Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs

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We describe a strategy for producing human monoclonal antibodies in mice by introducing large segments of the human heavy and κ light chain loci contained on yeast artificial chromosomes into the mouse germline. Such mice produce a diverse repertoire of human heavy and light chains, and upon immunization with tetanus toxin have been used to derive antigen-specific, fully human monoclonal antibodies. Breeding such animals with mice engineered by gene targeting to be deficient in mouse immunoglobulin (Ig) production has led to a mouse strain in which high levels of antibodies are produced, mostly comprised of both human heavy and light chains. These strains should provide insight into the adoptive human antibody response and permit the development of fully human monoclonal antibodies with therapeutic potential.

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Fully human antibodies, with lower immunogenicity and more desirable pharmacological properties than engineered mouse antibodies, may fulfill the enormous potential for monoclonal antibodies (mAbs) in treating human disease. As the use of human B cells as a source of rearranged human antibody genes may limit the generation of therapeutically useful specificities, particularly when the target antigen is of human origin, attention has focused on the use of transgenic mice bearing unrearranged human immunoglobulin (Ig) genes to exploit the adaptive immune response of the mouse[1–6]. So far, however, the ability to generate antigen-specific human antibodies in mice has proved elusive. Mice bearing minigene constructs rearrange and express human Ig genes, but the highly skewed or aberrant, fetal-like human Ig repertoires produced in such mice, and the low expression of human Ig relative to endogenous mouse Ig[2–4], has precluded the demonstration of antigen-specific human antibodies. Hence the need for large germline segments of human Ig genes with larger variable gene repertoire and critical regulatory elements to achieve normal levels of expression and diversity is suggested.

Here we describe a novel strategy which permits the generation of mouse hybridomas making antigen-specific human mAbs and the creation of a mouse strain in which the majority of Ig produced are fully human. Using technology we have recently developed[7], yeast artificial chromosomes (YACs) carrying large segments of the human heavy and κ chain loci have been introduced into the mouse germline via fusion of yeast spheroplasts with mouse embryonic stem (ES) cells. These mice produce a broad adult-like repertoire of human Ig and are capable of giving rise to antigen-specific human mAbs upon immunization. Breeding of such mice with those whose heavy and κ genes have been inactivated by gene targeting has led to the creation of a strain which primarily produces fully human antibodies. Such mice may be exploited to elucidate the nature of the human humoral immune response upon infection or immunization, and to develop fully human therapeutic mAbs.

Human Ig YACs in ES cells and mice

YACs containing sequences from the human heavy and kappa chain loci (Fig. 1) were shown to be in intact, germline configuration (M.J.M. *et al.*, manuscript in preparation). The cloned heavy chain YAC (220 kb insert) contains the mu (μ) and delta (δ) constant (C) regions, all six functional joining (J) regions, the major diversity (D) cluster, the intronic enhancer and five most proximal variable (V) genes from four V_H families: V_{VI}, $V_{I\text{-}2}$, $V_{I\text{-}3}$, $V_{IV\text{-}4}$ and $V_{II\text{-}5}$ (Fig. 1)[8,9]. The cloned κ YAC (170 kb insert) contains the κ deleting element (Kde), the intronic and 3' enhancers, the C_κ region, all five functional J regions and the three most proximal V_κ regions in the B cluster (B1, B2, B3) (Fig. 1)[10]. A human HPRT selectable marker[11] was targeted into the right vector arm of each YAC (Fig. 1; M.J.M. *et al.*, manuscript in preparation).

HPRT-targeted human heavy (yH1) and κ (yK1) chain YACs were introduced into the HPRT-deficient ES cell line E14.TG3B1 (H.T. *et al.*, manuscript in preparation) by yeast spheroplast-ES cell fusion[7]. Seven and 11 $HPRT^+$-ES clones obtained by fusion of yH1- or yK1-containing yeast, respectively, were analysed by Southern blotting for the integrity of the YACs. Five yH1-containing clones (2B, 2C, 3A, 125A, 125E) and 10 yK1-containing clones contained all *Hin*dIII fragments detected by probes

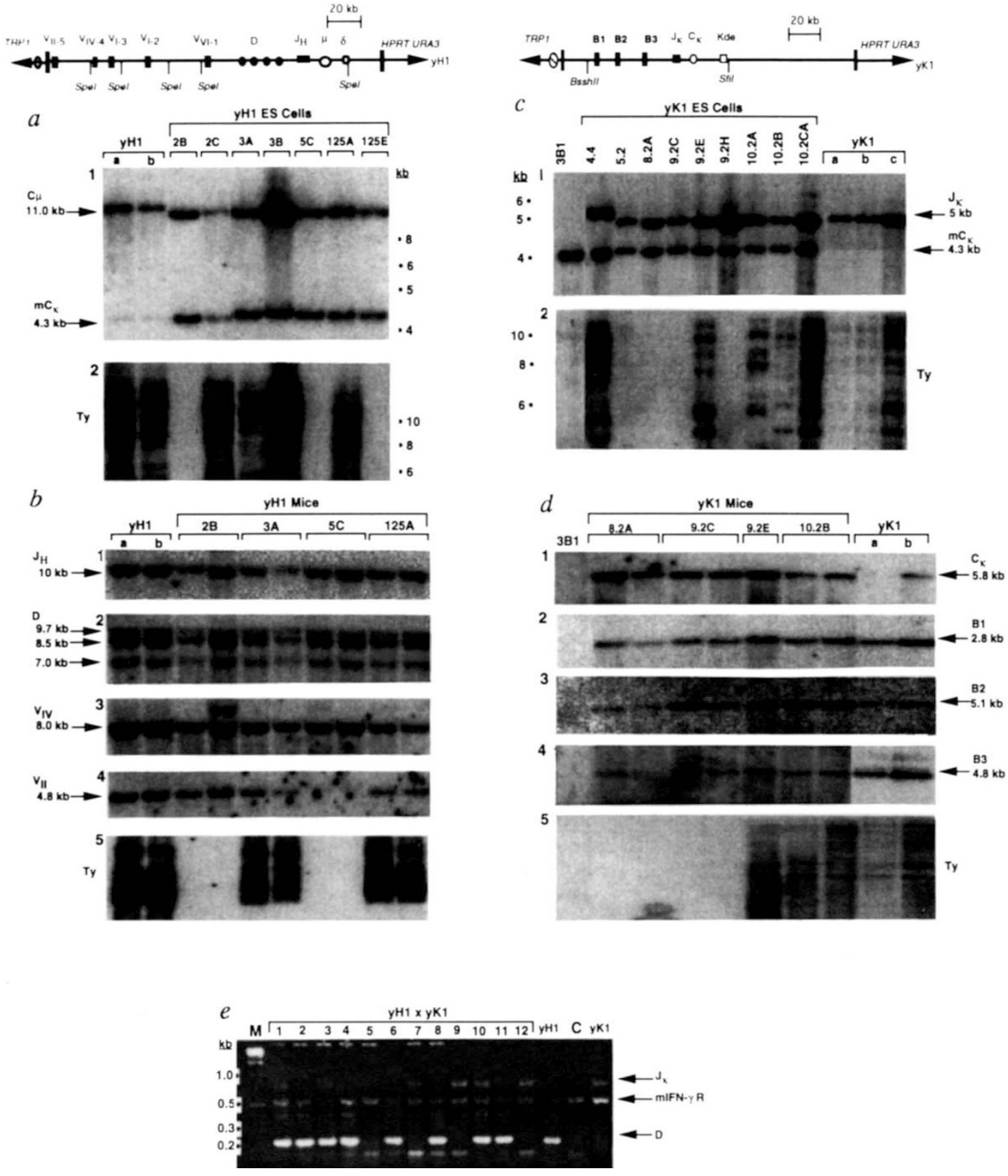

Fig. 1 Characterization of human heavy and κ light chain YACs integrated in ES cells and transgenic mice. Schematic representations of the human heavy (yH1) and kappa (yK1) YACs, retrofitted with a HPRT minigene, are shown above *a* and *c*, respectively. The locations of specific Ig regions are indicated along with YAC vector elements: ▶, telomere; ⬭, centromere; ■, *Eco*RI cloning site and yeast selectable markers, TRP1 and URA3. (YAC arms are not shown to scale.) *a*, *b*, Southern blot analysis of *Hin*dIII-digested DNA (10 μg) from: *a*, yH1-containing ES clones: 2B, 2C, 3A, 3B, 5C, 125A and 125E, probed with human Cμ and mouse Cκ (*a1*) and yeast Ty sequences (*a2*); *Hin*dIII-digested yH1, embedded in agarose, was used in *a*, resulting in slightly slower-migrating fragments than the corresponding fragments from yH1 in ES cells prepared in solution. *b*, mice (2 individual offspring from each strain) generated from clones: 2B, 3A, 5C, 125A, and yH1-containing haploid yeast DNA (a= 40 ng and b=20 ng corresponding to 2 and 1 YAC DNA copies, respectively). Probes: J_H (*b1*), D (*b2*), V_{IV} (*b3*, the band detected above the 8 kb V_{IV} fragment in the 2B lane represents a partial digest), V_{II} (*b4*) and yeast Ty sequences (*b5*). *c*, *d*, Southern blot analysis of *Hin*dIII-digested DNA (10 μg) from: *c*, unmodified E14.TG3B1 (3B1) and yK1-containing ES clones: 4.4, 5.2, 8.2A, 9.2C, 9.2E, 9.2H, 10.2A, 10.2B and 10.2CA and *d*, mice (2 individual offspring, except 9.2E) generated from clones 8.2A, 9.2C, 9.2E and 10.2B, yK1-containing haploid yeast DNA (a=10 ng, b=20 ng and c=40 ng (when shown), representing 0.5, 1 and 2 YAC DNA copies, respectively). Probes: $J_κ$ and mouse $C_κ$ (*c1, d1*), B1 (*d2*), B2 (*d3*), B3 (*d4*) and yeast Ty sequences (*c2, d5*). Fragment sizes (in kb) are indicated. *e*, Identification by PCR analysis of HuAb mice (1, 3 and 10) in a mouse litter (1–12) derived from the mating of yH1- and yK1-bearing mice. Specific human heavy chain (D, 230 bp), κ chain ($J_κ$, 860 bp) and mouse γ interferon receptor (INF-γR, 550 bp)-PCR products are indicated in control 129×C57Bl/6 mice (C), heavy (yH1)- or κ (yK1)-containing mice, or yH1;yK1 progeny.

Table 1 Structural integrity of human heavy and κ light chain YACs in ES clones and their expression in mice

a

yH1 ES Cell Clone	Cδ 7.8 kb	Cμ 11 kb	JH 10 kb	D 9.7, 8.5, 7 kb	V_{VI} ~ 28 kb	V_{I} >12, 1.7 kb	V_{IV} 8 kb	V_{II} 4.8 kb	Yeast Genomic Sequences	YAC copy number
2B	+	+	+	+	+	+	+	+	-	1
2C	+	+	+	+	+	+	+	+	+	2
125A	+	+	+	+	+	+	+	+	+	1
125E	+	+	+	+	+	+	+	+	-	1
3A	+	+	+	+	+	+	+	+	+	1
3B	+	+	+	9.7 kb 8.5 kb	+	+	+	+	+	~8
5C	+	+	+	+	+	+	+	-	-	1

b

yK1 ES Cell Clone	Kde 2.5 kb	Cκ 5.8 kb	Jκ 5 kb	B3 4.8 kb	B2 5.1 kb	B1 2.8 kb	Yeast Genomic Sequences	YAC copy number
4.4	+	+	5.5 kb	4.7 kb	4.9 kb	+	+	1
5.2	+	+	+	+	+	+	-	1
8.2A	+	+	+	+	+	+	-	1
9.2E	+	+	+	+	+	+	+	2
9.2C	+	+	+	+	+	+	-	1
10.2B	+	+	+	+	+	+	+	1
9.2H	+	+	+	+	+	+	-	~3
9.2F	+	+	+	+	ND	+	-	1
10.2C	+	+	+	+	ND	+	-	1
10.2A	+	+	+	+	+	+	+	2
10.2CA	+	+	+	+	+	+	+	2

c Human Heavy Chain

yH1 Mouse Clone	yH1 Copy Number	hμ Expression (μg/ml)
*3B	~8	3.8
125A	1	0.9
5C	1	0.8
2B	1	0.7
3A	1	0.4
2C	2	0.4
*125E	1	0.2
Control	0	0.0

d Human Kappa Chain

yK1 Mouse Clone	yK1 Copy Number	hκ Expression (μg/ml)
5.2	1	30.0
10.2B	1	27.5
9.2C	1	17.7
*9.2H	~3	15.5
*9.2F	1	11.0
9.2E	2	10.5
*10.2C	1	8.1
8.2A	1	8.0
4.4	1	0.0
Control	0	0.0

a, *b*, HPRT⁺-ES clones, generated by fusion of ES cells with yH1- (*a*) or yK1- (*b*) containing yeast spheroplasts, were analysed for the presence of human heavy or κ chain-specific sequences, and yeast genomic sequences (see Methodology). The presence of the expected fragment sizes for the specific probe (as shown), and of varied levels of yeast genomic sequences are indicated as '+'. Altered-sized fragments are indicated. In the case of yH1- ES clone 3B, the 7 kb doublet from the D region was deleted. ND-not determined. *c*, *d*, Detection of human heavy and κ chains in mouse sera. Serum samples derived from yH1- (*c*) or yK1- (*d*) containing transgenic or chimaeric (*) mice, from the indicated ES clones, or non-transgenic littermate mice (control), were analysed by ELISA for hμ or hκ. Shown are representative serum titrations for individual mice.

spanning the entire respective inserts (Fig. 1, Table 1). Deletions within the D or V_{II} regions of yH1 were detected in clones 3B and 5C, respectively, and altered Jκ, B3 and B2 fragments of yK1 were detected in clone 4.4. All clones retained the HPRT-containing right vector arm. All yK1-containing clones and four out of seven yH1-containing clones (2C, 3A, 3B, 125A) retained an intact left arm (data not shown). All clones contained a single YAC integration except 2C and 3B (yH1) and 9.2E, 9.2H, 10.2A and 10.2CA (yK1). Hybridization with yeast repetitive probes (Ty, Y', δ, rRNA) demonstrated the presence of varying amounts of yeast genomic sequences in 4 yH1 ES clones (2C, 125A, 3A, 3B) and 5 yK1 clones (4.4, 9.2E, 10.2B, 10.2A, 10.2CA) and their absence in the remaining ES clones (Fig. 1, Table 1).

The integrity of introduced YACs was further assessed by pulsed-field gel electrophoresis following digestion with *Not*I (a site absent in yH1) or *Sfi*I (a site occurring once in yK1, generating a 105 kb fragment containing the Kde-B1 region). A single, unique fragment hybridizing to multiple yH1 or yK1 specific probes was demonstrated in 5 yH1 (2B, 3A, 5C, 125A and 125E) and in 6 yK1 (5.2, 8.2A, 9.2C, 9.2F, 10.2B, 10.2C) ES clones (data not shown; M.J.M. *et al.*, manuscript in preparation). Together, these results strongly suggest that these clones contain a single, structurally intact YAC.

YAC-containing chimaeric mice were generated from 7 yH1 and 9 yK1 ES clones. Approximately 50% of their agouti offspring contained in their germline the respective YAC and yeast sequences (when present) in unaltered form (Fig. 1).

Human Ig production in mice

The expression of the human heavy mu (hμ) and kappa (hκ) chains on B cells and in serum of yH1- and yK1-containing mice was investigated by flow cytometry and

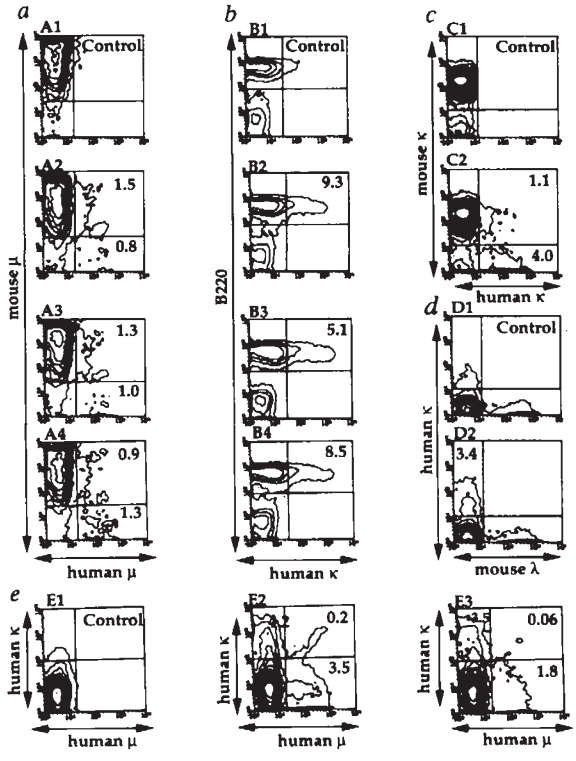

Fig. 2 Surface expression of human μ and κ chains on yH1- and yK1- containing mouse B cells. Blood or spleen lymphocytes derived from yH1-(*a*), yK1-(*b*,*c*,*d*) and yH1;yK1- (HuAb) (*e*) containing mouse strains or control mice (129xC57Bl/6:A1, B1, C1, D1, E1) were analysed by 2- or 3-colour flow cytometry for surface expression of human μ or κ chains, using antibodies to the B cell-specific marker B220 in combination with anti-human μ or κ, and anti-mouse μ, κ or λ, respectively. The net percentage of positively-stained cells (obtained by subtracting the background staining of each control) is shown in each quadrant. *a*, Blood samples from control (A1), or yH1-mice generated from clones 2B (A2), 5C (A3) and 125A (A4), were gated on B220⁺ cells and assayed for mIgM^a and mIgM^b, and hμ. *b*, Spleen lymphocytes from control (B1) or yK1-containing mice: 9.2C (B2), 8.2A (B3), and 10.2B (B4) were assayed for B220 and hκ. *c*, *d*, Spleen lymphocytes from control (C1, D1) or yK1-containing mice 8.2A (C2, D2), gated on B220⁺ cells and assayed for expression of hκ and mκ (*c*) or gated on B220⁺ cells and assayed for expression of hκ and mλ (*d*). The percentage of mλ⁺ B cells was similar (approximately 6%) in D1 and D2. *e*, Blood samples derived from control (E1) or HuAb strains (E2: 8.2A;125A, E3: 8.2A;5C) were analysed by 3-colour flow cytometry for surface expression of human μ and κ on B220⁺ cells. The net percentage of positively-stained cells is shown in each quadrant. The FACS profiles shown are representative of five experiments performed on these strains. Similar analysis indicated the presence of hμ⁺/hκ⁺ populations in other HuAb strains: 8.2A;2B (0.27%), 9.2C; 125A (0.13%) and 9.2C;2B (0.11%).

Table 2 Repertoire analysis of human heavy chain transcripts expressed in transgenic mice

a

Clone	Frame	V		N	D		N	J	
μ40	in	V_H4	GCGAGAGA	TCG	N1	GGGTATAG	TGGGAGCCC	J4	ACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ43	out	V_H4	GCGAGAGA	GGA	N1	GGTATAGCAGtgGC	CGGTACA	J3	CTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCA
μ46	in	V_H6	GCAA		21/9	TTACTATGATAGTAGTGGTTATTAC	TGAGCATTACT	J4	ACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ50	in	V_H1	GCGAGA	GGG	XP4	CGATTTTTGGAGTGGTTA	AAGAGGTCC	J6	TACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
μ55	in	V_H1	GCGAGAGA	GAGGC	M1	GTATAACTGGAACTAC	GGAG	J6	TACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
μ56	in	V_H6	GCA		M1	GGTATAACTGGAACT		J5	AACTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ65	in*	V_H1	GCG	GTCC	XP1	GTATTACGATATTTTGACTGGTTA	CCCCTGAT	J3	GCTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCA
μ73	in	V_H4	GCG	C	R1	GTGGATATAGTGGCTACGAT		J3	GCTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCA
μ80	out	V_H4	GCGAGAGA		M1	ACTGGAACTAC	CGGG	J4	ACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCGCCTCA
μ85	in	V_H1	GCGAG	GGGGGG	N1	TAGCAGtgGCTGGTAC	CTGA	J4	TTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ87	in	V_H1	GCGAGAGAG		N4	TATAGCAGCTCGTC		J4	CTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGgCTCCTCA
μ89	in	V_H1	GCGAGA	AGGGCG	N1	AGCAGCAGCT	TCTC	J4	CTTTGACTACTGGGGCCAGGGAACCCTGGTCgCCcTCTCCTCA
μ90	in	V_H1	GCGAGAG	GAGGGGCCCATT	LR2	GTGGTGGTgaCTGCT	CTACGTAC	J4	TACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ97	in	V_H6	GCAAGA		LR2	GTGGgAGCT	ACCCT	J4	CTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ98	out	V_H6	GCAAGAGA	TGAGGGGA	A4	TGACTACAGTAAC	CC	J4	GGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ100	in	V_H6	GCAAGAGA	GGGAGCAGTGGC	21/9	TGGTTATTAC		J6	TACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
μ102	in	V_H6	GCAAGAGA		M1	TAACTGGAAC	ACGT	J6	CTACTACGGTATGGACCGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
μ104	in	V_H6	GCA		A1	GACTACAGTAACT		J4	TTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA

b

Clone	FR3	CDR3	FR4
μ40	AR	DRGIVGAHFDY	WGQGTLVTVSS
μ46	AI	TMIVVVITEHYYFDY	WGQGTLVTVSS
μ50	AR	GRFLEWLKRSYYYYGMDV	WGQGTTVTVSS
μ55	AR	ERRITGTTEYYYYGMDV	WGQGTTVTVSS
μ56	AG	ITGTNWFDP	WGQGTLVTVSS
μ73	AR	GYSGYDAFDI	WGQGTMVTVSS
μ85	AR	GGSSGWYLIDY	WGQGTLVTVSS
μ87	AR	EYSSSSFDY	WGQGTLVTGSS
μ89	AR	RASSSFSFDY	WGQGTLVALSS
μ90	AR	GGAHCGGDCSTYYFDY	WGQGTLVTVSS
μ97	AR	VGATLFDY	WGQGTLVTVSS
μ100	AR	EGAVAGYYYYYYGMDV	WGQGTTVTVSS
μ102	AR	DNWNTSTTVWTV	WGQGTTVTVSS
μ104	AD	YSNFDY	WGQGTLVTVSS

c

	V_{II}	V_{IV}	V_I	V_{VI}	Total		Human PBL(ref.12)
J_1	0	0	1	0	1	(1%)	1%
J_2	0	0	1	0	1	(1%)	0%
J_3	0	5	7	3	15	(18%)	9.1%
J_4	0	8	16	21	45	(54%)	52.5%
J_5	0	0	3	1	4	(5%)	15.2%
J_6	0	3	7	7	17	(21%)	22.2%
Total	0 (0%)	16 (19%)	35 (42%)	32 (39%)	83	(100%)	100%

Human μ-specific mRNAs were amplified by PCR, cloned and analysed by sequencing or by hybridization to V_H- and J_H-region specific probes. *a*, Nucleotide sequences of 18 unique human heavy chain clones are divided into V_H, D, J_H and N segments, as identified by homology with published germline sequences[9,12,29–31]. Whether each V–D–J junction is in or out of open reading frame is indicated. Each D segment assignment is based on at least 8 bases of homology. Differences from the published sequences are in lower case. N-segment nucleotides were determined by their lack of sequence homology to V_H, D or J_H sequences. Clones chosen for sequencing had previously been shown to possess a V_H segment by colony hybridization (see part *c*). *b*, Predicted amino acid sequences of the 14 in-frame V–D–J junctions are divided into Framework Region 3 (FR3), CDR3 and FR4[14]. *c*, Results of colony hybridizations showing V_H and J_H gene utilization, as compared to that detected in human peripheral B cells[12]. Results are given only for colonies hybridizing to V_H, J_H and Cμ probes.

ELISA. Hμ was expressed on the surface of 1.5–2.5% of the B220$^+$ cells in blood (Fig. 2*a*) or spleen (not shown) in all 5 yH1 strains analysed (2B, 2C, 3A, 5C, 125A). Approximately half of the hμ$^+$ population had no detectable surface mouse mu (mμ) (Fig. 2*a*), indicating that hμ production can substantially exclude the expression of mμ. Hμ was detected in sera derived from all yH1 strains at 0.2–3.8 μg ml^{-1} (Table 1*c*). In all 3 yK1 strains examined (8.2A, 9.2C, 10.2B), 5–9% of the B220$^+$ splenocytes expressed surface hκ (Fig. 2*b*). In >80% of this population, hκ excluded the expression of mouse lambda (mλ) or kappa (mκ) (Fig. 2*c*,*d*). All yK1 mice derived from structurally intact YACs expressed hκ in serum at 8–30 μg ml^{-1} (Table 1*d*).

Human Ig-producing (HuAb) mice containing one copy each of yH1 and yK1 were generated (Fig. 1*e*). Three-colour flow cytometry of peripheral blood lymphocytes, derived from five HuAb strains (8.2A;125A, 8.2A;5C, 8.2A;2B, 9.2C;125A, 9.2C;2B) revealed B220$^+$ populations containing 0.8–3.9% hμ$^+$ or 1.4–4.8% hκ$^+$ cells (Fig. 2*e* and data not shown). Significantly, all five HuAb strains contained a population of B220$^+$ cells (0.06–0.27%) which simultaneously expressed both hμ and hκ (Fig. 2*e* and legend). The production of serum antibodies in HuAb mice containing both hμ and hκ (hμ/hκ) was demonstrated by an ELISA in which anti-hμ antibody was used to capture and anti-hκ antibody used to detect the product. Hμ/hκ antibodies were detected in HuAb strains 8.2A;2B and 8.2A;5C at 0.7 and 0.3 μg ml^{-1}, respectively (data not shown). Thus, in HuAb mice, human heavy and kappa genes on YACs were productively rearranged and expressed, leading to the presence of a significant population of B cells expressing both surface hμ and hκ and secreting antibodies containing both human heavy and light chains in the mouse serum.

Diverse adult-like human Ig repertoire in mice

To determine the diversity of the hμ and hκ repertoire in YAC-containing mice, hμ and hκ cDNAs were cloned from mouse spleen RNA. Hybridization analysis of hμ and hκ cDNA clones revealed broad usage of the V and J genes contained in the YACs (Tables 2*c*, 3*c*). All six J_H and five $J_κ$ segments were represented with a frequency comparable to that detected in adult human B cells[12,13]. Three of the four V_H families in yH1 were represented, with V_{IV} used about half as often as V_{VI} and V_I. No V_{II} transcripts (Table 2) or V_{II}–J_H rearrangement products (data not shown) were detected, suggesting that the proximity of V_{II} to the YAC vector cloning site may account for its inability to rearrange. All three $V_κ$ genes were represented, with B3 more frequently used than B2 or B1 (Table 3).

To analyse further the human Ig repertoire expressed in these mice, individual cDNA clones were sequenced. The 18 hμ cDNAs analysed displayed a V_H and J_H usage similar to that detected by hybridization (Table 2). Ten different D regions were represented, all with significant homology to known germline D genes. Some D sequences with less than 85% identity (for example, N1 in clones μ43 and μ85, and LR2 in clones μ90 and μ97) may represent novel D segments. For example, the C to T and A to G changes observed at the same position in the D segments of μ43 and μ85 (and ref. 12), suggest the existence of a new DN family member. Multiple reading frames were used in some of the D regions (μ90/μ97, μ55/μ102, μ46/μ100), suggesting a D usage more human-like than mouse[12,14]. Non-germlike nucleotides (N addition) were observed in 15 (83%) of the hμ cDNAs, with a majority having N additions at both the VD and DJ junctions. The length of N addition varied between 1–12 bp (average 6.1). The majority of the in-frame clones contained a complementarity determining region 3 (CDR3) of 10–18 amino acids (average 12). The $V_κ$ and $J_κ$ usage observed for 21 sequenced hκ cDNAs also agreed with hybridization analysis. In seven of the clones, N additions of 1–4 bp were found at the V–J junction. CDR3 sequences for in-frame κ transcripts were 9–10 amino acids in length. The $J_κ$ usage and CDR3 length observed are consistent with previous results for human B cells[13].

The pattern of V_H, D and J_H usage observed in human Ig YAC-containing mice is reminiscent of adult human B cells[12,13], in contrast to human Ig minigene-bearing mice[2–4]. There is an absence of position-biased V_H and D usage, in particular, for the V_{VJ} and DQ52 segments which are characteristic of human fetal development[15,16], with the latter dominating the in-frame repertoire observed in minigene-bearing mice[3,4]. Furthermore, the average length of N addition (6.1 bp), and thus the CDR3 region, closely approximates that seen in adult human B cells (7.7 bp)[12], while in minigene-bearing mice the average length (2.9 bp)[3,4] resembles that seen in adult mouse B cells (3.0 bp)[17]. These results suggest that the human Ig YACs contain sequences required to direct human-like repertoires in mice. Therefore, introduction of YACs

Table 3 Repertoire analysis of human κ transcripts expressed in transgenic mice

a

Clone	Frame	V		N	J	
K1	in	B3	AGTACTCCTC		J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K2	out	B3	AGTACTCCT	TTCT	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K3	in	B3	AGTACTCC		J1	GTGGACGTTCGGCCAAGGGACCATGGTGGAAATCAAACGA
K5	out	B3	TTTTCCTC		J3	ACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGA
K7	out	B3	AGTACTCCT		J1	GACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K8	in	B3	AGTACTCCTC		J2	GCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K9	in	B3	AGTACTT	T	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K10	in	B3	AGTACTCC		J1	GTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K13	out	B3	AGTACTCCTC	G	J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K14	in	B3	AGTACTCC	CAT	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K15	out	B3	AGTACTCC	AT	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K16	out	B2	TTCCC		J3	ATTCACTTCGGCCCTGGGACCAAAGTGATATCAAACGA
K18	in	B3	AGTACTCC		J4	GCTCACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K20	in	B2	TTCCCT		J1	TGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K22	out	B2	TTCCCTC		J4	ACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K25	in	B3	AGTACTCC		J3	ATTCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGA
K27	in	B3	AGTACTCC		J4	GCTCACTTTCGACGGAGGGACCAAGGTGGAGATCAAACGA
K28	out	B3	AGTACT	GTC	J3	TCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGA
K29	in*	B3	AGTACTCC	CAT	J2	GTGCAGTTTTGGCCAGGGGACCTAGCTGGAGATCAAACGA
K30	in	B1	TTTCCTC		J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K31	out	B3	AGTACTCCTCC		J4	ACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K32	in	B3	AGTACTCC		J3	ATTCACTTTCGGCCCTGGGATCAAAGTGGATATCAAACGA
K33	out	B3	AGTACTCCTCC		J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K34	in	B3	AGTACTCCTCC		J1	GACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA

b

Clone	FR3	CDR3	FR4
K1	YYC	QQYYSTPRT	FGQGTKVEIKR
K3	YYC	QQYYSTPWT	FGQGTMVEIKR
K8	YYC	QQYYSTPRS	FGQGTKLEIKR
K9	YYC	QQYYSTLCS	FGQGTKLEIKR
K10	YYC	QQYYSTPWT	FGQGTKVEIKR
K14	YYC	QQYYSTPMCS	FGQGTKLEIKR
K18	YYC	QQYYSTPLT	FGGGTKVEIKR
K20	YYF	CLQHDNFPWT	FGQGTKVEIKR
K25	YYC	QQYYSTPFT	FGPGTKVDIKR
K27	YYC	QQYYSTPLT	FDGGTKVEIKR
K30	YYC	LQSKNFPRT	FGQGTKVEIKR
K32	YYC	QQYYSTPPT	FGPGIKVDIKR
K34	YYC	QQYYSTPPT	FGQGTKVEIKR

c

	B1	B2	B3	Total
J_1	4	13	108	125 (47%)
J_2	10	5	53	68 (26%)
J_3	0	2	29	31 (12%)
J_4	1	9	25	35 (13%)
J_5	0	0	4	4 (1%)
Total	15 (6%)	29 (11%)	219(83%)	263 (100%)

mRNAs containing $hC_κ$ were amplified by PCR, cloned and analysed by sequencing or by colony hybridization to $V_κ$- and $J_κ$-region specific probes. *a*, Nucleotide sequences of V–J junctions of 21 independent human κ clones are shown, divided into $V_κ$, $J_κ$ and N segments and identified based on homology to published germline B1, B2 and B3, and $J_κ$ sequences[24–26]. Also indicated is whether each V–J junction is in or out of an open reading frame. N-segment nucleotides were determined by their lack of sequence homology to neither $V_κ$ nor $J_κ$ sequences. Differences from the published sequences are in lower case. The sequences shown are those which contained a V–J joining. The one sequenced B1-containing clone lacked a translational initiation site, as described[25]. *b*, Predicted amino acid sequences of in-frame V–J junctions are divided into FR3, CDR3 and FR4[14]. *c*, Results of colony hybridizations showing $V_κ$ and $J_κ$ gene utilization. Results are given only for colonies hybridizing to $V_κ$ and $J_κ$ probes.

with larger numbers of variable genes should ultimately recapitulate the diversity seen in humans.

Ag-specific fully human mAbs from mice

To determine whether HuAb mice can mount a specific human antibody response, mice were immunized with tetanus toxin C fragment (tet C). After immunization, tet C-specific hμ and hκ were readily detected in serum (Fig. 3*a*). The human origin of the tet C-specific antibodies was confirmed by using an ELISA in which tet C was used to capture and anti-hμ or anti-hκ used to detect the bound species. Thus, upon immunization, the HuAb mice are capable of producing antigen-specific human antibodies.

To determine whether antibodies containing both human heavy and light chains were produced, splenocytes derived from tet C-immunized HuAb mice (8.2A;5C) were fused with P3X63-Ag8.653 myeloma cells, and the resulting hybridomas screened for the production of tet C-specific fully human antibodies. Analysis of 678 hybridoma culture supernatants revealed 92 $hκ^+$ clones and 16 $hμ^+$ clones. Three clones were found to produce fully human mAbs specific for tet C. To confirm that all of the desired properties reside within the same antibody molecule, ELISAs were used in which either tet C was used to capture and anti-hκ used to detect the bound species (Fig. 3*b*), or anti-hμ used to capture and anti-hκ used to detect the bound species (Fig. 3*c*). All three clones were positive in both assays, indicating that HuAb mice, although containing only a small fraction of B cells co-expressing hμ and hκ, can mount an antigen-specific response leading to the generation of fully human mAbs. The nature of the human repertoire associated with the tet C-specific response and the extent to which somatic mutation plays a role in the maturation of tet C-specific human antibodies is currently under investigation.

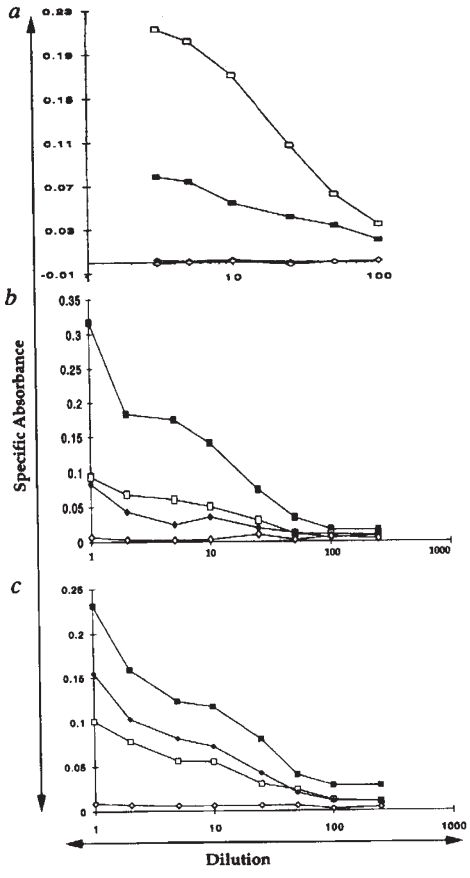

Fig. 3 Production of tet C-specific human polyclonal (*a*) or monoclonal (*b*, *c*) antibodies by HuAb mice. *a*, Sera obtained from HuAb strain 8.2A;125A, non-immune (◇,◆) or hyperimmune to tet C (□, ■) were analysed by ELISA for tet C-specific hμ (□) and hκ (■) antibodies. Similar results were obtained from HuAB strains 8.2A;2B, 8.2A;5C and 9.2C;2B. *b*,*c*, Supernatants derived from hybridomas (42C7-■,45B5-□, 45D9-◆, control 37G4-◇), generated by fusion of hyperimmune HuAb strain 8.2A;125A splenocytes with mouse myeloma cells, were analysed for the presence of fully (hμ/hκ) human monoclonal antibodies (*b*) and for specificity of the human antibodies to tet C (*c*), as detected in ELISA using tet C to capture and anti-hκ to detect. The hybridoma 37G4 (◇), secreting a tet C-specific monoclonal antibody containing only human heavy chain was used as a control in *b*, *c*.

Human YACs restore B cell and Ig production

While HuAb mice can mount an antigen-specific human antibody response, the preferential expression of mouse Ig genes suggested the potential value of their inactivation to increase the production of fully human antibodies in mice. Therefore, yH1- and yK1-expressing mice were bred with mice engineered by gene targeting to be deficient in mouse Ig production.

Initially, we examined the ability of yH1 to induce proper mouse B cell development and production of human Ig in a strain containing two functionally inactivated mouse heavy chain alleles ($yH1;\Delta J_H/\Delta J_H$). $\Delta J_H/\Delta J_H$ parental mice fail to rearrange their heavy chain genes, leading to a complete absence of mature B cells and a complete block in Ig production[18]. In $yH1;\Delta J_H/\Delta J_H$ mice, reconstitution of mature B cells ($B220^+$, $hμ^+$) was observed in bone marrow (Fig. 4*a*), spleen and blood (Fig. 4*e*) corresponding to 58%, 55% and 30% of the levels in wild-type mice, respectively. The majority of $B220^+/hμ^+$ cells in blood also expressed human delta (hδ) and all of the $hδ^+$ cells co-expressed hμ (data not shown), indicating proper expression and regulation of the human constant regions in these mice[19]. Remarkably, $yH1;\Delta J_H/\Delta J_H$ mice produced serum hμ at 350 μg ml^{-1}, a level 510-fold greater than parental 125A mice (Table 1*c*) and within 2–3-fold of normal human IgM serum levels. Hμ serum titres showed an age-dependent increase (not shown). These results demonstrate that yH1 can reconstitute B cell development in mice deficient in mouse heavy chains and direct the expression and assembly of high levels of hμ/mouse light chain antibodies.

The $yH1;\Delta J_H/\Delta J_H$ mice were further evaluated by analysing the orderly differentiation of bone marrow-derived B cells by flow cytometry using antibodies to the cell surface marker CD43, which defines early B cell subpopulations[20], in conjunction with antibodies to B220 and hμ. Bone marrow from $\Delta J_H/\Delta J_H$ mice contained no mature B cells ($B220^{bright/dull}$, $CD43^-$; R1, R2 populations) and a population (5.5%) of pro-B cells ($B220^{dull}$, $CD43^+$; R3 population), comparable to wild-type mice (Fig. 4*a*). In contrast, $yH1;\Delta J_H/\Delta J_H$ bone marrow contained a nearly normal R1, R2 population (41%) of mature B cells; however, the size of the pro-B cell population (12.4%) was increased (Fig. 4*a*). Surface hμ was detected only in the $B220^+$, $CD43^-$ population of $yH1;\Delta J_H/\Delta J_H$ mice, similar to mμ in wild-type mice (Fig. 4*b*). Thus, while B cell development and Ig production are substantially restored in $yH1;\Delta J_H/\Delta J_H$ mice, the maturation of bone marrow-derived B cells may be somewhat less efficient than in normal mice.

To delineate this partial block in B cell maturation, we examined the surface expression of HSA and BP-1 in bone marrow to resolve the pro-B and pre-B cell populations[20]. Large, primarily $CD43^-$ populations of $B220^+$, HSA^+ cells and $B220^+$, $BP\text{-}1^-$ cells as well as a smaller $CD43^-$, $B220^+$, $BP\text{-}1^+$ cell population confirmed that B cell development is largely normal in these mice (Fig. 4*c*,*d*). However,

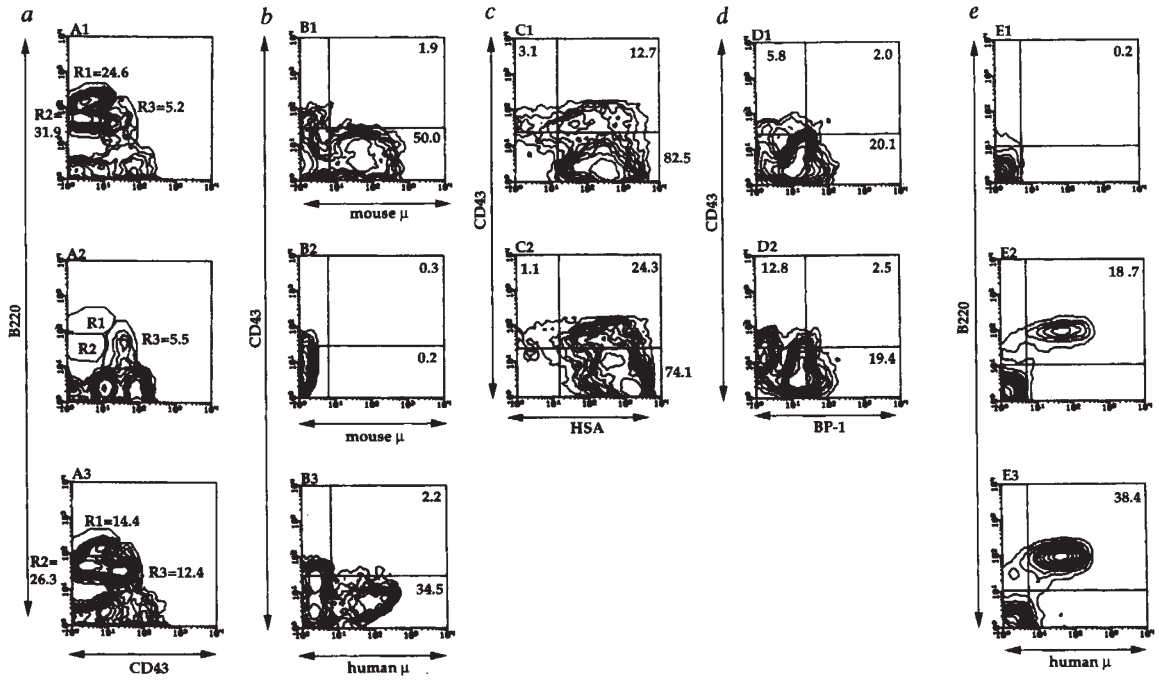

Fig. 4 Reconstitution of B cell development in ΔJ_H homozygous mutant mice by yH1 (125A strain) YAC. *a–d*, Bone marrow lymphocytes from normal control (129×B6: A1, B1, C1, D1), $\Delta J_H/\Delta J_H$ (A2, E1) or yH1;$\Delta J_H/\Delta J_H$ mice (A3, B2, B3, C2, D2) were assayed for surface expression of CD43 and B220 (*a*), or gated on B220⁺ cells and assayed for surface expression of mμ and CD43 (B1, B2), hμ and CD43 (B3), HSA and CD43 (*c*), or BP-1 and CD43 (*d*). The bone marrow B220⁺ sub-populations (R1, R2 and R3 (high (bright) or low (dull) levels of surface B220⁺ are delineated)) are indicated with their respective percentages of positively stained cells. *e*, Peripheral blood (E1, E2) and spleen (*E3*) lymphocytes were assayed for hm and B220 in $\Delta J_H/\Delta J_H$ (E1) and in yH1;$\Delta J_H/\Delta J_H$ mice (E2, E3). The net percentage of positively-stained cells is shown in each quadrant. In the normal 129×B6 mouse, 64% of the cells in blood and spleen were B220⁺. All animals used were 3 month old males.

increased populations of CD43⁺, HSA⁺ cells and CD43⁺, BP-1⁻ cells as well as smaller populations of CD43⁺, HSA⁻ cells and brighter CD43⁻, HSA⁺ cell populations indicated a small accumulation of pro-B cells, suggesting a less efficient pro-B cell to pre-B cell transition, the stage at which V to DJ joining occurs[20].

We next evaluated the ability of yH1 and yK1 together to restore B cell development and Ig production in a strain called Xenomouse, which also contains two functionally inactivated mouse heavy and kappa light chain alleles (yK1;yH1;$\Delta J_H/\Delta J_H$;$\Delta C_\kappa/\Delta C_\kappa$). In the $\Delta J_H/\Delta J_H$;$\Delta C_\kappa/\Delta C_\kappa$ parental mouse, called DI (for double-inactivated), the expression of both mouse heavy and κ was blocked (manuscript in preparation). In contrast to DI mice which did not produce any mature B220⁺ cells (Fig. 5*a*1–*c*1), mature B220⁺ cells were present in Xenomouse at 10% of the level seen in the wild-type. Approximately half of these cells (43%) co-expressed hμ and hκ, while the remainder (57%) co-expressed hμ and mλ (Fig. 5*a*2, *b*2). No co-expression of mλ and hκ was detected, indicating that each light chain completely excluded the expression of the other (Fig. 5*c*2). Higher levels of fully human hμ/hκ antibodies (10 μg ml^{-1}) than hμ/mλ antibodies (3 μg ml^{-1}) were detected in Xenomouse. The level of hμ/hκ was at least several hundredfold higher than the level detected in the parental HuAb strain (9.2C;2B), confirming that inactivation of the mouse heavy and κ genes greatly increased levels of fully human antibodies. In addition, the relative level of B cells expressing only hμ and hκ in Xenomouse was 100–200-fold higher than in the HuAb strains from which antigen-specific human antibodies were obtained, suggesting the usefulness of Xenomouse in deriving fully human mAbs. Higher levels of hμ/hκ antibodies (200 μg ml^{-1}) were detected in another Xenomouse strain (8.2A; 2B;$\Delta J_H/\Delta J_H$;$\Delta C_\kappa/\Delta C_\kappa$). As HuAb strains producing higher levels of hμ/hκ are bred with DI mice to create additional Xenomouse strains, it is anticipated that higher levels of B cell reconstitution and antibody production will be attained.

Discussion

We have produced antigen-specific, fully human mAbs in mice and created mouse strains in which the majority of antibodies produced are fully human. The ability to derive antigen-specific human antibodies upon immunization of mice may be related to the diverse human adult-like repertoire observed following the introduction of large, intact germline segments of the human heavy and κ loci contained on YACs, in contrast to the abnormal repertoires seen in human Ig minigene-bearing mice[2–4]. While the human Ig repertoire of our YAC-containing mice are characterized by the V, D and J usage, length of N addition and CDR3 size observed for adult human B cells[12,13], earlier studies with minigenes revealed fetal-like, position-biased usage of D segments and abnormally small N addition and CDR3 size[3,4], and abnormal CDR3 sequences[2]. These differences, as well as the higher, less position-dependent expression levels noted for YAC-containing mice, may reflect the greater size, variable gene content, structural integrity upon integration, and/or presence of unidentified regulatory elements needed for optimal expression and proper regulation.

The possibility that human Ig genes compete inefficiently with mouse antibody genes by virtue of intrinsic activity or fewer V segments, led us to investigate human Ig expression in mice with inactivated mouse Ig genes. Together, human heavy and κ YACs restored B cell development in mice with inactivated mouse heavy and κ genes, and the majority of antibodies produced are fully human. Indeed, as half of the mature B cells of these mice

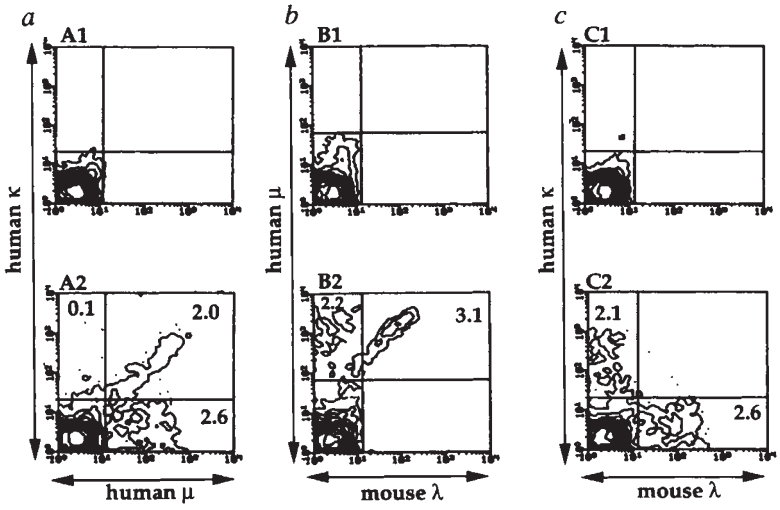

Fig. 5 Reconstitution of B cell development in Xenomouse. Peripheral blood mononuclear cells derived from a 3.5 week old DI ($\Delta J_H/\Delta J_H$; $\Delta C_\kappa/\Delta C_\kappa$) mouse (A1, B1, C1) or Xenomouse (9.2C; 2B; $\Delta J_H/\Delta J_H$; $\Delta C_\kappa/\Delta C_\kappa$) (A2, B2, C2) were assayed for hμ and h_κ (*a*), hμ and mλ (*b*) or hκ and mλ (*c*) as described in Methodology. The net percentage of positively stained cells is shown in each quadrant. All hμ⁺, hκ⁺ and hμ⁺, mλ⁺ cells were B220⁺. In the normal mouse, 47% of peripheral blood mononuclear cells were B220⁺. DI mice were generated by the breeding of homozygous ΔJ_H-mutant ($\Delta J_H/\Delta J_H$) mice[18] and homozygous ΔC_κ-mutant ($\Delta C_\kappa/\Delta C_\kappa$) mice. $\Delta C_\kappa/\Delta C_\kappa$ mice were derived by gene-targeted deletion of the mouse C_κ region (manuscript in preparation.)

express surface hμ and hκ, but not mλ, and the relative level of hμ/hκ B cells is at least 100-fold higher than in HuAb strains used to derive antigen-specific human antibodies, such mice should be extremely useful for obtaining human monoclonal antibodies upon immunization. The yH1 YAC induced the maturation of the growth-arrested B cell lineage in homozygous ΔJ_H-mutant mice, leading to the production of hμ levels approaching those in normal human serum. These results suggest that hμ can readily assemble with mouse B cell receptors, allowing the efficient development of mature, functional B cells. The analysis of bone marrow-derived B cells suggests that in yH1;$\Delta J_H/\Delta J_H$ mice the progression of late pro-B to pre-B cells is somewhat less efficient than in normal mice, a point at which V-DJ rearrangement is initiated[20]. The limited number of V genes in yH1 may result in a lower frequency of rearrangement and thus incomplete B cell development (analysis in progress), suggesting the value of a greater number of human V genes to support more complete B cell maturation and the generation of even more complex repertoires.

The ability to produce a diverse repertoire of fully human monoclonal antibodies may have significant application to human therapy. Unlike humanized mouse antibodies which contain a significant number of residues from murine hypervariable regions, fully human antibodies may be less immunogenic, and thus more suited for repeated administration, as they would present only minor idiotypic variations from any given patient. Such mice lack immunological tolerance to and thus readily yield antibodies to human proteins, which may constitute an important class of therapeutic targets. The introduction of larger portions of the human heavy and light chain loci may ultimately yield strains of mice capable of recapitulating the full repertoire characteristic of the human humoral response to infection or immunization. This strategy of introducing large segments of the human genome into mice coupled with inactivation of the corresponding mouse genes may also have applicability to the investigation of other complex or uncharacterized loci.

Note added in proof: Hybridomas producing human antibodies against a human protein, IgE antibody, have been generated.

Methodology

Generation and DNA analysis of yH1- and yK1-containing ES cells and mice. yH1 (240 kb) and yK1 (195 kb) were identified from the Washington U. human-YAC library DNA using V_{VI} PCR primers and a C_κ probe, respectively (M.J.M. *et al.*, manuscript in preparation). yH1- and yK1-containing yeast were fused with E14.TG3B1 cells as described[7] and HAT-resistant colonies were expanded for analysis. Probes used: D (detecting three expected *Hind*III bands, 9.7 kb, 8.5 kb and 7.0 kb (as a doublet), of D1–D4 family segments), Cμ, Cδ, J_H, V_{VI}, V_I and V_{IV} as described in ref. 21; V_{II}[22], C_κ (ATCC, 59173), J_κ[23,24], B1[25], B2[25], B3[26], mC_κ[27] and yeast Ty[7]. The number of integrated YACs per ES cell was determined by densitometric measurements of human Ig hybridization signals, normalized to those of mouse C_κ probe, and compared to the signal intensity of YACs in yeast. An equivalent hybridization signal should be obtained from a single-copy sequence in 20 ng of haploid yeast DNA and in 10 μg of diploid mammalian DNA.

Chimaeric mice were generated by microinjection of ES cells into C57Bl/6 blastocysts. HuAb offspring were identified by PCR analysis of tail DNA (30 cycles of 1 min at 94 °C; 2 min at 60 °C; 3 min at 72 °C) using human-specific primers for D: (DXPA: 5'–GCAATTACTAGTATAGACCCGAGTGTCCCC–3'; and DXPB: 5'–GCAATGGTCGACGGTTTTTGATGGACTCTG–3'; 230 bp fragment), and J_κ: (J_κ0: 5'–CTCGAAAAGGGAGTTGAGCT TCAGCAGCTG–3'; and J_κ3: 5'–GATACAATGGCACTAAAATC TCACG–3'; 860 bp fragment) and control primers for the mouse γ interferon receptor (IFN-γR) (5'–TTGGATTCTGGTG GTTGCTC–3' and 5'–GACCTATTTGTGCATTGGAAGC–3'; 550 bp fragment). The size markers (M) are a 1 kb DNA ladder (Gibco/BRL).

Flow cytometry analysis. Peripheral blood, spleen and bone marrow lymphocytes obtained from 4–12 week old transgenic or control mice were purified on Lympholyte M (Accurate) and treated with purified Fc γ II receptor (Pharmingen, 01241D) to block non-specific binding to Fc receptors, stained with antibodies and analysed on a FACScan (Becton Dickinson, LYSIS II software). Antibodies used: CyChrome anti-B220 (Pharmingen, 01124A); fluorscein isothiocyanate (FITC) anti-hIgM (Pharmingen, 08074D); phycoerythrin (PE) anti-mIgM^a (Pharmingen, 05095B) and PE anti-mIgM^b (Pharmingen, 05105B); biotin anti-hδ (Southern Biotechnology Association, 9030-08); biotin anti-hκ (Pharmingen, 08172D); FITC anti-mλ (Pharmingen, 02174D); FITC anti-CD43 (Pharmingen, 01604D); PE anti-CD43 (Pharmingen, 01605B); biotin anti-6C3/BP-1 (Pharmingen, 01282D); PE anti-HSA (Pharmingen, 01575A). PE-streptavidin (Pharmingen, 13025D) was used to detect biotinylated antibodies.

To assay hκ versus mκ expression in yK1 transgenic mice, mouse peripheral blood lymphocytes were first incubated with rat IgG (Pierce, 31233) then stained with PE-conjugated goat anti-mκ (Southern Biotechnology Association, 1050-09), washed, incubated with mouse IgG (Pierce, 31204), then stained with FITC-conjugated mouse anti-hκ (Pharmingen, 08174D) and CyChrome-conjugated anti-B220.

ELISA assays. Sera were obtained from 4–12 wk old transgenic or chimaeric (containing 40–90% of ES cell-derived B cells) mice. Human serum μ was assayed using mouse monoclonal anti-hμ (AMAC, Clone Af6) immobilized on Nunc Immuno plates (Maxisorp F96) and detected with biotinylated goat anti-hμ (Caltag, preabsorbed with normal mouse serum to lower background due to cross reactivity). Similarly, hκ was assayed using goat anti-hκ (Vector) to capture and detected with biotinylated goat anti-hκ (Vector). The standard used to determine hμ concentrations was hIgM (Sigma, I-

8260) shown to be equivalent to a chimaeric hIgM/mλ antibody (Serotec, MCA 446). The standard used to determine hκ concentrations was $hIgG_1$/hκ (Sigma, I-3889). Hμ/hκ antibodies were detected in serum by ELISA using mouse monoclonal anti-hμ (AMAC, Clone Af6) to capture and detected with biotinylated goat anti-hκ (Vector) using hIgM (Sigma, I-8260) as a standard. Tet C-specific antibodies (polyclonal or monoclonal) were assayed by coating plates (see above) with 100 ng tet C (Boehringer Mannheim, 1348655)/well, incubating with serial dilutions of the appropriate serum and then detecting with either biotinylated goat anti-hμ (Caltag, see above) or biotinylated goat anti-hκ (Vector). Human monoclonal antibodies were assayed the same as hμ/hκ antibodies above. Biotinylated antibodies were detected using ABC-HRP (Vector, PK-4000). Absorbance at A490 was measured using a UVmax spectrophotometer .

Immunization of mice and generation of hybridomas. Mice were immunized 4 times at about 2 wk intervals with tet C (Boehringer Mannheim 1348655) 50 μg/injection, in Freund's Complete Adjuvent (primary injection) or Freund's Incomplete Adjuvent (subsequent boosts) subcutaneously. The mice were bled 4 days after final boost and titered for human anti-tet C antibodies by plate ELISA. Serially diluted samples were incubated with 100 ng tet C bound/well of Nunc Immuno plate. The human antibody chains were then detected using the biotinylated anti-hμ and hκ antibodies (see "ELISA assays"). To generate mouse hybridomas, splenocytes from immunized mice were fused with nonproducer P3X63-Ag8.653 myeloma cells, 4–5 days following the final boost, using 50% PEG 4000 (Boehringer Mannheim). After 10–14 days the supernatants from hybrids growing in HAT-selected medium were screened for the presence of fully human antibodies and for tet C specificity as described above .

Repertoire of human Ig transcripts expressed in transgenic mice. poly(A)$^+$ mRNA was isolated from a yH1 spleen (strain 2B) or a yK1 spleen (strain 8.2A) transgenic mouse using a FastTrack kit (Invitrogen). Human μ-specific mRNAs amplified using a 5' Amplifinder RACE kit (Clontech), using oligonucleotide hμP1 (5'–TTTTCTTTGTTGCCGTTGGGGTGC–3') for reverse transcription and hμP2 (5'–GGGAAGCCCCGGGTGCTGCTGATG–3') for amplification. Human κ-specific cDNAs were amplified using hKP1 (5'-CTCTGTGACACTCTCCTGGGAGTT-3') for reverse transcription and hKP2 (5'–ACCCGATTGGAGGGCGTT-ATCCAC–3') for amplification. PCR products were cloned into pCRII using a TA Cloning kit (Invitrogen) and their sequences determined by double-stranded dideoxynucleotide sequencing. For hybridization experiments, individual colonies were picked onto multiple gridded LB-amp plates and grown at 37 °C. Colonies were transferred to Genescreen (DuPont) and lysed *in situ*[28] to yield replicas of the ordered grid. DNAs immobilized on filters were probed with ^{32}P-labelled V_H or Cμ fragments for yH1 cDNAs or with V_κ fragments for yK1 cDNAs (see above). ^{32}P-end labelled J_H- or J_κ-specific oligonucleotides were hybridized to DNAs on filters in 1% bovine serum albumin, 1.25 mM EDTA, 0.5 M $NaPO_4$ buffer, pH 7.2, and 7.1% SDS at 38 °C except J_H6 which was hybridized at 30 °C. Filters were washed three times in 6× SSC for 3 min at room temperature then washed once in 6× SSC for 3 min at 34 °C with the exceptions that J_H6-probed filters were washed at 30 °C and J_H5-probed filters were washed at 42 °C. J_H-specific oligonucleotides were: J_H1: 5'–ACTTCCAGCACTGGG–3' J_H2: 5'–ACTTCGATCTCT GG–3' J_H3: 5'–TTTTGATATCTGGGG–3' J_H4: 5'–TTTGACTACT–GGGGC–3' J_H5: 5'–TTCGACCCCTGGGGC–3' J_H6: 5'–TACTA–CTACTACTAC–3'. J_κ-specific oligonucleotides were: $J_\kappa1$: 5'–AGGTGGAAATCAAAC–3' $J_\kappa2$: 5'–TTTGGCCAGGGGACC–3' $J_\kappa3$: 5'–TTCGGCCCTGGGACC–3' $J_\kappa4$: 5'–TTTCGGCGGAGGGAC–3' $J_\kappa5$: 5'–AGGGACACGACTGGA–3'.

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