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Clinically significant anti-KEL RBC alloantibodies are transferred by breast milk in a murine model

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Abstract

Background and Objectives—Fetuses affected by maternal RBC alloantibodies may have prolonged anemia after birth, leading one to question whether maternal alloantibody transfer may occur outside the placenta. In response to a recent publication describing breast milk transfer of clinically significant amounts of maternal anti-platelet IgA antibodies from mother to nursing infant, we hypothesized that maternal RBC alloantibodies may also be capable of being transferred in breast milk.

Materials and Methods—The presence and clinical significance of breast milk alloantibody transfer was tested through a series of pregnancy, fostering, and transfusion experiments, using a murine model in which transgenic RBCs express the human KEL glycoprotein.

Results—Maternal anti-KEL immunoglobulins, induced through transfusion or pregnancy, were detected in the aqueous phase of breast milk. Further, efficient transfer of maternal anti-KEL IgG and IgA to nursing pups was observed in fostering experiments. The breast milk acquired alloantibodies were clinically significant in wild type pups in a transfusion setting, binding to “incompatible” KEL RBCs and leading to premature clearance from the circulation. Although breast milk acquired alloantibodies also bound to the RBCs of transgenic KEL positive fostered pups, no anemia resulted.

Conclusions—Taking these murine data in combination with recently published human data of maternal anti-platelet IgA antibodies in breast milk leading to sequelae in some infants, it is theoretically possible that maternal anti-RBC IgA alloantibodies may also be transferred in human breast milk and may lead to sequelae in some infants under some circumstances.

Keywords

RBC; alloimmunization; breast milk

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INTRODUCTION

Alloantibodies against RBC antigens, which may be generated following exposure to foreign antigens on transfused RBCs or on paternally derived antigens on fetal RBCs during pregnancy[1], can be clinically significant from the standpoint of morbidity and mortality [2]. In the transfusion setting, RBC alloantibodies can lead to premature clearance of transfused RBCs, resulting in hemolytic transfusion reactions and even death in severe cases [3]. In the pregnancy setting, these alloantibodies can cause hemolytic disease of the fetus and newborn (HDFN)[4–6].

Maternal alloantibodies against many different RBC antigens are known to be capable of leading to HDFN, with antibodies against Rh(D), antibodies against other antigens in the Rh family (C/c and E/e), and antibodies against antigens in the KEL family making up the majority of HDFN cases[5]. Whereas some maternal alloantibodies such as those against Rh(D) lead to hemolysis of fetal/neonatal RBCs in conjunction with hyperbilirubinemia, others such as those in the KEL family lead to fetal/neonatal anemia through suppression of erythropoiesis[7, 8]. In a transfusion setting, however, antibodies against antigens in the KEL family are also known to lead to hemolysis[9, 10].

Red blood cell alloantibodies may be detected in neonates for months after birth, with some affected neonates demonstrating more prolonged anemia and/or hyperbilirubinemia than others. These observations have led to the question of whether RBC alloantibodies continue to be transferred from mother to child after birth. Total IgA/G/M/D/E are known to be present in human breast milk,[11, 12] with maternal antibodies typically being protective to neonates. Levels of maternal immunoglobulins are significantly lower in breast milk than in serum[11], and absorption of orally ingested immunoglobulins in the neonatal GI tract is known to be inefficient[13]. However, a 2015 study of neonates with persistent thrombocytopenia demonstrated that maternal anti-platelet IgA antibodies are present in breast milk and are absorbed by neonates in sufficient amounts as to be clinically significant *in some instances*[14].

We hypothesized that like maternal anti-platelet antibodies, maternal anti-RBC alloantibodies may also be capable of being transferred via breast milk. To the best of the our knowledge, this question has never been investigated outside of the area of “naturally occurring” maternal hemagglutinins[15]. The presently described studies involve a model in which the entire human KEL glycoprotein is present on murine RBCs[16]. Antibodies are generated through transfusion[17] or pregnancy[18] in animals lacking the KEL glycoprotein, with the alloantibodies leading to clinically significant hemolytic transfusion reactions as well as HDFN. We report that maternal anti-KEL immunoglobulins (Igs) are present in breast milk and are capable of being transferred to nursing pups, with clinically significant sequelae *in some instances*.

METHODS

Mice

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). Transgenic mice expressing the human KEL glycoprotein, previously published as KEL2b, were generated and bred by our laboratory[16].

Murine blood collection and transfusion

Anti-KEL alloantibodies were generated through transfusion[17] or pregnancy[18] as previously described. In brief, the transfusion alloantibody induction experiments involved C57BL/6 recipients being serially transfused with the equivalent of 1 human unit of transgenic KEL RBCs that had been collected in the anticoagulant preservative solution CPDA-1 and filter leukoreduced over a Pall neonatal filter. The pregnancy alloantibody induction experiments involved C57BL/6 females being bred serially with KEL transgenic males. Alloantibodies in transfusion recipients and post-partum females were measured by flow cytometric crossmatch.

The post-transfusion survival and recovery of lipophilically labeled KEL RBCs was completed as previously described[19], with 20 μ l of lipophilically labeled leukoreduced KEL RBCs (equivalent of one human unit, adjusted for weight), mixed with control RBCs labeled with a different lipophilic dye, and transfused via the retro-orbital plexus into 10 day old nursing pups. Blood from the transfusion recipients was sampled from 10 minutes to 21 days post-transfusion, with survival and recovery of the KEL RBCs determined as a ratio of the survival and recovery of the control RBCs.

Flow cytometry and analysis

Breast milk was obtained after oxytocin administration (Henry Schein, Dublin, OH). The aqueous phase was separated via centrifugation and used for analysis. Anti-KEL glycoprotein total Igs, IgA, IgG, and IgG subtypes were evaluated in the aqueous phase of the breast milk and in the sera using target antigen positive (KEL) or negative (C57BL/6) RBCs. An adjusted mean fluorescence intensity (MFI) was calculated by subtracting the signal of the crossmatch with KEL negative control C57BL/6 RBCs from the signal of the crossmatch with KEL positive RBCs. This signal has previously been confirmed to be KEL specific through additional studies involving human RBCs expressing antigens in the KEL blood group[20]. Anti-KEL antibodies bound to transfused KEL RBCs were also evaluated, by first gating on the lipophilically labeled population of interest. Relevant secondary and tertiary antibodies were utilized (goat anti-mouse Igs (IgG/M/A) or IgA or IgG conjugated to a fluorophore (BD Biosciences, San Jose, CA), biotinylated IgG subtype antibodies (Bethyl Laboratories, Montgomery, TX), and streptavidin conjugated to a fluorophore (BD)). Samples were analyzed on a 4-color BD FACS Calibur. The Hct of the nursing KEL transgenic or wild type pups was measured by VetScan HM5 analysis (Abaxis, Union City, CA).

Statistics

All statistical analyses were performed using Graph Pad Prism software (San Diego, CA). A Mann Whitney U test was used to determine significant differences between two groups, and a 2 way ANOVA with Tukey's multiple comparisons test was completed in relevant experiments. Error bars represent one standard deviation, and significance was determined by a p-value of 0.05.

Study approval

All procedures and protocols were approved by Yale University's Institutional Care and Use Committee.

RESULTS

Anti-KEL immunoglobulins are present in serum and breast milk

Maternal anti-KEL glycoprotein antibodies, induced through transfusion[17] or pregnancy[18] in this murine model, are known to cross the placenta[18]. To investigate the timing of the transplacental transfer, alloimmunized C57BL/6 females were bred with C57BL/6 males. Serum was collected in parallel from mothers and fetuses on post-conception day #17 or at weaning. Serum anti-KEL immunoglobulin levels (IgG/M/A) were evaluated by flow cytometric crossmatch with KEL or C57BL/6 RBCs, with adjusted MFI representing the KEL specific alloantibody signal (Figure 1A).

Relatively low amounts of maternal anti-KEL were detected in the circulation of fetuses on post-conception day #17 (Figure 1B; the fetuses from mom #1 had 6.9% (SD 4.9) and the fetuses from mom #2 had 21.5% (SD 3.2) as much antibody as their mothers had, with testing completed in parallel between mothers and fetuses). Additional studies were completed on pups at earlier and later time points in gestation, with all studies showing that fetuses had just a fraction of the amount of anti-KEL detectable in the serum of their mothers.

In contrast, studies performed on pups at the time of weaning (e.g. approximately 3 weeks after birth) showed a substantially higher percentage of the anti-KEL present in the maternal serum also present in the serum of the nursing pups (Figure 1C). The pups from mom #3 had 73.4% (SD13.1), the pups from mom #4 had 86% (SD14.4), and the pups from mom #5 had 79% (SD 16.8) as much antibody as their mothers had, with testing completed in parallel between mothers and pups. Taken in combination, these data suggest that maternal anti-KEL is either transferred in significant amounts between mother and fetus in the last few days of pregnancy, or that maternal anti-KEL is transferred after delivery via breast milk.

To investigate the latter possibility, oxytocin was administered to nursing alloimmunized moms at approximately day #10 after delivery and breast milk was collected. The aqueous phase of the breast milk was separated out from the rest of the milk via centrifugation, and was then crossmatched with KEL positive or control RBC targets. Anti-KEL glycoprotein alloantibodies (total Igs, IgG/A/M) were detected in the aqueous phase of breast milk in 5/5 tested alloimmunized mothers (Figure 1D–E shows representative data), with no KEL

specific alloantibodies being present in the breast milk of control non-alloimmunized mothers. As has been described in humans[11] the relative amount of anti-KEL detected in breast milk was less than that detected in maternal serum (mean 44.7% compared to maternal serum anti-KEL), though the levels detected in breast milk were variable (range 12–100% compared to maternal serum anti-KEL). Additional studies, completed using secondary reagents specific for IgG or IgA, revealed the presence of anti-KEL IgG and IgA in the breast milk of alloimmunized mothers (Figure 1F).

Maternal anti-KEL immunoglobulins are transferred to fostered pups via breast milk

To isolate breast milk transfer as a potential source of maternal anti-KEL immunoglobulins, a fostering approach was taken. C57BL/6 females, alloimmunized through transfusion or pregnancy, were bred with C57BL/6 males and control C57BL/6 females were bred with C57BL/6 males. A subset of pups born to the control moms were fostered and nursed by the alloimmunized moms; these moms also nursed some of their own pups (Figure 2A depicts the experimental design). In 5/5 experiments, similar levels of total anti-KEL Igs (IgG/A/M) were detected at the time of weaning (e.g. approximately 21 days after birth) in the serum of the fostered pups as compared to the serum of the mom's own pups (Figure 2B shows a representative experiment, with the fostered pups exposed solely to anti-KEL Igs through breast milk having 71.2% (SD 13.5) of the alloimmunized foster mom's anti-KEL Ig level, and with the alloimmunized mom's own pups who were exposed to anti-KEL Igs through transplacental transfer as well as through breast milk having 73.8% (SD19) of their mom's anti-KEL Ig level). In contrast, pups born to alloimmunized mothers but nursed by non-alloimmunized foster mothers had essentially no anti-KEL Igs detected at the time of weaning (data not shown).

Given that maternal anti-platelet IgA has recently been shown to be transferred via breast milk in humans[14], additional studies were completed to investigate anti-KEL immunoglobulin subtypes transferred via breast milk in our murine model. Serum from fostered pups and serum from the mom's own pups were crossmatched with KEL positive or control RBC targets, with secondary reagents specific for IgG or IgA. Anti-KEL IgG and anti-KEL IgA were detected in the serum of all tested pups (Figure 2C shows a representative experiment). Additional flow cytometric studies, completed using secondary reagents specific for distinct IgG subtypes, revealed the presence of multiple anti-KEL IgG subtypes in the serum of all tested pups (Figure 2D shows a representative experiment, with anti-KEL IgG1, IgG2a, IgG2b, and IgG2c detected).

Maternal anti-KEL immunoglobulins, transferred to pups through breast milk, are detrimental in a transfusion setting

Anti-KEL glycoprotein or anti-Kell RBC alloantibodies, acquired through transfusion or pregnancy, lead to hemolytic transfusion reactions in mice and humans[18, 19]. To determine whether breast milk acquired anti-KEL could be clinically significant in a transfusion setting, leukoreduced KEL RBCs were labeled with the lipophilic dye DiO and control (tracker) KEL negative RBCs were labeled with a different lipophilic dye, DiI (Figure 3A shows labeling schematic). A mixture of KEL and control tracker RBCs was transfused to control pups, fostered pups, and the mom's own pups at 10 days of life, with

post-transfusion RBC recovery and survival measured at multiple time points through flow cytometric evaluation of the ratio of circulating DiO labeled KEL RBCs to control DiI labeled RBCs. In 2/2 experiments (n=18 pups), rapid clearance of approximately 50% of transfused DiO KEL RBCs was observed in fostered pups and in the mom's own pups, with stable RBC recovery being observed in control pups (Figure 3B shows a representative experiment). Although stable KEL antigen expression was detected on the DiO labeled KEL RBCs transfused into control pups, KEL antigen expression was essentially undetectable within 7 days of transfusion on the DiO KEL RBCs transfused into fostered pups with circulating anti-KEL antibody acquired solely through breast milk (Figure 3C shows a representative experiment). Of note, this pattern of rapid clearance of incompatible KEL RBCs followed by stable recovery of cells with undetectable levels of KEL antigen is similar to that which we have previously observed in adult mice transfused with incompatible RBCs that were alloimmunized through transfusion[19] or pregnancy[18].

Maternal anti-KEL immunoglobulins, transferred to foster pups solely through breast milk, bind to the RBCs of KEL positive pups but do not result in anemia

To test the impact of breast milk acquired anti-KEL on KEL positive pups, another set of experiments was completed. Pups from heterozygous KEL transgenic moms were fostered to alloimmunized moms (Figure 4A depicts the experimental design). Direct antiglobulin tests, completed by incubating fluorescently conjugated anti-mouse IgG to the RBCs of the fostered pups in an *in vitro* flow cytometry based assay, were positive on the RBCs of fostered KEL positive but not fostered KEL negative pups (Figure 4B shows representative data). However, no anemia was observed in any experiment at the time of weaning (3 weeks of age) in KEL positive pups compared to KEL negative pups that were exposed to anti-KEL glycoprotein antibodies solely through breast milk (Figure 4C).

DISCUSSION

These studies employ experimental designs not feasible in humans, to investigate the transfer and consequences of breast milk acquired anti-KEL RBC alloantibodies in a murine model. Our data demonstrate that maternal anti-KEL IgG and IgA RBC alloantibodies, generated in response to exposure to transgenic murine KEL RBCs during transfusion or pregnancy, can efficiently be transferred to nursing pups via breast milk. Breast milk acquired anti-KEL RBC alloantibodies are clinically significant in pups in a subsequent transfusion setting, binding to transfused KEL RBCs and leading to premature clearance of these cells. However, breast milk acquired anti-KEL RBC alloantibodies do not appear to be capable of causing anemia in transgenic KEL positive pups exposed to maternal alloantibody through breast milk for the first time after birth.

Breast milk acquired anti-KEL alloantibodies were as efficient as those acquired transplacentally at binding to transfused incompatible KEL RBCs and leading to post-transfusion RBC clearance. The pattern of rapid clearance of a portion of the incompatible KEL RBCs followed by stable circulation of approximately 40–50% of the KEL RBCs is one we have previously observed in adult animals immunized through transfusion or pregnancy, as well as in those adoptively transferred with anti-KEL serum prior to

transfusion with labeled KEL RBCs[18, 19]. As was observed in the current experiments, modulation of the KEL antigen has also been observed in past studies employing lipophilically labeled KEL of RBCs as well as naturally fluorescent KEL RBCs (uGFP × KEL F1 blood donors)[20] transfused into alloimmunized murine recipients. Modulation is not unique to the KEL antigen, to RBC antigens in general, or to mice, with other RBC antigens in murine models having been shown to undergo antibody mediated antigen modulation[21, 22], with multiple antibodies being reported to cause trophocytosis of WBC antigens in humans[23], and with multiple human cases of RBC antigen modulation reported[24]. The mechanism of antibody mediated KEL antigen modulation is not yet understood, though Fcγ receptors are critical in the modulation of WBC antigens[23].

The inability of breast milk acquired anti-KEL RBC alloantibodies to result in anemia in nursing KEL positive pups at the time of weaning was, at first glance, surprising given the clinical significance of these antibodies in a transfusion setting. These results were not due to “low” levels of anti-KEL in the foster moms, however, as no anemia was observed in experiments involving moms with a range of anti-KEL titers. It must be noted that the amount of KEL RBCs transfused (20 microliters packed RBCs) into wild type recipients was significantly smaller than the amount of KEL RBCs that make up a pup’s blood volume (blood volume is approximately 70 mL/kg, with the KEL RBC volume of a 10 gram pup thus being approximately 450 microliters). Thus, it is possible that there wasn’t enough breast milk derived anti-KEL antibody present in the circulation of the KEL positive pups to impact the endogenous KEL RBCs. It is also possible that anemia in the first few days of life might have gone unnoticed, as measuring Hcts in mice with such small blood volumes is difficult. Of note, maternal anti-Kell RBC alloantibodies are well known to suppress erythropoiesis[7, 8] and to lead to hydrops fetalis in human fetuses[5], and maternal anti-KEL RBC alloantibodies are also known to suppress erythropoiesis and to lead to hydrops fetalis in our murine HDFN model[18]. Thus, it must also be considered that fetal KEL RBC precursors, exposed to maternal anti-KEL alloantibodies in utero, are more sensitive than mature KEL RBCs to anti-KEL alloantibodies.

Our study results have clear implications for studies in our murine model. The finding that anti-KEL RBC alloantibodies are transferred via breast milk has impacted the experimental design of ongoing studies in our laboratory involving strategies to mitigate the dangers of maternal RBC alloantibodies to developing fetuses and neonates. For example, we now realize that the efficacy of strategies to prevent “transplacental” transfer must be evaluated prior to delivery to avoid the confounding effect of maternal antibody that continues to be transferred through breast milk after delivery. These findings also presumably have relevance to other animal models studying the effects of anti-platelet or anti-RBC antibody transfer in pregnancy settings[25–27].

Our study results may potentially have translational implications for some human infants in some settings, though multiple caveats must be considered. First and foremost, one must be cognizant of differences in the efficiency of FcRn-mediated intestinal absorption of immunoglobulins between mice and humans[28–30]. IgG absorption via the FcRns of rodents is significantly more efficient than IgG absorption via the FcRns of humans. This being realized, the clinical significance of low levels of breast milk acquired anti-RBC IgA

or IgG cannot be ruled out, especially given the recent report of breast milk acquired anti-platelet IgA antibodies being clinically significant in some infants[14]. One must also consider that many human infants exposed to maternal anti-RBC alloantibodies in utero will be positive for the cognate antigen, with prior pregnancies with the same partner often having stimulated the maternal antibody. Thus, the most relevant question in those settings--- whether continued breast milk exposure following in utero exposure may lead to anemia, hyperbilirubinemia, or other adverse effects--- is one not tested in our murine studies. In addition to the possibility of a mom's breast milk contributing alloantibodies to her own neonate, it is also theoretically possible that alloimmunized milk donors may contribute RBC alloantibodies to infants who drink donated "banked" milk. The known benefits of human breast milk are vast, however, and no change in breast feeding practice should be considered based on our murine studies. Rather, our study results could be pondered in instances of unexplained/persistent anemia in infants nursing from RBC alloimmunized mothers.

Limitations of any study must always be considered. Although our model allows for isolation of single variables and for study designs simply not feasible in humans (e.g. fostering pups immediately after birth with non-birth mothers to isolate the contribution of breast milk to the pup's serum anti-KEL Igs), the model is murine and as such has known limitations. As described, the major known limitation for these studies includes differences in FcRn between mice and humans. Another consideration includes the differences in IgG subtypes between mice and humans, though studies of RBC clearance following administration of monoclonal antibodies in humans[31] and mice alike[22, 32, 33] have shown the clinical significance of multiple different IgG subtypes. Next, these studies involved just one blood group antigen (KEL glycoprotein); thus, it is possible that different results may be observed in murine models expressing alternate RBC antigens or on human RBCs many alternate RBC antigens. Last, although we hypothesize that the IgG component of the maternal anti-KEL RBC alloantibodies resulted in the clinically significant sequelae observed, the fact that anti-KEL IgA was detected in the breast milk and in the serum of the fostered pups makes it difficult to rule IgA out as a contributing factor. Of note, testing completed in clinical transfusion services involves secondary reagents targeting IgG and as such, anti-RBC IgA antibodies may not be detected.

In sum, we have demonstrated that maternal anti-KEL RBC alloantibodies are transferred via breast milk to nursing pups in our murine model, with clinically significant sequelae in some settings. Being aware of the limitations of our studies, these data, in combination with a recently published study demonstrating the transfer of clinically significant amounts of anti-platelet IgA antibodies in human breast milk leading to prolonged neonatal thrombocytopenia[14], lead one to question whether clinically significant amounts of breast milk acquired anti-RBC IgA alloantibodies may also be transferred to some nursing infants in some situations.

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Figure 1. Anti-KEL immunoglobulins are present in serum and breast milk

(A) Representative histogram of serum from an alloimmunized animal crossmatched with KEL (solid) or C57BL/6 (shaded) RBCs. (B) Fetal serum anti-KEL immunoglobulins (IgG/M/A) as compared to maternal serum anti-KEL immunoglobulins at day 17 post-conception; all moms in these experiments are alloimmunized to the KEL antigen. (C) Neonatal serum anti-KEL immunoglobulins (IgG/M/A) as compared to maternal serum anti-KEL immunoglobulins at the time of weaning (approximately 21 days after birth). (D) Histogram of the aqueous phase of breast milk from an alloimmunized mom crossmatched with KEL (solid) or C57BL/6 (shaded) RBCs. (E) Comparison of anti-KEL immunoglobulins (IgG/M/A) in the serum and breast milk of a representative mom. (F) Comparison of anti-KEL immunoglobulins (IgG or IgA) in the serum and breast milk of a representative mom. Data in B and C reflect 5 pregnancies with sera from 20 fetuses/pups tested; data in D and E are representative of data from 5 experiments; data in F are representative of data from 2 experiments.

Figure 2. Maternal anti-KEL immunoglobulins are transferred to pups in breast milk
(A) Schematic of experimental design. **(B)** Serum anti-KEL immunoglobulins (IgG/M/A) in pups born to and nursed by an alloimmunized mom or in those born to a control mom but fostered and nursed by an alloimmunized mom, measured at the time of weaning (approximately 21 days after birth); representative data are shown as a percentage of the mom's serum anti-KEL level. **(C)** Serum anti-KEL IgG or IgA or **(D)** IgG subtypes in a representative mom, her own pups, and her fostered pups; adjusted mean fluorescence intensity (MFI) is the KEL specific antibody as determined by subtracting the signal of serum crossmatched with C57BL/6 RBCs from that of serum crossmatched with KEL RBCs. Data in B are representative of 5 experiments (n=28 pups total), and data in C and D are representative of 4 experiments (n=25 pups total).

Figure 3. Maternal anti-KEL immunoglobulins, transferred to pups through breast milk, are clinically significant in a transfusion setting

(A) Schematic of labeling approach for KEL and control tracker C57BL/6 RBCs, with post-transfusion RBC recovery and survival evaluated by examining the ratio of KEL to co-transfused control tracker C57BL/6 RBCs over time. (B) Post-transfusion recovery and survival of transfused KEL RBCs in pups with no alloantibodies at the initiation of the experiment (solid line), in pups born to and nursed by an alloimmunized mom (dashed line), and in pups born to a control mom and fostered by an alloimmunized mom (dotted line). At each time point measured, statistically significant differences were noted in KEL RBC survival between pups with no alloantibodies compared to those with antibodies (* $p < 0.001$), with no differences noted between the pups that acquired alloantibodies solely through nursing or through transplacental transfer plus through nursing. (C) KEL antigen detection on the DiO labeled KEL RBCs recovered at 10 minutes or 7 days post-transfusion. Data in B and C are representative of 2 experiments ($n = 15$ pups total).

Figure 4. Maternal anti-KEL immunoglobulins, transferred to foster pups solely through breast milk, bind to the RBCs of KEL positive pups but do not result in anemia

(A) Schematic of experimental design. (B) Direct antiglobulin tests of a representative KEL negative or KEL positive pup; shaded histogram is KEL negative pup and open histogram is KEL positive pup. (C) Hct of fostered KEL negative or KEL positive pups, measured at 3 weeks of age. Data in B are representative of 3 independent experiments and data in C are compiled from 20 pups.