RNA/Nucleotide Enhances Antibody Production *In Vitro* and Is Moderately Mitogenic to Murine Spleen Lymphocytes

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Abstract. **Suppression of immune functions was demonstrated in both humans and animals when exogenous RNA was eliminated from the diet. However, direct actions of RNA/nucleotide on the immune system are virtually unknown. Thus, in this study, we explored effects of RNA and nucleotide on lymphocyte functions *in vitro*. Yeast whole RNA, which is free of endotoxin, was supplemented to culture media, and changes in mitogen responses, thymocyte proliferation, or *in vitro* antibody production by murine spleen lymphocytes were analyzed. Yeast whole RNA potentiated the proliferation of spleen lymphocytes and it also strikingly enhanced *in vitro* antibody production in response to sheep red blood cells at least 10-fold. However, it did not potentiate the proliferation of thymocytes (immature lymphocytes). These enhancing activities of yeast RNA were significantly reduced by RNAse treatment, but not by treatments with DNAse or polymyxin B. Certain mononucleotides exhibited less, but similar, action on murine spleen lymphocytes. The whole yeast RNA employed was already degraded to small nucleotide (<1 kb). Therefore, it may be suggested that certain components of RNA degraders can function as powerful immunomodulators, indicating that exogenous RNA or nucleotide may be important in facilitating immune responses under certain circumstances.** [P.S.E.B.M. 1992, Vol 200]

The impact of nutrients on the immune system has been well documented in numerous investigations, and deficiency of certain nutrients can lead to profound immune dysfunction, as evidenced by zinc deficiency or vitamin A deficiency (1, 2). Since nucleotides affect a variety of biochemical and enzymatic reactions, nucleotides or preformed nucleic acids from dietary sources may be important to a number of bodily functions, including immune responses (3). However, until recently, the effects of exogenous nucleotides or nucleic acids on immune functions have not been studied in detail, partly because it was assumed that needs for nucleic acids or nucleotides are supplied primarily by *de novo* biosynthesis pathways (4).

Only recently have researchers begun to report the possible importance of RNA and nucleotides supplied by dietary sources on the immune system. It was reported that in patients who have been treated by renal allografting and have been on total parenteral nutrition (TPN), the immune responses to allografting was lower than those observed in patients on regular diets (5). This difference was attributed to the lack of RNA or nucleotide in the TPN fluid (5). In experiments with cardiac transplants of mice and rats, it was found that cardiac allografts survived longer when the animals were fed nucleotide-free diets in addition to being treated with cyclosporin (6, 7). In studies of infection models, animals fed nucleotide-free diets were shown to survive for shorter periods than control animals fed regular diets containing nucleotide (8, 9). These results indicate that nucleotide supplied exogenously may be important to optimize immune functions, especially under conditions of immunological stress.

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In spite of these interesting observations from comparative studies of different diets, little information is available with respect to actions of RNA preparations or nucleotide on lymphocyte functions *in vitro* and *in vivo*. Thus, as an initial approach to this question, we attempted to determine whether yeast whole RNA preparations could modify the lymphocyte functions *in vitro*. The results obtained indicate that RNA itself exerts mitogenic action on mature spleen lymphocytes, but not on immature lymphocytes, e.g., thymocytes. Additional RNA preparations enhanced *in vitro* antibody production in response to sheep red blood cells dramatically. These effects were substantially reduced after the treatment of RNAse and certain mononucleotides exhibited less, but similar, action on these assays. Since the RNA preparations used in this study have already been degraded to small nucleotides (<1 kb), these findings may suggest that certain small nucleotides or complexes of nucleotides in the products of RNA degraders may exert important immunomodulating actions. Thus, exogenous RNA/nucleotides from dietary sources may be of value to permit optimal functions of the immune system in certain circumstances.

Materials and Methods

Mice. In this study autoimmune-resistant inbred C57BL/6 (B6) mice were used as representative of normal strains of mice. Six 7-week-old female B6 mice were purchased from Jackson Laboratories, Bar Harbor, ME, and maintained in the animal facilities at the University of South Florida, St. Petersburg, FL, or at the University of Minnesota, Minneapolis, MN. The mice were housed as a group of five mice in each cage, fed regular lab chow (Purina Laboratory Chows; Purina Mills, Richmond, IN; 23.4% protein, 4.5% fat, 5.8% fiber, 7.3% ash, 76.0% total digestible nutrients, 4.25 kcal/g), and sacrificed at the age of 2–3 months to obtain spleen or thymus.

Cell Suspensions. Spleen cell suspensions were obtained by crushing the spleen between two sterile glass slides and suspending tissues in phosphate-buffered saline with 5% calf serum (CS). Debris was removed by allowing the cells to settle by placing the cell suspension on ice for 5 min. Thymocyte cell suspensions were obtained by gently squeezing cells out of the thymus using a cell scraper (Costar, Cambridge, MA). In each experiment pooled spleen or thymocyte cell suspensions obtained from 2–4 B6 mice were used.

Mitogen Responses (10). Prepared mouse spleen cells were washed twice and suspended in RPMI 1640 supplemented with 5% Nu serum (GIBCO-BRL, Gaithersburg, MD), penicillin and streptomycin, 1% Na pyruvate, 2% HEPES, 1% glutamine, and 2-mercaptoethanol. Cell suspensions were then adjusted by a final concentration of 2.5×10^6/ml in 96-well round-bottomed microtiter plates (Costar) in the presence of mitogens including phytohemagglutinin-P (PHA-P), concanavalin A (Con A), porkweed mitogen, and lipopolysaccharide (LPS) and/or RNA/nucleotide as indicated in Results. Then the cells were incubated for 2 days in CO_2 incubator (5%) at 37°C, pulsed with 1 μCi of [^{3}H]methylthymidine, and harvested 12–16 hr after the pulse. [^{3}H]Thymidine incorporation was detected by scintillation counter. In assays of thymidine incorporation, the results were expressed as stimulation index by dividing the values of average thymidine incorporation of triplicate samples for each specimen with control values.

Thymocyte Proliferation Assay. Thymocytes from B6 mice were washed twice and resuspended in the medium as described above. Then cells were placed in 96-well microtiter plates (Costar; either 10^6/ml or 5×10^6/ml as final concentration) based on bioassays for interleukin (IL)-1-like or IL-2-like activities (11, 12). Cells were stimulated with various amounts of nucleotide or RNA preparation in the absence or presence of PHA-P (0.5 μg/ml). As control, mouse recombinant IL-2 and recombinant human or mouse IL-1 (Genzyme, Boston, MA) was used as indicated in Results. Cells were incubated at 37°C for 3 days in 5% CO_2 incubator, pulsed with 1 μCi of [^{3}H]thymidine, and harvested 12–16 hr later.

***In Vitro* Antibody Production Assay.** Single cell suspensions from mouse spleen were washed twice in phosphate-buffered saline supplemented with 5% CS. Then the mouse cells were resuspended in RPMI 1640, 10% CS (Hyclone, Logan, UT), 1% Na pyruvate, penicillin and streptomycin, 2% HEPES, 1% glutamine, and 2-mercaptoethanol (10^{-6} *M*). Sheep red blood cells (SRBC) were washed three times, removed of the buffy coat, and resuspended in the same medium. Mouse spleen cells were then incubated in 24-well plates (Costar; 5×10^6/ml, 1 ml/well) for 5 days in 10% CO_2 at 37°C. Antigenic stimulation was provided by adding 50 μl of 1% SRBC to each 1-ml spleen cell suspension. Then cells were harvested, washed twice, and resuspended in 0.5 ml of Hanks' balanced salt solution (HBSS), and cell viability was examined. Fresh SRBC solution was prepared by washing SRBC twice and resuspending them in HBSS (30–40% SRBC). Then 50 μl of SRBC and 100 μl of harvested spleen cell suspensions were added quickly to the tubes containing 0.5 ml of preheated agar solution (0.5% agar [Sigma, St. Louis, MO] in complete HBSS), mixed well, and plated on microscope slides precoated with 0.025% agar. Then guinea pig complement solution (1:30 in HBSS; GIBCO-BRL) was applied to the trays in which slides were placed inverted to immerse the solidified agar-cell mixture in the complement solution. The slides were incubated at 37°C for 3 to 4 hr and the developed plaques were counted under a dissecting microscope.

The numbers of plaque-forming cells (PFC) were expressed as per 10^6 initially cultured cells.

RNA Agarose Gel. RNA samples were denatured with 1 *M* glyoxal in 10 m*M* NaH_2PO_4 buffer and 50% dimethyl sulfoxide for 1 hr at 50°C, and denatured RNA was electrophoresed in 1.2% RNA agarose gel (Sigma) at 40 V and visualized under the uv light (Foto/Phoresis I; Fotodyne, New Berlin, WI) after staining the gel with 0.5 μg/ml of ethidium bromide. The gel picture was taken with the Polaroid camera (Fotodyne).

DNAse/RNAse Treatment. Yeast whole RNA (10 mg/ml) was treated with 20 units of DNAse (R1 RNAse free DNAse; Progema, Madison, WI) in a reaction buffer of 40 m*M* Tris HCl (pH 7.2), 10 m*M* NaCl, and 10 m*M* $MgCl_2$ for 30 min at 37°C. Yeast whole RNA (10 mg/ml) in 50 m*M* Na acetate (pH 4.5) and 2 m*M* Na_2 EDTA were treated with 10 units of RNAse (Ribonuclease T2; GIBCO-BRL) at 37°C for 30 min. After DNAse or RNAse treatment, RNA samples were placed on ice until use on the same day.

Reagents. Rat IL-2 (Collaborative Laboratories), recombinant mIL-1 (Genzyme), PHA-P (Difco, Baxter Scientific, Ocala, FL), Con A (Pharmacia-LKB, Uppsala, Sweden), PWM (GIBCO-BRL), and LPS (Difco) were purchased from the commercial companies as indicated. Yeast RNA preparations used for the production of commercially available formula (IMPACT) were provided by Sandoz Nutrition Corp., Minneapolis, MN. The components of yeast whole RNA provided from Sandoz Nutrition were as follows: 91% RNA, 5.0% hydrolyzate of RNA, cold acid soluble fraction, which included 1.0% sodium salts of mononucleotide, 0.5% sugar, and 2.5% NaCl. Endotoxin levels were 0.748 pg/ml in 1% RNA solution, which is equivalent to the values detected in distilled water (Toxicolor system; Seikagaku Kogyo, Tokyo, Japan). These RNA compounds were kept at −20°C as in lyophilized forms and dissolved into diethylpyrocarbonate-treated sterile water on the day of the experiment. Adenine, uridine, and inosine were also purchased from Sigma and, after being dissolved into sterile, autoclaved water, were kept at −20°C in aliquot. FDA-certified AMP, CMP, GMP, IMP, and UMP (used for the production of the infant formula SMA) were kindly provided by Wyeth-Ayerst Research, Philadelphia, PA. The amount of free purine and pyrimidine bases contained in the CS and Nu sera employed in this study ranged from 0.1 μ*M* to 1.0 μ*M* according to the companies. The amount of RNA/nucleotide contained in the CS and Nu sera used in this study ranged from 5 to 10 ng/ml by the standard phenol/chloroform extraction (DNA DipSticks; Invitrogen, San Diego, CA).

Statistics. The significance of the data was assessed by comparing the data to control values in each experiment. After the F test, *P*-values were calculated by either Student's *t* test or Welch's test. $P < 0.05$ was considered to be significant.

Results

Mitogen Responses. Resting peripheral T cells were reported to have little *de novo* purine biosynthetic activity and, thus, are likely to be dependent upon exogenous nucleotide (13). Therefore, the effect of yeast RNA on mitogen responses by mouse spleen lymphocytes, most of which are considered to be not in S phase, was studied. Spleen cells were cultured with or without RNA (0.01% or 0.001%) in the presence of medium only or mitogens (PHA-P, Con A, pokeweed mitogen, and LPS). As shown in Table I, the supplementation of yeast whole RNA to the culture media increased [^{3}H]thymidine incorporation by spleen cells in the absence of mitogens or in the presence of suboptimal doses of mitogens (0.1 μg/ml). In the presence of 1–4 μg/ml of PHA-P, Con A, pokeweed mitogen, and LPS, the doses which usually give optimal responses, no significant enhancing effects by RNA were observed (Table I and data not shown).

Although the yeast whole RNA used in this study contained almost undetectable endotoxin, as stated in the Materials and Methods section, we also tested whether the mitogenic activity found in RNA preparation can be abolished by polymyxin B (Sigma), a specific inhibitor of endotoxin. The addition of polymyxin B to the culture media did not reduce the proliferative responses of spleen lymphocytes induced by yeast whole RNA (Table II). The mitogenic activity of LPS, a representative endotoxin, was completely lost with 10 or 50 μg/ml of polymyxin (Table II). Therefore, it is unlikely that enhanced proliferative responses of spleen

Table I. Yeast Whole RNA Enhances Proliferative Responses by Murine Spleen Lymphocytes

	Lymphocyte proliferation assay[a]		
	No Additive	0.01% RNA (SI)	0.001% RNA (SI)
Medium only[b]		10.1 ± 1.4	3.8 ± 0.5
Mitogens[c]			
PHA (1 μg/ml)	8.2 ± 0.9	7.7 ± 1.0	8.6 ± 0.7
Con A (1 μg/ml)	32.7 ± 13.0	28.1 ± 11.2	28.3 ± 11.9
PWM (1 μg/ml)	13.5 ± 1.0	13.7 ± 0.6	13.5 ± 2.6
LPS (1 μg/ml)	9.4 ± 1.2	8.9 ± 0.8	9.0 ± 0.8
PHA (0.1 μg/ml)	1.1 ± 0.2	8.4 ± 1.9	4.6 ± 1.1
Con A (0.1 μg/ml)	1.4 ± 0.5	8.7 ± 2.6	5.7 ± 2.1
PWM (0.1 μg/ml)	2.7 ± 0.8	8.6 ± 1.4	6.3 ± 1.8
LPS (0.1 μg/ml)	1.5 ± 0.3	8.4 ± 1.5	6.4 ± 1.6

[a] Results are expressed as stimulation index (SI) ± SE of several experiments.
[b] These are summaries of eight experiments. In each experiment, spleen cells were obtained from two to four young B6 female mice and pooled.
[c] These are summaries of three experiments. PWM, pokeweed mitogen.

Table II. Polymyxin B Do Not Abolish Mitogenic Action of Yeast Whole RNA[a]

	Lymphocyte proliferation assay (SI)
0.001% RNA	5.0 ± 1.3
0.001% RNA + polymyxin B (50 μg/ml)	4.1 ± 1.3
LPS (1 μg/ml)	4.6 ± 0.5
LPS (1 μg/ml) + polymyxin B (50 μg/ml)	0.8 ± 0.1[b]
LPS (1 μg/ml) + polymyxin B (10 μg/ml)	0.7 ± 0.3[b]
Polymyxin B (50 μg/ml)	0.7 ± 0.1

[a] The data are summaries of three experiments and results were expressed as stimulation index (SI) ± SE. Spleen cells were obtained from two to three B6 mice and pooled.
[b] Significantly low compared with the value obtained with LPS alone ($P < 0.005$).

Table III. Yeast Whole RNA Does Not Stimulate Thymocyte Proliferation

	Thymocyte proliferation assay[a] (SI)
0.01% RNA	0.67 ± 0.24
0.001% RNA	0.86 ± 0.19
PHA-P (0.1 μg/ml)	1.88 ± 0.30
PHA-P (0.1 μg/ml) + IL-2 (10 units/ml)	4.55 ± 0.39
PHA-P (0.1 μg/ml) + 0.01% RNA	0.39 ± 0.09
PHA-P (0.1 μg/ml) + 0.001% RNA	0.40 ± 0.04

[a] Thymocytes were obtained from three to four young B6 mice in each experiment and pooled. The results shown are the summaries of four experiments. Values of [^{3}H]thymidine incorporation are expressed as stimulation index (SI) ± SE.

lymphocytes by RNA preparation are secondary to the contamination of endotoxin.

Thymocyte Proliferation Assay. In contrast to spleen cells, thymocyte cell suspensions, are considered to contain many immature T cells in S phase, and Cohen *et al.* (13) reported that S phase-enriched large thymocytes are capable of *de novo* purine synthesis. For this reason, we examined the effects of exogenous RNA on the proliferation of thymocytes. In contrast to its effect on spleen cells, the presence of yeast whole RNA in the culture media did not enhance proliferation of thymocytes (Table III). In other experiments, we also observed that free nucleosides or nucleic acids (1–20 μM of uridine, inosine, or adenine) did not enhance thymocyte proliferation (data not shown). It may be suggested that yeast RNA provided exogenously can stimulate the proliferation of mature resting lymphocytes but not immature T cells *in vitro*.

***In Vitro* Antibody Production.** Since yeast RNA appears to enhance the lymphocyte proliferation moderately, we further attempted to test its effect on specific lymphocyte functions. For that purpose, we chose to study *in vitro* antibody production by spleen cells. Namely, B6 spleen lymphocytes were primed with SRBC *in vitro* for 5 days in the presence or absence of RNA, and antibody-secreting cells against SRBC were detected by PFC assay. PFC numbers (antibody-secreting B cells) increased strikingly when yeast whole RNA was present in the culture media during antigen priming compared with controls cultured without RNA (Table IV). This enhancing action of yeast whole RNA on *in vitro* antibody production appeared to be dose dependent (Fig. 1), and when the mouse spleen cells were primed in the culture supplemented with 0.01% RNA, usually 10-fold higher numbers of PFC were generated compared with controls cultured in the absence of

Table IV. Yeast Whole RNA Significantly Enhances *In Vitro* Antibody Production against SRBC

	In vitro antibody production[a]	
	No additive (PFC)	SRBC (PFC)
Medium only	4 ± 1	77 ± 51
0.01% RNA	7 ± 2	1736 ± 559[b]
0.001% RNA	3 ± 1	867 ± 219[b]
LPS (1 μg/ml)	51 ± 6[c]	90 ± 40

[a] In each experiment, spleen cells were obtained from two to three B6 mice and pooled. PFC numbers were expressed as average of triplicate samples ± SE/10^6 initially cultured cells in five experiments.
[b] Significantly high compared with control PFC numbers obtained when cells were primed with SRBC in the absence of RNA ($P < 0.05$).
[c] Significantly high compared with control PFC numbers obtained when cells were cultured in the medium alone, or RNA in the absence of SRBC ($P < 0.001$).

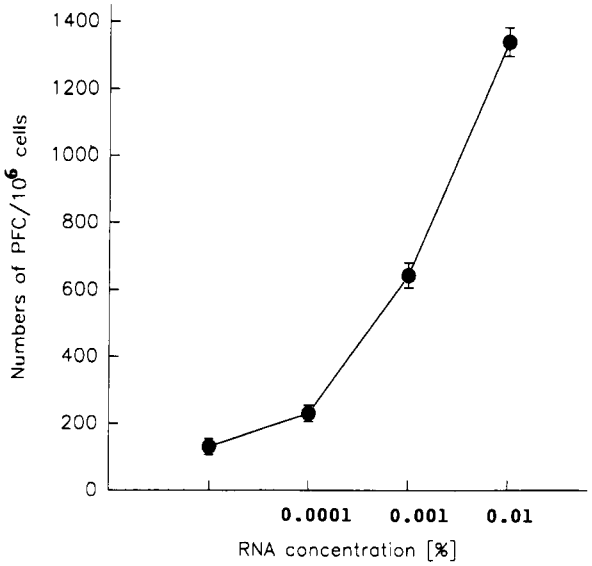

Figure 1. Numbers of PFC/10^6 initially cultured cells when spleen cells were primed with SRBC in the presence of various amounts of yeast whole RNA. Each data point corresponds to average numbers of PFC ± SE of triplicate samples. Spleen cells were obtained from three 10-week-old B6 female mice and pooled.

RNA. The recovery of spleen cells after being cultured for 5 days was usually slightly better (up to 20–30%) in the RNA-supplemented culture conditions compared with controls, which may reflect the moderate mitogenic actions of yeast RNA.

When cells were unprimed, the spontaneous formation of PFC did not increase at all in the presence of RNA (Table IV). In contrast, LPS, an endotoxin and a polyclonal B cell activator, seemed to enhance the PFC numbers formed in the absence of antigen stimuli, but did not increase those formed in response to SRBC (Table IV). The presence of goat anti-mouse IgG (1:1000) in the cell-agar mixture blocked the PFC formation approximately 70% irrespective of the presence of RNA in the culture (Fig. 2), suggesting that approximately 70% of PFC may be IgG-secreting cells, whereas 30% PFC may be IgM mediated or through other mechanisms and RNA may similarly influence both mechanisms.

Effects of RNAse/DNAse on the Immunomodulating Actions by Yeast RNA. When yeast whole RNA employed was subjected to gel electrophoresis, it was shown that the whole RNA used had all been degraded to <1 kb nucleotide (Fig. 3). Thus, we further tested the effects of RNAse and DNAse treatment on the immunomodulating actions of the yeast RNA to examine whether these actions of RNA can be attributable to RNA components. Yeast whole RNA was treated

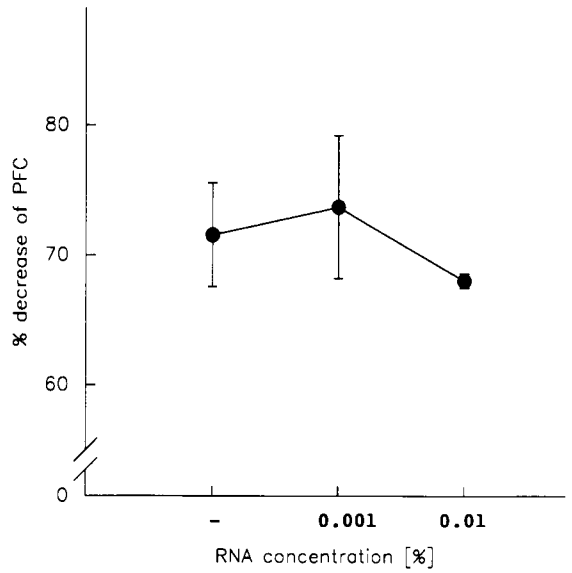

Figure 2. Percentage of decrease of PFC numbers formed in the presence of goat anti-mouse IgG antibody. Spleen cells were primed with SRBC in the presence of medium only or yeast whole RNA (0.001% or 0.01%) for 5 days and SRBC antibodies were determined by PFC assay in the absence or presence of goat anti-mouse IgG serum (1:1000). Each data point represents average percentage of decrease of PFC numbers ± SE in three separate experiments in the presence of anti-IgG antibody. In each experiment, spleen cells were obtained from two to three young B6 females and pooled. In each experiment, three slides per sample were prepared.

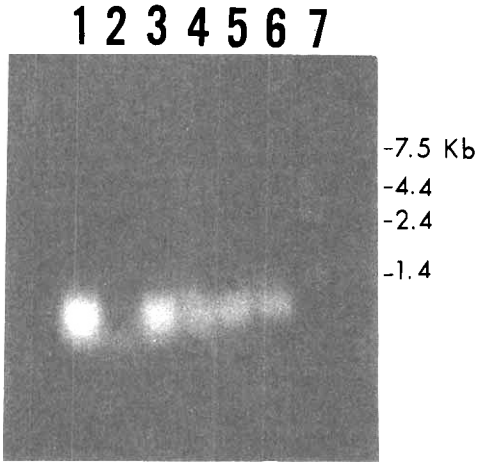

Figure 3. Yeast whole RNA and tRNA were examined in RNA agarose gel (1.2%) electrophoresis. Samples applied from Lane 1 to 7 were as follows: 1, 10 μg of yeast whole RNA; 2, 10 μg of yeast whole RNA treated with RNAse; 3, 10 μg of yeast whole RNA treated with DNAse; 4, 5 μg of yeast whole RNA; 5, 5 μg of yeast tRNA; 6, 5 μg of Escherichia coli tRNA; 7, 1 μg of molecular weight marker (1 kb ladder; GIBCO-BRL).

Table V. RNAse Treatment Significantly Reduces Immunomodulating Actions of Yeast Whole RNA

	In vitro antibody production[a] (PFC)	Lymphocyte proliferation assay[b] (SI)
Medium only	103 ± 14	
Untreated yeast 0.01% RNA	1409 ± 484	7.8 ± 0.4
RNAse-treated 0.01% RNA	257 ± 26	1.6 ± 0.2
DNAse-treated 0.01% RNA	1094 ± 366	7.4 ± 0.3
RNAse only[c]	103 ± 6	0.9 ± 0.1
DNAse only[c]	121 ± 17	0.9 ± 0.1

[a] This is the summary of four experiments. Results are expressed as mean PFC of triplicate samples ± SE.
[b] This is the summary of four experiments and results are expressed as stimulation index (SI) ± SE.
[c] Same amount of RNAse or DNAse included in RNAse- or DNAse-treated RNA added to the culture were supplemented without RNA as negative controls.

with either RNAse (40 units of RNAse/mg yeast RNA) or DNAse (2 units/mg yeast RNA). As shown in Figure 3, RNAse but not DNAse treatment considerably degraded the yeast whole RNA employed in this study. Enhancing actions of yeast whole RNA on *in vitro* antibody production and lymphocyte proliferation were significantly reduced after RNAse treatment, but not after DNAse treatment (Table V). Namely, the RNAse-treated RNA exerted less than 20% of mitogenic activity and less than one third of enhancing action on *in vitro* antibody production compared with the untreated yeast RNA. These results may indicate that the immuno-

modulating actions of yeast whole RNA are largely attributable to certain RNA degraders. The slight decrease in the stimulation index and PFC numbers formed after DNAse treatment may also be related to the contamination of RNAse remaining in the DNAse employed in this study, since the staining intensity of RNA slightly decreased after DNAse treatment.

Effects of Nucleosides and Mononucleotides on Mitogen Responses and Antibody Production by Spleen Cells. Since even after RNAse treatment immunomodulating actions of yeast RNA were not abolished completely, we also examined whether free nucleosides or mononucleotides exert similar actions as exhibited by yeast whole RNA preparations. Free nucleosides (uridine and inosine; 1–100 μM) did not significantly enhance the proliferation of spleen lymphocyte or *in vitro* antibody production by spleen cells (data not shown). Adenine, as well, did not show significant enhancing effects on *in vitro* antibody production by spleen cells (data not shown). However some mononucleotides (0.01%–0.001%) demonstrated less but similar immunomodulating actions in the same bioassays as summarized in Table VI. That is, GMP and IMP significantly enhanced *in vitro* antibody production at the concentration of 0.01%, and AMP and UMP also seemed to enhance *in vitro* antibody production, although the results obtained were not statistically significant. Lymphocyte proliferation was enhanced by AMP, GMP, IMP, and UMP up to 2-fold with the concentrations of 0.001% to 0.01% (Table VI). These results suggest that the remaining immunomodulating actions of yeast whole RNA after RNAse treatment may be attributable to certain mononucleotides, oligonucleotides, or complexes of both.

Table VI. Mononucleotides Exert Less but Similar Immunomodulating Actions on Murine Spleen Cells *In Vitro*[a]

	In vitro antibody production (PFC)	Lymphocyte proliferation assay (SI)
Medium only	27 ± 6	
AMP		
0.001%	32 ± 12	1.8 ± 0.3
0.01%	66 ± 18	2.0 ± 0.5
CMP		
0.001%	31 ± 12	1.4 ± 0.2
0.01%	40 ± 9	1.7 ± 0.2
GMP		
0.001%	81 ± 15[b]	2.5 ± 0.5
0.01%	88 ± 15[c]	1.9 ± 0.5
IMP		
0.001%	50 ± 7	2.0 ± 0.2
0.01%	108 ± 11[c]	1.8 ± 0.5
UMP		
0.001%	36 ± 14	1.8 ± 0.3
0.01%	56 ± 12	1.8 ± 0.2
RNA		
0.01%	738 ± 186[b]	9.7 ± 3.0

[a] Data are the summary of three experiments. Cells were primed with SRBC, and PFC numbers were expressed as average ± SE/10^6 initially cultured cells. The results of [^{3}H]thymidine incorporation were expressed as stimulation index (SI) ± SE.
[b] Significantly high compared with controls ($P < 0.05$).
[c] Significantly high compared with controls ($P < 0.005$).

Discussion

Exogenous supplementation of RNA and nucleotides has been considered to be not essential for normal individuals based on previous dietary studies (4, 14, 15). Cells with rapid turnover, such as mucosal cells or lymphocytes, have been assumed to be provided with the necessary amounts of purines or pyrimidines from other tissues, such as the liver, via the *de novo* synthesis pathways (4). The dietary nucleotides are absorbed mainly in the form of nucleosides and are utilized through the salvage pathway. The extent of the incorporation of exogenous nucleotides into cellular nucleotide pools and RNA/DNA molecules depends on dietary conditions. In animals fed normal regular diet, only about 2–5% of dietary nucleotide seemed to be incorporated into tissue nucleotide pools. This suggests a relatively insignificant role of exogenous nucleotides in nucleic acid metabolism in well-nourished individuals. However, Leleiko *et al.* (16) reported a significant decrease in RNA of small intestine of rats given purine antimetabolite (6-mercaptopurine) or fed with RNA-free diets, indicating the preservation of DNA content at the expense of RNA and protein pools in the intestine. This finding suggests that in certain clinical settings, exogenous RNA from dietary sources could be very important in maintaining normal body functions, including immune functions. A series of dietary studies has demonstrated decreased cellular immunity (delayed allograft rejection, decreased delayed hypersensitivity, and decreased T helper cell function), as well as decreased resistance to infection, in animals fed nucleotide-free diets (17, 18). This may be related to a disturbance in maturation of lymphocytes, since the maturation arrest of lymphocytes and bone marrow cells has been reported in mice fed RNA-free diets (19).

However, the effects of RNA or nucleotides on the immune system are not very well understood and the information regarding this issue is surprisingly scarce. Therefore, in the present study, we attempted to address the question of whether RNA/nucleotide could exert any effects on lymphocyte functions in bioassays of *in vitro* culture system where necessary energy or nutrients were well provided to sustain the growth of the lymphocytes.

It was found that the whole RNA used in the study was moderately mitogenic by itself to spleen lymphocytes (mature lymphocytes with a few cells in S phase). In contrast, exogenous RNA supplied to the culture

medium was not mitogenic at all to thymocytes, most of which are immature lymphocytes with many cells in S phase. The yeast RNA used in this study contained almost undetectable levels of endotoxin and its mitogenic actions were not abolished by polymyxin B, an endotoxin inhibitor. The contamination by yeast cell wall components was negligible in these RNA preparations. It has been reported that large thymocytes in S phase are capable of *de novo* synthesis of nucleotide, whereas mature lymphocytes in G_1 stage are largely dependent upon exogenous nucleotide (13). Thus, these results may suggest that certain components of yeast RNA/nucleotide in the culture medium may be capable of enhancing lymphocyte functions depending upon the stages of proliferation/maturation of cells.

A most significant finding in the present study was that yeast whole RNA enhanced *in vitro* antibody production in response to SRBC quite impressively. Murine spleen cells primed with SRBC in the culture medium supplemented with yeast whole RNA (0.01%) produced at least 10-fold more PFC than those cultured in the absence of RNA. This enhancing action of yeast whole RNA appeared to be dose dependent. However, spontaneous PFC production was not enhanced by addition of RNA preparations. That is, when spleen cells were not primed with SRBC *in vitro*, almost no PFC was formed irrespective of the presence of RNA, whereas the addition of LPS, an endotoxin and polyclonal B cell activator, produced higher amounts of PFC in the absence of SRBC. It should be emphasized that the culture conditions of the mitogen assay and PFC assay were not exactly the same as shown in the Materials and Methods to optimize the condition either for lymphocyte proliferation or for antibody production. Thus, the results of the mitogen study may not be proportionally applicable to the results of the PFC assay, although the RNA concentration in both the calf serum and Nu serum used in this study was minimal, as indicated in Materials and Methods. That is, after 5 days of culture, the numbers of recovered viable cells were only slightly higher in samples treated with RNA than in controls. In fact, the cellular recovery was generally higher in samples treated with LPS than those with RNA supplementation (up to 30–40%). The addition of PHA (1 μg/ml) or Con A (1 μg/ml) to the culture did not enhance PFC formation to SRBC significantly, as observed with LPS (unpublished observations). Therefore, it may be concluded that yeast RNA strongly enhances specific antibody production, which could be difficult to attribute solely to nonspecific mitogenic actions of RNA on spleen cells.

Although we do not know the mechanisms of the enhancing actions of RNA on *in vitro* antibody production, one possibility is that RNA may enhance *in vitro* antibody production to SRBC by augmenting the production of various soluble factors, including IL-2, IL-4, and IL-6 by T helper cells. It may also be possible that RNA enhances the cognitive interactions of T and B cells directly. In our preliminary experiments, yeast whole RNA preparations did not significantly increase IL-2-like or IL-4-like activity by itself. However, yeast RNA seem to expand Thy-1^+ and $CD5^+$ T lymphocyte populations, particularly the $L3T4^+$ T helper cell subset *in vitro*, and the removal of T cells from spleen cell suspensions seems to abolish this enhancing action of RNA completely. We also found that RNA only enhances antibody and Ig production to T-dependent antigen, but not to T-independent antigen or does not further facilitate the state of polyclonal B cell activation observed in the animal models (manuscript submitted for publication). The strong immunomodulating actions of RNA may be exerted on a particular T cell subset. Further studies will be of interest to assess the importance of exogenous RNA/nucleotide on immune functions.

The whole yeast RNA preparations used in this study were already degraded into small nucleotide (<1 kb), which may raise the question of whether these strong immunomodulating actions of whole yeast RNA are truly attributable to RNA components or other contaminations. As stated before, endotoxin contamination of this RNA preparation was unlikely, and we have shown that RNAse treatment but not DNAse treatment on yeast RNA prior to the supplementation to the culture significantly reduced its immunomodulating actions. It was also shown that certain mononucleotides exerted less, but similar, enhancing action on *in vitro* antibody production in B6 mice. Thus, RNA degraders may function as strong immunomodulators at least *in vitro*. Remaining immunomodulating actions of yeast RNA observed after RNAse treatment may be exerted by free mononucleotides, oligonucleotides, or complexes of both. In dietary studies, the supplementation of uridine to the diet can partly improve the suppressed immune functions in mice fed nucleotide-free diet, although we did not observe significant immunomodulating actions by free nucleosides (3, 7, 9). Nucleotide/nucleosides are known to be rapidly metabolized in intestinal mucosa to be degraded into uric acid or other metabolites or incorporated into nucleotide pool. It was shown that the removal of nucleotide from the diet led to the marked decrease of RNA content in the small intestine of rat but not in the liver (16), indicating that actively proliferating cells such as intestinal mucosal cells may preserve DNA content at the expense of RNA and protein in the state of RNA/nucleotide deprivation. This could significantly disturb their functions. Perhaps a similar reasoning may be applied to the lymphocyte population, That is, the supplementation of uridine to the diet may have been effective in improving immune functions in mice fed

nucleotide-free diet by increasing the nucleotide pool in the body of these animals.

In normal individuals on regular diets, such deprivation of exogenous RNA is highly unlikely to occur. However, in patients solely on TPN or on elementary formulas, the deprivation of exogenous RNA and nucleotide may occur since they are not supplemented in TPN solution or in most of these formulas. These patients are often already immunocompromised and may succumb to complications of infections. Although specific antibody can be provided using preparations of γ-globulin for intravenous use, it might be ideal to maintain the optimal functions of the immune system in these patients. Neonates and infants whose immune functions are relatively immature but rapidly developing may also require the dietary supplementation of nucleotides. In serum, most of the product of nucleic acid metabolism is present as nucleosides or free bases, since nucleotides are metabolized by 5′-nucleotidase and nucleoside phosphorylase while passing through cell membranes (20). However, human breast milk contains significant quantities of nucleotides, while most infant formulas are not fortified with nucleotides. Although little is known about the metabolism of dietary nucleotides in neonates or infants, our data may indicate the nutritional significance of nucleotides in breast milk in regard to enhancing immune functions.

Significant immunomodulating actions of yeast RNA/nucleotide can be demonstrated *in vitro*, suggesting the potential roles of RNA degraders in the regulation of immune functions. Our findings may also indicate the possible significance of RNA and nucleotide as immunomodulating nutrients. However, the mechanisms of their immunomodulating actions are poorly understood. Therefore, additional *in vivo* and *in vitro* studies are required to assess their significance as immunomodulating nutrients.

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