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Induction of IgM memory with RNA from the spleens of immunized mice

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Summary. Ribonucleic acid (immune RNA, *i*RNA), extracted from the spleens of mice immunized with sheep red blood cells (SRBC), induced specific immunological memory. The humoral immune responses of *i*RNA-treated animals were compared with *in vivo* and *in vitro* primary and secondary responses, using the haemolytic plaque-forming cell (PFC) assay. IgM antibody formation was markedly higher in *i*RNA-treated than in any other mice. The effect of *i*RNA was antigen-specific and similar materials from non-immunized mice had no activity.

INTRODUCTION

Immune RNA (*i*RNA) extracted from the spleens, peritoneal exudate cells or lymph nodes of immunized animals induced immunological memory as measured by *in vivo* serum antibody formation (Mitsuhashi, Kurashige, Kawakami & Nojima, 1968; Kurashige, Kitamura, Akama & Mitsuhashi, 1970; Yamaguchi, Kurashige & Mitsuhashi, 1971; Kurashige & Mitsuhashi, 1972; Kitamura, Kurashige & Mitsuhashi, 1973). Using the plaque-forming cell (PFC) technique, we now show that both *in vivo* and *in vitro*, the immunological memory induced with *i*RNA differs from that induced with antigen.

Abbreviations: *i*RNA, immune ribonucleic acid; *n*RNA, normal ribonucleic acid; SRBC, sheep red blood cells; HRBC, horse red blood cells; PFC, plaque-forming cells; 2-ME, 2-mercaptoethanol; FCS, foetal calf serum; SPC, spleen cells.

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MATERIALS AND METHODS

Animals

We used 8–12 week old BALB/c strain mice raised at the Central Animal Laboratory of Gunma University.

Antigens and immunization

Sheep red blood cells (SRBC), kindly supplied by the Central Diagnostic Laboratory of Livestock Diseases, Gunma Prefecture, and horse red blood cells (HRBC, Japan Biotest Laboratory, Tokyo) were stored in Alsever's solution until use. Unless otherwise stated, the mice were intraperitoneally (i.p.) injected with 0·1 ml of 10% SRBC or HRBC and boosted 4 weeks later.

Preparation of iRNA

RNA was extracted from the spleens of mice by a modification (Mitsuhashi *et al.*, 1968) of the method of Kidson, Kirby & Ralph (1963), except that bentonite (final concentration, 2·5%; Fraenkel-Conrat, Singer & Tsugita, 1961) was added to the RNA-extraction buffer. RNA preparations extracted from normal mice and mice immunized 5 days earlier were used as *n*RNA and *i*RNA, respectively.

Treatment of iRNA *preparation with amylase, proteinase, RNase or anti-SRBC antiserum*

Aliquots of the *i*RNA preparation (15 mg) in 3 ml of 0·85% NaCl were incubated for 30 min at 37° with amylase (100 mg/ml, α-Amylase, Sigma Chemical Co., Saint Louis), proteinase (600 μg/ml, Proteinase K, Merck) or RNase (50 μg/ml, RNase, recrystallized five times with ethanol and lyophilized, P-L Biochemicals, Inc., Milwakee). Another aliquot of the *i*RNA preparation was treated three times for 30 min at 37° with anti-SRBC mouse serum (IgM fraction). The enzymes and antiserum were removed by re-extraction of the treated RNA with phenol.

Culture medium and preparation of spleen cell suspension

RPMI 1640 (Nissui Seiyaku Co., Ltd, Tokyo) was used. For cell culture, the medium was supplemented with penicillin-G (50 u./ml), streptomycin sulphate (50 μg/ml), 2-mercaptoethanol (2-ME, 5×10^{-5} M) and decomplemented 20% foetal calf serum (FCS, Batch 90814, Microbiological Associates, Bethesda, Md).

The spleen cell suspension was prepared as follows. The spleens were dissected with curved forceps in a Petri dish containing the medium. The single cell suspension was washed three times with the medium by centrifugation and nucleated cells were counted in a haemocytometer. The trypan blue exclusion test was used to assay cell viability.

Culture method and in vitro *stimulation*

In a modification of the Marbrook culture system (Marbrook, 1967), we used the culture vessel shown in Fig. 1. A plastic disposable syringe (A) (5 ml, Terumo Co., Ltd, Tokyo) cut to a length of 41·5 mm was the inner tube, a glass vial (B) (PS-20, Nichiden Rika Garasu Co., Ltd, Tokyo) was the outer tube. The bottom of the inner tube was secured by a cellulose dialysis membrane (C) (Visking Company Co., Ltd) by means of a cut silicon tube (D) (12 mm in inner diameter, 7 mm in length). An aluminium cap (E) was used to cap it.

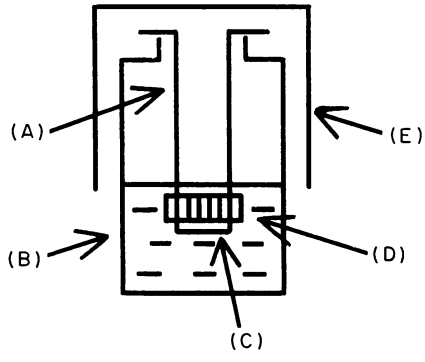

Figure 1. Modified Marbrook culture bottle. (A) plastic disposable syringe; (B) glass vial; (C) cellulose dialysis membrane; (D) silicon tube; (E) aluminium cap.

The spleen cell suspension (1 ml, 1×10^7/ml) in the culture medium was placed in the inner tube, 9 ml of the culture medium were inoculated into the outer tube. Culturing was at 37°–5% CO_2 in a humidified-air atmosphere. *In vitro* sensitization was by co-culturing 1×10^7 spleen cells and 1×10^6 RBC.

Detection of antibody-forming cells

Antibody-forming cells were detected as PFC according to the method of Jerne, Henry, Nordin, Fuji, Koros & Lefkovits (1974).

Determination of serum antibody

Mice were bled from the jugular vein. Serum (0·025 ml) was serially diluted by doubling dilutions with veronal buffer containing ethylenediamine tetracetic acid (EDTA, 0·01 M) and normal mouse serum (0·5%) on a microtitration plate. Equal volumes (0·025 ml) of 0·5% RBC suspensions in the buffer were added to each well. The mixture was thoroughly shaken and the settled pattern was read after leaving the plates at room temperature for approximately 24 hr.

2-ME treatment of the serum was as follows. Serum (0·025 ml) was mixed in the first well of the microtitration plate with an equal volume of veronal buffer containing 2-ME (0·2 M) and then the plate was allowed to stand at room temperature for 2 hr. By this procedure, most of the IgM antibody was inactivated. All sera were heated at 56° for 30 min before titration.

RESULTS

Distinction between primary and secondary *in vivo* responses

Normal and SRBC-primed mice were immunized with SRBC, and a kinetic study of the PFC-response was performed. As shown in Fig. 2, the primary and secondary responses were easily distinguishable by determining the PFC on day 4 after *in vivo* antigenic stimulation.

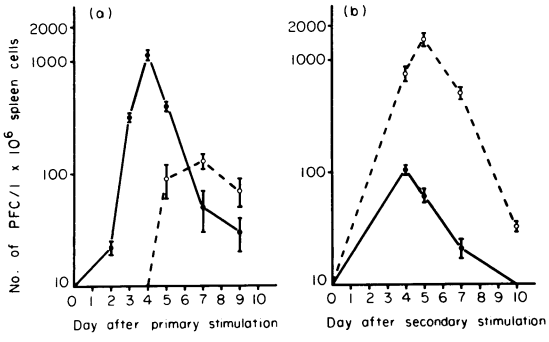

Figure 2. *In vivo* antibody formation against SRBC. (a) Primary response; (b) secondary response. Normal (a) and SRBC-primed mice (b) were i.p. immunized with SRBC. IgM-PFC (●———●) and IgG-PFC (o- - - -o) in spleen cells were assayed on the indicated days after immunization. The values represent the mean number of PFC/1 x 10^6 spleen cells ± SD of five mice assayed individually using SRBC.

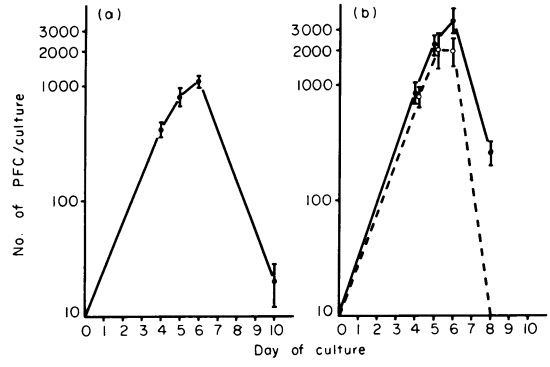

Figure 3. *In vitro* antibody formation against SRBC. (a) Primary response; (b) secondary response. Spleen cells from normal mice (a) and SRBC-primed mice (b) were cultured with or without SRBC. IgM-PFC (●———●) and IgG-PFC (o- - - -o) in cultured spleen cells were assayed on the indicated days of culture. Spleen cells from five mice of each group were pooled; triplicate or quadruplicate cultures were performed. The values represent the mean number of PFC/culture ± SD assayed individually using SRBC.

In vivo induction of immunological memory with *i*RNA

The antibody response of mice primed with various doses of *i*RNA was compared with that of normal and SRBC-primed mice. As shown in Table 1, the IgM antibody-formation response of *i*RNA-primed mice was markedly higher than that of the other tested mice. Concerning the IgG antibody-formation response, *i*RNA priming produced only a slight increase. Based on these results, 500 μg *i*RNA per mouse was chosen as the priming dose for detecting IgM and IgG memory.

Distinction between *in vitro* primary and secondary responses

Normal and SRBC-primed spleen cells were stimulated *in vitro* with SRBC and the kinetics of the PFC response were studied (Fig. 3). IgM-PFC were observed in both the primary and secondary responses, but IgG-PFC were present only in the secondary responses. Therefore, the primary and secondary responses were easily distinguishable by assaying the PFC on days 4, 5 or 6 of culture. Hereafter, we usually assayed PFC on day 5 of culture.

In vitro induction of immunologic memory with *i*RNA

The PFC response of spleen cells primed with various

Table 1. Effect of the *i*RNA-priming dose on *in vivo* antibody production

Mice	No. of PFC/1 x 10^6 SPC*		Serum agglutinin titre†	
	IgM	IgG	Total	2-ME resistant
Normal	200 ± 28	0 ± 0	7	< 2
*i*RNA 25 μg-primed	690 ± 42	0 ± 0	8	4
*i*RNA 100 μg-primed	1100 ± 99	98 ± 64	9	3
*i*RNA 300 μg-primed	430 ± 57	115 ± 78	8	3
*i*RNA 500 μg-primed	600 ± 14	265 ± 21	10	4
SRBC-primed	93 ± 14	1662 ± 106	11	11

* Mean ± SD of five mice. PFC in spleen cells were assayed individually on day 4 after secondary stimulation, at the same time, the serum antibody titre was determined.

† Log_2 titre of pooled serum.

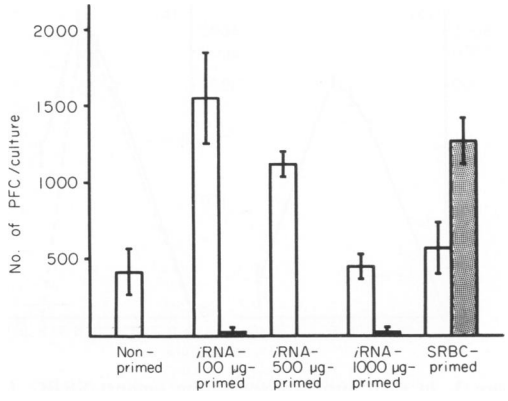

Figure 4. Effect of the *i*RNA-priming dose on *in vitro* antibody production. Spleen cells from normal mice, antigen-primed mice and mice injected 4 weeks earlier with *i*RNA (100 μg, 500 μg or 1000 μg per mouse) were stimulated *in vitro* with SRBC. Spleen cells from ten mice of each group were pooled; triplicate or quadruplicate cultures were performed. IgM-PFC (□) and IgG-PFC (□) in cultured spleen cells were assayed on day 5 of culture. The values represent the mean number of PFC/culture ± SD assayed individually using SRBC.

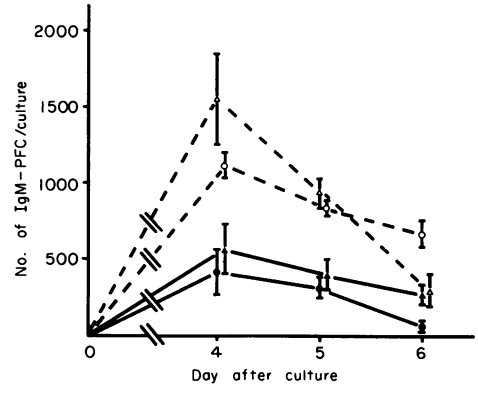

Figure 5. Immunological memory induced with *i*RNA. Spleen cells from normal mice (●———●), SRBC-primed mice (▲———▲) and mice injected 4 weeks earlier with 100 μg (△ - - - - △) or 500 μg (○ - - - - ○) *i*RNA were co-cultured with SRBC. Spleen cells from five mice of each group were pooled; triplicate or quadruplicate cultures were performed. IgM-PFC in cultured spleen cells were assayed on the indicated days of culture. The values represent the mean number of IgM-PFC/culture ± SD assayed individually using SRBC. IgG-PFC were detected only in SRBC-primed spleen cells.

Table 2. Antigen specificity of *i*RNA

Group	Cells cultured*		No. of anti-SRBC PFC/culture†		No. of anti-HRBC PFC/culture‡	
	Spleen cells	RBC	IgM	IgG	IgM	IgG
A	normal	(—)	47 ± 14	0 ± 0	15 ± 12	0 ± 0
		SRBC	533 ± 39	0 ± 0	ND§	ND
		HRBC	ND	ND	200 ± 91	0 ± 0
B	*i*RNA (against SRBC)-primed	SRBC	1993 ± 193	0 ± 0	ND	ND
		HRBC	ND	ND	253 ± 59	0 ± 0
C	*i*RNA (against HRBC)-primed	SRBC	635 ± 44	17 ± 32	ND	ND
		HRBC	ND	ND	480 ± 57	0 ± 0
D	SRBC-primed	(—)	43 ± 13	6 ± 9	ND	ND
		SRBC	170 ± 28	290 ± 84	0 ± 0	0 ± 0
		HRBC	35 ± 16	10 ± 10	23 ± 19	1 ± 2
E	HRBC-primed	(—)	ND	ND	50 ± 21	0 ± 0
		SRBC	500 ± 57	0 ± 0	8 ± 10	4 ± 7
		HRBC	22 ± 12	2 ± 3	110 ± 14	55 ± 21

* Spleen cells from seven mice of each group were pooled, and 1×10^7 cells were cultured with or without 1×10^6 RBC. The priming dose of *i*RNA was 500 μg/mouse.

† Mean ± SD of triplicate cultures assayed individually on day 5 of culture, using SRBC.

‡ Mean ± SD of triplicate cultures assayed individually on day 5 of culture, using HRBC.

§ Not done.

Table 3. Effect of normal RNA

Spleen cells cultured*	No. of anti-SRBC PFC/culture†	
	IgM	IgG
Normal	232±45	10±13
*n*RNA-primed‡	285±89	28±49
*i*RNA-primed§	1520±57	0±0
SRBC-primed	793±149	1202±220

* Spleen cells from seven mice of each group were pooled, and 1×10^7 cells were cultured with 1×10^6 SRBC.

† See footnote † of Table 2.

‡ Spleen cells from mice primed with 500 μg *n*RNA.

§ Spleen cells from mice primed with 500 μg of *i*RNA against SRBC.

doses of *i*RNA was compared with that of normal and SRBC-primed spleen cells (Fig. 4). At *i*RNA doses of 100 or 500 μg, the number of IgM-PFC was markedly higher in *i*RNA-primed spleen cells, IgG-PFC were detected only in SRBC-primed cells. Although weak IgG memory was transferred by *i*RNA in the *in vivo* experiment, this could not be detected *in vitro*.

Kinetic study of the PFC response of *i*RNA-primed spleen cells also revealed that IgM memory was induced with *i*RNA, but IgG-PFC were not detected except in SRBC-primed spleen cells (Fig. 5).

Antigen specificity of *i*RNA against *in vitro* antibody formation by cultured spleen cells

Immune RNA against SRBC and against HRBC was

Table 4. Antigenicity of *i*RNA

In vitro stimulation*	No. of anti-SRBC PFC/culture†	
	IgM	IgG
(—)‡	200±89	162±85
*i*RNA 20 μg/ml	160±89	160±41
*i*RNA 100 μg/ml	233±153	113±60
*i*RNA 500 μg/ml	183±93	200±80
*i*RNA 2500 μg/ml	180±84	187±85
SRBC 1×10^6‡	1033±208	1675±287
(—)	308±162	290±119
SRBC 1×10^2	366±153	310±89
SRBC 1×10^3	367±58	267±76
SRBC 1×10^4	350±58	333±58
SRBC 1×10^5	725±189	1150±282
SRBC 1×10^6	1380±130	1400±136

* SRBC-primed spleen cells from twenty mice were pooled and cultured with or without SRBC. In the *i*RNA-stimulated groups, spleen cells (1×10^7/ml, in serum-free medium) were preincubated for 30 min at 37° with the indicated *i*RNA concentration and recultured in culture medium containing FCS.

† See footnote † of Table 2.

‡ SRBC-primed spleen cells (1×10^7/ml) suspended in serum-free medium were preincubated without *i*RNA and 1×10^7 cells were recultured.

Table 5. Effect of the SRBC-priming dose on the *in vitro* secondary response

Priming dose of SRBC*	No. of anti-SRBC PFC/culture†	
	IgM	IgG
0	1431±257	13±28
1×10^1	882±128	4±8
1×10^2	540±143	3±6
1×10^3	57±44	3±6
1×10^4	1176±163	6±13
1×10^5	1158±93	108±21
1×10^6	1394±338	360±52
1×10^8	1268±266	316±73

* Mice were primed with the indicated SRBC dose. Spleen cells from five mice of each group were pooled and cultured with SRBC.
† See footnote † of Table 2.

prepared and injected into mice. Four weeks later their spleen cells were cultured with or without RBC. As shown in Table 2, IgM memory induced with *i*RNA was antigen-specific.

Effect of normal RNA

Normal, *n*RNA-primed, *i*RNA-primed, and SRBC-primed spleen cells were cultured with SRBC. The PFC response of *n*RNA-primed spleen cells was very similar to that of normal spleen cells (Table 3). This indicates that the ability of *i*RNA to induce IgM memory is not due to an adjuvant action of the RNA *per se*.

Evidence that *i*RNA does not contain any detectable antigen

SRBC-primed spleen cells were cultured with or without various doses of *i*RNA or SRBC. As shown in Table 4, at the doses used, *i*RNA did not stimulate SRBC-primed spleen cells.

The minimal SRBC-priming dose which can induce immunological memory was 1×10^5 per mouse, less than 1×10^4 SRBC could not induce this memory and 1×10^2 to 1×10^3 SRBC induced low-dose tolerance (Table 5).

Based on these results of our memory induction experiments, the antigenicity, if present, of 500 μg

Table 6. Treatment of *i*RNA preparations with amylase, proteinase, RNase or anti-SRBC antiserum

Spleen cells cultured*	No. of anti-SRBC PFC/culture†	
	IgM	IgG
Normal	2680±499	3±3
Non-treated *i*RNA-primed	5238±799	3±5
RNase-treated *i*RNA-primed	2772±331	6±14
Proteinase-treated *i*RNA-primed	4295±490	0±0
Amylase-treated *i*RNA-primed	4258±384	0±0
Anti-SRBC-treated *i*RNA-primed	5110±396	0±0
SRBC-primed	6335±710	4245±473

* Mice were primed with 500 μg of re-extracted *i*RNA or SRBC. Spleen cells from seven mice of each group were pooled, and 1×10^7 cells were cultured with 1×10^6 SRBC.
† See footnote † of Table 2.

*i*RNA is equivalent to or more than 1×10^5 SRBC (Table 5). If so, then 500 to 2500 μg of *i*RNA must induce the secondary response of SRBC-primed spleen cells. However, as shown in Table 4, these doses did not, indicating that *i*RNA does not contain SRBC antigen.

Further evidence that *i*RNA does not contain antigen

Immune RNA preparations were treated with amylase, proteinase, RNase, or anti-SRBC antiserum and transferred to mice to compare their activity with that of non-treated *i*RNA. As shown in Table 6, the ability of *i*RNA to induce IgM memory was totally abrogated by RNase treatment; the other treatments showed no detectable effect. These results indicate that the activity of the *i*RNA preparation requires intact RNA, and also that *i*RNA does not contain antigen.

DISCUSSION

The present study showed that immunological memory induced with *i*RNA differred from that induced with antigen both *in vivo* and *in vitro*. The immunological memory induced with *i*RNA was mainly IgM memory. The activity of *i*RNA to induce IgM memory was antigen-specific, normal RNA had no activity, indicating that the activity of *i*RNA was not an adjuvant action of RNA itself. Although in the *in vivo* experiment, weak IgG memory was induced with *i*RNA, it could not induce complete IgG memory at the stage of memory cell induction. Although the mechanism that switches IgM production to IgG production is not completely understood, we hypothesize that IgM memory is manifested at first; thereupon a conversion from IgM to IgG production may occur. Namely, if there were antigen at the stage of memory induction, IgM memory cells would be changed into IgG memory cells. Based on these considerations, we suggest that *i*RNA induces a certain degree of antigen-specific differentiation of immunocytes and that, cells are committed to respond to subsequent antigenic stimulation as IgM memory cells or helper cells.

Concerning the nature of *i*RNA, sucrose density gradient centrifugation detected the active *i*RNA fraction in subfractions 4 to 10S, and 10 to 18S (manuscript in preparation). Furthermore, oligo d(T)-cellulose chromatography showed that the poly (A)-minus as well as the poly (A)-plus fraction could induce IgM memory (in preparation). Therefore, *i*RNA could be messenger RNA (mRNA) which, after uptake by cells, could attach to the ribosomes and be then used to translate a protein which may be antibody or have a regulatory role. Another possibility is the reverse transcription of this RNA to form DNA copies which can then be integrated into the cell's genome. This latter process might account for the selectivity of the RNA preparation with respect to the competence of only immunologically committed cells to respond in the manner shown.

Based on the results of the present study, this RNA did not contain antigen and its activity to induce IgM memory was not due to contamination by SRBC antigen. At the doses used (20 μg to 2500 μg), *i*RNA did not induce an *in vitro* PFC response of SRBC-primed spleen cells (Table 4). There exists an optimal *i*RNA dose for the induction of IgM memory (Table 1 and Fig. 4) and while low dose of SRBC induced tolerance (Table 4), none of the used *i*RNA doses did. The activity of *i*RNA was not abrogated by anti-SRBC treatment (Table 6) although it was totally abrogated by RNase treatment (Table 6). This indicates that intact *i*RNA is necessary for the induction of IgM memory. In a separate investigation, we have found that the injection of a massive dose of *i*RNA (12·5 mg/mouse) into SRBC-primed mice did not elicit a PFC response, nor was tolerance induced (submitted for publication).

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