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Properties of Human IgG1s Engineered for Enhanced Binding to the Neonatal Fc Receptor (FcRn)*

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We describe here the functional implications of an increase in IgG binding to the neonatal Fc receptor. We have defined in a systematic fashion the relationship between enhanced FcRn binding of a humanized anti-respiratory sincytial virus (RSV) monoclonal antibody (MEDI-524) and the corresponding biological consequences in cynomolgus monkeys. The triple mutation M252Y/S254T/T256E (YTE) was introduced into the Fc portion of MEDI-524. Whereas these substitutions did not affect the ability of MEDI-524 to bind to its cognate antigen and inhibit RSV replication, they resulted in a 10-fold increase in its binding to both cynomolgus monkey and human FcRn at pH 6.0. MEDI-524-YTE was efficiently released from FcRn at pH 7.4 in both cases. We show that MEDI-524-YTE consistently exhibited a nearly 4-fold increase in serum half-life in cynomolgus monkeys when compared with MEDI-524. This constituted the largest half-life improvement described to date for an IgG in a primate. For the first time, we demonstrate that these sustained serum levels resulted in an up to 4-fold increase in lung bioavailability. Importantly, we also establish that our non-human primate model is relevant to human. Finally, we report that the YTE triple substitution provided a means to modulate the antibody-dependent cell-mediated cytotoxicity (ADCC) activity of a humanized IgG1 directed against the human integrin $\alpha_v\beta_3$. Therefore, the YTE substitutions allow the simultaneous modulation of serum half-life, tissue distribution and activity of a given human IgG1.

Conclusive evidence indicates that the neonatal Fc receptor (FcRn)[3] plays a central role in the regulation of serum IgG levels in mammals (reviewed in Ref. 1). The Fc-binding FcRn is a heterodimer, which comprises β_2-microglobulin and a membrane-anchored α chain that is related to the α chain of major histocompatibility complex class I molecules. The corresponding sequences and overall structures are well conserved across species such as in human, mouse, rat, and cow (2, 3, 4, 5, 6, 7), as well as in non-eutherian mammals (8). However, differences in their binding specificity to various IgG exist (9), indicating the presence of species-specific binding mechanisms.

The IgG-FcRn interaction exhibits a remarkable pH dependence, varying from strongest at slightly acidic pH to marginal under neutral and basic conditions (10, 11). This crucial characteristic is intricately linked to the IgG salvage mechanism, which involves recycling of FcRn-bound molecules from within acidic lysosomes back to the general circulation (1). As a result, recycled IgGs exhibit a significantly prolonged serum half-life when compared with other serum proteins. It is thought that this rescue takes place in the endothelial cells of adult tissues and particularly in small arteries and capillaries (12, 13). IgGs that retain significant binding to FcRn at neutral pH have a significantly decreased serum persistence (14).

At present, no direct evidence exists for a correlation between increased IgG binding to FcRn at pH 6.0 and longer serum half-life in human. However, previous work carried out in mouse (15, 16) and non-human primates (17, 18) suggest that engineering IgGs for better binding to FcRn may represent a viable strategy to modulate their half-lives in human. Therapeutic antibodies that exhibited longer half-lives likely would be of benefit with increased efficacy because of sustained serum concentrations, decreased dosing frequency and/or lower cost of goods.

While the judiciousness of engineering the Fc-FcRn interaction to prolong serum half-lives seems to be well-established, much remains to be known about the potential biological consequences related to the IgG-transportation role of FcRn. Most notably, this function includes the transcytosis of IgGs through human placenta (19) and intestine (20, 21), as well as the reabsorption of IgG in the human kidneys (22). FcRn has also been reported to be actively involved in the transport of IgG from the lumen of the lungs to the systemic circulation in both mice (23) and cynomolgus monkeys (24). However, the *in vivo* consequences of increasing the affinity of IgGs to FcRn on their distribution to FcRn-expressing tissues are still unknown.

We describe here the behavior of a humanized IgG1 Fc variant dosed in a non-human primate. The Fc changes consisted of a triple substitution (M252Y/S254T/T256E; EU numbering as reported in (25); referred to as YTE thereafter) in the C_H2 domain of MEDI-524 (26, 27). We have shown previously that these mutations increase the binding of palivizumab to human FcRn by about 10-fold at pH 6.0 while allowing efficient release at pH 7.4 (14). However, the *in vivo* behavior of such a mutated human IgG in a relevant model (non-human primate) was unknown. Thus, detailed pharmacokinetics studies in cynomolgus monkeys were specifically designed in an effort to further clarify the relationship between the affinities of the IgG/

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[3] The abbreviations used are: FcRn, neonatal Fc receptor; PBS, phosphate-buffered saline; i.v., intravenous; PBMC, peripheral blood mononuclear cell; HEK, human embryonic kidney; ADCC, antibody-dependent cell-mediated cytotoxicity; BAL, bronchio-alveolar lavage; CMV, cytomegalovirus; RU, resonance unit.

FcRn interaction and the corresponding IgG serum half-lives and lung distribution properties. For the first time, we describe here the consequence of an increased binding to FcRn on the distribution of IgGs from the systemic circulation to respiratory airways. We also report the largest serum half-life increase to date in a primate. Finally, the ability of YTE to modulate the antibody dependent cell-mediated cytotoxicity (ADCC) activity of an anti-$\alpha_v\beta_3$ humanized IgG1 (MEDI-522, formerly known as Vitaxin®; Ref. 28) is described, and new insights into the molecular basis of the Fc-FcRn interaction revealed. Based on the established relevance of our animal model to human, we propose that our set of substitutions will be useful in the development of the next generation of therapeutic antibodies.

EXPERIMENTAL PROCEDURES

Reagents—All chemicals were of analytical grade. Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs, Inc. (Beverly, MA). Oligonucleotides were purchased from Invitrogen (Carlsbad, CA).

Generation of MEDI-524-YTE—The heavy and light chains of the humanized anti-RSV monoclonal antibody MEDI-524 were cloned into a mammalian expression vector encoding a human cytomegalovirus major immediate early (hCMVie) enhancer, promoter and 5′-untranslated region (29). In this system, a human γ1 chain is secreted along with a human κ chain (30). A combination of three mutations (M252Y/S254T/T256E; EU numbering, (25) was introduced into the heavy chain of MEDI-524. Generation of these mutations was carried out by site-directed mutagenesis using a QuickChange XL mutagenesis kit (Stratagene) according to the manufacturer's instructions, and the primers: 5′-GCATGTGACCTCAGGTTCCCGAGTGATATAGAGGGTGTCCTTGGG-3′ and 5′-CCCAAGGACACCCTCTATATCACTCGGGAACCTGAGGTCACATGC-3′. This generated MEDI-524-YTE. The sequence was verified using an ABI 3100 sequencer. NS0 (murine myeloma) cells were then stably transfected with the corresponding antibody constructs, and the secreted immunoglobulins were purified using protein A and standard protocols.

Generation of MEDI-522-YTE, MEDI-522-S239D/A330L/I332E, and MEDI-522-YTE/S239D/A330L/I332E—The heavy and light chains of the humanized anti-integrin $\alpha_v\beta_3$ monoclonal antibody MEDI-522 were cloned into the mammalian expression vector described in the previous section. Combinations of three (M252Y/S254T/T256E or S239D/A330L/I332E (31); EU numbering) or six (S239D/A330L/I332E/M252Y/S254T/T256E; EU numbering) individual mutations were introduced into the heavy chain of MEDI-522. Generation of these mutations was carried out by site-directed mutagenesis using a QuickChange XL mutagenesis kit according to the manufacturer's instructions, and the primers: 5′-GCATGTGACCTCAGGTTCCCGAGTGATATAGAGGGTGTCCTTGGG-3′ and 5′-CCCAAGGACACCCTCTATATCACTCGGGAACCTGAGGTCACATGC-3′ for M252Y/S254T/T256E and 5′-CTGGGGGGACCGGACGTCTTCCTCTTC-3′, 5′-GAAGAGGAAGACGTCCGGTCCCCCCAG-3′, 5′-AAAGCCCTCCCACTGCCCGAGGAGAAA-3′, 5′-TTTCTCCTCGGGCAGTGGGAGGGCTTT-3′ for S239D/A330L/I332E.

This generated MEDI-522-YTE, MEDI-522-S239D/A330L/I332E and MEDI-522-YTE/S239D/A330L/I332E. The corresponding antibody constructs were then transfected into NS0 or human embryonic kidney (HEK) 293 cells and the secreted immunoglobulins were purified using protein A and standard protocols.

Cloning, Expression, and Purification of Cynomolgus Monkey and Human FcRn—Human FcRn was cloned, expressed, and purified as described in Ref. 14. The β_2-microglobulin chain of cynomolgus monkey FcRn was cloned from cynomolgus monkey lung following mRNA isolation and cDNA synthesis using a Straight A mRNA isolation kit and First Strand cDNA synthesis kit, respectively, according to the manufacturer's instructions (Novagen, San Diego, CA). A 3′-His_6-tagged version of the cynomolgus monkey β_2 chain was subsequently generated using standard PCR protocols. The extra cellular domain of the α chain of cynomolgus monkey FcRn (ECD-α) was obtained by mutating its cloned human counterpart (14) at 6 positions (N39D/E44Q/G99S/S189G/S190N/L217M), thereby creating a perfect match to the corresponding cynomolgus monkey sequence (GenBank™ accession number AAL92101). This was carried out using PCR by overlap extension (32). Both the cynomolgus monkey ECD-α and β_2 chains were then cloned into the mammalian expression vector described in the previous sections. HEK-293 cells were transiently transfected with the cynomolgus monkey FcRn constructs in 35 mm, 6-wells dishes using Lipofectamine and standard protocols. Supernatants were harvested thrice at 72, 144, and 216 h post-transfection. The secreted, soluble cynomolgus monkey FcRn was then purified directly on an IgG Sepharose 6 column (APBiotech, Piscataway, NJ) as described in Ref. 14. An additional purification step was included, whose aim was to remove any FcRn molecule potentially containing endogenously produced human β_2 microglobulin. This consisted in applying the IgG Sepharose-purified cynomolgus monkey FcRn to a 1-ml nitrilotriacetic acid (Ni^{2+}-NTA) affinity column according to the manufacturer's instructions (Qiagen, Valencia, CA). Purified cynomolgus monkey FcRn (>95% homogeneity as judged by SDS-PAGE) was dialyzed against phosphate-buffered saline (PBS), flash frozen, and stored at −70 °C.

Determination of the Dissociation Constants (K_D) of the Various IgG/FcRn Pairs—The interaction of soluble human and cynomolgus monkey FcRn with immobilized MEDI-524 and MEDI-524-YTE was monitored by surface plasmon resonance detection using a BIAcore 3000 instrument (Biacore International AB, Uppsala, Sweden). Protein concentrations were calculated by the bicinchoninic acid (BCA) method for both human and cynomolgus monkey FcRn or using the 1% extinction coefficient at 280 nm of 1.47 for MEDI-524 and MEDI-524-YTE Both IgGs were coupled to the dextran matrix of a CM5 sensor chip (Biacore International AB) using an Amine Coupling Kit as described (33) at a surface density of ~1000 RU. Excess reactive esters were quenched by injection of 70 μl of 1.0 M ethanolamine hydrochloride, pH 8.5. Human and cynomolgus monkey FcRn were buffer-exchanged against 50 mM PBS, pH 6.0 containing 0.05% Tween 20 and used in equilibrium binding experiments. All measurements were performed at 25 °C with FcRn concentrations typically ranging from 2.86 μM

to 6 nM at a flow rate of 5 μl/min; data were collected for ~50 min and three 1-min pulses of PBS, pH 7.4 containing 0.05% Tween 20 were used to regenerate the surfaces. Human and cynomolgus monkey FcRn were also flowed over an uncoated cell and the sensorgrams from these blank runs subtracted from those obtained with IgG-coupled cells. Dissociation constants were determined by fitting the binding isotherms using GraphPad Prism (GraphPad Software, Inc.).

The interaction of soluble human FcRn with immobilized MEDI-522, MEDI-522-S239D/A330L/I332E, MEDI-522-YTE, and MEDI-522-YTE/S239D/A330L/I332E was monitored using a BIAcore 3000 instrument as described above. IgGs were coupled to the surface of a CM5 sensor chip at a surface density of ~8000 RU. Human FcRn was buffer-exchanged against 50 mM PBS, pH 6.0 containing 0.05% Tween 20 and used in equilibrium binding experiments at concentrations typically ranging from 2.86 μM to 8 nM at a flow rate of 5 μl/min. Data were collected as described in the previous paragraph. Values for K_D were determined by fitting the binding isotherms using GraphPad Prism.

Determination of the FcRn pH Dependence of Binding toward Various Fc Variants—Comparison of the interaction of soluble human and cynomolgus monkey FcRn with immobilized MEDI-524 and MEDI-524-YTE at acidic and neutral pH was carried out using a BIAcore 3000 in 50 mM PBS, pH 6.0 containing 0.05% Tween 20 or 50 mM PBS, pH 7.4 containing 0.05% Tween 20, respectively. IgGs were coupled to the surface of a CM5 sensor chip at a surface density of ~1000 RU whereas human and cynomolgus monkey FcRn were used at a typical concentration of 500 nM. Analysis of the pH dependence of binding for MEDI-522, MEDI-522-S239D/A330L/I332E, MEDI-522-YTE, and MEDI-522-YTE/S239D/A330L/I332E was carried out in a similar fashion after coupling the IgGs at a surface density of ~8000 RU and flowing human FcRn at a typical concentration of 250 nM. In all cases, FcRn from both species was also flowed over an uncoated cell, and the sensorgrams from these blank runs subtracted from those obtained with IgG-coupled cells.

Microneutralization Assays—The microneutralization assays were carried out essentially as described (30). Briefly, dilutions of MEDI-524 or MEDI-524-YTE were made in quadruplicate in a 96-well plate. RSV Long (ATCC, Manassas, VA) was added to each well and incubated for 2 h at 37 °C in 5% CO_2. 2×10^4 HEp-2 cells (ATCC, Manassas, VA) were then added to each well and incubated for 5 days at 37 °C in 5% CO_2. Cells were then washed three times with PBS containing 0.1% Tween 20 and fixed with acetone. Viral replication was quantified by successive incubations with a mouse anti-RSV monoclonal antibody (Chemicon, Temecula, CA) and a horseradish peroxidase conjugate of a goat anti-mouse IgG (TAGO, Burlingame, CA). Peroxidase activity was detected with 3,3′,5,5′-tetramethylbenzidine (TMB), and the reaction was quenched with 2 M H_2SO_4. The absorbance was read at 450 nm and means plotted for each antibody concentration.

Pharmacokinetics Studies in Cynomolgus Monkeys—A first non-GLP (Good Laboratory Practice) pharmacokinetics study (Study A) was approved by GeneLogic's Institutional Animal Care and Use Committee (IACUC) and conducted at GeneLogic (GeneLogic Laboratories, Gaithersburg, MD). Twenty male cynomolgus monkeys were randomized using computer-generated random numbers and assigned to one of two study groups. Each animal received a single intravenous (i.v.) dose of MEDI-524 or MEDI-524-YTE at 30 mg/kg. Blood samples were drawn prior to dosing on day 0, at 1 and 4 h after dosing, and at 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 20, 24, 31, 41, and 55 days after dosing. The serum concentrations of MEDI-524 or MEDI-524-YTE were determined using the anti-MEDI-524 ELISA described in the next section. For each i.v. infusion, a non-compartmental model was fitted for the serum concentration data of each animal using SAS 8.0 (SAS Institute, Cary, NC). Descriptive statistics for several pharmacokinetics parameters were then calculated.

A second non-GLP pharmacokinetics study (Study B) was approved by the GeneLogic IACUC and conducted at GeneLogic (GeneLogic Laboratories, Gaithersburg, MD). Twelve male and twelve female cynomolgus monkeys were randomized separately using computer-generated random numbers and assigned to one of two study groups (6 males and 6 females per group). Each animal received a single i.v. dose of MEDI-524 or MEDI-524-YTE at 30 mg/kg. Bronchio-alveolar lavages (BALs) were conducted at 4 and 24 days after dosing. More precisely, 6 animals (3 males and 3 females) of each group were sacrificed by barbiturate overdose and exsanguinations at each time point. Chests were opened and the lungs were removed. The distal trachea of each animal was opened to visualize the bifurcation of the bronchi. Lavages were then carried out using a syringe fitted with an appropriate tube. A fresh syringe and tubing was used for each collection. Approximately 8 ml of physiological saline warmed for at least 1 h prior in a water bath set to maintain 39 ± 3 °C was introduced into the main bronchus of the right lung until a slight expansion of the lung was noted. Aspirates were transferred in a tube and the procedure was carried out again on the right lung. The procedures were then repeated for the left lung. Blood samples were also drawn on surviving animals prior to dosing on day 0 and at 1, 4, 8, 14, and 24 days after dosing. The concentrations of MEDI-524 or MEDI-524-YTE in serum and BAL samples were determined using the anti-MEDI-524 ELISA described in the next section. Pharmacokinetics parameters were calculated as described above for Study A.

Analysis of the Various Cynomolgus Monkey Biological Samples—The concentrations of MEDI-524 or MEDI-524-YTE in the serum and BAL samples derived from Study A and B were determined using an anti-human IgG ELISA and appropriate standards. In this assay, MEDI-524 and MEDI-524-YTE were captured by a goat anti-MEDI-524 antibody (anti-idiotype, MedImmune, Inc.) coated to a microtiter plate. Any bound MEDI-524 or MEDI-524-YTE was detected using a goat anti-human IgG antibody linked to biotin. Streptavidin conjugated to horseradish peroxidase followed by TMB (KPL, Gaithersburg, MD) as substrate were used for the colorimetric reaction. Total protein levels in the BAL samples from Study B were also determined by the BCA method.

The possible presence of anti-MEDI-524 antibodies in the serum samples derived from Study A (pre-dose and at days 6, 14, 31, and 55 post-infusion) and B (pre-dose and at days 4, 8,

and 24 post-infusion) was determined using an ELISA as follows: typically, 15 ng of MEDI-524 or MEDI-524-YTE were coated onto the wells of a microtiter plate, which were then blocked with 0.1% Tween 20/0.5% BSA/PBS and incubated with appropriate dilutions of serum samples. Plates were then incubated with horseradish peroxidase-conjugated MEDI-524. Horseradish peroxidase activity was detected with TMB substrate, and the reaction quenched with stop solution (KPL). Plates were read at 450 nm.

Comparison of the Binding of Human and Cynomolgus Monkey IgG to Human and Cynomolgus Monkey FcRn—The interaction of soluble human and cynomolgus monkey IgG with immobilized human and cynomolgus monkey FcRn was monitored as follows: briefly, human and cynomolgus monkey FcRn were coupled to the surface of a CM5 sensor chip at a surface density of ~2500 RU. MEDI-524, MEDI-524-YTE and purified cynomolgus monkey IgG (Antibodies, Inc., Davis, CA) were used in equilibrium binding experiments at concentrations typically ranging from 3 μM to 16 nM at a flow rate of 5 μl/min. Dilutions were made in 50 mM PBS, pH 6.0 containing 0.05% Tween 20. Data were collected for ~50 min and three 1-min pulses of PBS, pH 7.4 containing 0.05% Tween 20 were used to regenerate the surfaces. IgGs were also flowed over an uncoated cell and the sensorgrams from these blank runs subtracted from those obtained with FcRn-coupled cells.

Comparison of the Binding of MEDI-524 and MEDI-524-YTE to Human and Cynomolgus Monkey Sera—The interaction of MEDI-524 and MEDI-524-YTE with human and cynomolgus monkey sera was monitored after coupling the IgGs to the surface of a CM5 sensor chip at a surface density of ~2100 RU. Human (MedImmune, Inc.) and cynomolgus monkey (Antibodies, Inc., Salem, NH) sera were then used in binding experiments at dilutions of 1:10 and 1:2500 at a flow rate of 5 μl/min. Dilutions were made in 50 mM PBS, pH 7.4 containing 0.05% Tween 20. Data were collected for ~50 min and three 1-min pulses of PBS, pH 7.4 containing 0.05% Tween 20 were used to regenerate the surfaces. Sera were also flowed over an uncoated cell and the sensorgrams from these blank runs subtracted from those obtained with IgG-coupled cells.

Cloning, Expression, and Purification of FLAG-tagged Human FcγRIIIA—The C-terminal end of the extracellular domain of human FcγRIIIA (F158 allotype) was fused with a FLAG tag. Cloning and expression were carried out as follows: briefly, the EA1/EA2 (see below) oligonucleotides combination was used to PCR amplify the extracellular domain of human FcγRIIIA using a cDNA library of human bone marrow (Clontech, Mountain View, CA) as the template and standard protocols. The PCR product was then cloned as an XbaI/NotI fragment into the mammalian cell expression vector described earlier for IgG and FcRn expression. HEK-293 cells were transiently transfected with this construct using Lipofectamine and standard protocols. Supernatants were harvested three times at 72, 144, and 216 h post-transfection and pooled. The secreted, soluble human FLAG-tagged FcγRIIIA was purified from the conditioned medium directly on an anti-FLAG M2 agarose column according to the manufacturer's instructions (Sigma). The FLAG-tagged FcγRIIIA was then dialyzed against PBS buffer and stored at −70 °C. EA1: 5′- TCCACAGGTGTCCACTCCCGGACTGAAGATCTCCCAAAG-3′, EA2: 5′-GGGAGAATTCCGCGGCCGCTTATTTGTCATCGTCATCTTTGTAGTCATGGTGATGGTGATGGTGTGCGCCTGCCAAACCTTGAGTGATGGT-3′.

TABLE 1
Dissociation constants for the binding of MEDI-524, MEDI-522, and their Fc variants to human and cynomolgus monkey FcRn

Molecule	K_D-cynomolgus monkey FcRn[a]	K_D-human FcRn
	nM	nM
MEDI-524	1,196 ± 170[b]	2,249 ± 53
MEDI-524-YTE	134 ± 5	210 ± 56
MEDI-522	ND[c]	1,270 ± 100
MEDI-522-YTE	ND	197 ± 4
MEDI-522-S239D/A330L/I332E[d]	ND	1,345 ± 105
MEDI-522-YTE/S239D/A330L/I332E	ND	225 ± 20

[a] Affinity measurements were carried out by BIAcore at pH 6.0, as described under "Experimental Procedures."
[b] Errors were estimated as the standard deviations of at least two independent experiments for each interacting pair.
[c] ND, not determined.
[d] Residue numbering is according to EU (25).

Analysis of Human FcγRIIIA Binding to MEDI-522 and Its Fc Variants—The interaction of soluble human FLAG-tagged FcγRIIIA (F158 allotype) with immobilized MEDI-522, MEDI-522-YTE, MEDI-522-S239D/A330L/I332E, and MEDI-522-YTE/S239D/A330L/I332E was monitored by surface plasmon resonance detection using a BIAcore 3000 instrument. Protein concentrations were calculated by the BCA method. The different humanized IgG1s were coupled to the dextran matrix of a CM5 sensor chip at a surface density of between 7745 and 10426 RU. FLAG-tagged human FcγRIIIA was used in equilibrium binding experiments at concentrations ranging from 16 μM to 31.3 nM at a flow rate of 5 μl/min. Dilutions and binding experiments were carried out in 50 mM HBS buffer containing 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% P-20. Data were collected for about 50 min, and one 30-s pulse of 5 mM HCl was used to regenerate the surfaces. FLAG-tagged human FcγRIIIA was allowed to flow over an uncoated cell, and the sensorgrams from these blank runs subtracted from those obtained with IgG-coupled cells. Values for K_D were determined from the corresponding binding isotherms using GraphPad Prism.

ADCC Assays—Typically, the ADCC activity of MEDI-522 and of its Fc variants was assessed as follows: human blood samples were collected from independent healthy volunteers using heparinized syringes, diluted with twice the volume of PBS buffer, layered onto a Lymphoprep gradient (ICN, Irvine, CA), and centrifuged at 400 × *g* for 30 min at room temperature. Peripheral blood mononuclear cells (PBMCs) were harvested from the interface, washed three times with PBS, and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (Invitrogen) supplemented with 10% fetal bovine serum (FBS). 40 ng/ml of recombinant human IL-2 (R&D Systems, Minneapolis, MN) was then added to the PBMCs followed by incubation overnight of these cells at 37 °C in T-175 flasks (BD Biosciences, Bedford, MA). Cultured M21 (mouse melanoma) cells, which exhibit high surface expression of $\alpha_v\beta_3$, were harvested the following day and resuspended in RPMI 1640 supplemented with 5% FBS (assay buffer) at a density of 2×10^5 cells/ml. These were then added to a 96-well

round bottom tissue culture plate (BD Biosciences, Bedford, MA) at 50 μl/well along with various concentrations of antibody at 50 μl/well in assay buffer (see above), and the mixtures were preincubated at 37 °C for 30 min. PBMCs were then harvested from their overnight incubation and resuspended at 5×10^6 cells/ml (for an Effector (E):Target (T) ratio of 50:1) and 2.5×10^6 cells/ml (for an E:T ratio of 25:1) in assay buffer (see above) and added at 100 μl/well to the assay plate. 25 μl/well of 9% Triton X-100 (Promega, Madison, WI) was added as a control for complete lysis. The plates were centrifuged at 300 $\times$ *g* for 3 min, and incubation at 37 °C was continued for 4 h. Plates were then centrifuged at 300 $\times$ *g* for 10 min, and 50 μl of supernatant from each well was transferred to MaxiSorp 96-well plates (BD Biosciences, Bedford, MA). 50 μl of reconstituted substrate mix (CytoTox 96 Non-Radioactive Cytotoxicity assay kit, Promega) was then added to all wells and incubated in the dark at room temperature for 30 min. 50 μl of stop solution (Promega) was added to each well and lactate dehydrogenase (LDH) release was quantified by measuring the absorbance at 490 nm. % cytotoxicity was calculated using Equation 1.

$$\%\ \text{cytotoxicity} = (\text{Experimental} - \text{Effector}_{\text{Spontaneous}} - \text{Target}_{\text{Spontaneous}})/(\text{Target}_{\text{Maximum}} - \text{Target}_{\text{Spontaneous}}) \times 100 \quad \text{(Eq. 1)}$$

Experimental corresponds to the signal measured in one of the conditions of interest described above, $\text{Effector}_{\text{Spontaneous}}$ corresponds to the signal measured in the presence of PBMCs alone, $\text{Target}_{\text{Spontaneous}}$ corresponds to the signal measured in the presence of M21 cells alone and $\text{Target}_{\text{Maximum}}$ corresponds to the signal measured in the presence of detergent-lysed M21 cells.

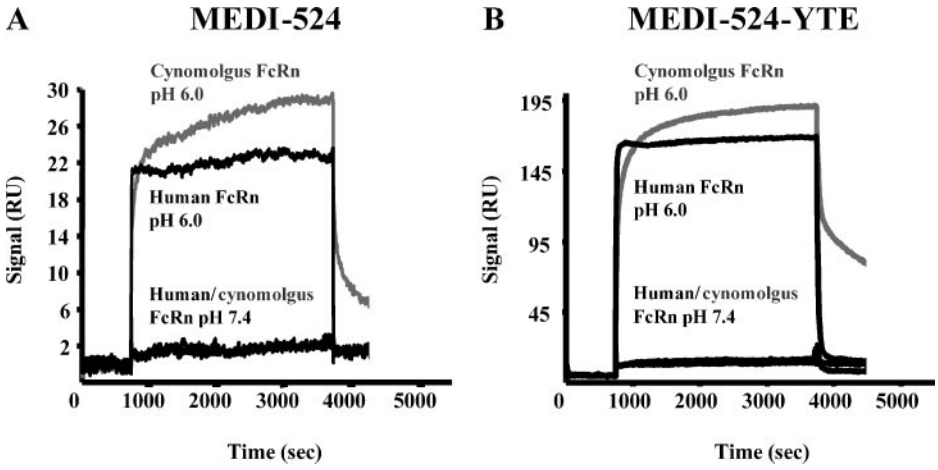

FIGURE 1. **Biacore analysis of the binding of (*A*) MEDI-524, and (*B*) MEDI-524-YTE to human and cynomolgus monkey FcRn at pH 6. 0 and 7.4 after correction for nonspecific binding.** Human and cynomolgus monkey FcRn were injected at a concentration of 500 nM on surfaces onto which 1,017 and 1,050 RU of MEDI-524 and MEDI-524-YTE, respectively, had been coupled.

RESULTS

Characterization of the IgG Variants—Binding of MEDI-524 and MEDI-524-YTE to both cynomolgus monkey and human FcRn was analyzed by BIAcore as described under "Experimental Procedures." Results are shown in Table 1. The K_D for the interaction of MEDI-524 with human FcRn agrees well with previous values determined for other human IgG1s (14, 19). Similarly, the affinity determined for the binding of MEDI-524-YTE to human FcRn is in complete agreement with that measured for the same triple mutation in a different humanized IgG1 background (namely palivizumab, Ref. 14). Interestingly, the observed affinities for the binding of MEDI-524 and MEDI-524-YTE to cynomolgus monkey FcRn were ~2-fold higher than seen for human FcRn. However, when compared with the unmodified IgG, the introduction of YTE into MEDI-524 resulted in a very similar affinity increase of IgG binding to both cynomolgus monkey and human FcRn at pH 6.0 (9- and 11-fold, respectively). No such increase was detected at pH 7.4. In the latter condition, both MEDI-524 and MEDI-524-YTE did not exhibit any significant binding to either cynomolgus monkey or human FcRn (Fig. 1, *A* and *B*).

The ADCC-enhancing triple mutation S239D/A330L/I332E (31) was introduced into both MEDI-522 (formerly known as Vitaxin®, Ref. 28) and MEDI-522-YTE. Binding of MEDI-522, MEDI-522-S239D/A330L/I332E, MEDI-522-YTE, and MEDI-522-YTE/S239D/A330L/I332E to human FcRn was then analyzed as described previously for MEDI-524 and the corresponding K_D values are shown in Table 1. MEDI-522 exhibited an about 2-fold lower K_D when compared with MEDI-524 despite their identical Fc regions. MEDI-522-S239D/A330L/I332E and MEDI-522-YTE/S239D/A330L/I332E had a similar affinity to human FcRn as MEDI-522 and MEDI-522-YTE, respectively, indicating that S239D/A330L/I332E did not significantly affect the IgG/FcRn interaction. When compared with MEDI-522 and MEDI-522-S239D/A330L/I332E, both MEDI-522-YTE and MEDI-522-YTE/S239D/A330L/I332E, respectively, exhibited a similar increase in binding to human FcRn of about 6-fold. When tested at pH 7.4, no significant binding of MEDI-522-YTE or MEDI-522-YTE/S239D/A330L/I332E to human FcRn could be detected.[4] Taken together, our binding data demonstrated the general applicability of YTE to increase binding of human IgG1s to FcRn in a pH-dependent manner.

An RSV microneutralization assay was conducted to investigate the effect of YTE on antibody function. As seen in Fig. 2, both MEDI-524 and MEDI-524-YTE exhibited undistinguishable RSV microneutralization properties, indicating that the triple Fc mutation did not result in major structural changes of the IgG molecule or in significant alteration of its functional activity. This is in complete agreement with data showing that the affinity of MEDI-524-YTE for its cognate antigen (RSV F protein) is not significantly different to that

[4] W. F. Dall'Acqua, unpublished observations.

of MEDI-524 (data not shown). The lack of significant YTE-related structural effects in the Fc region is further confirmed by the ability of MEDI-524-YTE to bind and be purified by protein A (see "Experimental Procedures, Generation of MEDI-524-YTE").

Pharmacokinetics Studies—The *in vivo* consequences of increasing the binding of MEDI-524 to FcRn were investigated by two separate pharmacokinetics studies in cynomolgus monkeys. More precisely, Study A was designed to examine changes in IgG serum half-life, whereas Study B proposed to investigate changes in both IgG serum half-life and distribution to the lungs following systemic administration.

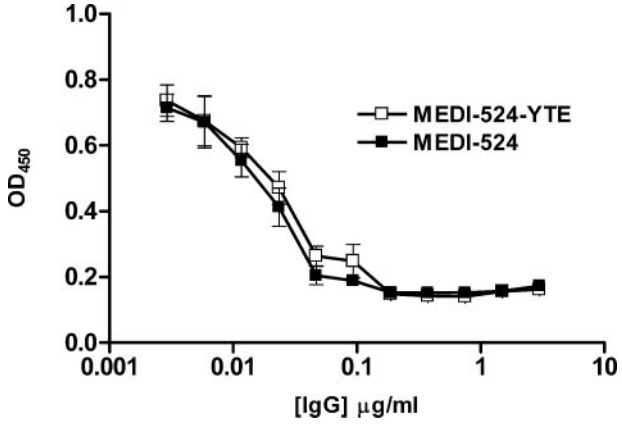

FIGURE 2. **RSV neutralization curves of MEDI-524 and MEDI-524-YTE.** Both IgGs were tested for their ability to inhibit the infection of HEp-2 cells by RSV Long. Each data point represents the average of four measurements. S.E. are indicated by *error bars*.

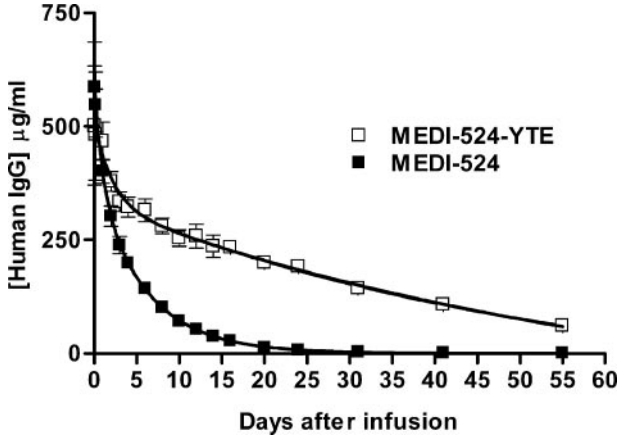

FIGURE 3. **Clearance curves of MEDI-524 and MEDI-524-YTE following i.v. infusion of 30 mg/kg in cynomolgus monkeys (Study A).** Each time point represents the average serum concentration for 10 animals. S.E. are indicated by *error bars*.

The serum pharmacokinetics profiles derived from Study A for MEDI-524 and MEDI-524-YTE are shown in Fig. 3. MEDI-524-YTE exhibited a significantly increased serum persistence when compared with MEDI-524. Indeed, although the mean serum concentration of MEDI-524 and MEDI-524-YTE were similar during the first day post-dose, the serum levels of MEDI-524 decreased rapidly from ~300 μg/ml at day 2 to below the limit of detection at the last day of the study (day 55). In contrast, the serum levels of MEDI-524-YTE gradually decreased from ~380 μg/ml at day 2 to ~60 μg/ml at day 55. This was further confirmed by the determination of the corresponding pharmacokinetics parameters reported in Table 2. As shown in this table, the serum elimination (β phase) half-life of MEDI-524-YTE was nearly four times greater than that of MEDI-524. Likewise, the area under the curve (AUC) for MEDI-524-YTE was nearly five times larger than for MEDI-524. The Wilcoxon test (34) was used to compare these two parameters between the two treatment groups. It suggested that the group differences in half-lives and AUCs were statistically significant ($p < 0.001$). The mean maximum serum antibody concentration (C_{MAX}) was very similar for MEDI-524 and MEDI-524-YTE and was achieved by 1-h post-infusion in most of the animals.

The serum pharmacokinetics profiles and corresponding parameters derived from Study B are shown in Fig. 4 and Table 2, respectively. The same trends as in Study A were observed. Specifically, the serum elimination (β phase) half-life and AUC of MEDI-524-YTE were over three and eight times, respectively, as much as that of MEDI-524. Here again, the mean maximum serum antibody concentrations were not significantly different between MEDI-524 and MEDI-524-YTE.

To determine whether changes in affinity for FcRn would alter the distribution of antibodies to the lungs, BALs were collected at two time points for both MEDI-524 and MEDI-524-YTE and the corresponding human IgG levels measured. Data are reported in Fig. 5*A* and show that the MEDI-524-YTE levels in BALs were significantly increased at days 4 (2.6-fold) and 24 (4.1-fold) post-infusion when compared with MEDI-524. A similar increase was observed when the BAL IgG levels were normalized for total protein concentration (Fig. 5*B*). Finally, [BAL]/[serum] ratios at days 4 and 24 post-dose were very similar between MEDI-524 and MEDI-524-M252Y/S254T/T256E (Fig. 6), indicating that the inherent ability of MEDI-524-YTE to traffic to the lungs was not altered.

None of the animals from Studies A and B which were infused with MEDI-524 or MEDI-524-YTE tested positive for the presence of anti-MEDI-524 antibody (at a 1:640 serum dilu-

TABLE 2
Descriptive summary of the pharmacokinetics parameters for MEDI-524 and MEDI-524-YTE in cynomolgus monkeys

Molecule	β phase $t_{1/2}$[a]		C_{MAX}[b]		AUC[c]	
	Study A	Study B	Study A	Study B	Study A	Study B
	days		*μg/ml*		*h·μg/ml × 10⁴*	
MEDI-524	5.7 ± 1.4[d]	6.1 ± 1.2	644 ± 211	366 ± 77	6.1 ± 1.5	2.5 ± 0.9
MEDI-524-YTE	21.2 ± 9.1	21.2 ± 1.6	639 ± 248	489 ± 64	29.4 ± 9.5	20.2 ± 4.7

[a] IgG serum elimination half-life.
[b] IgG peak serum concentration.
[c] Area under the curve to infinity.
[d] Errors for each parameter were estimated from the corresponding S. D.

tion; data not shown). Although the ELISA used for this purpose could not detect antibodies specifically recognizing the YTE motif, this result suggested that MEDI-524-YTE was not immunogenic in cynomolgus monkeys. This conclusion is in good agreement with the corresponding individual pharmacokinetics profiles in which no precipitous clearance of MEDI-524 or MEDI-524-YTE was observed in any of the animals up to the last day post-infusion (day 55 and 24 for study A and B, respectively). Our data also agree with previous reports showing that human and humanized IgGs are only slightly immunogenic (if at all) in cynomolgus monkeys and other non-human primates (35–37).

Comparative Binding of Human/Cynomolgus Monkey IgG to Human/Cynomolgus Monkey FcRn—To investigate the influence of endogenous IgG on the clearance rate of human IgGs in cynomolgus monkeys, binding of purified MEDI-524 and cynomolgus monkey IgG to cynomolgus monkey FcRn was studied. As shown in Fig. 7*A*, human and cynomolgus IgGs bound similarly well to cynomolgus monkey FcRn. More specifically, the ratio of the human over cynomolgus IgG concentrations at $R_{eq50\%}$ (corrected equilibrium response corresponding to 50% of the maximum signal) was estimated at 1.2. Thus, our data indicated that the strength of the endogenous IgG/FcRn interactions in cynomolgus monkeys was similar to that found in human, thereby validating our choice of this particular non-human primate model for pharmacokinetics studies. The same observations were made when binding of human and cynomolgus IgGs to human FcRn was studied (Fig. 7*B*). In this situation, the ratio of the human over cynomolgus IgG concentrations at $R_{eq50\%}$ was estimated at 0.9.

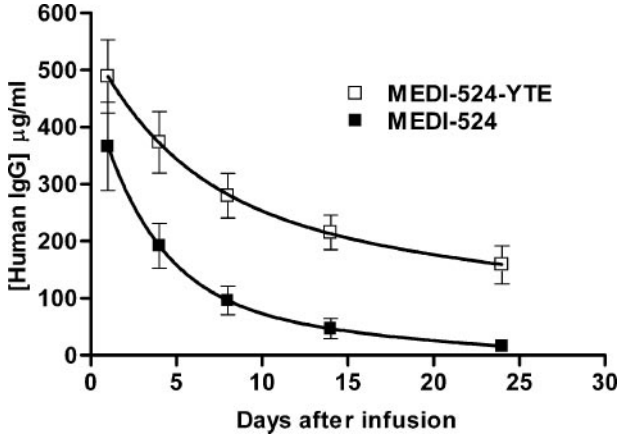

FIGURE 4. **Clearance curves of MEDI-524 and MEDI-524-YTE following i.v. infusion of 30 mg/kg in cynomolgus monkeys (Study B).** One animal infused with a suboptimal dose of MEDI-524-YTE due to a technical error was excluded from the data set. Each time point represents the average serum concentration for at least three animals. S.E. are indicated by *error bars*.

Serum Binding Abilities of MEDI-524 and MEDI-524-YTE—Comparison of the serum binding abilities of MEDI-524 and MEDI-524-YTE was assessed using BIAcore. As shown in Fig. 8, *A* and *B*, both IgGs exhibited no significant binding to cynomolgus monkey and human sera, respectively. Although the sensorgrams corresponding to the interaction of MEDI-524 and MEDI-524-YTE with human serum did not perfectly overlap, we did not consider this difference to be significant. Indeed, complex mixtures such as serum typically exhibit a substantial bulk effect; therefore, corrections to remove this nonspecific component often result in signal variations spanning several RUs (as exemplified by the negative corrected signal exhibited by MEDI-524-YTE; Fig. 8*B*). Thus, our data indicated that: (i) the introduction of YTE into MEDI-524 did not modify its binding properties toward human or cynomolgus monkey serum components, and (ii) the extended serum half-life seen for MEDI-524-YTE in cynomolgus monkeys was not because of a serum protein carrier effect.

FcγRIIIA Binding and ADCC Activity—To investigate any YTE-related effect on antibody effector functions, binding of MEDI-522, MEDI-522-YTE, MEDI-522-S239D/A330L/I332E, and MEDI-522-YTE/S239D/A330L/I332E to FcγRIIIA (F158 allotype) was analyzed using BIAcore (Table 3). MEDI-522-S239D/A330L/I332E exhibited an about 80-fold better affinity than MEDI-522, in good agreement with the enhanced binding to FcγRIIIA seen for various S239D/A330L/I332E-modified human IgGs (31). Conversely, introduction of YTE into MEDI-522 resulted in a nearly 2-fold decreased affinity to FcγRIIIA when compared with MEDI-522. However, the addition of S239D/A330L/I332E to YTE restored the IgG affinity to FcγRIIIA within 2-fold of that exhibited by MEDI-522-S239D/A330L/I332E, and resulted in an overall affinity increase of nearly 40-fold when compared with MEDI-522. Thus, YTE allowed for a reversible modulation of human IgG binding to FcγRIIIA.

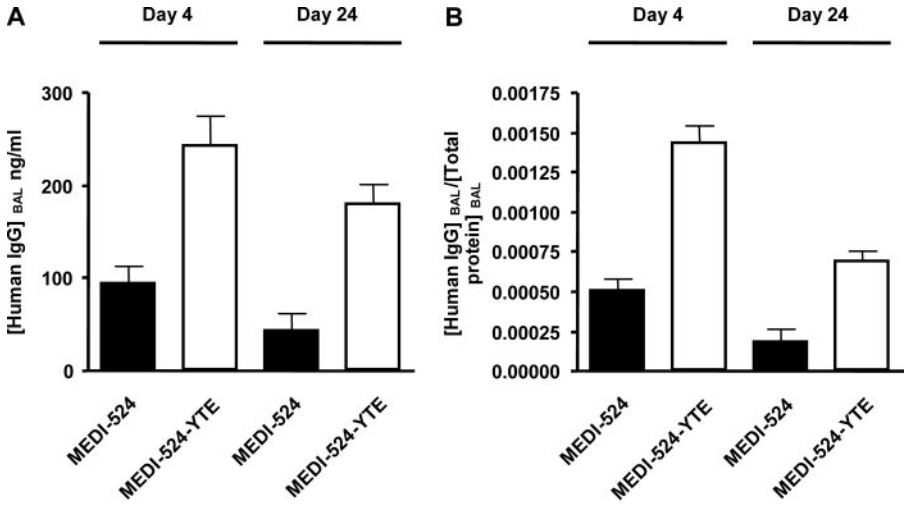

FIGURE 5. **Localization of MEDI-524 and MEDI-524-YTE in bronchio-alveolar lavage of cynomolgus monkeys.** Human IgG levels were determined at days 4 and 24 post-i.v. infusion and are reported without (*A*), or with (*B*) normalization for total protein levels. One animal was excluded from the MEDI-524-YTE data set (see legend to Fig. 4). Each time point represents the average human IgG concentration of the first and second lavage of both right and left lungs for at least five animals. S.E. are indicated by *error bars*.

MEDI-522, MEDI-522-YTE, MEDI-522-S239D/A330L/I332E, and MEDI-522-YTE/S239D/A330L/I332E were further selected characterized by ADCC. The results are in

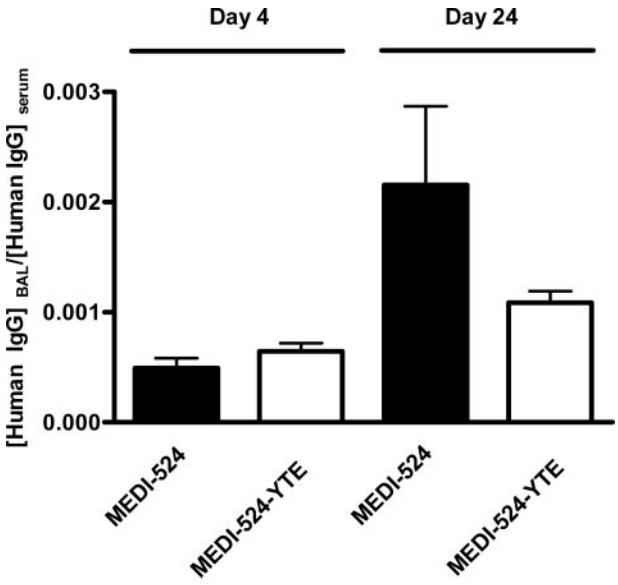

FIGURE 6. **Ratios of MEDI-524 and MEDI-524-YTE concentrations in bronchio-alveolar lavage and serum.** Ratios were determined at days 4 and 24 post-i.v. infusion. Because of possible blood contamination, the first lavage of the right lung of one animal infused with MEDI-524 as well as one animal from the MEDI-524-YTE data set (see legend to Fig. 4) were excluded. Each time point represents the average [BAL]:[serum] ratio for the first and second lavage of both right and left lungs for at least four animals. S.E. are indicated by *error bars*.

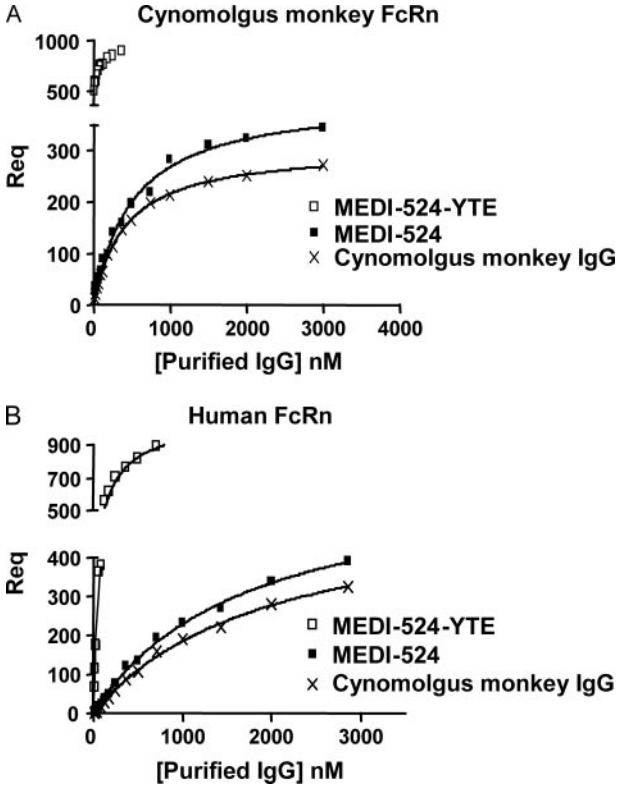

FIGURE 7. **Biacore analysis of the binding of MEDI-524, MEDI-524-YTE, and purified cynomolgus monkey IgG to (*A*) cynomolgus monkey FcRn and (*B*) human FcRn.** Measurements were carried out at pH 6.0 after correction for nonspecific binding. IgGs were injected at varying concentrations on a surface onto which 2593 RU of cynomolgus monkey FcRn (*A*) or 2439 RU of human FcRn (*B*) had been coupled. R_{eq} is the equilibrium response after correction for nonspecific binding. Data shown are representative of two series of experiments.

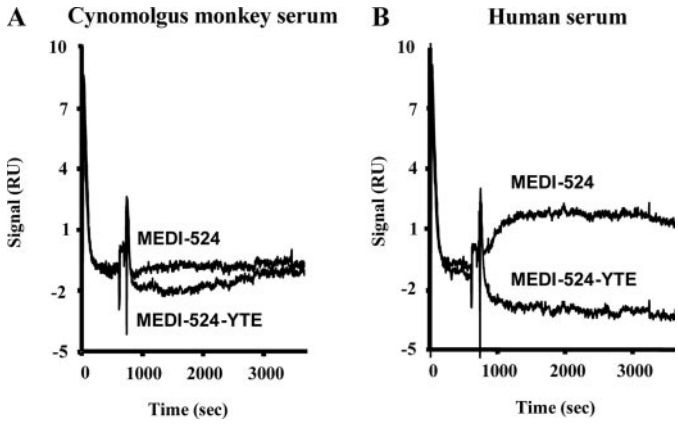

FIGURE 8. **Biacore analysis of the binding of (*A*) cynomolgus monkey serum and (*B*) human serum to MEDI-524 and MEDI-524-YTE.** Measurements were carried out at pH 7.4 after correction for nonspecific binding. Human and cynomolgus monkey sera were injected at a 1:2500 dilution on a surface onto which 2103 RU of MEDI-524 and 2124 RU of MEDI-524-YTE had been coupled. When sera were used at a 1:10 dilution, no significant difference in the binding activity of both IgGs was observed (data not shown).

TABLE 3

Dissociation constants for the binding of MEDI-522 and its Fc variants to human FcγRIIIA (F158)

Molecule	K_D-FcγRIIIA[a]
	nM
MEDI-522	33,600 ± 14,300[b]
MEDI-522-YTE	68,200 ± 16,700
MEDI-522-S239D/A330L/I332E[c]	430 ± 140
MEDI-522-YTE/S239D/A330L/I332E	910 ± 390

[a] Affinity measurements were carried out by BIAcore as described under "Experimental Procedures."
[b] Errors were estimated as the S. D. of at least two independent experiments for each interacting pair.
[c] Residue numbering is according to EU (25).

agreement with the corresponding FcγRIIIA binding data. Indeed, in terms of estimated active concentration, MEDI-522-YTE exhibited an > 100-fold reduction in ADCC activity on M21 cells when compared with MEDI-522 as shown in Fig. 9. However, the addition of S239D/A330L/I332E into MEDI-522-YTE resulted in an over 10- and 100-fold increase in ADCC activity when compared with MEDI-522 and MEDI-522-YTE, respectively (Fig. 9).

DISCUSSION

Several crucial points of intervention exist to engineer the potency of therapeutic antibodies. Most notably, these include serum half-life, tissue distribution and effector functions. The ability to modulate these different properties, individually or together, could lead to the generation of novel, improved therapeutics. First, multiple benefits could be derived from immunoglobulins exhibiting very long serum persistence. Among those benefits are the possibility of decreasing their administration frequency, while maintaining or improving their efficacy. Second, the optimization of the tissue distribution of a given therapeutic antibody would also potentially enhance its efficacy. Indeed, the latter is directly linked to the efficiency with which the molecule reaches its site of action, such as the respiratory tract (38) and rheumatoid joints (39). Third, the ability to up-regulate effector functions could be an important tool in increasing the potency of various therapeutic antibodies, to the

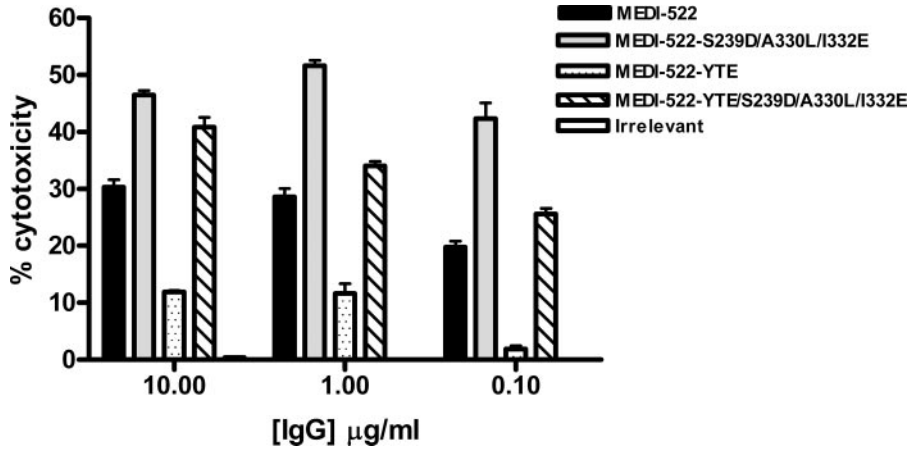

FIGURE 9. **Antibody-dependent cell-mediated cytotoxicity (ADCC) activity of MEDI-522, MEDI-522-YTE, MEDI-522-S239D/A330L/I332E, and MEDI-522-YTE/S239D/A330L/I332E using PBMCs from a single donor and a effector:target ratio of 50:1 against M21 cells.** S.E. are indicated by *error bars* and represent triplicate measurements within the same experiment. Data shown are representative of two independent series of experiments using PBMCs from different individual donors.

extent that their killing properties toward tumors can often be correlated with efficient ADCD/CDC activities (40, 41). Fourth and last, the ability to decrease ADCC and/or CDC could be beneficial in terms of minimizing toxicity in a therapeutic setting (42, 43). The triple Fc mutation M252Y/S254T/T256E (YTE) was previously shown by us to increase the binding of a humanized anti-RSV monoclonal antibody to both murine and human FcRn by ~10-fold (14). In an effort to further characterize this set of mutations and modulate the different properties discussed above, we describe here the behavior of two correspondingly mutated humanized IgG1s, first in terms of serum pharmacokinetics and lung penetration in cynomolgus monkeys, and second in terms of effector functions.

We have introduced YTE into the Fc portion of MEDI-524, a humanized monoclonal antibody (IgG1, κ) derived by *in vitro* affinity maturation of palivizumab. MEDI-524 exhibits potent anti-RSV neutralizing activity (26, 27) and is currently undergoing clinical trials in human for prevention of severe RSV infection in high-risk infants. This particular background was chosen so that the interpretation of the *in vivo* data were not complicated by significant binding of the IgG to an endogenous antigen. In this study, we have shown that: (i) MEDI-524 and MEDI-524-YTE both exhibited a similar affinity to human and cynomolgus monkey FcRn (within 2-fold of each other), (ii) the YTE triple mutation resulted in the same binding affinity increase of MEDI-524 to human and cynomolgus monkey FcRn (~10-fold), and (iii) MEDI-524-YTE retained its pH dependence of binding, showing no significant interaction with human and cynomolgus monkey FcRn at pH 7.4. This last point was of particular importance since it was previously noted that a lack of efficient IgG release at neutral pH lead to their decreased serum persistence in mouse (14). Because of these similarities between human and cynomolgus monkey FcRn in terms of binding to MEDI-524 and MEDI-524-YTE, we concluded that this particular non-human primate was a relevant and predictive pharmacokinetics model for studying the functional effects of increasing binding of IgG to FcRn. Importantly, we have also shown that the interaction of cynomolgus monkey FcRn with endogenous (cynomolgus) immunoglobulin G was similar to the corresponding interaction with human IgG (see "Results" and Fig. 7*A*). This further established that the cynomolgus monkey *in vivo* context closely resembles that found in human; therefore, the pharmacokinetics properties, which we describe here for our YTE-modified IgG, are likely to be seen in the human.

We have shown here that the serum elimination half-life of MEDI-524-YTE was increased by a factor of up to ~4-fold when compared with the unmodified version of MEDI-524. The serum half-life of MEDI-524 was typical of that seen for a human IgG1 in cynomolgus monkeys (44, 45). To our knowledge, this constitutes the largest serum half-life increase for a human IgG in a primate. The mean maximum serum antibody concentrations were indistinguishable between MEDI-524 and MEDI-524-YTE, indicating that the increased serum levels seen for MEDI-524-YTE were not due to altered distribution properties in the general circulation. MEDI-524-YTE also exhibited a significant increase in its total exposure, as its area under the curve extrapolated to infinity (AUC) was 5–8-fold larger than seen for MEDI-524. Interestingly, Hinton *et al.* (17, 18) have described a different set of mutations (T250Q/M428L) which, when introduced into the Fc portion of human IgG1 and IgG2, result in a significant increase in both their binding to rhesus FcRn and serum half-life in rhesus monkeys (nearly 40- and 2.5-fold, respectively, for IgG1 and 25- and 2-fold, respectively, for IgG2). These author's data, when compared with ours, seem to indicate the lack of a direct correlation between increase in FcRn binding and increase in serum-half-life. This may be attributable to inherently different IgG recycling mechanisms across primates. Alternatively, T250Q/M428L and/or YTE may invoke alternate, FcRn-independent IgG rescue or clearance strategies. To this end, it is worth noting that MEDI-524-YTE did not exhibit significantly enhanced binding to cynomolgus monkey serum when compared with MEDI-524, suggesting that its enhanced serum half-life was not mediated through specific interaction with serum components. The same lack of reactivity with human serum will be important in the interpretation of future *in vivo* data in human. It is also possible that endogenous IgGs affected the clearance rate of the Fc-modified human IgGs to a different extent in cynomolgus and rhesus monkeys. Therefore, it would be interesting to compare the relative strength of the interactions between human IgG/rhesus FcRn and rhesus IgG/rhesus FcRn, as was done in our study for the cynomolgus monkeys. Conceivably, a large difference in the corresponding affinities could result in an under- or over estimation of the predicted serum half-life enhancement of the test human IgGs in human because of varying competition levels between endogenous IgGs and endogenous FcRn.

It has been reported that reduced binding of an immunocytokine to mouse FcγR could lead to its increased serum half-life

in mice (46). When compared with MEDI-524, the binding affinity of MEDI-524-YTE to recombinant human FcγRIIA, FcγRIIB, and FcγRIIIA was decreased by 3-, 2-, and 3-fold, respectively, whereas binding to human FcγRI was not significantly affected (data not shown). Therefore, in light of the high similarity between human and cynomolgus monkey Fcγ receptors (>85% identity at the amino acid level for FcγRI, FcγRIIA, FcγRIIB, and FcγRIIIA; US patent number 6,911,321), we cannot rule out that this phenomenon might play, along with FcRn, a role in increasing the serum half-life of MEDI-524-YTE. However, we note that the model used by Gillies *et al.* (46) significantly differs from ours because of the potential ability of immunocytokines to co-cross-link Fcγ receptors with cytokine receptors and trigger their own internalization. A decrease in the ability of the fusion protein to bind FcγR would decrease co-cross-linking of the receptors and thus alter its rate of clearance.

The increased persistence of MEDI-524-YTE in serum was reflected by up to 4-fold higher lung levels as measured in bronchio-alveolar lavages. Essentially similar results were observed when the IgG levels in BALs were normalized for total protein concentration, suggesting no particular bias during collection of the corresponding samples. Importantly, the [BAL]/[serum] ratios were similar between MEDI-524 and MEDI-524-YTE, suggesting that the higher BAL levels exhibited by MEDI-524-YTE were a direct reflection of its increased serum persistence as opposed to a specific accumulation of this variant. It is worth noting that FcRn has been implicated in the active transport of Fc-containing molecules from the lumen of the central airways to the systemic circulation in cynomolgus monkeys (24). Thus, the BAL concentrations of MEDI-524-YTE may represent the snapshot of a phenomenon in which an enhanced passive transport from the serum to the lumen is counterbalanced by an active transport pathway in the opposite direction. To date however, little is known about the role of FcRn in a potential IgG active transport mechanism from the general circulation to the lungs. Therefore, the BAL levels observed for MEDI-524-YTE could conceivably be the result of an equilibrium between two active, opposite transport pathways.

Our study provided new insights on the nature of the Fc/FcRn interaction. Although the binding affinities of palivizumab (14), MEDI-524, and MEDI-522 (Table 1) to human FcRn were similar, some significant differences were nevertheless observed. Whereas palivizumab and MEDI-524 exhibited undistinguishable dissociation constants (2.2–2.5 μM), MEDI-522 bound more tightly by a factor of about 2-fold (1.3 μM). We primarily attribute this result to binding variations caused by different lots of human FcRn. However, because these IgGs all had identical Fc regions, it may also suggest that the Fab portion plays a discernible (though minor) role in the Fc-FcRn interaction. It is likely that such a role would be mediated through long range interaction(s) as the FcRn binding site is located at the interface between the IgG C_H2 and C_H3 domains (5, 47).

We have also studied the effect of YTE on effector functions. To this end, we introduced YTE into the Fc portion of MEDI-522, a humanized, affinity-optimized monoclonal antibody (IgG1, κ) directed against the human $\alpha_v\beta_3$ integrin complex (28). The indications of this molecule include the treatment of rheumatoid arthritis and of solid tumors because of its anti-inflammatory and anti-angiogenic properties (48, 49). This particular IgG1 was chosen because of its ability to mediate efficient ADCC. We have shown that YTE significantly reduced the binding of MEDI-522 to human FcγRIIIA (F158 allotype) as well as its ADCC activity. Our results are in good agreement with the fact that Fc positions 254 and 256 have previously been shown to be implicated in FcγRIIIA/F158 binding (50). It is likely that MEDI-522-YTE would exhibit different binding affinities toward other FcγRIIIA allotypes, such as V158 whose inherent affinity to human IgGs is higher than F158 (51). Likewise, the various donors used here were not genotyped in terms of their FcγRIIIA allotypic diversity; therefore, it is possible that the ADCC activities we derived did not exactly reflect our binding data in which the F158 allotype was used. Importantly, the ADCC activity of MEDI-522-YTE as well as its binding to FcγRIIIA can be restored at a level significantly higher than the unmodified MEDI-522 through the introduction of an ADCC-enhancing triple substitution (S239D/A330L/I332E; Ref. 31). Thus, YTE provides a reversible way to modulate the ADCC function of a human IgG1. In contrast, the serum half-life-enhancing double mutation T250Q/M428L described by Hinton *et al.* (17, 18) was shown to minimally impact the CDC and ADCC activities of a humanized anti-HLA-DR IgG1.

In summary, we have dissected the functional consequences of a pH-dependent increase in IgG binding to FcRn and shown that the YTE triple mutation is generally applicable to human IgG1s for this purpose. It will be interesting to know if our approach can be used in conjunction with the strategy described by Hinton *et al.* (17, 18) to further increase serum half-life. Additional characterization of our Fc mutations will require a more detailed analysis, such as the determination of the pharmacokinetics properties of YTE-containing IgGs in the context of their binding to an endogenously expressed antigen. Likewise, the impact of YTE on IgG distribution to the general circulation following aerosol administration into the airways will provide new insights on the nature of FcRn-mediated transport pathways. Conceivably, this could also constitute a novel method to increase the serum bioavailability of IgG or Fc fusion proteins after deposition into the upper respiratory tract. We believe that our set of mutations will be a valuable addition to the antibody therapy field.

Acknowledgments—We thank Robert M. Woods and Jia Li for BIAcore measurements and purifying human and cynomolgus monkey FcRn. We are also grateful to Nita K. Patel for help in the microneutralization assays and Edward O'Connor for technical assistance in the pharmacokinetics study.

REFERENCES

1. Ghetie, V., and Ward, E. S. (2000) *Annu. Rev. Immunol.* **18,** 739–766
2. Simister, N. E., and Mostov, K. E. (1989) *Nature* **337,** 184–187
3. Ahouse, J. J., Hagerman, C. L., Mittal, P., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Simister, N. E. (1993) *J. Immunol.* **151,** 6076–6088
4. Story, C. M., Mikulska, J. E., and Simister, N. E. (1994) *J. Exp. Med.* **180,** 2377–2381
5. Burmeister, W. P., Gastinel, L. N., Simister, N. E., Blum, M. L., and Bjorkman, P. J. (1994) *Nature* **372,** 336–343
6. West, A. P., and Bjorkman, P. J. (2000) *Biochemistry* **39,** 9698–9708

7. Kacskovics, I., Wu, Z., Simister, N. E., Frenyo, L. V., and Hammarstrom, L. (2000) *J. Immunol.* **164,** 1889–1897
8. Adamski, F. M., King, A. T., and Demmer, J. (2000) *Mol. Immunol.* **37,** 435–444
9. Ober, R. J., Radu, C. G., Ghetie, V., and Ward, E. S. (2001) *Int. Immunol.* **13,** 1551–1559
10. Rodewald, R. (1976) *J. Cell Biol.* **71,** 666–669
11. Raghavan, M., Bonagura, V. R., Morrison, S. L., and Bjorkman, P. J. (1995) *Biochemistry* **34,** 14649–14657
12. Ghetie, V., Hubbard, J. G., Kim, J. K., Tsen, M. F., Lee, Y., and Ward, E. S. (1996) *Eur. J. Immunol.* **26,** 690–696
13. Borvak, J., Richardson, J., Medesan, C., Antohe, F., Radu, C., Simionescu, M., Ghetie, V., and Ward, E. S. (1998) *Int. Immunol.* **10,** 1289–1298
14. Dall'Acqua, W. F., Woods, R. M., Ward, E. S., Palaszynski, S. R., Patel, N. K., Brewah, Y. A., Wu, H., Kiener, P. A., and Langermann, S. (2002) *J. Immunol.* **169,** 5171–5180
15. Medesan, C., Radu, C., Kim, J. K., Ghetie, V., and Ward, E. S. (1996) *Eur. J. Immunol.* **26,** 2533–2536
16. Ghetie, V., Popov, S., Borvak, J., Radu, C., Matesoi, D., Medesan, C., Ober, R. J., and Ward, E. S. (1997) *Nat. Biotechnol.* **15,** 637–640
17. Hinton, P. R., Johlfs, M. G., Xiong, J. M., Hanestad, K., Ong, K. C., Bullock, C., Keller, S., Tang, M. T., Tso, J. Y., Vasquez, M., and Tsurushita, N. (2004) *J. Biol. Chem.* **279,** 6213–6216
18. Hinton, P. R., Xiong, J. M., Johlfs, M. G., Tang, M. T., Keller, S., and Tsurushita, N. (2006) *J. Immunol.* **176,** 346–356
19. Firan, M., Bawdon, R., Radu, C., Ober, R. J., Eaken, D., Antohe, F., Ghetie, V., and Ward, E. S. (2001) *Int. Immunol.* **13,** 993–1002
20. Israel, E. J., Taylor, S., Wu, Z., Mizoguchi, E., Blumberg, R. S., Bhan, A., and Simister, N. E. (1997) *Immunology* **92,** 69–74
21. Dickinson, B. L., Badizadegan, K., Wu, Z., Ahouse, J. C., Zhu, X., Simister, N. E., Blumberg, R. S., and Lencer, W. I. (1999) *J. Clin. Investig.* **104,** 903–911
22. Haymann, J. P., Levraud, J. P., Bouet, S., Kappes, V., Hagege, J., Nguyen, G., Xu, Y., Rondeau, E., and Sraer, J. D. (2000) *J. Am. Soc. Nephrol.* **11,** 632–639
23. Spiekermann, G. M., Finn, P. W., Ward, E. S., Dumont, J., Dickinson, B. L., Blumberg, R. S., and Lencer, W. I. (2002) *J. Exp. Med.* **96,** 303–310. Erratum in: *J. Exp. Med.* (2003) **197,** 1601
24. Bitonti, A. J., Dumont, J. A., Low, S. C., Peters, R. T., Kropp, K. E., Palombella, V. J., Stattel, J. M., Lu, Y., Tan, C. A., Song, J. J., Garcia, A. M., Simister, N. E., Spiekermann, G. M., Lencer, W. I., and Blumberg, R. S. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 9763–9768
25. Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller. (1991) *Sequences of Proteins of Immunological Interest,* U.S. Public Health Service, National Institutes of Health, Washington, D. C.
26. Wu, H., Pfarr, D. S., Tang, Y., An, L. L., Patel, N. K., Watkins, J. D., Huse, W. D., Kiener, P. A., and Young, J. F. (2005) *J. Mol. Biol.* **350,** 126–144
27. Mejias, A., Chavez-Bueno, S., Rios, A. M., Aten, M. F., Raynor, B., Peromingo, E., Soni, P., Olsen, K. D., Kiener, P. A., Gomez, A. M., Jafri, H. S., and Ramilo, O. (2005) *Antimicrob. Agents Chemother.* **49,** 4700–4707
28. Wu, H., Beuerlein, G., Nie, Y., Smith, H., Lee, B. A., Hensler, M., Huse, W. D., and Watkins, J. D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6037–6042
29. Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B., and Schaffner, W. (1985) *Cell* **41,** 521–530
30. Johnson, S., Oliver, C., Prince, G. A., Hemming, V. G., Pfarr, D. S., Wang, S. C., Dormitzer, M., O'Grady, J., Koenig, S., Tamura, J. K., Woods, R., Bansal, G., Couchenour, D., Tsao, E., Hall, W. C., and Young, J. F. (1997) *J. Infect. Dis.* **176,** 1215–1224
31. Lazar, G. A., Dang, W., Karki, S., Vafa, O., Peng, J. S., Hyun, L., Chan, C., Chung, H. S., Eivazi, A., Yoder, S. C., Vielmetter, J., Carmichael, D. F., Hayes, R. J., and Dahiyat, B. I. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 4005–4010
32. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* (*Amst.*) **77,** 51–59
33. Johnsson, B., Lofas, S., and Lindquist, G. (1991) *Anal. Biochem.* **198,** 268–277
34. Wilcoxon, F. (1945) *Biometrics* **1,** 80–83
35. Stephens, S., Emtage, S., Vetterlein, O., Chaplin, L., Bebbington, C., Nesbitt, A., Sopwith, M., Athwal, D., Novak, C., and Bodmer, M. (1995) *Immunology* **85,** 668–674
36. Shinkura, H., Imazeki, I., Fukushima, N., Chiba, N., Takahashi, F., Aikawa, H., Kitamura, H., Furuichi, T., Horiba, N., and Ohsugi, Y. (1997) *Toxicology* **122,** 163–170
37. Fishwild, D. M., Hudson, D. V., Deshpande, U., and Kung, A. H. (1999) *Clin. Immunol.* **92,** 138–152
38. Cardenas, S., Auais, A., and Piedimonte, G. (2005) *Expert Rev. Anti. Infect. Ther.* **3,** 719–726
39. Choy, E. H., Pitzalis, C., Cauli, A., Bijl, J. A., Schantz, A., Woody, J., Kingsley, G. H., and Panayi, G. S. (1996) *Arthritis Rheum.* **39,** 52–56
40. Anderson, D. R., Grillo-Lopez, A., Varns, C., Chambers, K. S., and Hanna, N. (1997) *Biochem. Soc. Trans.* **25,** 705–708
41. Green, M. C., Murray, J. L., and Hortobagyi, G. N. (2000) *Cancer Treat. Rev.* **26,** 269–286
42. Raasveld, M. H., Bemelman, F. J., Schellekens, P. T., van Diepen, F. N., van Dongen, A., van Royen, E. A., Hack, C. E., and ten Berge, I. J. (1993) *Kidney Int.* **43,** 1140–1149
43. Vallhonrat, H., Williams, W. W., Cosimi, A. B., Tolkoff-Rubin, N., Ginns, L. C., Wain, J. C., Preffer, F., Olszak, I., Wee, S., Delmonico, F. L., and Pascual, M. (1999) *Transplantation* **67,** 253–258
44. Adams, C. W., Allison, D. E., Flagella, K., Presta, L., Clarke, J., Dybdal, N., McKeever, K., and Sliwkowski, M. X. (2006) *Cancer Immunol. Immunother.* **55,** 717–727
45. Lin, Y. S., Nguyen, C., Mendoza, J. L., Escandon, E., Fei, D., Meng, Y. G., and Modi, N. B. (1999) *J. Pharmacol. Exp. Ther.* **288,** 371–378
46. Gillies, S. D., Lan, Y., Lo, K. M., Super, M., and Wesolowski, J. (1999) *Cancer Res.* **59,** 2159–2166
47. Martin, W. L., West Jr., A. P., Gan, L., and Bjorkman, P. J. (2001) *Mol. Cell.* **7,** 867–877
48. Gutheil, J. C., Campbell, T. N., Pierce, P. R., Watkins, J. D., Huse, W. D., Bodkin, D. J., and Cheresh, D. A. (2000) *Clin. Cancer Res.* **6,** 3056–3061
49. Wilder, R. L. (2002) *Ann. Rheum. Dis.* **61,** Suppl. 2, ii96–ii99
50. Shields, R. L., Namenuk, A. K., Hong, K., Meng, Y. G., Rae, J., Briggs, J., Xie, D., Lai, J., Stadlen, A., Li, B., Fox, J. A., and Presta, L. G. (2001) *J. Biol. Chem.* **276,** 6591–6604
51. Koene, H. R., Kleijer, M., Algra, J., Roos, D., von dem Borne, A. E., and de Haas, M. (1997) *Blood* **90,** 1109–1114