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Evaluation of the Bactericidal Activity of Galectins

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Abstract

Over a century ago, Karl Landsteiner discovered that blood group antigens could predict the immunological outcome of red blood cell transfusion. While the discovery of ABO(H) blood group antigens revolutionized transfusion medicine, many questions remain regarding the development and regulation of naturally occurring anti-blood group antibody formation. Early studies suggested that blood group antibodies develop following stimulation by bacteria that express blood group antigens. While this may explain the development of anti-blood group antibodies in blood group negative individuals, how blood group positive individuals protect themselves against blood group positive microbes remained unknown. Recent studies suggest that several members of the galectin family specifically target blood group positive microbes, thereby providing innate immune protection against blood group antigen positive microbes regardless of the blood group status of an individual. Importantly, subsequent studies suggest that this unique form of immunity may not be limited to blood group expressing microbes, but may reflect a more generalized form of innate immunity against molecular mimicry. As this form of anti-microbial activity represents a unique and unprecedented form of immunity, we will examine important considerations and methodological approaches that can be used when seeking to ascertain the potential anti-microbial activity of various members of the galectin family.

Keywords

galectin; blood group positive microbes; innate immune lectin; molecular mimicry; anti-microbial

INTRODUCTION

While the discovery of ABO(H) blood groups by Karl Landsteiner over a century ago provided an unprecedented ability to predict the immunological outcome of red blood cell transfusion, many questions remain regarding the development and regulation of naturally occurring anti-blood group antibodies. Several early studies suggested that naturally occurring antibody formation results from the colonization of microbes that express blood group antigens within the first few months of life[1–3]. While the ability of blood group

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positive microbes to stimulate naturally occurring antibodies provides a mechanism for naturally occurring antibody formation, the factors that regulate immunity to blood group positive microbes in blood group positive individuals remains undefined[4]. The ability of tolerance to prevent adaptive immunity against blood group antigens in blood group positive individuals not only reduces the likelihood of autoimmunity, but also leaves an important gap in adaptive immunity. These results suggest that protection against blood group positive microbes in blood group positive individuals likely resides within the realm of innate immunity.

As ABO(H) blood group antigens reflect carbohydrate post-translational modifications [5], factors responsible for providing immunity toward blood group positive microbes likely possess carbohydrate binding activity. As a result, over one hundred different carbohydrate-binding proteins with putative immunological activity were examined on a glycan microarray in an effort to determine whether innate immune factors exist that possess significant blood group binding activity [6–9]. While many carbohydrate proteins displayed reactivity toward non-blood group antigens[10–12], several members of the galectin family, in particular galectin-4 (Gal-4) and galectin-8 (Gal-8), exhibited significant preference for blood group antigens[13,14,9]. Gal-4 and Gal-8 not only recognized blood group antigens on the glycan microarray, but these innate immune proteins exhibited similar specificity toward blood group positive microbes[14]. These results strongly suggested that galectins might be uniquely poised to provide innate immunity against blood group positive microbes.

Although galectins have been implicated in a wide variety of biological processes[15], including anti-microbial immunity against mycotic organisms[16], whether galectins could directly impact bacterial viability remained untested. Gal-4 and Gal-8 not only recognized blood group positive microbes, but recognition resulted in direct killing[14]. These results suggest that Gal-4 and Gal-8 provide innate immunity against blood group positive microbes and thus fill an important gap in adaptive immunity against blood group positive microbes in blood group positive individuals. In addition to recognizing blood group positive microbes, recent studies using microbial glycan microarrays suggest that Gal-4 and Gal-8 display very specific interactions with a variety of microbes that share the common feature of expressing mammalian-like carbohydrate antigens, while failing to recognize microbes with unrelated antigens[17,18]. Similar to the impact of Gal-4 and Gal-8 on blood group positive microbes, galectin engagement of additional gram negative and gram positive microbes that express other mammalian-like antigens likewise results in impaired microbial viability[17,18]. These results strongly suggest that galectins may provide a unique and broad form of innate immunity against molecular mimicry. In contrast to their ability to recognize and kill microbes expressing self-like antigens, Gal-4 and Gal-8 recognize the same antigenic determinants on mammalian cells, yet fail to induce detectable changes in membrane integrity or cellular viability[9,14,18]. While complex pathways, such as those that regulate complement, typically protect mammalian cells from injury[19], Gal-4 and Gal-8 appear to intrinsically possess the unique ability to recognize the exact same antigenic determinant on a microbe and mammalian cell, while only impacting microbial viability. As a result, galectin anti-microbial activity represents a unique form of immunity with significant implications in innate immune protection against molecular mimicry (Figure 1).

As Gal-4 and Gal-8-mediated immunity requires recognition of self-like antigens on microbes, this form of immunity fundamentally differs from defensins and other cationic anti-microbial peptides that engage features unique to an intact microbial membrane [20,21]. The requirement of Gal-4 and Gal-8 for distinct antigen recognition not only suggests their potential role in immunological protection against molecular mimicry, but also requires unique considerations when assessing microbial recognition and killing. In this work, we will describe the methods employed to examine the anti-microbial activity of Gal-4 and Gal-8 toward microbes, using blood group positive microbes as a model system.

2. Materials

2.1 Bacteria preparation

1. Luria Burtani (LB) media (Difco)
2. E. coli O86 (ATCC 12701)
3. E. coli K12 (ATCC 29425)
4. Luria Burtani agar (Difco)
5. 100 × 15mm sterile disposable petri dishes (Fisher)
6. 17 × 100 mm polystyrene tubes, dual position cap, sterile (USA Scientific)
7. 100 mL Erlenmeyer flask
8. 200 mL Erlenmeyer flask
9. Bacteria cell spreader (sterile)
10. Spectrophotometer
11. 37°C degree incubator/shaker
12. 37°C incubator (stationary)
13. Autoclave

2.2 Galectin preparation

1. Recombinant galectin
2. PD10 column (GE Healthcare)
3. 0.2 micron filter (Fisher)
4. Phosphate buffered saline standard pH 7.4 (Hyclone)
5. Spectrophotometer

2.3 Assessing anti-microbial activity

- 1 Luria Burtani media (Difco)
- 2 Luria Burtani agar (Difco)
- 3 Phosphate buffered saline standard pH 7.4 (Hyclone)

- 4 Bacteria cell spreader (sterile)
- 6 Sterile 96 well, round bottom plate
- 7 12 × 75 mm polystyrene tubes with dual position polyethylene caps (USA Scientific)
- 8 1.7 mL snap cap microcentrifuge tube (Sigma-Aldrich)
- 9 37°C degree incubator/shaker
- 10 37°C incubator (stationary)

3. Methods

3.1 Bacteria preparation

- 1 Bacterial glycerol stocks should be stored at −80°C.
- 2 Using a sterilized loop under a sterile hood, transfer a loop of glycerol stock to 1ml of sterile LB media in a sterile 15mL culture tube (17 × 100 mm polystyrene tube).
- 3 Incubate culture at 37°C overnight on an orbital shaker at 250 revolutions per minute (rpm).
- 4 Following the overnight incubation, dilute culture 1:100 into LB media by placing 0.05 mLs of the overnight culture in 5 mLs of sterile LB media.
- 5 Allow this culture to incubate at 37°C on an orbital shaker for 2.5–3 hours.
- 6 Remove 0.5mL of culture from the sample and determine the OD at 600nm (OD600). If the OD600 is between 0.3 and 0.5 then proceed to step 7. If the value is below 0.3, continue to check the culture every 30 minutes until an OD600 of between 0.3 and 0.5 is achieved. If the culture is over 0.5 OD600 then repeat step 4 with examination of the OD600 at 30 min intervals until an OD600 of 0.3 is achieved.
- 7 Dilute the culture 1:100 by placing 0.5mL of the culture into 50mL of sterile LB media in an autoclaved 200mL Erlenmeyer flask.
- 8 Every 30 min, remove 0.6mL of this culture. Place 100µL of this sample into a sterile eppendorf tube and use the rest to record the OD600 of the culture.
- 9 To determine the corresponding CFU value of the culture to the OD600 reading, perform six 10-fold serial dilutions in sterile LB media.
- 10 Plate 50µL of each dilution onto three separate LB agar plates and spread the suspension over the entire face of the agar plate using a sterile bacterial cell spreader.
- 11 Allow plate to dry for 2–3 min under tissue culture hood.
- 12 Incubate plates face down at 37°C for 12 hours or overnight and then remove plates from incubator.

- 13 Repeat steps 7–10 every 30 minutes to obtain an OD600 value with a corresponding set of dilutions plated on LB agar for CFU enumeration.
- 14 Following step 12, count the number of colony forming units on the corresponding plate. (*see* Note 1)
- 15 Use the dilution calculations to extrapolate the number of CFUs in the starting material of each sample.
- 16 Once the a relationship between OD600 and CFU has been established, use the OD600 readings to provide a estimate of the number of bacteria typically present at a particular OD600 value. Additionally, use this data to determine the normal growth kinetics of the bacterial culture.

Preparing E. Coli O86 for bacteria killing assay

- 17 Inoculate a fresh overnight culture of E. coli O86 and grow overnight shaking at 37°C as outlined in steps 1–3 of **Section 3.1**.
- 18 Use 100uL of this overnight culture to re-inoculate 2 mL of sterile culture media.
- 19 Grow bacteria to midlog phase (approximately OD600 0.3–0.5, or as determined empirically in steps 1–16)
- 20 Re-inoculate fresh sterile culture media for killing assay at a 1:100 dilution. (*see* Note 2)
- 21 Grow fresh culture to midlog phase.
- 22 Dilute culture in sterile LB mediate to 10^8 cells/mL (as determined empirically in steps 1–16). (*see* Note 3)

3.2 Galectin preparation

1. Thaw frozen galectin stocks on ice at 4°C.
2. To remove β ME and lactose, prepare a PD10 column for gel filtration by equilibrating the column with 5 column volumes of cold PBS (pH 7.4) (*see* Note 4)

¹Meticulous care should be employed when enumerating CFU using these methods to ensure that the potential anti-microbial activity of galectins is not over or underestimated. In addition, the drop plate method can be used in place of the spread method for CFU enumeration[22]. Each of these methods possess intrinsic advantages and limitations[22].

²The number of samples needed to do a particular experiment will dictate the amount of media used for this step. When examining alternative microbes, follow recommended guidelines for ideal growth conditions and empirically determine the optimal conditions for examining potential galectin anti-microbial activity.

³Bacteria used in killing assays should be in mid log phase. This is the reason for repeat inoculations. Inoculation with overnight culture will contain mostly dead or stationary post-log phase bacteria. However, following a second inoculation, bacteria that are mostly in the log phase of growth will be present. This may be important during the killing assay, as many bacteria are susceptible to particular killing mechanisms during active growth. In addition, examination of microbes harvested from post-log growth conditions often results in cultures with significant lipopolysaccharide (LPS) contamination. As the blood group antigen on blood group positive microbes resides on LPS, significant increases in LPS concentrations secondary to overgrowth conditions may artificially inhibit galectin-mediated anti-microbial activity.

⁴ β ME may interfere with the biological activity of the microbe. In addition, lactose can readily inhibit galectin-carbohydrate interactions. Inclusion of lactose can be used as a control but must be removed to assess the potential biological activity of a particular galectin in the absence of hapten inhibitors. When assessing the potential dependency of galectin microbial interactions or anti-

3. Add 1 mL of the recombinant galectin solution to the PBS re-equilibrated PD10 column and collect 0.5mL fractions.
4. Following complete penetration of the galectin solution into the PD10 column, add 2 mLs of cold PBS.
5. Continue to collect 0.5mL fractions while adding additional PBS as needed to prevent the column from drying.
6. To determine which fractions may have galectin protein, examine protein content by simply measuring the OD at 280nm (OD280) of each fraction. This is typically done by diluting each fraction 10 fold (i.e. 10 μ L of each fraction in 90 μ L PBS) followed by examination of the OD280. (*see Note 5*)
7. Once positive fractions are identified, pool galectin containing fractions and re-evaluate the OD280 to determine the final concentration of pooled galectin
8. Filter-sterilize galectin using 0.2 μ m filter. Maintain galectin sterility after this point. (*see Note 6*)
9. Once the galectin has been filtered, re-evaluate the OD280 to determine the final galectin concentration.
10. Dilute galectin to the appropriate concentration with sterile PBS using aseptic technique under a sterile hood. (*see Note 7*)
11. In addition, hapten inclusion control solutions, which consist of LB solution containing 100mM TDG (galectin inhibitor) or 100 mM Sucrose (non-inhibitor control) may added to control samples. Control solutions are diluted five-fold to achieve a final concentration of 20mM in control samples. (*see Note 8*)

3.3 Assessing anti-microbial activity

1. Once galectin is diluted to the appropriate concentration and bacterial cultures are grown to mid-log phase, a final bacteria concentration of 10⁸ bacteria/mL should be obtained.
2. Mix galectin and bacteria and place in optimal growth conditions for the microbe being examined.

microbial activity, thiodigalactoside (TDG) may be employed as an inhibitor of galectin-carbohydrate interaction instead of lactose, as it is relatively inert to metabolism.

⁵To extrapolate the protein concentration from the OD280 values, use the extinction coefficient for the particular galectin being examined to calculate actual concentration in mg/ml. The following websites <http://www.basic.northwestern.edu/biotools/proteincalc.html> and <http://web.expasy.org/protparam/protparam-doc.html>, offer explanation and assistance in calculating extinction coefficient and using this calculation to determine the actual concentration of a given protein in mg/ml, including caveats as to how these numbers may differ from the actual values. As methods of calculating the extinction coefficient only provide estimates, alternative approaches can be used to empirically determine these values. These include using a Bradford assay to calculate protein concentration or simply re-equilibrating the recombinant protein directly into water, lyophilizing, weighing and then resuspending in the buffer of choice followed by empirically determining the extinction coefficients for a particular galectin family member.

⁶This should be done before a final concentration is determined and final dilutions are made since some galectin could be lost during the sterile filtration step.

⁷Typically a target concentration of 5 times the desired final concentration is employed to allow for a 1:5 dilution of galectin with the target bacteria.

⁸Both lactose and TDG are effective inhibitors of galectin carbohydrate binding. Lactose is typically easier to obtain, but also can be metabolized with a variety of potential alterations in the test system. Incubation of hapten solutions with recombinant galectins prior to introducing microbes should facilitate hapten engagement of galectin prior to galectin incubation with microbes.

3. Following 2 hours incubation, bacteria should be immediately removed. (*see* Note 9)
4. To determine the corresponding CFU value of each condition, perform six 10-fold serial dilutions in sterile LB media.
5. Plate 50 μ L of each dilution onto three separate LB agar plates and spread the suspension over the entire face of the agar plate using a sterile bacteria cell spreader.
6. Allow plate to dry for 2–3 min under tissue culture hood.
7. Incubate plates face down at 37°C for 12 hours or overnight and then remove plates from incubator.
8. Count the number of colony forming units for each dilution
9. Use the dilution calculations to extrapolate the number of CFUs in the starting material of each sample. (*see* Note 10 and 11)

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⁹A 2 hour incubation is not necessary to observe anti-microbial activity. Shorter intervals can be used and should be empirically determined. Longer intervals allow easy visual determination of the potential anti-microbial activity of a particular galectin for a given microbe before plating.

¹⁰Plating accuracy is the most challenging step of this assay. As a result, significant care should be taken when plating samples on LB agar to ensure even distribution of the sample across the entire agar plate.

¹¹CFU counts in PBS treated control samples can be used to represent 100% of normal growth for a given bacteria. Galectin anti-microbial activity may be shown as an extrapolation of the difference in CFU between PBS treated and galectin treated bacteria, typically shown as “CFU remaining (%)” (*see* Figure 2)

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Figure 1. Galectins provide innate immunity against blood group positive microbes

While elimination of self-reactive immune cells reduces the probability of autoimmunity, a fitness cost would be anticipated due to the reduced ability of an individual to respond to self-like antigens on pathogens. However, compensatory mechanisms at the level of innate immunity may exist whereby individuals protect themselves against self-like antigens. Gal-4 and Gal-8 uniquely recognize pathogens bearing self-antigens, providing a possible mechanism whereby individuals protect themselves against self-antigen positive pathogens despite reduced adaptive immunity toward these microbes.

Figure 2. Galectin-4 and Galectin-8 killing blood group positive microbes requires carbohydrate recognition

Quantification of viable bacteria after BGB⁺ *E. coli* were mixed with 5 μM Gal-1, Gal-3, Gal-4 or Gal-8 (A), 5 μM Gal-4 with or without 20 mM lactose (Lac) or 20 mM sucrose (Sucr.) (B), 5 μM Gal-8 with or without 20 mM lactose (Lac) or 20 mM sucrose (Sucr.) (C) or the indicated concentrations of Gal-1, Gal-3, Gal-4 and Gal-8 (D). In each experiment, bacteria were quantified by dilution plating. Error bars represent means ± s.d. This research was originally published in Nature Medicine. Stowell SR, Arthur CM, Dias-Baruffi M, Rodrigues LC, Gouridine JP, Heimburg-Molinaro J, Ju T, Molinaro RJ, Rivera-Marrero C, Xia B, Smith DF, Cummings RD. Innate immune lectins kill bacteria expressing blood group antigen. 2010 Mar;16(3):295–301