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Immunological complex for enhancement of innate immune response in passive vaccination

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ABSTRACT

Passive vaccination is used to treat a wide range of infections and cancer. However, this approach has some limitations. An immune complex termed Y-complex was developed to intensify the effect of the passive vaccine. The complex is composed of a microbead that carries specific antibodies and an inducer. It enables targeting of pathogen or abnormal cells, and stimulation of a desired response by innate immune cells, depending on the inducer. The production and efficacy of Y-complex as a passive immune prophylaxis is demonstrated in this study by its use in treating cow mastitis. In an in vitro assay, Y-complex inhibited propagation and induced phagocytosis of bacteria. In challenge experiments, cows were inoculated through the udder with *Escherichia coli* or *Streptococcus dysgalactiae*. Following treatment with Y-complex, no bacteria were isolated in the milk and N-acetyl-ß-D-glucosaminidase activity had returned to normal levels. Thus the Y-complex approach can be used as an effective treatment for mastitis. Due to its modularity, this approach may serve as a treatment for a variety of disease agents.

1. Introduction

In human, passive vaccination is used in cancer immunotherapy and in a wide range of infections and poisoning, e.g. measles, rabies, hepatitis A and hepatitis B virus (HBV) [1,2]. Passive vaccination is generally used when active immunization is either unavailable or was not given before exposure as in the case of cow mastitis.

Mastitis is one of the most prevalent diseases in dairy cows [3]. The clinical symptoms of the disease include changes in milk composition, in the animal's physiological parameters and a significant decrease in milk yield [4–6]. Mastitis is caused mainly by *Escherichia coli*, [7,8], *Streptococcus agalactiae*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* [9,10], *Staphyloccocus aureus* [11], or by coagulase-negative staphylococci strains [12]. Mastitis diagnosis is based on the elevation of somatic cell count (SCC), N-acetyl-ß-D-glucosaminidase (NAGase) activity [13,14] and bacterial isolation in milk.

The main treatment for clinical mastitis is the administration of antibiotic drugs [15], after which the milk cannot be used for at least 5 to 7 milking days due to drug residues [16,17]. Vaccines against mastitis-causing bacteria do exist, but are rarely used [18]. The use of soluble IgY as a treatment for mastitis has also been previously reported [19]. However, these immunological treatments are only partially effective due to the nature of the immune response in the udder.

The response to bacteria in the mammary gland is mediated mainly by neutrophils and macrophages. However, the phagocytic potential of those cells is significantly reduced in the inflamed udder relative to their counterparts in the blood [20,21].

The approach developed in this study was aimed at targeting and activating innate immune cells against a specific disease-causing agent, in this case mastitis-causing bacteria. A multifunctional immunocomplex (termed Y-complex) was constructed and comprise of a microbead carrying immunoglobulins targeted against specific bacteria, and an inducer of innate immune cells. This methodology provides a novel medical use of passive vaccination in case of infections as well as cancer [22].

2. Materials and methods

2.1. Y-complex assembly

2.1.1. Bacteria strains immunization of hens

S. dysgalactiae (ATCC 27957) was purchased from Hy Lab (Rehovot, Israel). *E. coli* was obtained from the stocks of the National Mastitis Reference Center, Kimron Veterinary Institute, Israel.

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White Leghorn hens were intramuscular immunized twice in 2 weeks interval, with 0.5 mg of bacterial protein in incomplete Freund's adjuvant (IFA) (1:1 v/v), in a total volume of 1.0 mL. Eggs were collected daily, starting 2 weeks after the second vaccination.

2.1.2. Extraction of IgY from egg yolk and titer determination

IgY was extracted from eggs according to [23]. Titer was determined by ELISA [24]. Briefly, ELISA plates were coated with polylysine, washed and 200 μL of live bacteria (1×10^5 cell/mL) were added. After 24 h, 100 μL glutaraldehyde (0.07% v/v) was added for 15 min and washed with PBS. Wells were blocked, IgY was applied at 1:10 serial dilutions in duplicates and incubated for 2 h, washed and incubated with rabbit anti-chicken IgY conjugated with horseradish peroxidase (HRP, Sigma, Rehovot, Israel), washed and 100 μL/well of substrate solution was added. The optical density at 450 nm (OD_{450}) was determined.

2.1.3. Production of bovine anti-avidin IgG and titer determination

A cow was immunized intramuscularly twice at a 3-week interval with 1 mL of 50 μg avidin mixed 1:1 with IFA. Blood was drawn two weeks after the second injection. Anti-avidin IgG was determined by ELISA [24]. Briefly, avidin was incubated in an ELISA plate, washed and serum from the immunized cow diluted in blocking buffer [24] was added and incubated for 1 h, washed and a secondary antibody, HRP-conjugated rabbit anti-bovine IgG (Sigma) was added and incubated for 1 h, washed and substrate was added. $OD_{450\ nm}$ was determined.

2.1.4. Formation of Y-complex

Polystyrene carboxylated microparticles (1 μm) (PolySciences Inc., Warrington, PA) were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and avidin was added. EDC couples the carboxyls to primary amine residues in avidin, forming amide bonds. IgY antibodies were biotinylated using sulfo-NHS-biotin reagent according to the manufacturer's instructions (Pierce). The coupling of IgY to the microbeads was based on the high-affinity binding of biotin or biotinylated protein to avidin. The biotinylated anti-bacterial IgY and the specific cow anti-avidin IgG were attached to the avidin-coated particles in a two-step procedure: first, 1×10^6 microparticles were incubated (1 h, 37 °C) with various concentrations of the chicken IgY targeted against bacteria; they were washed twice with PBS and then incubated with various concentrations of cow anti-avidin IgG. The assembly procedure enables to produce Y-complex with various ratios of the anti-mastitis pathogen IgY and anti-avidin IgG.

2.2. In vitro assays

2.2.1. The effect of specific IgY on bacterial propagation

E. coli (1×10^7 CFU/mL) was inoculated in tripticase soy broth (TSB), and 650 μg/mL IgY was added. As negative controls, non-specific IgY or PBS were added. The mixture was incubated at 37 °C. The OD_{590} of the cultures was measured up to 4 h post-incubation and the bacterial growth curve was calculated.

2.2.2. Phagocytosis assay

E. coli (6.5×10^6 CFU) was mixed with fresh bovine whole blood and 1×10^6 Y-complex particles coated with various ratios of anti-*E. coli* IgY and anti-avidin cow IgG. The mixture was incubated for 1 h at 37 °C, stopped by the addition of ice cold PBS and centrifuged (1000 *g*, 10 min). Supernatant aliquots were serially diluted; 10 μL from each dilution was inoculated on an agar plate and incubated for 24 h at 37 °C. CFU was determined.

2.3. In vivo assays—challenge experiments

The studies were performed at the Agricultural Research Organization (ARO), Volcani Center, Israel, using six Holstein cows in mid-lactation, producing 25 to 35 L/day in three milking. All cows were free of infection, with SCC lower than 100×10^3 cell/mL and normal NAGase activity.

In experiment 1, two quarters of each of four cows were inoculated with *S. dysgalactiae* strain VL1989 by intramammary injection (1750 CFU in a volume of 5 mL per quarter). Seventy two and 80 h after inoculation 20×10^6 of the relevant particles of Y-complex were administered intramammary.

In experiment 2, two quarters of each of four cows were infected with *E. coli* strain P4 by intramammary injection (500 CFU in a volume of 5 mL per quarter). Six h and 30 h after infection, 20×10^6 of the relevant particles of Y-complex were administered intramammary into one infected quarter while the second get PBS.

Cows were inspected for clinical symptoms and elevated body temperature. Milk was collected separately from each of the four quarters every 24 h for the first 5 days and every 48 h for the following 10 days. All milk samples were tested for bacteriology, SCC and NAGase activity.

All protocols were approved by the Institutional Animal Care Committee of the ARO.

2.4. Milk-quality parameters

Bacteriological analysis was performed according to the US National Mastitis Council [25]. SCC was determined with a Coulter® Counter model Z1 (Coulter Electronics, High Wycombe, UK), and the concentration of NAGase, was fluorometrically determined according to the ADL MILK NAGase test (ADC Applied Diagnostics, Helsinki, Finland) [26].

3. Results

3.1. Y-complex assembly

3.1.1. Antibody production

A total of 90 to 100 mg IgY was extracted per egg. The level of the specific anti-bacteria IgYs, reach OD_{450} of 1.4 in 1:1000 dilution. Similar levels were inspected for cow specific IgG anti-avidin. IgY biotinylation did not impair antigen recognition (data not shown).

3.1.2. Antibody attachment to Y-complex

Biotinylated IgY (anti-*E. coli* or anti-*S. dysgalactiae*) and bovine anti-avidin IgG antibodies were successfully attached to avidin-coated polystyrene microparticles. The OD_{450} reflect the numbers of treated microbeads (Fig. 1).

3.2. In vitro assays

3.2.1. The effect of IgY on bacterial propagation

Specific IgY completely inhibited the proliferation of *E. coli* whereas the non-specific control IgY had only a minor insignificant effect on bacterial growth. Representative values from three independent experiments with *E. coli* are shown in Fig. 2.

3.2.2. Phagocytosis enhancement by Y-complex

E. coli was incubated with fresh cow blood in the presence of microbeads coated with anti-*E. coli* IgY to cow anti-avidin IgG ratios of 0:0 (non-coated microbead), 0:1, 1:1, 7:3, 9:1 and 1:0, respectively.

The number of bacteria that escaped phagocytosis was clearly influenced by the IgY:IgG ratio on the bead (Fig. 3). The highest number of bacteria was counted in the treatment with particles