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A hemolysis–hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds

Kevin D. Matson[a,*], Robert E. Ricklefs[a], Kirk C. Klasing[b]

[a]Department of Biology, University of Missouri, 8001 Natural Bridge Road, St Louis, MO 63121-4499, USA
[b]Department of Animal Science, University of California, Davis, CA 95616, USA

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Abstract

Methods to assess immunocompetence requiring only a single sample are useful in comparative studies where practical considerations prevent holding or recapturing individuals. The assay for natural antibody-mediated complement activation and red blood cell agglutination described here, requiring ~100 μl of blood, is highly repeatable. The effects of complement deactivation, 2-mercaptoethanol (2-ME), age, and lipopolysaccharide (LPS)-induced sickness response were examined to validate comparisons among diverse avian species. Complement deactivation by heating significantly reduces lysis and treatment with 2-ME reduces both lysis and agglutination. Lysis and agglutination both increase with age in chickens; LPS treatment does not influence these variables in 11-week-old chickens. In a comparison of 11 species, both lysis (0.0–5.3 titers) and agglutination (1.8–8.0 titers) vary significantly among species. Accordingly, this assay can be used to compare constitutive innate humoral immunity among species and with respect to age, sex, and experimental treatments within populations.
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1. Introduction

A subset of immune defenses can be categorized as both constitutive and innate [1]. Constitutive innate immunity provides the first-line of protection against invading microbes. Among these defenses two interrelated humoral components: natural antibodies (NAbs) and complement. On one hand, NAbs serve as a recognition molecules capable of opsonizing invading microorganisms and initiating the complement enzyme cascade, which ends in cell lysis [2]. On the other hand, levels of complement and the expression of its receptors by B cells are positively correlated with NAb diversity and B-1 cell number [2]. This interaction of NAbs

Abbreviations: 2-ME, 2-mercaptoethanol; CV, coefficient of variation; LPS, lipopolysaccharide; NAb, natural antibody; PBS, phosphate buffered saline; PHA, phytohemagglutinin; RBC, red blood cell; SD, standard deviation; SE, standard error.

* Corresponding author. Tel.: +1-314-516-5488; fax: +1-314-516-6233.
E-mail address: kdm50c@umsl.edu (K.D. Matson).

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and complement is an important link between innate and adaptive immunity [2–4].

Details of the role of complement have been elucidated through decades of research on the large group of proteins that make up the system. Because complement deficiencies are associated with a range of infectious and non-infectious diseases, including lupus and arthritis, assessment of the complement system has become commonplace in humans [5], as well as in a range of domestic animals [6–8], including ducks [9], chickens [10,11], and turkeys [12]. In non-domestic animals, hemolytic activity associated with complement has been identified in higher vertebrates [13] as well as lower vertebrates (including three species of fish [14]).

NAbs are unique among immunoglobulin molecules because their presence does not require previous exposure to a particular antigen. Circulating NAbs have been described in naïve animals, including those raised in germ-free environments [4,15,16]. Additionally, NAbs are encoded directly by the germ-line genome [15,17,18] and do not require somatic hypermutation and recombination during ontogenesis as in the case of the adaptive antibody repertoire [19,20].

NAbs react with various affinities to a wide variety of epitopes on macromolecular and particulate antigens including foreign red blood cells (RBCs), bacteria, viruses, and toxins [15,21,22]. In mammals, the main sources of NAbs are peritoneal $CD5^+$ B-1 cells, a subset of the antibody-producing B cells [23]. Most NAb molecules are pentameric IgM, but some IgG (monomeric) and IgA (dimeric) forms have been reported [17]. A variety of functions have been proposed for NAbs, including directly controlling novel bacterial and viral disease challenges, enhancing antigen presentation and initiating specific responses of B and T cells, regulating self reactive B and T cells, and clearing damaged or transformed cells [4,24].

Because a large fraction of all antibody-secreting B cells are located in gut-associated lymphoid tissue [25], and many of these are peritoneal B-1 cells that constitutively secrete NAbs, these molecules may be important in clearing commensal microflora that leak across the gut epithelial barrier [26]. While NAbs also might play an immunoregulatory role, the 'evolution-evolutionary important physiological role for enhancing survival of the host seems to be in early resistance against infection' [4]. For example, NAbs against the parasite *Plasmodium lophurae* have been identified in White Leghorn chickens (*Gallus domesticus*) [27]. Also, NAbs at naturally occurring concentrations have been shown to kill bacteria and spirochetes in vitro [15,22] and to promote clearance of bacterial cell wall components (e.g. lipopolysaccharides) in vivo [26].

Assessment of immune function in free-living vertebrates is emerging as an important tool in evolutionary and ecological research. The types of assays that can be employed with wild animals, however, are constrained by the stress that results from capture and handling, the unreliability of recapturing animals, the prohibition of terminal studies, the lack of specialized reagents, and the small size of many study species. Conventional assays of B-cell function, such as lymphocyte phenotyping and specific antibody responses to vaccinations, are usually not possible. The levels of NAbs and complement in small blood samples taken at single capture events may provide important information about immunocompetence. The direct, genotype-dependent expression of NAb genes makes the assessment of innate humoral immunity particularly appealing for addressing evolutionary questions. As the first line of defense against initial infection, germ-line encoded NAb genes are subject to natural selection. Indeed, NAb levels in chickens have responded over 20 generations of artificial selection on the primary antibody response [28]. Also, NAbs should be less sensitive than acquired antibody responses to short-term variations in environmental conditions, nutritional status, or stress levels [29]. Moreover, NAb production appears to represent a functionally distinct (though genetically related [28]) component of humoral immunity in which the cells producing NAbs are not influenced by experimental infection and initiation of a specific antibody response [30].

Here, we describe a highly repeatable assay for characterizing NAb-mediated complement activation and RBC agglutination titers that is useful for comparing innate humoral immunity among species and with respect to age, sex, and experimental treatments within populations. This assay uses a single small blood sample (roughly 100 μl, which can

be safely drawn from birds as small as 10 g) collected upon capture. This uncomplicated sampling makes the method ideal for comparative immunological studies requiring numerous samples from small birds, and where recapture is difficult.

2. Materials and methods

2.1. Subjects and samples

Plasma samples were collected from both captive and free-living birds. The captive species were jungle fowl (*Gallus gallus*, ~3 years old, $n=4$, all males), Cobb broiler chicken (*G. domesticus*, four 2-week-old chicks and 20 11-week-old chicks, all males), mallard duck (*Anas platyrhynchos*, ~6 months old, two females, three males, $n=5$), Japanese quail (*Coturnix coturnix japonica*, ~1 year old, all males, $n=6$), American kestrel (*Falco sparverius*, average 11 years old, sexes unknown, $n=5$), and zebra finch (*Taeniopygia guttata*, ages unknown, all female, $n=4$). The wild species were mourning dove (*Zenaida macroura*, ages and sexes unknown, $n=4$), house sparrow (*Passer domesticus*, ages and sexes unknown, $n=4$), common grackle (*Quiscalus quiscula*, ages and sexes unknown, $n=6$), gray catbird (*Dumetella carolinensis*, ages and sexes unknown, $n=11$), and waved albatross (*Phoebastria irrorata*, ages unknown, seven males, six females, one unknown sex, $n=14$). Blood samples were collected using heparinized syringes or heparinized microcapillary tubes. With one exception, blood samples were kept on ice for periods less than 1 h until centrifugation, after which the plasma fraction was removed and frozen for future analysis. Due to field constraints, the waved albatross blood samples remained un-centrifuged and at ambient temperatures for periods not exceeding 6 h, at which point the plasma was collected and frozen.

Blood collection protocols for all captive birds were approved by the UC Davis Campus Committee on Animal Care and Use. The work with all wild birds was approved by the UM Saint Louis Institutional Animal Care and Use Committee.

Plasma enriched in IgM for use as a positive control was collected from three adult Leghorn chickens. Each chicken was subcutaneously injected with 50 μl of whole rabbit blood in Alsever's solution (HemoStat Laboratories #RBA050, Dixon, CA) in four places in the pectoral region. After approximately 90 h, when specific IgM levels had increased but specific IgY levels had not, blood from each chicken was collected in heparinized syringes. The blood was centrifuged, and plasma was collected, pooled, and frozen for later use. Prior to freezing, a portion of the pooled plasma was heated to 56 °C in order to deactivate complement.

2.2. General hemolysis–hemagglutination assay

The assay is carried out in 96-well (eight rows by 12 columns) round (U) bottom assay plates (Corning Costar #3795, see [31]). Twenty-five microliters of eight plasma samples are pipetted into columns 1 and 2 of the plate and 25 μl of 0.01 M phosphate buffered saline (PBS; Sigma #P3813, St Louis, MO) are added to the columns 2–12. Using a multi-channel pipetter the contents of the column 2 wells are serially diluted (1:2) through column 11. This results in dilutions ranging from 1 to 1/1024 and 25 μl in every well. The 25 μl of PBS only in column 12 serves as a negative control. For the assay itself, 25 μl of a 1% rabbit blood cell suspension is added to all wells, effectively halving all plasma dilutions. Each plate is then sealed with ParafilmM (Pechiney Plastic Packaging, Neenah, WI) and covered with a polystyrene plate lid. Plates are gently vortexed for 10 s prior to incubation during which they are floated in a 37 °C water bath for 90 min.

Upon completion of the incubation, the long axis of each plate is tilted to a 45° angle for 20 min at room temperature in order to enhance visualization of agglutination. Plates are then scanned (full size image at 300 dpi) using the positive transparency (top-lit) setting of a flatbed scanner (Microtek Scan-maker 5900). Afterward, plates are kept at room temperature for an additional 70 min and scanned for a second time to record maximum lytic activity. From the digitized images, lysis and agglutination are scored for each sample. Lysis reflects the interaction of complement and NAbs, whereas agglutination results from NAbs only. Both variables are recorded as the negative $\log_2$ of the last plasma dilution exhibiting each behavior, i.e. column 9 is a score of 9

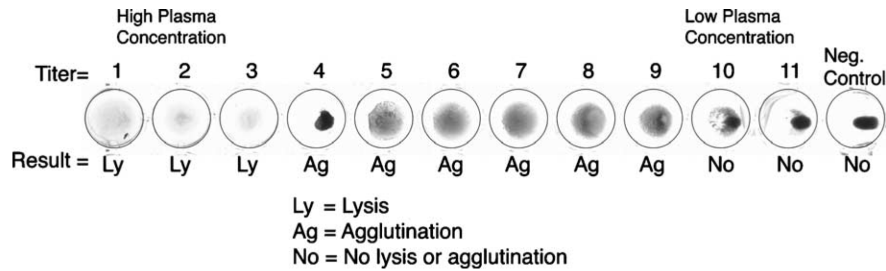

Fig. 1. An example scan. Scan shows results of the hemolysis–hemagglutination assay using serially diluted plasma from one 11-week-old Cobb broiler chicken (*Gallus domesticus*). Titers 1–3, show hemolysis. Titer 4 shows a tight or compact form of agglutination, while titers 5–9 show a more flocculent form of agglutination. In addition to the negative control (PBS only), the clear dripping in titers 10 and 11 demonstrate a lack of lysis and agglutination. This example scan would be given a lysis score of 3 and an agglutination score of 9.

[32] (see Fig. 1). Half scores between two titers are recorded when the termination of lysis or agglutination is intermediate or is ambiguous [32].

2.3. Rabbit blood cell suspension

Because chickens exhibit high levels of natural hemagglutinins for rabbit RBCs compared to RBCs from four other mammals [33], this assay was developed to use a commercially available suspension of whole rabbit blood in Alsever's solution (HemoStat Laboratories #RBA050, Dixon, CA; supplied as 50% whole blood, 50% Alsever's). The RBCs were washed four times with PBS (ca. $275\times g$ for 5 min). The hematocrit was checked in duplicate using capillary tubes and the RBCs were adjusted to a final cell concentration of 1% in PBS. At 25 μl per well, each assay plate requires 2.4 ml of the prepared 1% cell suspension. To ensure that the 1% cell suspension remains well mixed, the suspensions were vortexed immediately prior to addition to the assay plate. Fresh cell suspensions were prepared daily.

2.4. Plasma treatments

Two plasma treatments are referred to throughout this manuscript: heat and 2-mercaptoethanol (2-ME). Heat-treated plasma was de-complemented at 56 °C for 30 min [34]. 2-ME-treated plasma had 2-ME added to a final concentration of 0.1 M in order to break up polymeric immunoglobulins (primarily IgM) [34]. 2-ME-treated plasma samples were incubated at 37 °C for 30 min [32].

2.5. Assay repeatability, rabbit blood age effects, and scorer effects

To examine assay repeatability, 16 assays using the Leghorn positive control plasma pool were run over a 2-day period when the rabbit blood was freshest (days 1 and 2). Two individuals (KM and A Scheuerlein) scored these assays to test for scorer effects on lysis and agglutination titers. Further, to examine effects of storage time of the rabbit blood in Alsever's solution, four additional assays were run on days 6, 10, and 15 after receipt of the shipment of fresh blood. Every assay plate contained four repeats of both unheated and heated plasma.

2.6. LPS-induced sickness response

Fifteen 11-week-old male Cobb broiler breeder chicks were injected subcutaneously with 1 mg lipopolysaccharide (LPS; Sigma #L7261, St Louis, MO) from *Salmonella typhimurium* per kilogram body mass. At 4, 8, and 16 h post injection, groups of five chickens were bled from the jugular vein. One mililiter of blood was collected in heparinized microcentrifuge tubes from each chicken. Control samples were collected in a similar manner from five non-injected 11-week-old male Cobb broiler breeder chicks.

2.7. Statistical analyses

Within- and among-assay variation was calculated from four repeats for each of two types of plasma (heated and unheated pooled chicken plasma) run in

16 different assay plates. Within-assay variation is reported as the mean, minimum, and maximum values of the standard deviations (SD), standard errors (SE), and coefficients of variation (CV) ($n=4$ for each type of plasma on each plate) across the 16 plates, whereas among-assay variation is reported as the SD, SE, and CV of the 16 intra-plate means. Variation due to rabbit blood storage time was tested using a linear regression on the five daily means for lysis and agglutination with unheated and heated plasma during the 15-day test period. A one-sample *t*-test was used to assess the difference between scorers.

Variation in mean lysis and agglutination titers was tested independently using univariate general linear models (SPSS, Release 9.0.0, 1998). To test the effect of species and plasma treatment, we used a GLM with species and plasma treatment as fixed factors, individual as a random factor, and the interaction between species and plasma treatment. The effects of chick age and interval following LPS injection were analyzed separately for unheated and heated plasma using GLMs with only a single fixed factor. Post-hoc multiple comparisons were made using Tukey's test.

3. Results and discussion

3.1. Assay design: repeatability, scorer effects, and rabbit blood storage effects

Within- and among-assay variation was calculated for lysis and agglutination titers using both unheated and heated plasma pools. Across all plates, the agglutination titer averaged 10.0 for unheated plasma and 9.8 for heated plasma and the lysis titer averaged 3.9 for unheated plasma. Lysis was absent from the heated plasma. The mean within-assay variation (SD) was ±0.2 titers for agglutination and ±0.1 titers for lysis (Table 1). The mean among-assay variation (SD) was ±0.4 titers for agglutination and ±0.3 titers for lysis (Table 2). The CV within and among assays is frequently reported, but SD is more meaningful when comparing values on a logarithmic scale because the mean value, by which the SD is divided to obtain the CV, is arbitrary and can be 0 or negative.

The assay data revealed a small, but significant scorer effect. Samples were scored blindly, and one individual recorded significantly lower values than

Table 1
Within-assay variation reported as the mean, minimum, and maximum values of the standard deviations (SD), standard errors (SE), and coefficients of variation (CV)

Plasma treatment	Variable	n_{sample}	Mean	SD				SE				CV			
				Mean	SD	High	Low	Mean	SD	High	Low	Mean	SD	High	Low
Chicken Pos. std, unheated	Agglutination	4	10.0	0.2	0.3	0.9	0.0	0.1	0.1	0.4	0.0	2.0	2.5	8.1	0.0
Chicken Pos. std, heated	Agglutination	4	9.8	0.2	0.2	0.5	0.0	0.1	0.1	0.3	0.0	2.1	2.2	5.7	0.0
Chicken Pos. std, unheated	Lysis	4	3.9	0.1	0.1	0.5	0.0	0.0	0.1	0.2	0.0	2.0	3.9	13.2	0.0
Chicken Pos. std, heated	Lysis	4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				

Across the 16 plates, $n=4$ for each type of plasma on each plate.

Table 2
Among-assay variation reported as the standard deviations (SD), standard errors (SE), and coefficients of variation (CV) of the 16 within-assay-plate means for each type of plasma

Plasma treatment	Variable	n_{plate}	Mean	SD	SE	CV
Chicken Pos. std, unheated	Agglutination	16	10.0	0.5	0.1	4.7
Chicken Pos. std, heated	Agglutination	16	9.8	0.4	0.1	4.2
Chicken Pos. std, unheated	Lysis	16	3.9	0.3	0.1	7.0
Chicken Pos. std, heated	Lysis	16	0.0	0.0	0.0	

the other. The mean difference was 0.2 titers (SD = 0.5; $n = 128$, $p < 0.001$) for agglutination and 0.04 titers (SD = 0.2; $n = 128$, $p < 0.001$) for lysis. The smaller difference between scorers for lysis is likely due to the fact that the endpoint for lysis is clearer than that of agglutination.

The rabbit blood cells as packaged by the supplier are given an expiration date of 2 weeks after collection and shipment. The storage time of the blood cells (up to 15 days) did not significantly affect the outcomes of the assays with either heated or unheated plasma (Table 3). Thus, it appears that one batch of rabbit blood cells can be safely used until its expiration date.

Due to concerns regarding the anti-complementary effects of heparin, lysis titers in plasma (collected in heparinized microcapillary tubes) and serum were compared in three species. No significant effects were found (unpublished data). Further, to ensure lytic activity was not limited by the concomitant serial dilution of endogenous divalent cations, the effects on lysis of two different dilutants (the standard PBS dilutant and a dilutant with Mg^{+2} and Ca^{+2}) were compared in two species. Again, no significant effects were found (unpublished data). Across species, the functional importance of heparin interference and of endogenous cationic concentration on in vitro complement activation is not well understood, and, therefore, both warrant further investigation.

3.2. Plasma treatments

The effects of heating plasma to deactivate complement and treating plasma with 2-ME to disaggregate IgM were tested in five species: mourning dove, Japanese quail, mallard duck, jungle fowl, and 11-week-old Cobb chicken. Because the interaction between species and plasma treatment was significant for both lysis ($F(8,38) = 48.3$, $p < 0.001$) and agglutination ($F(8,38) = 61.8$, $p < 0.001$), the effect of plasma treatment was examined independently for each species. Thus, data for each species

Table 3
Results of linear regressions on the five daily means for lysis and agglutination with unheated and heated plasma during the 15-day test period

Plasma treatment	Variable	Beta	d.f.	t	p
Chicken Pos. std, unheated	Agglutination	−0.097	3	−0.168	0.877
Chicken Pos. std, heated	Agglutination	0.521	3	1.058	0.368
Chicken Pos. std, unheated	Lysis	−0.719	3	−1.793	0.171
Chicken Pos. std, heated	Lysis				

The storage time of the blood cells did not significantly affect the outcomes of the assays with either heated or unheated plasma.

were analyzed using plasma treatment as a fixed factor and individual as a random factor in a repeated-measures GLM. Simple contrasts were conducted to compare lysis and agglutination values for both heated plasma and 2-ME-treated plasma with values for untreated plasma.

A significant decrease in agglutination titer was observed when plasma from 11-week-old Cobb chicks was heated ($p=0.001$) or treated with 2-ME ($p<0.001$; Fig. 2). Significant reductions in agglutination were exhibited in jungle fowl ($p<0.001$), mallard ducks ($p<0.001$), and Japanese quail ($p=0.001$) only when the plasma was treated with 2-ME (Fig. 2). Neither heat nor 2-ME significantly reduced the low levels of agglutination observed in mourning doves (Fig. 2). Lysis was significantly reduced in both heated and 2-ME treated plasma in 11-week-old Cobb chickens, jungle fowl, mallard ducks, and Japanese quail ($p<0.001$ for both treatments for each species; Fig. 2). Because lysis is absent from untreated mourning dove plasma, no comparisons were made.

Plasma samples were heated to deactivate complement and then scored for lysis to ensure that complement, and not a heat stable serum factor (e.g. an acute phase protein), was responsible for the lysis titers that were determined in untreated plasma. Only a small (<1 $\log_2$ unit) amount of lytic activity remained in heat-treated plasma from Cobb chickens and mallards and none occurred in most species. This indicates that all, or almost all, of the lysis observed with unheated plasma was due to complement. Additionally, the lysis titer was almost always below the agglutination titer, indicating that immunoglobulin was not limiting for measurement of complement levels. The exception was the common grackle, where the lysis and agglutination titers were similar. Thus, it is not possible to determine whether the measured complement titer in this species was accurate because it may have been constrained by NAb levels. One potential side effect of the heat inactivation of complement is a significant reduction in agglutination titers (e.g. 11-week-old Cobb chicken

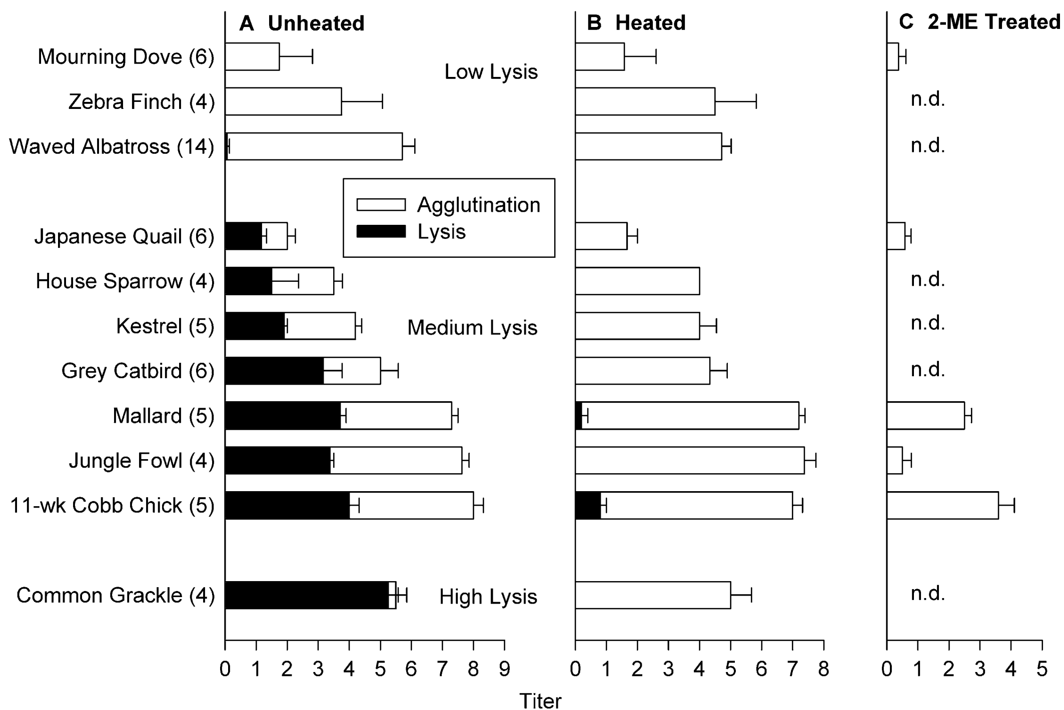

Fig. 2. Lysis and agglutination titers in unheated (A), heated (B), and 2-ME treated (C) plasma from 11 species of birds. Filled bars represent lysis; open bars, agglutination. Error bars represent SE. Numbers in parentheses indicate number of individuals per species. Due to plasma volume limitations, not all species were treated with 2-ME (n.d., no data).

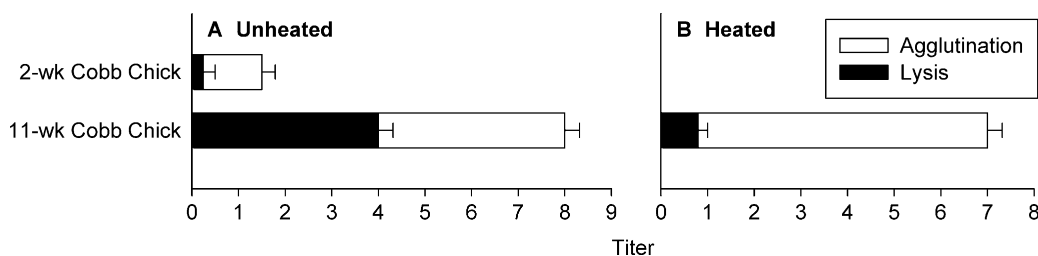

Fig. 3. Lysis and agglutination titers in unheated (A) and heated (B) plasma from four 2-week-old and five 11-week-old Cobb broiler chickens (*Gallus domesticus*). Filled bars represent lysis; open bars, agglutination. Error bars represent SE.

samples). In this case, some NAb fraction appears to be denatured by the heat treatment.

We added 2-ME to plasma samples to break polymeric NAbs into monomeric units, such that a common currency could be used to compare agglutination across species. However, the ability of the immunoglobulin fragments to agglutinate is questionable [34], and the ME-resistant titers more likely represent IgY molecules that cross-react with rabbit RBCs. Though 2-ME treatment reduces titers by one-half or more, the interspecific pattern of agglutination seen with 2-ME resistant antibodies parallels the pattern of agglutination with untreated plasma and provides little additional information. One notable exception, jungle fowl plasma, exhibits a more severe reduction in agglutination titers. This reduction suggests that jungle fowl may rely more on polymeric forms of NAbs than the other species. In addition to reducing overall agglutination levels, 2-ME treatment totally eliminates all hemolytic activity. This elimination of lysis could result from the destruction of disulfide bonds in complement components or from the inability of 2-ME-resistant antibodies to initiate the complement cascade.

Many Passeriformes (songbirds) and Apodiformes (swifts and hummingbirds) are too small ($<$20 g) to collect sufficient blood to test all plasma treatments. In such cases, untreated plasma should be the highest priority because it provides the most information (i.e. lysis and agglutination titers).

3.3. Effects of age in young birds

With respect to lysis and agglutination in unheated and heated plasma, 11-week-old Cobb broiler chicks exhibited significantly higher titers than 2-week-old chicks (Fig. 3). In unheated plasma, lysis titers increased from 0.3 ± 0.3 (SE) in 2-week-old birds to 4.0 ± 0.3 in 11-week-old birds ($F(1,7)=79.5$, $p<0.001$) and agglutination titers increased from 1.5 ± 0.3 to 8.0 ± 0.3 ($F(1,7)=219.1$, $p<0.001$). In heated plasma, lysis titers increased from 0.0 ± 0.0 in 2-week-old birds to 0.8 ± 0.2 in 11-week-old birds ($F(1,7)=12.4$, $p=0.010$) and agglutination titers increased from 0.0 ± 0.0 to 7.0 ± 0.3 ($F(1,7)=381.1$, $p<0.001$). Seto and Henderson [33] found low levels of NAbs in embryos and in chicks up to 20 days of age. In their experiment, NAb levels increased rapidly during the next several weeks and reached a plateau at around 12 weeks of age. The low level of agglutination by plasma from young chicks indicates that maternal IgY contributes little to the NAb titer as measured with RBC agglutination. The timing of the increase and plateau of NAbs follows a time course that is similar to that of plasma IgM levels, further implicating this form as the responsible Ig.

Changes in immune function in developing birds are expected, but the generality of this result across species is unknown. The rates of increase of circulating NAb levels likely depend on the development period and vary over the altricial-precocial development spectrum. For practical purposes, these results suggest the need to limit comparisons to birds of similar age groups (e.g. hatchling, fledgling, hatch year or older) whenever possible.

3.4. Effects of LPS-induced sickness response

Lysis and agglutination titers were not greatly affected by LPS injection in 11-week-old Cobb chicks (Fig. 4). Only in one case a significant effect was shown: lysis in heated plasma decreased from

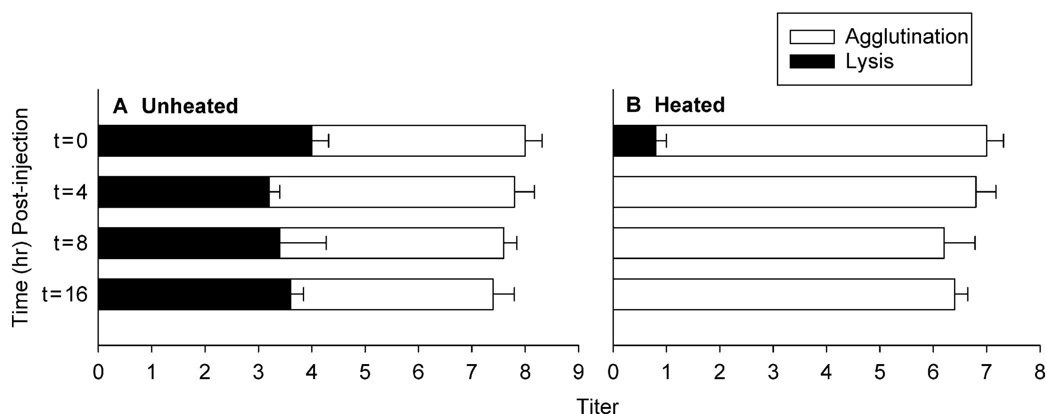

Fig. 4. Lysis and agglutination titers in unheated (A) and heated (B) plasma from 11-week-old Cobb broiler chickens (*Gallus domesticus*). Each group was composed of five different birds. Filled bars represent lysis; open bars, agglutination. Error bars represent SE.

0.8 ± 0.2 in the control birds to 0.0 ± 0.0 in the 4, 8, and 16 h birds ($F(3,16) = 16.0$, $p < 0.001$). The non-significant decreasing trend in agglutination with time may, in fact, be the result of NAbs binding and clearing LPS, as suggested by Reid [26].

Because some acute phase proteins are likely able to lyse RBCs in a complement-dependent manner, one might predict higher levels of lysis in birds undergoing an acute phase response. On the other hand, the lack of substantial changes in lysis and agglutination titers after LPS injection may be less surprising in light of the finding of Baumgarth et al. (i.e. that infection does not affect NAb levels [30]) and our finding that vaccination with keyhole limpet hemocyanin has no effect on either titer (unpublished data). Nevertheless, that the acute sickness response with its symptomatic anorexia, elevated acute phase proteins, and periodic hypo- and hyperthermia did not significantly affect lysis or agglutination is noteworthy, particularly in relation to the tradeoffs that are made between other physiological stresses and immunocompetence [29]. Thus, with wild birds it can be assumed that short-term fluctuations in health status, which are difficult to determine when a bird is in hand, do not strongly affect these measures of innate immunity.

3.5. Inter-species comparisons

Because the interaction of species and heat treatment was significant in the case of lysis ($F(10,61) = 37.7$, $p < 0.001$) and agglutination ($F(10,61) = 2.5$, $p = 0.012$), and because the effects of heat treatment were documented in a subset of the species in this study, we examined the effect of species independently within each plasma treatment type. The data for heated and unheated plasma were analyzed using a GLM with species as a fixed factor.

Lysis titers in unheated plasma (Fig. 2A) ranged from 0.0 ± 0.0 (SE) in the mourning dove to 5.3 ± 0.3 in the common grackle ($F(10,57) = 38.9$, $p < 0.001$). Agglutination titers for unheated plasma (Fig. 2A) ranged from 1.8 ± 0.7 in the mourning dove to 8.0 ± 0.3 in the 11-week-old Cobb chickens ($F(10,57) = 8.4$, $p < 0.001$). Lysis titers in heated plasma (Fig. 2B) ranged from 0.0 ± 0.0 in 9 different species to 0.8 ± 0.2 in the 11-week-old Cobb chickens ($F(10,57) = 10.8$, $p < 0.001$). Agglutination titers for heated plasma (Fig. 2B) ranged from 1.6 ± 1.0 in the mourning dove to 7.4 ± 0.4 in the jungle fowl ($F(10,57) = 9.3$, $p < 0.001$).

With the range in agglutination titers observed in the 11 species, we conclude that different species rely on the constitutive innate humoral branch of the immune system to differing degrees. The level of this reliance may reflect the underlying differences in the biology or life history of each species. Furthermore, the differences across species may represent tradeoffs with other branches of the immune system, so that birds with high NAb levels may rely less on induced and/or specific responses. This, however, does not appear to be the case *within* the humoral branch.

Chickens artificially selected for high or low primary antibody responses exhibited parallel changes in NAb levels (and disease resistance) [28,35,36]. Thus, despite a functional partitioning, constitutive innate and induced specific humoral immunities appear to be genetically related to one another and to disease resistance within a species. The extent of these relationships across bird species, such that NAb variables could be measured as a proxy for overall humoral immunity, remains to be examined.

In addition to the variation in agglutination across species, the 11 studied species also varied with respect to average lysis titers. When the two variables are examined together, the species can be placed in three groups based on the amount of complement (lysis) relative to NAb (agglutination). In the low group, lysis titers are less than 5% of total agglutination titers; in the medium group, they are 40–65%; and in the high group, greater than 95%. Both overall agglutination and lysis titers could vary to balance the robustness of other branches of the immune system; the particular pattern of immune defenses might reflect differences in the life history and ecology of species, but resolving these relationships will require carefully designed experiments and broader comparative studies.

In birds, the total fitness costs of maintaining NAbs and complement are not well understood. In mammals, however, pathological autoimmunity appears to be related to anomalous NAb production in some cases [37–39]. Ultimately, the degree of investment in innate humoral immunity should reflect a number of factors, including life span and the need for long-term memory, exposure to pathogenic organisms in the environment, and the coevolutionary responses of pathogens to a host's immune response.

Use of the assay described in this paper for characterizing constitutive innate humoral immunity has clear advantages over several methods currently employed to assess immune function in comparative and experimental studies. The most common assay of immune system function in the ecological literature, the swelling to a subcutaneous injection of phytohemagglutinin (PHA), quantifies non-specific cellular immunity [40,41]. This technique requires measurements over a 24-h period. Moreover, the PHA response does not represent a single clearly definable immune phenomenon but a suite of responses that results in swelling. A second technique, involving specific antibody responses following vaccinations by particular antigens (such as sheep RBCs [42] and keyhole limpet hemocyanin [43]), measures specific, humoral immunity. As this technique requires either recapturing individuals or holding individuals over periods of up to 30 days, the tests are logistically complicated and the results are confounded by other physiological phenomena such as stress responses or changes in breeding status. However, if the conjecture of Kohler et al. [44] that NAb levels against particular pathogens approximate the specific antibody response to those same pathogens is true across avian species, then minor modifications of our assay (i.e. replacement of RBCs with specific pathogens) could permit a comparative study of adaptive immunity.

4. Conclusions

The hemolysis–hemagglutination assay described here can be effectively used to characterize and quantify constitutive innate humoral immunity in birds. The assay is highly repeatable and the results are unambiguous. Agglutination and lysis titers vary significantly between species. In chickens, agglutination and lysis titers were affected by age, but were not affected by an LPS-induced sickness response. When plasma sample volume is limited, we recommend untreated plasma for use in this assay, as this option is most informative.

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