Neutrophil function and plasma opsonic capacity in colostrum-fed and colostrum-deprived neonatal kittens

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Objective—To determine whether passive transfer of IgG in neonatal kittens affects plasma opsonic capacity and neutrophil phagocytic and oxidative burst responses to bacteria in vitro.

Animals—22 kittens from 6 specific pathogen-free queens.

Procedure—Kittens were randomized at birth into the following treatment groups: colostrum-fed, colostrum-deprived, or colostrum-deprived supplemented with feline or equine IgG. Blood samples were collected at intervals from birth to 56 days of age. Plasma IgG concentrations were determined by radial immunodiffusion assay. Neutrophil function was assessed by a flow cytometry assay providing simultaneous measurement of bacteria-induced phagocytosis and oxidative burst. The opsonic capacity of kitten plasma was determined in an opsonophagocytosis assay with bacteria incubated in untreated or heat-inactivated plasma.

Results—Among treatment groups, there were no significant differences in neutrophil phagocytic and oxidative burst responses to bacteria or opsonic capacity of plasma. In all samples of plasma, inactivation of complement and other heat-labile opsonins significantly reduced the opsonic capacity. Plasma IgG concentrations in kittens did not correlate with simultaneous measurement of bacteria-induced phagocytosis and oxidative burst. The alternate complement pathway or other heat-labile opsonins may be more important than IgG in bacterial opsonization and phagocytosis. (Am J Vet Res 2003;64:538–543)

Conclusions and Clinical Relevance—The plasma opsonic capacity and neutrophil phagocytic and oxidative burst responses in vitro of kittens receiving passive transfer of IgG via colostrum intake or IgG supplementation and those deprived of colostrum were similar. The alternate complement pathway or other heat-labile opsonins may be more important than IgG in bacterial opsonization and phagocytosis.

Materials and Methods

Reagents—Commercial preparations of propidium iodide (PI; P4170), dihydrorhodamine 123 (DHR; D1054),...
0.4% trypan blue (T8154), paraformaldehyde (P6148), and tryptic soy broth (T8907) were used in our study. Magnesium- and calcium-free PBS (PBSS; pH 7.2) was prepared by conventional technique. Propidium iodide was dissolved in PBSS to a stock concentration of 1 mg/mL and stored at 4°C in a lightproof container. Dihydrorhodamine was dissolved in dimethyl sulfoxide to a stock concentration of 500 µM and stored at 4°C in a lightproof container.

**Animals**—Six blood type-A specific pathogen-free queens and their 22 kittens from 6 litters were included in the study. Queens were under constant observation during the final days of pregnancy, and all kitten deliveries were attended. The research protocol was approved by the Institutional Animal Care and Use Committee and conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

**Treatment groups**—Kittens were removed from the queens immediately after birth to prevent suckling and ingestion of colostrum. After collection of blood samples for baseline plasma IgG determination, the kittens in each litter were randomly assigned to 1 of 4 treatment groups; the groupings included kittens that were colostrum-fed (n = 6), colostrum-deprived without IgG supplementation (6), colostrum-deprived but supplemented SC with 400 mg of feline IgG (5), and colostrum-deprived but supplemented SC with 400 mg of equine IgG (5). Kittens in the colostrum-fed group were returned to their queens to nurse after blood sampling. All other kittens were fed a kitten milk replacer for 48 hours to assure colostrum deprivation then returned to their queens and allowed to suckle normally for the duration of the study.

**IgG supplementation**—A pooled collection of serum (22 mg of IgG/mL) was prepared from blood samples obtained from adult cats in the research colony. Lyophilized equine IgG was resuspended in sterile physiologic saline (0.9% NaCl) solution to a concentration of 22 mg of IgG/mL. In a previous study in colostrum-deprived kittens, parenteral supplementation with approximately 400 mg of IgG resulted in serum IgG concentrations comparable to that measured in kittens that had nursed from birth. Colostrum-deprived kittens that received IgG supplementation were administered a 6-ml dose (133 mg of IgG) SC of either the feline or equine IgG preparations every 12 hours from birth for a total of 3 doses (approx 400 mg of IgG) within 24 hours.

**Blood sample collection**—At birth (day 0), blood samples (0.4 mL) were collected from each kitten by jugular venipuncture for plasma IgG analysis. On days 2, 7, 14, 28, 42, and 56, blood samples (0.8 mL) were collected from each kitten and placed into tubes containing lithium heparin. A 25-µL aliquot was removed from each sample for determination of the WBC concentration and differential count on a stained blood smear. A 400-µL aliquot of blood was removed for the intrinsic neutrophil function assay. Plasma was obtained from the remaining blood for IgG analysis and opsonophagocytosis assays.

**Measurement of IgG**—Plasma IgG concentrations were determined by radial immunodiffusion assay as described.  

The concentration of feline IgG standards ranged from 32 to 2,000 mg of IgG/dL of plasma. The concentration of equine IgG standards ranged from 200 to 1,600 mg of IgG/dL of plasma. Samples with plasma concentrations < 32 mg of feline IgG/dL and 200 mg of equine IgG/dL were recorded as 0 mg of IgG/dL of plasma for statistical purposes. Both feline and equine IgG concentrations were determined in plasma from colostrum-deprived kittens supplemented with equine IgG.

**Preparation of bacterial targets**—Staphylococcus aureus and *E coli* were obtained from kittens with naturally occurring sepsis that was confirmed at necropsy by the Veterinary Medical Teaching Hospital’s Clinical Microbiology, Parasitology, and Serology Service. These isolates were grown in tryptic soy broth for 18 hours at 37°C, and the concentration was determined by colony counts from serial dilutions of the broth culture. The bacteria were heat-killed at 70°C for 60 minutes, washed, resuspended to the original volume in sterile PBSS, and added to an equal volume of PI diluted to 100 µg/mL in sterile PBSS. After mixing by continuous rotation for 60 minutes at 23°C, the PI-labeled bacteria were washed and resuspended in sterile PBSS to a concentration of 1 x 10⁷ cells/mL. Aliquots of the labeled bacteria were protected from light exposure during storage at 4°C and warmed to 23°C prior to use.

**Intrinsic neutrophil function assay**—Neutrophil phagocytosis and oxidative burst were assessed by means of a modified dual-color flow cytometry assay. In a preliminary study involving 20 adult cats from the research colony, the DHR loading dose, bacterial target to neutrophil ratio, and incubation time were optimized for maximal neutrophil phagocytosis and oxidative burst responses. In the modified assay, leukocytes in 100-µL aliquots of blood from each kitten were loaded with DHR (5µM) for 15 minutes at 37°C with continuous mixing. Aliquots of PI-labeled *S aureus* or *E coli* were added to achieve a bacteria to neutrophil ratio of 30:1. A control tube contained DHR-loaded leukocytes in blood without bacteria. After incubation at 37°C for 30 minutes with continuous mixing, the tubes were placed on ice to stop phagocytic and oxidative burst activity and processed for flow cytometry with a reagent kit and an automated lysing system. After the addition of 500 µL of water to each tube for completion of hemolysis, extracellular fluorescence was quenched with 10 µL of 0.4% trypan blue solution. The tubes were acquired on the flow cytometer within 2 hours of preparation.

**Opsonophagocytosis assay**—The intrinsic neutrophil function assay was used to measure phagocytosis of bacteria exposed to all opsonins present in kitten blood, including IgG and complement. To determine the opsonic capacity of IgG independent of complement and other heat-labile opsonins, kitten plasma samples were heated at 36°C for 30 minutes prior to analysis in an opsonophagocytosis assay. In this assay, 15 µL of untreated plasma or heat-inactivated plasma was incubated at 37°C for 30 minutes with continuous mixing, then placed on ice at 4°C for 15 minutes, and 15 µL of PI-labeled *S aureus* or *E coli* (1.5 x 10⁵ cells) with continuous mixing. Donor leukocytes were prepared by density gradient centrifugation of heparinized blood collected from an adult cat in the research colony. Donor leukocytes were suspended in sterile PBSS to a concentration of 5 x 10⁵ cells/mL, loaded with DHR (5µM) as described, and 100-µL aliquots (5 x 10⁵ cells) were added to tubes containing the bacteria. The samples were incubated at 37°C for 30 minutes with continuous mixing, then placed on ice, and 500 µL of 0.5% paraformaldehyde in PBSS and 10 µL of 0.4% trypan blue solution were added. The tubes were acquired on the flow cytometer within 2 hours of preparation.

To investigate the possibility that a toxic effect of heat-inactivated plasma could alter neutrophil phagocytosis, the opsonophagocytosis assay was performed as described using 5 µL bacteria incubated in untreated plasma, heat-inactivated plasma, and serial 2-fold dilutions of untreated plasma in heat-inactivated plasma. This assay was performed with untreated and heat-inactivated plasma from three 2-day-old colostrum-fed kittens and three 2-day-old colostrum-deprived kittens.

**Flow cytometry analysis**—The samples from the intrinsic neutrophil function and opsonophagocytosis assays were acquired on a flow cytometer equipped with a 488-nm laser.
argon-ion laser for excitation and 15 mW of power. Density cytograms were generated by linear amplification of the signals in the forward scatter and side scatter channels. Fluorescence cytograms were based on a 4-decade logarithmic amplification of the signals in the FL1 (530-nm filter with a 30-nm band) and FL2 (585-nm filter with a 42-nm band) channels. Neutrophils were selectively analyzed on the basis of their size and complexity in the density cytogram, and data from acquisition of 10,000 events/sample were processed by computer software. Parameters quantified from the fluorescence cytograms included the percentage of neutrophils that phagocytosed bacteria and the percentage of neutrophils with a phagocytosis-induced oxidative burst.

Statistical analyses—Data were reported as mean ± SD. The Kruskal-Wallis test was used for comparisons among treatment groups of plasma IgG concentrations, percentage of neutrophils that phagocytosed bacteria, and percentage of neutrophils with a phagocytosis-induced oxidative burst. Simple linear regression analysis was used to estimate correlation coefficients (r) for plasma IgG concentrations versus percentage of neutrophils that phagocytosed bacteria and the percentage that had an oxidative burst response in the intrinsic neutrophil function and opsonophagocytosis assays. For all tests, a value of P < 0.05 was considered significant.

Results

Plasma IgG concentrations in kittens—None of the kittens had detectable plasma IgG at birth. The mean peak plasma IgG concentration on day 2 was significantly (P < 0.01) higher in colostrum-fed kittens (4,092 ± 1,260 mg/dL) than that observed in colostrum-deprived kittens (0 mg/dL) and colostrum-deprived kittens supplemented with feline IgG (1,768 ± 517 mg/dL) or equine IgG (1,838 ± 1,293 mg/dL). Compared with values in all other kittens, mean plasma IgG concentration in the colostrum-fed kittens remained significantly (P < 0.01) higher for 28 days. Mean plasma IgG concentration in the IgG-supplemented kittens was significantly (P < 0.01) higher than that of colostrum-deprived kittens for 14 days. The mean concentration of maternal or exogenous IgG in plasma steadily declined to a nadir at 28 days in colostrum-fed kittens and IgG-supplemented kittens. After 28 days, there was a gradual increase in mean plasma IgG in all treatment groups from endogenous synthesis, resulting in similar mean concentrations in all kittens by 56 days of age.

Intrinsic neutrophil function in kittens—Scatterplots were used to determine the percentage of neutrophils that phagocytosed bacteria and the percentage that had a phagocytosis-induced oxidative burst response (Fig 1). The mean percentage of neutrophils that phagocytosed S aureus and the mean percentage that had an oxidative burst response were similar (P ≥ 0.8) in the colostrum-fed, IgG-supplemented, and colostrum-deprived kittens, despite significant differences in mean plasma IgG concentrations among the treatment groups from 2 to 28 days of age (Fig 2). Similarly, there were no significant (P ≥ 0.4) differences between treatment groups in the mean percentage of neutrophils that phagocytosed and the mean percentage that had an oxidative burst response to E coli. Mean plasma IgG concentration in kittens from 2 to 56 days of age was not significantly correlated with the neutrophil phagocytic and oxidative burst response to either S aureus (r = –0.1, P = 0.08; Fig 2) or E coli (r = –0.2, P = 0.06).

For all kittens, the mean percentage of neutrophils that phagocytosed S aureus increased from 44 ± 19% at 2 days of age to 74 ± 14% at 8 weeks, whereas the mean percentage that phagocytosed S aureus and had an oxidative burst response increased from 21 ± 13% at 2 days to 53 ± 11% at 56 days. The neutrophil responses in 56-day-old kittens were comparable to those previously reported in adult cats, in which the mean percentage of neutrophils that phagocytosed S aureus was 78 ± 15%, and the mean percentage that phagocytosed S aureus and had an oxidative burst response was 54 ± 14%.

Plasma opsonic capacity—Since the intrinsic neutrophil function assay measures phagocytosis of bacte-

Figure 1—A representative scatterplot illustrating analysis of neutrophil phagocytosis and oxidative burst by flow cytometry generated by the intrinsic neutrophil function assay performed on whole blood from a 2-day-old, colostrum-fed kitten. A—Neutrophils were gated for analysis on the basis of cellular size (forward scatter) and complexity (side scatter). In the other panels, the propidium iodide (PI)-labeled y axis represents the intensity of red fluorescence from bacteria labeled with PI. The dihydrotriamine (DHR)-labeled x axis represents the intensity of green fluorescence generated by conversion of nonfluorescent DHR to fluorescent rhodamine by the oxidative burst response in neutrophils. Fluorescence intensity is measured on a log scale with arbitrary units. B—The lower left quadrant contains DHR-loaded neutrophils in the absence of PI-labeled bacteria. C—Quadrant 1 contains DHR-loaded neutrophils that did not phagocytose PI-labeled bacteria or undergo an oxidative burst response. Quadrant 2 contains DHR-loaded neutrophils that have phagocytosed PI-labeled bacteria but have not yet undergone an oxidative burst response to generate fluorescent rhodamine. Quadrant 3 contains DHR-loaded neutrophils that have phagocytosed PI-labeled bacteria and undergone a subsequent oxidative burst response to generate rhodamine. The percentage of neutrophils that phagocytosed bacteria is the sum of the percentages in quadrants 2 and 3, and the percentage of neutrophils with a phagocytosis-induced oxidative burst response is the percentage of total neutrophils in quadrant 2.

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ria exposed to all opsonins present in blood, the contribution of IgG to bacterial opsonization may be masked by the presence of other opsonins such as complement. In the opsonophagocytosis assay, opsonization attributable to IgG was determined by comparison of donor neutrophil phagocytosis of bacteria opsonized in kitten plasma before and after heat inactivation of complement and other heat-labile opsonins. There were no significant ($P = 0.4$) differences in the mean percentage of neutrophils that phagocytosed $\textit{S. aureus}$ and had an oxidative burst response for colostrum-deprived kittens in panel B. The percentages of neutrophils that phagocytosed and had an oxidative burst response to $\textit{S. aureus}$ for IgG-supplemented kittens were similar to those for colostrum-fed and colostrum-deprived kittens.

**Figure 2**—Relationship of mean plasma IgG concentrations to intrinsic neutrophil function in colostrum-fed (n = 6) and colostrum-deprived (6) kittens from birth to 56 days of age. The percentage of neutrophils (mean ± SD) that phagocytosed $\textit{Staphylococcus aureus}$ (black squares) and had an oxidative burst response (white squares) for colostrum-fed kittens is shown in panel A. The percentages of neutrophils that phagocytosed $\textit{S. aureus}$ and had an oxidative burst response for colostrum-deprived kittens are shown in panel B. The dashed line represents the mean plasma IgG concentration (mg/dL) at each time point. The percentages of neutrophils that phagocytosed and had an oxidative burst response to $\textit{S. aureus}$ for IgG-supplemented kittens were similar to those for colostrum-fed and colostrum-deprived kittens.

**Figure 3**—Bacterial opsonic activity in untreated plasma and heat-inactivated plasma from 6 colostrum-fed (panel A) and 6 colostrum-deprived (panel B) kittens at birth and 2, 28, and 56 days of age. The percentages (mean ± SD) of donor neutrophils that phagocytosed $\textit{S. aureus}$ (% phagocytosis) that had been opsonized with untreated plasma (dark bars) or heat-inactivated plasma (white bars) are shown. The percentages of neutrophils that phagocytosed $\textit{S. aureus}$ opsonized with untreated or heat-inactivated plasma from IgG-supplemented kittens were similar to those calculated for colostrum-fed and colostrum-deprived kittens. The dashed line represents the mean plasma IgG concentration (mg/dL) at each time point. *Values for percentage phagocytosis of bacteria opsonized in untreated plasma are significantly ($P < 0.05$) higher than values for bacteria opsonized in heat-inactivated plasma.

Heat inactivation of plasma resulted in significant ($P < 0.05$) loss of opsonic capacity for $\textit{S. aureus}$ (Fig 3) and $\textit{E. coli}$ opsonized by untreated plasma samples from colostrum-fed, IgG-supplemented, or colostrum-deprived kittens. Consistent with the results of the intrinsic neutrophil function assay, the mean concentration of IgG in untreated plasma was not correlated with phagocytosis of opsonized bacteria ($r = 0.01, P = 0.9$).

Heat inactivation of plasma containing increasing proportions of untreated plasma. There was a dose-dependent linear relationship between the number of neutrophils that phagocytosed bacteria and the proportion of untreated plasma. Inclusion of as little as 3% untreated plasma significantly ($P < 0.05$) increased bacterial phagocytosis from 19 to 31%, suggesting that a toxic effect of heat-inactivated plasma on neutrophil phagocytic capacity was unlikely. After inactivation of heat-labile opsonins, there was no correlation between the IgG concentration and residual opsonic activity in the plasma ($r = 0.2, P = 0.1$).

**Discussion**

Several studies in large animal neonates have shown that passive transfer of IgG correlated with increased bacterial opsonization and neutrophil function in vitro. In contrast, recent studies in neonatal foals found that plasma IgG concentrations did not correlate with neutrophil function and plasma opsonic capacity. In the study of this report, passive transfer of IgG in neonatal kittens, either by colostrum ingestion or parenteral supplementation, did not enhance their neutrophil phagocytic and oxidative burst responses to...
bacterial challenge in vitro, compared with those responses in kittens with FPT. In fact, the neutrophil phagocytic and oxidative burst responses were more dependent on age than plasma IgG status, as evidenced by increasing functional capacity from birth to 56 days of age. A similar age-dependent maturation in intrinsic neutrophil function has been reported for foals, calves, lambs, lambs, puppies, and infants. Many studies suggest that effective neutrophil function in neonates depends more on the type and amount of opsonins, such as IgG and complement in the plasma, than on the intrinsic capacity of neutrophils per se. In the opsonophagocytosis assay, the bacterial opsonization capacity in plasma was similar for all kittens from birth to 56 days of age, regardless of plasma IgG concentration. In a study to investigate the contribution of IgG and complement in the plasma, than on the intrinsic capacity of neutrophils per se. In the opsonophagocytosis assay, the bacterial opsonization capacity in plasma was similar for all kittens from birth to 56 days of age, regardless of plasma IgG concentration. Furthermore, the bacterial opsonization capacity in plasma was similar for all kittens from birth to 56 days of age, regardless of plasma IgG concentration. In a study to investigate the contribution of IgG and complement to opsonization in horses, the alternative pathway of complement activation accounted for most of the activity in opsonophagocytosis assays that involved high (30%) serum concentrations or long (30 minutes) incubation times. Because the opsonophagocytosis assay for kittens incorporated high plasma concentrations (100%) and a long incubation time (30 minutes), it was conceivable that the contribution of IgG to bacterial opsonization may have been overwhelmed by activation of the alternative complement pathway. Heat inactivation of the alternative complement pathway in plasma resulted in a marked reduction in plasma opsonic capacity in all kittens, as reported in other species. The importance of complement activation for effective opsonization and phagocytosis has recently been demonstrated by minimal phagocytosis of yeast organisms opsonized by IgG or C3b in the absence of other plasma proteins.

When the alternative pathway is inactivated, neutrophil phagocytosis is dependent on activation of the classical complement pathway by IgG bound to the bacterial surface. In our study, IgG concentrations were not correlated with the remaining opsonic activity in heat-inactivated kitten plasma. This suggested that either the amounts of IgG antibodies specific for S. aureus and E. coli were insufficient to support activation of the classical complement pathway or that heat treatment of the bacteria altered surface antigens and resulted in lack of recognition by IgG antibodies. Alternatively, heat-stable opsonins other than IgG may have been involved in promoting bacterial phagocytosis in the absence of the alternative complement pathway. Compared with the role of IgG, the alternate complement pathway or other heat-labile opsonins appear to contribute more to bacterial opsonization and phagocytosis.

References