Developmental Immunotoxicology Assessment of Rituximab in Cynomolgus Monkeys

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Rituximab is a chimeric murine/human-engineered immunoglobulin (Ig) G1 anti-CD20 monoclonal antibody, selectively depleting CD20-expressing cells in peripheral blood and lymphoid tissues. As part of the rituximab registration-enabling program for rheumatoid arthritis, cynomolgus monkey embryo-fetal development and pre- and postnatal developmental toxicity studies were performed. In both studies, female cynomolgus monkeys were administered rituximab iv at doses of 0/0, 15/20, 37.5/50, and 75/100 mg/kg (loading dose/study dose) from gestation day (GD) 20 to 50 for the embryo-fetal development study and GD 20 to postpartum (pp) day 28 for the pre- and postnatal study. In the embryo-fetal development study, although maternal dosing ended during the first trimester at GD 50, placental transfer of rituximab to fetuses was demonstrated at GD 100. Consequently, fetuses demonstrated B-cell depletion in lymphoid tissues at GD 100. Repletion of B cells was demonstrated in infants in a follow-up pre- and postnatal study following fetal and neonatal exposure. In the pre- and postnatal study, despite B-cell depletion, there was no significant functional consequence on the infant’s ability to mount T-cell–dependent antibody responses following vaccination or antigenic challenge. Overall, rituximab was well tolerated at maximum feasible doses up to 100 mg/kg in pregnant cynomolgus monkeys and their infants after exposure from the period of organogenesis throughout pregnancy, parturition, and postnatal development. Importantly, the preclinical data have been concordant with the clinical data in children for cases where rituximab was administered during pregnancy.

Key Words: cynomolgus monkey; anti-CD20; embryo-fetal development; pre- and postnatal development; developmental immunotoxicology.

Rituximab is a chimeric murine/human-engineered immunoglobulin (Ig) G1 anti-CD20 monoclonal antibody, which selectively depletes CD20-expressing cells in peripheral blood and lymphoid tissues (Fig. 1) (Reff et al., 1994). Originally approved for non-Hodgkins lymphoma, its use has been expanded to include rheumatoid arthritis, and clinical trials are underway for additional non-oncology indications. CD20 expression is stable and is not shed from the cell surface and does not internalize upon antibody binding. These characteristics allow for immune processes and induction of apoptosis, thereby making rituximab a successful treatment for B-cell–related diseases (Einfeld et al., 1988). CD20 is expressed at key stages in B-cell development, and although not expressed on precursor lymphoid stem cells or pro-B cells, CD20 is expressed on pre-B cells in bone marrow and on immature and mature B cells in blood and lymphoid tissues (Nadler et al., 1981). Additionally, although not expressed on normal plasma cells, plasma blasts and stimulated plasma cells may express CD20 at relatively lower expression levels (Treon et al., 2000). Targeting the CD20 antigen on B lymphocytes has been shown to be a relatively safe therapy (Maloney et al., 1997) hypothesized to be because of the absence of CD20 antigen expression on stem cells. Based on these mechanisms and clinical experience, rituximab is a targeted B-cell therapy that has not exhibited a permanent effect on the subsequent ability to produce normal B lymphocytes.

With the exception of infusion reactions associated with administration, rituximab has generally been well tolerated clinically with limited reported incidence of opportunistic infection or toxicity. This may be in part because of the body’s apparent ability to continue to mount immune responses during and after administration of rituximab (Bingham et al., 2009; Kolk et al., 2002; Oren et al., 2008; Vøllerskig et al., 2006). Specifically, existing antibody titers against mumps, rubella, varicella, tetanus toxoid (TTx), influenza, and pneumococcus remained stable over 6 months while exposed to rituximab (Bingham et al., 2009; Kolk et al., 2002; Oren et al., 2008; Vøllerskig et al., 2006). Additional evidence to support the limited impact on humoral immunity by rituximab includes the formation of neutralizing antibodies to immunogenic proteins despite the absence of circulating antibody-producing B cells (Frankel, 2004; Hassan et al., 2004).

Although clinical data exist regarding the effect of rituximab administration on the adult human immune system, an outstanding question regarding the pharmacologic action of...
rituximab is the impact of B-cell depletion on the developing immune system. This is of particular interest as a variety of immune organs are active during early development. These include key immune organs such as the thymus and lymph node as well as organs with partial immune functions (spleen, bone marrow, and skin) (Buse, 2005).

Consistent with clinical data, toxicology studies with rituximab have identified effects limited to the expected pharmacology of B-cell depletion in adult cynomolgus monkeys. These findings have not been identified as a significant toxicity to the immune system, partly because of the mechanism of action of rituximab and target cell population. To address the question regarding the impact of rituximab on the developing immune system, nonclinical reproductive toxicity studies in monkeys were designed to include an evaluation of the impact of B-cell depletion on the developing fetal and neonatal immune system as well as the ability of infants that were exposed to rituximab during gestation to mount an appropriate immune response after antigenic challenge or vaccination.

The developmental and reproductive toxicology program for rituximab was specifically designed based upon previous toxicology data, specific concerns with immunogenicity, and consideration of the mechanism of action of rituximab. The cynomolgus monkey was chosen as the most appropriate toxicology species based on the ability of rituximab to bind the CD20 antigen and effectively deplete B cells in this species (Vugmeyster et al., 2003). Rituximab exhibits a mean terminal elimination half-life of approximately 8 days in cynomolgus monkey. An extended dosing duration in the pre- and postnatal study was utilized to maintain exposures and determine reversibility of B-cell depletion observed in the embryo-fetal development study. The incidence of immunogenicity in previous general toxicology studies (~40%) as well as the extended dosing duration in the pre- and postnatal study were also taken into consideration by including a staggered dosing schedule into the pre- and postnatal study. Based on the mechanism of action of rituximab, the developmental and reproductive toxicology package to support clinical use in autoimmune indications included a robust developmental immunotoxicology assessment consisting of immunophenotyping, immunohistochemistry (IHC), microscopic evaluation of immune organs, and immune cell function analysis. Combined, the two studies encompass the critical period of immune system development as well as demonstrate the reportedly increased late gestational maternal IgG placental transfer of rituximab to the fetus (Fujimoto et al., 1983).

MATERIALS AND METHODS

Test Article

Rituximab contains the complementarity determining regions of the murine anti-CD20 antibody 2B8 in conjunction with human kappa and IgG1 heavy chain sequences.
chain constant region sequences (Reff et al., 1994). The vector was cloned into Chinese hamster ovarian cells as the production source of Ig (Reff et al., 1994). Rituximab is composed of two heavy chains of 451 amino acids and two light chains of 213 amino acids with a molecular weight of 145 kDa. The sequence homology of the CD20 receptor-binding site in human and cynomolgus monkey shares 100% amino acid identity. Rituximab (Lot no. N9182AX, 10 mg/ml) and rituximab vehicle (Lot no. M3-TOX53, 0 mg/ml) were supplied as clear liquids and were stored under refrigerated conditions.

Animal Care and Use, and Regulatory Compliance

Toxicology studies were conducted at Shin Nippon Biomedical Laboratories, Ltd in Kagoshima, Japan (embryo-fetal development study), and Covance Laboratories GmbH, Munster, Germany (pre- and postnatal development study); all animals were experimentally naive, purpose-bred cynomolgus monkeys (Macaca fascicularis) originating from China and Mauritius, respectively. Breeder males were of similar origin as females used for each study.

All study procedures were conducted according to a written study protocol and facility standard operating procedures in strict compliance with national legal regulations on animal welfare and accepted animal welfare standards.

Study Designs

The iv route of administration was based on the intended clinical route. The cynomolgus monkey was selected for these studies based on the ability of rituximab to bind to the CD20 antigen and effectively deplete B cells in this species (Vugmeyster et al., 2003). The dosing paradigm utilized loading doses (LD) for 3 consecutive days at study start to achieve steady-state exposures followed by weekly study doses (SD) to maintain exposures throughout the study duration. Doses for both studies were selected to achieve safety factors of approximately 1.5- to 10-fold the target clinical dose. The maximum feasible dosing volume of 5 ml/kg was used for both studies.

Embryo-fetal developmental study. Female cynomolgus monkeys showing a regular menstrual cycle (vaginal bleeding on two occasions separated by 20 days) were mated with males of established fertility for 3 days. This 3-day mating period took place between the 12th and 14th day of the menstrual cycle. During the mating period, coitus was confirmed visually or by the presence of sperm in a vaginal smear. The second day of the mating period was designated as gestation day (GD) 0. On GD 18, pregnancy was confirmed by ultrasound. Twelve pregnant females (dams) were assigned at random using a table of random numbers to study groups.

Pregnant female cynomolgus monkeys between the ages of 3–14 years old, weighing between 2.25 and 4.5 kg on GD 0 were administered rituximab iv at doses of 0, 15/20, and 75/100 (LD/SD, groups 1–3) from GD 20 until day 28 postpartum (pp). The rationale for dosing past parturition was to provide rituximab exposure to the newborn in an attempt to maintain drug levels throughout the lactation period. Figure 2 represents the dosing and sample collection paradigm for the study. Based on previous observations from general toxicology studies and the duration of the dosing period for the pre- and postnatal study (GD 20 until day 28 pp), a high incidence of ATA formation was anticipated for rituximab-treated animals. As the generation of ATAs is often accompanied by a decreased exposure to rituximab, a staggered dosing regimen was utilized for groups 4–7 to maintain trough concentrations of rituximab during critical fetal and neonatal development periods. Groups 4 and 5 were dosed from GD 76 to GD 134 and groups 6 and 7 were dosed from GD 132 to day 28 pp at rituximab doses of 15/20 (groups 4 and 6) and 75/100 (groups 5 and 7) mg/kg, respectively. The number of pregnant females assigned to groups 1–7 ranged from 12 to 17.

Experimental observations during the in-life period included clinical signs, monitoring of pregnancy by vaginal smears (daily from GD 20 until delivery) and ultrasound (from GD 30 until –GD 156), body weights, food consumption, hematology and chemistries, fluid cytometry (CD40+ immunophenotyping in dams and infants), PK, and ATA (dams, infants, and maternal milk). Maternal milk from the breast (at least 1 ml) was collected from full-term maternal animals on day 28 pp and stored at –80°C to –60°C until analysis. CD40 antigen was used for immunophenotyping based upon limitations with detecting CD20 antigen on B cells in the presence of rituximab. Additionally, CD40 antigen has a high correlation of expression with CD20 (Vugmeyster et al., 2005).

On day 1 pp, neonatal gender and general health were recorded. Weight and examination of external abnormalities occurred weekly until day 28 pp, followed by monthly intervals for up to approximately 7 months (study termination).

As part of the developmental immunotoxicology assessment of rituximab, neonatal T-cell–dependent antibody responses (TDAR) were measured. Keyhole limpet hemocyanin (KLH) and TTx were utilized as part of the TDAR assessment based upon the availability of well-validated methods at the testing facility (Grote-Wessels et al., 2010). Antigen challenge with class-specific Ig assessment was conducted to characterize rituximab-related effects on secondary Ig response or primary Ig response. To measure secondary IgG-specific responses, infants received 100 µg KLH in 0.2 ml of a 1:1 emulsion of sterile water for injection and Freund’s incomplete adjuvant on days 90 and 180 pp. Blood samples were collected for evaluation of anti-KLH IgG titers prior to antigenic challenge on days 90 and 180 pp and weekly from days 97 to 215 pp. To measure primary IgM and IgG responses, infants were immunized
with Daptacel (diphtheria, pertussis, and TTXs) on day 180 pp. Daptacel (0.5 ml) was injected im. Blood samples were collected for evaluation of both anti-TTX IgG and anti-TTX IgM titer prior to injection on day 180 pp and weekly from days 187 to 215 pp.

In addition to antigen challenge, T-cell function was evaluated as part of the developmental immunotoxicology assessment of rituximab. T-cell function was determined using an ex vivo proliferation assay utilizing isolated peripheral blood mononuclear cells (PBMC) from rituximab-exposed infants collected on days 90 and 180 pp. PBMC samples were stimulated for 76 h with nonspecific T-cell mitogens (i.e., interleukin 2, concanavallin A, and phytohemagglutinin). T-cell proliferation was quantified by 3H-thymidine incorporation.

In addition to the above analyses, in order to measure if existing B cells could generate IgM and IgG, blood samples were collected from infants on approximately days 89 and 179 pp for measurement of nonspecific total serum IgG and IgM. In order to determine the effect of B-cell depletion on tissues, terminal procedures were performed on all infants on day 217 ± 1. Timing of terminal procedures was based on repletion of peripheral blood B cells in infants as well as completion of antigenic challenge-related measurements. Terminal procedures included full external and internal examination, organ weights, histopathology, and IHC (for lymphoid markers using anti-CD2, CD3, CD4, CD8, and noncompeting CD20 antibodies).

Body weights, body weight gain, neonatal/fetal body weight, and infant organ weights were analyzed as parametric data using standard software package Statistical Analysis System (SAS release 8.2). Hematology data, clinical chemistry data, Ig data, and flow cytometry measurement data were analyzed as nonparametric data using standard software package SAS release 8.2.

RESULTS

Embryo-Fetal Development

Maternal findings. Administration of rituximab to pregnant cynomolgus monkeys during the critical period of organogenesis did not result in maternal death, adverse clinical signs, or changes in body weight or food consumption (data not shown). There were no noteworthy hematology or serum chemistry changes in maternal animals (data not shown). There was limited to no incidence of ATA formation in rituximab-treated dams. Rituximab exposure (area under the curve, AUC) in dams increased proportionally with dose (Fig. 3).

Fetal findings. There were no treatment-related embryonic deaths or abortions as compared with both control cohorts and the historical incidence for the testing facility. In fetuses obtained by cesarean section, there were no test article–related abnormalities in fetal external measurements, fetal body weight, and placental weight. Rituximab-related effects in fetuses were limited to expected pharmacology of B-cell depletion. There was a statistically significant decrease in absolute and relative spleen weights in the high-dose group only as compared with concurrent study control fetuses. Routine histologic evaluation revealed no splenic abnormalities associated with these decreased weights; however, IHC analysis revealed a dose-dependent decrease in CD20+ B cells as characterized by a decrease in the size and cellularity of lymphoid follicles of the spleen and lymph node (spleen, Fig. 4). In all fetal tissues evaluated for IHC with evidence of B-cell depletion, the degree of depletion was consistently more pronounced in sections of spleen relative to the lymph nodes.

Rituximab had no teratogenic effects on the developing fetus. There were no off-target drug-related visceral, skeletal, or histopathologic abnormalities or variations noted in any of the fetuses (data not shown).

PK analysis of day 100 serum samples revealed that fetal serum drug concentrations were approximately 35–74% of maternal concentrations at GD 100 (Table 1).

Pre- and Postnatal Study

Maternal findings. There were no treatment-related deaths or impact on pregnancy outcome, as measured by prenatal loss, when compared with both control cohorts and the normal ranges for the testing facility (Table 2). There were no drug-related maternal clinical observations, changes in maternal

FIG. 3. Mean rituximab serum concentration (µg/ml) versus time (GD) in dams treated at 20, 50, and 100 mg/kg (GD 20–50). Symbols represent mean observed concentrations, and lines represent a two-compartment model fit to the mean observed data.

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FIG. 4. Representative IHC sections of the spleen from fetuses in vehicle (panel A), 20 mg/kg rituximab (panel B), 50 mg/kg rituximab (panel C), and 100 mg/kg rituximab (panel D). Tissues were processed by standard techniques for IHC using mouse IgG2 anti-human CD20. Murine IgG2 was used as a negative control. Staining indicates CD20+ B cells.
body weight or body weight gain, hematology, or clinical chemistry (data not shown). Rituximab-related effects in dams were limited to expected pharmacology of B-cell depletion.

The incidence of ATA formation in dams did not increase in dosing cohorts with the longest rituximab dosing duration (groups 2 and 3; dosed from GD 20 to day 28 pp) as compared with groups with staggered dosing (groups 4–7) (Table 3). Antibodies were first detected on day 90 pp in group 2, day 180 pp in group 3, day 28 pp in group 4, day 90 pp in group 5, and day 180 pp in group 6. The presence of ATAs did not affect serum rituximab concentrations. Maternal B-cell depletion was comparable at all dose levels and dosing schedules. For these purposes, data from groups 1, 2, and 3 will be highlighted.

Findings in dams consisted of rituximab-related B-cell depletion as measured by immunophenotyping (CD40+ B cells). Maternal CD40+ B-cell depletion was similar among the different dose groups and dosing regimens. Repletion of B cells was defined as B cells reaching 25% of baseline levels. This number in peripheral blood corresponds to complete repletion of B cells in lymphoid organs. After reaching 25% of baseline, B-cell counts in peripheral blood rapidly rise to predose levels. CD40+ B cells were repleted to 25% of baseline levels by day 90 pp at 20 mg/kg rituximab and by day 180 pp at 100 mg/kg (Fig. 5). By day 180 pp, group 1, 2, and 3 had similar absolute CD40+ B-cell counts.

PK assessment of maternal rituximab concentrations revealed a dose proportional increase in $C_{\text{max}}$ and AUC. The PK and pharmacodynamic relationship of maternal exposure to rituximab and B-cell depletion at the highest dose tested in this study (100 mg/kg) is shown in Figure 6.

Rituximab levels in milk were low but tended to be dose dependent (Table 4). Detectable levels were present in 4/15 and 7/12 animals in groups 2 and 3, respectively. Rituximab levels in milk (expressed as percent serum) in animals with detectable milk concentrations were 0.19 and 0.26% for groups 2 and 3, respectively. Because of the limited lactational transfer of rituximab, dosing into the parturition period may not be needed.

ATAs to rituximab were detected in 2/15 and 1/12 maternal animals exposed to 20 and 100 mg/kg rituximab, respectively. The presence of circulating rituximab in serum samples can interfere with detection of an antibody response in the assay, which may have resulted in the limited incidence of ATA formation. When present, ATAs did not appear to affect serum concentrations or the B-cell depletion profile of rituximab. ATAs to rituximab were detected in milk in 0 of 5 group 2 animals and 0 of 6 group 3 animals.

**Infant findings.** There were no drug-related effects on clinical signs, external findings, gestation length, or parturition and no changes in body weight, hematology, or clinical chemistry in the infants (data not shown).

### TABLE 1
Comparison of Maternal and Fetal Rituximab Serum Concentrations at Cesarean Section (GD 100)

<table>
<thead>
<tr>
<th>Group</th>
<th>Rituximab (mg/kg)</th>
<th>Maternal serum (µg/ml)</th>
<th>Fetal serum (µg/ml)</th>
<th>% Maternal concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>20</td>
<td>8.25 ± 6.0 (1.20–20.2) n = 9</td>
<td>3.05 ± 2.4 (0.461–7.82) n = 9</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>7.51 ± 3.8 (1.27–13.9) n = 11</td>
<td>5.59 ± 3.6 (0.687–12.9) n = 11</td>
<td>74</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>18.5 ± 15 (1.29–48.6) n = 12</td>
<td>13.6 ± 9.7 (0.496–27.7) n = 11</td>
<td>74</td>
</tr>
</tbody>
</table>

*Note. Values represented as mean ± SD. Values in parentheses represent the range of concentration values.*

**TABLE 2**
Incidence of Prenatal Loss/Death

<table>
<thead>
<tr>
<th>Group</th>
<th>Pregnant</th>
<th>Incidence of dams with prenatal loss</th>
<th>Total live birth infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>15/20 mg/kg</td>
<td>14</td>
<td>6</td>
<td>43</td>
</tr>
<tr>
<td>75/100 mg/kg</td>
<td>12</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td>15/20 mg/kg</td>
<td>14</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>75/100 mg/kg</td>
<td>14</td>
<td>4</td>
<td>29</td>
</tr>
<tr>
<td>15/20 mg/kg</td>
<td>15</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>75/100 mg/kg</td>
<td>17</td>
<td>5</td>
<td>29</td>
</tr>
<tr>
<td>Historical control</td>
<td>13 ± 6 (6–24)</td>
<td>3 ± 2 (1–6)</td>
<td>22 ± 9 (7–33)</td>
</tr>
</tbody>
</table>

*aControl data from 13 studies at Contract Research Organization represented as mean ± SD.*

**TABLE 3**
Incidence of Maternal ATA

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of dams</th>
<th>Rituximab dose (mg/kg)</th>
<th>Dosing period</th>
<th>Maternal incidence of ATA (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>0</td>
<td>GD 20 to day 28 pp</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>20</td>
<td>GD 20 to day 28 pp</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>100</td>
<td>GD 20 to day 28 pp</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>20</td>
<td>GD 76 to GD 134</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>100</td>
<td>GD 76 to GD 134</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>50</td>
<td>GD 132 to day 28 pp</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>100</td>
<td>GD 132 to day 28 pp</td>
<td>0</td>
</tr>
</tbody>
</table>

*aVariation in number of dams per group because of abortions after dose administration or replacement of animals after study start because of false pregnancies.*
There was a marked depletion of peripheral blood CD40+ B cells as measured by flow cytometry in rituximab-exposed infants (Fig. 7). By day 180 pp, all infants demonstrated repletion of CD40+ B cells to counts that were similar to study control values.

Rituximab concentrations were generally higher in maternal serum as compared with infant serum as measured during the postparturition period (Table 5). The presence and concentration of rituximab in infant serum correlated with pharmacodynamic effect of peripheral blood B-cell depletion and subsequent repletion. The dosing regimens were appropriate to demonstrate placental transfer of IgG during development of the fetal immune system.

Antibodies to rituximab were detected in sera of infants from rituximab-treated dams in 0 of 5 in group 2, 0 of 7 in group 1, and 0 of 7 in group 3.
group 3, 1 of 8 in groups 4 and 5, 3 of 7 in group 6, and 3 of 9 in group 7.

In general, infant total serum IgM levels were not affected by maternal rituximab administration (Fig. 8A). Total serum IgG levels of infants from maternal rituximab-dosed groups were significantly decreased at day 89 pp compared with study controls but were comparable to study controls by day 179 pp (Fig. 8B).

In order to assess effects on immune tissue development, infant necropsies were conducted on approximately day 217 pp. At necropsy, there were no adverse effects of maternal rituximab treatment identified based on infant organ weights, gross, or histopathologic findings. There were no treatment-related findings from IHC evaluation in lymphoid tissues. By IHC, cells positive for T-cell markers (CD2, CD3, CD4, and CD8) were distributed in similar quantities and patterns in all infant tissues of all groups. CD20⁺ B cells in rituximab-exposed infants were distributed in quantities and patterns similar to the vehicle control group.

Infant immune function analysis. All rituximab-exposed infants were able to generate anti-KLH IgG antibodies by day 104 following primary administration of KLH (on day 90 pp) and to generate a memory response with a significant titer increase following secondary administration (on day 180 pp) (Fig. 9). However, there were moderate decreases in primary and secondary humoral responses to KLH challenge in rituximab-exposed neonates as compared with age-matched concurrent study controls; these differences were not statistically significant. In group 3 (100 mg/kg), 3 of 7 infants did not generate detectable levels of anti-KLH IgG antibodies after the first administration of KLH but showed clear response following secondary administration. Moderate decreases in primary and secondary humoral responses to KLH challenge in rituximab-exposed neonates may have been a result of initial administration of antigenic challenge at a time when neonates were B-cell depleted (day 90), which subsequently resulted in a muted secondary or memory response. A clear response following secondary administration correlates with repletion of B cells at the time when secondary challenge was administered (day 180).

All rituximab-exposed infants developed anti-TTx IgG responses following vaccination with Daptacel, with the onset of response similar to control infants. The anti-TTx IgM responses were generally low in all groups and did not reveal any effect of maternal treatment (data not shown).

There was no evidence of substantial immunosuppression or other immunotoxicity in offspring of rituximab-treated dams. T cells (using PBMC from rituximab-exposed infants) appeared to respond and function normally after stimulation with mitogens and cytokines (data not shown).

DISCUSSION

These studies examined the effects of rituximab, a chimeric monoclonal IgG1 antibody, on the developing fetus when administered during pregnancy, parturition, and lactation in cynomolgus monkeys. In general, rituximab at doses up to 100 mg/kg was well tolerated and did not elicit maternal toxicity, embryotoxicity, or teratogenicity when administered throughout the period of organogenesis in the cynomolgus monkey. Rituximab-related effects were limited to expected pharmacology of B-cell depletion in fetuses. B-cell depletion in fetuses correlated with exposure to rituximab. PK analysis of GD 100 serum samples showed maternal rituximab concentrations at the low dose were much higher than fetal concentrations. At the mid and high dose, rituximab concentrations were only slightly higher in maternal animals, demonstrating placental transfer of rituximab from GD 20 to GD 100. Administration of rituximab during gestation, parturition, and lactation did not produce overt maternal toxicity. With the exception of expected pharmacologic activity of B-cell depletion, there were no other drug-related effects observed on growth, development, or immune function in the F1 generation.

Pharmacologic activity related to rituximab was confirmed by depletion of B cells both in the peripheral blood (dams and infants) and lymphoid tissues (fetuses). Fetal B-cell depletion was characterized by IHC in the lymphoid tissues of the spleen and lymph node at GD 100. Importantly, B-cell depletion
observed in the embryo-fetal development study was a reversible event as demonstrated in the pre- and postnatal study where peripheral blood and lymphoid tissue B cells repleted by 6 months pp in infants.

Given the concordance of B-cell development in human and monkey immune systems, the cynomolgus monkey is a suitable model for reproductive/developmental toxicologic assessment for anti-CD20 therapies like rituximab. Immune system evaluation was not limited to rituximab-related effects on B cells but also included a thorough evaluation of infant immune function as measured by TDAR and T-cell proliferation assays measuring responsiveness to mitogens and cytokines. The studies were specifically designed to examine critical events in cynomolgus monkey immune system development to identify rituximab-related adverse effects as they may relate to humans. For example, in humans, B lymphocytes develop in the liver by week 9 of gestation and are present in the blood and spleen by week 12 (Hayward, 1983). In cynomolgus monkey fetuses, CD20⁺ B lymphocytes are present in the lymphoid organs and blood by 14 weeks (Buse, 2005).

### TABLE 5
Comparison of Maternal and Infant Rituximab Serum Concentrations on pp days 28, 90, and 180

<table>
<thead>
<tr>
<th></th>
<th>Maternal serum</th>
<th>Infant serum</th>
<th>% Maternal concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 28 pp</td>
<td>Day 90 pp</td>
<td>Day 180 pp</td>
</tr>
<tr>
<td>2</td>
<td>326 ± 78.7</td>
<td>11.4 ± 7.76</td>
<td>LTR</td>
</tr>
<tr>
<td>3</td>
<td>1520 ± 747</td>
<td>44.3 ± 16.7</td>
<td>2.15 ± 0.148</td>
</tr>
</tbody>
</table>

Note. LTR, less than reportable (< 1.00 μg/ml in maternal serum; < 0.250 μg/ml in infant serum); values represented as mean ± SD.

**FIG. 8.** Infant IgM (panel A) and IgG (panel B) levels in serum following exposure to maternal dosing of rituximab up to day 28 pp. Ig levels were measured in infants on day 89 and day 179 pp (n = 4–7 per time point per dosing cohort). Asterisk indicates statistically significant (p < 0.01) as compared with study controls.

**FIG. 9.** Primary (panel A) and secondary (panel B) anti-KLH infant immune responses after KLH challenge; infants were administered antigen challenge with KLH on day 90 and day 180 pp.
There was no significant functional consequence on the infant cynomolgus monkey immune system based on the retained ability to mount TDAR following vaccination or antigenic challenge in the pre- and postnatal study. Infants developed anti-Daptacel IgG responses following vaccination with onset of response in rituximab-treated animals similar to control infants. The timing of Daptacel challenge was at a time when infants had repleted B cells. Rituximab treatment of maternal animals during gestation did not affect humoral immune function in infants as measured by primary anti-TTx IgG and anti-TTx IgM response to Daptacel immunization. Similarly, nonspecific total serum IgG levels of infants from maternal rituximab-dosed groups were significantly decreased at day 89 pp but were comparable to study controls by day 179 pp. The initial decrease on day 89 and increase on day 179 in serum IgG levels in infants could be directly correlated with B-cell depletion and repletion in infants.

Infants were able to generate anti-KLH antibodies shortly after primary administration of KLH (day 90 pp) and to generate a booster response following the secondary administration (day 180 pp). Primary and secondary responses to KLH challenge were lower in $F_1$ animals from rituximab-dosed dams relative to study control responses; however, these differences were not statistically different. The decrease in the primary antibody responses in rituximab groups was likely due in part to the initial administration of antigens during B-cell depletion resulting in a suboptimal primary immune response subsequently resulting in a muted secondary anamnestic response. An important factor when designing infant immune function evaluation with therapies like rituximab is the consideration of drug exposures, toxicokinetics pertaining to drug half-life, and expected pharmacodynamic effects at the time of antigen challenge (i.e., administration of primary antigenic challenge when infants are not B-cell depleted to measure a true memory response). The absence of opportunistic infections in the rituximab-exposed infants may reflect a consequence of targeting CD20+ B cells because CD20 is, in general, not expressed on precursor lymphoid stem cells, pro-B cells, and plasma cells (Nadler et al., 1981). The expression pattern of CD20 on B cells allows for Ig production by plasma cells and the continued ability to mount immune responses during and after administration of rituximab.

Administration of rituximab during pregnancy either in the nonclinical setting or in the clinical setting demonstrates similar developmental and reproductive findings that are consistent with the expected pharmacologic action of rituximab. Maternal exposure to rituximab during the first, second, or third trimester of pregnancy has resulted in transient B-cell depletion in infants at birth (Decker et al., 2006; Friedrichs et al., 2006; Herold et al., 2001; Kimby et al., 2004; Klink et al., 2008). When mothers were exposed to rituximab during the first trimester of pregnancy, infants were B-cell depleted at birth with repletion of B cells occurring at 5 weeks after birth (Herold et al., 2001; Kimby et al., 2004). Similarly, infants of rituximab-treated mothers during the second or third trimester of pregnancy repleted B cells after 4–6 months (Klink et al., 2008). Immune responses in children inadvertently exposed to rituximab in utero have been assessed after vaccinations for tetanus, diphtheria, hepatitis B, measles, mumps, and rubella along with pertussis and influenza. Children showed normal immune responses at approximately 8–20 months after birth with no other observable adverse effects on the immune system (Decker et al., 2006; Friedrichs et al., 2006; Kimby et al., 2004; Klink et al., 2008). For these reasons, rituximab is considered to be well tolerated preclinically and clinically with limited reported incidence of opportunistic infection or related toxicities.

Based on the supporting toxicokinetics in the embryo-fetal development study, rituximab administration to maternal animals during GD 20–50 resulted in significant placental transfer of rituximab to the fetus prior to GD 100. At GD 100, fetuses were exposed to approximately 35–74% of maternal rituximab concentrations in utero. Based on published literature, placental transfer of endogenous IgG1 to the fetus in non-human primate and human occurs primarily during late gestation with maternofetal ratios ≤ 0.25 at GD 100 (Coe et al., 1993; Fujimoto et al., 1983; Malek et al., 1996; Pentus and Van der Laan, 2009). Our embryo-fetal development study demonstrated placental transfer of our IgG1 monoclonal antibody rituximab with fetal to maternal ratios in the 0.4–0.7 range at GD 100. Because of the paucity of data related to maternal transfer of endogenous IgG, these data help develop a better understanding of placental transfer of recombinant human monoclonal antibody therapeutics. Fetal exposures in the embryo-fetal development study were accompanied by concurrent pharmacodynamic effect of B-cell depletion in the lymphoid tissues. In the pre- and postnatal study, exposure to rituximab during the pp period was accompanied by peripheral blood B-cell depletion in infants up to day 90 pp (100 mg/kg group only). Repletion of peripheral blood B cells in 100 mg/kg group infants was similar to control by day 180 pp; B-cell repletion paralleled rituximab clearance.

There was a very limited incidence of ATA formation in the pre- and postnatal study in contrast to previous general toxicology studies with rituximab. When present, the limited incidence of ATAs (predominantly at the lower rituximab doses) may have in part been because of the dose-related B-cell depletion in lymphoid tissues in maternal animals or the general hyporesponsiveness associated with pregnancy (Stahn et al., 1978). Previous general toxicology studies with rituximab reveal an inverse dose relationship of ATA incidence and B-cell depletion, which may be the result of decreased antigenic responsiveness at higher rituximab doses, although drug interference in the ATA assay may also contribute to this observation.

Overall, rituximab was well tolerated at pharmacologically active doses up to 100 mg/kg in pregnant cynomolgus monkeys and their infants after exposure from the period of organogenesis throughout pregnancy, parturition, and postnatal
development. Toxicologic findings in fetuses and infants were consistent with primary pharmacology of B-cell depletion. Importantly, the preclinical data have been predictive of clinical data in children when rituximab is administered during pregnancy.

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