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Effect of the calcium phosphate-mediated RNA uptake on the transfer of cellular immunity of a synthetic peptide of HIV-1 to human lymphocytes by exogenous RNA

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Abstract

It is known that exogenous RNA molecules can be taken up by eukaryotic cells and can exert a variety of biological effects both *in vitro* and *in vivo*. The modulation of human lymphocytes by exogenous RNAs has medical implications. The exogenous RNA used in this study was obtained from lymphoid organs of animals immunized with the synthetic peptide p12 of HIV-1 and was referred to as p12-RNA. Human lymphocytes were transfected with the p12-RNA and the transfer of immunoreactivity of p12 was assessed by the lymphocyte proliferation and the leukocyte adherence inhibition assays. Our results indicate that the transfer of cellular immune response to the p12 occurred in 9 donors (60%) who were named responsive individuals whereas 6 donors (40%) were non-responsives. We also found that the calcium phosphate-mediated RNA uptake method is effective in converting non-responsive into responsive donors. The calcium phosphate-mediated RNA uptake may also be used to increase the efficiency of RNA transfection in other models with medical implications and to contribute to a better understanding of the molecular events involved in the uptake of RNA. Our findings give support for the use of exogenous RNAs obtained from lymphoid organs of immunized animals with synthetic peptides of HIV-1 in the immune reconstitution of individuals infected with HIV-1. (Mol Cell Biochem **228**: 9–14, 2001)

Key words: RNA transfection, calcium phosphate-mediated uptake, peptide, HIV-1, lymphocyte

Introduction

The ability of eukaryotic cells to take up exogenously supplied RNA molecules has been described for more than three decades [1]. However, it was only a few years ago that this phenomenon was first used as a biochemical tool. Thus, the role of the oncogene *Wtn* in development was elucidated by the injection of the *Wtn* mRNA into *Xenopus laevis* embryos [2]. More recently, it was shown that dendritic cells synthesized tumor specific antigens after incubation with RNA from tumor cells [3]. Interestingly, dendritic cells pulsed with RNA are potent antigen-presenting cells which is relevant for the immunotherapy of human cancer [4].

It is well known that DNA uptake is greatly increased by calcium phosphate and this transfection method has been widely used in the protocols of gene transfer into target cells. To date, there is only one report on the effect of calcium phosphate on the RNA uptake where the authors proposed the RNA transfection with calcium phosphate as a method for studying RNA processing in human cells [5].

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In the present work, the exogenous RNA was obtained from lymphoid organs of animals immunized with a synthetic peptide (p12) of HIV-1 referred to as p12-RNA. The p12 is a peptide of 12 amino acid residues corresponding to amino acids 600–611 of the precursor envelope protein gp-160 of the HIV-1 which is one of highly conserved epitopes of gp-41 in mature viral particles [6]. We found that the p12-RNA is active in transferring cellular immune responses of p12 to human lymphocytes. However, the percentage of cellular immunoreactivity transfer was only 60% (responsive donors) whereas 40% were non-responsive individuals. It is important to note that this finding represents a drawback for the use of p12-RNA as an alternative for immunomodulation in HIV-1 infection. Therefore, it is of interest to investigate the possibility of converting non-responsive into responsive donors by using methods that could increase the uptake of the p12-RNA by human lymphocytes. In this study, we have used the calcium phosphate-mediated p12-RNA uptake method in an attempt to induce the conversion of non-responsive into responsive individuals.

Material and methods

Animals

The BALB/c mice weighing 20–25 g were raised by the Central Animal Laboratory of the School of Medicine University of São Paulo, Ribeirão Preto, SP, Brazil. Care and treatment of experimental animals received prior institutional approval.

Immunization of RNA donors

The antigen used to immunize the BALB/c mice is a peptide of 12 amino acid residues (p12) with the sequence LGIWGCSGKLIC, corresponding to amino acids 600–611 of the precursor envelope protein gp-160 of the HIV-1 which is one of highly conserved epitope of gp-41 [6]. The p12 was synthesized by the Merrifield solid-phase method [7] and was a gift from Dr. Nelson F. Mendes (Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil). The immunization of the BALB/c mice with the p12 was as previously described [8].

Extraction of RNA

The RNA was extracted from the lymphoid organs of non-immunized (N-RNA) and immunized with p12 (p12-RNA) mice as described by White and De Lucca [9]. The B16-RNA was obtained from lymphoid organs of guinea pigs immunized with B16/F10 melanoma cells as reported earlier [10]. The B16-RNA was used as control in the immunological assays since we have previously demonstrated that the B16-RNA is able to transfer immunoreactivity to lymphocytes [10]. The integrity of RNA preparations was routinely evaluated by electrophoresis on 1% agarose.

Treatment of the p12-RNA with RNase

Pancreatic RNase (type I-A, Sigma Chemical Co.) was added to a solution of p12-RNA in 0.85% NaCl at the ratio of 1 μg of RNase to 10 μg of p12-RNA and the mixture was incubated at 37°C for 30 min.

Lymphocyte preparation

The peripheral blood mononuclear cells of individuals seronegative for HIV-1 were obtained by Ficoll–Hypaque density gradient centrifugation. The mononuclear cells were collected, washed and incubated at 37°C for 1 h on plastic dishes to remove monocytes. The purity (95%) of the human lymphocyte preparations was obtained from a smear of the lymphocyte fraction treated with Wright's stain. Lymphocyte viability (95%) was determined by the Trypan blue exclusion test. The lymphocyte preparations were used in the experiments of RNA transfection by the method of calcium phosphate.

RNA transfection by the calcium phosphate method

The p12-RNA transfection was essentially as described by Kleinschmidt and Pederson [5]. After formation of calcium phosphate-bound N-RNA or p12-RNA, this mixture was added to human lymphocytes (150 μg of N-RNA or p12-RNA/10^6 cells) and incubated at 37°C for 16 h in an atmosphere of 5% of CO_2. After incubation, the cells were harvested, washed 2 times with RPMI medium and used in the lymphocyte proliferation and the leukocyte adherence inhibition assays.

Lymphocyte proliferation assay

Quadruplicates cultures containing 5×10^5 lymphocytes in 0.1 ml of RPMI medium, supplemented with 10% fetal calf serum and 0.1% gentamicin, were maintained in plastic microtiter plates with a U-shaped bottom. To measure blastogenesis, 1 μCi of ^{3}H-thymidine (specific activity 65 Ci/mmol) was added after 5 days in culture for an 8 h pulse. Cultures were harvested on glass fiber filters and radioactivity determined by liquid scintillation. The antigen used in all experi-

ments was the synthetic peptide p12. The results are expressed as the stimulation index, which is the ratio: c.p.m. in the presence of antigen/c.p.m. in the absence of antigen. In some experiments, the synthetic peptide with the sequence ISRPPFGSPFR corresponding to IS-bradykinin was used as non-related antigen.

Leukocyte adherence inhibition (LAI) assay

The LAI assay was performed according to Artigas *et al.* [11]. Briefly, aliquots of 0.1 ml of the lymphocyte suspension (10^7 cells/ml) were placed in glass test tubes (16 × 150 mm) and p12 was added to each tube at a final concentration of 2 µg/ml. The mixture was brought to a final volume of 0.5 ml by the addition of Eagle's minimum essential medium. The tubes were agitated and incubated horizontally so that the contents covered 4/5 of the lower surface of each tube. After incubation at 37°C for 2 h, the tubes were placed vertically and their contents were gently aspirated with a Pasteur pipette. The non-adherent cells were counted in a hemocytometer and all assays were done in triplicate. The results were expressed as a non-adherence index (NAI) = [(A – B)/B] × 100, where A is the number of non-adherent cells in the presence of p12 and B is the number of non-adherent cells in the presence of medium alone. NAI values equal to or greater than 30 were taken to indicate a positive response. In some experiments, the synthetic peptide with the sequence ISRPPFGSPFR corresponding to IS-bradykinin was used as a non-related antigen.

Statistical analysis

Data were analyzed statistically by Student's *t*-test with the level of significance set at $p < 0.05$.

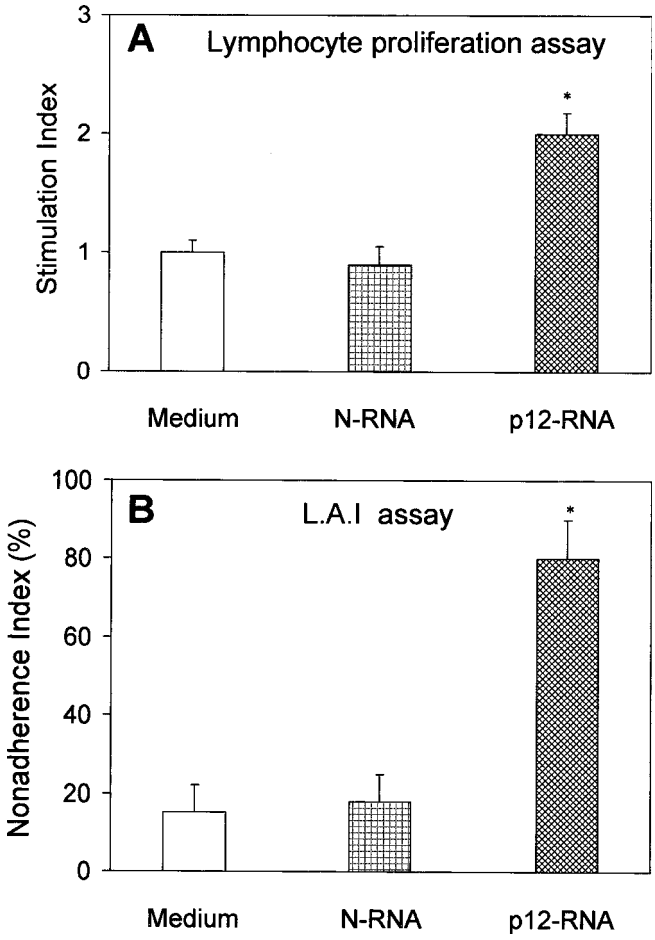

Fig. 1. Transfer of cellular immune response of p12 to human lymphocytes with p12-RNA. The transfer of cellular immunity was assessed by the lymphocyte proliferation assay (Panel A) and the leukocyte adherence inhibition assay (Panel B). Human lymphocytes incubated with medium alone or N-RNA were used as controls. Values represent means ± S.D. of three independent assays. *Significantly different from control values ($p < 0.05$).

Results

It is known that the integrity of exogenous RNA is essential in order to exert its biological effect [12]. Because RNA is easily degraded by mechanical shearing and by RNase cleavage, we always tested the integrity of our p12-RNA preparations before using in the immunological assays. The electrophoretic profiles obtained with the p12-RNA samples were always characteristic of non-degraded RNA preparations of eukaryotic cells.

We found by using the lymphocyte proliferative assay that the p12-RNA is active in transferring cellular immune response of the p12 to human lymphocytes whereas the N-RNA had no effect (Fig. 1, panel A). These results were confirmed using the leukocytes of the same donor and the LAI assay as a parameter to detect cellular immunity (Fig. 1, panel B). The ability of the p12-RNA in transferring cellular immunity to the p12 is abolished by treatment with RNase (Table 1). We also observed that the transfer of cellular immune response to the p12 is not detected when we used non-related antigen (Table 2) or different type of exogenous RNA but the p12 as antigen (Table 3).

The results depicted in Fig. 2 indicated that the p12-RNA is not able to transfer immunoreactivity to the p12 to all donors. Thus, we found that there is a group of individuals that is responsive to the p12-RNA (Fig. 2, panel A) and the other group is referred to as non-responsive donors (Fig. 2, panel B). Our findings indicated that 9 donors (60%) were responsives whereas 6 individuals (40%) were non-responsives as determined by the lymphocyte proliferative assay (Fig. 2) and by the LAI assay (data not shown).

Table 1. Effect of RNase treatment on p12-RNA transfer of cellular immunity of the p12 to human lymphocytes

Immunological assay	Experiment	Treatment with RNase	
		p12-RNA	p12-RNA+ RNase
LAI assay (N.A.I.)**	1	50 ± 3*	16 ± 1
	2	48 ± 2*	12 ± 1
Lymphocyte proliferation assay (S.I.)***	1	2.60 ± 0.18*	0.80 ± 0.05
	2	2.10 ± 0.12*	1.00 ± 0.02

Values represent means ± S.D. of three independent assays. *p < 0.01 compared to P12-RNA + RNase; **N.A.I. = non-adherence index (%); ***S.I. = stimulation index.

Table 2. Detection of the cellular immunity transfer of p12 to human lymphocytes with p12-RNA in the presence of specific and non-specific antigens

Immunological assay	Experiment	Antigen	
		Specific (p12)	Non-related
LAI assay (N.A.I.)**	1	70 ± 5*	25 ± 2
	2	62 ± 3*	16 ± 1
Lymphocyte proliferation assay (S.I.)***	1	2.30 ± 0.15*	0.90 ± 0.05
	2	1.90 ± 0.09*	1.00 ± 0.01

The synthetic peptide ISRPPFGSPFR was used as a non-related antigen. Values represent means ± S.D. of three independent assays. *p < 0.01 compared to non-related peptide; **N.A.I. = non-adherence index (%); ***S.I. = stimulation index.

Table 3. Transfer of cellular immunity of the p12 to human lymphocytes by the p12-RNA and B16-RNA

Immunological assay	Experiment	Exogenous RNA	
		p12-RNA	B16-RNA
LAI assay (N.A.I.)**	1	58 ± 4*	23 ± 1
	2	58 ± 3*	18 ± 1
Lymphocyte proliferation assay (S.I.)***	1	1.80 ± 0.09*	0.90 ± 0.04
	2	1.90 ± 0.07*	1.10 ± 0.03

Values represent means ± S.D. of three independent assays. * p < 0.01 compared to B16-RNA. ** N.A.I. = non-adherence index (%); ***S.I. = stimulation index.

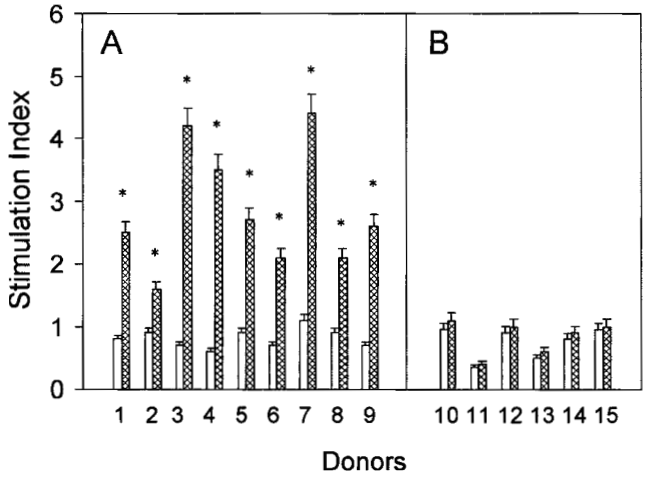

Fig. 2. Transfer of cellular immune response of p12 to human lymphocytes with p12-RNA assessed by lymphocyte proliferation assay. Panel A: Responsive donors. Panel B: Non-responsive donors. Human lymphocytes were incubated with medium ( ) and with the p12-RNA ( ). Lymphocytes incubated with medium alone were used as control. Values represent means ± S.D. of three independent assays. *Significantly different from the corresponding control value (p < 0.05).

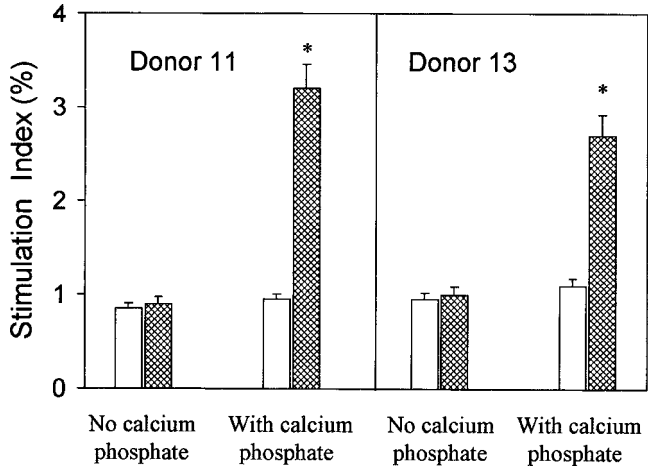

Fig. 3. Effect of the calcium phosphate on the transfer of cellular immune response of p12 to human lymphocytes with p12-RNA assessed by lymphocyte proliferation assay. The experiments were performed with lymphocytes from the donors 11 and 13 which are non-responsive individuals according to Fig. 2. Human lymphocytes were incubated with medium ( ) and with the p12-RNA ( ). Lymphocytes incubated with medium alone were used as control. Values represent means ± S.D. of three independent assays. * Significantly different from control value (p < 0.05).

The next step was to examine the possibility of converting non-responsive individuals into responsive donors by incubating human lymphocytes with the p12-RNA in the presence of calcium phosphate. Figure 3 shows that the calcium phosphate-mediated p12-RNA transfection is efficient to convert the non-responsive donors 11 and 13 (Fig. 2) into responsive donors. Similar results were obtained with lymphocytes from the non-responsive donors 10, 12, 14 and 15 (Fig. 2) and the conversion of non-responsive into responsive donors was also assessed by the lymphocyte proliferative assay (data not shown).

Discussion

The possibility of using RNA molecules as therapeutic agents such as ribozymes and antisense RNAs stimulates the search for new methods in order to increase the uptake of RNA. It is well documented that exogenous RNA molecules can be taken up by eukaryotic cells and can exert a variety of biological effects both *in vitro* and *in vivo* [13]. Thus, the cellular uptake of RNA has been reported to result in induction of differentiation [14], synthesis of specific proteins [15] and secretion of cytokines by macrophages [16, 17]. It has been also demonstrated that exogenous RNAs are active in transferring cellular immune responses to a variety of antigens [12, 13, 18–22].

The results presented here showed that the p12-RNA is effective in transferring cellular immunoreactivity of the synthetic peptide p12 of HIV-1 to human lymphocytes. It should be noted that the lymphocyte proliferative and LAI assays used in this study are reliable and reproducible tests to detect cellular immune responses [10, 11]. We also found that this effect was abolished by the treatment of p12-RNA with RNase, indicating that the integrity of the exogenous RNA is essential for its immunological activity. It was also possible to confirm that the transfer of cellular immunity with p12-RNA is specific in terms of antigen and type of exogenous RNA as previously demonstrated by another investigator [13] and by our group [12].

We have already reported that exogenous RNAs obtained from lymphoid organs of immunized animals with synthetic peptides of HIV-1 are able to induce immunological memory, secretion of IFN-γ and to generate specific HIV-1 cytotoxic T lymphocytes [8, 19]. Recently, we demonstrated that these effects are mediated by the RNA-dependent protein kinase (PKR) and the transcription factor NF-κB [23, 24]. These findings represent the first example of elucidation of molecular mechanisms underlying the action of an exogenous RNA. The concept of regulatory RNA is now emerging based on findings showing that there are transcripts that do not encode proteins but rather function directly as RNAs [25]. It is possible that p12-RNA constitutes a small fraction of our total RNA preparations which acts as a regulatory RNA.

It should be emphasized that the phenomenon of modulation of human lymphocytes by exogenous RNAs has potential medical applications. Thus, the manipulation of lymphoid cells with an exogenous RNA named anti-tumor RNA was already used in the treatment of human cancer [26, 27].

We have proposed the use of these exogenous RNA obtained from lymphoid organs of immunized animals with synthetic peptides of HIV-1 as an alternative for immunomodulation in HIV-1 infection [8, 19]. However, our data with p12-RNA indicate that the transfer of cellular immune response to p12 was negative in 40% of the individuals tested and this group was referred to as non-responsive donors. Therefore, this finding can be considered as a drawback for the use of p12-RNA as a immunomodulator in patients infected with HIV-1. However, in the present work we succeeded in converting non-responsive into responsive donors using the p12-RNA transfection of human lymphocytes with the calcium phosphate method. Thus, our results give support for the use of exogenous RNAs obtained from lymphoid organs of immunized animals with synthetic peptides of HIV-1 in the immune reconstitution of individuals infected with HIV-1. There is a need for immunomodulators to treat patients infected with HIV-1 due to the fact that the highly active antiretroviral therapy (HAART) restores the number of CD4+ and CD8+ lymphocytes but remains uncertain whether the functions of these cells are normal or impaired [28]. Moreover, it was demonstrated that the HAART is not able to eradicate latent HIV-1 [28].

Despite the great amount of work with DNA transfection, the molecular events involved in uptake of DNA are still poorly understood. It is well known that the use of the calcium phosphate method increases the efficiency of the transfection by DNA. One possible speculation is that this method could increase the interaction of the DNA with a protein recently described in the plasmatic membrane, which is involved in the binding and internalization of exogenous DNA [29]. However, the mechanism responsible for the RNA uptake by eukaryotic cells remains an open challenge. The calcium phosphate-mediated RNA uptake may be used not only to convert non-responsive into responsive donors but also to increase the efficiency of RNA transfection in other models with medical implications [30] and to contribute to a better understanding of the molecular events involved in the uptake of RNA.

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