

SPECIFIC V GENE USAGE IN ANTI-PHOSPHORYLCHOLINE IgE ANTIBODIES*

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Abstract—Three hybridomas from phosphorylcholine(PC)-KLH immunized BALB/c mice producing IgE antibodies against the PC hapten were investigated for their fine specificity to the hapten and usage of V gene segments in H- and L-chains. All three IgE antibodies recognize the entire azophenyl-PC hapten. They are T15 Id negative and do not bind to the natural PC determinant expressed by the *Streptococcus* carbohydrate R36A. T15 Id positive IgE antibodies could neither be elicited by immunization in detectable amounts nor generated by the cell fusion technique. By using the Southern blot technique and nucleotide sequence analysis of PCR amplified V_HDJ_H and V_LJ_L rearrangements, we have demonstrated that the three IgE anti-PC hybridomas use the V_H1 -DSP2- J_H2 , the V_HOX1 -DSP2- J_H3 or the V_H36 -60-D- J_H2 gene segment combinations for the H chain together with the $V_{\kappa}1C$ - $J_{\kappa}1$, $V_{\kappa}1C$ - $J_{\kappa}2$ or $V_{\lambda}1$ - $J_{\lambda}1$ genes for the L chains. Except for the V_H36 -60, the same gene segments were found in different combinations in anti-PC antibodies of other Ig classes than IgE. However, high rates of somatic mutations are expressed in both V_H1 of the H chain and in $V_{\kappa}1C$ of the L chain. The V_H36 -60 is expressed in antibodies with the major Id of the azophenyl-arsonate (Ars) response and V_HOX1 generally contributes to the phenyl-oxazolone specificity. This suggests that these V genes are involved in the recognition of the azophenyl moiety of the coupled PC hapten. Thus PC-KLH specific IgE antibodies utilize mutated V_H1 and/or V_H/V_L gene segment combinations which are involved in binding of the azophenyl spacer. These IgE are therefore specific for azophenyl-phosphorylcholine, unlike antibodies normally expressed against the *Streptococcus* PC determinant in mice. The genetic diversity and the high mutation rates indicate that the specific B cells develop later in the immune response. Thus, they represent newly generated specificities of so-called group II anti-PC antibodies and are not isotype-switch descendants from already existing T15 Id positive IgM antibodies.

INTRODUCTION

Primary and memory antibody responses to phosphorylcholine (PC)-conjugated antigenic protein carrier differ from each other in fine specificity, idiotype (Id) expression and isotype preference (Blaser and Heusser, 1987; Weber *et al.*, 1986; Chang and Rittenberg, 1981; Lötscher *et al.*, 1990; Stenzel-Poore and Rittenberg, 1989; Heusser *et al.*, 1984; Bruderer *et al.*, 1988). In the primary response, >90% of the anti-PC antibodies display the T15 Id and express the V_H1 -FL16.1- J_H1 H chain rearrangement in combination with $V_{\kappa}22$ - $J_{\kappa}5$ L chain (Clafin and Cubberley, 1980). In contrast, memory antibodies to PC are more diverse and consist of three main specificities directed to the entire azophenyl-PC hapten, to its PC or the azo-phenyl moiety, respectively (Lötscher *et al.*, 1992, 1993). These sub-specificities

to one and the same hapten result from the usage of particular combinations of V gene segments involved in PC binding with others usually involved in phenyl or azo-phenyl recognition (Stenzel-Poore and Rittenberg, 1989; Lötscher *et al.*, 1992, 1993; Todd *et al.*, 1984; Chang *et al.*, 1984; Stenzel-Poore *et al.*, 1987). The phenyl-PC and azo-phenyl specificities are predominantly associated with the IgG1, IgG2a, IgG2b and IgE classes (Weber *et al.*, 1986; Heusser *et al.*, 1984; Chang and Rittenberg, 1982). IgE antibodies produced excessively against allergens play an important role in the pathogenesis of immediate-type allergic diseases. Phenyl-PC-specific IgE can be elicited by PC coupled to a T-dependent protein carrier, but not by inoculation of *Pneumococci* or *Streptococcus pneumonia* R36A carbohydrate (Blaser and Heusser, 1987; Heusser *et al.*, 1984).

Despite the fact that the comprehension of the regulatory mechanism involved in specific IgE formation is of great interest, only little is known about the specific V-gene usage in anti-PC IgE antibodies. So far, partial N-terminal amino-acid and nucleotide sequence analysis revealed that IgE aPC 12-3 utilizes $V_{\kappa}1$ -3 L chains, while H chains, as in T15 Id⁺ anti-PC antibodies, are inaccessible to Edman degradation (Heusser *et al.*, 1984; Bruderer *et al.*, 1988; Chang *et al.*, 1984).

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Abbreviations: PC, phosphorylcholine; NPPC, *p*-nitro-phenyl-phosphorcholine; KLH, keyhole limpet hemocyanin.

Although 2/3 of the anti-PC IgE response could be suppressed in BALB/c mice which were actively or passively immunized with isologous anti-Id to the TEPC-15 myeloma protein (Blaser and Heusser, 1984, 1987; Blaser *et al.*, 1979; Blaser and de Weck, 1982), T15 Id positive IgE was neither detectable in serum nor could be generated by the cell fusion technique (Blaser and Heusser, 1987; Weber *et al.*, 1986; Blaser and de Weck, 1982). The serum IgE anti-PC antibodies are phenyl-PC or azophenyl specific, as are most other memory response antibodies to PC-KLH (Blaser and Heusser, 1987; Weber *et al.*, 1986). In order to investigate the functional V-region gene segment usage in H and L chains of IgE anti-PC in comparison to the other isotypes described in the literature (Lötscher *et al.*, 1990, 1992, 1993; Stenzel-Poore and Rittenberg, 1989), IgE secreting hybridomas were generated from the memory response to PC-KLH in BALB/c mice and subjected to Southern blot and nucleotide sequence analysis.

MATERIALS AND METHODS

Production and selection of anti-PC IgE hybridomas

The IgE anti-PC hybridomas aPC-12-3, aPC-4-33 and aPC-6-6-4 were produced from different BALB/c mice immunized i.p. three times in 3 week intervals with 2 μ g PC₇₀-KLH in 2 mg Al₂(OH)₃. One week later, 10⁸ spleen cells were transferred i.v. into a 650 R X-irradiated recipient immunized i.p. with 2 μ g PC₇₀-KLH in 2 mg Al₂(OH)₃ one day after the transfer. The spleen cells were fused five days later with the myeloma SP2/0 at a spleen cell to SP2/0 ratio of 2.3:1 in 0.9 ml polyethylene glycol 1500. The supernatants were screened for anti-PC IgE antibodies by ELISA using PC-BSA coated microtiter plates. Bound IgE antibodies were detected with the biotinylated C_H ϵ specific monoclonal rat anti-mouse IgE antibody Ram 638-18-20. This antibody reacted with a streptavidin-biotinylated horseradish peroxidase complex (Amersham International plc, U.K.). The nature of the IgE antibodies was confirmed by passive cutaneous anaphylaxis (PCA) titration in rat skin using PC-BSA for challenge (Blaser and Heusser, 1987; Weber *et al.*, 1986). Hybridoma cells secreting anti-PC IgE antibodies were cloned and recloned by limiting dilution. They were grown in Dulbecco's modified Eagle's medium (Seromed) supplemented with 10% FCS, 100 μ g/ml gentamycin, 0.4% sodium bicarbonate, 1mM sodium pyruvate, 4mM L-glutamine and 0.01mM 2-mercaptoethanol (GIBCO).

L chain isotype analysis, fine specificity and T15 Id expression

The L chain isotype was determined by ELISA using monoclonal anti- κ , anti- λ 1 and anti- λ 1, λ 2, λ 3 Ram antibodies as described by Lötscher *et al.* (Lötscher *et al.*, 1990). The fine specificity of the anti-PC IgE antibodies was established in a solid phase ELISA by comparing the capacity of free PC-chloride and *p*-nitro-phenylphosphorylcholine (NPPC) to inhibit binding of antibody to PC-BSA or HSA (Weber *et al.*, 1986; Lötscher

et al., 1990; Chang and Rittenberg, 1982). The molar concentration required for 50% inhibition of the maximal antibody binding (IC₅₀) was determined. The fine specificity is defined by the IC₅₀ NPPC/PC quotient, which is typically >1 for PC-specific and <10⁻¹ for phenyl-PC-specific anti-PC antibodies (Weber *et al.*, 1986; Chang and Rittenberg, 1981; Heusser *et al.*, 1984; Lötscher *et al.*, 1992). The binding of anti-PC antibodies to *Streptococcus pneumoniae* R36A and the expression of the T15 Id was determined by solid phase ELISA as described (Lötscher *et al.*, 1990, 1992, 1993).

Hybridization probes and Southern blot analysis

The H chain-specific probe was a 1.4 kb Pst I germline fragment including J_H4 and its 3' flanking region (Lötscher *et al.*, 1990, 1992). As κ -chain-specific probe, a 720 bp Bst EII germline fragment from the intron between J _{κ} 5 and the C _{κ} exon was employed (Sahagan, 1986). Both probes were kindly provided by Dr S. Morrison (Columbia University, NY). The C _{λ} 1 probe was a 450 bp XhoI-Pst I cDNA fragment from the rearranged λ gene of MOPC-104 E (Bothwell *et al.*, 1981) kindly provided by Dr S. Weiss (Institute for Immunology, Basel).

All hybridizing probes were labeled with a ³²P-dGTP (NEN Du Pont, Boston, MA) by the random primed labelling method, as described by Feinberg and Vogelstein (Feinberg and Vogelstein, 1983). The labellings were performed with a reagent kit from Böhringer Mannheim GmbH (Mannheim). Specific activities of about 10⁹ cpm/ μ g DNA were obtained. Genomic DNA was isolated from the anti-PC IgE hybridomas according to standard protocols. Southern blotting and hybridization was performed as described (Lötscher *et al.*, 1990, 1992; Wahl *et al.*, 1979).

Oligonucleotide primers and polymerase chain reaction

The following oligonucleotide primers were used to amplify the H and L chain V gene rearrangements of the anti-PC IgE hybridomas: (a) V_H1 primer: 5'-GGGATCCGAATTCGCAGGTATCCAGTGTGAGGTG-3'; located from bp 15 to bp 6, exon V_H1. Restriction sites: Bam HI and Eco RI. (b) V_H BACK primer: 5'-GGGAGCTCAAGCTTAGGT(C/G)(A/C)A(A/G)CTGCAG(C/G)AGTC(A/T)GG-3' (Orlandi *et al.*, 1989); located from bp 2 to bp 23, exon V_H. Restriction sites: Pst I, Hind III and Sac I. (c) V_H FOR (J_H) primer: 5'-ATGTCGACTCTAGACGGTGACCGTGGTCCCTTGCC-3' (Orlandi *et al.*, 1989); located in the second half of the four J_H germ-line segments. Restriction sites: Sal I, XbaI and Bst EIII. (d) V _{κ} 1 primer: 5'-GGGATCCGAATTCACCCAACTCCACTCTCCCTG-3'; located from bp13 to bp33, exon V _{κ} 1. Restriction sites: Bam HI and Eco RI. (e) κ 3' primer: 5'-GGGAAGCTTCATGAAACCTGTGTCTTACAC-3'; located from bp28 to bp49, intron 3' side of J _{κ} 5. Restriction site: Hind III. (f) V _{λ} primer: 5'-GGGATCCGAATTCAGGCTGTTGTGACTCAGGAATCTGC-3'; located from bp1 to bp26, exon V _{λ} . Restriction sites: Bam HI and Eco RI. (g) C _{λ} 1 primer: 5'-GGGA-

AGCTTCTAGAGGGTGACTGATGGCGAAGACT-TGGG-3'; located from bp7 to bp31, exon C_λ1. Restriction sites: Hind III and XbaI.

Total RNA was prepared from 5×10^7 hybridoma cells according to standard procedures. First-strand cDNA synthesis was performed in a 50 μ l reaction mixture containing 100mM Tris-HCl (pH 8.3), 10 mM MgCl₂, 140mM KCl, 7mM dithiothreitol, 6 units RNase inhibitor (Böhringer Mannheim), 250 μ M of each dNTP (Pharmacia, Uppsala, Sweden), 10 μ g total RNA (heated at 90°C for 2 min) and 2 μ M of appropriate primer. 45 units of AMV reverse transcriptase (Pharmacia) were added and the samples were incubated at 42°C for 1 h. Amplification was performed by PCR in a final volume of 100 μ l containing either 15 μ l of the first-strand cDNA synthesis reaction or 4 μ g genomic DNA as template (Table 1), 1 μ M of appropriate primers and 1.5mM MgCl₂, 68mM Tris-HCl (pH 8.8), 50mM KCl, 20 μ g gelatine, 0.2% Triton-X-100, 250 μ M of each dNTP and 1 unit of Taq polymerase (Böhringer Mannheim) were further added. The reaction mixture was overlaid with 80 μ l paraffin oil and subjected to 30 rounds of temperature cycling on a Thermocycler 60 heating block (Bio-med). Each cycle consisted of 1 min at 95°C (denaturation), 1 min at 65°C to anneal primers and 2 min at 72°C for strand synthesis. After purification, the PCR products of suitable size were cloned into Gene Scribe-Z vectors pTZ 18/19 (USB, Cleveland, OH) or into the sequencing vector M13mp18. Ligations were performed according to standard protocols. Competent *E. coli* JM101 cells were transfected by electroporation with a Biorad gene pulser following the

Table 2. Properties of monoclonal IgE anti-PC antibodies

Cell lines	aPC-4-33	aPC-12-3	aPC-6-6-4
Isotype	ϵ/κ	ϵ/κ	ϵ/λ
IC ₅₀ NPPC/PC	0.04	0.02	0.08
Specificity	Phe-PC	Phe-PC	Phe-PC
PN ^a binding	Negative	Negative	Negative
T15 Id	Negative	Negative	Negative

^aPN: *Streptococcus pneumoniae* R36A.

recommendations of the manufacturers. Recombinant ssDNA was produced and isolated as described (Dente *et al.*, 1983; Sanger *et al.*, 1977) and sequenced according to the di-deoxy method of Sanger *et al.* (Sanger *et al.*, 1977) using a T7 sequencing kit (Pharmacia). To rule out nucleotides misincorporated during PCR, the sequences of at least four independent clones were compared.

RESULTS

Binding characteristics of IgE anti-PC hybridomas

The IgE antibody secreting hybridomas aPC-4-33, aPC-12-3 and aPC-6-6-4 were obtained from two fusions with SP2/0 cells. The properties of the monoclonal IgE anti-PC antibodies are summarized in Table 2. Isotype determination by solid-phase ELISA with L chain-specific monoclonal antibodies showed that aPC-4-33 and aPC-12-3 express the ϵ isotype in association with a

Table 1. V region characterization of the monoclonal IgE anti-PC antibodies

Cell lines	aPC-4-33	aPC-12-3	PC-6-6-4
V _H family	S107	Q52	36-60
V _H	V _H 1	V _H -OX1	V _H 36-60
D _H	SP2.2	SP2.6	— ^a
J _H	J _H 2	J _H 3	J _H 2
V _L	V _L 1C	V _L 1C	V _L 1
J _L	J _L 1	J _L 2	J _L 1
H CDR3 length	8	10	6
Mutations V _H ^b	14	2	11
Mutations V _L	11	2	7
Silent V _H	4	1	3
Silent V _L	5	1	3
R/S V _H CDR ^c	7/1	1/0	2/1
R/S V _H FR ^c	3/3	0/1	6/2
R/S V _L CDR	5/3	1/1	4/1
R/S V _L FR	1/2	0/0	0/2
Source for PCR H	cDNA	cDNA	cDNA
Source for PCR L	DNA	DNA	DNA

^aGerm-line and family assignment undetermined.

^bTotal number of mutations in the V_H region. Nucleotide changes within the D_H segments and those caused by the 5' primer in PCR are not considered.

^cReplacement/silent mutation quotient in CDR and FR regions (without D_H segment).

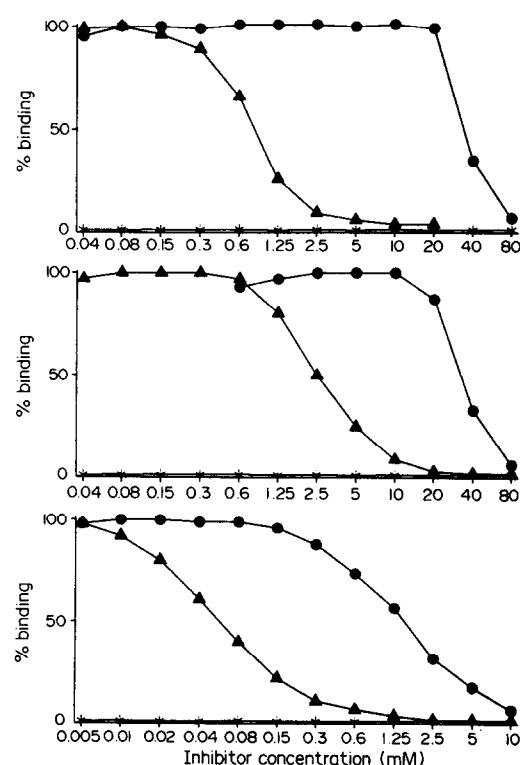


Fig. 1. Inhibition of IgE anti-PC antibody binding PC-HSA in ELISA. The binding of anti-PC antibodies aPC-12-3 (top), aPC-4-33 (middle) and aPC-6-6-4 (bottom) to PC-HSA was inhibited by PC-Cl (●) and NPPC (◆), respectively. Culture medium was used as a background control (*).

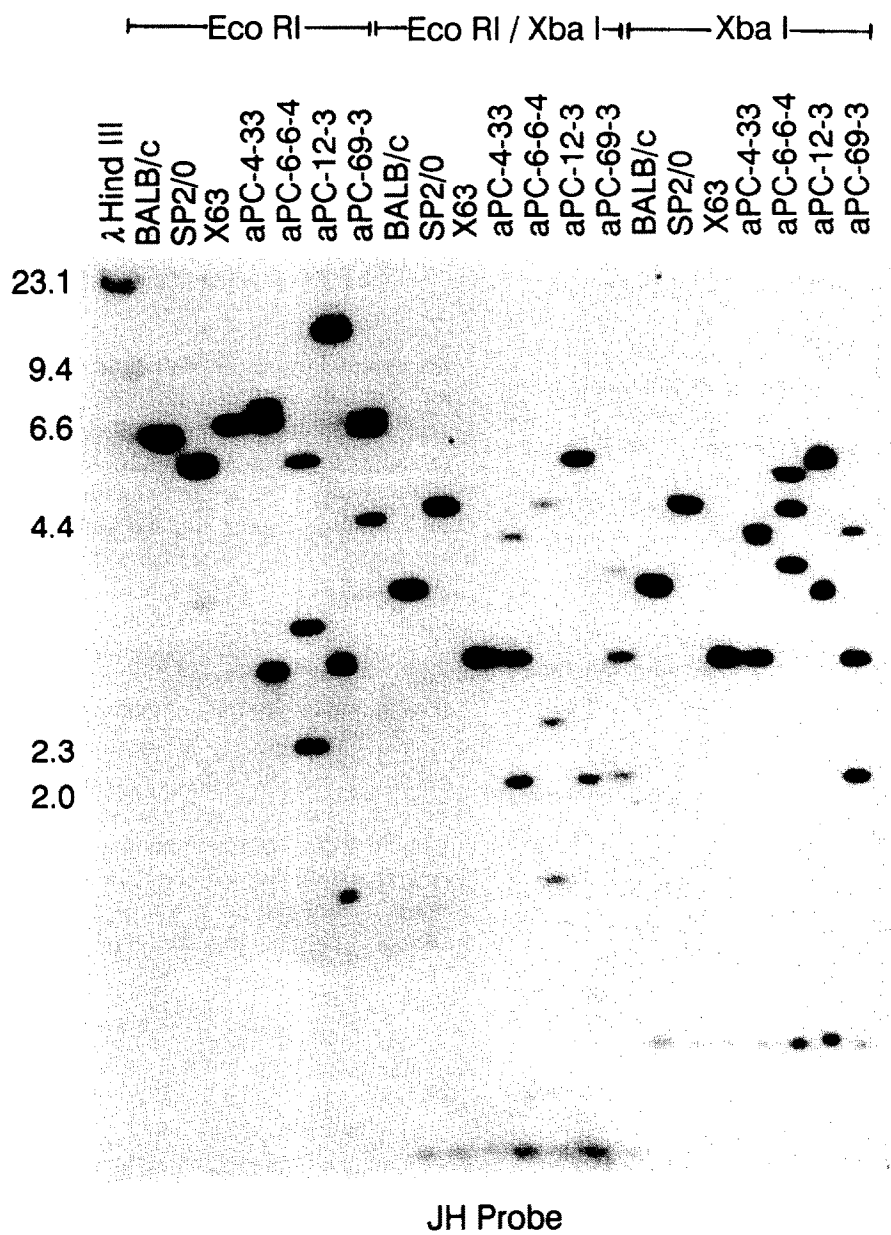


Fig. 2. Southern blot analysis of IgE anti-PC hybridomas. The size of Hind III digested λ phage DNA on the left side of the panels is indicated in kb.

κ-L chain and aPC-6-6-4 with λ1. The fine specificity was determined by ELISA inhibition with PC-chloride and NPPC as inhibitors. As demonstrated in Fig. 1, all three IgE antibodies revealed the typical binding characteristics towards phenyl-PC. Furthermore, they did not bind to *Streptococcus pneumoniae* strain R36A and were T15 Id negative (Table 2).

V gene usage of anti-PC IgE

Southern blot analysis using the J_H probe revealed different hybridizing band patterns in the three IgE anti-PC hybridomas suggesting the usage of different V regions (Fig. 2). The V_H gene rearrangements of these cells were amplified by PCR, and subsequently cloned and sequenced. The aPC-4-33 cells expressed the V_H1 germ-line gene combined with J_H2 (Fig. 3, Table 1). This functional rearrangement is comprised of the 7.2 kb Eco RI and in the 4.2 kb XbaI and Eco RI/XbaI bands on

the Southern blot (Fig. 2). The V_H1 segment of aPC-4-33 has undergone extensive mutational processes. Compared to the V_H1 germ-line gene, 14 nucleotide substitutions could be detected (Fig. 3, Table 1).

Most replacement mutations took place in the CDR 2 region, where Ala52 C was exchanged by Val, Asn53 by Tyr, Ser60 by Asn, Ala61 by Ser and Val63 by Leu. The ratio between replacement and silent mutations (R/S) in the H chain regions is high (7/1), indicating that the alterations were antigen driven. The D segment of aPC-4-33 resembles DSP2.2, which is also used in combination with V_H1 in memory anti-PC antibodies of other isotypes (Stenzel-Poore and Rittenberg, 1989). The CDR3 region consists of eight amino acid residues, of which four are encoded by the J_H2 segment (Fig. 3). The aPC-4-33 expresses V_H1-DSP2.2-J_H2 in association with the V_κ1C-J_κ1 rearrangement (Fig. 4) which is contained in the 13 kb Bam HI band in the Southern blot using the

This rearrangement is comprised in the 2.9 kb Eco RI, 3.5 kb XbaI and 2.1 kb Eco RI/XbaI bands in Fig. 2. The V_H-OX1 gene used in aPC-12-3 shows only two nucleotide substitutions (Table 1, Fig. 6). The only replaced amino acid in CDR2 is Ser56, which is substituted by Asn. The CDR3 region is longer than in aPC-4-33 and consists of 10 amino acids. The L chain V

	10										20																		
V1	Glu	Val	Lys	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Thr	Ser	Gly	Phe	Thr	Phe
S107 ^a	GAG	GTG	AAG	CTG	GTG	GAA	TCT	GGA	GGA	GGC	TTG	GTA	CAG	CCT	GGG	GGT	TCT	CTG	AGA	CTC	TCC	TGT	GCA	ACT	TCT	GGG	TTC	ACC	TTC
	-----VH BACK primer-----																												
	Gln Gln																												
4-33	--C C-A--CA--G-----A-----																												
	Glu Val Gln Leu Gln Glu Ser Gly Pro Ser Leu Val Lys Pro Ser Gln Thr Leu Ser Leu Thr Cys Ser Val Thr Gly Asp Ser Ile																												
VH36-60 ^b	GAG	GTG	CAG	CTT	CAG	GAG	TCA	GGA	CCT	AGC	CTC	GTG	AAA	CCT	TCG	CAG	ACT	CTG	TCC	CTC	ACC	TGT	TCT	GTC	ACT	GGC	GAC	TCC	ATC
6-6-4	--C--G-----T-----																												
	-----CDR 1-----																												
	30 40 50 52A 52B 52C																												
V1	Ser	Asp	Phe	Tyr	Met	Glu	Trp	Val	Arg	Gln	Pro	Pro	Gly	Lys	Arg	Leu	Glu	Trp	Ile	Ala	Ala	Ser	Arg	Asn	Lys	Ala	Asn	Asp	Tyr
S107	AGT	GAT	TTC	TAC	ATG	GAG	TGG	GTC	CGC	CAG	CCT	CCA	GGG	AAG	AGA	CTG	GAG	TGG	ATT	GCT	GCA	AGT	AGA	AAC	AAA	GCT	AAT	GAT	TAT
	Asn Val Tyr																												
4-33	--A--T--T--																												
	Thr Ser Gly Tyr Trp Asn Trp Ile Arg Lys Phe Pro Gly Asn Lys Leu Glu Tyr Met Gly Tyr Ile Ser ... Tyr Ser Gly																												
VH36-60	ACC	AGT	GGT	TAC	TGG	AAC	TGG	ATC	CGG	AAA	TTC	CCA	GGG	AAT	AAA	CTT	GAG	TAC	ATG	GGG	TAC	ATA	AGC	...	...	...	TAC	AGT	GGT
	Val																												
6-6-4	--G--A--C--																												
	-----CDR 2-----																												
	60 70 80 82A 82B																												
V1	Thr	Thr	Glu	Tyr	Ser	Ala	Ser	Val	Lys	Gly	Arg	Phe	Ile	Val	Ser	Arg	Asp	Thr	Ser	Gln	Ser	Ile	Leu	Tyr	Leu	Gln	Met	Asn	Ala
S107	ACA	ACA	GAG	TAC	AGT	GCA	TCT	GTG	AAG	GGT	CGG	TTC	ATC	GTC	TCC	AGA	GAC	ACT	TCC	CAA	AGC	ATC	CTC	TAC	CTT	CAG	ATG	AAT	GCC
	Asn Ser Leu Asp																												
4-33	--A-T--T--A--A--																												
	Ser Thr Tyr Tyr Asn Pro Ser Leu Lys Ser Arg Ile Ser Ile Thr Arg Asp Thr Ser Lys Asn Gln Tyr Tyr Leu Gln Leu Asn Ser																												
VH36-60	AGC	ACT	TAC	TAC	AAT	CCA	TCT	CTC	AAA	AGT	CGA	ATC	TCC	ATC	ACT	CGA	GAC	ACA	TCC	AAG	AAC	CAG	TAC	TAC	CTG	CAG	TTG	AAT	TCT
	Phe Val																												
6-6-4	--T--G--																												
	-----CDR 3-----																												
	82C 90 JH-----																												
V1	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Ile	Tyr	Tyr	Cys	Ala	Arg	D segment					Tyr Trp Tyr Phe Asp Val Trp Gly										
S107	CTG	AGA	GCT	GAG	GAC	ACT	GCC	ATT	TAT	TAC	TGT	GCA	AGA	G						TAC TGG TAC TTC GAT GTC TGG GGC JH1									
	Asp Tyr Tyr Gly Ser Ser																												
4-33	--AT TAC TAC GGT AGT AGC-----Tyr Phe Asp Tyr Trp Gly																												
	Val Val Gly Asn Tyr Asp Phe																												
6-6-4	--T--G--AAT TAT GAC-----T--G--																												
	Val Thr Thr Glu Asp Thr Ala Thr Tyr Tyr Cys Ala																												
VH36-60	GTG	ACT	ACT	GAG	GAC	ACA	GCC	ACA	TAT	TAC	TGT	GCA	A																
	Ser Phe Ile Asn Thr Gly Lys Gly																												
6-6-4	--T--T--AT--AT ACT GGA AAG-----G--																												

Fig. 3. V_H nucleotide sequences of two hybridomas secreting IgE anti-PC antibodies. The V_H sequence of aPC-4-33 is compared with the V1 germ-line gene (Kim *et al.*, 1981) and the one of aPC-6-6-4 with the V_H36-60 germ-line sequence (Near *et al.*, 1984). Nucleotide identity is indicated by a dash. Gaps introduced in CDR3 to allow the assignment of the J_H segments are indicated by dots. The position of the V_HBACK primer is mentioned. The remaining part of the J_H segments is identical to the analogous germ-line sequence. The numbering of the residues is according to Kabat *et al.* (Kabat *et al.*, 1987).

		10										20										27A 27B									
		Asp Val Leu Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Ile										Gln Ala Val Val Thr Gln Glu Ser Ala ... Leu Thr Thr Ser Pro Gly Glu Thr Val Thr Leu Thr Cys Arg Ser Ser Thr Gly Ala										27D 27E									
V _K 1C		GAT GTT TTG ATG ACC CAA ACT CCA CTC TCC CCG CTT GTC AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT										CAG GCT GTT GTG ACT CAG GAA TCT GCA ... CTC ACC ACA TCA CCT GGT GAA ACA GTC ACA CTC ACT TGT CGC TCA AGT ACT GGG GCT																			
4-33		-----V _K 1 primer-----										-----V _K 1 primer-----																			
12-3		-----V _K 1 primer-----										-----V _K 1 primer-----																			
		-----CDR 1-----										-----CDR 2-----																			
		27C 27D 27E										50																			
V _K 1C		Val His Ser Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn										GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC CTG CAG AAA CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC																			
4-33		A-C										A																			
12-3		Tyr										T																			
		27F																													
V _K 1		Val Thr ... Thr Ser Asn Tyr Ala Asn Trp Val Gln Glu Lys Pro Asp His Leu Phe Thr Gly Leu Ile Gly Gly Thr Asn Asn										GTT ACA ... ACT AGT AAC TAT GCC AAC TGG GTC CAA GAA AAA CCA GAT CAT TTA TTC ACT GGT CTA ATA GGT ACC AAC AAC																			
6-6-4		Arg										C																			
		---CDR 2---																													
		60										70										80									
V _K 1C		Arg Phe Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp										CGA TTT TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT																			
4-33		Leu										C										G									
12-3																															
		Arg Ala Pro Gly Val Pro Ala Arg Phe Ser Gly Ser Leu Ile Gly Asp Lys Ala Ala Leu Thr Ile Thr Gly Ala Gln Thr Glu Asp										CGA GCT CCA GGT GTT CCT GCC AGA TTC TCA GGC TCC CTG ATT GGA GAC AAG GCT GCC CTC ACC ATC ACA GGG GCA CAG ACT GAG GAT																			
V _K 1																															
6-6-4																															
		-----CDR 3-----																													
		90										J _K																			
V _K 1C		Leu Gly Val Tyr Tyr Cys Phe Gln Gly Ser His Val Pro										Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg																			
4-33		CTG GGA GTT TAT TAC TGC TTT CAA GGT TCA CAT GTT CCT										TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGT										J _K 1									
12-3		Leu										Ser										J _K 1									
		Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg										TAC ACG TTC GGA GGG GGC ACC AAG CTG GAA ATA AAA CGT										J _K 2									
V _K 1		Glu Ala Ile Tyr Phe Cys Ala Leu Trp Tyr Ser Asn His										Trp Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly										J _K 1									
6-6-4		GAG GCA ATA TAT TTC TGT GCT CTA TGG TAC AGC AAC CAT										TGG GTG TTC GGT GGA GGA ACC AAA CTG ACT GTC CTA GGT										J _K 1									
		Asn Asp										Leu																			
		A G										T										C									

Fig. 4. V_L nucleotide sequences of IgE aPC-4-33, aPC-12-3 and aPC-6-6-4. The V_K1C (Corbett *et al.*, 1987) and the V_K1 (Bernard *et al.*, 1978) germ-line sequences are shown for comparison. The position of both 5' primers are presented in the figure. Nucleotide identity is indicated by a dash. Gaps in the CDR1 region of V_K1 are indicated as dots. The residues are numbered according to Kabat *et al.* (Kabat *et al.*, 1987).

region in aPC-12-3 consists of the V_K1C segment combined with J_K2 (Fig. 4). Only one replacement mutation took place in this functional rearrangement, leading to the exchange of Asn28 for Tyr in CDR1.

In the third IgE anti-PC hybridoma aPC-6-6-4, a member of the 36-60 V_H gene family, probably the V_H36-60 germ-line gene is expressed (Fig. 3). This segment was subjected to extensive nucleotide changes (Table 1, Fig. 3). In contrast to aPC-4-33, most of the substitutions took place in the FR regions. The only

replacement mutation concerning the CDR regions of the V_H36-60 segment led to the exchange of Ser62 by Phe. Considering the nucleotide sequence alone, it remains unclear whether the V_H36-60 gene is joined to J_H2 or J_H4 (Fig. 3). Southern blot analysis answered this question. The band pattern of aPC-6-6-4, using the J_H-specific probe for hybridization, is shown in Fig. 2. Rehybridization of the same blot, using the cloned functional VDJ region as probe, revealed that the 2.3 kb Eco RI, 5.1 kb XbaI and 1.6 kb Eco RI/XbaI bands in

Fig. 2 contain the functional H chain rearrangement (data not shown). The expressed V_H36-60 segment possesses an internal Eco RI site in FR3 and the next Eco RI and XbaI sites 3' of J_H4 are known (Sakano *et al.*, 1980). This allowed us to correlate the 2.3 kb Eco RI and 1.6 kb Eco RI/XbaI band sizes with the usage of J_H2 (Fig. 2). The J_H2 segment is strongly truncated at the DJ recombination site, thus leading to a relatively short CDR3 region which is characterized by only six amino acid residues (Fig. 3). Clear identification of the specific germ-line D gene was not possible, since the one used in aPC-6-6-4 is not perfectly homologous to the reported germ-line D sequences. Therefore, D family and member assignment was not performed. In aPC-6-6-4, a nonfunctional $V_H-OX1-D_H-J_H2$ rearrangement is transcribed that is contained in the 3.3 kb Eco RI, 3.9 kb XbaI and 2.4 kb Eco RI/XbaI bands in Southern blot (Fig. 2). The difference in size of about 400 bp between the corresponding fragments in aPC-6-6-4 and aPC-12-3 correlates with the distance between J_H2 and J_H3 as demonstrated in Fig. 2. (Sakano *et al.*, 1980). The cells of aPC-6-6-4 use the $V_{\lambda 1}-J_{\lambda 1}$ rearrangement which was mutated at seven nucleotide positions (Table 1, Fig. 4). Three replacement mutations led to the exchange of Gly50 by Arg in CDR2, and of Ser93 and Val94 in CDR3 by Asn and Asp, respectively (Fig. 4). Trp, the first amino acid in $J_{\lambda 1}$ is replaced by Leu. The $\lambda 1$ L chain isotype determination by ELISA was confirmed by Southern blot analysis using a $C\lambda 1$ probe (Fig. 7). This probe cross-hybridizes to $C\lambda 4$ (Blomberg *et al.*, 1981).

The 8.6 kb fragment in Fig. 7 contains the unrearranged $C\lambda 1$ and the 2.8 kb band of the germ-line $C\lambda 4$ gene segment (Blomberg *et al.*, 1981). aPC-6-6-4 shows a unique 7.5 kb band containing the functional $V_{\lambda 1}-J_{\lambda 1}-C_{\lambda 1}$ rearrangement (Fig. 7) (Weiss *et al.*, 1985).

DISCUSSION

We have investigated the contribution of H and L chain gene segments to the genetic diversity in three anti-PC IgE secreting hybridomas. Whereas aPC-4-33 expresses the dominant V_H1 segment of the anti-PC response to *Streptococcus pneumoniae*, aPC-6-6-4 utilizes the V_H36-60 germ-line gene. The 36-60 V_H gene family participates to a considerable extent in the anti-azophenylarsonate (Ars) response in BALB/c mice (Near *et al.*, 1984). Most mouse strains possess five highly homologous members in this V_H gene family, but only one is utilized in memory anti-Ars antibodies which bear the major Id.

The most obvious difference between the V_H region used by anti-Ars antibodies and in aPC-6-6-4 concerns the participation of different D_H and J_H genes. The D_H segment in anti-Ars is derived from the DFL16 family and codes for only two amino acids. In aPC-6-6-4, this segment is of unknown origin and encodes four amino acid residues. In addition to the functional V region, a non-productive VDJ rearrangement is transcribed in aPC-6-6-4, consisting of the V_H-OX1 germ-line gene joined to an unknown D_H segment and J_H2 (Fig. 6). This rearrangement is not functional due to the presence of the stop codon TAA within the D_H segment in the reading frame (Fig. 6). These nucleotide substitutions in the V_H-OX1 gene also indicate that unproductive rearrangements are subjected to somatic mutational events.

The IgE aPC-12-3 expresses a member of the Q52 V_H family in association with a κ -L chain. The participation of the Q52 V_H gene family in memory anti-PC antibodies of other isotypes has been reported (Stenzel-Poore and Rittenberg, 1989; Stenzel-Poore *et al.*, 1987). Whereas these antibodies use the PJ14 germ-line gene, aPC-12-3 expresses the V_H-OX1 germ-line gene which plays a major role in the response of BALB/c mice to phenyl-oxazolone (phOx) (Kaartinen *et al.*, 1986). It has been shown that the H chain residues His35, Val37 and Ala93 are conserved among available V_H-Ox1 sequences (Berek *et al.*, 1985). The side chain of His35 is in close vicinity to the phenyl group of phOx, whereas Val37 and Ala93 are involved in forming the hydrophobic pocket interacting with the whole phOx hapten (Alzari *et al.*, 1990). These amino acids are also present in aPC-12-3 at the corresponding sites and may be directed towards the phenyl moiety of the PC hapten. Although aPC-12-3 expresses another member of the Q52 V_H gene family from the memory anti-PC antibodies of other isotypes (Stenzel-Poore and Rittenberg, 1989), it uses the same SP2.6- J_H3 rearrangement as the IgG1 anti-PC hybridoma PCG1-1 which employs the PJ14 germ-line gene (Stenzel-Poore and Rittenberg, 1989; Stenzel-Poore

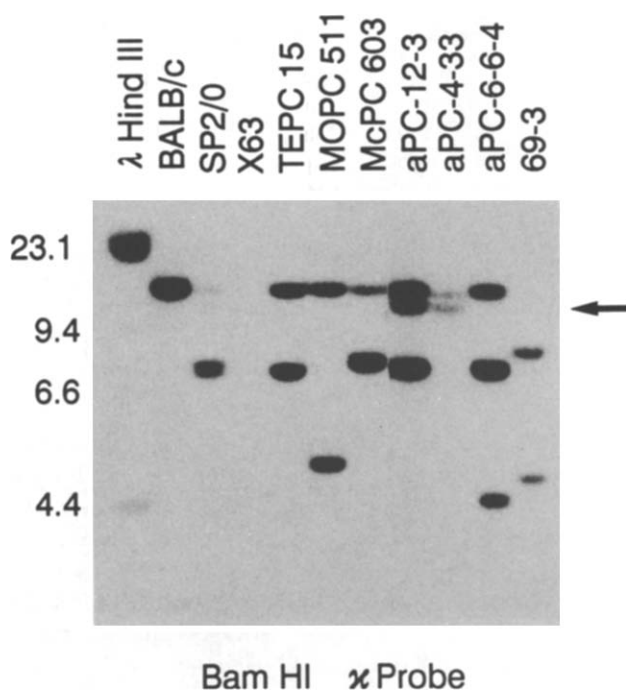


Fig. 5. Southern blot analysis of L_{κ} chain rearrangements in Bam HI digested genomic DNA of Group II hybridomas secreting IgE anti-PC antibodies. The molecular size on the left side of the panels is indicated in kb. The arrow points to the fragments containing the functional VJ rearrangement in aPC-12-3 and aPC-4-33. The myeloma X63-Ag8.653 has lost the DNA segment, corresponding to the κ chain-specific probe.

[illegible]

Fig. 6. V_H nucleotide sequences of aPC-12-3 and aPC-6-6-4. (a) This VDJ rearrangement of aPC-6-6-4 is nonfunctional due to a chain termination codon in the D segment. The section coding for the V region of the H chain is compared with the V_H -OX1 germ-line gene (Kaartinen *et al.*, 1983). The PJ14 germ-line sequence is taken from Sakano *et al.* (Sakano *et al.*, 1980). Nucleotide identity is indicated by a dash. Gaps have been introduced in the CDR3 region to permit the alignment of the J_H segments and the primers are indicated. The part of the J_H segments not considered is identical to the corresponding germ-line J_H sequence. Residues are numbered according to Kabat *et al.* (Kabat *et al.*, 1987).

et al., 1987). Also the V-D and D-J recombination sites are identical among aPC-12-3 IgE and PCG1-1 IgG1. Thus, both reveal a CDR3 region consisting of 10 amino acid residues. Most amino acid residues which were shown to interact with the PC molecule (Padlan *et al.*, 1976) are not expressed at corresponding sites in aPC-12-3 and aPC-6-6-4. This may indicate that these IgE antibodies recognize a site of the hapten in which the PC moiety is less involved than in those expressing V_H1. The weak affinity of aPC-12-3 and aPC-6-6-4 to the PC molecule is revealed by their inhibition curves (Fig. 1). Compared to aPC-4-33, higher concentrations of free PC are required to reach IC₅₀. A further relationship between anti-phOx and anti-PC antibodies concerns the L chain usage, since

members of the V_κ 1 gene family being dominantly recruited in κ chains of memory anti-PC antibodies (Stenzel-Poore and Rittenberg, 1989; Lötscher *et al.*, 1993) are also commonly observed in anti-phOx antibodies.

The production of IgE antibodies requires T cell help and is regulated by antigen-, isotype- and idiotype-specific interactions (Blaser and Heusser, 1987; Blaser and de Weck, 1982; Ishizaka, 1984). IgE antibodies are highly susceptible to anti-Id suppression and it was demonstrated that 2/3 of the IgE anti-PC response can be suppressed by isologous anti-T15 antiserum, although IgE reacting with specific monoclonal anti-T15 Id was not found (Blaser *et al.*, 1979; Blaser and de Weck, 1982; Blaser and Heusser, 1984). This could result from anti-Id

which recognize idiotopes in the V_{H1} region of the aPC-4-33 IgE antibody. Indeed it was found that aPC 4-33 IgE binds to anti-T15 antiserum and to a number of mAb which recognize T15 but not the true T15 Id (Blaser and Heusser, 1987). This may indicate that IgE antibodies using the V_{H1} gene are substantially expressed in IgE anti-PC responses in serum (Blaser and Heusser, 1987; Blaser and de Weck, 1982; Cerny *et al.*, 1982).

The segment diversity used to encode the V region of the IgE anti-PC antibodies is high, although only a limited number of IgE anti-PC hybridomas has been investigated. The recruitment of such a variety of H and L chain gene segments contributing to the V region of these IgE antibodies, the different length of the H chain CDR3 segment and the lack of shared sets of antigen-contacting amino acids in the CDR regions of their H chain suggest that each of these IgE antibodies recognize a different site of the PC hapten. Two V_H genes are used, which have not been detected in anti-PC antibodies of other Ig classes, but participate in responses to other phenyl-containing haptens. Thus, novel germ-line gene combinations contribute to the IgE related anti-PC immune response. Therefore, IgE antibodies against PC-KLH do not represent true anti-phosphorylcholine specificity as the antibodies normally expressed in mice against the Streptococcus PC determinant. They develop in the secondary response and express the characteristics

of group II anti-PC antibodies. (Weber *et al.*, 1986; Blaser *et al.*, 1987; Chang and Rittenberg 1981). On the other hand, the same V_H and V_L gene region segments as in the other isotypes (Lötscher *et al.*, 1992; Lötscher *et al.*, 1993) are involved in IgE anti-PC antibodies. The high rate of mutations and the fact that T15 Id⁺ IgE antibodies could not be elicited indicates that B_e cells are activated later in the response and that IgE antibodies are not derived from preexisting primary T15⁺ IgM antibodies.

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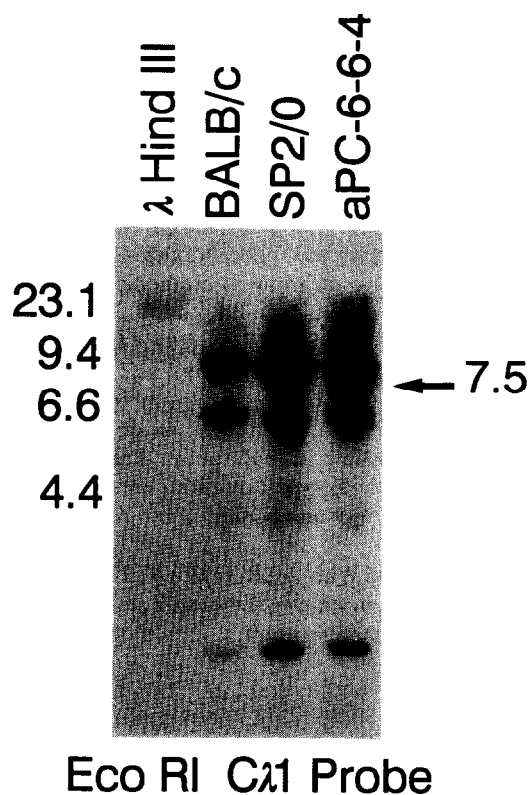


Fig. 7. Southern blot analysis of the Group II hybridoma aPC-6-6-4 producing an IgE anti-PC antibody with a λ 1 chain. Eco RI digested DNA was hybridized with a $C_{\lambda}1$ probe. The arrow points to the 7.5 kb band containing the functional $V_{\lambda}1$ -J λ 1- $C_{\lambda}1$ rearrangement (Weiss *et al.*, 1985). The 6 kb band results from partial digestion of the Eco RI site between $C_{\lambda}2$ and $C_{\lambda}4$ (Blomberg *et al.*, 1981; Weiss *et al.*, 1985).

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