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

Michael J. Osborn,*1 Biao Ma,*1 Suzanne Avis,* Ashleigh Binnie,* Jeanette Dilley,† Xi Yang,‡ Kevin Lindquist,‡ Séverine Ménoret,‡ Anne-Laure Iscache,‡ Laure-Hélène Ouisse,‡ Arvind Rajaipal,† Ignacio Anegon,‡ Michael S. Neuberger,§ Roland Buelow,*2, and Marianne Brüggemann*2,‡

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{K}s linked to J\text{C}-C\text{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: 1481–1490.

Human mAbs account for an increasing proportion of new drugs (1, 2). There have been major recent improvements in the way in which genetic information to encode Ag-specific mAbs can be obtained directly from human B cells (3–5). However, such approaches are largely restricted to Ags such as infectious agents where individuals mounting a specific immune response can be identified and provide a source of Ag-specific B cells. For other Ags, approaches have been developed that either involve individual humanization of Ag-specific rodent Abs (which needs to be carried out on a case-by-case basis) or involve the selection of Ag-specific binders from human Ab repertoires performed outside the human body. The individual humanization of rodent mAbs is laborious because it needs to be carried out on a case-by-case basis with the V region sequence being manipulated (as well as the C region exchanged) to minimize immunogenicity (6–8). The alternative strategy of obtaining Ag-specific human Abs by use of selections performed outside the human body typically employs either in vitro selection technologies (e.g., phage display) or in vivo Ag-mediated selection (i.e., immunization) in genetically engineered animals that express human Ab repertoires (9–14).

Thus, following immunization, Ag-specific human mAbs can be obtained by conventional hybridoma technology from transgenic mice whose B cell populations express human Ab repertoires. Indeed, in light of the potential importance of human mAbs as therapeutics, much effort has been devoted to creating improved mouse strains from which human mAbs can be more readily elicited ever since the first description of a mouse strain carrying an artificially constructed rearranging human IgH minilocus (15). In most of the published mouse strains currently under use, segments of the human IgH and IgL loci comprising differing numbers of human V, D, and J segments linked to human C regions have been integrated into the mouse germline (16, 17) with the endogenous mouse Ig loci having been rendered nonfunctional through targeted gene disruption (18).

Many human mAbs have been generated from transgenic lines by this strategy with six of eight fully human mAbs approved by the U.S. Food and Drug Administration (panitumumab, ofatumumab, golimumab, denosumab, ustekinumab, ipilimumab) and with further such mAbs currently being tested in phase II or III trials (http://en.wikipedia.org/wiki/List_of_monoclonal_antibodies) (1).
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 mice 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-JH segments 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 CITB) 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 containing the authentic region spanning from VH3-11 to VH6-1 (811L16 RPCI-11) and BAC3 with full enhancer sequences (9).

For the construction of the rat strain, three overlapping BAC libraries were used: BAC6-VH3-11 with VH4-39 to VH3-23, BAC3 with VH6-1 to VH3-11, and Annabel. To construct BAC6-VH3-11, initially two fragments, a 115-kb NotI-PmeI and a 110-kb RsHI-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-PmeI fragment was ligated to a NotI-PmeI 9 AvrII fragment containing 14-kb overlap in the VH region and 1, Cg2b, Cg2a, Cg1b, Cg1a, Cg1, Cg0, Cg, Cb, Ca, Cy2b, Cy1, Cy1, Cy1, C, B, A, IgHV-KAC, KDE

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 Jg segments followed by the rat C region (Cp, Cy1, Cy2b, Ce, Co) with full enhancer sequences. (B) The human Igk BACs with 12 Vks and all Jks provide an ~14-kb overlap in the Vc region and ~40 kb in Ck to include the KDE. (C) The human Igλ region with 17 VAs and all J-CAs, including the 3' enhancer, is from a YAC (24).
with a 5′ tail matching the sequence upstream of Vp3-39 and a 3′ tail matching downstream of Vε1-23 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 Vκ3-23 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 3′ end of the 1.6-kb fragment at the 5′ end of human Vp4-39 as described above, as well as PCR fragments, were joined upstream with a tail matching the 5′ end of Vp4-39 as described above, and pBelo-CEN-HYG as template. Subsequently, the three PCR fragments were assembled into a small cYAC conferring HYGΔ and TRPΔ 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 YAC6, and through homologous recombination YAC BAC−V3-31 containing part of rat μ coding sequence (using oligonucleotides 488 and 346, and rat genomic DNA as template), an ∼25-kb NotI-Pfnel fragment containing the authentic rat μ, δ, and ϵ2 region cut out from BAC MA (CH230-408M5) and the pBelo-CEN-URA vector with the URA3 joined downstream with a homology tail matching the 3′ end of the rat ϵ2 region and the CEN4 joined upstream with a tail matching the 5′ region of human Vp6-1 as described (using oligonucleotides 485 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 the C region with the Vp4 overlap, the human Vp6-1-Ds-JHs region had to be joined with the rat genomic sequence immediately downstream of the last JH followed by rat Cs to yield a C YAC/BAC. To achieve this, five overlapping restriction as well as PCR fragments were prepared: a 6.1-kb fragment 5′ of human Vp6-1 (using oligonucleotides 383 and 384, and human genomic DNA as template), an ∼78-kb PvuI-PacI fragment containing the human Vp6-1-Ds-JHs region cut out from BAC1 (RP161465E6), a 8.7-kb fragment joining the human JH with the rat genomic sequence immediately downstream of the last JH, and the pBelo-URA3 vector with the URA3 joined downstream with a homology tail matching the 3′ end of the rat Cs and the CEN4 joined upstream with a tail matching the 5′ region of human Vp6-1 as described (using oligonucleotides 485 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 humanVp6-1-Ds-JHs followed by the authentic rat μ, δ, and ϵ2 region, as well as PCR fragments, were used. Five overlapping fragments contained the 5′ region of human Vp6-1 as described (using oligonucleotides 485 and 550, and rat genomic DNA as template), an ∼52-kb NotI-Pfnel fragment containing the authentic rat μ, δ, and ϵ2 region cut out from BAC MA (CH230-408M5) and the pBelo-CEN-URA vector with the URA3 joined downstream with a homology tail matching the 3′ end of the rat ϵ2 region and the CEN4 joined upstream with a tail matching the 5′ region of human Vp6-1 as described (using oligonucleotides 485 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.
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 isolate 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 IgH 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 429–430) confirmed that RNA extraction and cDNA synthesis were successful. RT-PCRs were set up using V4 leader primers with rat μC2 or rat γC2 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; BAc, Naarden, The Netherlands), as described in the protocol. Similarly, human Igk 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 7.4 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 (BI-30366; Bioline).

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

Cell suspensions were washed and adjusted to 5 × 10^5 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 prgmun (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 GERBU adjuvant (http://www.Gerbu.com) were administered i.p. as follows: days 0, 14, 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 VHk, D, and JH segments followed by rat C genes) and human Igk BACs as well as of the human Igλ YACs are depicted in Fig. 1. The integrated IgH and Igλ transloci were then generated by injecting multiple BACs into fertilized rat oocytes, exploiting the previous finding that coinjection of overlapping DNA constructs often leads to cotargeting into the genome (29). Thus, the IgH translocus was created by coinfection of BAC6-VH3-11 (a 128-kb AscI-SacI fragment containing 13 VH9s) with BAC3 (a 173-kb NotI fragment containing 10 V16s) and BAC3-1N12MS18 (human/rat Amnbell, a 193-kb NotI fragment containing human V16-1 and all Ds and J16s followed by the rat C region). This resulted in the reconstitution of a fully functional IgH locus in the rat genome. Similarly, the human Igk locus was integrated by homologous overlaps (D9 containing Vκ genes, a 150-kb NotI fragment; E24, containing Vκs, Jκs, and Cκ on a 150-kb NotI fragment; and F17, a 40-kb PacI 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 VDJ-C recombinations from the most 5' to the most 3' end of the locus injected. Multiple BAC insertions were identified by quantitative PCR using Vκ- and Cκ-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, Igκ, and Igλ loci (the inactivation of the rat IgH locus was described in Ref. 35, and a manuscript describing inactivation of rat Igk 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 Cκ and Cα 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 crossbred successfully to homozygosity with Ig locus KO rats. This produced a highly efficient new multifluorene line (OmniRat) with human Vκ-D-Jκ regions of >400 kb containing 22 functional Vκ8s 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 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 VH</th>
<th>Rat Cκ</th>
<th>Human Igκ</th>
<th>Human Igλ</th>
<th>ZFN KO</th>
<th>Rat Chromosome (FISH)</th>
</tr>
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<tbody>
<tr>
<td>BAC6-VH3-11 (182 kb)</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>5q22 Homozygous KO</td>
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<tr>
<td>BAC3 (Annabel) (193 kb)</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>6q23</td>
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<tr>
<td>Human Igκ BACs (300 kb)</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>6q32</td>
</tr>
<tr>
<td>Human Igλ YAC (300 kb)</td>
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<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
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<td>OmniRat No. 117</td>
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<tr>
<td>OmniRat No. 35</td>
<td>Homozygous KO</td>
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</table>

OmniRat (HC14JKOJKO/KKO/KKO/LKOLKO/79/6262) is the product of breeding three translocus features (human/rat IgH, human Igκ, and human Igλ) with three KO lines (rat JH, Cκ, and JCκ).

**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+ expressers in OmniRat animals compared with wt. In bone marrow: A, pro/pre–B cells (CD45R+IgM+); B, immature B cells (CD45R+IgM+). In spleen: A, lymphocyte precursors (CD45R+IgM+); B, follicular B cells (CD45R+IgM+); C, marginal zone B cells (CD45R+IgM+).
**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 Igκ 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 μCH2 or γCH2 for IgH, and to Cl and Ck for IgL.**

<table>
<thead>
<tr>
<th>IgH V</th>
<th>IgH D</th>
<th>IgH J</th>
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<th>IgL V</th>
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<th>IgK V</th>
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* 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 γCH2 reverse primer; IGLV2, 3, and 4 with reverse Cα 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 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 Vλ-D-Jκ human Vκ-Jκ, or Vκ-Jλ 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 Vκ, 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.** 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 (8531, 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 Igκ 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 rat IgH C regions should ensure efficient physiological interaction with the various host Fc receptors and other host Ag receptor-associated molecules that are necessary for efficient B cell activation. The use of rat IgH C regions will also allow physiological interaction with the various host Fc receptors implicated in immune response regulation (49, 50).

However, a distinct attraction of using an IgH translocus in which the IgH C regions are of endogenous origin is that regions toward the 3′ end of the IgH locus are known to play a major role in immune response regulation (49, 50). As optimal IgV gene somatic hypermutation (19, 51, 52), There are substantial differences between mouse/rat and human in the se-

| Table III. Ag-specific rat IgG hybridomas with fully human idiotypes |
|-----------------|-------|---------|-------|----------|-------|
| Animal | Ag | Cells | Fusions | Titer | Hybrids | IgGs |
| SD | PGRN | LN | 1 | 38,400 | 3,520 | 38 | 0.3–1.0 |
| OmniRat | PGRN | LN | 1 | 12,800 | 1,600 | 148 | 0.7–2.4 |
| SD | PGRN | SP | 1 | 51,200 | 8,000 | 29 | ND |
| OmniRat | PGRN | SP | 1 | 51,200 | 36,000 | 24 | ND |
| OmniRat | hGHR | LN | 3 | 4,800 | 704–1,024 | 18.3, 2 | ND |
| SD | hGHR | SP | 1 | 204,800 | 53,760 | 230 | <0.07–0.4 |
| OmniRat | hGHR | SP | 1 | 76,800 | 53,760 | 7 | 0.16–2.4 |
| SD | TAU/KLH | LN | 1 | 20,000 | 1,728 | 99" | 0.6–2.4 |
| OmniRat | TAU/KLH | LN | 1 | 4,800 | 1,880 | 118" | 0.5–3.2 |
| SD | HEL | LN | 1 | 12,800 | 1,564 | 26 | 0.02–0.1 |
| OmniRat | HEL | LN | 3 | 25,600 | 288–640 | 0.2, 7 | 0.6–1.5 |
| SD | HEL | SP | 1 | 6,400 | 30,720 | 0 | ND |
| SD | OVA | LN | 1 | 9,600 | 1,488 | 10 | 1.1–4.8 |
| OmniRat | OVA | LN | 4 | 8,000 | 512–2240 | 0, 30, 0, 1 | 0.7–1.5 |

Omnirat animals (HC14/Huc and/or Huo/JKOKJ/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.

°Cell numbers were 3–9 × 10⁷/fusion.

"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 IgGκ 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>IGJH</th>
<th>CDR3</th>
<th>IGκ/λV</th>
<th>Amino Acid Changes</th>
<th>IGκ/αJ</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>CATQTGQEDAFPDW</td>
<td>LV3-10</td>
<td>1</td>
<td>2 or 3</td>
</tr>
<tr>
<td>PGRN</td>
<td>LN</td>
<td>8080.2B3</td>
<td>1</td>
<td>3-23</td>
<td>1</td>
<td>3-3</td>
<td>4</td>
<td>CAGIQSSLTPFFPDW</td>
<td>LV3-19</td>
<td>2</td>
<td>2 or 3</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>CAVGQMLHAFPDW</td>
<td>LV2-14</td>
<td>9</td>
<td>2 or 3</td>
</tr>
<tr>
<td>hGHR</td>
<td>LN</td>
<td>9046.8E10</td>
<td>4.2</td>
<td>1-2</td>
<td>7</td>
<td>3-16</td>
<td>4</td>
<td>CARKGDQAPFDW</td>
<td>LV2-23</td>
<td>5</td>
<td>2 or 3</td>
</tr>
<tr>
<td>TAU/KLH</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>CARRHYYSDEWYFYFPDW</td>
<td>KV4-1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>OVA</td>
<td>LN</td>
<td>9477.2F4</td>
<td>2.7</td>
<td>3-23</td>
<td>6</td>
<td>1-26</td>
<td>4</td>
<td>CAKERQSGYGGPDW</td>
<td>KV1-17</td>
<td>1</td>
<td>5</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>CARAYYGSGLSFDYW</td>
<td>KV1-6</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>HEL</td>
<td>SP</td>
<td>1H2</td>
<td>0.9</td>
<td>3-23</td>
<td>15</td>
<td>6-19</td>
<td>4</td>
<td>CAKREYSSYWYFPDW</td>
<td>KV3-11</td>
<td>1</td>
<td>2</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>CAKERGSQYWFFQHW</td>
<td>KV1-5</td>
<td>0</td>
<td>5</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>CARRFRLGLPFDW</td>
<td>KV1-12</td>
<td>0</td>
<td>4</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-
brination 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-
bridomas 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 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
clonal 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 Igα 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 IgG 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(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 differen-
tiation, high Ab expression, and diversification. For therapeutic
applications, the rat C<sub>H</sub> regions in mAbs obtained from OmniRat
can readily be replaced by human C<sub>H</sub> regions without compro-
mising Ag-specificity during the bulking up phase of mAb pro-
duction.

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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 Cy1 should have been labeled sy1 and not sy2c. The corrected Fig. 1 is shown below. The figure legend was correct as published and is shown below for reference.

![Integrated human Ig loci](http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1390026)

**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 Vks and all Jks provide an ~14-kb overlap in the Vk region and ~40 kb in Ck to include the KDE. (C) The human Igλ region with 17 Vls and all J-Cλs, including the 3’ enhancer, is from a YAC (24).