High-Affinity IgG Antibodies Develop Naturally in Ig-Knockout Rats Carrying Germline Human IgH/Igκ/Igλ Loci Bearing the Rat C_H Region

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Errata
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High-Affinity IgG Antibodies Develop Naturally in Ig-Knockout Rats Carrying Germline Human IgH/Igκ/Igλ Loci Bearing the Rat CH Region

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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 VHs, all human D and JH segments in natural configuration linked to the rat CH locus) together with fully human IgL loci (12 Vks linked to Jc-Cc and 16 VAs 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.

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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 have been 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 IgV regions, D, and J regions have been linked to germline-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 VH3-11 to VH6-1 (811L16 RPCI-11). A BAC termed Annabel was constructed by joining rat CH containing the authentic region spanning from VH4-39 to VH3-23 followed by VH3-11 (modified from a commercially available BAC clone 3054M17 CITB) and BAC3 containing full enhancer sequences. A PmlI–SphI fragment containing the authentic region with 17 Vlkl, 9 Vlkl, and a hygromycin-resistance expression cassette (HygR) was cut out and ligated to ApaLI and BamHI or HpaI and SphI doubly digested pBAC-1380 cells via spheroplast transformation (22), and S. cerevisiae Subsequently, the ligation mixture was transformed into S. cerevisiae CEN4, which can also be romp YAC-RC. The resulting ligation mixture was transformed into S. cerevisiae CEN4 cloned in between S. cerevisiae URA3 and a hygromycin-resistance expression cassette (HygR). From this plasmid, an ApaLI-BamHI fragment containing URA3 followed by CEN4 or a PmlI–SphI fragment containing CEN4 followed by HygR was cut out and ligated to ApaLI and BamHI or HpaI and SphI doubly digested pBAC-Belo11 (New England BioLabs) to yield pBAC-CEN-URA and pBelo-CEN-HYG.

To construct BAC6-VH3-11, initially two fragments, a 115-kb NotI-Pmel and a 110-kb Rsfl-SgraI, were cut out from the BAC clone 3054M17 CITB. 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-BglII YAC arm containing S. cerevisiae CEN4 as well as TRP1/ARS1 from pYAC-RC, and the Rsfl-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 URA3/TRP1 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 cYAC. 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 HpaI sites...
with a 5′ tail matching the sequence upstream of Vp6-39 and a 3′ tail matching downstream of Vp6-23 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 Vp6-23 as described above and a unique Ascl 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 3′-end fragment with a tail matching the sequence upstream of Vp6-39 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 TrpR 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 HpaI-digested pBeloHYG-YAC6+ BAC3(5′) was used to transform yeast cells carrying YAC6C, and through homologous recombination a YAC BAC La2-3-11 containing part of the Hyg end R plus 238. Plugs were made (26) and yeast chromosomes were separated from BAC vector DNA (27) and eluted as described in the NucleoBond method for a low-copy AX silica-based anion-exchange resin (Macherey-Nagel, Düren, Germany). Bacterial colonies were collected for subsequent microinjection. Manipulated embryos were reared for 10–18 wk age. The animals were bred at Charles River under specific pathogen-free conditions.

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 8˚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.

For the assembly of the C region with the V3 overhang, the human Vp6-1-Ds-JH6 regions were ligated to the authentic rat μa, θc, and ϵc region as well as PCR fragments, were used. Five overlapping fragments were identified: from Vp6-1 to Vp6-23 (23); from Vp6-23 to 3′ of Cεε2b (158-kb NotI with ~14-kb overlap), from Vp6-3′ to 3′ of Cε (158-kb NotI with ~40-kb overlap), and from Cε to 3′ of the KDE (55-kb Pacl with 40-kb overlap) (Fig. 1B). Overlapping regions may generally favor joint integration when co-injected into oocytes (29).

Derivation of rats and breeding

Purified DNA encoding recombinant Ig 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 Ig 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. Outfed SD/Hsd (wild-type [wt]) strain animals were housed in standard microisolator cages under approved animal care protocols. Animals that are specific pathogen-free have received specific training from the Assessment 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 within 24 h after copulation for subsequent microinjection. Manipulated embryos were transferred to pseudopregnant 501 Hsd female rats to be carried to parturition.

Multifunctional human rat lgs (human IgH, IgG, and IgA in combination with rat J knockout [KO], κKo, and κKo 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 assessment 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

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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 Isogenomic 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 IgG PCRs >1 kb KOD polymerase (Novagen) was used with standard cycling conditions but with an extension time of 90 s. The Igks and IgG 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 RNA spin mini kit (GE Healthcare). cDNA was made using oligo(dT) and Promega reverse transcriptase at 42°C for 1 h. GAPDH PCR reactions (oligonucleotides 429–430) confirmed that RNA extraction and cDNA synthesis were successful. RT-PCRs were set up using Vх leader primers with rat μCp2 or rat γCp2 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; BAAC, Naarden, The Netherlands), as described in the protocol. Similarly, human Igk and Igλ was purified on anti-λ chain affinity matrix (CaptureSelect anti-IgX 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).

Electrophoresis 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). For rat IgG purification (34) protein A and protein G-agarose was used (Innova Biosciences, Cambridge, U.K., nos. 851-0024 and 895-0024).

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

Cell suspensions were washed and adjusted to 5 × 10^6 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 (MARM4; Jackson ImmunoResearch 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 C region BAC (36).

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

Immunizations were performed with 125 μg programulin (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 CD14 in GE/RBU 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 VH, D, and JH segments followed by rat C genes) and human Igk BACs as well as of the human Igλ YAC are depicted in Fig. 1. The integrated IgH and IgL transloci were then generated by coinserting 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 coinjection of BAC6-VH3-11 (a 182-kb AsI-I-AseI fragment containing 13 VхJs) with BAC3 (a 173-kb NotI fragment containing 10 Vυs) and BAC3-1N2M518 (human/rat Annabel, a 193-kb NotI fragment containing human Vυ6-1 and all Ds and Jυ8 followed by the rat C region). This resulted in the reconstitution of a fully functional IgH locus in the rat genome. Similarly, the human Igx locus was integrated by homologous overlaps (D9 containing Vk genes, a 150-kb NotI fragment; E24, containing VyS, JxS, and Cx on a 150-kb NotI fragment; and F17, a 40-kb PaeI fragment containing JxS, Cx, 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 VyF- and Cγ3-specific oligonucleotides (not shown) and it is likely that head-to-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, Igk, and Igλ loci (the inactivation of the rat IgH locus was described in Ref. 35, and a manuscript describing inactivation of rat Igx 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 sequencing analysis can be better achieved in BACs than in YACs. Further detailed restriction mapping and sequencing analysis can be better achieved in BACs than in YACs.

**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 λKO/λKO 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&lt;sub&gt;H&lt;/sub&gt;</th>
<th>Rat C&lt;sub&gt;H&lt;/sub&gt; (Annabel)</th>
<th>Human Ig&lt;sub&gt;k&lt;/sub&gt; BACs (300 kb)</th>
<th>Human Ig&lt;sub&gt;λ&lt;/sub&gt; YAC (300 kb)</th>
<th>ZFN KO</th>
<th>Rat Chromosome (FISH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OmniRat</td>
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<td>✓</td>
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<td></td>
</tr>
</tbody>
</table>

OmniRat (HC14JKOJKO/KKOKKO/LKOLKO/79/6262) is the product of breeding three translocus features (human/rat IgH, human Ig<sub>k</sub>, and human Ig<sub>λ</sub>) with three KO lines (rat JH, C<sub>k</sub>, and JC<sub>λ</sub>).

**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 JKOJKO/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<sup>+</sup> expressers in OmniRat animals compared with wt. In bone marrow: A, pro/pre–B cells (CD45R<sup>+</sup>IgM<sup>-</sup>); B, immature B cells (CD45R<sup>+</sup>IgM<sup>-</sup>); C, marginal zone B cells (CD45R<sup>+</sup>IgM<sup>-</sup>). In spleen: A, lymphocyte precursors (CD45R<sup>+</sup>IgM<sup>-</sup>); B, follicular B cells (CD45R<sup>+</sup>IgM<sup>-</sup>); C, marginal zone B cells (CD45R<sup>+</sup>IgM<sup>-</sup>).
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 Cm 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 μCγ2 or γCγ2 for IgH, and to Cl and Cκ for IgL.

<table>
<thead>
<tr>
<th>IgH V</th>
<th>IgH D</th>
<th>IgH J</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC14</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>JC14</td>
<td>×</td>
<td>×</td>
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<table>
<thead>
<tr>
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<th>IgL J</th>
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</thead>
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<table>
<thead>
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<th>IgK V</th>
<th>IgK J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hu K #79</td>
<td>1 2 3 4 5</td>
</tr>
</tbody>
</table>

*: Unproductive.
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 Cγ2a 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 Igλ, 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 serum 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 Vκ-D-Jκ, human Vκ-Jκ, or Vκ-Jα 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 CIH region has resulted in a highly efficient and near-normal expression level of Abs with human idiotypes. The combination of this chimeric IgH
Table III. Ag-specific rat IgG hybridomas with fully human idiotypes

<table>
<thead>
<tr>
<th>Animal</th>
<th>Ag</th>
<th>Cells</th>
<th>Fusions</th>
<th>Titer</th>
<th>Hybrids</th>
<th>IgGs</th>
<th>Kd (nM)</th>
<th>Range of five highest affinities</th>
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<td>SD</td>
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<td></td>
<td></td>
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<tr>
<td>OmniRat</td>
<td>PGRN LN 1</td>
<td>12,800</td>
<td>1,600</td>
<td>148</td>
<td>0.7–2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>PGRN SP 1</td>
<td>51,200</td>
<td>8,000</td>
<td>29</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>OmniRat</td>
<td>PGRN SP 1</td>
<td>51,200</td>
<td>36,000</td>
<td>24</td>
<td>ND</td>
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<tr>
<td>OmniRat</td>
<td>hGHR LN 3</td>
<td>4,800</td>
<td>704–1,024</td>
<td>18.3</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>hGHR SP 1</td>
<td>204,800</td>
<td>53,760</td>
<td>230</td>
<td>&lt;0.07–0.4</td>
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<tr>
<td>OmniRat</td>
<td>hGHR SP 1</td>
<td>76,800</td>
<td>53,760</td>
<td>7</td>
<td>0.16–2.4</td>
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<td></td>
<td></td>
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<tr>
<td>OmniRat</td>
<td>CD14 SP 2</td>
<td>102,400</td>
<td>2,800–3500</td>
<td>54,14</td>
<td>&lt;0.1–0.2</td>
<td></td>
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<td></td>
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<tr>
<td>SD</td>
<td>TAU/KLH LN 1</td>
<td>20,000</td>
<td>1,728</td>
<td>99</td>
<td>0.6–2.4</td>
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<td></td>
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<tr>
<td>OmniRat</td>
<td>TAU/KLH LN 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 LN 1</td>
<td>12,800</td>
<td>1,564</td>
<td>26</td>
<td>0.02–0.1</td>
<td></td>
<td></td>
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<tr>
<td>OmniRat</td>
<td>HEL LN 3</td>
<td>25,600</td>
<td>288–640</td>
<td>0.2–7</td>
<td>0.6–1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>HEL SP 1</td>
<td>6,400</td>
<td>30,720</td>
<td>0</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>OVA LN 1</td>
<td>9,600</td>
<td>1,488</td>
<td>10</td>
<td>1.1–4.8</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>OmniRat</td>
<td>OVA LN 4</td>
<td>8,000</td>
<td>512–2240</td>
<td>0</td>
<td>0.7–1.5</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

OmniRat animals (HC14/Huc and/or Huo/JKOJKO/KKOKKO) and control SD rats were immunized with human PGRN, hGHR, human CD14, TAU peptide (TAU/KLH), HEL, or OVA, LN, lymph node; SP, spleen cell.

aCell numbers were 3–9 × 10⁶/fusion.
bAg specificity confirmed by biosensor analysis.
cRange of five highest affinities.
dEight mAbs were specific for Tau peptide.

E. coli engineering of large transloci containing multiple repeats, and the use of E. coli allowed the production of high DNA yields, which aided clonal characterization as well as oocyte microinjection. This circumvented both the often problematic engineering of large transloci that harbor multiple repeat sequences in E. coli and obtaining high concentrations of translocus DNA, which is not readily accomplished from yeast.

The assembling of the transgenic IgH locus in the rat genome was also facilitated by the finding that different DNA constructs carrying distinct parts of the IgH locus can cointegrate at a single site of genomic integration, thereby allowing a large translocus to be recreated by cointegration of smaller sections. Overlapping integration has been reported previously, but for much smaller regions (<100 kb) (29, 54). Our results in this study suggest that cointegration of simultaneously injected construct, probably by homology but possibly in tandem, is quite a frequent event. This is very helpful because there is a limit to the size of individual DNA molecules that can be manipulated in vitro and microinjected into eggs without risk of shearing. The usual alternative strategy for generating animals containing very large transloci would be via

Table IV. Affinity and V gene diversity of IgG1 hybridoma clones

<table>
<thead>
<tr>
<th>Ag</th>
<th>Fusion Cells</th>
<th>Clone</th>
<th>Kd (nM)</th>
<th>IGHV</th>
<th>Amino Acid Changes</th>
<th>IGHD</th>
<th>IGH1</th>
<th>CDR3</th>
<th>IgG/kV</th>
<th>Amino Acid Changes</th>
<th>IgG/kA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGRN</td>
<td>LN 1</td>
<td>0.7</td>
<td>4-31</td>
<td>2</td>
<td>7-27</td>
<td>3</td>
<td>CATGQDEADFWD</td>
<td>LV3-10</td>
<td>2 or 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGRN</td>
<td>LN 2</td>
<td>1.4</td>
<td>3-23</td>
<td>1</td>
<td>3-3</td>
<td>4</td>
<td>CAGGISSLIPFDW</td>
<td>LV3-19</td>
<td>2 or 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hGHR</td>
<td>LN 1</td>
<td>2.4</td>
<td>1-2</td>
<td>6</td>
<td>19-1</td>
<td>3</td>
<td>CARYQQLNAFDW</td>
<td>LV2-14</td>
<td>9 or 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hGHR</td>
<td>LN 2</td>
<td>4.2</td>
<td>1-2</td>
<td>7</td>
<td>16-4</td>
<td>4</td>
<td>CARWGQAPDFW</td>
<td>LV2-23</td>
<td>5 or 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAU/KLH</td>
<td>LN 1</td>
<td>0.8</td>
<td>4-39</td>
<td>5</td>
<td>22-23</td>
<td>4</td>
<td>CARHYYDSEQFDVY</td>
<td>KV1-1</td>
<td>0 or 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA</td>
<td>LN 2</td>
<td>2.7</td>
<td>3-23</td>
<td>6</td>
<td>26-4</td>
<td>4</td>
<td>CAKEWYGQGPFDFW</td>
<td>KV1-17</td>
<td>1 or 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA</td>
<td>LN 3</td>
<td>3.9</td>
<td>3-11</td>
<td>5</td>
<td>10-4</td>
<td>4</td>
<td>CARARYGSGSLPDY</td>
<td>KV1-6</td>
<td>12 or 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEL</td>
<td>SP 1</td>
<td>0.9</td>
<td>3-23</td>
<td>15</td>
<td>19-4</td>
<td>4</td>
<td>CAKEYSSNFWPFH</td>
<td>KV3-11</td>
<td>1 or 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEL</td>
<td>SP 1</td>
<td>0.8</td>
<td>6-1</td>
<td>1</td>
<td>19-1</td>
<td>4</td>
<td>CARESVGWQGPFQH</td>
<td>KV1-5</td>
<td>0 or 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-gal</td>
<td>SP 1</td>
<td>0.8</td>
<td>6-1</td>
<td>5</td>
<td>21-4</td>
<td>4</td>
<td>CARTFRGELPDY</td>
<td>KV1-12</td>
<td>0 or 4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

A further major aspect of the technical strategy that had facil-
itated the creation of OmniRat was the use of ZFN technology in
fertilized rat oocytes to inactivate the endogenous rat Ig loci (35, 42).
Because there is no established method for targeted gene recom-
bination 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 hy-
briddomas expressing high-affinity human mAbs could be obtained
following immunization. Thus, in these rats containing a chimeric
human/rat IgH loci togetherness with human IgL translocus, the transloci
compete very effectively in terms of performance with the endo-
genous 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 selec-
tive 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 hu-
manized 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
junctonal differences in OmniRat animals as observed in wt
control rats. When identical combinations of V, D, and J seg-
ments were very occasionally observed, differences between these
sequences due to either N sequence addition/deletion or hyper-
mutation were nevertheless observed. Extensive diversity was also
seen for the introduced human Igk and Igλ loci, similar to what
has previously been observed with mice transgenic for human Ig
loci (17, 19, 24). Hence, the compromised efficiency in the pro-
duction 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 accom-
plished 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(DJ) gene
segments, so as to yield human Ag-specific binding sites, but rodent
C genes and control sequences to ensure efficient B cell differen-
tiation, high Ab expression, and diversification. For therapeutic
applications, the rat C4 regions in mAbs obtained from OmniRat
can readily be replaced by human C4 regions without compro-
mising Ag-specificity during the bulking up phase of mAb pro-
duction.

Acknowledgments
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NJ. Breeding and genotyping of the Open Monoclonal Technology, Inc.
OmniRat strain was performed at Charles River Laboratories (Wilmington,
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Disclosures
The authors have no financial conflicts of interest.

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Corrections


In Fig. 1A, the switch region upstream of C_y1 should have been labeled s_y1 and not s_y2c. The corrected Fig. 1 is shown below. The figure legend was correct as published and is shown below for reference.

![Integrated human 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 V_H segments. BAC6-3 has been extended with V_H3-11 to provide a 10.6-kb overlap to BAC3, which overlaps 11.3 kb via V_H6-1 with the C region BAC human/rat Annabel. The latter is chimeric and contains all human D and J_H segments followed by the rat C region (C_m, C_g1, C_g2b, C_ε, C_a) with full enhancer sequences. (B) The human Igκ BACs with 12 V_κs and all J_κs provide an ~14-kb overlap in the V_κ region and ~40 kb in C_κ to include the KDE. (C) The human Igλ region with 17 V_λs and all J_C_λs, including the 3’ enhancer, is from a YAC (24).

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