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Original article

Humoral and cellular immune response of mice challenged with *Yersinia pestis* antigenic preparations

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ABSTRACT

Objectives: The plague, which is an infectious disease caused by *Yersinia pestis*, still threatens many populations in several countries. The worldwide increase in human plague cases and the potential use of the bacteria as a biological weapon reinforce the need to study the immunity that is induced by potential vaccine candidates. To determine the immunogenicity of antigenic preparations based on the F1 protein and the total extract from *Y. pestis*, we assessed the role of these antigens in inducing an immune response.

Methods: The immunogenicity of antigenic preparations based on the *Y. pestis* (YP) total extract and the *Y. pestis* fraction 1 capsular antigen protein (F1) was determined in Swiss-Webster mice immunized with 40 μg or 20 μg for each preparation. Immunophenotyping was performed by flow cytometry.

Results: Animals immunized with the YP total extract did not elicit detectable anti-F1 antibodies (Ab) in the hemaglutination/inhibition (HA/HI) test. Animals immunized with 40 μg or 20 μg of the F1 protein produced anti-F1 Abs, with titres ranging from 1/16 to 1/8132. The average of $CD3^+$–$CD4^+$ and $CD3^+$–$CD8^+$ T cells did not differ significantly between the groups. Neither YP total extract nor F1 protein induced a significant expression of IFN-γ and IL-10 in $CD4^+$ T lymphocytes. In addition, F1 failed to induce IFN-γ expression in $CD8^+$ T cells, unlike the YP total extract.

Conclusion: The results showed that F1 protein is not an immunogenic T cell antigen, although the YP total extract (40 μg dose) favoured $CD8^+$ T cell-mediated cellular immunity.

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Introduction

The plague, which is caused by *Yersinia pestis*, is essentially a rodent-flea-transmitted disease that affects man and other mammalian species.[1] Due to the possibility of using *Y. pestis* for bioterrorism purposes and the high mortality rate of the disease,[2] there is a crucial need to study the immunity induced by potential vaccine candidates for future use as immunoprophylaxis. In recent years, effort has focused on developing a subunit vaccine based on virulence factors of this bacterium.[3]

Y. pestis F1 capsular antigen protein (Fraction 1 or F1) has been evaluated in several immunization studies that used experimental animals.[4–6] Furthermore, vaccines that are formulated with whole cells may contribute to the induction of an effective immune response.[7] The mechanism of protection conferred by these preparations has not been fully elucidated, but it has already been shown that an effective vaccine against the plague must induce both humoral immunity and Th1 type cellular response.[8]

Aiming to determine the immunogenicity of the antigenic preparations based on the total extract from *Y. pestis* and the F1 protein, we assessed the role of these antigens in inducing the production of antibodies, determining the phenotype of splenic T-lymphocytes and stimulating the production of IFN-γ and IL-10 by subpopulations of $CD4^+$ and $CD8^+$ T cells.

Materials and methods

Animals

Female, 6–8-week-old, Swiss-Webster mice (20–24 g) were obtained from the Universidade Federal de Minas Gerais (UFMG) facilities. Four animals per cage were maintained with temperatures at 21–24 °C, a 12 h light/dark cycle, and fed with pelletized food and water *ad libitum*. Due to the policy of reducing the number of animals used in research protocols, only the minimum number of animals per group was utilized.

Preparation of whole cell extract and Y. pestis F1 antigen

Y. pestis strain CYP 0579 from the culture collection Fiocruz-CYP was reactivated by inoculation in brain heart infusion broth media (Difco, USA) and incubated overnight at 37 °C. The presence of the genes *caf1*, *lcr*, *pla*, and *irp2*, which are prominent pathogenicity markers,[9] was determined by M-PCR according to Leal and Almeida.[10] The culture was inactivated by the addition of 2% formaldehyde (Sigma–Aldrich, USA), incubated overnight at room temperature, 23–25 °C, and plated on blood agar base (Difco, USA) to confirm bacterial death. The F1 antigen was extracted from the *Y. pestis* strain A1122 as previously described.[11]

The *Y. pestis* formaldehyde-killed suspension (YP) was fragmented by sonication for 90 s (2 cycles of 30 Hz and 2 cycles of 60 Hz intercalated with an ice bath). The YP total extract and F1 protein were subjected to gamma radiation. The protein concentration of the preparations was determined by the Lowry method[12] and the products were suspended in PBS at pH 7.2–7.4.

Immunization

Four groups of four female Swiss-Webster mice were immunized with 40 μg or 20 μg of the YP total extract and the *Y. pestis* F1 protein suspensions in PBS plus 25% (v/v) aluminium hydroxide adjuvant, administered in two doses with a 21-day interval. Each animal was injected intramuscularly in the posterior thigh with a total volume of 0.1 mL. The control group received the same volume of aluminium hydroxide adjuvant in PBS. After primary immunization, on day 42, the mice received a booster intravenous injection at the base of the tail with 4 μg or 2 μg doses of the antigens without adjuvant.[13] On day 45 after primary immunization, mice were euthanized by anaesthetic overdose. Blood samples and spleens of the immunized and control mice were collected.

Hemaglutination/inhibition test (HA/HI)

Sera were collected from immunized animals throughout the study and assayed for the presence of anti-F1 Abs by the HA/HI test.[11] The test was considered positive when the HA endpoint titre was depressed by three or more dilutions in the HI test. A titre of 1/16 was considered positive.

Preparation of spleen cell suspensions

Single cell suspensions were prepared from each 2/3 spleen in RPMI 1640 (Gibco, Germany). Each spleen was aseptically collected, and the mononuclear cells were filtered through sterile nylon. The cells were washed in RPMI 1640 and spun down at 440 × *g* for 10 min at 18 °C. The erythrocytes were lysed with an ammonium chloride solution, and the cells were washed three times in RPMI 1640 and resuspended into RPMI 1640 supplemented with 5% heat-inactivated foetal calf serum (Cultilab, SP, Brazil), penicillin (100 UI/mL) and streptomycin (50 pg/mL; Sigma–Aldrich, USA).

Spleen cell culture and flow cytometry analysis

Mononuclear cells were isolated from the spleens of immunized and control mice by Ficoll-Hypaque (Sigma–Aldrich, USA) density gradient centrifugation. After counting the viable lymphocytes, suspensions were prepared containing 1×10^6 splenic cells/mL in RPMI supplemented with 5% foetal bovine serum, 2 mM glutamine, 50 UI penicillin, and 0.05 mg streptomycin (Sigma–Aldrich, USA). Cell suspensions were used for the phenotypic analysis of lymphocytes and for intracellular cytokine analysis.

Cell surface marker staining was performed by adding the previously prepared cell suspension (0.1 mL) to flow cytometry tubes containing combinations of the following fluorochrome-labelled Abs: CD3-FITC (145-2C11), CD4-PercpCy55 (RM4-5) and CD8-APC (53–6.7) (Becton Dickinson, USA). Samples were vortexed gently and incubated for 30 min at room temperature. The fixing solution containing 4% paraformaldehyde was added to the samples, which were refrigerated until flow cytometric analysis. Lymphocytes were specifically analyzed by selective gating based on size and granularity of the cells using a flow cytometer (Forteza, Becton Dickinson, USA). As negative controls, the respective isotype control (BD) labelled with the

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