The effect of total RNA on splenocyte response to allogenic cells

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In this study we have estimated the ability of total RNA to change the activity of splenocytes in a mixed cell culture where stimulator cells are not inactivated, similar to cell interactions *in vivo*. According to our experimental results, activity of splenocytes was diminished in all cases when RNA and stimulator spleen cells had been taken from the same mouse line, even if responder spleen cells were from mice immunized against antigens of stimulator cells. On the contrary, we could not find RNA downregulating effect, if stimulator cells and RNA were from different mouse lines. Therefore, here we found a strictly genetically specific downregulating effect of RNA. Since only messenger RNA carries information about allogenic antigens, we can suspect that it is messenger RNA that should be the main downregulator of responder spleen cells. Moreover, the effect of mRNA on different immune cells (antigen presenting cells, T cells) suggests a novel mechanism of self-antigen recognition through mRNA, which remains to be elucidated.

Key words: RNA, mixed splenocyte culture, genetically specific downregulation

INTRODUCTION

Transfused blood before transplantation can serve as a tolerogenic material [1, 2]. Erythrocytes retain an appreciable amount of poly (A) + RNA, and cell-free translation studies indicate that this material is translationally active [3]. On the other hand, erythrocytes and other blood cells comprise the ribonuclease inhibitors that prolong survival of RNR [4]. For that reason, we hypothesize that RNA can serve as a tolerogen. The purpose of this study was to test the ability of total RNA to change the activity of splenocytes in a mixed cell culture where stimulator cells are not inactivated, similarly to cell interactions *in vivo*. We are presenting primary data before developing a full-length paper.

MATERIALS AND METHODS

The female mice were obtained from the Institute of Immunology, Vilnius, Lithuania. Six months old animals were killed by cervical dislocation. After the mice were killed, the spleen was removed aseptically and was mechanically disrupted in 5 ml of the used medium. The spleen cells were cultivated in the flat-bottom 96-microwell plates in 10% CO_2-in-air atmosphere at a temperature of 28 °C. Some of responder spleen cells were obtained from CC57W mice which were immunized by C57BL/6 skin transplant (1 x 1 cm) four months before the experiment. The used medium was based on RPMI 1640, supplemented with sodium pyruvate (1 mM), heat-inactivated fetal calf serum (10%), 2-mercaptoethanol (1 mM), antibiotics (penicillin 50 μg/ml and streptomycin 50 μg/ml) and 10 mM Hepes.

RNA was extracted from mouse livers by a phenol-method [5]. Six months old female mice were killed by ether. The livers were excised aseptically and frozen at –20 °C in EDTA-Tris buffer (0.1 M NaCl, 0.001 M EDTA, 0.02 M Tris-HCl pH 7.4). The frozen livers were ground with pestle and homogenized in phenol saturated with EDTA-Tris buffer containing 0.1% SDS. After homogenization, the mixture was centrifuged at 4 °C, 800 x g for 20 min to separate the phases. The aqueous phase was extracted again with phenol saturated with buffer. Phenol was removed from aqueous phase with ether at 4 °C. DNA was removed by Deoxyribonuclease I from bovine pancreas free of RNase (Fluka 31135). 3000 units of Deoxyribonuclease in 5 ml Tris buffer (0.12 M Tris-HCl pH 7.4; 0.03 M $MgCl_2$) was added to 10 ml aqueous phase. After 15 min of cultivation with Deoxyribonuclease at 37 °C, Deoxyri-

bonuclease was removed by double extraction with phenol saturated with buffer. Phenol was removed with ether. RNA was precipitated overnight at –20 °C by absolute ethanol. The precipitate was collected by centrifugation at 4 °C and washed twice with a 3:1 ethanol-water mixture. The absorbance of the RNA solution was estimated on a Perkin-ELMER 550. The purity of RNA was controlled by the ratio of absorbances at 260 and 280 nm (A_{260}:A_{280} > 1.80). The amount of RNA recovered was determined from the absorbance of RNA solution at 260 nm.

50 µl of medium containing 200 µg/ml of total RNA was added to 100 µl of responder spleen cells (20 x 10^6 cell/ml) incubated in 96 microwell plates. At the third day of incubation 50 µl of stimulator cells (20 x 10^6 cell/ml) was added to the same microwells plate. Six days later the medium in each well was changed to 100 µl RPMI-PR$^-$ medium with MTT (Sigma Chemical Co.) (1 mg/ml) for additional 3 h. After the medium was removed, 50 µl isopropanol was added into each well. The optical density of dissolved formazan in each well was measured by an automatic plate reader (Multiscan EX) with 540 nm test-wavelength and 690 nm control-wavelength. The difference of measured optical density in 540 nm and 690 nm shows the activity of cell proliferation [6]. Statistical analysis of experiments was done by t test for independent samples.

RESULTS AND DISCUSSION

As one can see from experimental results presented in Table, OD was diminished in all cases if RNA and stimulator spleen cells were taken from the same mouse line (#2, #5, #13, #15). The downregulating effect of RNA was the same even if responder spleen cells had been taken from mice immunized against antigens of stimulator cells (#12 compared with #13). On the contrary, we could not find the downregulating effect of RNA if stimulator cells and RNA had been taken from different mouse lines (#1, #3, #4, #6, #12, #14) or if stimulator cells were the same as responder spleen cells (#8 compared with #9, P = 0.779). No differences were observed in the downregulating effect of RNA on allogenic or syngenic spleen cells in our primary experiments (#10 compared with #11, P = 0.117). Therefore here we found a strictly genetic specific downregulating effect. Since only messenger RNA carry information about allogenic antigens, we can suspect that it is messenger RNA that should be the main downregulator. Furthermore, the effect of mRNA on different immune cells (antigen presenting cells, T cells) suggest a novel mechanism of self-antigen recognition through mRNA, which remains to be elucidated.

At the end of our discussion, we would like to suggest mRNA as the main immunotolerogen in case the further experiments confirm that mRNA *in vivo* carries antigenic information from nonimmune cells to antigen-presenting cells and if this antigen-presenting cell is able to produce and present epitopes of nonself antigens as self.

ACKNOWLEDGMENTS

We sincerely thank Dr Dalia Juzulienė, Dr Violeta Vaičiuvienė, Gintautas Višniauskis and Dr Vytautas Mažeika for their technician support. We also thank Dr Daiva Bironaitė for critically reviewing the

Table. **Genetic specific downregulating effect of total RNA on splenocyte response to allogenic spleen cells**
OD – the mean of difference of measured optical density in 540 nm and 690 nm – the activity of splenocyte proliferation; SD – standard deviation of OD. CC57W-s – spleen cells from CC57W mice, which were immunized by C57BL/6 skin transplant.

#	Responder	RNA from	Stimulator	Number of well	OD	SD
1	CC57W	CC57W	CBA	32	0.089	0,015
2	CC57W	CBA	CBA	31	0.037	0,009
3	CC57W	C57BL/6	CBA	8	0.086	0.020
4	CC57W	CC57W	C57BL/6	16	0.090	0.018
5	CC57W	C57BL/6	C57BL/6	16	0.040	0.009
6	CC57W	CBA	C57BL/6	16	0.092	0.020
7	CC57W	–	C57BL/6	16	0.089	0.018
8	CC57W	C57BL/6	CC57W	16	0.062	0.016
9	CC57W	–	CC57W	16	0.060	0.010
10	–	C57BL/6	CC57W	8	0.022	0.003
11	–	C57BL/6	C57BL/6	8	0.020	0.003
12	CC57W-s	CC57W	C57BL/6	32	0.153	0.014
13	CC57W-s	C57BL/6	C57BL/6	48	0.036	0.006
14	CC57W-s	CC57W	CBA	16	0.092	0.005
15	CC57W-s	CBA	CBA	32	0.031	0.006

manuscript. This work was supported by grant 97P-06 from Lithuanian Innovation Centre.

Received
8 February 2001

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RNR POVEIKIS SPLENOCITŲ ATSAKUI Į ALOGENINES LĄSTELES

S a n t r a u k a

Šiame darbe mes nustatėme, kaip bendra RNR keičia splenocitų aktyvumą mišrioje ląstelių kultūroje, kurioje stimuliuojančių ląstelių proliferacinis aktyvumas nėra supresuotas, panašiai kaip ląstelių sąveikoje *in vivo*. Eksperimentų rezultatai parodė, kad splenocitų aktyvumas sumažėjo visais atvejais, kai RNR ir stimuliuojančios blužnies ląstelės buvo paimtos iš vienos linijos pelės, net jei atsakančios ląstelės buvo iš pelių, imunizuotų prieš stimuliuojančių ląstelių antigenus. Ląstelių aktyvumą slopinančio RNR poveikio nenustatėme, kai stimuliuojančios ląstelės ir RNR priklausė skirtingoms pelių linijoms. Remiantis šiais rezultatais galima daryti prielaidą, kad čia stebime genetiškai specifinį imuniteto ląstelių aktyvumą reguliuojantį RNR poveikį. Kadangi informaciją apie alogeninius antigenus perneša tik iRNR, galime tikėtis, kad būtent iRNR galėtų būti pagrindiniu atsakančių ląstelių reguliatoriumi. Aprašytas RNR poveikis suponuoja naują kelią, savų antigenų atpažinimo reguliavimą iRNR, kuris turėtų būti patikrintas tolesniais eksperimentais.

Raktažodžiai: RNR, maišyta splenocitų kultūra, genetiškai specifinis imuniteto reguliavimas