

# *In vivo* immunomodulation with poly(A)<sup>+</sup> RNA against a murine Balb/3T3 fibrosarcoma transfected with a mutant Ha-ras-1 oncogene

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## ABSTRACT

Balb-c mice injected subcutaneously with a mouse embryo-derived Balb/3T3 cell line transfected with a mutant form of the oncogene c-Ha-ras-1, developed a neoplastic mass, classified as fibrosarcoma, at the site of injection. The cytoplasmic RNA from the spleens of these tumor-bearing mice was prepared and further separated into poly(A)<sup>+</sup> and poly(A)<sup>-</sup> RNA fractions. These RNA preparations were separately injected into normal Balb-c mice. The total cytoplasmic RNA and the poly(A)<sup>+</sup> RNA fraction were able to induce *in vivo* reactivity against a challenge with viable neoplastic cells. The Poly(A)<sup>-</sup> RNA fraction had no effect, nor did the control RNA extracted from the spleens of normal Balb-c mice.

## INTRODUCTION

The use of exogenous RNA preparations, extracted from tumor-immunized or tumor bearing animals, in experimental research and human tumor immunotherapy (Steele Jr. *et al.*, 1982) has provided evidence that RNA can be used as an antitumor immunomodulator. When injected *in vivo* into animals these RNA preparations can induce specific immunological memory, and it has been demonstrated that the active component is the poly(A) containing RNA (messenger RNA fraction) (Passos Jr. and De Lucca, 1988). These preparations may contain specific RNA messages which are translated into proteins involved in immune recognition in the receptor cells.

In human tumors expressing *ras* oncogene family members, mutations that result in transforming proteins with amino acid substitutions in positions 12, 13 or 61 are frequently found (Barbacid, 1987; Barbacid, 1988; Bos *et al.*, 1987 and Bos, 1989). Recently, it was demonstrated that synthetic peptides harboring these substitutions are able to generate *in vitro* specific sensitized human T-lymphocytes clones (Gedde-Dahl III *et al.*, 1993). These positions in the p21 *ras* protein are considered as mutational hot-spots and are immunogenic in man. The peptides encompassing these substitutions are candidate tumor antigens (Gedde-Dahl III *et al.*, 1993).

The mouse embryo derived cell strain Balb/3T3 transfected with a mutated form of the human c-Ha-ras-1 (EJ-ras) oncogene expresses the p21 *ras* protein with a substitution gly → val 12 (cell strain B61) (Kovary *et al.*, 1989). Our objective was to organize a model-system for studies on the *in vivo* immuno-

modulation by exogenous RNA against a fibrosarcoma generated in Balb-c mice by injection of the B61 cell strain.

## MATERIAL AND METHODS

### Transformed cells

Mouse Balb/3T3 fibroblasts were transfected with the mutated form of the human c-Ha-ras-1 (EJ-ras) oncogene (pEJ plasmid expressing the p21 ras protein with a substitution gly → val 12) (Shih and Weinberg, 1982; Kovary *et al.*, 1989). This transformed cell strain, called B61, bears 50-100 copies of the active c-Ha-ras-1 oncogene and was generated by Kovary *et al.* (1989).

### *In vivo* generation of the tumor

The transformed cells (clone B61) were cultured in HAM F-10 medium plus 10% fetal bovine serum, 5% CO<sub>2</sub>, 95% air at 37°C for one week. The cultures were trypsinized and supplemented with fresh medium at intervals of three days. We injected the transformed cells subcutaneously in the scapular region of each Balb-c four to six week-old male or female immunocompetent mouse. We inspected for palpable tumors at the site of the injection: Once detected, the tumors were removed surgically and the diameters measured with a caliper. Some tumors were fixed in 70% cold ethanol, sectioned, and stained with hematoxylin-eosin for routine histological examination.

### RNA extraction and fractionation

Total cytoplasmic RNA was extracted from the spleens of tumor-bearing Balb-c mice by the cold phenol method (White and De Lucca, 1977). These samples were referred to as anti-B61-RNA. Samples of cytoplasmic RNA from normal Balb-c mice extracted by the same procedure were referred to as normal-RNA (N-RNA). We used only preparations that were free of phenol ( $A_{220}/A_{260} = 0.7$ ) and free of protein ( $A_{260}/A_{280} = 1.9-2.0$ ) and non-degraded, as judged by SDS-polyacrylamide cylindrical gel electrophoresis (Sambrook *et al.*, 1989). The gels were stained with ethidium bromide and the positions of ribosomal and transfer RNAs were visualized under ultraviolet illumination. Some anti-B61-RNA samples were fractionated into poly(A)<sup>+</sup> and poly(A)<sup>-</sup> RNA by oligo dT-cellulose chromatography (Aviv and Leder, 1972).

### Digestion of RNA with ribonuclease

Pancreatic ribonuclease (RNase type I-A, Sigma Chemical Co.) was dissolved in 10 mM TRIS pH 7.5 plus 1 mM EDTA and pre-heated at 70°C for 10 minutes to inactivate the possible traces of DNase. The enzyme was added to a solution of anti-B61-RNA in 0.85% NaCl at the ratio of 1 µg RNase to 10 µg RNA and the mixture was incubated at 37°C for 30 minutes.

### *In vivo* RNA immunomodulation assay

The total cytoplasmic anti-B61-RNA samples and N-RNA were dissolved in sterile 0.85% NaCl under aseptic conditions, the poly(A)<sup>-</sup> and poly(A)<sup>+</sup> RNA samples were maintained in 10 mM TRIS-HCl pH 7.5 as recovered from the oligo dT-cellulose chromatography, and adjusted to 0.85% NaCl. Groups of 30 female four to six week-old Balb-c mice were injected with 150 µg each of anti-B61-RNA, N-RNA or poly(A)<sup>-</sup> anti-B61-RNA through intravenous, intramuscular and intraperitoneal routes (50 µg RNA/0.1 ml/route). A total of 15 µg of the poly(A)<sup>+</sup> anti-B61-RNA fraction was injected into each mouse. About ten minutes after the RNA injection, all groups of mice received 10<sup>6</sup> B61 tumor cells in culture medium subcutaneously in the scapular region. Fifteen days later, all the mice were sacrificed by ether inhalation and inspected surgically at the site of the injection of the tumor cells. The diameter of each tumor was determined using a caliper. Control groups were mice that received injections of 10 mM TRIS-HCl pH 7.5 plus 0.85% NaCl and challenged with 10<sup>6</sup> B61 cells in culture medium. All the groups were analysed in a single blind manner.

### Statistics

The comparisons of the average number of tumors, and tumor diameters, were analyzed by the Student's *t* test.

## RESULTS

### Tumorigenicity of the B61 cell line transfected with EJ-ras oncogene

The B61 cell clone bearing the active EJ-ras oncogene was found to be highly tumorigenic when injected in Balb-c mice. All the injected animals developed a tumor histologically classified as

fibrosarcoma (data not shown). The latency time to detect a tumor mass with two mm diameter, at the site of the injection, was dependent on the number of B61 cells injected (Table I).

**Table I** - Tumorigenicity assay of B61 cell line in Balb-c mice.

Number of cells injected/ animal	Animals with tumor/ injected animals	Latency period (days) (2 mm diameter tumor)
10 <sup>4</sup>	10/10	20
10 <sup>5</sup>	8/8	5-8
10 <sup>6</sup>	15/15	5

### Antitumor effect of RNA preparations

It was possible to control the tumorigenic power of B61 cells *in vivo* in Balb-c mice by means of injections of RNA preparations. Table II shows that in the control group, the tumorigenicity was 100%. In the groups treated with anti-B61-RNA or with the poly(A)<sup>+</sup> RNA, the tumorigenicity decreased. Poly(A)<sup>-</sup> anti-B61-RNA and N-RNA were not effective and ribonuclease abolished the effect of the anti-B61-RNA.

**Table II** - Effect of RNA treatments on the tumorigenicity of the B61 cell line in Balb-c mice 15 days after the injection of 10<sup>6</sup> B61 cells and RNA.

Animal group	Animals with tumor/ injected animals
Control	50/50
Anti-B61-RNA	19/50
Poly(A) <sup>+</sup> anti-B61 RNA	10/50
Poly(A) <sup>-</sup> anti-B61 RNA	49/50
Anti-B61-RNA (RNase digested)	50/50
N-RNA	50/50

### *In vivo* control of B61 tumor growth

The remaining tumor-bearing animals from the RNA immunomodulation experiments exhibited a tumor mass quite different from the controls (Table III). In the experimental groups treated with anti-B61-RNA or with poly(A)<sup>+</sup> anti-B61-RNA, the tumor diameters were considerably decreased. Poly(A)<sup>-</sup> anti-B61-RNA, N-RNA and ribonuclease digested anti-B61-RNA

treated groups did not exhibit this effect as the tumor diameters were similar to those of the control group.

**Table III** - *In vivo* control of the growth of the B61 tumor in mice by means of RNA 15 days after the injection of 10<sup>6</sup> B61 cells and RNA.

Animal group	Tumor diameter (mm)
Control	6 ± 2
Anti-B61-RNA	2 ± 1
Poly(A) <sup>+</sup> anti-B61 RNA	2 ± 1
Poly(A) <sup>-</sup> anti-B61 RNA	6 ± 2
Anti-B61-RNA (RNase digested)	6 ± 2
N-RNA	6 ± 2

## DISCUSSION

The ability of RNA preparations, extracted from specifically immunized experimental animals, to transfer both *in vitro* and *in vivo* immunological reactivity to a great variety of antigens has been well demonstrated. These RNA-mediated transfers of immunity include reactivity against *T. cruzi* (De Lucca *et al.*, 1982; Bertolini and De Lucca, 1986), tumors (Steele *et al.*, 1982; Richie *et al.*, 1984; Passos Jr., and De Lucca, 1988) and hepatitis B virus (Shi-Shan *et al.*, 1982) and HIV-1 (Mendes *et al.*, 1986; Passos Jr. and De Lucca, 1991; Passos Jr., 1991). Moreover, RNA from LPS stimulated macrophages can induce the release of cytokines by cultured control macrophages, suggesting a participation of RNA message(s) during the inflammation process (Ribeiro *et al.*, 1993). However, the precise cellular and molecular mechanisms of these phenomena are still unknown.

The *in vivo* control of tumor growth is mediated by the T-lymphocytes that simultaneously recognize the MHC and tumor antigen(s) on the surface of tumor cells by means of T-cell receptors (Williams and Barclay, 1988; Raulet, 1989; Chien and Davis, 1993). We describe a syngeneic model in relation to the tumor and the host mouse strain, to study the *in vivo* immunomodulation with RNA. We studied a Balb fibrosarcoma (tumor B61) generated by transfection of an expression vector containing the activated human Ha-ras-1 oncogene (Shih and Weinberg, 1982; Kovary *et al.*, 1989). The transfected B61 tumor cells expressed the human p21 ras protein with a substitution in position 12, gly → val (Shih and Weinberg, 1982). To investigate the possible participation of the specific antitumor T-lymphocytes during the RNA-mediated immunomodulation, it is necessary to establish a syngeneic model-system, that

is, with both the host and the tumor expressing the same MHC.

The data presented here relate to the syngeneic model-system, since the B61 cell line arose from Balb-3T3 origin (Shih and Weinberg, 1982) and to this end we assayed the tumorigenic potential of B61 cells in Balb-c mice (Table I). The cytoplasmic RNA preparations from spleens of these tumor-bearing animals showed antitumor effects when injected *in vivo* in Balb-c mice.

The protection against the tumorigenic power of B61 cells *in vivo* conferred by the injections of anti-B61-RNA (Table II) demonstrates that the tumor bearing mice mounted an immune response to the tumor and that the messages for this response can be recovered in the form of RNA.

The murine B61 tumor is of particular interest due to its expression of the human p21 ras protein mutated in one of the hot-spot positions (gly → val), characterizing an animal model to study the *in vivo* specific immune response to this protein.

The fractionation of cytoplasmic anti-B61-RNA by means of affinity chromatography on oligo-dT-cellulose and its subsequent injection, demonstrated that the message(s) responsible for the antitumor effect reside in the messenger RNA fraction, since this type of RNA from eukariotic cells is polyadenylated (Darnell *et al.*, 1982). The absence of effect observed with the N-RNA and the sensitivity of anti-B61-RNA to RNase, suggest that the RNA messages arise only from the tumor-bearing animals and are active only as entire RNA chains. The differences between the tumor diameters of the control groups and the anti-B61-RNA, as well as poly(A)<sup>+</sup> RNA-treated groups (Table III), suggest that the RNA can act as modulator of the immune response against this tumor.

This model-system represents a new tool which can be used in further studies analyzing the participation of particular messenger RNAs and cells in the *in vivo* RNA-mediated immunomodulation.

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## RESUMO

Camundongos Balb-c injetados subcutaneamente com uma linhagem celular de origem embrionária (Balb/3T3) transfectada com uma forma mutante do oncogene c-Ha-ras-1, desenvolveram massa neoplásica, classificada como fibrossarcoma, no local da injeção. O RNA citoplasmático dos baços desses camundongos portadores de tumor foi preparado e fracionado em RNA poli(A)<sup>+</sup> e RNA poli(A)<sup>-</sup>. Essas preparações de RNA foram separadamente injetadas em camundongos Balb-c normais. Demonstramos que o RNA citoplasmático total tem a capacidade de induzir *in vivo* reatividade contra um desafio com as células neoplásicas viáveis, sendo que o componente ativo está na fração de RNA poli(A)<sup>+</sup>. A fração de RNA poli(A)<sup>-</sup> bem como o RNA controle extraído de baços de camundongos Balb-c normais não apresentaram efeito antitumor. Estes dados representam um novo sistema-modelo para estudos sobre as bases celulares e moleculares da imunomodulação mediada por RNA.

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