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Molecular and Functional Characterization of Cynomolgus Monkey IgG Subclasses

Frederick W. Jacobsen,*† Rupa Padaki,*† Arvia E. Morris,† Teri L. Aldrich,†,‡ Richard J. Armitage,‡ Martin J. Allen,†,‡ Jennifer C. Lavallee,* and Taruna Arora*

Studies for vaccine and human therapeutic Ab development in cynomolgus monkeys (cynos) are influenced by immune responses, with Ab responses playing a significant role in efficacy and immunogenicity. Understanding the nature of cyo humoral immune responses and characterizing the predominant cyo IgG types produced and the Fc–FcεR interactions could provide insight into the immunomodulatory effects of vaccines. Anti-drug Ab responses against human IgG therapeutic candidates in cynos may affect efficacy and safety assessments because of the formation of immune complexes. There is, however, limited information on the structure and function of cyo IgG subclasses and how they compare with human IgG subclasses in Fc-dependent effector functions. To analyze the functional nature of cyo IgG subclasses, we cloned four cyo IgG C regions by using their sequence similarity to other primate IgGs. The four clones, cyo (cy)IGG1, cyIGG2, cyIGG3, cyIGG4, were then used to construct chimeric Abs. The sequence features of cyo IgG subclasses were compared with those of rhesus monkey and human IgG. Our data show that rhesus monkey and cyo IgG C regions are generally highly conserved, with differences in the hinge and hinge-proximal CH2 regions. Fc-dependent effector functions of cyo IgG subclasses were assessed in vitro with a variety of binding and functional assays. Our findings demonstrate distinctive functional properties of cyo IgG subclasses. It is notable that human IgG1 was less potent than cyo IgG1 in cyo FcεR binding and effector functions, with the differences emphasizing the need to carefully interpret preclinical data obtained with human IgG1 therapeutics. The Journal of Immunology, 2011, 186: 000–000.

Animals serve as representative models in biomedical research involving target assessment and in studies of immune modulation. Their phylogenetic proximity to humans makes nonhuman primates, such as macaques (rhesus monkeys [rhesus] and cynomolgus monkeys [cynos]), preferred hosts in vaccine development, transplantation research (1), studies of viral diseases, and also for pharmacodynamics and pharmacokinetic and safety assessments of therapeutic candidate Abs. Ab responses in cynos play a key role in development of vaccine and Ab therapeutics, as anti-drug Ab responses can mask efficacy, affect pharmacokinetics, and influence toxicity profiles (2).

Ab responses are linked to cellular immune responses through the interaction of Ig Fc with FcRs (3). These interactions are well characterized for mouse and human Igs and their respective FcRs. In mice and humans, different subclasses of IgG are known to carry unique C region features that enable distinct interactions of the Fc region with various types of FcεRs (4–6).

The human IgG subclasses act through specific FcεRs on immune cells to mediate immune functions such as cell killing, complement activation, opsonization, and phagocytosis (4). Further studies have shown a contribution of IgG sequences from CH2 and CH3 regions in differing effector functions of human IgG subclasses (7, 8). However, limited information exists about human Fc interactions and functions in cyno species.

In addition to understanding the function of human IgG subclasses in cyno species, the role of the cyo immune response itself in vaccine studies and immunogenicity to therapeutic biologics also underscores the need to study sequence, structure, and functional nature of cyo IgG subclasses. Knowledge of the structure and function of cyo IgG constant domains can contribute to improved understanding of preclinical studies that use this model for drug development. Four IgG genes from the closely related rhesus have been cloned: RH1, RH2, RH3, and RH4 (http://www.ncbi.nlm.nih.gov/nuccore accession numbers AF045537, AF045539, AF045538, and AY292520.1, respectively) (9–12). Three rhesus IgG constant domain subclasses have also been characterized by using subclass-specific Abs, further demonstrating that there are at least three CH chain (HC) subclasses expressed in rhesus (12). Additional studies have revealed at least one IgG C region sequence for the cynos as well as a κ CH chain (LC) C region gene (GenBank number CS101580.1) (11).

The present study was designed to characterize cyo IgG C region genes and to describe their relationship to rhesus and human IgG sequences. The functional nature of cyo IgG subclasses was elucidated by engineering these C regions into whole Abs and assessing their physical properties and their ability to bind FcRs and induce effector functions.

Materials and Methods

Amplification and cloning of cyo IgG C region DNAs
Cyo IgG C regions were cloned by using PCR amplification of sequences derived from cyo cDNA or genomic DNA. Specifically, DNA isolated from an EBV-transformed cyo B cell line was used for the cloning of
cyto 2–4 (GenBank number CS101556.1) and cyto 33 (GenBank number CS101554.1). The cytocyto sequence cyto 3–16 was cloned from cDNA synthesized by using the Amersham Biosciences (Piscataway, NJ) first-strand cDNA synthesis kit and RNA from freshly isolated B cells from two different monkeys. RNA was isolated by using an RNasey kit (Qiagen, Valencia, CA). The cyto 686 IgG (GenBank number CS101564.1) was cloned from mixed cyto lymphoid tissue cDNA, and all four cyto C regions were confirmed to be present in this cDNA.

The pGEM-T Easy Vector was moved from the vector backbone of pDC414-N. The 2.1-kb pair EBV replication origin in pDC412. The expression vector pDC414-N, which is a modified version of pDC412 (13). PCR primers were used: 5′-IGGRH3′ following the manufacturer’s instructions and were sequenced. An initial CD20 anti-CD20 cyno DNA constructs that comprised the variable regions of an anti-human (hu) IGHG3 chain were cloned into the pGEM-T Easy Vector (Promega, Madison, WI) following the manufacturer’s instructions and were sequenced. An initial cyto IgG clone 2–4 was selected on the basis of its homology with rhesus IGHGRH3 (GenBank number AF045538). The initial cyto IgG2–4 C region was reamplified by PCR and was subsequently recloned. The following PCR primers were used: 5′-GCTAGCACAAGGGGATCCGTCTT-3′ and 5′-GGCCGCCGCTTACCTACCCGGAGACGAGGAG-3′. The 3′ primer included a Leu to Val mutation in the CH3 domain near the 3′ end of the C region. This mutation was introduced because other cloning efforts that used cyto templates and primers downstream of the stop codon produced clones that encoded the sequence SVSPGK instead of SLSPGK. Thus, the Leu was thought to be an artifact of the 3′ primer on the basis of a comparison with GenBank number AF045536. The initial cyno IgG2–4 C region was amplified by PCR. The primer was used to Val mutation was not introduced into the cyto 33 clone. Cyno 33 was cloned by using primers 5′-GTCACATGGCAACCACCTCT-3′ and 5′-GGTACGTGCAAGCAGTCTC-3′. These primers were designed on the basis of their homology to the human IGG sequences, and the 3′ primer is located 3′ of the stop codon.

To clone the cyto 686 IGG, we prepared a cdNA library from RNA isolated from mixed cyto lymphoid tissues. PCR was carried out with the human IGG C region primers 5′-GCTAGCACAAGGGGATCCGTCTT-3′ and 5′-GGCCGCCGCTTACCTACCCGGAGACGAGGAG-3′. The 3′ primer included a Leu to Val mutation in the CH3 domain near the 3′ end of the C region. This mutation was introduced because other cloning efforts that used cyto templates and primers downstream of the stop codon produced clones that encoded the sequence SVSPGK instead of SLSPGK. Thus, the Leu was thought to be an artifact of the 3′ primer on the basis of a comparison with GenBank number AF045536. The initial cyno IgG2–4 C region was amplified by PCR. The primer was used to Val mutation was not introduced into the cyto 33 clone. Cyno 33 was cloned by using primers 5′-GTCACATGGCAACCACCTCT-3′ and 5′-GGTACGTGCAAGCAGTCTC-3′. These primers were designed on the basis of their homology to the human IGG sequences, and the 3′ primer is located 3′ of the stop codon.

The cyto (c)IgG4cys (GenBank number CS101558.1) constant domain was constructed by PCR site-directed mutagenesis of the final cyto 2–4 sequence; a QuiChange site-directed mutagenesis kit (Stratagene) was used. The fourth Ser in the CH1 domain was converted to a Cys by in vitro site-directed mutagenesis with the PCRII TOPO TA cloning system (K4600-01SC; Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions.

**Sequencing and analysis of cyto IGG DNA sequences**

Individual cloned DNAs were sequenced by using Applied Biosystems DNA sequencing instruments (PerkinElmer, Waltham, MA); and consensus sequences were determined. Sequences were aligned with human and rhesus IgG peptides by using the ClustalW function of the AlignX program in the Vector NTI analysis software (Invitrogen).

**Construction of anti-CD20 cyto IGG expression vectors**

By using standard cloning methods, we made full-length IGHG and IGG DNA constructs that comprised the variable regions of an anti-human (hu) CD20 mAb and the cyto IGG C regions described above. DNA fragments encoding full-length IGHG and IGG chains were cloned into the transient expression vector pDC414-N, which is a modified version of pDC412 (13). This vector contains a 120-bp EBV replication origin (14) in place of the 2.1-kb pair EBV replication origin in pDC412. The NheI site is also removed from the vector backbone of pDC414-N.

**Transient expression**

A Plasmid Mega kit (Qiagen) was used to prepare DNA for transfection. The anti-CD20 cyto IGG expression vectors containing HC and LC were cotransfected into COS-1 cells (ATCC CRL 1650). On the day of the transfection, a DNA/DEAE-dextran mixture was prepared and incubated with the cells in culture for 4.5 h at 37˚C and 5% CO2 in the presence of chloroquine. The cells were then exposed to a 10% DMSO solution for 5 min. Low serum-containing medium (0.5% low IgG) was then added to the cultured cells. The cells were incubated at 34˚C for 7 d, at which point the conditioned medium was harvested for purification.

**Gel analysis**

A protein A resin column was used to purify Abs from cell supernatants. Purified Abs were then analyzed under denaturing nonreducing conditions on an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA) by using the protein 200 assay according to the manufacturer’s instructions.

**Reagent Abs**

Anti–CD3-PE (clone SP34-2) and anti–CD14-PE (clone M5E2) were obtained from BD Biosciences (San Jose, CA) and used at 20 μl per test. Anti–CD19-allophycocyanin (clone J3-119) was obtained from Beckman Coulter (Fullerton, CA) and used at 10 μl per test. Anti-human κ LC-FITC (affinity-isolated Ab) was obtained from Sigma-Aldrich (Saint Louis, MO) and was used at 20 μl per test. As described earlier, anti-CD20 cyto IgG chimeric Abs and anti-CD20 human IgG Abs were constructed and purified in-house.

**Binding to antigenic targets on human B cells**

Raji cells (ATCC CCL-86) were plated at 5 × 105 cells/well in flow buffer in each well of a 96-well plate and were incubated for 1 h at 4˚C with anti-CD20 cyto Abs or anti-CD20 human Abs. Following incubation, the cells were washed and resuspended in 100 μl buffer. Anti–human κ LC-FITC (Sigma-Aldrich, catalog number F3761) secondary Ab was added and incubated for 40 min at 4˚C. The cells were washed and fixed in 2% formalin. B cell depletion was quantified by flow cytometry. The fourth Ser in the CH1 domain was converted to a Cys by in vitro site-directed mutagenesis with the PCRII TOPO TA cloning system (K4600-01SC; Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions.

**Ab binding analysis to cyto monocytes**

Heparinized cyto blood was received from Bioreclamation (Westbury, NY). Ammonium chloride lysis buffer was used to lyse RBCs. Cyno leucocytes were incubated with anti-CD20 cyto Abs and anti-CD20 human Abs. After washing, the cells were resuspended in 100 μl flow buffer. A 30 μl Ab mixture containing anti–CD14-PE, anti–CD19- allophycocyanin, and anti-human κ LC-FITC (Sigma-Aldrich) Ab was then added to the cells and incubated for 40 min at 4˚C. The cells were then washed and fixed. Fluorescence emission in duplicate wells was analyzed via flow cytometry (FACSCalibur; BD Biosciences). We used GraphPad Prism software (GraphPad Software, La Jolla, CA) to plot the binding curve of the mean fluorescence index (MFI).

**Whole blood matrix B cell depletion assay**

Heparinized blood of four juvenile cynos (age, 2–4 y) was received from Bioreclamation at room temperature via overnight shipping. Triplicate samples of cyto whole blood were treated with PBS or 2 μg/ml various anti-CD20 cyto Abs and anti-CD20 human Abs for 1 h. Following treatment, 100 μl blood was stained with anti-CD19-allophycocyanin and anti-CD3-Pe mixture for 15 min at room temperature. RBCs were lysed with ammonium chloride lysis buffer, and cells were then washed and fixed with 2% formalin. B cell depletion was quantified by flow cytometry. The percentage of B cell depletion was determined with the following equation: [1 – (% CD19+ cells in IgG-treated samples/% CD19+ cells in PBS-treated samples)] × 100.

**Complement-dependent cytotoxicity assays**

A total of 2.5 × 104 human B cells (Raji) were incubated in the presence of different concentrations of anti-CD20 Abs for 1 h at 4˚C. The cells and Abs were then incubated in the presence of complement-preserved cyno serum (Bioreclamation) at a final concentration of 20% for 2 h at 37˚C. Material was frozen immediately after collection and shipped on dry ice to preserve complement. The degree of cell death was determined by analysis of propidium iodide uptake (Invitrogen) by flow cytometry. Experiments were performed in duplicate.
Sequence characterization: four classes of cyno IGG identified

By using primers designed to identify homologs to the human and rhesus IGHG C regions, we identified what we believe are four distinct cyno IGHG C region sequences: cyno 3–16, cyno 33, cyno 686, and cyno 2–4. All four C regions were cloned using cDNA as the template, and cyno 33 and cyno 2–4 were also identified using genomic DNA as the template. The sequences were designated cyIgG1 (cyno 3–16), cyIgG2 (cyno 33), cyIgG3 (cyno 686), and cyIgG4 (cyno 2–4) on the basis of their homology to the hinge region of the previously reported rhesus sequence and human sequences (Fig. 1B, Table I) (9, 12).

Comparison of cyno, rhesus, and human C region domains

Sequence alignment between human, rhesus, and cyno IgG C region sequences indicates that there are more differences in human and macaque than between the two macaque species (Fig. 1). Cyno and rhesus IGHG C region sequences are highly conserved. The rhesus and cyno CH1 domains are identical for all the isotypes except IgG3. Cyno has an Ala to Val change at position 12, and

![FIGURE 1](http://www.jimmunol.org/DownloadedFrom)

A. Alignment of human, cyno, and rhesus CH1 domains. Note that the presumed first residues of the rhesus CH1 sequences have not been reported. B. Alignment of human, cyno, and rhesus hinge domains. C. Alignment of human, cyno, and rhesus CH2 domains. D. Alignment of human, cyno, and rhesus CH3 domains. Note that the presumed last nine residues of the rhesus CH3 sequences have not been reported.
rhesus has a Glu to Gln change relative to the cyno at position 20 (Fig. 1A). Of note, the IgG4 isotype sequence in the rhesus and cyno CH1 both have a Ser at position 13 (Fig. 1A), whereas human IgG4 CH1 has a Cys at this position. In human IgG4 the Cys at this position forms a bridge with a Cys in the LC to form covalently bonded Ab molecules. The macaque IgG4 appears to lack the ability to form covalently bonded HC to LC species (Fig. 1B).

The hinge regions for the cyno IgG1–4 were identical to their rhesus counterparts. Upper hinge region sequences of cyno and human IgG subclasses contain the most differences.

As in humans, the lengths of the cyIgG1 and cyIgG3 upper hinge regions are similar and longer than the upper hinges of huIgG2/4 and cyIgG2/4. There is a Pro in cyo and rhesus IgG3/4 at position 16 that is not found in human IgG1 (Fig. 1B). The cyo and rhesus IgG4 have a conserved core hinge sequence of CysProProCysPro, while human IgG4 has CysProSerCysPro as the core hinge sequence (Fig. 1B, Table 1).

The CH2 domain is also well conserved between rhesus and cyno IgG subclasses: changes in IgG3 include Leu to Val at position 12 and Thr (cyo), Arg (rhesus), and Glu (human) in IgG3 CH2 at position 64 (Fig. 1C). Cyno IgG3 CH2 also has a Glu to Gln difference at position 103 and an Ala to Asp difference at position 109 relative to rhesus. The macaque IgG4 CH2 domains differ at position 56: rhesus has a His and cyno has an Asn (Fig. 1C). In human IgG, the CH2 domain has several amino acids known to interact with FcγRs (8, 15). When compared with huIgG CH2, analysis of cyno CH2 shows a deletion of the Pro from AlaPro-GluLeuLeu (position 2, Fig. 1C) in cyIgG2, and the Leu is changed to Val in cyIgG3. The CH2 domain N-glycosylation site there are two amino acids, GlnTyr, reported to play a role in FcγRIII binding in huIgG3 (15). The cyIgG1 and cyIgG3 share the GlnTyr of huIgG1, whereas the cyIgG2 and cyIgG4 have the GlnPhe that is found in huIgG2/4 (position 66, Fig. 1C).

The CH3 domains of the IgG3 isotype show differences across all three species (Fig. 1D). This region has the greatest divergence between cyo and rhesus IgG, with seven amino acid differences at positions 10, 14, 15, 30, 38, 44, and 82. The CH3 domain of the IgG4 isotype also showed differences between the two macaque species, with six amino acid differences at positions 65, 71, 73, 79, 82, and 84. A similar level of sequence divergence was observed between human and the two macaque IgG4 CH3 regions. The 3′ sequences for rhesus are not shown in Fig. 1 since the primers used for PCR precluded direct cloning of those sequences.

Despite the differences between the human and macaque sequences, many of the amino acids that have structural or functional significance have been conserved between macaques and humans. For example, the N-glycosylation site at position 67 in CH2 (Fig. 1C) is conserved. This site is an absolute requirement for FcγR interactions (16). Moreover, the GluLeuGlyGly motif in the CH2 domain (positions 3–7, Fig. 1C), also associated with effector function in humans (7, 17, 18), is conserved in the macaques and is invariant between macaque isotypes. All the Cys residues are responsible for intrachain disulfide bonding: CH1 positions 14 and 83, CH2 positions 31 and 83, CH3 positions 27 and 85 have been conserved.

CynoK is the κ sequence cloned in this study. Fig. 2 compares cynoK with a previously reported cyno κ sequence (L13317), as well as with the human κ sequence. The two cyno κ sequences differ at two positions, that is, Ala or Val at position 46 and Asp or Gly at position 50 (Fig. 2). These amino acid changes may represent allotypic differences.

**Predicted disulfide bonding for cyo HC and κ LC and assembly into multimeric Abs**

In Fig. 3A, the amino acid sequence for CH1 and the hinge for huIgG1/2/4 and cyIgG4 are aligned, with the Cys residues predicted for disulfide bonds shown in a red font. The intrachain HC disulfide bonds in the CH1 domain are aligned across isotypes,

<table>
<thead>
<tr>
<th>Table 1. Derived amino acid sequences of the upper, core, and middle hinge of IgG molecules from human, cyno, and rhesus</th>
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<tbody>
<tr>
<td>Upper Hinge</td>
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<tr>
<td><strong>Hu-IgG1</strong></td>
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<tr>
<td><strong>Cyno-IgG1</strong></td>
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<tr>
<td><strong>Rhesus-IgG1</strong></td>
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<tr>
<td><strong>Hu-IgG2</strong></td>
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<tr>
<td><strong>Cyno-IgG2</strong></td>
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<tr>
<td><strong>Rhesus-IgG2</strong></td>
</tr>
<tr>
<td><strong>Hu-IgG3</strong></td>
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<tr>
<td><strong>Cyno-IgG3</strong></td>
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<tr>
<td><strong>Rhesus-IgG3</strong></td>
</tr>
<tr>
<td><strong>Hu-IgG4</strong></td>
</tr>
<tr>
<td><strong>Cyno-IgG4</strong></td>
</tr>
<tr>
<td><strong>Rhesus-IgG4</strong></td>
</tr>
</tbody>
</table>

*Human IgG3 may exhibit two to five repetitions in the hinge, with four shown in this example.*
and species and are indicated by the bar above the sequences. The predicted inter-HC/LC disulfide bonds for huIgG1/2/4 are indicated by the “to LC” above the Cys predicted for that bond. The inter-HC Cys residues for bonding in the hinge region are indicated by “Hinge S-S.” The intra- and interchain disulfide bonds are shown in line drawings above the sequences for the huIgGs. The cyIgG4 sequence has Cys residues predicted for inter-HC and intra-HC bonding, but no Cys residues for inter-HC/LC bonding. This finding indicates that cyIgG4 cannot form a covalent bond between HC and LC via HC Cys (residue 14, Fig. 1A, EU 131) and Cys at the C terminus of the LC C region. To enable inter-HC/LC bonding in cyIgG4, a single amino acid change was introduced into the cyIgG4 to mutate the Ser to a Cys at the position. This engineered molecule was named cyIgG4cys.

The huIgG Ab (huIgG1/2/3) is predominantly a tetrameric protein consisting of two HCs and two LCs. To produce cyo IgG C regions as full-length Abs for functional assessment and further understanding of their multimeric state, the variable heavy region from an anti-CD20 Ab was attached to each of the cyo HC C regions or to the human IgG1, IgG2, or IgG4 C regions and was cloned into a transient expression vector by using standard methods. An LC was constructed by attaching the anti-CD20 variable light region to the cyo or human κ C regions. These constructs were transiently expressed, protein A purified, and analyzed on denaturing nonreduced gels to investigate the multimeric state of the expressed recombinant Abs.

As expected, when investigated on nonreduced gels, the huIgG1-, cyIgG1-, and cyIgG2-containing Abs formed tetrameric Ab molecules consisting of 2HC and 2LC running as ~150 kDa species (Fig. 3A).
3B, lanes 1, 3, and 4, respectively). Also as expected, the huIgG4 molecule (Fig. 3B, lane 2) formed two covalent species of ~150 and 75 kDa. HuIgG4 is known to have a propensity to form two half molecules consisting of a tetramer that contains 2HC and 2LC (150 kDa) and dimers that contain one HC and one LC (75 kDa) (19). As predicted from the sequence data, the cyIgG4 did not form tetramers but did appear to form dimers of either HC-HC (100 kDa) or LC-LC (50 kDa), as shown in Fig. 3B, lane 5. The cyIgG4 cyst mutant appeared to have improved HC-HC binding relative to wild-type cyIgG4, and the mutant also formed dimers (75 kDa) similar to huIgG4 (Fig. 3B, lane 6).

**Binding of anti-CD20 Abs to human B cells**

Anti-CD20 Abs engineered with cyno C regions were examined for binding to human B cells (Raji) to determine whether they retained parental specificity after conversion to whole Abs. The cyno C region-containing Abs along with the different isotypes of human C region Abs were used in this binding assay at a final concentration of 0–1.0 \( \mu \text{M} \) to determine the differences in binding of the various anti-CD20 IgGs. We observed that anti-CD20 huIgG1 and huIgG4 and the four different anti-CD20 cyno C region-engineered Abs exhibit similar binding properties to human B cells, demonstrating that the chimerization did not alter parental specificity of these Abs (data not shown). Anti-CD20 huIgG2 showed slightly less binding to B cells when compared with the rest of the Abs. The EC\(_{50}\) was determined for each Ab, and they fell within the range of 0.1–0.3 \( \mu \text{M} \) except for anti-CD20 huIgG2, which had an EC\(_{50}\) of 0.8 \( \mu \text{M} \) (data not shown).

**Cyco chimeric Abs bind differentially to cyco monocytes**

FcyRs play an important role in binding Fc and in mediating Fc-dependent effector functions of IgG subclasses (4–6). To determine the effect of cyno FcyR binding to the Fc portion of chimeric cyno Abs, a monocyte FcyR binding assay was performed with cyno PBLs. Cells were gated for the CD14\(^+\) population (monocytes), and binding differences were evaluated between the cyno IgG subclasses. Anti-CD20 Abs with cyno IgG3 and cyno IgG1 C regions showed the greatest binding to cyno monocytes, while all four human anti-CD20 Abs and anti-CD20 cyno IgG2 Ab showed 4- to 6-fold lower MFI of binding, and anti-CD20 cyno IgG4 Abs completely lacked binding (Fig. 4). Similar observations for cyno monocyte binding were made for another Ab engineered as a human-cyno chimera (data not shown). Investigation of the type of FcyRs engaged by cyno IgG Fc is ongoing.

**Susceptibility to anti-CD20 depletion of cyo B cells in vitro**

The cyno and human anti-CD20 Abs were investigated for their ability to mediate Fc effector functions by performing B cell depletion assays in whole blood matrix isolated from four individual cynos. The percentage of B cell depletion was calculated as described in Materials and Methods. Anti-CD20 Abs containing the cyIgG3 and cyIgG1 C regions induced 70–100% B cell depletion in all four blood samples. Depletion mediated by anti-CD20 human IgG1 ranged from 20 to 85%, while the other human and cyno isotypes showed negligible effector function (Fig. 5).

**Effect of anti-CD20 Abs with cyno C regions on complement-dependent cytotoxicity**

Ab-mediated cell cytotoxicity can also occur by activation of the complement pathway via binding of C1q to Ag-bound Ab complexes. To assess the degree of complement-induced cell death associated with these Abs, the human B cell line Raji was incubated with Abs and cyno serum. In the presence of cyno serum, cell death associated with complement-dependent cytotoxicity (CDC) was observed in a dose-dependent manner with anti-CD20 human IgG1, anti-CD20 cyIgG1, anti-CD20 cyIgG3, and anti-CD20 cyno IgG4 (Fig. 6). In contrast, other mAbs were not as effective at inducing CDC. In this assay, no Ab-dependent cell death was observed when Raji cells were incubated with cyno serum that was depleted of complement component C5 or in complement that was heat-inactivated (data not shown), confirming that cell death occurred via CDC. A summary of all effector functions is found in Table II.

**Discussion**

In this study, our primary objective was to gain insight into sequence and functional relationships between cyno and human IgG subclasses. Our aim was to guide and enhance understanding of preclinical studies of efficacy and safety in cyno species for development of vaccines or human Fc-based therapeutics. We have described the cloning of four cyno C regions and examined sequence homology to C regions of other nonhuman primate and human IgG subclasses. With respect to C region sequences, our data indicate that cyno and rhesus show greater homology than do cyno and human. The estimated homology between the macaques was ~95–99%. It is noteworthy that the actual homology may be even higher since the observed amino acid changes may represent allotypic differences between the compared species isotypes. As seen in humans, these differences may be geographic in origin (20). Our data also show that, as with rhesus, four cyno C regions were clearly distinguishable by what we consider the distinct sequence of the hinge regions. On the basis of identity between cyno and rhesus IgGs, we classified four C region sequences representing four cyno IgG subclasses: cyIgG1, cyIgG2, cyIgG3, and cyIgG4. Additionally, sequencing of CA-primed cyno cDNA clones identified three distinct clones encoding peptides with >90% homology to rhesus Ca peptides. An IGL cDNA library was not generated, and thus a \( \alpha / \gamma \) mRNA ratio was not determined.

To understand the significance of the differences among cyno IgG sequences, we used knowledge of the biological mechanisms of B cell depletion by anti-CD20 mAbs in designing chimeric molecules consisting of anti-CD20 Ab variable domains and cyno constant domains. These chimeras were used to assess the effector functions of cyno IgG subclasses by in vitro B cell depletion assays and CDC assays. Our data show that cyno IgG1 and IgG3 subclasses have the most potent effector functions of those we studied. Cyno IgG2 displayed levels of activity in effector functions that were lower and more variable than those of IgG1 and IgG3. The isotype with the least potent effector functions of those we studied...
was cyno IgG4. One could conceive that engineering of the anti-CD20 V region on a different IgG may affect CD20 binding, which in turn may affect B cell depletion by the chimeric Abs. We have confirmed that all four chimeric Abs bind equivalently to CD20 on Daudi cells and primary cyno B cells (data not shown). Previously, four C regions for rhesus species were cloned but their functions were not studied. To our knowledge, this is the first report of a study demonstrating that there are four functional subclasses of cyno IgG.

The observations regarding Fc-dependent functions of the cyno IgG subclasses are somewhat different from those of the human IgG subclasses. When examined for its ability to induce Fc-dependent effector functions in cyno whole blood or FcγR binding on cyno monocytes, human IgG1 was less potent than cyno IgG1 and cyno IgG3 in these assays. This distinction between human and cyno IgG1 in cyno effector functions reinforces the need to understand Fc–FcγR interactions for interpretation of preclinical studies evaluating human IgG1-based therapeutics. We also noted distinctions between human and cyno IgG4 effector functions. In humans, the IgG4 immune complex is known to bind human FcγRs (21), which means that this subclass potentially has effector functions. In contrast, huIgG4 had negligible binding to cyno monocytes as either monomers or immune complexes (data not shown) and did not induce any B cell depletion. This discrepancy of huIgG4–Fc interactions in human versus cyno may also partly explain the lack of adverse events observed with anti-CD28 huIgG4 in cynos, which contrasts with the life-threatening events seen in humans (22). Differences in these effector functions may be accounted for by sequence features of cyno IgG Fc that distinguish the cyno IgG class from human IgG and may influence interactions of Fc with cyno FcγRs. These data further emphasize the need for careful study designs, increased knowledge of cyno Fc–cyno FcγR interactions, and improved understanding of data with huIgG-based therapeutics that are tested in animals.

Indeed, our data clearly demonstrate that differential binding of IgG subclasses to cyno FcγRs (as determined indirectly by binding to monocytes) correlates with an ability to mediate B cell depletion. Notably, results of a cyno complement-dependent cyto-

FIGURE 5. Ability of anti-CD20 constructs to mediate B cell depletion in cyno whole blood. Four cyno whole blood samples were treated with different anti-CD20 Abs for 1 h at 37°C. The percentage of B cell depletion was calculated as described in Materials and Methods. Data show that cyIgG1 and cyIgG3 anti-CD20 Abs are most efficacious in mediating effector functions in cyno whole blood matrix, followed by huIgG1 anti-CD20 Ab.

![Figure 5](http://www.jimmunol.org/Downloadedfrom)

FIGURE 6. Complement-dependent cell cytotoxicity was measured for these Abs by incubating Raji cells in cyno serum containing complement, and cell death was calculated as the percentage of propidium iodide uptake. CDC activity was measured by flow cytometry.

![Figure 6](http://www.jimmunol.org/Downloadedfrom)
toxicity assay ranked cyIgG1 and huIgG1 as the most potent followed by huIgG4 and then cyIgG3. This discrepancy in CDC and B cell depletion implies a partial, subdominant contribution of the cyno complement system to both cyno and human IgG effector functions in cyno species.

In contrast with the abundance of information on human FcγRs, the information on the distribution, allelic polymorphism, and functional role of cyno FcγRs is scarce. For example, in human NK cells, FcγRIIIa or CD16 is the only known FcγR that mediates Ab-dependent cell-mediated cytotoxicity (ADCC) (23–26). The huIgG1 and huIgG3 show the highest affinity for CD16, which correlates with ability to mediate ADCC; varying affinity for human FcγRIIa polymorphic variants (at position 158) determines ADCC potency of huIgG1 (24, 27, 28). We are investigating whether differences in cyo CD16 binding by the four types of cyno Fc are associated with differential effector function activity. Future studies with recombinantly expressed cyno FcRs can help decipher FcγR interactions with various cyno IgG subclasses. At this time, our results suggest a weaker binding affinity of human IgG1 to the monocytes. Recently, Rogers et al. (29) cloned cyno FcγRIIIa (CD16), which shows 91% homology to human CD16 and lacks polymorphism at position 158. Of note, cyno CD16 has Iso158 instead of the Phe158 or Val158 present in huCD16, further supporting the notion of a dissimilar nature of FcγRs.

We found that sequence divergence of cyno and human IgGs was concentrated mostly in the upper hinge region where most C region sequences (cyo IgG1, IgG2, and IgG3) have additional Pro residues that are not present in the human upper hinge region. It is possible that the prolines allow for increased bend or flexibility of the V region arms or that they can also help stabilize a conformation (31). The core hinge sequence CysProProCysPro was conserved among all four cyno IgGs. In contrast, this feature is not conserved in all human IgG isotypes, as huIgG2 has extra Cys residues in the core hinge, and huIgG3 has a longer core hinge that is caused by exon duplication (32, 33). The presence of extra Cys residues in the huIgG2 core hinge is thought to contribute to varying disulfide isoforms in IgG2 preparations (32, 34). The hypothesis of whether the absence of extra Cys residues in cyno IgG may avoid disulfide shuffling is currently under investigation. Also, cyno and rhesus IgG4 do not have covalently bonded HC and LC because of a Cys to Ser change at EU position 131 (residue 14, Fig. 1A) in the CH1 domain of the monkey sequences, raising the question of whether cyno and rhesus IgG4 may exchange arms with other circulating IgG4. Such exchange can be envisioned to occur either for half molecules or even for the LC itself. We observed half-molecule formation in the cyno IgG4 Ab (cyIgG4cys) despite the presence of a core hinge sequence, CysProProCysPro, suggesting a role of other regions of the cyno IgG4 C region in providing stability for Fe-Fc dimerization. As noted by van der Neut Kolfschoten et al. (35) in regard to the role of the CH3 domain in dynamic Fab arm exchange of human IgG4, our data also point to a contribution of residues from other domains of C regions in influencing the inherent instability of the cyno IgG4 molecule.

In this study, we extended our analysis of C region sequences to the CH2 motifs that are known to contribute to effector functions of huIgG1 and huIgG3. Substitutions or deletions within the sequence motif GluLeuGlyGlyProSer (residues 3–9, Fig. 1C, EU position 233–239) affect binding of huIgG1 to FcγRs (7, 36). HuglG2, known for its weak binding to FcγRs, has a substitution and a deletion in this motif, and huIgG4, which shows modest binding to FcγRs, carries a substitution of Leu134 with Phe. In contrast, all four cyno subclasses carry this motif in the CH2 region (Table I) but differ in their ability to engage cyno FcγRs. It is possible that, in contrast to human FcγR interactions, this hinge-proximal CH2 motif in cyno Fe may not be sufficient for determining binding to FcγRs. Moreover, both huIgG1 and huIgG2 bind either weakly or negligibly to the cyno monocytes, further supporting a lesser role of the residues at EU position 233–239 in FcγR interactions and pointing to dissimilarity of human and cyno effector systems. Previous studies also mapped residues at EU positions 295–297 (residues 65–67, Fig. 1C) in huIgG1 to contribute to FcγRIIa binding and ADCC activity. Substitution of Gln295 or Tyr296 reduces FcγRIIa binding and ADCC activity of huIgG1 (7, 15). Similar to human IgG1, cyno IgG1 and cyno IgG3 carry amino acids GlnTyr at EU positions 295–296, whereas cyno IgG2 and IgG4 carry a substitution of Tyr296 with Phe296. Whether this difference contributes to the distinct cyno Fc-dependent effector functions is a subject for future investigation. Additionally, the N-glycosylation site, AsnXxxThr, at EU positions 297–299 (residues 65–67, Fig. 1C) in human IgG Fe was also found in all cyno IgG subclasses. This conserved N-glycosylation site in the CH2 domain is known to be an absolute requirement for huFcγR binding (33, 37). On the basis of this comparison, we hypothesize that the differences in the hinge and CH2 domains of the four types of cyno IgG Fe may influence cyno FcγR binding and thus affect the ability to induce FcγR-dependent effector functions in cyno species.

Table II. Functional profiles of each of the characterized cyno isotypes and their human counterparts

<table>
<thead>
<tr>
<th>Cyno C Region</th>
<th>FcγR Binding</th>
<th>Effector Function (B Cell Depletion)</th>
<th>Cyco CDC</th>
<th>Equivalency to Human IgG Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyIgG1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>≥huIgG1</td>
</tr>
<tr>
<td>cyIgG3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>≥huIgG1/huIgG3*</td>
</tr>
<tr>
<td>cyIgG2</td>
<td>(very weak)</td>
<td>-</td>
<td>-</td>
<td>=huIgG2</td>
</tr>
<tr>
<td>cyIgG4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>≤huIgG4*</td>
</tr>
</tbody>
</table>

*aHuIgG3 was not tested in our experiments.

bMinimal effector function for cyIgG4.
Further studies are needed to assess the immunomodulatory effects of cyto IgGs through design of human-cyto chimeric Abs.

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Disclosures
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References