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Short communication

The form of NY-ESO-1 antigen has an impact on the clinical efficacy of anti-tumor vaccination

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ABSTRACT

Anti-tumor vaccination is being evaluated as a prophylactic and therapeutic strategy against cancer growth, dissemination (spreading) or recurrence. Although a large number of studies investigate in detail the identity of antigens to be used for efficacious immune intervention, there have been few studies investigating the optimal form for antigen to be used in the vaccine. Here we show in a mouse H-2^d MHC background and for NY-ESO-1 that genetic (plasmid DNA) but not full length protein vaccine is capable of inducing a protective prophylactic anti-tumor cytotoxic T-cell immune response *in vivo*. Peptide vaccination using nominal MHC class I epitope adjuvanted with a Toll Like Receptor agonist such as stabilized RNA can also provide some anti-tumor protection. Our results highlight the idea that when evaluating the clinical efficacy of a cancer vaccine, not only the identity of the antigen but also the format of the vaccine is of the utmost importance.

1. Introduction

Spontaneous and vaccine-induced anti-tumor immune responses can help in controlling cancer development [1]. The molecular identification of tumor antigens has provided the basis for the engineering of sub-component cancer vaccines dedicated to specifically induce *de novo* or strengthen pre-existing tumor-specific immune responses. Five categories of tumor antigens have so far been used in immunotherapy: mutated antigens (e.g. P53 or Ras), over-expressed self antigens (e.g. Her2-neu or Mucin-1), differentiation antigens (e.g. gp100 or tyrosinase), cancer-testis antigens (e.g. members of the MAGE, BAGE or GAGE families, NY-ESO-1) and viral antigens (e.g. HPV16-E6 or -E7). The frequency at which those antigens are found in specific tumor types is the main criterion determining which tumor antigen will be tested in a particular patient sub-population. Thus, for example, Non Small Cell Lung Cancer patients are currently involved in Phase III trials testing MAGE-3- and Mucin-1-based vaccines [2], those antigens being widely expressed among that patient population. Although component test vaccines can consist of polypeptide (recombinant full length proteins or synthetic peptides) or genetic information (recombinant viruses, plasmid DNA, messenger RNA), the relative ease of production of synthetic peptides and the wealth of expertise in protein vaccines have thus far favoured the development of polypeptide-based anti-cancer experimental vaccines. Genetic vaccines have appeared only later in clinical studies, being less well controlled and subject to demanding regulatory issues. Direct clinical efficacy to vaccination can be demonstrated in relevant animal models of tumor challenge, while immunomonitoring of vaccination further provides a variety of surrogate markers to qualitatively analyse response. However, there is little data comparing the various modes of vaccination – recombinant protein, synthetic peptide or genetic material as far as intracellular antigens are concerned (therapeutic effects for those antigens rely on T-lymphocyte's activity). Nonetheless, in human studies, clinical observations point to qualitative differences in the induced immunity depending on the format of the antigen. Looking at the immune response against NY-ESO-1, a strongly immunogenic member of the cancer-testis tumor antigen family (review by Gnjatic et al. [3]), it was shown that the spontaneous cellular cytotoxic immune response measurable in some cancer patients with an NY-ESO-1-expressing tumor [4] was qualitatively distinct and potentially more therapeutically relevant (higher tumor reactivity) than the anti-NY-ESO-1 response induced by full length protein [5] or peptide-based vaccines [6–8]. Additionally, the induction of anti-NY-ESO-1 CTL responses was found only in half of full length protein-vaccinated patients and shown to depend on the expression of the HLA-class I molecules HLA-B35 and HLA-Cw3 [6]. In contrast, the gene-therapy-based endogenous expression of NY-ESO-1 by viral vectors has led in vaccinated patients to cellular immune responses that efficiently recognize NY-ESO-1-expressing tumor cells *in vitro* and correlate with tumor protection *in vivo* [9,10]. Our aim was to identify the most

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efficacious format of vaccination against NY-ESO-1. Thus, we first optimized vaccine formulation *in vitro* and established a protocol which was consistent across human and mouse immune cells. We then compared adjuvanted (RNA-nanoparticles [11]) NY-ESO-1 protein or nominal MHC class I peptide epitope and plasmid DNA-based vaccine formulations as inducers of protective anti-cancer immunity in BALB/c mice.

2. Material and methods

2.1. Preparation and measurement of the immunological activity of Protamine–RNA nanoparticles

An unmodified synthetic RNA oligonucleotide (RNA18: AGUGUUAUUCUUGUAUGG, produced by Thermo) was diluted to 1 mg/ml in pure water and mixed with the equivalent or a four times higher amount of Protamine (Protamine 5000, Valeant) diluted at 1 mg/ml in pure water. Under such conditions, homogenous Protamine–RNA nanoparticles of approximately 250 nm in diameter are spontaneously formed [11]. Human PBMC were isolated from the blood of various healthy donors by Ficoll density gradient centrifugation (PAA Laboratories). Similarly, mouse splenocytes were prepared from BALB/c or C57Bl/6 spleens using Ficoll density gradient centrifugation. After washing in PBS, cells were resuspended at 1 million per 200 μl of complete medium (RPMI, 10% FCS, 2 mM L-glutamine, 10 μg/ml streptomycin, 10 U/ml penicillin). Cells were distributed in 96 well plates (200 μl per well) containing 4 μg (2 μg of Protamine in 2 μl plus 2 μg of RNA in 2 μl: 1–1 ratio) or 10 μg (8 μg of Protamine in 8 μl plus 2 μg of RNA in 2 μl: 4–1 ratio) of Protamine–RNA nanoparticles. After 24 h' incubation at 37 °C, the amount of cytokine in 10 or 20 μl of supernatant was quantified using ELISA kits (human interferon-alpha ELISA kit, Bender; human TNF-alpha ELISA kit, Abazyme, mouse interferon-alpha ELISA kit, Antigenix; mouse TNF-alpha ELISA kit, eBioscience) according to the instructions of the manufacturers.

2.2. Plasmid DNA vaccination

The pDNA coding for NY-ESO-1 has been described previously [12]. Loading on 1.6 μm gold particles and the preparation of cartridges to be used in Particle-Mediated Epidermal Delivery were performed as recommended by the manufacturer (Biorad). Four to eight week old BALB/c mice were bombarded with plasmid DNA-loaded gold particles on the shaved peritoneal area skin using the Helios Gene Gun (Biorad). Each shot was delivered at 250 psi and administered 1 μg of DNA. Each mouse received three shots for a total of 3 μg of DNA. Two to three weeks after the prime, a boost was performed under the same conditions as the prime; removal of underbelly fur followed one day later by Gene Gun treatment. The resulting anti-NY-ESO-1 immune response was monitored by detection of specific antibodies in serum and specific T-cells in the peripheral blood.

2.3. Polypeptide vaccination

Protamine–RNA nanoparticles (equivalent to 20 μg of RNA per mouse and either 20 μg or 80 μg of Protamine per mouse) were mixed with 1 μg of NY-ESO-1 full length protein or 20 μg of the synthetic H2-D^d NY-ESO-1 epitope (RGPESRLL) [13]. PBS was added to generate a volume of 200 μl per mouse and injected intraperitoneally. Protamine–RNA nanoparticles alone (equivalent to 20 μg of RNA per mouse and either 20 μg or 80 μg of Protamine per mouse), NY-ESO-1 protein alone (1 μg per mouse) and synthetic H2-D^d epitope alone (20 μg per mouse) were diluted in PBS (final volume 200 μl per mouse) and injected as controls. In all cases, two weeks after a first injection, a boost injection was performed.

2.4. Immunomonitoring

Mouse antibodies against NY-ESO-1 in serum were quantified two weeks after boost using ELISA as previously described [14]. Secondary antibodies (anti-mouse IgG1 and anti-mouse IgG2a) were from BD-Pharmingen. Serum dilution was started at 1 in 20 and proceeded by serial dilution. T-cell monitoring was performed on peripheral blood before tumor grafting. Total cells were stained using mouse CD4 FITC, mouse CD8 APC and PE-labelled MHC tetramers comprising H2-D^d monomers loaded with the RGPESRLL peptide, the immunodominant NY-ESO-1 epitope in the H-2^d haplotype [13]. Cells were then acquired using a Cyan FACS (Beckman Coulter) and data were analysed using Flowjo software (Treestar).

2.5. Tumor challenge

Two weeks after boost immunisation, mice received 2 million CT26/NY-ESO-1 cells (CT26 colon carcinoma cells stably expressing NY-ESO-1, kindly provided by the Ludwig Institute for Cancer Research [15]) sub-cutaneously at the base of the neck in a volume of 200 μl of PBS. Tumor size was recorded using a caliper every over day starting at day 9 post-tumor implantation. In this BALB/c-CT26/NY-ESO-1 tumor model, cytotoxic T-lymphocytes are responsible for tumor elimination after adequate vaccination with NY-ES0-1 [13].

3. Results and discussion

3.1. Protamine–RNA ratios affect the immunostimulating capacities of nanoparticles

Nucleic acids associate spontaneously with the cationic peptide Protamine to form aggregates [16] or nanoparticles [11], depending on the conditions of formulation. In this stabilized form, RNA signals through Toll Like Receptors-7 and -8 and induces the maturation of antigen presenting cells as well as cytokine production, thereby providing an adjuvant effect to vaccines [17,18]. Mixing Protamine and RNA at a 1–1 ratio facilitates the generation of nanoparticles with a nearly neutral surface charge and we have previously characterised the immunological activity of those particles [11]. Protamine–RNA nanoparticles and CpG oligodeoxynucleotides (ODN) have similar adjuvant activities as far as can be recorded *in vitro* using mouse cells [19]. They stimulate functionally similar TLR-7 and -9, respectively. Towards further pre-clinical and clinical use of this adjuvant formulation, we tested in more detail the immunostimulating capacity of nanoparticles with varying Protamine–RNA ratios on mouse and human immune cells. Previously, we could not detect mouse interferon-alpha in the cell culture supernatant of mouse bone marrow derived dendritic cells [11], and for this reason we used here mouse splenocytes. In such cultures a low but detectable amount of mouse interferon-alpha could be detected after overnight incubation with Protamine–RNA nanoparticles, the 1–1 ratio being more efficacious than the 4–1 ratio (Fig. 1, left panel). However, for mouse TNF-alpha, both nanoparticles prepared at 1–1 and particles prepared at 4–1 ratios induced similar amounts of cytokine in mouse splenocytes. The situation is different in human immune cells (Fig. 1, right panel): a 4–1 ratio induces more TNF-alpha and interferon-alpha in human PBMCs than a 1–1 ratio. Thus, while in mouse a 1–1 ratio may be optimal for the adjuvant activity of Protamine–RNA nanoparticles, a 4–1 ratio may be best in human. Consequently, we have evaluated the adjuvant capacities of par-