High-Affinity IgG Antibodies Develop Naturally in Ig-Knockout Rats Carrying Germline Human IgH/Igκ/Igλ Loci Bearing the Rat C\textsubscript{H} Region

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*J Immunol* published online 9 January 2013
http://www.jimmunol.org/content/early/2013/01/09/jimmunol.1203041

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/01/09/jimmunol.1203041.1.DC1.html

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Mice transgenic for human Ig loci are an invaluable resource for the production of human Abs. However, such mice often do not yield human mAbs as effectively as conventional mice yield mouse mAbs. Suboptimal efficacy in delivery of human Abs might reflect imperfect interaction between the human membrane IgH chains and the mouse cellular signaling machinery. To obviate this problem, in this study we generated a humanized rat strain (OmniRat) carrying a chimeric human/rat IgH locus (comprising 22 human V\_\text{H}S, all human D and J\_\text{H} segments in natural configuration linked to the rat C\_\text{H} locus) together with fully human IgL loci (12 V\_\text{L}s linked to Jc-C\_\text{C}c and 16 V\_\text{A}s linked to JA-Ca). The endogenous Ig loci were silenced using designer zinc finger nucleases. Breeding to homozygosity resulted in a novel transgenic rat line exclusively producing chimeric Abs with human idiotypes. B cell recovery was indistinguishable from wild-type animals, and human V(D)J transcripts were highly diverse. Following immunization, the OmniRat strain performed as efficiently as did normal rats in yielding high-affinity serum IgG. mAbs, comprising fully human variable regions with subnanomolar Ag affinity and carrying extensive somatic mutations, are readily obtainable, similarly to conventional mAbs from normal rats. The Journal of Immunology, 2013, 190: 000–000.

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Received for publication November 6, 2012. Accepted for publication December 4, 2012.

This work was supported by Open Monoclonal Technology, Inc., Biogenouest, and Région Pays de la Loire, France.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BAC, bacterial artificial chromosome; cYAC, circular yeast artificial chromosome; FISH, fluorescence in situ hybridization; HEL, hen egg lysozyme; hGH, human growth hormone receptor; KLH, keyhole limpet hemocyanin; KO, knockout; PFGE, pulsed field gel electrophoresis; PGRN, progranulin; wt, wild-type; YAC, yeast artificial chromosome; ZFN, zinc finger nuclease.

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http://jimmunol.org/cgi/doi/10.4049/jimmunol.1203041

www.jimmunol.org

The Journal of Immunology

Published January 9, 2013, doi:10.4049/jimmunol.1203041

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However, there is clearly room for improvement. Indeed, it has been suggested that suboptimal performance of these humanized mouse strains with regard to the efficacy with which they yield human mAbs might result from imperfect interaction between the C region of the human Ig expressed on the B cell membrane and the mouse cellular signaling machinery (19). Because the transgenic mouse can essentially be viewed as a source of Ag-specific IgV genes (with the desired IgC region provided at a later stage during the creation of cell lines for bulk Ab production), we wondered whether the transgenic approach could be improved if the germline configuration human IgV-D-J regions were linked to endogenous (rather than human) IgCH regions.

In this study, we describe a rat strain carrying entirely human Ig transloci but with an IgH translocus in which human IgVH, D, and JH segments have been linked to germine-configured rat IgCH regions. We find that this rat strain gives highly efficient chimeric Ab expression with serum IgM and IgG levels similar to those obtained with normal rats. Large numbers of high-affinity chimeric mAbs can also be readily established from these animals.

Materials and Methods

Construction of modified human Ig loci on yeast artificial chromosomes and bacterial artificial chromosomes

IgH loci. The human IgH V genes were covered by two bacterial artificial chromosomes (BACs): BAC6-VH3-11 containing the authentic region spanning from VH4-39 to VH3-23 followed by VH3-11 (modified from a commercially available BAC clone 3054M17 CB1T) and BAC3 containing the authentic region spanning from VH3-11 to VH6-1 (811L16 RPCI-11). A BAC termed Annabel was constructed by joining rat CH region genes immediately downstream of the human VH6-1-Ds-JH region (Fig. 1A). All BAC clones containing part of the human or rat IgH locus were purchased from Invitrogen. Oligonucleotides and PCR conditions are listed in the Supplemental Material.

Both BAC6-VH3-11 and Annabel were initially constructed in Saccharomyces cerevisiae as circular yeast artificial chromosome (cYACs) and further checked and maintained in Escherichia coli as BACs. Unlike YACs, BAC plasmid preps yield large quantities of the desired DNA. To convert a linear YAC into a cYAC or to assemble DNA fragments with overlapping ends into a single cYAC in S. cerevisiae, which can also be maintained as a BAC in E. coli, two self-replicating S. cerevisiae E. coli shuttle vectors, pBelO-CEN-URA and pBelO-CEN-HYG, were constructed. Briefly, S. cerevisiae CEN4 was cut out as an AvrII fragment from pYAC-RC (20) and ligated to SpeI linearized pAP599 (21). The resulting plasmid contains CEN4 cloned between S. cerevisiae URA3 and a hygromycin-resistance expression cassette (HygR). From this plasmid, an ApaI-BamHI fragment containing URA3 followed by CEN4 or a PmlI-Sphl fragment containing CEN4 followed by HygR was cut out and ligated to ApaI and BamHI or HpaI and Sphl doubly digested pBAC-Belo11 (New England Biolabs) to yield pBelO-CEN-URA and pBelO-CEN-HYG.

To construct BAC6-VH3-11, initially two fragments, a 115-kb NotI-Pmel and a 110-kb RsiII-SgrAI, were cut out from the BAC clone 3054M17 CB1T. The 3’ end of the former fragment overlaps 22 kb with the 5’ end of the latter. The NotI-Pmel fragment was ligated to a NotI-BamHI YAC arm containing S. cerevisiae CEN4 as well as TRP1/ARS1 from pYAC-RC, and the RsiII-SgrAI fragment was ligated to an SgrAI-BamHI YAC arm containing S. cerevisiae URA3, also from pYAC-RC. Subsequently, the ligation mixture was transformed into S. cerevisiae AB1380 cells via spheroplast transformation (22), and URA+ TRP+ yeast clones were selected. Clones, termed YAC6, containing the linear region from human VH4-39 to VH3-23 were confirmed by Southern blot analysis. YAC6 was further extended by addition of a 10.6-kb fragment 3’ of VH3-23 and conversion to a CAC. The 10.6-kb extension contains the human VH3-11 and also occurs at the 5’ end of BAC3. We constructed pBelOHyg-YAC6+ BAC3(5’) for the modification of YAC6. Briefly, three fragments with overlapping ends were prepared by PCR: 1) a “stuff” fragment containing S. cerevisiae TRP1/ARS1 flanked by Hpal sites

![Diagram of Ig loci](image)

**FIGURE 1.** Integrated human Ig loci. (A) The chimeric human/rat IgH region contains three overlapping BACs with 22 different and potentially functional human VH segments. BAC6-3 has been extended with VH3-11 to provide a 10.6-kb overlap to BAC3, which overlaps 11.3 kb via VH6-1 with the C region BAC human/rat Annabel. The latter is chimeric and contains all human D and JH segments followed by the rat C region (Cμ, Cλ, Cγ2b, Cε, Cα) with full enhancer sequences. (B) The human Igκ BACs with 12 Vκs and all Jκs provide an ~14-kb overlap in the Vκ region and ~40 kb in Cκ to include the KDE. (C) The human Igλ region with 17 Vλs and all Jλs, including the 3’ enhancer, is from a YAC (24).
with a 5′ tail matching the sequence upstream of V_{H}6-39 and a 3′ tail matching downstream of V_{H}6-23 in YAC6 (using long oligonucleotides 561 and 562, and pYAC-RC as template); 2) the 10.6-kb extension fragment with a 5′ tail matching the sequence downstream of V_{H}6-23 as described above and a unique AscI site at its 3′ end (using long oligonucleotides 570 and 412, and human genomic DNA as template); and 3) pBelo-CEN-HYG vector with the CEN4 joined downstream with a homology tail matching the 3′ end of the 10.6 extension fragment and the pBelo-CEN-URA vector with a tail matching the sequence upstream of V_{H}6-39 as described above (using long oligonucleotides 414 and 566, and pBelo-CEN-HYG as template). Subsequently, the three PCR fragments were assembled into a small cYAC conferring HYGR and TRP* in *S. cerevisiae* via homologous recombination associated with spheroplast transformation, and this cYAC was further converted into the BAC pBeloHYG-YAC6-BAC3(5′). Finally, the HpaII-digested pBeloHYG-YAC6+ BAC3(5′) was used to transform yeast cells carrying YAC6, and through homologous recombination, a YAC BAC4-V_{H}3-11 containing part of rat μ coding sequence (using oligonucleotides 488 and 346, and rat genomic DNA as template), an ~52 kb NotI-Pfnel fragment containing the authentic rat μ, α, and δ2 region cut out from BAC5 (CH230-408MS) and the pBelo-CEN-URA vector with the URA3 joined downstream with a homology tail matching the 3′ end of the rat CΕ region and the CEN4 joined upstream with a tail matching the 5′ region of human V_{H}6-1 as described (using oligonucleotides 500 and 550, and pBelo-CEN-URA as template). Correct assembly via homologous recombination in *S. cerevisiae* was analyzed by PCR and purified cYAC from the correct clones was converted into a BAC in *E. coli*.

For the assembly of Annabel, parts of the above cYAC/BAC containing humanV_{H}6-1-Ds-JHs followed by the authentic rat μ, α, and δ2 region, as well as PCR fragments were used. Five overlapping fragments contained the 5′ end of human V_{H}6-1 joined with the rat genomic sequence immediately downstream of the last JH followed by rat Cs to yield a cYAC/BAC. To achieve this, five overlapping restriction as well as PCR fragments were prepared: a 6.1-kb fragment 5′ of human V_{H}6-1 (using oligonucleotides 383 and 384, and human genomic DNA as template), an ~78-kb PvuI-PcaI fragment containing the human V_{H}6-1-Ds-JHs region cut out from BAC1 (RP11-164E6/5), a 8.7-kb fragment joining the human JH6 with the rat genomic sequence immediately downstream of the last JH followed by rat Cs as described above, an ~83 kb SpeI fragment comprising human V_{H}6-1-Ds-JHs immediately followed by the rat genomic sequence downstream of the last JH and containing part of rat Cα, a 5.2-kb fragment joining the 3′ end of rat μ with the 5′ end of rat γ1 (using oligonucleotides 490 and 534, and rat genomic DNA as template), an ~118-kb NotI-SgrAI fragment containing the authentic rat γ1, χ2b, ε, α, and 3′ Eε Iγ enhancer region cut out from BAC4 (CH230-597M) and the pBelo-3′Eγ vector with the URA3 vector with the CEN4 joined downstream with a homology tail matching the 3′ end of rat Eε and the CEN4 joined upstream with a tail matching the 5′ end of human V_{H}6-1 as described above. There is a 10.3-kb overlap between the human V_{H}6-1 regions in both the BAC3 and Annabel. The human V_{H}6-1-Ds-JHs followed by the rat CΕ region together with the *s. cerevisiae* URA3 in Annabel can be cut out as a single ~193-kb NotI fragment (see Fig. 1A).

For the assembly of cYACs and BACs from V_{H}3-11, the VH region was extended extensively by restriction analysis and partial sequencing for their authenticity.

Igκ locus. The human Igκ locus on an ~410-kb YAC was obtained by recombination assembly of a VA YAC containing 3 Cκ containing cosmids (23). Rearrangement and expression was verified in transgenic mice derived from ES cells containing one copy of a complete human Igκ YAC (24). This Igκ YAC was shorted by the generation of a cYAC removing ~100 kb of the region 5′ of V_{κ}3-27. The vector pYAC-RC was digested with ClaI and BspEI to remove URA3 and ligated with pUCMinIV MF, a vector containing a NotI apiginc (Invitrogen), which covered a region over 300 kb from 5′ V_{κ}3-17 to 3′ KDE (28). In digests and sequence analysis three overlapping fragments were identified: from V_{κ}3-17 to V_{κ}3-2 (159-kb NotI with ~14-kb overlap), from V_{κ}3-2 to 3′ of Cκ (158-kb NotI with ~40-kb overlap), and from Cκ to 3′ of the KDE (55-kb Pca1 with 40-kb overlap) (Fig. 1B). Overlapping regions may generally favor joint integration when cojected into oocytes (29).

**Gel analyses and DNA purification**

Purified YAC and BAC DNA were analyzed by restriction digest and separation on conventional 0.7% agarose gels (30). Larger fragments (50–200 kb) were separated by PFGE (Bio-Rad CHEF Mapper) at 4°C using 0.8% pulsed field–certified agarose in 0.5 TBE, at 2–20 s switch time for 16 h, 6 V/cm, 10 mA. Purification allowed a direct comparison of the resulting fragments with the predicted size obtained from the sequence analysis. Alterations were analyzed by PCR and sequencing.

Linear YACs, cYACs, and BAC fragments after digests were purified by electroelution using Elutrap (Schleicher and Schuell) (31) from strips cut from 0.8% agarose gels ran conventionally or from PFGE. The DNA concentration was usually several nanograms per microliter in a volume of ~100 nl. For fragments up to ~200 kb the DNA was precipitated and redissolved in microinjection buffer (10 mM Tris-HCl [pH 7.5], 100 mM EDTA [pH 8], and 100 mM NaCl but without spermine/spermidine) to the desired concentration.

The purification of cYACs from yeast was carried out using NucleoBond AX silica-based anion-exchange resin (Macherey-Nagel, Düren, Germany). Briefly, spheroplasts were made using zymolyase or lyticase and pelleted (32). The cells then underwent alkaline lysis, binding to AX100 column and elution as described in the NucleoBond method for a low-copy plasmid.

Contaminating yeast chromosomal DNA was hydroyzed using Plasmid-Safe ATP-dependent DNase (Epicentre Biotechnologies) followed by a final cleanup step using SureClean (BioLine). An aliquot of DH10 electrocompetent cells (Invitrogen) was then transformed with the cYAC to obtain BAC colonies. For microinjection, the insert DNA (150-200 kb) was separated from BAC vector DNA (~10 kb) using a filtration step with Sepharose 4B-CL (33).

**Derivation of rats and breeding**

Purified DNA encoding recombinant Igκ loci was resuspended in microinjection buffer with 10 mM spermine and 10 mM spermidine. The DNA was injected into fertilized oocytes at various concentrations from 0.5 to 3 ng/μl. Plasmid DNA or mRNA encoding zinc finger nucleases (ZFNs) specific for rat Igκ genes were injected into fertilized oocytes at various concentrations from 0.5 to 10 ng/μl.

Microinjections were performed at the Caliper Life Sciences facility and Rat Transgenic Nantes facilities. Outebred SD/Hsd (wild-type [wt]) strain animals were housed in standard microisolator cages under approved animal care protocols in an animal care facility (Nantes) that is accredited by the Association and Accreditation for Laboratory Animal Care. The rats were maintained on a 14–10 h light/dark cycle with ad libitum access to food and water. Four to 5-wk-old SD/Hsd female rats were injected with 20–25 IU pregnant mare serum gonadotropin (Sigma-Aldrich) followed 48 h later with 20–25 IU human chorionic gonadotropin (Sigma-Aldrich) before breeding to outbred SD/Hsd males. Fertilized single-cell stage embryos were collected from a Clad/Tag blot and collected for subsequent microinjection. Manipulated embryos were transferred to pseudopregnant SD/Hsd female rats to be carried to parturition.

Multifunction human Ig rats (human IgH, Igκ, and Igλ in combination with rat J knockout, κKO, and ακO) and wt, as control, were analyzed at 10–18 wk age. The animals were bred at Charles River under specific pathogen-free conditions.

For the generation of the care and use of OmniRat were in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals (available at: http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf), which are
adapted from the requirements of the Animal Welfare Act or regulations concerning the ethics of science research in the INSERM Unité Mixte de Recherche 1064 animal facility and approved by the regional ethics and veterinary commissions (no. F44011).

**PCR and RT-PCR**

Transgenic rats were identified by PCR from tail or ear clip DNA using an Isolgenic genomic DNA mini kit (Bioline). For IgH PCRs ≤1 kb GoTaq Green Master mix was used (Promega) following the general guidelines provided for this enzyme, with details given in Supplemental Table I. For IgL PCRs >1 kb KOD polymerase (Novagen) was used with standard cycling conditions but with an extension time of 90 s. The Igk and Igλ PCR used Green Master mix as described above.

RNA was extracted from blood using the RiboPure blood kit (Ambion) and from spleen, bone marrow, or lymph nodes using the RNAspin mini kit (GE Healthcare). cDNA was made using oligo(dT) and Promega reverse transcriptase at 42˚C for 1 h. GAPDH PCR reactions (oligonucleotides DH1 and DH2) confirmed that RNA extraction and cDNA synthesis were successful. RT-PCRs were set up using Vh leader primers with rat µCH2 or rat γCH2 primers (Supplemental Table I), and GoTaq Green Master mix PCR products of the expected size were either purified by gel or QuickClean (Bioline) and sequenced directly or cloned into pGem-T (Promega).

**Protein purification**

IgM was purified on anti-IgM affinity matrix (CaptureSelect no. 2890.05; BCN, Naarden, The Netherlands) as described in the protocol. Similarly, human Igκ and Igλ was purified on anti-L chain affinity matrix (CaptureSelect anti-Igκ no. 0833 and anti-Igλ no. 0849) according to the protocol. For rat IgG purification (34) protein A and protein G-agarose was used (Innova Biosciences, Cambridge, U.K., nos. 851-0024 and 895-0024). Serum was incubated with the resin and binding was facilitated at 0.1 M sodium phosphate pH 7 for protein G and pH 8 for protein A under gentle mixing. Poly-Prep columns (Bio-Rad) were packed with the mixture and washed extensively with PBS (pH 7.4). Elution buffer was 0.1 M sodium citrate (pH 2.5) and neutralization buffer was 1 M Tris-HCl (pH 9). Electroelution was performed on 4–15% SDS-PAGE and Coomassie brilliant blue was used for staining. Molecular mass standards were HyperPAGE prestained protein marker (BIO-33066; Bioline).

**Flow cytometry analysis and fluorescence in situ hybridization**

Cell suspensions were washed and adjusted to 5 × 10⁷ cells/100 µl in PBS/1% BSA/0.1% sodium azide. Different B cell subsets were identified using mouse anti–rat IgM FITC-labeled mAb (MAM4; Jackson Immuno-Research Laboratories) in combination with anti–B cell CD45R (rat B220; PE-conjugated mAb (His24; BD Biosciences). A FACSCanto II flow cytometer and FlowJo software (Becton Dickinson, Pont de Claix, France) were used for the analysis (35). Fluorescence in situ hybridization was carried out on fixed blood lymphocytes using purified IgH and IgL region BAC (36).

**Immunization, cell fusion, and affinity measurement**

Immunizations were performed with 125 µg pro目gramulin (PGRN) in CFA, 150 µg human growth hormone receptor (hGHR) in CFA, 200 µg TAU/ keyhole limpet hemocyanin (KLH) in CFA, 150 µg hen egg lysozyme (HEL) in CFA, and 150 µg OVA in CFA at the base of the tail and medial iliac lymph node cells were fused with mouse P3X63Ag8.653 myeloma cells 22 days later as described (37). For multiple immunizations, protein, 125 µg PGRN or hen egg lysozyme, or 100 µg human growth hormone receptor or CD45 in GERBU adjuvant (http://www.Gerbu.com) were administered i.p. as follows: days 0, 14, and 28, and day 41 without adjuvant, followed by spleen cell fusion with P3X63Ag8.653 cells 4 days later (3).

Binding kinetics were analyzed by surface plasmon resonance using a Biacore 2000 with the Ags directly immobilized as described (19).

**Results**

The human IgH and IgL loci

Construction of the human Ig loci employed established technologies to assemble large DNA segments using YACs and BACs (23, 29, 38–40). As multiple sequential BAC modifications in *E. coli* frequently led to the deletion from the BAC of repetitive regions such as Ig switch sequences or of elements in the vicinity of the IgH 3′ enhancers, a strategy was developed to assemble these large transloci by homologous recombination in *S. cerevisiae* as cYAC and, subsequently, converting such a cYAC into a BAC. The advantages of YACs include their large size, their sequence stability, and the ease of homologous alterations in the yeast host. BACs propagated in *E. coli* offer the advantages of easy preparation and large yield. Furthermore, detailed restriction mapping and sequencing analysis can be better achieved in BACs than in YACs.

The structures of the assembled chimeric IgH (human V₃, D, and J₃ segments followed by rat C genes) and human Igκ BACs as well as of the human Igλ YAC are depicted in Fig. 1. The integrated IgH and IgL transloci were then generated by coinjecting multiple BACs into fertilized rat oocytes, exploiting the previous finding that coinjection of overlapping DNA constructs often leads to cointegration into the genome (29). Thus, the IgH translocus was created by coinfection of BAC6-VH3-11 (a 182-kb AsnI-AscI fragment containing 13 V₃(18) with BAC3 (a 173-kb NolI fragment containing 10 V₃(18) and BAC3-1N12M518 (human/rat Annabel, a 193-kb NolI fragment containing human V₃(6)-1 and all Ds and J₃(8) followed by the rat C region). This resulted in the reconstitution of a fully functional IgH locus in the rat genome. Similarly, the human Igκ locus was integrated by homologous overlaps (D9 containing Vκ genes, a 150-kb NolI fragment; E24, containing Vκs, Jκs, and Cκ on a 150-kb NolI fragment; and F17, a 40-kb Pael fragment containing Jκs, Cκ, and the KDE). The human Igλ locus was isolated intact as an ~300-kb YAC and also fully inserted into a rat chromosome. The integration success was identified in several founders each by transcript analysis that showed V(D)J-C recombinations from the most 5′ to the most 3′ end of the locus injected. Multiple BAC insertions were identified by quantitative PCR using V₃(18) and C₃(18-specific oligonucleotides (not shown) and it is likely that head-tail integrations occurred. In all cases, transgenic animals with single-site integrations were generated by breeding.

Breeding to homozygosity

The derivation of the transgenic rats by DNA microinjection into oocytes, as well as their breeding and immunization, were carried out by a strategy similar to that previously used with the humanized mice (15, 16, 41). However, a different approach was needed to achieve inactivation of the endogenous rat Ig loci because targeted gene inactivation in embryonic stem cells is not a technology that has been developed in the rat. We therefore used ZFN technology, an approach that has only been reported recently (42, 43), to obtain rat lines with targeted inactivation of their IgH, Igκ, and Igλ loci (the inactivation of the rat IgH locus was described in Ref. 35, and a manuscript describing inactivation of rat Igκ and Igλ is in preparation [by M.J. Osborn, S. Avis, R. Buelow, and M. Brüggemann]).

Analysis of the translocus integration by PCR as well as by fluorescence in situ hybridization (FISH) (Table I) revealed integration of all injected BACs in completion. Several founder rats carried low translocus copy numbers, with the rat C gene BAC in OmniRat likely to be fully integrated in five copies as determined by quantitative PCR of C₃μ and C₄α products (not shown). Identification by FISH of single position insertion in many lines confirmed that multiple integration of BAC mixtures into different rat chromosomes was rare. Rats carrying the individual human transloci (IgH, Igκ, and Igλ) were crossed successfully to homozygosity Igλ locus KO rats. This produced a highly efficient new multilineage line (OmniRat) with human V₃-D-J₃ regions of ~400 kb containing 22 functional V₃(18) and a rat C region of ~116 kb.

**B cell development in the KO background**

Flow cytometric analyses were performed to assess whether the introduced human Ig loci were capable of reconstituting normal B cell development. Particular differentiation stages were analyzed...
in spleen and bone marrow lymphocytes (Fig. 2), which previously showed a lack of B cell development in JKO/JKO rats (35), as well as no respective IgL expression in kKO/kKO and lKO/lKO animals (data not shown). Most striking was the complete recovery of B cell development in OmniRat compared with wt animals, with similar numbers of B220(CD45R)+ lymphocytes in bone marrow and spleen. IgM expression in a large proportion of CD45R+ B cells marked a fully reconstituted immune system. Separation of spleen cells was indistinguishable between OmniRat and wt animals and thus was successfully restored in the transgenic rats expressing human idiotypes with rat C region. Moreover, a small population of surface IgG+ spleen lymphocytes was present in OmniRat (Fig. 2, right).

Other lymphoid populations (as judged by flow cytometric staining for CD3, CD4, and CD8) were unaltered in OmniRat as compared with control animals (data not shown), which further supports the notion that optimal immune function has been completely restored.

Table I. Generated rat lines: transgenic integration, KO, and gene usage

<table>
<thead>
<tr>
<th>Rat Line</th>
<th>Human V\textsubscript{H}</th>
<th>BAC6-VH3-11 (182 kb)</th>
<th>BAC3 (173 kb)</th>
<th>Rat C\textsubscript{\lambda} (Annabel) (193 kb)</th>
<th>Human Igk BACs (300 kb)</th>
<th>Human Ig\textsubscript{\lambda} YAC (300 kb)</th>
<th>ZFN KO</th>
<th>Igk KO</th>
<th>Ig\textsubscript{\lambda} KO</th>
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</table>

OmniRat (HC14JKOJCO/KKOKKOLKOLKO/79/6262) is the product of breeding three translocus features (human/rat IgH, human Igk, and human Ig\textsubscript{\lambda}) with three KO lines (rat JH, Ck, and JC\textsubscript{\lambda}).

FIGURE 2. Flow cytometry analysis of lymphocyte-gated bone marrow and spleen cells from 3-mo-old rats. Surface staining for IgM and CD45R (B220) revealed a similar number of immature and mature B cells in bone marrow and spleen of OmniRat (HC14 JKOKKO/LKOLKO HuL) and wt animals, whereas JKO/JKO animals showed no B cell development. Plotting forward scatter (FSC) against side scatter (SSC) showed comparable numbers of lymphocyte (gated) populations, concerning size and shape. Surface staining of spleen cells with anti-IgG (G1, G2a, G2b, G2c isotype) revealed near normal frequency of IgG+ expressers in OmniRat animals compared with wt. In bone marrow: A, pro/pre–B cells (CD45R\textsuperscript{+}IgM\textsuperscript{2}); B, immature B cells (CD45R\textsuperscript{+}IgM\textsuperscript{+}). In spleen: A, lymphocyte precursors (CD45R\textsuperscript{+}IgM\textsuperscript{+}); B, follicular B cells (CD45R\textsuperscript{+}IgM\textsuperscript{+}); C, marginal zone B cells (CD45R\textsuperscript{+}IgM\textsuperscript{+}).
Diverse human H and L chain transcripts

Analysis of Ig V, D, and J gene usage by RT-PCR of transcripts present in splenic or PBLs revealed that all of the human VH and VL genes present in the Ig transloci in OmniRat and regarded as functional (44) were used (Table II). Human VH genes were associated with diverse human D and JH segments linked to both rat Cμ and CG. Similarly, RT-PCR analysis of L chain transcripts showed extensive use and diversity.

The analysis of class switch and hypermutation (Fig. 3) showed that both of these processes are operating effectively on the OmniRat IgH locus. Amplification of IgG switch products from PBLs revealed an extensive rate of mutation (>2 aa changes) in most cells (~80%) and in near equal numbers of γ1 and γ2b H chains. A small percentage of trans-switch sequences, γ2a and 2c, were also identified, which supports the observation that the translocus is similarly active, but providing human (VH-D-JH)s, as the endogenous IgH locus (45). The number of mutated human IgL and Igk L chain sequences is ~30% and thus appears to be less pronounced than what has been found for IgG H chains. The reason is that L chain RT-PCR products are amplified from both IgM, which is less mutated, and IgG-producing cells rather than from IgG+ or differentiated plasma cells.

Table II. Productive V, D, and J usage in PBL transcripts obtained by RT-PCR with group-specific V oligonucleotides to μCμ2 or γCγ2 for IgH, and to Cλ and Cκ for IgL.

<table>
<thead>
<tr>
<th>VH/D/JH</th>
<th>VH/D/JH</th>
<th>VH/D/JH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hu L #6.2</td>
<td>1 2 3</td>
<td>1 2 3</td>
</tr>
<tr>
<td>Hu K #79</td>
<td>1 2 3 4 5</td>
<td>1 2 3</td>
</tr>
</tbody>
</table>

* Unproductive.

FIGURE 3. Mutational changes in IgH and IgL transcripts from PBLs. Germline Vs are listed on the horizontal axes and amino acid changes on the vertical axes. Unique (VH[DJH])s and VLs were from amplifications with V group–specific primers: IGHV1, 2, 3, 4, and 6 in combination with the universal γCγ2 reverse primer; IGLV2, 3, and 4 with reverse Cλ primer; and IGKV1, 3, 4, and 5 in with reverse Cκ primer (Supplemental Table I). Mutated trans-switch products were identified for human VH-rat Cγ2a (4) and human VH-rat Cγ2c (2).
Ig levels in serum

To gain unambiguous information about Ab production we compared quality and quantity of serum Ig from ~3-mo-old OmniRat and normal wt animals housed in pathogen-free facilities. Purification of IgM and IgG separated on SDS-PAGE under reducing conditions (Fig. 4) showed the expected size, that is, ~75 kDa for μ, ~55 kDa for γ H chains, and ~25 kDa for L chains, and was indistinguishable between OmniRat and wt animals. The yield of Ig from serum was found in both OmniRat and wt animals to be 100–300 μg/ml for IgM and 1–3 mg/ml for IgG. However, as rat IgG purification on protein A or G is seen as suboptimal (34), rat Ig levels may be underrepresented. The results from these naive animals compares well with the IgM levels of 0.5–1 mg/ml and IgG levels of several milligrams per milliliter reported for rats kept in open facilities (46, 47). Interestingly, we were able to visualize class-specific mobility of rat IgG isotypes on SDS-PAGE (34) with a distinct lower size band for γ2a H chains (Fig. 4B). This band is missing in OmniRat owing to the lack of γ2a in the translocus. However, because the IgG levels were similar between OmniRat and wt animals, we assume class switching is similarly efficient, albeit using different C genes. Purification of human IgG and IgA by capturing with anti–L chain was also successful (Fig. 4C, 4D) with H and L chain bands of the expected size. Confirmation of the IgM/G titers was also obtained by ELISA, which determined wt and OmniRat isotype distribution and identified comparable amounts of IgG1 and IgG2b (not shown).

A direct comparison of human Ig L chain titers in solid phase titrations (Fig. 4E, 4F) revealed 5- to 10-fold lower levels in OmniRat animals than in human serum. However, this was expected, as human control serum from mature adults can sometimes contain >10-fold higher Ig levels than in children up to their teens (48), which would be similar to the human IgG and IgA titers in young rats. Although wt rats produce very little endogenous IgA, transgenic rats can efficiently express both types of human L chain, Igκ and Igλ.

Fully human Ag-specific IgG

Several cell fusions were carried out using either a rapid one immunization scheme and harvesting lymph nodes or, alternatively, using booster immunizations and spleen cells (Table III, Table IV). For example, a considerable number of stable hybridomas were obtained after one immunization with human PGRN and myeloma fusion 22 d later. In this study, cell growth was observed in ~3520 and ~1600 wells in SD control and OmniRat hybridoma clones, respectively. Anti–PGRN-specific IgG, characterized by biosensor measurements, was produced by 148 OmniRat clones. Limiting dilution, to exclude mixed wells, and repeat affinity measurements revealed that OmniRat clones retain their Ag specificity. A comparison of association and dissociation rates of Abs from SD and OmniRat clones showed similar affinities between 0.3 and 74 nM (Tables III, IV, and data not shown). Single immunizations with hGHR, TAU receptor coupled to KLH (TAU/KLH), HEL, or OVA, followed by lymph node fusions, also produced many high-affinity human Abs often at similar numbers compared with wt.

Furthermore, conventional booster immunizations with human PGRN, hGHR, human CD14, and HEL resulted in high affinities (picomolar range) of IgG with human idiotypes. OmniRat animals always showed the expected 4- to 5-log titer increase of Ag-specific IgG, similar to and as pronounced as wt rats (Table III). Although the results could vary from animal to animal, comparable numbers of hybridomas producing Ag-specific Abs with similarly high affinities were obtained from wt animals (SD and other strains) and the OmniRat strain. A summary of individual IgG-producing lymph node and spleen cell fusion clones, showing their diverse human VH-D-JH, human Vk-Jκ, or Vκ-Jκ characteristics and affinities, are presented in Table IV. The immunization and fusion results showed that affinities well <1 nM (determined by biosensor analysis) were frequently obtained from OmniRat animals immunized with PGRN, CD14, TAU, HEL, and OVA Ags. In summary, Ag-specific hybridomas from OmniRats could be as easily generated as from wt animals yielding numerous mAbs with subnanomolar affinity even after a single immunization.

Discussion

Assembling a novel IgH locus comprising human VH, D, and JH gene segments linked to a large part of the rat CH region has resulted in a highly efficient and near-normal expression level of Abs with human idiotypes. The combination of this chimeric IgH

![FIGURE 4](http://jimmunol.org/)

Purification of rat Ig with human idiotypes and comparison with human and normal rat Ig levels. OmniRat serum and human or rat wt control serum, 100 μl each, was used for IgM/G purification. (A) IgM was captured with anti-IgM matrix, which identified 14 μg in wt rat and 30 and 10 μg in OmniRat animals (HC14(a) and HC14(b)). (B) IgG was purified on protein A and protein G columns, with a yield of up to ~3 mg/ml for OmniRat (protein A: HC14(a), 1000 μg/ml; HC14(b), 350 μg/ml; wt rat, 350 μg/ml; protein G: HC14(a), 2970 μg/ml; HC14(b), 280 μg/ml; wt rat, 1010 μg/ml). (C) Human Igκ and (D) human Igλ was purified on anti-Igκ and anti-Igλ matrix, respectively. No purification product was obtained using wt rat serum (not shown). Purified Ig, ~3 μg (concentration determined by NanoDrop), was separated on 4–15% SDS-PAGE under reducing conditions. Comparison by ELISA titration of (E) human Igκ and (F) human Igλ levels in individual OmniRat animals (~3531, 8322, 8199, 8486, 8055), human, and wt rat serum. Serum dilution (1:10, 1:100, 1:1,000, 1:10,000) was plotted against binding measured by adsorption at 492 nm. Matching name/numbers refer to samples from the same rat.
locus with human Igκ and Igλ loci has further revealed that chimeric Ab with fully human specificity is readily produced by the rats and that these chimeric IgH chains associate well with human IgL chains.

The excellent performance of these transgenic Ig loci with respect to the reconstitution of B cell development, the high titers of serum Ig, and the efficacy with which high-affinity Abs are obtained most probably derives from the fact that the C region of the IgH translocus is of endogenous (rat) origin. This could be reflected in several aspects of its performance. The quality of an immune response is known to rely on the combined actions of many signaling and modifier components associated with the B cell Ag receptor (see: http://www.biocarta.com/pathfiles/h_bcrpathway.asp). The use of endogenous rat sequences (53). The use of endogenous rat sequences

### Table III. Ag-specific rat IgG hybridomas with fully human idiotypes

<table>
<thead>
<tr>
<th>Animal</th>
<th>Ag</th>
<th>Cells</th>
<th>Fusion</th>
<th>Titer</th>
<th>Hybrids</th>
<th>IgGs</th>
<th>K_{D} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>PGRN</td>
<td>LN</td>
<td>1</td>
<td>38,400</td>
<td>3,520</td>
<td>38</td>
<td>0.3–1.0</td>
</tr>
<tr>
<td>OmniRat</td>
<td>PGRN</td>
<td>LN</td>
<td>1</td>
<td>12,800</td>
<td>1,600</td>
<td>148</td>
<td>0.7–2.4</td>
</tr>
<tr>
<td>SD</td>
<td>PGRN</td>
<td>SP</td>
<td>1</td>
<td>51,200</td>
<td>8,000</td>
<td>29</td>
<td>ND</td>
</tr>
<tr>
<td>OmniRat</td>
<td>PGRN</td>
<td>SP</td>
<td>1</td>
<td>85,600</td>
<td>40,000</td>
<td>24</td>
<td>ND</td>
</tr>
<tr>
<td>OmniRat</td>
<td>hGHR</td>
<td>LN</td>
<td>3</td>
<td>4,800</td>
<td>704–1,024</td>
<td>18.3</td>
<td>2</td>
</tr>
<tr>
<td>SD</td>
<td>hGHR</td>
<td>SP</td>
<td>1</td>
<td>204,800</td>
<td>53,760</td>
<td>230</td>
<td>&lt;0.07–0.4</td>
</tr>
<tr>
<td>OmniRat</td>
<td>hGHR</td>
<td>SP</td>
<td>1</td>
<td>76,800</td>
<td>53,760</td>
<td>7</td>
<td>0.16–2.4</td>
</tr>
<tr>
<td>OmniRat</td>
<td>CD14</td>
<td>SP</td>
<td>2</td>
<td>102,400</td>
<td>2,800–3,500</td>
<td>54</td>
<td>14</td>
</tr>
<tr>
<td>SD</td>
<td>TAU/KHL</td>
<td>LN</td>
<td>1</td>
<td>20,000</td>
<td>1,728</td>
<td>99</td>
<td>0.6–2.4</td>
</tr>
<tr>
<td>OmniRat</td>
<td>TAU/KHL</td>
<td>LN</td>
<td>1</td>
<td>4,800</td>
<td>1,880</td>
<td>118</td>
<td>0.5–3.2</td>
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<tr>
<td>SD</td>
<td>HEL</td>
<td>LN</td>
<td>1</td>
<td>12,800</td>
<td>1,564</td>
<td>26</td>
<td>0.6–0.1</td>
</tr>
<tr>
<td>OmniRat</td>
<td>HEL</td>
<td>LN</td>
<td>3</td>
<td>25,600</td>
<td>288–640</td>
<td>0.2</td>
<td>7</td>
</tr>
<tr>
<td>SD</td>
<td>HEL</td>
<td>SP</td>
<td>1</td>
<td>6,400</td>
<td>30,720</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>SD</td>
<td>OVA</td>
<td>LN</td>
<td>1</td>
<td>9,600</td>
<td>1,488</td>
<td>10</td>
<td>1.1–4.8</td>
</tr>
<tr>
<td>OmniRat</td>
<td>OVA</td>
<td>LN</td>
<td>4</td>
<td>8,000</td>
<td>512–2,240</td>
<td>0</td>
<td>30, 0, 1</td>
</tr>
</tbody>
</table>

OmnIRat animals (HC14/Hus and/or HuQJ/KOKO/KKOKKO) and control SD rats were immunized with human PGRN, hGHR, human CD14, TAU peptide (TAU/KHL), HEL, or OVA, LN, lymph node; SP, spleen cell.

*Ag specificity confirmed by biosensor analysis.

**Range of five highest affinities.

†Eight mAbs were specific for Tau peptide.

### Table IV. Affinity and V gene diversity of IgG \(^{+}\) hybridoma clones

<table>
<thead>
<tr>
<th>Ag</th>
<th>Fusion Cells</th>
<th>Clone</th>
<th>K_{D} (nM)</th>
<th>IGHV</th>
<th>IGHJ</th>
<th>IGHD</th>
<th>IGK</th>
<th>CDR3</th>
<th>IGe/kJ</th>
<th>Amino Acid Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGRN</td>
<td>LN</td>
<td>8080.1B2</td>
<td>0.7</td>
<td>4-31</td>
<td>2</td>
<td>7-27</td>
<td>3</td>
<td>CATGTCEDAFDW</td>
<td>LV3-10</td>
<td>1</td>
</tr>
<tr>
<td>PGRN</td>
<td>LN</td>
<td>8080.2B3</td>
<td>1.4</td>
<td>3-23</td>
<td>1</td>
<td>3-4</td>
<td>3</td>
<td>CAGKGSLSITTPFDYW</td>
<td>LV3-19</td>
<td>2</td>
</tr>
<tr>
<td>hGHR</td>
<td>LN</td>
<td>9046.8A3</td>
<td>2.4</td>
<td>1-2</td>
<td>6</td>
<td>1-19</td>
<td>3</td>
<td>CARVQWMJNADFW</td>
<td>LV2-14</td>
<td>9</td>
</tr>
<tr>
<td>hGHR</td>
<td>LN</td>
<td>9046.6E10</td>
<td>4.2</td>
<td>1-7</td>
<td>7</td>
<td>3-16</td>
<td>4</td>
<td>CARKGDAFDYW</td>
<td>LV2-23</td>
<td>5</td>
</tr>
<tr>
<td>TAU/KHL</td>
<td>LN</td>
<td>8898.2B10</td>
<td>0.8</td>
<td>4-39</td>
<td>5</td>
<td>3-22</td>
<td>4</td>
<td>CARHRYYARDSGTYTPFDYW</td>
<td>KV4-1</td>
<td>0</td>
</tr>
<tr>
<td>OVA</td>
<td>LN</td>
<td>9477.2A9</td>
<td>3.9</td>
<td>3-11</td>
<td>5</td>
<td>3-10</td>
<td>4</td>
<td>CARAYYYGSGSSLFDYW</td>
<td>KV1-17</td>
<td>1</td>
</tr>
<tr>
<td>HEL</td>
<td>SP</td>
<td>H2</td>
<td>0.9</td>
<td>3-23</td>
<td>15</td>
<td>6-19</td>
<td>4</td>
<td>CARKRESWSNYPFDYW</td>
<td>KV3-11</td>
<td>1</td>
</tr>
<tr>
<td>HEL</td>
<td>SP</td>
<td>3C10</td>
<td>0.8</td>
<td>6-1</td>
<td>1</td>
<td>6-19</td>
<td>4</td>
<td>CARGSSSSGFFQFW</td>
<td>KV1-5</td>
<td>0</td>
</tr>
<tr>
<td>β-gal</td>
<td>SP</td>
<td>5005.6C1</td>
<td>ND</td>
<td>6-1</td>
<td>5</td>
<td>2-21</td>
<td>4</td>
<td>CARTPRGLPFDYW</td>
<td>KV1-12</td>
<td>0</td>
</tr>
</tbody>
</table>

*Individual clones from the fusions in Table III.
integration of YACs into stem cells and subsequent animal derivation (39, 55); this can prove quite laborious, especially in animals such as rats where there is limited experience with stem cell technology.

A further major aspect of the technical strategy that had facilitated the creation of OmniRat was the use of ZFN technology in fertilized rat oocytes to inactivate the endogenous Ig loci (35, 42). Because there is no established method for targeted gene recombination in rat embryonic stem cells, we had to devise a strategy distinct from that which has been previously used for target gene inactivation in the mouse. However, the ready success of this application of ZFN technology in rat eggs suggests that this may well be the future technology of choice for gene disruptions and replacement.

The diverse high expression of the transgenic Ig loci in OmniRat is further demonstrated in rats in which an endogenous Ig locus was intact and good titers of Ag-specific human Ig as well as hybridomas expressing high-affinity human mAbs could be obtained following immunization. Thus, in these rats containing a chimeric human/rat IgH locus together with human IgL translocus, the transloci compete very effectively in terms of performance with the endogenous rat Ig loci. A comparison of immunization results, based on Ag-binding and isotype (see Tables III, IV), would make it near impossible to identify whether the results were obtained from normal wt rats or from OmniRat. This appears to be very different from the selected transgenic human Ab results made available and from the experience we had respective to the relative performance of the transloci and endogenous loci in mice carrying fully human IgH transloci (15, 19, 55).

Following fusions of spleen and lymph node cells, OmniRat yielded a range of specific IgG Abs in response to immunization with a variety of Ags. These Abs displayed a diversity in epitope recognition comparable to that obtained using wt control rats. The molecular diversity of the Abs produced was considerable, with contributions as anticipated (44) from nearly all the V, D, and J gene sequences on the transloci segments. This was in stark contrast to some mice carrying fully human transloci where selective clonal expansion of relatively few precursor B cells was found to yield only limited molecular diversity (19, 55). Thus, for example, five-feature mice expressing fully human Ab repertoires showed a substantial reduction in the frequency of IgM* B cells in the bone marrow from the pre–B-cell stage onward: frequencies were 21% of those observed in wt mice (56). The five-feature mice also showed a substantial reduction of splenic IgM* B cells (~35% of controls) (17). Furthermore, although the extent of this reduction was variable, the frequency of splenic IgM* B cells in the humanized five-feature mice was always less than that in controls, whereas OmniRat consistently gave the same frequency of splenic IgM* cells as observed in wt animals.

The fact that the number of transplanted V genes in OmniRat is only about half of those present in humans does not appear to have led to any significant restriction in the diversity of the immune response. Comparison of the CDR3 diversity in >1000 B cell clones (sequences can be provided) revealed the same extensive junctional differences in OmniRat animals as observed in wt control rats. When identical combinations of V, D, and J segments were very occasionally observed, differences between these sequences due to either N sequence addition/deletion or hypermutation were nevertheless observed. Extensive diversity was also seen for the introduced human Igs and IgA loci, similar to what has previously been observed with mice transgenic for human Ig loci (17, 19, 24). Hence, the compromised efficiency in the production of human Abs observed with mice carrying fully human Ig transloci (13) has been overcome in OmniRat: these rats generate enormous diversity of V(D)J gene rearrangements from their transloci with efficient subsequent somatic hypermutation and class switching, leading to the production of high-affinity IgG Abs as a matter of routine. The yield of transgenic serum IgG and the level of IgV gene somatic hypermutation observed in the Ag-specific mAbs obtained from the OmniRat strain revealed that clonal diversification and levels of serum Ab production were similar in OmniRat and control animals. Routine generation of high-affinity specificities in the subnanomolar range was accomplished by different single immunizations and compared favorably with wt animals.

In summary, this reveals that to maximize human Ab production, the best approach is to use an IgH locus with human V(D)J gene segments, so as to yield human Ag-specific binding sites, but rodent C genes and control sequences to ensure efficient B cell differentiation, high Ab expression, and diversification. For therapeutic applications, the rat C\textsubscript{H} regions in mAbs obtained from OmniRat can readily be replaced by human C\textsubscript{H} regions without compromising Ag-specificity during the bulking up phase of mAb production.

Acknowledgments

We acknowledge that some microinjections for the generation of transgenic rats were performed at the Taconic Farms, Inc. facility located in Cranbury, NJ. Breeding and genotyping of the Open Monoclonal Technology, Inc. OmniRat strain was performed at Charles River Laboratories (Wilmington, MA), and FISH analysis was carried out by Cell Line Genetics (Madison, WI). We are grateful to G. Davis for critical discussion and comments on the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


