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Development of serum antibodies during early infancy in rhesus macaques: implications for humoral immune responses to vaccination at birth

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Abstract

Background—A better understanding of immune responses in human infants could lead to more effective immunization and vaccination strategies in early life.

Methods—Since antibodies are key components of protective vaccine responses, we examined developmental changes in serum levels of immunoglobulins (IgG, IgA, IgM) in infant rhesus macaques from birth through 6-months of age.

Results—As in human infants, macaques are born with high levels of IgG in sera, with rapid increases in serum IgM, yet very slow increases in levels of IgA from birth. We also examined levels of anti-tetanus antibodies in infants born to vaccinated dams to distinguish and track maternal and infant antibodies. These data suggest essentially all serum IgG in newborn infants is derived from the dams, which gradually wanes over a few weeks. In contrast, levels of IgM and IgA appear to all be infant-derived, as evidenced by their low to undetectable levels at birth. In addition, abnormally high levels of serum IgM and IgA were detected in a few infants, which correlated with specific, yet clinically silent disease processes.

Conclusions—Our data indicate that newborn macaques have competent immune systems, and are able to produce their own antibodies in response to exposure to environmental antigens immediately upon birth.

Keywords

development; serum antibody; infant; rhesus macaques

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Introduction

Vaccination in early life provides significant protection against vaccine-preventable diseases and significant decreases in infant mortality [1-4]. However, most vaccines are not administered in the first month of life due to a variety of factors including an “immature” immune system and the interference of passively acquired maternal antibodies. Generally, serum immunoglobulin (Igs) levels provide key information on the status of the humoral immune system, and it is well established that normal serum Igs concentrations in young children are considerably lower than those of adults [5-8]. However, very limited data on serum antibodies has been reported in normal newborn or infant primates.

Antibodies, also known as immunoglobulins (Igs), are proteins produced by plasma cells to defend the host infections by identifying and neutralizing foreign objects such as bacteria and viruses [9]. Among the five classes of antibodies, IgG, IgA and IgM are the most abundant in serum [9, 10]. At birth, newborns have an increased susceptibility to infectious agents due to functional immaturity of the immune system, and early protection initially relies on the presence of maternal antibodies, especially maternal IgG antibodies transferred to the fetus from the placenta in utero [11], and IgA through colostrum and breast milk to breastfed infants after birth [12]. Although maternal antibodies can help prevent newborns from developing diseases and infections, their levels will not be sustained and wane within weeks to months. Therefore, neonates are thought to become vulnerable to infections due to their immature immune system being incapable of developing their own adaptive immune responses during early infancy [13-17].

Non-human primates (NHP) are necessary for testing the safety and efficacy of new vaccines and treatment strategies, especially for diseases in which other models do not exist. Primates also closely resemble humans in their physiology and immune system development. Thus, here we examined the development of antibodies in normal, uninfected neonatal rhesus macaques from birth through 6 months of age for comparison with their mothers (dams). Since only IgG, but not IgA and IgM antibodies can cross the placenta [18, 19], and secretory IgA (sIgA) is mainly passed through breast milk in humans [12], we examined and tracked serum IgA, IgM, and IgG levels in 10 formula-fed infant rhesus macaques from birth through 6 months of age, and levels of maternally-derived anti-tetanus toxoid (anti-TT) antibodies in infants born to vaccinated dams to distinguish and track levels of both maternal and infant antibodies.

Materials and methods

Animals and ethics statement

A total of 10 female Indian-origin rhesus macaques (*Macaca mulatta*) and their paired 10 infants were examined in this study as shown in Table 1. Ten infants (5 female and 5 male) were obtained from their dams ranging from 3.94 to 9.02 years of age (mean age=5.5 years) at birth, and nursery-reared on formula thereafter without exposure to breast milk. To examine mucosal and systemic lymphoid tissues, infants were sacrificed at 2 weeks (n=3), 1 month (n=3) and 6 months (n=4) after birth. All animals were housed at the Tulane National Primate Research Center (TNPRC) in accordance with the regulations of the Association for

Assessment and Accreditation of Laboratory Animal Care (AAALAC), and all experiments were reviewed and approved by the Tulane Institutional Animal Care and Use Committee (IACUC).

Vaccination and serum sample collection

Ten dams were intramuscularly vaccinated twice with 0.5 mL tetanus toxoid (TT) (Statens Serum Institute, Copenhagen) as shown as in Table 1. Serum samples were collected from all dams and their newborns at birth. By staggering the blood sampling, we were also able to collect sufficient blood at 2 weeks of birth, and monthly thereafter from all remaining infants. All serum samples were kept at -80°C till use.

Quantitation of total Igs by sandwich ELISAs

Serum total IgG, IgM and IgA levels were measured by optimal sandwich ELISAs as previously described [20, 21]. Briefly, plates were coated with either goat anti-monkey IgG Fc/7s (Accurate Chemicals, NY, USA), monoclonal anti-IgM (2C11-1-5, Life Diagnostics, PA, USA), or rabbit anti-IgA Fc (Accurate Chemicals, NY, USA) antibodies at a final concentration of $2\text{ }\mu\text{g/mL}$ in bicarbonate/carbonate coating buffer (100 mM , $\text{PH}=9.6$) overnight at 4°C . Standard controls included purified reference-rhesus IgG, rhesus IgA (NIH-Nonhuman Primate Reagent Resource) and monkey IgM (Rockland, PA, USA), which were run in parallel with each plate. Serum samples were diluted to optimal concentrations and tested in duplicate. Total IgG, IgM, and IgA were detected with biotin-conjugated goat anti-monkey IgG (Rockland, PA, USA), anti-monkey IgM Fc (Accurate Chemicals, NY, USA), and anti-monkey IgA (Alpha Diagnostic, TX, USA) respectively, and then incubated with HRP-streptavidin (Alpha Diagnostic, TX, USA). Plates were developed with 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate (Alpha Diagnostic, TX, USA) for 20 min, and reactions stopped with $2\text{ N H}_2\text{SO}_4$, and optical density (OD) was measured at 450 nm with a BioTek™ Synergy™ H4 Hybrid Microplate Reader. Standard curves were determined from controls, and antibody levels in experimental serum specimens were quantified by interpolation from controls.

Quantification of specific anti-TT IgG, IgG1 and IgM antibodies in sera of rhesus macaques

Serum anti-TT IgG levels were measured quantitatively using a commercial Tetanus IgG ELISA kit (IBL international, GMBM, Germany). According to manufacturer's instructions, each serum sample was diluted to 1:101 with diluent buffer. Optical densities (OD) were measured with a Microplate Reader at 450 nm. The results were expressed as international units per milliliter (IU/mL).

Monkey Anti-TT IgG1 and Monkey Anti-TT IgM ELISA kits (Life Diagnostics, PA, USA) were used to quantify serum anti-TT IgG1 and IgM levels respectively in rhesus macaques. According to manufacturer's instructions, each serum sample was diluted to the optimal concentration with provided diluent buffer, and run in duplicate on the plates. After development with TMB substrate, OD values were read at 450 nm. Seropositivity for anti-TT IgG1 and anti-TT IgM antibodies were defined by detection of concentrations $\geq 50\text{ }\mu\text{g/mL}$ and $\geq 75\text{ }\mu\text{g/mL}$ respectively.

Histopathology

Histopathologic assessments of all major tissues from infant macaques were performed by a board certified pathologist. Specifically, multiple sections of intestine, brain, liver, thymus, bone marrow, peripheral lymph nodes, and kidney were collected at necropsy, routinely fixed in neutral buffered 10% formalin, paraffin-embedded, sectioned and stained with hematoxylin and eosin (H&E) and examined for inflammation, changes in tissue architecture (organized lymphoid tissues and GALT) or other abnormalities.

Statistical analysis

Graphical presentation and statistical analysis of the data were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA). A paired t test was used to compare differences in levels of total antibodies between dams and their infants. Correlations between dams and corresponding infants were analyzed using Spearman's coefficient of correlation. For all analyses, P values <0.05 were considered significant.

Results

Comparison of total IgG, IgM, and IgA levels in sera between dams and their newborns at birth

At birth, there were significant differences between dams and their newborns in levels of serum total IgA and IgM, but not total IgG (Fig 1). Newborn sera contained very little total IgA (average 0.10 mg/mL; range from 0.03 to 0.15) or IgM (average 0.12 mg/mL; range 0.03 to 0.24). In contrast, there were high levels of serum IgG (average 10.3 mg/mL; range 7.3 to 16.1) in newborn sera. In dams, the highest levels of antibodies were IgG (averaged 12.6 mg/mL, range from 7.3 to 24.4) followed by IgM (6.4 mg/mL; range from 2.0 to 14.3), and IgA levels were lower (4.2 mg/mL; range from 1.4 to 8.8). Levels of total IgG in newborns highly correlated with their respective dams (Pearson $r=0.8390$, $P=0.0024$), suggesting most to all IgG in newborns was maternal in origin. Since minimal amounts of serum total IgM and IgA were detected in newborn macaques, despite high levels in their dams, this suggested that among the three classes of antibodies examined (IgG, IgA, and IgM), only IgG is transferred across the placenta to the fetus in macaques, which is consistent with previous reports in human infants [18, 19].

Distinct changes in maternal anti-TT IgG and serum total IgG with age in infant rhesus macaques

As shown in Table 1, all dams were vaccinated twice with TT, and 80% of dams had high levels of anti-TT IgG antibody. Similar to total IgG, both anti-TT IgG and anti-TT IgG1 showed significant correlations between dams and their respective newborn infants (Pearson $r=0.9665$, $P<0.0001$ for anti-TT IgG, and Pearson $r=0.8523$, $P=0.0017$ for anti-TT IgG1). Moreover, the data suggested different placental transport rates between anti-TT IgG and anti-TT IgG1 (averaged 83.9% vs averaged 46.7%), indicating preferential and selective transfer patterns may occur in IgG subclasses. Similar to serum total IgG, only anti-TT IgG but not anti-TT IgM antibodies were present in newborn sera (Data not shown).

To estimate the duration of maternally derived antibodies in infant macaques, we examined sequential changes in total serum IgG, and antigen specific antibodies (anti-TT IgG and IgG1) in infants with age, and found distinct changes in levels of these antibodies. As shown in Fig. 2A-B, both maternal anti-TT IgG and IgG1 declined rapidly within 1 month after birth, and continuously declined to undetectable levels by one month of age in most infants. In contrast, total serum IgG initially declined for 2-4 weeks, then gradually increased throughout the 6-month duration of the study, as levels of neonatal derived IgG increased (Fig. 2C).

Changes in total IgM and IgA in infants with age

Since all infants were formula-fed from birth in this study, and since levels of both serum IgM and IgA were undetectable at birth in newborns, IgM and IgA antibodies detected in infants after birth indicated these were autologously-derived antibodies. As shown as in Fig 3, high levels of both IgM and IgA were detected in older infants compared to newborns, and IgM levels rapidly increased and peaked within one month of age (Fig 3A). In contrast, IgA levels were barely detectable through 1 month of age, and gradually increased through the 6 months of study (Fig 3B). Interestingly however, we observed a sharp peak in IgM levels in all neonates by 2 weeks of birth (Fig 3A), probably due to the fact IgM is the first antibody generated in response to antigen stimulation.

Abnormally high levels of serum IgA and IgM correlate with infection or inflammation in tissues

Although we found increasing levels of IgM and IgA in blood of infants with age, infant levels at 6 months of age still remained much lower when comparing to their adult dams. Surprisingly however, we found two infants with particularly high IgM levels; one at 2 weeks and another at 1 month of age, as well as two with particularly high levels of IgA spiking at 1 month and 6 months of age (Fig 3). These abnormally high levels of IgM and IgA were all from three infants (KK21, KN99 and KJ38), none of whom showed any clinical signs of illness. However, histologic examination of tissues revealed focal lesions in all 3 infants. As shown in Fig 3, infant KN99 had moderate thymic atrophy (Fig 3C1) and mild, multifocal lymphoplasmacytic pancreatitis (Fig 3C2), suggestive of a viral infection. Infant KK21 had mild to moderate periportal hepatitis, characterized by infiltration of lymphocytes and plasma cells into portal triads (Fig 3D). Finally, infant KJ38 had focal suppurative inflammation in the ileocecal junction, suggestive of an acute bacterial infection (Fig 3E). No significant lesions were detected in tissues of the other neonates examined, suggesting the spikes in IgM and IgA were associated with specific disease processes. Moreover, these findings indicated neonates were able to generate humoral immune responses in the face of infections, even in very early neonatal life.

Discussion

These data in ten infant rhesus macaques are consistent with those reported in human infants, demonstrating that only IgG, and not IgM and IgA can cross the placenta, by showing formula-fed macaques are born with high levels of serum IgG, yet minimal to undetectable IgA and IgM at birth. Since all infants were hand reared, these antibodies were

not derived from breast milk. Finally, although levels of IgA and IgM were generally lower in infants, including those 6 months old, high levels of IgA and/or IgM in three infants show that neonates are capable of generating high levels of these antibodies under certain conditions (i.e., infections).

It has been well documented that maternal antibodies are transferred to infants via placental transport [22, 23]. Although maternal antibodies are important for protection of infants in the early months of life, evidence suggests they can also dampen or interfere with the infant's primary antibody response to infection or immunization [24-26]. The neonatal immune system has long been thought to be "immature" and unable to generate robust humoral immune responses, as antigen exposure in early life results in delayed or undetectable antibody responses to infections and immunizations [27-29]. However, some studies have demonstrated that adult-like B- and T-cell responses could be generated in early life under specific conditions of antigenic stimulation [30, 31], suggesting the humoral immune system of newborns is capable of mounting effective antibody responses. Since antibodies are the main mediators of humoral defenses, measurement of serum antibody levels generally reflects the ontogeny of the humoral immune response in early life. Here we show infant macaques demonstrate a rapid increase in serum IgM after birth, and a slow, yet progressive increase in IgA production, and both antibodies are derived from the infants themselves, evidenced by undetectable levels of either in sera on the day of birth. Surprisingly however, we observed an apparent peak in levels of IgM at 2 weeks of age (Fig 3A). Since IgM is the first antibody to appear in response to initial antigenic exposure, this suggests infants mount a marked, early humoral immune response to environmental antigens immediately after delivery from the sterile intra-uterine environment. In summary, these data provide evidence that from the moment of birth, the infant immune system is capable of responding to antigenic challenges by generating humoral responses.

We also showed, as in humans, that both total and vaccine induced, antigen specific IgG can be transferred from dams to their fetus in rhesus macaques, as demonstrated by the presence of high levels of both total IgG and TT-specific IgG at birth (Fig 1 and 2). Further, we showed that maternally derived TT-specific IgGs waned with age, and disappeared within 2-4 weeks (Fig. 2). Although total IgG levels declined at these points as well, total IgG levels were maintained and increased with age, presumably due to generation of infant derived IgG antibodies due to exposure to numerous environmental antigens since birth. Together, our data indicate infants are able to synthesize their own antibodies including IgM, IgG, as well as IgA in early life.

Despite differences in levels and production rates of IgG, IgM and IgA during early life, by 6 months of age, the highest levels of serum antibodies were IgG. Although IgM levels peaked first, mean IgA levels eventually exceeded those of IgM, by 3 to 6 months of age in serum (Fig. 3A). Further, levels in serum and the dynamics of changes in serum levels are consistent with those reported in studies of human infants. In humans, infant IgG and IgA concentrations show a gradual rise with age, yet IgM concentrations remain constant and distinctly lower than those of adults [32]. Although mean levels of all three antibody classes examined were lower in infant macaques than adults, levels in the three infants at 2 weeks of age were comparable to those of adults. Although we could not determine the specificity of

these antibodies, these findings suggest that neonates have the capacity to generate strong, adult-like antibody responses when faced with specific, yet clinically silent disease processes. Although the numbers of infants examined at each timepoint in this study were few (3-4 per group) the consistency in the development of IgM and IgA after birth in all infants, plus the correlation of maternal and infant antibody levels, and finally, detection of infant IgM/IgA spikes only in infants with disease processes, suggest these data are representative of normal immunologic development and responses.

Taken together, our data demonstrate that neonatal macaques have antibody levels and responses that parallel those of human infants, and the newborn immune system is competent, and able to respond to antigenic challenges by generating IgG, IgA, and IgM responses. Although maternal antibodies are somewhat variable in both their levels and rates of decay [22, 33], our data here suggest that proper immunization administered closer to the time of birth could theoretically generate effective and protective immune responses against many fatal diseases. Among number of biological and ethical factors that influence the administration or evaluation of vaccines administered in infants [34], optimal neonatal immunization including the timing of the first dose of vaccine at birth is not only feasible but also desirable, and the rhesus macaque appears optimal for testing new vaccination strategies in newborns.

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Highlights

- Essentially all serum IgG in newborn macaques is derived from the dams.
- IgG, IgA and IgM is differentially produced in neonatal macaques with age.
- Newborn immune system is competent to respond to foreign antigens and vaccines.
- Infant humoral responses may contribute to the clinically silent disease processes.

Figure 1.

Levels of total IgG, IgM, and IgA antibody levels in serum of adult dams (black bars) and their newborn infants (grey bars). Bars represent mean concentrations (mg/mL) of total antibody in serum from 10 dams and their paired newborns (n=10). Two-tailed paired t tests were used for comparison of total antibody levels between dams and newborns. Note that total IgG levels are similar between newborn infants and adult dams, whereas IgA or IgM levels are undetectable in newborns at birth. P values <0.05 were considered significant.

Figure 2. Changes in maternally-derived total IgG and antigen specific IgGs (anti-TT IgG and IgG1) in infant rhesus macaques with age. Note waning of maternal antigen specific IgGs (A and B) but not total IgG (C) suggests infant macaques are already producing their own IgGs within a few days or weeks of life.

Figure 3.

Dynamic of total IgM and total IgA with age in infant rhesus macaques and association of abnormally high levels of serum IgA and IgM with infection or inflammation in tissues of infant macaques. There are rapid increases in serum IgM spiking 2 weeks after birth (A), yet very slow increases in levels of IgA (B) from birth. Also note particularly high levels of total IgM and IgA in infants KK21, KN99 and KJ38. Histopathology of tissues collected at necropsy from these three infant macaques as shown as in C to E. Thymus of the 1-month old infant KN99 shows lymphocytes in the cortex are depleted and replaced by many tingible-body macrophages in the remaining, shrunken thymic lobules, indicative of thymic atrophy (C1), and his pancreas of KN99 showing atrophy of acinar cells and replacement with numerous infiltrates of lymphocytes and plasma cells (C2). Liver of the 2-

week old infant KK21 shows marked infiltration of portal triads by numerous lymphocytes and plasma cells. Adjacent hepatocytes were often pale swollen and vacuolated (degenerate) (<) or even necrotic (piecemeal necrosis) (arrow), indicative of portal hepatitis (D). Ileoceco-junction of KJ38 shows erosion of the mucosal epithelium with numerous degenerate neutrophils in the lumen and lamina propria consistent with acute suppurative enteritis (E). All photomicrographs were taken from H&E stained slides at an original magnification of 200X for C1, C2 and E, and 400X for D.

Table 1

Rhesus macaques used in this study and titers of serum anti-TT IgG at birth.

Dams (n=10)				Newborns (n=10)				Serum Tetanus IgG at birth (IU/mL) *	
Animal N.O.	D.O.B.	Date of TT Vaccination		Animal N.O.	SEX	D.O.B.	Date of Necropsy	Dam	Newborn
		Primary	Boost						
IH36	4/14/09	11/18/10	2/13/12	KK21	F	4/14/13	4/18/13	0.01	0.01
IM47	6/12/09	6/3/10	5/16/12	KK88	F	4/14/13	4/29//13	0.12	0.10
IM56	6/13/09	4/14/10	10/11/12	KL63	M	4/21/13	5/6/13	3.02	3.21
GJ15	5/5/06	9/7/06	4/3/12	KK11	F	4/2/13	5/1/13	0.83	0.73
HP50	4/22/08	7/15/08	1/19/12	KN86	F	5/10/13	6/12/13	0.99	1.08
HN66	4/16/08	7/15/08	2/28/12	KN99	M	5/12/13	6/13/13	0.31	0.26
FR93	5/5/05	11/10/05	5/15/12	KJ26	M	3/19/13	9/19/13	0.08	0.05
FF66	5/14/04	10/18/05	10/20/11	KJ32	M	3/20/13	9/23/13	1.47	0.41
HV60	5/28/08	8/12/08	10/2/11	KJ38	M	3/24/13	9/25/13	0.45	0.29
IG19	3/22/09	3/17/10	6/4/12	KJ39	F	3/24/13	9/25/13	0.28	0.06

* Interpretation of IU/mL: <0.1 suggesting basic immunization recommended.