions of nutritional stress, when glutamine was removed from the medium, their degree of differentiation was increased by nucleotide supplementation. IEC-6 cells, on the other hand, do not spontaneously differentiate, but brush border enzyme activity can be induced by growing them on Matrigel (Carroll et al. 1988).

After 4 d on Matrigel the IEC-6 cells began to differentiate, which was indicated by an increase in alkaline phosphatase and sucrase activity. As shown in **Figure 4**, the activities of alkaline phosphatase and sucrase increased over the period of incubation when the cells were plated on Matrigel. Significantly increased activities of both enzymes were noted over the next several days in the cells grown in the medium supplemented with nucleotides. In contrast, no changes in alkaline phosphatase activity were noted and no sucrase activity was detected in the IEC-6 cells grown on plastic plates under similar culture conditions, with or without nucleotide supplements.

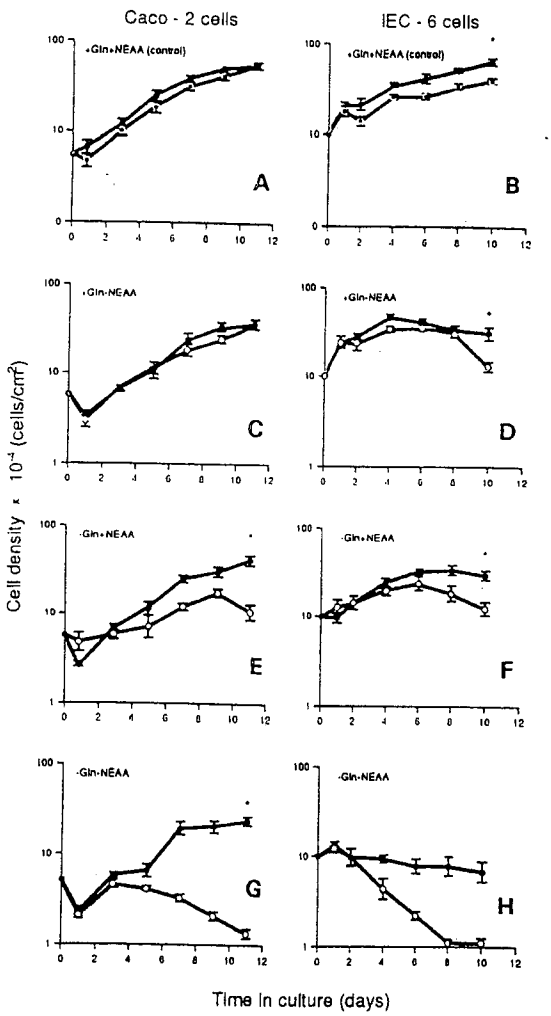

FIGURE 3 Effects of nucleotide supplementation on the growth of Caco-2 (*left*) and IEC-6 (*right*) cells. A nucleotide mixture (AMP, CMP, GMP, IMP and UMP, 10 mg/L each) was added to the culture medium on d 2 after seeding. The medium contained glutamine (Gln; 2 mmol/L), MEM nonessential amino acids solution (NEAA; 10 mL/L), both or neither. Serum-free medium (DMEM/MITO+) was used throughout the study for the Caco-2 cells. Dialyzed fetal bovine serum (5 mL/L) and insulin (1.72 μmol/L) were added to the serum-free medium for the IEC-6 cells. Values are means ± SD of three determinations. Significance levels were estimated by two-way ANOVA analysis at the end of the study. Open circles (○), no nucleotide supplementation; solid circles (●), with nucleotide supplementation. $^{*}P < 0.05$ vs. nonnucleotide-supplemented. Adapted from He et al. (1993).

These experiments show that the de novo biosynthesis of nucleotides is sufficient to support the proliferation of the neoplastic Caco-2 cell line but not

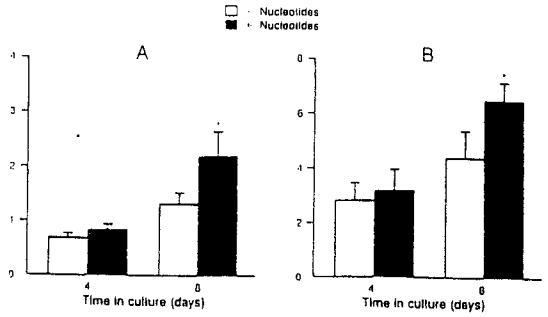

FIGURE 4 Effects of nucleotide supplementation on differentiation of IEC-6 cells grown on an extracellular matrix. IEC-6 cells ($1 \times 10^6/cm^2$), passages 15–17, were plated onto the Matrigel-coated six-well plastic plates in the standard medium containing 50 mL/L fetal bovine serum and 1.72 μmol/L insulin, with or without added nucleotides, at d 0. Enzyme activities were determined in the particulate fraction of the cells collected at d 4 and d 8. Values are means ± SD from two different studies. Enzyme activities are expressed as mU/mg protein for alkaline phosphatase (*A*) and as nU/mg protein for sucrase (*B*). One unit of activity is defined as 1 μmol of substrate hydrolyzed per min. $^*P < 0.05$ vs. nonnucleotide-supplemented. Data from He et al. 1993.

that of the normal rat cell line. Nucleotide supplements may therefore increase the growth and maturation of normal enterocytes as well as reduce their dependence on exogenous glutamine.

UPTAKE, TRANSPORT AND METABOLISM OF EXOGENOUS NUCLEOSIDES

The absorption, transport and use of exogenous nucleotides and nucleosides are complex processes. Both pyrimidine and purine nucleotides are constantly being synthesized and degraded as dictated by biological and physiological demands. The mechanism of nucleotide digestion and absorption has been principally studied in vitro by using the everted gut sacs of animals. The transport and uptake of purines and pyrimidines have also been studied in isolated rat intestine (Bronk and Hastewell 1988), in rabbit ileum (Harms and Stirling 1977), in cultured cell lines, such as Novikoff rat hepatoma cells (Marz et al. 1979, Plagemann et al. 1978, Zylka and Plagemann 1975) and in Chinese hamster ovary cells (Marz et al. 1979, Plagemann et al. 1978, Wohlhueter et al. 1978), as well as in other epithelial cell lines (Plagemann et al. 1978). There is no evidence for transcellular transport of nucleotides or nucleosides across Caco-2 cells. The high negative charge of nucleotide phosphate groups and the absence of general nucleotide transport systems limit the ability of most nucleotides to pass through cell membranes under physiological conditions. Nucleoside uptake is the major pathway for the entry of purines and pyrimidines into intestinal epithelial cells. Work performed in our laboratory has characterized the uptake and transport of six nucleosides, adenosine, guanosine, inosine, cytidine, uridine and thymidine, in two intestinal epithelial cell lines, Caco-2 and IEC-6 (He et al. unpublished data). Differentiated Caco-2 cells were used for examining transepithelial transport. Radiolabeled nucleotides at 30 μmol/L were added to the culture medium and samples were obtained at 20, 40, 60, 120 and 180 min (Fig. 5). Samples of the medium were collected from both the apical and the basolateral sides of the inserts and the cells were counted. The metabolites of the transported nucleosides from the apical to the basolateral side and from the basolateral to the apical side were determined by HPLC. The transport of purine and pyrimidine nucleosides across cultured Caco-2 monolayers was estimated from the cumulative radioactivity in the medium. In general, the transport of nucleosides from the apical to the basolateral side was faster than the reciprocal transport (Fig. 5), except with adenosine, for which the basolateral-to-apical transport was faster during the initial incubation. There were differences in the rate of transport of the different nucleosides, guanosine being the one most rapidly taken up. The relation of uptake with time also varied for each nucleoside.

METABOLITES OF TRANSPORTED NUCLEOTIDES

The amounts of nucleoside metabolites that were transported from both sides of the Caco-2 monolayers were estimated in samples of the medium by means of HPLC. Purine nucleosides were not transported intact across both aspects of the cell monolayers but appeared as nucleobases, including uric acid, hypoxanthine, xanthine and guanine, with no free purine nucleoside remaining in the medium. Similarly, pyrimidines were not transported intact, and appeared as nucleobase metabolites, except for small quantities of uridine (10–15%) that were released from both sides. The predominant metabolites of thymidine that appeared after basolateral-to-apical transport were thymine and uracil, but no thymidine was detected on either side. However, most of the metabolites transported, such as guanine and hypoxanthine, were in a form appropriate for reuse by cellular salvage pathways. The cumulative apical radioactivity from the basolateral side was less than that from the apical side, suggesting that the rate and the capacity of uptake and transport of nucleosides from the apical side were greater than those from the basolateral side.

In summary, nucleosides are transported across intestinal epithelial cells but are partially metabolized

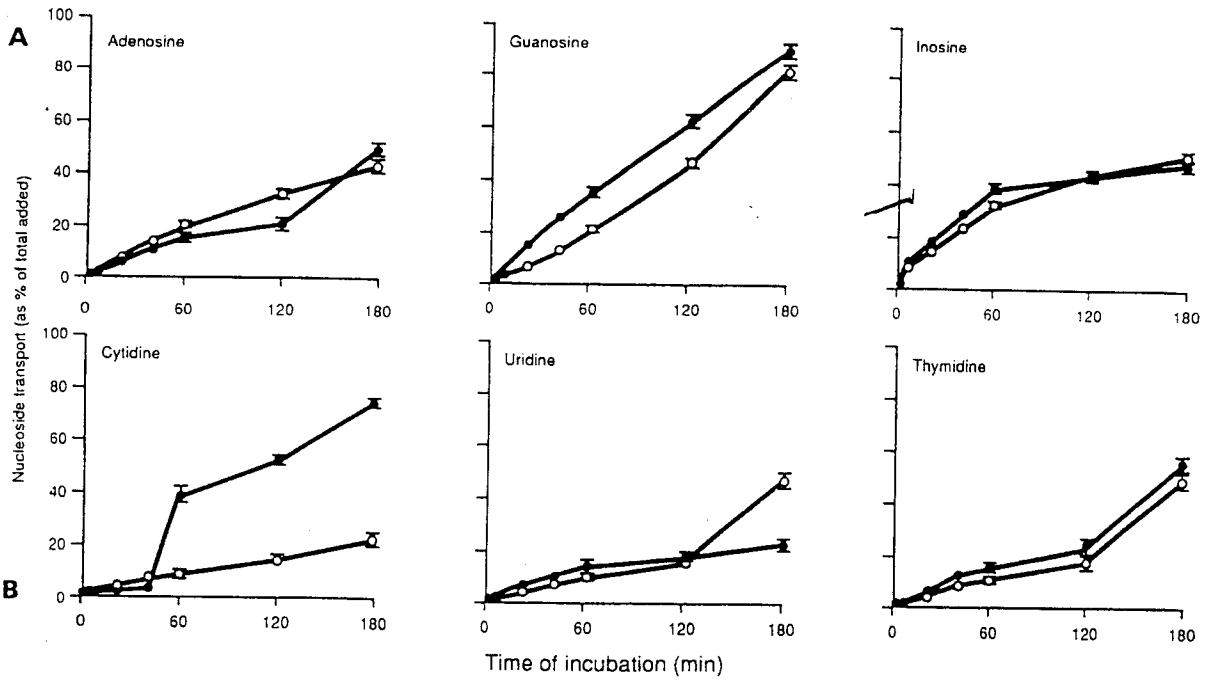

FIGURE 5 Transport of nucleosides across differentiated Caco-2 cell monolayers. The transport of nucleosides was calculated from the radioactivity in the medium samples and is expressed as a percentage of the nucleoside added. *A*, purine transport; *B*, pyrimidine transport; solid circles (●), apical-to-basolateral transport; open circles (○), basolateral-to-apical transport. He et al. (unpublished data).

en route. The metabolites transported are in a form that can be readily salvaged by other cell types after absorption by the intestinal epithelium.

FUTURE DIRECTIONS

The importance of the steps in the interaction between nucleotides and the intestinal epithelium has still to be determined. In particular, little is known about how these steps are influenced by growth processes or by disease. The role of brush border alkaline phosphatase in nucleotide transport is one area in which research is needed. Could alkaline phosphatase be important in the absorption of nucleotides by removing phosphate groups and thus rendering the nucleotides more available for transport? The tools to answer this question are now available. Alkaline phosphatase expression of Caco-2 cells could be reduced by transfecting cells with antisense mRNA for alkaline phosphatase. The ratios of nucleotide to nucleoside uptake by monolayers could then be compared with those in nontransfected or dummy-transfected Caco-2 cells. In vivo experiments to examine this question could be performed by using transgenic techniques; specifically, eliminating brush border alkaline phosphatase activity in transgenic mice by homologous recombination is theoretically possible. If such a technique were effective in reducing nucleotide absorption, further insights into the body's dependence on exogenous nucleotides could be gained.

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Nucleotide Actions on Humoral Immune Responses[1,2,3]

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ABSTRACT Previous studies indicate the importance of dietary nucleotides in maintaining optimal cellular immunity. Our studies using murine spleen cells showed that polynucleotides significantly increase in vitro antibody production in response to T-cell–dependent antigen. They seem to exert actions on T-helper cells at antigen presentation, perhaps during cognitive cell-cell interactions. They do not augment the actions of cloned, antigen-specific, activated T-helper cells, nor do they increase antibody production in response to T-cell–independent antigen or polyclonal B-cell activation. Polynucleotides increase in vitro human immunoglobulin production in response to T-cell–dependent stimuli and antigen. Humoral immune responses to T-cell–dependent antigen were depressed in mice fed a nucleotide-free diet, but were restored by a mononucleotide-nucleoside mixture. Responses to T-cell–independent antigen remained intact in these mice. The mononucleotide-nucleoside mixture had no effect on in vitro antibody production and did not further increase humoral immune responses in mice fed regular lab chow. These results suggest that the in vivo actions of polynucleotides on humoral immune responses may reflect local immune responses, perhaps at the site of inflammation. Mononucleotides and nucleosides may be incorporated into the tissue nucleotide pool fairly rapidly in a state of relative nucleotide deficiency and help restore T-cell–dependent humoral immune responses. Our findings may further support the importance of dietary nucleotides. J. Nutr. 124: 138S–143S, 1994.

INDEXING KEY WORDS:

- *nucleotide* • *T-cell–dependent*
- *humoral* • *T-helper cell*
- *immunity*

Nucleotides and their metabolites are key components in several processes in the human body. Because of active de novo synthesis of nucleotides, mainly in the liver, healthy persons appear to be almost independent of exogenous nucleotides, using <5% of their dietary nucleotides. However, the body's requirements for exogenous nucleotides may vary considerably and may increase under certain conditions. For example, a need for exogenous nucleotides may be expected when recovering from major tissue injury, such as in major surgery, systemic infection or extensive burn injury, or when the body is growing rapidly, such as in early infancy or the growth spurt of adolescence. The body's requirements for dietary nucleotides may also increase considerably when the liver function is suppressed. In rats fed a nucleotide-free diet, a significant decrease in the RNA and protein levels in the small intestine has been shown, indicating the preservation of the DNA content at the expense of the RNA and protein pools in the intestine in the state of relative nucleotide deficiency (Leleiko et al. 1987).

Human breast milk is known to contain a significant amount of nucleotides, which are implicated in the increased growth of the gastrointestinal tract in breast-fed infants (Uauy 1989). These findings may further indicate the importance of dietary nucleotides in maintaining optimal functions of the body in certain conditions.

The importance of dietary nucleotides to the immune system is not well understood. However, lymphocytes, a key component of the immune system, are not capable of effectively synthesizing nucleotides when in the G_1 phase of the cell cycle, and they depend on nucleotides synthesized de novo

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by other organs, mainly the liver (Rudolph et al. 1990). Consequently, under stressful conditions such as those described above, dietary nucleotides may have a significant role in maintaining optimal lymphocyte functions when the body's requirements for nucleotides surpass what is available through de novo synthesis.

The immune system is generally divided into two major components: cellular and humoral immunity. It is important that both systems function properly for the body to effectively fight invading pathogens and suppress tumorigenesis. In a series of studies, Rudolph and colleagues showed that feeding animals a nucleotide-free diet for a prolonged period could significantly suppress their cellular immunity (Rudolph et al. 1990, Van Buren et al. 1985 and 1987). Through studies employing murine spleen cells, we have also demonstrated that yeast RNA preparations strikingly increase in vitro antibody production in response to T-cell–dependent antigens (Jyonouchi et al. 1992). This finding led us to further investigate the effects of nucleotides on the humoral immune responses in both animals and in vitro human systems. In this review I briefly summarize the results of these studies.

IN VITRO ACTION OF NUCLEOTIDES ON HUMORAL IMMUNE RESPONSES IN EXPERIMENTAL ANIMAL MODELS

When murine spleen cells were primed with T-cell–dependent antigens, the numbers of antibody-producing cells detected by a plaque-forming-cell assay increased strikingly in RNA-supplemented culture conditions (Jyonouchi et al. 1992, Jyonouchi et al. 1993a). These actions of yeast RNA were also observed in several normal strains of mice, and were nullified by the depletion of T cells (Jyonouchi et al. 1993a). In contrast, antibody production in response to T-cell–independent antigens was not significantly increased in the presence of RNA (Jyonouchi et al. 1993a). Polyclonal B-cell activation induced by lipopolysaccharide, a nonspecific B-cell stimulant, was not increased in RNA-supplemented culture conditions, and spontaneous polyclonal B-cell activation was observed in autoimmune-prone strains of mice (Jyonouchi et al. 1994). These results may indicate that yeast RNA preparations exert their actions partly through T cells. To further study the mechanisms of these enhancing actions of RNA, we examined the following: *1*) which components of yeast RNA preparations are responsible for this action, *2*) which T-cell subsets are affected, and *3*) what T-cell functions are modified in RNA-supplemented culture conditions.

The fetal calf serum employed in the in vitro antibody-production assay contained degraded products of RNA, mainly in the form of nucleosides and nucleic acid. Because RNA exerted these enhancing actions in fetal calf serum–supplemented culture conditions, its action on in vitro production of antibodies is likely to be attributed to the larger components of RNA. The results indicate that the action of yeast RNA on specific antibody responses (to T-cell–dependent antigens) is largely attributable to polynucleotides. This conclusion is based on the following findings: *1*) the action of RNA was nullified by ribonuclease treatment but not by deoxyribonuclease treatment (Jyonouchi et al. 1992); *2*) the yeast RNA preparations employed were already degraded to nucleotides smaller than 1000 base pairs (molecular weight, <33,000); *3*) chemical modification or degradation of RNA significantly reduced its actions, and *4*) removing small oligonucleotides (molecular weight, <1000) did not reduce the actions of RNA (Zhang et al. 1993).

These results naturally raise the question of how polynucleotides could modulate specific antibody responses in vitro, because it is very likely that they are degraded rapidly by the ribonuclease that is abundant in cell membranes. In subsequent studies, we have found that depleting T-helper cells with antibody to L3T4 (equivalent to human CD4) plus complement completely nullifies the actions of polynucleotides and that polynucleotides do not enhance in vitro antibody production if added at d 3 or later to the culture of antigen priming (Jyonouchi et al. 1993a). It was also shown that this increasing action of polynucleotides requires cognitive cell-cell interactions between T cells and other lineage cells (Jyonouchi et al. 1994). It was also inhibited by antibody to the CD18/CD11 molecule, one of the adherent molecules expressed on the cell surface of lymphocytes and macrophage-monocyte lineage cells (**Table 1**). The CD18/CD11 molecules consist of α and β chains, and their expression is significantly upregulated upon the activation of T cells (Springer 1990). The antibody to CD18 reacts to the β chain of this molecule; the antibody to CD11a reacts to the α chain of this molecule expressed on lymphocytes, and the antibody to CD11b reacts to the α chain of this adherent molecule expressed on macrophage-monocyte lineage cells (Springer 1990). That antibody to the CD11a molecule expressed on lymphocytes blocked the actions of polynucleotides indicates that polynucleotides may directly exert their actions on T-lineage cells. These results also suggest that the actions of polynucleotides on in vitro antibody production depend on the presence of T-helper cells at the initial stages of antigen presentation. It may also be speculated that polynucleotides modulate humoral immune responses by interacting with cell-surface molecules of T cells or other lineage cells, perhaps in the processes of cognitive cell-cell interactions.

We have also observed that when murine spleen cells are briefly incubated with polynucleotides in the absence of antigens before the culture of antigen priming, T-helper cells appear to be activated nonspecifically, resulting in nonspecific increase of antibody

TABLE 1

Effects of antibodies to CD18, CD11a and CD11b on the actions of polynucleotides on in vitro antibody production[1]

Cells primed with	In vitro antibody production in the presence of			
	—	anti-CD18	anti-CD11a	anti-CD11b
	PFC/10^6 viable cells			
—	0	n.d.	n.d.	n.d.
Sheep RBC (0.05%)	50	32	22	77
Sheep RBC (0.05%) + RNA (100 mg/L)	1244	343	213	1215

[1]In vitro antibody production in response to sheep RBC was examined in the presence of yeast RNA preparations and antibodies to mouse CD18, CD11a and CD11b (25 mg/L). Spleen cells were obtained from 2-mo-old female B6 mice and were subjected for in vitro antibody production, as previously reported (Jyonouchi et al. 1992). These are the results of one representative experiment. n.d. = not done; PFC = plaque-forming cells.

and immunoglobulin production irrespective of the antigen stimulus employed (**Table 2**). Thus, the immunomodulating actions of polynucleotides on humoral immune responses may depend on the nature of the antigen (T-cell–dependent vs. –independent) and on the presence or absence of antigen stimulus in vitro. We have hypothesized that, in the presence of antigen stimulus, nonspecific T-cell activation by polynucleotides may be suppressed in the initial stages of antigen presentation, perhaps to facilitate specific antibody responses.

It was reported previously that double-stranded RNA could induce a significant production of interferon gamma in an antigen-independent manner (Levy and Salazar 1992). Some of the effects of polynucleotides we have observed could be attributable to the changes in certain cytokines, including interferon-γ. Therefore, we also studied the levels of total immunoglobulins and certain cytokines, including interferon-γ, in the culture supernatant of antigen priming. However, the results of such studies were inconclusive, showing only a moderate increase in the production of immunoglobulin M and subtle changes in the production of interferon-γ in an RNA-supplemented culture (unpublished observations). To further investigate the actions of polynucleotides on specific antibody responses in detail, we then turned to cloned, antigen-specific, T-helper cells. In rodents, the presence of T-helper cell subsets is now firmly established, on the basis of the patterns of lymphokine production (Mosmann and Coffman 1989, Swain et al. 1991). Namely, resting T cells produce only interleukin-2, activated type 1 T-helper cells produce interleukin-2 and interferon-γ and mainly participate in cellular immune responses and type 2 T-helper cells produce interleukin-4, interleukin-5 and interleukin-10 and are mainly involved in humoral immune responses (Mosmann and Coffman 1989, Swain et al. 1991). We have used cloned type 1 T-helper cells (A.E.7 cells, provided by Marc K. Jenkins, University of Minnesota, specific for pigeon cytochrome C, MHC-restricted to B10 mice [Mosmann et al. 1986]) and type 2 T-helper cells (CDC35 cells, provided by D. C. Parker, University of Massachusetts, specific for rabbit gamma globulin, MHC-restricted to DBA/2 mice [Tony et al. 1985]) for that purpose. By using these two cloned T cells, we initially examined *1*) changes in cytokine production, *2*) changes of helper functions for antibody and immunoglobulin production by host spleen B cells and *3*) proliferation of cloned T-helper cells upon antigen stimulus. Murine spleen cells obtained from DBA/2 and B10 mice were used as the source of resting T cells. To compare the specific antibody responses in this setting, we primed the cells with either rabbit gamma globulin or pigeon cytochrome C, and the specific antibody responses to these antigens were assessed by detecting antigen-specific immunoglobulin M- and immunoglobulin G-secreting cells with antigen-specific solid-phase enzyme-linked immunospot assay (Czerkinsky et al. 1983, Sedgwick and Holt 1983).

Our preliminary results are summarized in **Table 3**. When T-helper cells are already activated, polynucleotides do not appear to further increase specific antibody responses. Interestingly, the proliferation of T-helper cell clones was moderately suppressed in the presence of polynucleotides. While nonspecific immunoglobulin M and immunoglobulin G production was significant in the presence of activated cloned T-helper cells, it was again moderately suppressed in the presence of polynucleotides. In contrast, when murine spleen cells were stimulated with these antigens, the numbers of antigen-specific immunoglobulin-secreting cells increased significantly in the presence of polynucleotides. Most T cells in the spleen cell suspension are considered to be resting T cells. Therefore, these preliminary results may further support our initial hypothesis that polynucleotides suppress nonspecific activation of T cells in the presence of antigen stimulus. They may also indicate that polynucleotides increase specific

TABLE 2

Effects on antibody and immunoglobulin M production of preincubation of cells with RNA before culture of antigen priming[1]

Cells primed with	Unseparated cells preincubated with —	Unseparated cells preincubated with RNA (100 mg/L)	T-helper cell-depleted cells preincubated with RNA (100 mg/L)
		Antibody production *PFC/10^6 viable cells*	
—	23 ± 5	71 ± 20	3 ± 1
Sheep RBC (0.05%)	94 ± 11	784 ± 129***	3 ± 2
TNP-LPS (2 mg/L)	97 ± 26	274 ± 67 *	87 ± 22
		Immunoglobulin M production *μg/L*	
—	110.2 ± 17.4	213.1 ± 22.4**	49.8 ± 14.4
Sheep RBC (0.05%)	105.7 ± 23.8	178.4 ± 42.6	38.5 ± 15.5
TNP-LPS (2 mg/L)	233.3 ± 48.3	281.5 ± 44.4	137.3 ± 24.7

[1]Values are means ± SEM for levels in the culture supernatant. Murine spleen cells were obtained from 2- to 3-mo-old female B6 mice. Unseparated or L3T4$^+$ (CD4) T-helper cell–depleted cell suspensions were incubated with RNA for 3 h before the culture, washed extensively and then subjected for in vitro antibody and immunoglobulin M production. PFC = plaque-forming cells; TNP-LPS = total parenteral nutrition–lipopolysaccharide. *P < 0.05, **P < 0.01, ***P < 0.001 compared with nonRNA-preincubated values.

antibody responses in vitro mainly through affecting resting T cells. However, because these preliminary results were obtained in serum-supplemented culture conditions, it is possible that activated T cells may depend on exogenous nucleotides or nucleosides in nutritionally deprived culture conditions. We are now testing the effects of serum-free medium on the actions of polynucleotides on cloned T-helper cells.

IN VIVO ACTIONS OF NUCLEOTIDES ON HUMORAL IMMUNE RESPONSES

As stated above, our studies indicate that the enhancing actions of yeast RNA preparations on in vitro antibody production are largely attributable to polynucleotides. This raises the question of whether dietary nucleotides, which often have already been degraded substantially and are degraded further to nucleosides or nucleic acids in the gastrointestinal tract, can modulate immune functions in vivo. It may be argued that, in a state of relative nucleotide deficiency, dietary nucleotides and nucleosides may be rapidly incorporated into the tissue nucleotide pool and can exert significant actions on the immune system in vivo, similar to those observed in vitro.

To answer this question, we studied specific antibody responses to T-cell–dependent and –independent antigens in mice fed a nucleotide-free diet for a prolonged period (>3 wk), and observed a profound decrease of specific antibody responses to T-cell–dependent antigens when the mice were primed with antigens i.p. (Zhang et al. 1993). The responses to T-cell–independent antigens and lipopolysaccharide, a nonspecific polyclonal B-cell activator, appeared to be retained. The mononucleotide-nucleoside mixture, which was originally developed for supplementing total parenteral nutrition solutions, restored the humoral immune responses in response to T-cell–dependent antigens in mice fed a nucleotide-free diet (Adjei et al. 1993, Ogoshi et al. 1990, Zhang et al. 1993). This was achieved in a relatively short period (1 wk) when these mice were given this mixture i.p. However, this mononucleotide-nucleoside mixture showed virtually no effect on the in vitro specific antibody production in response to T-cell–dependent antigens and also did not further increase the in vivo humoral immune responses in mice fed regular lab chow.

The distinction between the actions of the nucleotide-nucleoside mixture on antibody production in vivo and in vitro may be confusing; nevertheless, it may be explained if we propose that the actions of polynucleotides to increase the local immune responses could be observed only locally and briefly in the body, perhaps at the site of tissue injury or inflammation, and then they are degraded rapidly. However, this speculation is yet to be proved by employing a further refined experimental model system. In summary, the results of our in vivo studies suggest that, in a state of relative nucleotide deficiency, mononucleotides and nucleosides may be rapidly incorporated into the tissue nucleotide pool and may restore the immune function. That the humoral immune responses were restored in a relatively short period in mice fed a nucleotide-free diet may be encouraging, because this may indicate the rapid restoration of immune functions by exogenous nucleotides in certain clinical settings.

TABLE 3

Effects of polynucleotides on clones types 1 and 2 T-helper cells and resting spleen T cells

	T-helper cells		DBA/2 and B10 spleen cells
	Type 1 (A.E. 7 cells)	Type 2 (CDC35 cells)	
Cytokine production[1]			
Interleukin-2 and interleukin 4	→→	→→	→→
Interferon-γ	→→	→→	→→
Cell proliferation[2]	→ or ↓	→ or ↓	slightly ↑
Specific antibody production[3]			
Antigen-specific immunoglobulin M-secreting cells	→→	→→	↑↑
Antigen-specific immunoglobulin G-secreting cells	→→	→→	↑↑↑
Total immunoglobulin production[4]			
Immunoglobulin M	→→	→→	↑
Immunoglobulin G	→	→→	→→

[1]Cytokine production by type 1 and 2 T-helper cell cloned cells were examined when T-helper cells were stimulated with antigens in the presence of irradiated B10 or DBA/2 spleen cells for 2 d. Interleukin-2 and interleukin-4 activity was assessed by cytotoxic T lymphocyte cell assay (Mosmann et al. 1986) and interferon-γ levels were measured by ELISA (Jyonouchi et al. 1993a, Jyonouchi et al. 1993b).

[2]Cell proliferation by type 1 and 2 T-helper cell clones were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay when T-helper cells were stimulated by antigens in the presence of irradiated B10 or DBA/2 spleen cells for 2 d (Mosmann et al. 1986).

[3]Specific solid phase enzyme-linked immunospot (ELISPOT) antibody responses were assessed by detecting antigen-specific immunoglobulin M- or immunoglobulin G-secreting cells by an ELISPOT assay as reported elsewhere (Czerkinsky et al. 1983, Sedgwick and Holt 1983) when cloned T-helper cells were incubated with T-helper cell–depleted murine spleen cells for 5 d in the presence of antigens.

[4]Total immunoglobulin production by T-helper cell–depleted murine spleen cells in the presence of antigen-specific T-helper cell clones with antigen stimulus was assessed by measuring immunoglobulin M and G levels in the culture supernatant by ELISA.

IN VITRO ACTIONS OF NUCLEOTIDES IN HUMAN SYSTEMS

We have also studied the effects of yeast RNA preparations on immunoglobulin production in in vitro human systems. Our initial studies employing adult peripheral blood mononuclear cells have demonstrated that yeast RNA could increase immunoglobulin M and immunoglobulin G production in response to T-cell–dependent stimuli and T-cell–dependent antigens and that this action of RNA is largely attributable to polynucleotides (Jyonouchi et al. 1993c). The production of immunoglobulins in response to T-cell–independent stimuli and T-cell–independent antigens was not significantly altered in the presence of polynucleotides. As observed in rodents, polynucleotides increase immunoglobulin production by peripheral blood mononuclear cells partly through exerting actions on T cells in the initial stage of the culture of the immunoglobulin production assay (Jyonouchi et al. 1993b). In contrast, a brief incubation of T cells with RNA before the culture seemed to result in nonspecific activation of T cells (unpublished observations). We have also observed that, when cognitive cell-cell interactions were permitted between T cells and non-T cells in peripheral blood mononuclear cells, nonspecific activation of T cells again appeared to be suppressed, as observed in experimental animal models (unpublished observations).

It has been shown that immature human lymphocytes appear to be poorly responsive to T-cell–dependent stimuli (Israel et al. 1991, Splawski et al. 1991, Watson et al. 1991), which potentially undermines the nutritional significance of the nucleotides in human breast milk on humoral immune responses. Umbilical cord blood T cells are phenotypically and functionally immature, lacking the features of memory T cells and providing poor helper functions to B cells (Israel et al. 1991, Splawski et al. 1991, Watson et al. 1991). Cord blood B cells are also phenotypically distinct from mature adult peripheral blood B cells and are probably functionally immature (Splawski et al. 1991). Therefore, as the next step, we examined the effects of polynucleotides on immunoglobulin production by cord blood mononuclear cells. We have found that polynucleotides also increase immunoglobulin M production by these cells when they are potentiated with pokeweed mitogen, a T-cell–dependent stimulant (unpublished observations). The production of immunoglobulin G by cord blood mononuclear cells is negligible and is not significantly altered in the presence of polynucleotides. Spontaneous immunoglobulin M production by cord blood mononuclear cells also appeared to be increased by polynucleotides, relatively independent of T cells (unpublished observations).

However, polynucleotides increased immunoglobulin production nonspecifically when the cord blood mononuclear cells were preincubated with them for a short period before the culture, an action that appears to depend on the presence of T cells. It may be concluded that polynucleotides increase immunoglobulin M production partly through T cells in cord blood mononuclear cells, but they could also exert actions on other lineage cells in cord blood mononuclear cells. In summary, our results demonstrate that polynucleotides could modulate in vitro humoral responses of both mature and immature lymphocytes in humans.

CONCLUSION

Our results suggest that exogenous nucleotides are important in maintaining optimal humoral immune responses. Along with evidence of the impaired cellular immunity in a state of relative nucleotide deficiency, these results support the necessity and importance of nucleotide supplementation.

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Dietary Nucleotides: Cellular Immune, Intestinal and Hepatic System Effects[1,2]

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ABSTRACT Investigations with animals demonstrate that dietary nucleotides influence immune function. Restriction of dietary nucleotides in mice decreases several indices of cell-mediated immunity as well as resistance to challenge with *Staphylococcus aureus* or *Candida albicans*. Spleen cells of mice maintained on nucleotide-free diet produce less interleukin-2 and have lower natural killer cell cytotoxicity and macrophage activation than those of animals fed nucleotide-supplemented diets. In vivo lymphoproliferative response, macrophage phagocytic activity and expression of interleukin-2 receptor and lyt1 surface marker are also lower in animals fed nucleotide-free diets. At 2 mo of age, infants fed breast milk or nucleotide-supplemented infant formula exhibit increased natural killer cell activity compared with infants fed unsupplemented formula. Dietary nucleotide restriction in animals may also result in hepatic lipid accumulation and decreased mucosal height and gut wall thickness. Adenosine monophosphate, a mediator of hepatic and small bowel blood flow, may play a unique role among the nucleotides studied. In conclusion, de novo synthesis and salvage of nucleotides is a metabolically costly process. An exogenous source of nucleotides from the diet may optimize the function of rapidly dividing tissues, particularly when growth is rapid and the diet is low in nucleotides. J. Nutr. 124: 144S–148S, 1994.

INDEXING KEY WORDS:

- *dietary* • *nucleotides*
- *cellular* • *immunity*

The endogenous supply of nucleotides is maintained through de novo synthesis and the salvage pathway. Because these are metabolically costly processes, it is more efficient to use already formed nucleotides. This is particularly true in rapidly dividing tissues, such as lymphoid and intestinal tissues, which require nucleotides for the synthesis of nucleic acids. One DNA replication requires at least 10^9 nucleotide molecules (Roux 1973). The effects of dietary nucleotides have been demonstrated primarily in these rapidly dividing tissues. An exogenous source of nucleotides, such as a dietary supplement, could optimize the tissue function by sparing the cost of de novo synthesis or salvage. This may be especially important during periods of rapid growth and when the diet is low in nucleotides; for example, in infants who are given cow's milk–based commercial formulas, most of which have significantly lower levels of nucleotides than does human milk (Janas and Picciano 1982).

EFFECTS OF DIETARY NUCLEOTIDES ON CELLULAR IMMUNITY

The precise mechanism of the effects of dietary nucleotides on cellular immunity is not clear, but the data suggest that the exogenous nucleotides supplied by the diet increase immunity by contributing to the pool of nucleotides available to the leukocytes. Barankiewicz and Cohen (1987) demonstrated that the activation of T lymphocytes causes a rapid increase in the synthesis of nucleotides, which are required immediately for the increase in energy metabolism and later as precursors for the synthesis of nucleic acids. Pérignon et al. (1987) reported that lymphocytes have limited capacity to salvage pyrimidines, and suggested that rapidly dividing lymphoblasts have a greater need for pyrimidine nucleotides.

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Studies conducted at the University of Texas and Rice University demonstrated that restricting dietary nucleotides decreases cell-mediated immunity. The incidence of allogeneic graft rejection (Van Buren et al. 1983a and 1983b), alloantigen-induced lymphoproliferation (Van Buren et al. 1983b) and delayed cutaneous hypersensitivity (Van Buren et al. 1983b) is lower in animals fed a nucleotide-free diet than in animals fed a nucleotide-supplemented diet. The spleen cells of mice fed a nucleotide-free diet produce less interleukin-2 (Van Buren et al. 1985) and express lower levels of interleukin-2 receptor and lyt1 surface marker (Kulkarni et al. 1989). This diet also decreases the resistance to challenge with *Staphylococcus aureus* (Kulkarni et al. 1986a and 1986b) and *Candida albicans* (Fanslow et al. 1988) and decreases the ability of macrophages to phagocytize *S. aureus* (Kulkarni et al. 1986a). These data suggest that restriction of dietary nucleotides influences immune responsiveness primarily by acting on the T-helper/inducer cell population (Van Buren et al. 1985) and predominantly by affecting the initial phase of antigen processing and lymphocyte proliferation. The higher levels of an intracellular marker specific for immature lymphocytes in the primary lymphoid organs of mice fed a nucleotide-free diet suggests that the mechanism is suppression of uncommitted T-lymphocyte responses (Rudolph et al. 1986). Adding RNA or uracil to the diet restored most immune functions. We have found that the natural killer cell activity and macrophage activation in the spleen cells of weanling mice fed a nucleotide-free diet are lower than those in animals fed a diet supplemented with five nucleotides (Carver et al. 1990).

Additionally, the effects of nucleotide supplementation on natural killer cell activity and interleukin-2 production in healthy term infants were studied (Carver et al. 1991) (**Table 1**). Natural killer cells are capable of spontaneous cytolytic activity against a variety of cells, and interleukin-2 is a growth factor for T lymphocytes and it stimulates natural killer cell activity. The infants were fed human milk or commercially available infant formula with or without added nucleotides at a level of 32 mg/L. The hematologic profiles did not differ between the infants fed the two formulas. At 4 mo of age, plasma chemistry profiles of the infants, including urea nitrogen, uric acid, creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, total protein, albumin and globulin, did not differ. The incidence of documentable infections was low in all groups, but slightly lower in the breast-fed infants. All infants grew at equivalent rates, and there was no evidence of toxicity or intolerance to the nucleotide-supplemented formula. At 2 mo of age, natural killer cell activity was significantly higher in the breast-fed and nucleotide supplemented groups compared with the unsupplemented group at the 50:1 and 25:1 effector-to-target cell ratios. Although the variability associated with the measurement of interleukin-2 makes interpretation of results difficult, the production of interleukin-2 by stimulated mononuclear cells was significantly higher in the nucleotide-supplemented compared with the unsupplemented group. At 4 months of age, natural killer cell activity and production of interleukin-2 remained consistently, although not always significantly, higher in the breast-fed and nucleotide-supplemented groups compared with the unsupplemented group. Although the sample size was small, we conclude that the nucleotides in human milk may contribute to the greater immunity in breast-fed infants.

EFFECTS OF DIETARY NUCLEOTIDES ON HEPATIC AND INTESTINAL COMPOSITION AND MORPHOLOGY

We have studied the effects of dietary nucleotides on hepatic (Novak et al. 1993) and intestinal (Carver et al. 1993) composition and morphology in weanling mice fed for 5 wk either *1)* rodent laboratory chow 5001 (Ralston Purina, Richmond, IN 47374), *2)* Basal Diet 5755 (Ralston Purina), *3)* Basal Diet 5755 supplemented with 0.0425% (wt/wt) each of AMP, CMP, UMP, IMP and GMP, for a total of 0.21% (wt/wt) nucleotides or *4)* Basal Diet 5755 supplemented with 0.0425% (wt/wt) AMP. The rodent chow contains approximately 0.25% (wt/wt) nucleotides, and the basal diet provides negligible amounts of nucleotides (Kulkarni et al. 1986b).

The liver weight as a percentage of the body weight was significantly lower in the animals fed the basal diet than in those fed chow or the two supplemented diets. The total serum bilirubin level was significantly higher in the animals fed the basal diet than in those fed the nucleotide-supplemented diet, and the levels of cholesterol, creatinine, uric acid, calcium, phosphorous, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total proteins, albumin and globulins did not differ among the groups. The total lipid level in the livers of the animals fed the basal diet was higher than that in those fed the AMP-supplemented diet or chow, and the cholesterol and lipid phosphorous levels were higher and the glycogen level lower in the livers of the animals fed the basal diet than in those of all other groups. The percentage of distribution of the phospholipid subclasses was not affected by the diet, and no differences in the fatty acid composition were found among the livers from the basal diet, nucleotide-supplemented and AMP-supplemented groups.

The ratio of the small bowel weight to length was lower in the group fed the basal diet than in all other groups. In the jejunum, the villus height and the

TABLE 1

Natural killer cell activity and interleukin-2 production of peripheral blood mononuclear cells from infants fed breast milk, nucleotide-supplemented formula or unsupplemented formula[1]

	Diet		
	Breast milk (n = 9)	NT+ formula (n = 13)	NT− formula (n = 15)
	At 2 mo of age		
Natural killer cell activity[2], *% cytotoxicity*			
50:1	41.74 ± 4.75**	32.24 ± 3.40*	21.65 ± 2.20
25:1	21.05 ± 3.20*	19.48 ± 2.51*	13.48 ± 1.67
12.5:1	7.56 ± 1.75	9.01 ± 1.59	6.13 ± 1.04
Interleukin-2 production, *U/mL*	0.37 ± 0.12	0.90 ± 0.28*	0.27 ± 0.11
	At 4 mo of age		
Natural killer cell activity[2], *% cytotoxicity*			
50:1	25.20 ± 2.78*	23.73 ± 4.02	14.07 ± 1.26
25:1	15.47 ± 0.77**	14.94 ± 3.47	7.90 ± 0.86
12.5:1	5.97 ± 0.10	6.66 ± 1.94	3.43 ± 1.03
Interleukin-2 production, *U/mL*	1.84 ± 0.35	1.53 ± 0.21	0.75 ± 0.21

[1]Values are means ± SEM. NT+ = nucleotide-supplemented; NT− = unsupplemented. *P < 0.05 vs. NT−; **P < 0.01 vs. NT−; P values were obtained using one-way ANOVA followed by a series of Fisher's least-squares difference comparisons. Adapted from Carver et al. (1991).
[2]Effector-to-target cell ratio.

villus-crypt ratio were greater in the chow and AMP-supplemented groups than in the basal diet and nucleotide-supplemented groups. The gut wall thickness and protein content were greater in the AMP-supplemented group than in all other groups. Unlike Uauy et al. (1990), we did not find significant effects of dietary nucleotides on disaccharidase activities, which may be because of differences in the levels of nucleotides fed, length of the study and species studied.

Dietary intake and pair-feeding experiments showed that the effects of the dietary nucleotides were not due to the quantity of the diet consumed.

The mechanism by which dietary nucleotides exert their effects on the liver and the intestine is not clear. Most of the ingested nucleotides are degraded in the intestine to nucleosides by alkaline phosphatase and nucleotidases, and may be further broken down by nucleosidases to produce purine and pyrimidine bases; however, investigations in animals suggest that nucleosides are the primary form absorbed (Uauy 1989). Most of the absorbed nucleosides and bases are rapidly degraded within the enterocytes (Sonoda and Tatibana 1978), but ~5% may be incorporated into the tissue pools, primarily in the small intestine, liver and skeletal muscle (Burridge et al. 1976, Saviano and Clifford 1978).

In the liver, extracellular nucleotides and nucleosides are reported to modulate hepatocyte growth (Ohyanagi et al. 1989) and regeneration (Yamaguchi et al. 1985) and to play an important role in the synthesis of glycogen. Ogoshi et al. (1985 and 1988) reported that parenterally administered nucleotides improved the hepatic function and promoted earlier restoration of the nitrogen balance after liver injury or partial hepatectomy.

In the intestine, exogenous nucleotides may be important for rapidly dividing mucosal cells, owing to absent (MacKinnon and Deller 1973, Saviano and Clifford 1981) or limited (Leleiko et al. 1983) de novo nucleotide synthesis. Uauy et al. (1990) reported that nucleotide supplementation increases the mucosal protein levels, DNA levels, villus height and disaccharidase activities in the intestine of weanling rats, and Nuñez et al. (1990) reported faster intestinal recovery after chronic diarrhea.

Of particular interest is the role of AMP supplementation, which in our studies was associated with a greater difference from the basal diet group than was supplementation with the mixture of nucleotides. The metabolism of dietary adenine is different from that of other purines in that a greater portion is absorbed and incorporated into the tissues (Burridge et al. 1976, Saviano and Clifford 1978, Saviano et al. 1980), and up to 20% may be recovered unmetabolized in the portal vasculature (Salati et al. 1984). Adenosine modulates an array of physiological processes, including increased blood flow to various tissues, such as those of the small intestine (Granger et al. 1978, Lautt et al. 1985, Sawmiller and Chou 1990) and the liver (Lautt et al. 1985). Extracellular adenosine stimulates glucose production in the perfused rat liver (Buxton et al. 1986) and induces System A amino acid transport in cultured hepatocytes (Kiyokawa et al. 1991). In the gut, adenosine plays a role in postprandial and reactive hyperemia and ar-

rests inflammatory changes associated with reperfusion (Kaminski and Proctor 1992). The effects in animals fed diets supplemented with AMP alone may relate to one or more of these phenomena.

SUMMARY

Studies in animals suggest that restriction of dietary nucleotides decreases several indices of cell-mediated immunity, perhaps because of effects upon the initial phase of antigen processing and lymphocyte proliferation. In addition, infants fed breast milk or nucleotide-supplemented infant formula have increased natural killer cell activity compared with infants fed unsupplemented formula. We have also found that dietary nucleotide restriction in animals results in hepatic lipid accumulation and decreased mucosal height and gut wall thickness. Adenosine monophosphate, a mediator of hepatic and small bowel blood flow, may play a unique role among the nucleotides studied.

Because the de novo synthesis and salvage of nucleotides is a metabolically costly process, an exogenous source of nucleotides from the diet may optimize the function of rapidly dividing tissues. This may be particularly important for the formula-fed infant for whom growth is rapid and whose diet is low in nucleotides. The mechanisms underlying dietary nucleotide effects and their significance in the infant remain to be determined. Areas for future study in dietary nucleotide effects include determination of dietary nucleotide effects on gut associated lymphoid tissues, the metabolic fate of dietary nucleotides, nucleosides and nucleic acids in humans, particularly in neonates; bioavailability of human milk cellular nucleic acids to the breast-fed infant; and the relative contribution of individual nucleotides to observed biologic effects.

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Dietary Nucleotides: Effects on the Gastrointestinal System in Swine[1,2]

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ABSTRACT Nucleotides in the intestinal lumen may decrease the inflammatory response to ischemia-reperfusion. In a newborn-swine model, we showed that perfusion of the intestinal lumen with nucleotides in concentrations similar to those in human milk induced hyperemia. The levels of hypoxanthine (and xanthine) were not increased in the presence of nucleotides during ischemia-reperfusion, and the number of leukocytes accumulated in the intestine was reduced in the presence of nucleotides. Furthermore, nucleotides may have decreased protein leak and the production of nitric oxide during ischemia. These effects are not changed significantly in the presence of an adenosine antagonist. We interpreted our results to indicate that the protective effects of nucleotides in the intestinal lumen are not due to adenosine alone. J. Nutr. 124: 149S–156S, 1994.

INDEXING KEY WORDS:

- *nucleotides* • *swine*
- *ischemia-reperfusion*

The effect of dietary nucleotides on the gastrointestinal system of the newborn is the subject of active research. Uauy et al. (1990) and Uauy and Stringel (1988) studied the growth of rat intestinal mucosa and found that the protein and DNA levels in animals fed a nucleotide-supplemented diet were 50–70% greater than those in animals fed a nucleotide-free diet. The authors also found that intestinal disaccharidase activity—especially that of maltase—was increased in the animals fed the nucleotide-supplemented diet. The villus height, as measured by cell counts of enterocytes, was 25% greater in the animals fed the supplemented diet. Carver and Bustamante showed that supplementing the diet with adenosine causes a significant increase in the length of the villus as measured by both total protein and cell number (unpublished data); however, they were unable to demonstrate any significant effect on disaccharidase activity.

Grisham et al. (1989) reported that adenosine interferes with leukocyte adherence and granulocyte extravasation in the intestinal mucosa during ischemia and reperfusion. Their findings have been confirmed by other investigators (Asako et al. 1993), who have found that adenosine attenuates the platelet-activating factor–induced adhesion of leukocytes and endothelial cells in postcapillary venules. This effect can be obtained by using other inhibitors, such as methotrexate.

Sawmiller and Chou (1991) reported that adenosine is a vasodilator in the canine intestinal mucosa. On the basis of these observations, we examined the possible roles of dietary nucleotides in concentrations similar to those found in human milk on hemodynamics and accumulation of hypoxanthine and granulocytes in an animal model (Bustamante and Lundgren 1989). Three series of experiments were performed. The first series addressed the accumulation of hypoxanthine (and xanthine) in the intestinal mucosa during ischemia and reperfusion and the possible effects of a countercurrent exchange of oxygen in the microcirculation of the villus crypt units. A top-to-bottom gradient of oxygenation is expected to have an effect on the accumulation of hypoxanthine in the intestinal mucosa during partial ischemia. The second series addressed the effects of nucleotides on the hemodynamics of the intestinal circulation and the accumulation of hypoxanthine

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(and xanthine) as well as granulocytes within the intestinal mucosa during ischemia and reperfusion. The third series examined the intraluminal effects of nucleotides with and without an adenosine antagonist.

MATERIALS AND METHODS

A modification of this animal model has been described previously (Bustamante and Lundgren 1989). We obtained piglets from a commercial farm in two age groups, 3–6 d (newborn) and 14–17 d (suckling). The characteristics of the study animals are presented in **Tables 1, 2 and 3**. The piglets were anesthetized with phenobarbital 30 mg/kg i.p. and chloralose 25 mg/kg i.v. followed by maintenance chloralose 6 $mg \cdot kg^{-1} \cdot h^{-1}$ i.v. by continuous infusion. A tracheostomy was performed, and ventilation was controlled with a respirator to maintain a normal pH (pCO_2). Femoral venous and arterial catheters were placed. The arterial catheter was used for measuring the systemic blood pressure and for infusing a solution of 7.5% glucose and bicarbonate 1.0 mmol/L, to decrease the tendency to develop acidosis, associated with the surgical procedure, as previously reported (Bustamante et al. 1989b). The femoral vein was used for administering the drugs. Paramedial laparotomy was performed. Loops of the ileum were isolated and denervated with intact circulation, and all other parts of the small intestine were removed. These loops were then perfused with an isotonic electrolyte solution (modified Krebs solution with 0.5% glucose). After a resting period, the various experimental protocols were performed.

In the first series of experiments, the loop of intestine was perfused with electrolyte solution for ~30 min (at rest period). The superior mesenteric artery (cranial mesenteric artery) was then clamped with a screw clamp to reduce the blood flow to ~10–15% of that at rest. This partial ischemia was maintained for 30 min, after which the clamp was removed and reperfusion was allowed for 10 min. Specimens of the intestinal loop were obtained at rest, during ischemia and after reperfusion for measuring the levels of hypoxanthine in the tissues. Using a cryostat, 15-μm cuts were made following the horizontal plane of the mucosa cutting the villi across. The slices thus obtained divided the mucosa into top and bottom halves.

In the second series of experiments, two loops of ileum were isolated and perfused with the modified Krebs isotonic electrolyte solution until a steady state was obtained and recorded. Then, one of the loops was perfused with the electrolyte solution containing nucleotides in similar concentrations as those found in commercial formula containing nucleotides. Ischemia was induced, reperfusion was allowed and

TABLE 1

Study group characteristics: Experiment 1[1]

	Age	Weight	Blood pressure	Blood flow	Peripheral resistance	Partial blood flow	Ischemia
	d	*kg*	*mm Hg*	*mL·100 g^{-1}·min^{-1}*	*PRU*	*mL·100 g^{-1}·min^{-1}*	*PRU*
Group 3–6 d (n = 20)	4.65 ± 0.30	1.83 ± 0.09	73 ± 1.99	83 ± 3.34	0.86 ± 0.03	13.1 ± 1.2	1.47 ± 0.12
Group 14–17 d (n = 20)	15 ± 0.18	3.56 ± 0.24	86.2 ± 2.56	53.7 ± 2.8	1.60 ± 0.09	8.9 ± 1.04	2.97 ± 0.32

[1]Values are means ± SEM. PRU = peripheral resistance units = blood pressure/blood flow.

TABLE 2

Study group characteristics: Experiment 2[1]

	Age	Weight	Blood pressure	Blood flow	Peripheral resistance
	d	*kg*	*mm Hg*	*mL·100 g^{-1}·min^{-1}*	*PRU*
Group 3–6 d (*n* = 8)	4.25 ± 0.41	1.70 ± 0.15	90.0 ± 6.2	104.0 ± 20.0	0.82 ± 0.05
Group 14–17 d (*n* = 8)	14.90 ± 0.26	3.86 ± 0.34	106.2 ± 13.1	82.4 ± 16.6	1.24 ± 0.10

[1]Values are means ± SEM. PRU = peripheral resistance units = pressure/blood flow.

specimens for biopsy were obtained as in the first series of experiments.

In the third series of experiments, isolated loops of ileum were perfused with electrolyte solution for a resting period and then perfused with solutions containing physiologic levels of nucleotides. Adenosine antagonist (CGS 15943A, Ciba-Geigy, Summit, NJ) was added to the perfusion fluid when ischemia was induced. Ischemia was maintained for 30 min and reperfusion allowed as in the first two series of experiments.

Specimens for biopsy were obtained at the beginning of the experiment in the undisturbed intestine and at the end of the experiment from the two loops of intestine. Samples of the perfusate were obtained every 15 min for measuring protein and nitrite content. The histologic examinations included granulocyte counts (the means of four counts per villus crypt unit) and assessments of damage, graded on a scale of 0 to 4+ by an independent observer who was not aware of the nature of the experiments. In all of the experiments in which hypoxanthine levels were measured, the measurement was done against DNA estimation by using cryostat sections cut across the axis of the villus crypt unit to divide it into top and bottom levels. Leukocyte accumulation and extravasation were studied by counting the granulocytes in 2-μm-thick fixed sections of intestinal mucosa stained with hematoxylin-eosin. Counts were made provided that both the top and the bottom of the villus crypt units could be seen in one cut. These specimens were examined at 450× magnification.

The protocol was reviewed and approved by the Louisiana State University Medical Center Institutional Animal Care and Use Committee. Statistical analysis was performed by ANOVA and differences between means by Student's *t* test when appropriate.

RESULTS

As shown in Tables 1–3, in all experimental groups the blood flow was greater and the blood pressure lower, with a consequently lower peripheral resistance, in the younger animals than in the older ones. In the first series of experiments, the accumulation of hypoxanthine relative to DNA was evident (**Fig. 1**), with hypoxanthine accumulating to a greater extent in the top than in the bottom of the villus (**Fig. 2**), a finding that we interpret to suggest the effect of a countercurrent exchange of oxygen at the base of the villus that causes the top of the villus to suffer a greater degree of hypoxia during partial ischemia. This effect was demonstrated in both the younger and the older newborn swine. In the second series of experiments, we demonstrated that nucleotides do not contribute to the accumulation of hypoxanthine in the intestinal mucosa during ischemia or reperfusion (**Fig. 3**) and that the number of leukocytes accumulated during ischemia and reperfusion may in fact be reduced by nucleotides in the lumen (**Fig. 4**). In the last series of experiments, we demonstrated that adenosine antagonist has inconsistent effects, which can be interpreted to indicate that the protective effects of the nucleotides during ischemia and reperfusion are not due to adenosine alone. We also demonstrated that the protein and nitrite levels, as evidence of the inflammatory response to ischemia and reperfusion (Miller et al. 1993), are not increased (**Fig. 5, Fig. 6**). In fact, in some experiments the inflammatory response could have been decreased by the presence of nucleotides in the intestinal lumen. Characteristic postischemic hyperemia was noted in both the younger group and the older group. In further analyzing the blood pressure and flow relationship, we observed the usual higher blood pressure in the older animals. Evidently, peripheral resistance develops in piglets within the 2-wk time of our study. Peripheral resistance development appears to be responsible for the greater extent of change observed in the older piglets, as vascular resistance decreases with postischemic hyperemia.

There was no difference in the accumulation of leukocytes in the intestine between the nucleotide and the adenosine antagonist group (**Fig. 7**), suggesting

TABLE 3

Study group characteristics: Experiment 3[1]

	Age	Weight	At rest			Nucleotides in lumen			Reperfusion	
			Blood pressure	Blood flow	Peripheral resistance	Blood pressure	Blood flow	Peripheral resistance	Blood flow	Peripheral resistance
	d	*kg*	*mm Hg*	*mL·100 g⁻¹·min⁻¹*	*PRU*	*mm Hg*	*mL·100 g⁻¹·min⁻¹*	*PRU*	*mL·100 g⁻¹·min⁻¹*	*PRU*
Group 3–6 d (*n* = 7)	4.86 ± 0.46	1.57 ± 0.17	94.7 ± 3.7	100 ± 13.3	0.98 ± 0.11	92 ± 7.7	110 ± 18	0.90 ± 0.12	125 ± 27	0.88 ± 0.15
Group 14–17 d (*n* = 7)	15 ± 0.44	3.19 ± 0.30	109 ± 7.4	88 ± 4.6	1.22 ± 0.13	112 ± 7.4	93 ± 6.4	1.20 ± 0.16	121 ± 12.7	0.96 ± 0.06

[1]Values are means ± SEM. PRU = peripheral resistance units = blood pressure/blood flow.

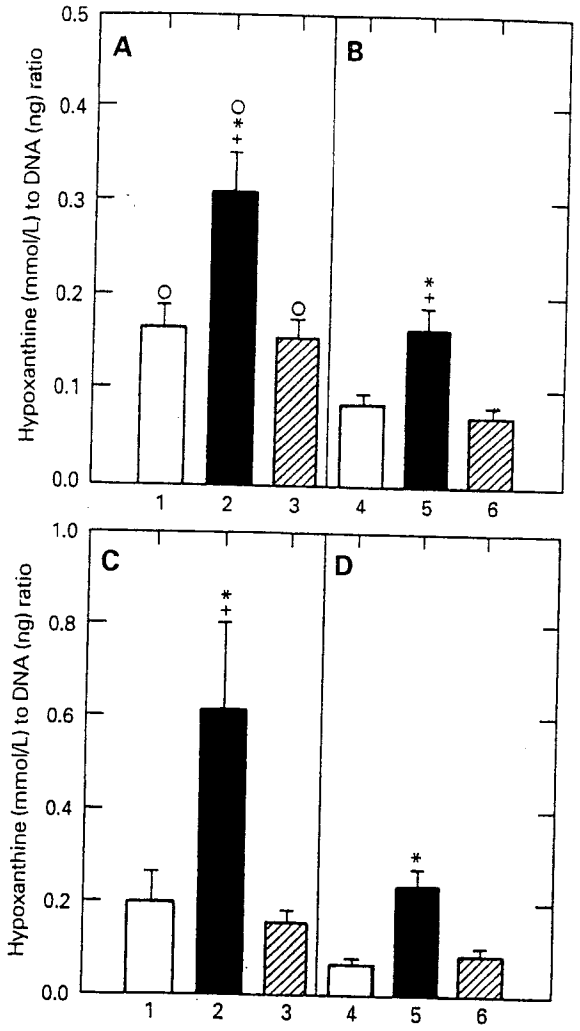

FIGURE 1 Hypoxanthine-DNA ratio in intestinal mucosa of piglets at rest (□), during ischemia (■) and during reperfusion (right diagonal hatching) at 3–6 d of age (*A,B; n* = 20) and 14–17 d of age (*C,D; n* = 20). Top half of villus = *A, C;* bottom half of villus = *B, D.* $^{*}P < 0.05$ vs. at rest; $^{+}P < 0.05$ vs. reperfusion; $^{o}P < 0.05$ vs. bottom half of villus.

that adenosine is not the only factor involved in the decreasing accumulation of leukocytes in the intestine during ischemia-reperfusion.

DISCUSSION

Our experiments have confirmed previous studies in which hypoxanthine has been found to accumulate during hypoxia (Saugstad 1975). The rapid disappearance of accumulated hypoxanthine (and xanthine) was also observed. The top-to-bottom differential ac-

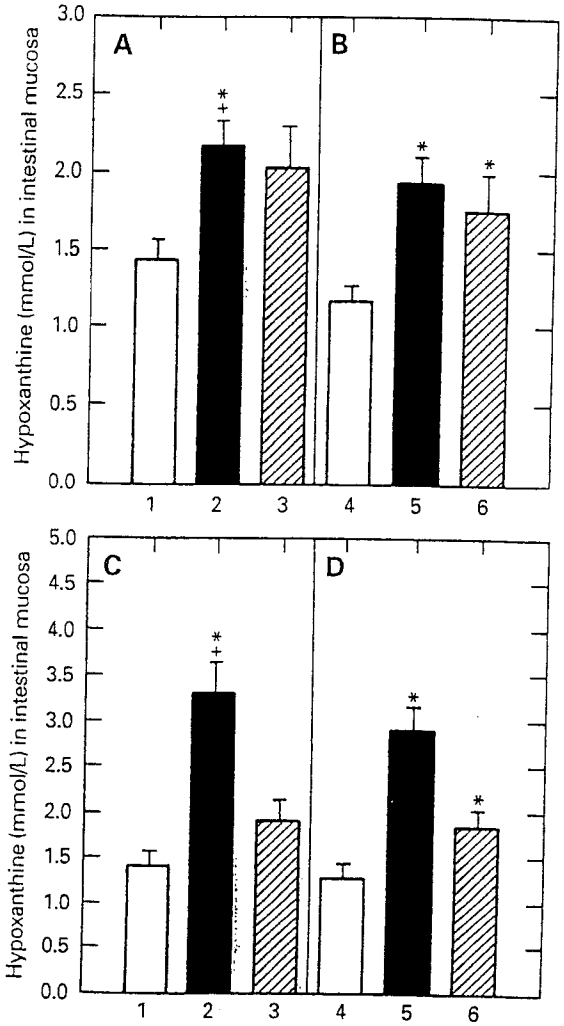

FIGURE 2 Hypoxanthine concentration in intestinal mucosa of piglets at rest (□), during ischemia (■) and during reperfusion (right diagonal hatching) at 3–6 d of age (*A,B*; n = 20) and 14–17 d of age (*C,D*; n = 20). Top half of villus = *A, C*; bottom half of villus = *B, D*. $^{*}P < 0.05$ vs. at rest; $^{+}P < 0.05$ vs. bottom half of villus.

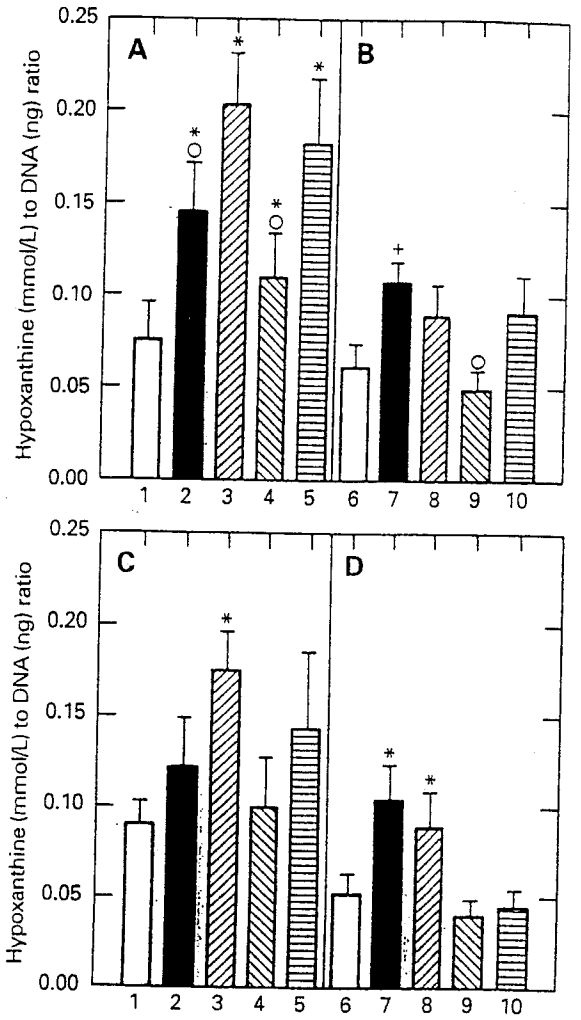

FIGURE 3 Hypoxanthine-DNA ratio in intestinal mucosa of piglets at rest (□), during ischemia with nucleotides (■), during ischemia without nucleotides (control; right diagonal hatching), during reperfusion with nucleotides (left diagonal hatching) and during reperfusion without nucleotides (control, horizontal hatching) at 3–6 d of age (*A,B*; n = 8) and at 14–17 d of age (*C,D*; n = 9). Top half of villus = *A, C*; bottom half of villus = *B, D*. $^{*}P < 0.05$ vs. at rest; $^{o}P < 0.05$ vs. control.

cumulation of hypoxanthine during hypoxia is in agreement with the hypothesis that a countercurrent exchange of oxygen takes place at the base of the villus crypt unit (Kampp et al. 1968). The tops of the villi get less oxygen during partial ischemia because the arteriole and venous capillaries are in close proximity, with blood flowing in opposite directions and allowing an oxygen shunt along each villus crypt unit (Bustamante et al. 1989a).

Nucleotides in physiologic concentrations induced hyperemia in the intestine (Table 3). This finding is not surprising, because adenosine, a known vasodilator (Sawmiller and Chou 1991), is one of the nucleosides involved. We could not demonstrate a definite vasodilator effect in the suckling piglets (14–17 d old). The effect is more consistently found in the newborn piglets (3–6 d old) despite the naturally greater blood flow in that age group.

Recently, Uauy et al. (1993) speculated that excessive supplementation of the diet with nucleotides could lead to the production of free radicals of oxygen

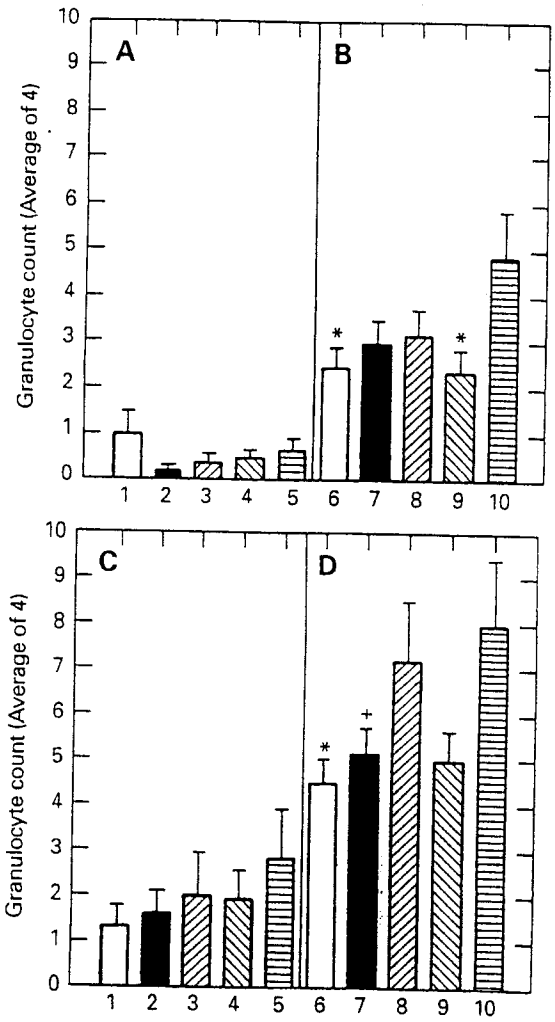

FIGURE 4 Granulocyte counts (means of four counts) in intestinal villus crypt units of piglets at rest (□), during ischemia with nucleotides (■), during ischemia without nucleotides (control; right diagonal hatching), during reperfusion with nucleotides (left diagonal hatching), during reperfusion without nucleotides (control; horizontal hatching) at 3–6 d of age (*A,B*; $n = 8$) and at 14–17 d of age (*C,D*; $n = 9$); top half of villus = *A, C*; bottom half of villus = *B, D*. $^{*}P < 0.05$ vs. at rest and vs. reperfusion with nucleotides; $^{+}P < 0.05$ vs. control.

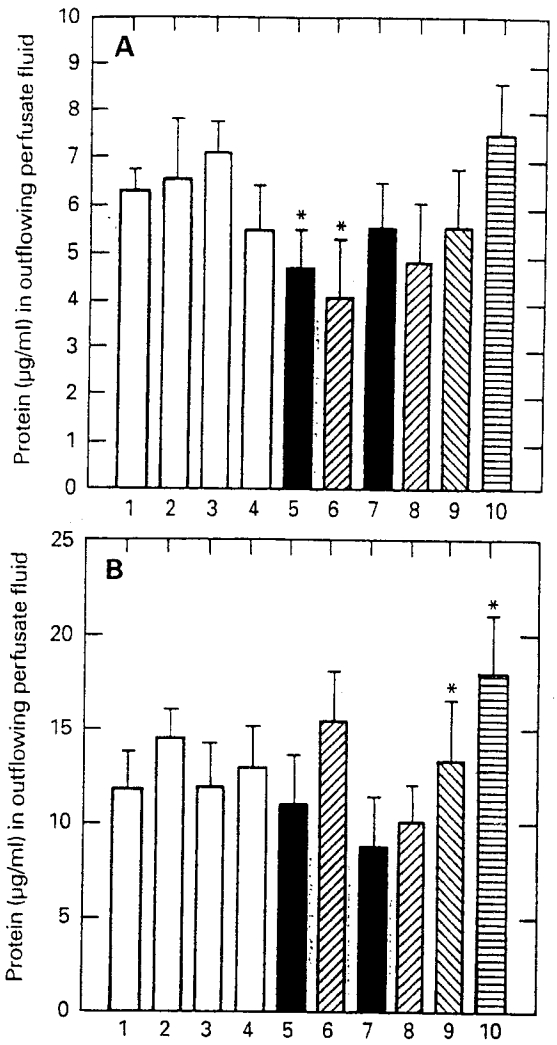

FIGURE 5 Exudate protein in 3–6-d-old (*A*; $n = 7$) and 14–17-d-old piglets (*B*; $n = 7$) at rest (□), during ischemia in the presence of adenosine antagonist (■), during ischemia in the presence of nucleotides (right diagonal hatching), during reperfusion in the presence of adenosine antagonist (left diagonal hatching) and during reperfusion in the presence of nucleotides (horizontal hatching). $^{*}P < 0.05$ vs. at rest (two leftmost columns); $^{+}P < 0.05$ vs. ischemia in the presence of adenosine antagonist (seventh column from left) and of nucleotides (ninth column from left).

from the conversion of hypoxanthine to urate by xanthine oxidase. The hypothesis that dietary nucleotides could contribute to a hypoxanthine pool during ischemia and to the formation of oxygen radicals at reperfusion was tested in our experiments. It is evident that dietary nucleotides in concentrations similar to those in human milk would not increase the hypoxanthine pool but would actually decrease it (Fig. 3). This seemingly protective effect of nucleotides should be studied in detail. Another source of free radicals of oxygen could be the granulocytes that accumulate in the intestinal mucosa during ischemia and reperfusion (Grisham et al. 1989). Considering that adenosine inhibits the ischemia and reperfusion-induced adherence and extravasation of leukocytes, we studied the numbers of granulocytes in the villus crypt units in our model. Although ischemia and reperfusion caused an increase in the number of leu-

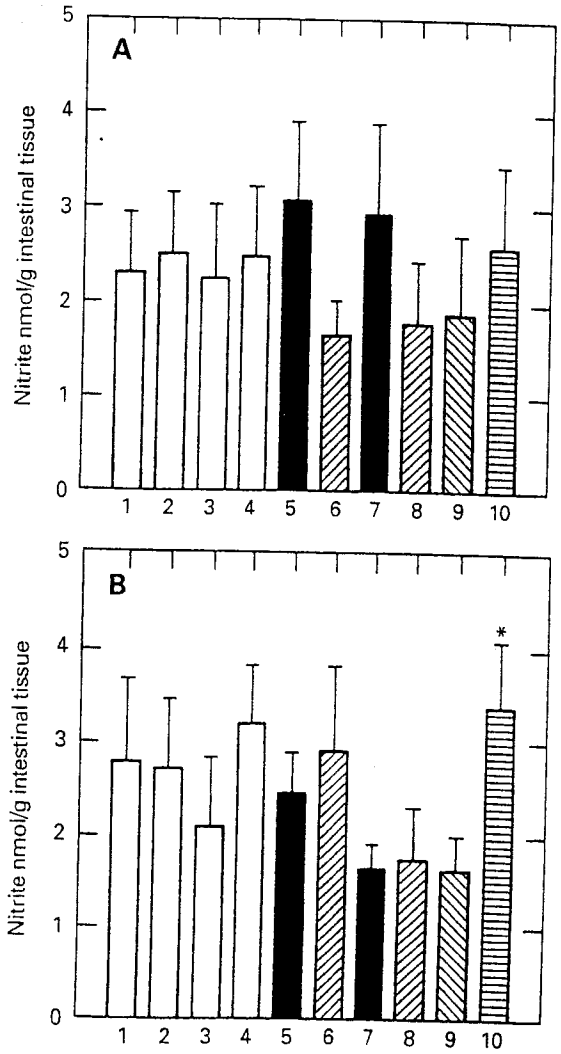

FIGURE 6 Nitrite in outflow perfusate in piglets 3–6 (*A*; $n = 7$) and 14–17 (*B*; $n = 7$) d of age at rest (□), during ischemia in the presence of adenosine antagonist (■), during ischemia in the presence of nucleotides (right diagonal hatching), during reperfusion in the presence of adenosine antagonist (left diagonal hatching) and during reperfusion in the presence of nucleotides (horizontal hatching). $^*P < 0.05$ vs. reperfusion in the presence of adenosine antagonist (ninth column from left).

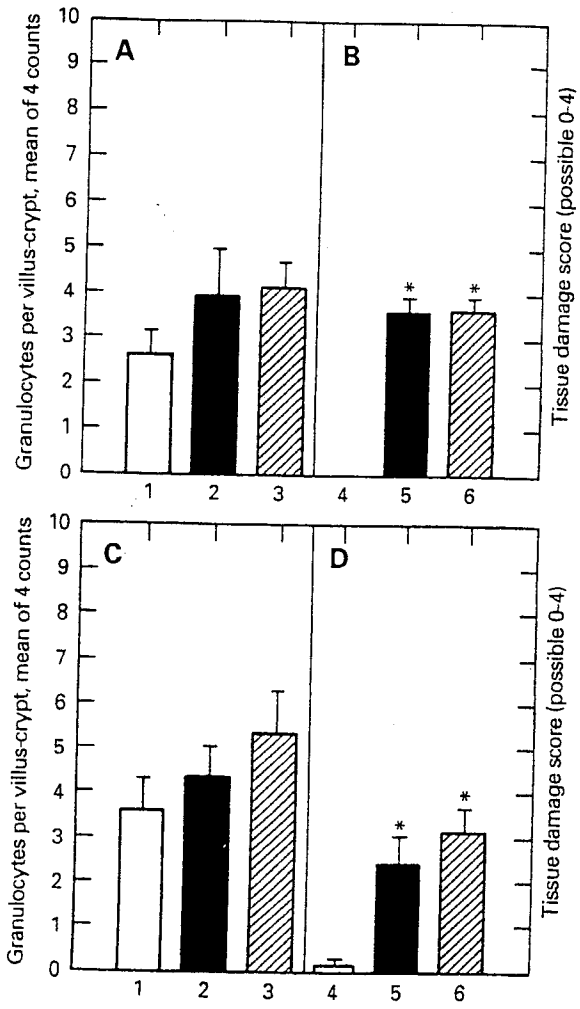

FIGURE 7 Granulocyte accumulation (means of 4 counts) (*A*, *C*) and tissue damage (*B*, *D*) after ischemia-reperfusion in piglets 3–6 (*A*,*B*; $n = 7$) and 14–17 (*C*,*D*; $n = 7$) d of age at rest (□), after ischemia-reperfusion in the presence of adenosine antagonist (■) and after ischemia-reperfusion in the presence of nucleotides (right diagonal hatching). $^*P < 0.05$ vs. at rest.

kocytes, there were fewer cells in the nucleotide-perfused intestine than in the controls (Fig. 4). This effect persisted when we tried to nullify it with an adenosine antagonist (Fig. 7), perhaps because of an action other than that of adenosine on the adherence and extravasation of leukocytes.

We further studied the effects of nucleotides on the inflammatory process of ischemia and reperfusion by measuring the protein and nitrite levels in the perfusion fluid used in our experiments (Figs. 5 and 6). Our design did not allow observation without nucleotides, but we interpreted the results to indicate that nucleotides in the intestinal lumen during ischemia and reperfusion do not increase these markers of inflammation and furthermore, it seems that the nucleotides might have decreased the protein and nitrite levels. The results of our experiments with the adenosine antagonist suggest that adenosine is not the active nucleotide in the seemingly protective effects we observed.

Under the conditions of our laboratory experiments, perfusing the intestinal lumen with nucleo-

tides in concentrations similar to those found in human milk may induce hyperemia. Adenosine antagonists do not nullify reperfusion hyperemia. Nucleotides do not increase the pool of hypoxanthine (and xanthine) during ischemia and reperfusion. The accumulation of leukocytes during ischemia and reperfusion is reduced with nucleotides in the lumen, and this reduction is not due to adenosine alone. Finally, nucleotides may decrease protein leak and the production of nitrites during ischemia, and those effects are also not due to adenosine alone.

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Symposium: Nucleotides and Nutrition

Nonimmune System Responses to Dietary Nucleotides[1,2]

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ABSTRACT Studies have demonstrated that a fecal flora with a predominance of bifidobacteria develops in infants fed a nucleotide-supplemented commercial formula, closer to that in breast-fed infants. In contrast, enterobacteria predominate in the fecal flora of infants fed an unsupplemented formula. When given parenterally, nucleotides promote recovery from injuries caused by hepatotoxic agents. These results suggest that dietary nucleotides may potentially play a significant role in nutrition. J. Nutr. 124, 157S–159S, 1994.

INDEXING KEY WORDS:

- *nucleotides* • *microflora*
- *injury* • *nonimmune*

Dietary nucleotides can be used through the salvage pathway in the synthesis of nucleic acids or can be catabolized to urates and excreted. It is important to understand the relative importance of these pathways when evaluating the role of nucleotides and nucleosides in cellular development and function. Experimental models that seek to define the role of nucleotides and nucleosides have generally involved administering large doses of them to mature animals, and the majority of these nucleotides and nucleosides end up as urates. We were interested in the amount of nucleotides and nucleosides that would be used in the synthesis of nucleic acids by rapidly growing animals given physiologic doses.

Different cell types have different capacities for nucleotide uptake and use. Rapidly growing cells such as enterocytes have limited capacity for de novo synthesis of purine and pyrimidine bases and require exogenously supplied bases that can be used through the salvage pathway as their predominant means of maintaining nucleotide pools. Liver cells, on the other hand, synthesize pyrimidine and purine bases by salvage synthesis if dietary nucleotides are available or activate de novo synthesis in the absence of dietary nucleotides. Different needs for and capabilities to use nucleotides, as well as the physiology and pathology of the tissue, influence the effects of nucleotides on nonimmune system functions. This article will review studies that demonstrate the effects of nucleotides on the composition of the intestinal microflora, liver regeneration, and lipoprotein metabolism.

EFFECTS OF NUCLEOTIDES ON INTESTINAL MICROFLORA

Tanaka and Mutai (1980) studied the effect of various culture medium supplements on the growth of bifidobacteria. As shown in **Table 1**, bifidobacteria plated on a selective medium grew better than those plated on a basal control medium. Adding nucleotides to the medium resulted in even greater growth.

Gil et al. (1986) examined the fecal flora of infants fed human milk, commercial formula or nucleotide-supplemented commercial formula and found a higher concentration of bifidobacteria than enterobacteria in the stools of the infants fed human milk and an inverse ratio of bifidobacteria to enterobacteria in the stools of the infants fed commercial formula (**Table 2**). The fecal microbial counts in the infants fed nucleotide-supplemented formula were intermediate, with a ratio closer to that in the infants fed human milk. The fecal composition in the human-milk and nucleotide-supplemented–formula groups differed from that in the standard-formula group but

[1]Presented at the symposium "Nucleotides and Nutrition" held in New Orleans, LA, March 28, 1993. The symposium was cosponsored by the American Institute of Nutrition and Wyeth-Ayerst International. Funding for the symposium and the publication of the supplement was provided through an educational grant from Wyeth-Ayerst International. Guest editor for this supplement was W. Allen Walker, Massachusetts General Hospital, 149 13th Street, Charleston, MA 02129.

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TABLE 1

Growth promoters of bifidobacteria

Species (*n*)	Increase from control value: Selective medium[1]	Increase from control value: Selective medium + nucleotides
	%	
Bifidobacterium bifidum (33)	85	97
B. breve (20)	65	90
B. parvulorum (56)	66	95
B. adolescentis (18)	28	78
Total (162)	70	95

[1]Selective medium = lactose, ammonium acetate, cystine, biotin and panthotenate. Adapted from Tanaka and Mutai (1980).

not from each other. These results suggest that dietary nucleotides may stimulate the growth of bifidobacteria in vivo, as has been demonstrated in vitro. It cannot be determined from these data whether the decreased percentage of enterobacteria in the stools is due to a direct effect of nucleotides or is a result of growth competition by the bifidobacteria.

EFFECTS OF NUCLEOTIDES ON RECOVERY AFTER LIVER INJURY

Ogoshi et al. (1988) studied the effects of standard total parenteral nutrition and total parenteral nutrition supplemented with a mixture of nucleotides and nucleosides or with uridine only in D-galactosamine–induced liver injury in rats. As shown in **Figure 1**, the aspartate aminotransferase and alanine aminotransferase levels were significantly lower in the animals given either of the supplemented preparations than in those given the standard total parenteral nutrition, indicating faster recovery from liver injury.

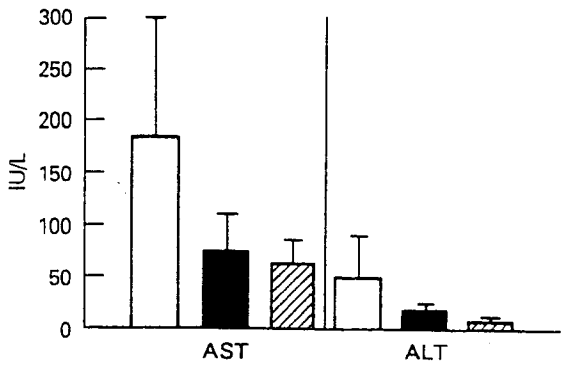

FIGURE 1 Effects of nucleotides on recovery from liver injury. Rats were subjected to D-galactosamine–induced liver injury and then given either standard total parenteral nutrition (TPN), TPN with a mixture of nucleotides and nucleosides or TPN with uridine only. The levels of aminotransferases in the serum of the animals given the mixture of nucleotides and nucleosides or uridine were lower than those in the standard-TPN group ($P < 0.05$), indicating faster recovery of liver function. [white bar], standard TPN ($n = 6$); [black bar], TPN + nucleotides and nucleosides ($n = 9$); [cross-hatched bar], TPN + uridine ($n = 7$); AST = aspartate aminotransferase; ALT = alanine aminotransferase. Values are means ± SD. Adapted from Ogoshi et al. (1988).

EFFECTS OF NUCLEOTIDES ON PLASMA LIPOPROTEIN LEVELS

Sánchez-Pozo et al. (1986) measured the levels of plasma lipoproteins in babies fed either human milk, commercial formula or nucleotide-supplemented formula, and found that the HDL levels increased in the first 4 wk of life and that they were higher in the infants fed human milk or nucleotide-supplemented formula than in those fed the commercial formula. These data suggest that dietary nucleotides may have

TABLE 2

Fecal microbial counts in neonates[1]

	Age	Diet: Human milk	Diet: Nucleotide-supplemented formula	Diet: Commercial formula
	wk			
Bifidobacteria	1	10.79 ± 0.10	10.26 ± 0.25	9.73 ± 0.42
	4	10.58 ± 0.24	10.17 ± 0.42	9.56 ± 0.42
Enterobacteria	1	10.41 ± 0.20	10.41 ± 0.21	10.30 ± 0.20
	4	10.01 ± 0.50	10.01 ± 0.23	10.08 ± 0.11

[1]Values are logarithmic counts, means ± SEM. Adapted from Gil et al. (1986).

a physiologic effect on lipoprotein metabolism in the neonatal period.

SUMMARY

Increased attention is being paid to the role of dietary nucleotides in infant nutrition. The studies reviewed here suggest that dietary nucleotides are important to maintaining normal growth and development in infants. Dietary supplementation with nucleotides was shown to modify the composition of the intestinal microflora, improve liver function after damage, and modulate lipoprotein metabolism.

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Symposium: Nucleotides and Nutrition

The Role of Nucleotides in Adult Nutrition[1,2]

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ABSTRACT Dietary nucleotides, found in normal diets, have been recently determined to be required for normal immune defenses. Rejection of cardiac transplants, graft-vs.-host disease, and delayed cutaneous hypersensitivity in animal models are all suppressed by a diet deficient in nucleotides. T lymphocytes seem to require dietary nucleotides for normal maturation and function. Host resistance to bacterial and fungal infections is decreased in mice on nucleotide free diets; addition of RNA or uracil prevents this vulnerability to infection. Dietary RNA is required to restore lost immune function after protein deprivation. Adequate calories and protein alone do not return immune function to normal. Dietary nucleotides can restore lost immune function even during protein starvation and weight loss. Because all parenteral and most enteral nutrient solutions are nucleotide free, clinical studies were undertaken comparing a new nucleotide containing diet (Impact) to a standard high protein enteral feeding. In two separate double blind clinical studies the patients fed the enteral diet containing nucleotides had improved immune function compared with patients receiving a nucleotide free diet. In addition, infectious complications and length of hospital stay were reduced in postoperative cancer patients fed Impact compared with a control group. J. Nutr. 124: 160S–164S, 1994.

INDEXING KEY WORDS:

- *dietary* • *nucleotides*
- *lymphocyte* • *immunity*

Conditional requirements for dietary substrates have become important considerations in human nutrition. The best defined of these substrates are amino acids, such as arginine, which are required for growth in infants and for maintaining host immune responses in animals. Another amino acid, histidine, is required by persons with chronic renal failure. Nucleotides are ubiquitous in cells, in either monomeric or polymeric form, and are vital for the function of the organism. The metabolism and importance of these substrates have been reviewed in this supplement (Bustamante et al. 1994, Carver 1994, Jyonouchi 1994, Rudolph 1994, Sanderson and He 1994, Uauy 1994) and elsewhere (Rudolph et al. 1990). Recent literature in experimental transplantation has demonstrated that exogenous nucleotides are important for maintaining host immunity to allogeneic tissues; restoring specific immune responses to foreign antigens requires providing exogenous nucleotides. Thus, exogenous nucleotides appear to be required for maintaining specific host immunity.

INFLUENCE OF NUCLEOTIDES ON LYMPHOCYTE FUNCTION AND CELLULAR IMMUNITY

These observations, while significant, have been relevant primarily to clinicians in suppressing the immune responses for successful organ transplantation. To test the hypothesis that dietary nucleotides might influence nonspecific host responses to infective organisms, the contralateral footpads of BALB/c mice fed a protein-free (PF)[3] diet were injected with BALB/c spleen cells (Kulkarni et al. 1989). Syngeneic inoculation was chosen for controlling for nonspecific

[1]Presented at the symposium "Nucleotides and Nutrition" held in New Orleans, LA, March 28, 1993. The symposium was cosponsored by the American Institute of Nutrition and Wyeth-Ayerst International. Funding for the symposium and the publication of the supplement was provided through an educational grant from Wyeth-Ayerst International. Guest editor for this supplement was W. Allen Walker, Massachusetts General Hospital, 149 13th Street, Charleston, MA 02129.

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[3]Abbreviations used: NF diet, nucleotide-free diet; NFA diet, nucleotide-free diet plus 0.06% adenine; NFR diet, nucleotide-free diet plus 0.25% RNA; NFU diet, nucleotide-free diet plus 0.06% uracil; PF diet, protein-free diet.

immune stimulation. After inoculation, the mice were either continued on the PF diet or switched to a nucleotide-free (NF), nucleotide-free plus 0.25% RNA (NFR), nucleotide-free plus 0.06% uracil (NFU), nucleotide-free plus 0.06% adenine (NFA) or commercial diet (containing ~0.25% RNA by weight based on analysis in our laboratories). The mice were weighed daily, and were killed 7 d after inoculation. The popliteal lymph nodes from both hind limbs were harvested, and a stimulation index of immune responsiveness was calculated by dividing the weight of the allogeneically stimulated nodes by the weight of the contralateral popliteal nodes.

All mice that were switched from a PF diet regained the weight that they had lost. No difference was noted in the weight restoration between these groups. In contrast, the mice that were maintained on the PF diet continued to lose weight, and their weight loss was 30% when they were killed.

The restoration of immunity depended on the presence of either RNA or uracil in the diet. In the mice maintained on the NF diet, even though they regained the lost weight, the immune response was equivalent to that in the mice continued on the PF diet (**Table 1**). Thus, providing calories and protein alone is insufficient to reverse the immunosuppression induced by protein starvation. Dietary nucleotides or dietary pyrimidines were necessary to restore the lost immune function (Pizzini et al. 1990). These findings, if extended to humans, may help explain the lack of association in previous clinical studies of an improved nitrogen balance after nutritional support with any change in the infection or mortality rates.

INFLUENCE OF NUCLEOTIDES ON HOST RESPONSE TO INFECTION

The studies discussed above focused on the influence of dietary nucleotides on lymphocyte function and cellular immunity. To test the hypothesis that these findings altered the host response to infective organisms, we intravenously inoculated BALB/c mice that were fed an NF, NFR, NFU, NFA or commercial diet with either fungal (*Candida albicans*) or bacterial (*Staphylococcus aureus*) pathogens. The amount of RNA, pyrimidine (uracil) or purine (adenine) was calculated on the basis of the amounts of the nucleotides or nucleobases present in a normal commercial diet. All mice were maintained on the assigned diets for 3 wk before inoculation. In the mice inoculated with *C. albicans*, survival was significantly greater ($P < 0.02$) in those fed the NFR and NFU diets than in those fed the NF and NFA diets (Fanslow et al. 1988). Decreased resistance to fungal infection was reflected in the ability to culture more viable organisms from the spleens of the animals fed the NF and NFA diets, a finding that, because of the importance of cellular immunity in resistance to fungal infections, might be expected.

In a study of the influence of the diet on resistance to bacterial infection, a dose of *S. aureus* that was lethal in 50% of mice fed a commercial diet resulted in 100% mortality when it was given to mice fed an NF diet. Supplementation with RNA or uracil resulted in increased survival after inoculation with the bacteria, but supplementation with adenine had no beneficial effect (Kulkarni et al. 1986). Because cellular immunity is not known to have an important role in host defenses against gram-positive bacterial infections, the macrophage responses were examined. The phagocytic ability of the macrophages could not account for the differences in survival. Macrophages isolated from the NFR group had a greater ability to engulf radiolabeled bacteria and no difference was observed in the phagocytic ability of macrophages isolated from the NF and commercial groups, yet the mortality was higher in NF diet fed mice. Instead, the bactericidal ability of splenic macrophages after phagocytosis appeared to correlate with in vivo resistance to *S. aureus*. The ability to kill engulfed bacteria was greater in the mice fed the NFR, NFU and commercial diets than in those fed the NF and NFA diets. Further studies showed that the production of superoxides in macrophages from animals fed the NF diet was less than that in animals fed the NFR and chow diets (Kulkarni et al. 1994). Thus, the requirement for dietary nucleotides appeared to be important for nonspecific as well as specific host defenses.

These studies are significant for two reasons. First, bacteria and fungi are important pathogens in critically ill hospitalized patients and are major contributors to morbidity and mortality. Second, all total parenteral nutrition solutions and most enteral feeding solutions are nucleotide-free. These studies suggest that depriving these critically ill patients of dietary nucleotides might adversely affect their ability to fight infection.

INFLUENCE OF NUCLEOTIDES ON LYMPHOCYTE RESPONSIVENESS

The finding that macrophage as well as lymphocyte function appears to depend on exogenous nucleotides has suggested a more basic role for these substrates. In a mouse model of bone marrow transplantation to study acute graft-vs.-host disease, the ability to cause graft-vs.-host disease in H_2-incompatible irradiated recipients was less in donors maintained on an NF diet than in donors fed commercial diet (Kulkarni et al. 1984). The influence is not absolute. The ability to cause graft-vs.-host disease was suppressed in radiation chimera donors fed the NF diet for 6–8 wk after

TABLE 1

Effect of various diets on in vivo popliteal lymph node (PLN) response[1]

Diet[2]	Allogeneic (allo) PLN	Syngeneic (syn) PLN	Delta (allo-syn)	Solid Impact (allo/syn)
		mg		
PF-PF	3.1 ± 0.6	1.2 ± 0.2	1.2 ± 0.6	2.9 ± 0.7
PF-NF	2.9 ± 0.2	1.4 ± 0.1	1.5 ± 0.1	2.2 ± 0.2
PF-NFR (0.025%)	7.4 ± 0.6	1.7 ± 0.2	5.7 ± 0.5	4.3 ± 0.3*
PF-NFR (2.5%)	7.0 ± 0.8	1.4 ± 0.2	5.6 ± 0.9	5.4 ± 0.8*
PF-NFU (0.6%)	9.8 ± 1.0	1.7 ± 0.3	7.9 ± 1.0	5.8 ± 1.0*
F-F	8.6 ± 1.2	1.5 ± 0.3	7.1 ± 1.2	6.7 ± 1.3*

[1]Values are means ± SEM. *PF, NF vs. NFR (0.025%), NFR (2.5%), NFU (0.6%), F $P < 0.05$.

[2]Diet abbreviations used: F, commercial rodent diet; NF, nucleotide-free diet; NFR, nucleotide-free diet + RNA; NFU, nucleotide-free diet + uracil; PF, protein-free diet.

bone marrow infusion; normal alloreactive ability was regained in radiation chimera donors that were fed commercial diet for 15 wk. These findings are most consistent with a maturation arrest of T lymphocytes or their precursors. Supporting this hypothesis is the finding that the responsiveness to interleukin-3, an important cytokine that directs lymphocyte maturation, is decreased in bone marrow harvested from mice fed an NF diet and is normal in that harvested from mice fed an NFR diet (Kulkarni et al. 1992). The ability to generate splenic colonies of bone marrow cells that are transfused into hosts fed an NF diet is less than that of bone marrow cells from donors fed a commercial diet or an NFR diet. Hosts fed an NFU diet support normal bone marrow engraftment, but engraftment is suppressed in hosts that are fed an NFA diet. Thus, the influence of dietary nucleotides on the immune system may be much broader, and they may affect a more primitive population of immunopotent cells than was initially believed.

Nonspecific host defense mechanisms appear to require dietary nucleotides for optimal function. The bactericidal activity of macrophages is suppressed in animals fed an NF diet, resulting in increased mortality from bacterial challenge. Supplementation with dietary RNA prevents the compromise of host defenses. In these studies and in evaluations of lymphoproliferative responses, pyrimidines mimic the action of RNA and purines are usually ineffective in maintaining immune responsiveness.

CLINICAL EFFECTS OF NUCLEOTIDES

For clinical studies, dietary nucleotides have been incorporated into casein-based enteral feeding formula with two other immunopotent substrates, fish oil and arginine. Fish oil, which is rich in (*n*-3) fatty acids, has been shown to increase lymphoproliferative responses to lectins (Kinsella et al. 1990). The suggested mechanism for this effect is the increased production of prostaglandin E_3 rather than E_2, the latter of which is synthesized from arachidonic acid. Prostaglandin E_2 appears to suppress lymphoproliferative responses (Goodwin and Ceuppens 1985). Arginine is a dibasic amino acid that promotes thymic development, increasing lymphocyte numbers and response. The mechanism by which it exerts these effects remains to be elucidated.

In an animal model that examined the effects of single substrates on the responses of popliteal lymph nodes to injected allogeneic spleen cells, fish oil and dietary RNA appeared equivalent in maintaining the lymphoproliferative responses and arginine appeared less effective (Kulkarni et al. 1989). Furthermore, when RNA and fish oil were combined with arginine, an additive effect on the immune function was seen. These effects were also seen in the previously described model of challenge with *S. aureus* (**Fig. 1**). This research laid the foundation for the development of an enteral formula that combines nucleotides, fish oil and arginine (Impact, Sandoz Pharmaceuticals, East Hanover, NJ).

In two clinical studies, Impact was compared with an isocaloric casein-based high-nitrogen enteral formula (Osmolite, Ross Laboratories, Columbus, OH). In a double-blind study conducted at the University of Minnesota with septic or critically ill patients in the intensive care unit, 11 patients were randomly assigned to Impact and 9 to Osmolite (Cerra et al. 1990). Both patient groups were similar with respect to demographics, prestudy nutritional assessment and severity of illness. Nitrogen delivery was controlled for, and both groups received equivalent amounts of nitrogen. Because Impact contains nearly 12 g/L more amino acids and/or protein than Osmolite, the caloric intake in the Impact group

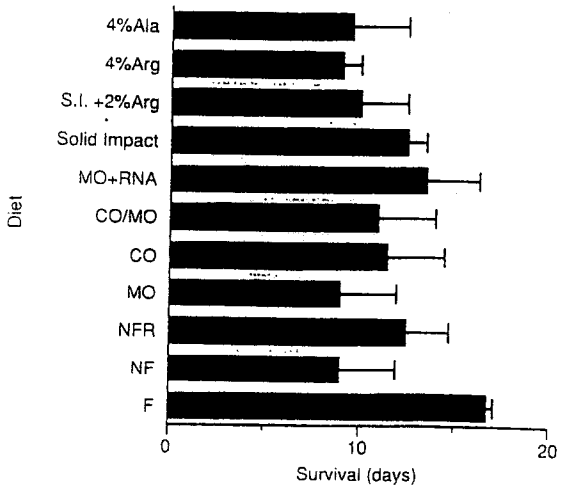

FIGURE 1 Mean survival (± SEM) in BALB/c mice fed various diets after inoculation with 1×10^8 *S. aureus* organisms. Abbreviations used: Ala, alanine; Arg, arginine; CO, basal diet supplemented with corn oil; F, commercial rodent diet; MO, basal diet supplemented with fish oil; NF, nucleotide-free casein-based diet; NFR, nucleotide-free casein-based diet supplemented with 0.25% RNA; S.I., Solid Impact.

was less. The patients were examined before enteral feeding and throughout the course of the study for lymphoproliferative responses to phytohemagglutinin, concanavalin A and tetanus toxoid protein. Both groups of patients remained on study for an average of 9 d. The average total duration of hospitalization was 37 d in the Impact group and 55 d in the Osmolite group. Because of the small number of patients and the wide standard error, this difference was not statistically significant. However, the trend is interesting in light of subsequent clinical studies.

The difference between the groups in the immune response to supplementation was statistically significant. There was no change in the immune response throughout the study in the Osmolite group, with the lymphoproliferative responses at the study's end being practically identical to those before enteral feeding. In contrast, there was statistically significant and progressive improvement in the lymphoproliferative responses to phytohemagglutinin, concanavalin A and tetanus toxoid protein in the Impact group, with the greatest responses being observed at the study's conclusion. Tube feeding with Osmolite failed to improve the initial immune suppression noted in the critically ill patients, while the Impact did improve it significantly. This improvement occurred despite there being no difference in nitrogen balance between the two groups.

In the second study, Daly et al. (1992) studied Osmolite and Impact in 85 postoperative gastrointestinal cancer patients. The caloric intake was controlled for in this study, which resulted in the Impact group receiving significantly more nitrogen than the Osmolite group, and, as expected, the nitrogen balance was better in the Impact group. However, no matter how large the positive nitrogen balance in the Osmolite group, no improvement in the immune response and no association between the nitrogen balance and the immune function were noted in this group. In contrast, a statistically significant improvement in the immune function was demonstrated in the Impact group. The clinical outcome associated with this improved immune response was a 70% reduction in infectious and wound complications in the Impact group (11% incidence) from that in the Osmolite group (37% incidence). This reduced complication rate resulted in a 22% reduction in the length of hospitalization in the Impact group.

The results of these two comparative clinical studies suggest that specially formulated enteral solutions that contain nucleotides favorably influence the outcome in hospitalized patients. Enteral feeding solutions that have not been evaluated for their effects on the immune response may not be optimum for critically ill patients.

SUMMARY

Exogenous nucleotides appear to support normal nonspecific and specific immune defenses. Investigations in animal models and recent clinical literature suggest that administering dietary nucleotides will help to minimize infectious complications, improve clinical outcomes and provide the most cost-effective nutritional support available.

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