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Generation and characterization of hybridoma antibodies for immunotherapy of tularemia

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Abstract

Tularemia is caused by the Gram-negative facultative intracellular bacterium *Francisella tularensis*, which has been classified as a category A select agent—a likely bioweapon. The high virulence of *F. tularensis* and the threat of engineered antibiotic resistant variants warrant the development of new therapies to combat this disease. We have characterized 14 anti-*Francisella* hybridoma antibodies derived from mice infected with *F. tularensis* live vaccine strain (LVS) for potential use as immunotherapy of tularemia. All 14 antibodies cross-reacted with virulent *F. tularensis* type A clinical isolates, 8 bound to a purified preparation of LVS LPS, and 6 bound to five protein antigens, identified by proteome microarray analysis. An IgG2a antibody, reactive with the LPS preparation, conferred full protection when administered either systemically or intranasally to BALB/c mice post challenge with a lethal dose of intranasal LVS; three other antibodies prolonged survival. These anti-*Francisella* hybridoma antibodies could be converted to chimeric versions with mouse V regions and human C regions to serve as components of a recombinant polyclonal antibody for clinical testing as immunotherapy of tularemia. The current study is the first to employ proteome microarrays to identify the target antigens of anti-*Francisella* monoclonal antibodies and the first to demonstrate the systemic and intranasal efficacy of monoclonal antibodies for post-exposure treatment of respiratory tularemia.

Keywords: *Francisella tularensis*; Antibodies; Lipopolysaccharide; Proteome microarray; Mice

1. Introduction

Francisella tularensis, a Gram-negative facultative intracellular bacterium that causes tularemia, has been classified as a category A select agent—a likely biowepon, due to its high infectivity (10 inhaled CFU can cause respiratory disease in humans) and high mortality rate (up to 30% of cases for untreated respiratory tularemia) [1,2]. *F. tularensis* comprises four subspecies: *tularensis* (type A), *holarctica* (type B), *novicida*, and *mediasiatica*. *F. tularensis tularensis* (type A) and *F. tularensis holarctica* (type B) cause most cases of human disease; type A, found predominantly in North America, being the more virulent of the two [1,2]. An attenuated *F. tularensis* type B strain, designated live vaccine strain (LVS), is partially protective against pathogenic *F. tularensis* in humans [3] but is highly virulent in mice [4].

The genomes of two type A strains, Schu S4 and FSC198, two type B strains, OSU 18 and LVS, and the *novicida* strain U112 have been sequenced (BioHealthBase BioDefense Public Health Database, http://www.biohealthbase.org). Schu S4 was found to contain 1804 predicted coding sequences [5], most of which have been expressed as recombinant proteins and used to generate a proteome microarray chip. A smaller chip, containing 244 proteins most often identified by human and mouse anti-tularemia immune sera, has also been generated [6] The current study provides a first demonstration of the use of these chips to identify the target antigens of *F. tularensis*-specific monoclonal antibodies.

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Tularemia is usually treated by i.v. then oral administration of antibiotics but infection is still associated with considerable morbidity and up to 2–% mortality in treated patients [7] (http://www.uos.harvard.edu/ehs/fs_tularemia.shtml). And LVS, the partially protective vaccine, is not currently licensed [8]. These considerations, combined with the threat of engineered multiple antibiotic-resistant strains for bioterrorism, suggest the need for additional strategies to combat tularemia. One such strategy could be antibody immunotherapy.

Antibodies, in the form of serum from immunized goats or horses, were used in the management of tularemia in the pre-antibiotic era [9]. Although some reports claimed that antibodies were ineffective and even harmful, others described a significant reduction in both morbidity and mortality, with the greatest benefit obtained when the antibodies were given within 2 weeks of the onset of symptoms [9]. Mice treated with human or mouse immune serum to LVS showed protection from infection with LVS [4,10–12] and mice treated with mouse immune serum to *F. tularensis* LPS showed protection from infection with LVS and delay in time of death after infection with the type A *F. tularensis* strain Schu S4 [13]. LVS-specific mouse serum and LVS-specific human IgG were also shown to partially protect mice against virulent type B strains [3,12]. Similarly, pre-treatment with the mouse IgG2a hybridoma antibody FB11, specific for *F. tularensis* LPS, was shown to partially protect mice and guinea pigs against subcutaneous (s.c.) infection with a virulent *F. tularensis* type B strain [14]. And recently, LVS-specific mouse serum was shown to confer protection against intranasal LVS infection even when given 24–48 h post-exposure [15].

These reports are encouraging, even though no immune serum or IgG protection of mice against type A *F. tularensis* strains has been shown [3]; because only a fraction of antibodies in immune sera and IgG preparations is likely to be specific for *F. tularensis* and an even smaller fraction is expected to comprise antibodies to protective antigens or of an optimal isotype. Single hybridoma antibodies, even of an optimal isotype, are also not expected to protect against type A *F. tularensis* strains because all antibody molecules would compete against the same epitope.

To further explore the possibility of using antibodies as therapy for tularemia, we have generated anti-*Francisella* hybridomas from LVS-infected mice, to identify antibodies that could constitute components of a therapeutic recombinant chimeric polyclonal antibody with mouse V regions and human C regions for clinical use. Unlike immune serum or IgG preparations, all antibodies in a recombinant polyclonal antibody preparation would be of the optimal isotype and specific for protective *F. tularensis* antigens. In the current study, we analyzed 14 anti-LVS hybridoma antibodies for their target antigens and for their efficacy against intranasal LVS infection in mice, and identified four antibodies with therapeutic or prophylactic potential.

2. Materials and methods

2.1. Generation of hybridomas

All animal studies have been reviewed and approved by the Boston University Medical Center Institutional Animal Care and Use Committee. BALB/cJ and C57BL/6J female mice were obtained from the Jackson Laboratory, and 8–10-week-old mice were infected with LVS by the intranasal (i.n.), intradermal (i.d.), or i.p. routes. Splenocytes were prepared from euthanized mice by lysis of erythrocytes with 0.83% NH_4Cl, and used in polyethylene glycol-mediated fusions with Sp2/0-Ag14 mouse myeloma cells [16] as previously described for spheroplast fusions with mouse myeloma cells [17]. Hybridomas were obtained in 96-well tissue culture plates in IMDM (GIBCO) supplemented with 20% FBS, 10% hybridoma enhancing supplement (Sigma–Aldrich) and HAT (13.9 μg/ml hypoxanthine, 6 μg/ml aminopterin, and 7.2 μg/ml thymidine), and cell supernatants were screened for binding to whole LVS bacteria by ELISA as described below. Cells from positive wells were subcloned in 96-well plates, and single clones recovered and gradually adapted to growth in serum free medium (SFM, HYQ® SFM4Mab™-Utility, HyClone) supplemented with 2% FBS.

2.2. Cell lines, monoclonal antibodies, and bacterial strains

Hybridoma cell lines generated in our laboratory were cultured in IMDM/10% FBS or SFM/2% FBS. Mouse hybridoma cell line CO17-1A [18], producing an IgG2a antibody specific for a glycoprotein on human colorectal cancer cells [19] was obtained from Dr. Dorothee Herlyn of the Wistar Institute (Philadelphia, PA) and cultured in IMDM/10% FBS. All cell cultures were maintained at 37 °C in a humidified environment of 5% CO_2/95% air. Protein G-purified mouse IgG2a monoclonal antibody FB11, specific for the O-antigen polysaccharide chain of *F. tularensis* LPS [20], was purchased from GeneTex® Inc. *F. tularensis holarctica* strain LVS was obtained from Jeannine Petersen (Centers for Disease Control and Prevention, Fort Collins, CO). It was certified to be type B (*F. tularensis* subspecies *holarctica*) based on the amplification of PCR products [21] with primer set pdpDh but not with primer sets hyp4, hypR, hyp6 or pdpDn (Madico G, et al., unpublished data). To prepare LVS stocks, bacteria were grown on chocolate agar plates (REMEL Inc.) at 37 °C in a humidified environment of 100% air for 2.5 days and pools of single colonies were scraped and resuspended in PBS. Eight heat-inactivated (2 h 80 °C) *F. tularensis* clinical isolates (strains KU29, KU37, KU40, KU42, KU44, KU47, KU49 and KU54) [22] were the gift of Michael Parmely and Rebecca Horvat (University of Kansas Medical Center, Kansas City, KS). To identify their subspecies, a sample of DNA from each strain was prepared and analyzed by PCR (Madico G. et al., unpublished data). The following species- or subspecies-specific primers were used: hyp4 (*F. tularensis* subsp. *tularensis*), pdpDh (*F. tularensis* subsp. *holarctica*), hypR (*F. tularensis* subsp. *mediasiatica* and *F. tularensis* subsp. *tularensis*), pdpDn (*F. tularensis* subsp. *novicida*), and hyp6 (*F. tularensis* subsp. *tularensis* and *Francisella philomiragia*). Strains KU29, KU37, KU40, KU42, KU44, KU47, KU49 and KU54 were designated type A (*F. tularensis* subsp. *tularensis*) based on the amplification of PCR products with primers hyp4, hypR, and hyp6 but not with primer pdpDh or pdpDn. Prepara-