Polyazolidinammonium as an adjuvant in immunization with lipopolysaccharide of Yersinia pseudotuberculosis

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Abstract. The use of polyazolidinammonium modified with iodine hydrate ions (PAAG) as an adjuvant made it possible to obtain rabbit hyperimmune sera for the pseudotuberculosis microbe lipopolysaccharide (LPS) with specific specificity. The optimal immunizing dose for obtaining hyperimmune pseudotuberculous sera was a dose of 0.25 mg of LPS Yersinia pseudotuberculosis O:3 serovariant per rabbit. When immunizing rabbits with LPS of the pseudotuberculosis microbe, PAAG showed more pronounced adjuvant properties compared to Freund’s complete adjuvant.

1. Introduction

Rabbit hyperimmune pseudotuberculosis sera are necessary for the diagnosis of pseudotuberculosis in humans and farm animals. They are used in serological reactions such as indirect hemagglutination reaction, approximate agglutination reaction, fluorescent antibody method, enzyme-linked immunosorbent assay (ELISA) [1].

One of the important requirements for diagnostic sera is specificity. Among specific antigens, the most widely used are lipopolysaccharide (LPS), which is located on the surface of a microbial cell and is an endotoxin. The toxicity of LPS pseudotuberculosis microbe for many rodents, including rabbits, requires careful selection of the immunizing dose. In this case, focusing only on literary data is not permissible, since toxicity depends not only on the type of bacteria, but also on the specific strain of the pseudotuberculosis microbe [1, 2].

In addition to antigen, an adjuvant is required to obtain diagnostic serum. This substance non-specifically enhances the immune response to the antigen. Freund's complete adjuvant (FCA) can be used as an adjuvant to obtain pseudotuberculosis diagnostic hyperimmune serum. This is one of the most effective adjuvants for obtaining hyperimmune sera. According to the classification, it refers to oil-particle adjuvants. However, it contributes to the formation of significant foci of inflammation in the subcutaneous tissue of laboratory animals and is of high cost. All this makes it relevant to search for new adjuvants to obtain pseudotuberculous hyperimmune sera [3].

Recently, synthetic polyelectrolytes have gained popularity as adjuvants. The simplicity of chemical synthesis, solubility in water, the ability to bind to antigen particles, including LPS bacteria, opened the prospect of their use as adjuvants [4]. One of the representatives of this group of chemical compounds is polyazolidinammonium modified with iodine hydrate ions (PAAG). It has a wide range of antimicrobial properties [5] and is safe for warm-blooded animals [6]. For the first time, the study of the possibility of using it as an adjuvant in the preparation of hyperimmune sera was carried out in...
combination with microparticles of calcium carbonate and a protein-containing antigen. This experiment showed the promise of using PAAG for hyperimmunization [7, 8]. However, the study of the possibility of using PAAG in conjunction with LPS has not been previously conducted.

The aim of our study was to determine the possibility of using PAAG in combination with LPS of Yersinia pseudotuberculosis to obtain rabbit hyperimmune pseudotuberculous serum, as well as a comparative assessment of antibody activity of sera obtained using PAAG + LPS and FCA + LPS.

Experiment Design:
1. Obtaining LPS of Yersinia pseudotuberculosis.
2. The study of the activity of the cellular and humoral subsystems of immunity during the immunization of white mice with different doses of LPS to determine its immunizing dose.
3. Repeated immunization of LPS rabbits in combination with PAAG and FCA to obtain and further study the hyperimmune sera through ELISA, as well as leukocyte counting.
4. Based on the analysis of the results of the experiments, draw a conclusion about the effectiveness of using PAAG and FCA in combination with LPS.

2. Materials and methods
To obtain LPS, we used the microbial culture of Yersinia pseudotuberculosis III O:3 serovariant (Y. pseudotuberculosis O:3) from the state-owned collection of pathogenic microorganisms (SCPM) of the Federal state healthcare institution antiplague research Institute "Microbe", which possesses characteristic morphological, cultural, bio-chemical and serological properties.

LPS was isolated from acetone-dried pseudotuberculosis microbe cells with a hot 45% aqueous phenol solution without layer separation [9]. Protein impurities were precipitated from the LPS solution by adding 40% trichloroacetic acid to a final pH of 2.7.

To study the effects of various doses of LPS on the activity of the cellular and humoral subsystems of immunity, 0.5 ml of LPS solution was administered intraperitoneally to white mice in doses: 125, 63, 31, 16, 8, 4, 2 μg / animal (3 mice per dose). Group 8 mice were injected with 0.01 M phosphate buffered saline (negative control). The mass of mice was 20-22 g. Immunization was carried out two times with an interval of 10 days. Ten days after the second immunization, mice were decapitated with peritoneal macrophages removed from the abdominal cavity and blood was taken from the cut neck vessels.

The activity of cellular immunity in mice was determined by measuring the respiratory activity of peritoneal macrophages [10].

The activity of humoral immunity in mice, the effectiveness of adjuvants for immunization of rabbits, as well as the specificity of the obtained blood serum was determined by the number of antibodies in the blood serum using the solid-phase ELISA [11].

Rabbits were immunized subcutaneously along the back at 3-4 points in a volume of 1 ml of the mixture. When using an adjuvant, the ratio of adjuvant to antigen solution was 1:1. 5 immunizations were carried out with an interval of 2 weeks. Blood for the study was taken from the ear vein in a volume of 5 ml a day before the introduction of antigen, starting with the 1st immunization.

The number of leukocytes in the blood of rabbits was determined on a hematological analyzer.

Histological studies of rabbit subcutaneous tissue densification were performed at the Federal State Budgetary Institution Saratov Interregional Veterinary Laboratory.

To determine the specificity of the obtained sera, microbial cultures of Y. pseudotuberculosis I, III, IV, V were used (O:1, O:3, O:4, O:5 serovariants, respectively), Y. enterocolitica 66-82, 383 (O:3, O:9 serovariants, respectively) from the SCPM of the Federal state healthcare institution antiplague research Institute "Microbe", as well as cultures isolated from young farm animals: Y. pseudotuberculosis 67 (O:3 serovariant, from a calf) and Y. enterocolitica 58 (O:3 serovariant, from a piglet). Yersinia cultures were treated with formalin, washed, and suspensions with a concentration of 10⁹ microbial bodies / ml were prepared.
3. Results

The results of the studies of mice are shown in table 1. As can be seen from the results, the greatest respiratory activity was observed in peritoneal macrophages with immunizing doses of 8-16 μg of LPS per mouse.

Elevated antibody genesis in mice was observed with immunizing doses of LPS of 8-125 μg / animal. Thus, taking into account all the studies carried out, the optimal immunizing dose of LPS for mice was a dose of 8-16 μg per animal. When converted to a rabbit, this dose was 103-206 mcg per animal. Doses close to these doses we took to obtain hyperimmune blood serum in rabbits.

| Immunizing doses of LPS μg / mouse | Respiratory activity peritoneal macrophages | Antibodies to LPS |
|-----------------------------------|------------------------------------------|-----------------|
|                                   | Formazan Concentration on 1 macrophage, g | ELISA antibody titers with disintegrated membranes of Y. pseudotuberculosis O:3 |
| 125                               | 1.4*10^{-10}                              | 1:3200          |
| 63                                | 2.7*10^{-10}                              | 1:1600          |
| 31                                | 16.9*10^{-10}                             | 1:1600          |
| 16                                | 62.6*10^{-10}                             | 1:1600          |
| 8                                 | 68.5*10^{-10}                             | 1:1600          |
| 4                                 | 19.1*10^{-10}                             | 1:800           |
| 2                                 | 8.4*10^{-10}                              | 1:800           |
| 0 (control)                       | 1.9*10^{-10}                              | 1:400           |

At the next stage of the study, we repeatedly immunized rabbits with four doses of LPS (1; 0.5; 0.25; 0.12 mg / animal) to obtain hyperimmune sera.

12 groups of rabbits were formed:
- Groups 1, 2, 3, 4 were immunized with four doses of LPS;
- 5, 6, 7, 8 groups – four doses of LPS in combination with FCA;
- 9, 10, 11, 12 groups – four doses of LPS in combination with PAAG.

In each group there were 3 animals. The research results are shown in table 2.

The results show that at a dose of 1 mg without the use of an adjuvant, rabbits died after the third immunization. In dead rabbits, pathological changes characteristic of LPS lesion were noted: moderate granular dystrophy of the liver and kidneys, swollen spleen, moderate hyperemia of the vessels of the internal organs, pulmonary edema, and death from a stop of the respiratory center. This indicated a higher toxicity of LPS for rabbits compared to mice. The use of adjuvants allowed to reduce the toxic effect of LPS. However, in all surviving rabbits, a dose of 1 mg LPS caused a moderately depressed state and decreased appetite. The optimal immunizing dose of LPS for rabbits, judging by the magnitude of antibody titers, was a dose of 0.25 mg / animal. Moreover, the use of PAAG as an adjuvant significantly increased antibody production. FCA did not allow to increase the number of antibodies
above the control level, table 2. Another drawback of FCA was the formation of subcutaneous tissue densities of 1-2 cm in diameter at the sites of injection in rabbits, consisting of immature lymphoblastic and dividing cells surrounded by a connective tissue capsule and having small areas of hemorrhage. These seals hindered subsequent immunization and caused a painful reaction in animals.

**Table 2.** The results of determining the effectiveness of PAAG and PAF for immunization of rabbits with different doses of LPS by antibody activity of blood serum in ELISA with disintegrated membranes (DM) Y. pseudotuberculosis O:3.

| Serum Collection Time      | Antibody titers of the obtained sera in ELISA with DM | mg of LPS / rabbit |
|----------------------------|------------------------------------------------------|--------------------|
|                            | LPS (control)                                        | LPS + FCA          | LPS + PAAG         |
| Before immunization        | 1:800                                                | 1:800              | 1:800              |
|                            | 0.5:800                                              | 0.25:800           | 0.12:800           |
|                            | 1:800                                                | 0.5:800            | 0.25:800           |
| After 1st immunization     | 1:200                                                | 1:800              | 1:800              |
|                            | 0.5:800                                              | 1:400              | 1:800              |
| After 2nd immunation       | 1:200                                                | 1:800              | 1:1600             |
| After 3rd immunation       | died                                                 | 1:800              | 1:1600             |
| After 4th immunation       | died                                                 | 1:800              | 1:1600             |
| After 5th immunation       | died                                                 | 1:800              | 1:1600             |

The number of leukocytes was studied in rabbits before immunization and 12 days after 3 and 5 immunizations, table 3.

**Table 3.** The results of determining the number of leukocytes in the blood of immunized rabbits.

| Serum Collection Time      | White blood cell count (10^9 cells / liter) when immunizing rabbits with the following formulations | LPS mg / rabbit |
|----------------------------|--------------------------------------------------------------------------------------------------|-----------------|
|                            | LPS (control)                                      | LPS + FCA       | LPS + PAAG         |
| Before immunization        | 7.0                                                | 7.0             | 7.3               |
|                            | 7.2                                                | 7.3             | 7.2               |
|                            | 7.1                                                | 7.2             | 7.3               |
| After 3rd immunation       | 10.0                                               | 9.6             | 9.2               |
|                            | 8.0                                                | 9.0             | 12.0              |
|                            | 7.8                                                | 9.0             | 12.0              |
| After 5th immunations      | 12.1                                               | 11.7            | 13.1              |
|                            | 9.1                                                | 10.9            | 12.2              |
|                            | 8.5                                                | 10.3            | 11.1              |
|                            | 11.7                                               | 13.1            | 12.2              |

As can be seen from table 3, the increase in the number of leukocytes was influenced by an increase in the immunizing dose of LPS, as well as the use of PAAG. FCA when used as an adjuvant, reduced the dependence of the number of leukocytes on the dose of LPS. Apparently, leukocytes are actively involved in the neutralization of LPS and antibody synthesis [12].
In the study of specificity in ELISA, rabbit serum obtained using PAAG and LPS of Y. pseudotuberculosis III showed a positive reaction with Y. pseudotuberculosis I, III, IV, V, 67 cells in titers of 1:50-1:100 and a negative reaction with cells of Y. enterocolitica 66-82, 383, 58, which indicates the species specificity of these sera. The lack of strict specificity of antibodies to O:3 cells of the Y. pseudotuberculosis serovariant is consistent with data from other authors [13].

4. Conclusion

Based on the work done, the following conclusions can be drawn:

1. The optimal immunizing dose of LPS of Y. pseudotuberculosis O:3 with two times intraperitoneal administration to white mice is 8-16 μg / animal.
2. LPS Y. pseudotuberculosis O:3 serovariants at a dose of 1 mg / animal causes the death of rabbits. This indicates its higher toxicity to rabbits compared to mice.
3. The optimal immunizing dose to obtain hyperimmune pseudotuberculosis serum is a dose of 0.25 mg of LPS Y. pseudotuberculosis O:3 serovariant per rabbit.
4. The use of PAGE as an adjuvant allows the production of antibodies to LPS of a pseudotuberculosis microbe with species specificity.
5. During immunization of rabbits with LPS of the pseudotuberculosis microbe, PAAG shows more pronounced adjuvant properties compared to FCA.

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