Sequence changes at the V–D junction of the V_{H}1 heavy chain of anti-phosphocholine antibodies alter binding to and protection against *Streptococcus pneumoniae*

Wei-Xing Guo, Angelika M. Burger, Randy T. Fischer, Donna G. Sieckmann, Dan L. Longo and James J. Kenny

Laboratory of Biochemical Physiology, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD 21702-1201, USA

Clinical Oncology Unit, University of Bradford, Bradford, UK

Naval Medical Research Institute, Bethesda, MD 20889-5607, USA

National Institutes of Health, National Institute on Aging, Gerontology Research Center, 4940 Eastern Avenue, Baltimore, MD 21224, USA

Keywords: junctional diversity, pneumococcal vaccine, T15 idiotype

Abstract

X-linked immune deficient (Xid) mice fail to produce anti-phosphocholine (PC) antibodies even after immunization with *Streptococcus pneumoniae*. Consequently, Xid mice are extremely susceptible to infection with *S. pneumoniae*. PC-specific B cells appear to undergo clonal deletion in Xid mice; however, a new thymus-dependent form of PC, 6-(O-phosphocholine)hydroxyhexanoate (EPC), can rescue PC-specific B cells from the bone marrow presumably by providing T cell help before clonal deletion. Analysis of PC-specific IgG hybridomas from Xid mice revealed utilization of several V–D junctional variants of the V_{H}1 gene segment rearranged to different D and J_{H} gene segments. The majority of Xid anti-PC antibodies exhibit an Asp$\rightarrow$Gly95H replacement at the V–D junction. These Gly95H V_{H}1 variants associate with κ_{1}C L chains to produce anti-PC antibodies that: (i) have low relative affinity for PC, (ii) are heteroclitic for nitrophenylphosphocholine and (iii) fail to bind to or provide protection against *S. pneumoniae*. Single prototypic V–D variants of the T15 idiotype (Asp95H), M603 idiotype (Asn95H) and M167 idiotype (Asp95H–Ala96H) were also induced in Xid mice. The M603-like and M167-like antibodies bound to and protected against *S. pneumoniae* even though they exhibited $K_{a}$s for PC which were lower than T15 idiotype$^{+}$ antibodies. These data demonstrate that small changes in the V–D junctional sequence of the T15 (V_{H}1) heavy chain alter L chain usage and the structure of the PC binding site so that the PC expressed on *S. pneumoniae* is no longer recognized.

Introduction

CBA/N mice have a point mutation in the pleckstrin homology domain of their Bruton’s tyrosine kinase (*btk*) gene (1,2). The expression of this mutant *btk* results in an X-linked immune defect (Xid) characterized by an inability to respond to thymus-independent (TI) type 2 antigens (3). Mice expressing the xid gene also fail to respond to phosphocholine (PC) following immunization with diazophenylphosphocholine (DPPC) conjugated to thymus-dependent (TD) carriers (4,5). Inasmuch as PC is one of the immunodominant epitopes on *Streptococcus pneumoniae* (6,7), the failure to respond to PC or to pneumococcal capsular polysaccharides (8,9) makes Xid mice highly susceptible to infection with *S. pneumoniae* (10–12). Previous studies from our laboratory suggested that PC-specific B cells are either clonally deleted via an antigen-specific, receptor-mediated process (13), or they fail to be positively selected from the bone marrow into the peripheral lymphoid tissues of Xid mice (14). However, we have recently demonstrated (15,16) that T15 idiotype$^{+}$, PC-specific B cells can be rescued in Xid mice following immunization with a novel TD form of PC, 6-(O-phosphocholine) hydroxyhexanoate (EPC), conjug-
ated to keyhole limpet hemocyanin (KLH) (EPC–KLH) and that these antibodies can protect in vivo against infection with \textit{S. pneumoniae}. In the absence of competition from the higher affinity PPC-specific B cells which dominate the immune response of Xid mice to DPPC-conjugated proteins (17,18), cognate interactions with KLH-specific T helper cells appear to rescue PC-specific bone marrow B cells and thus prevent their antigen-driven clonal deletion.

To elucidate the structural and functional characteristics of the T15 idiotype: anti-PC antibodies induced in Xid mice by EPC–KLH, we generated PC-specific hybridomas from C3.BA/N mice. Twelve hybridomas were analyzed for: (i) binding affinity and specificity, (ii) passive protection against at room temperature, washed and 100°C. CBA/N mice. Twelve hybridomas were analyzed for: (i) for each antibody. Plates were again incubated overnight with 100 µl of this antibody–hapten mixture was put onto testing their binding to DPPC-conjugated BSA in the EPC–BSA-coated wells at room temperature presence and absence of 10–2 M PC. The production and overnight. Plates were developed using 100 µl/well EPC–BSA (5 µg/ml) in PBS–0.1% NaH2O4, EPC-BSA-coated plates were washed with Tris–HCl buffer, pH 7.2, containing 0.05% Tween-80 using a Skatron plate washer (Skatron, Lier, Norway) and blocked with 200 µl/well of 5% Hapril liquid gelatin (Norland Products, New Brunswick, NJ) in BBS for 2 h. Purified antibodies (100 µl/well) were added as 2-fold serial dilutions (1.0 µg/ml to 3.9 ng/ml protein) to generate standard curves for each antibody. Plates were again incubated overnight at room temperature, washed and 100 µl of biotinylated mononclonal rat anti-idiotype (Biosource, Camarillo, CA) or anti-idiotype antibodies, at predetermined concentrations, were added to individual standard curves of each antibody. Plates were then incubated for 2 h at 37°C, washed, and 100 µl of a 1:1000 dilution of streptavidin–alkaline phosphatase (SAAAP) (Calbiochem, La Jolla, CA) was added to all wells for 1 h at 37°C. The reaction was developed by adding \(\rho\)-nitrophenylphosphonate (PNPP) substrate (Sigma, St Louis, MO) (1 mg/ml in 1 M diethanolamine buffer, pH 9.8). After 20–40 min at room temperature, ODs were read using a Bio-Tec plate reader (Bio-Tek Instruments, Burlington, VT) set at 405 nm.

\section*{Methods}

\subsection*{Animals and immunization protocol}

Breeding pairs of consomic C3.BA/N Xid mice were obtained from Dr Carl Hansen, Division of Veterinary Medicine, National Institutes of Health, Bethesda, MD. These mice were derived as previously described for the C3.BA/N strain (19) and were maintained in our colony by brother/sister mating. Amino-phenolphosphocholine and EPC were synthesized and coupled to KLH at molar coupling ratios of 30:1 and 100:1 respectively, as previously described (15). Immunizations were performed i.p. using 200 µg of EPC–KLH in complete Freund’s adjuvant for the primary response and another 100 µg in incomplete Freund’s adjuvant for the day 14 secondary immunizations. Three days before spleen cell fusion, mice were given 50 µg of soluble EPC–KLH i.v. in saline.

\subsection*{Hybridoma production and antibody purification}

Hybridomas were generated from spleen cells of Xid mice immunized with EPC–KLH as previously described (20). Hybridomas secreting PC-specific antibodies were selected by testing their binding to DPPC-conjugated BSA in the presence and absence of 10–2 M PC. The production and partial characterization of hybridoma 1B8E5 has been previously described (21), and hybridoma 31-23-1 was previously tested for binding and protection to \textit{S. pneumoniae} (16). Hybridoma cells (5×10^5) were injected into Pristane primed C3.BA/N to produce ascites fluid. Ascites fluid was delipidated with Lipid Clearing Solution (Clinetics, Tustin, CA) and passed over PPC-tyr-gly-Sepharose columns equilibrated with borate-buffered saline, pH 8.4 (BBS) (22). The columns were washed with BBS, followed by 0.5 M NaCl until the OD was <0.05 and the bound antibodies were then eluted using 10–2 M PC in BBS. The eluted antibodies were extensively dialyzed against BBS and the protein concentration determined using the BCA protein assay (Pierce, Rockford, IL). Purity of the antibodies was determined by SDS-PAGE.

\subsection*{Antibody analysis: isotype, idiotype and PC-inhibition assays}

H chain isotype expression was determined using 96-well flexible plates (MIC 2000, Dynatech, Chantilly, VA) coated overnight at room temperature with 100 µl/well EPC–BSA (5 µg/ml) in PBS–0.1% NaH2O4. EPC-BSA-coated plates were washed with Tris–HCl buffer, pH 7.2, containing 0.05% Tween-80 using a Skatron plate washer (Skatron, Lier, Norway) and blocked with 200 µl/well of 5% Hapril liquid gelatin (Norland Products, New Brunswick, NJ) in BBS for 2 h. Purified antibodies (100 µl/well) were added as 2-fold serial dilutions (1.0 µg/ml to 3.9 ng/ml protein) to generate standard curves for each antibody. Plates were again incubated overnight at room temperature, washed and 100 µl of a 1:1000 dilution of streptavidin–alkaline phosphatase (SAAAP) (Calbiochem, La Jolla, CA) was added to all wells for 1 h at 37°C. The reaction was developed by adding \(\rho\)-nitrophenylphosphonate (PNPP) substrate (Sigma, St Louis, MO) (1 mg/ml in 1 M diethanolamine buffer, pH 9.8). After 20–40 min at room temperature, ODs were read using a Bio-Tec plate reader (Bio-Tek Instruments, Burlington, VT) set at 405 nm.

\subsection*{Inhibition assays}

Inhibition assays were performed using EPC–BSA (5 µg/ml)-coated 96-well plates as described above. Antibodies at concentrations (100–400 ng/ml) that would give between 1 and 2 OD units in a direct binding ELISA developed with biotinylated anti-\(\kappa\) antibody were diluted in 5% Hapril containing PC or NPPC (10–2 to 10–8 M) in a 3-fold serial dilution. Then, 100 µl of this antibody–hapten mixture was put onto EPC–BSA-coated plates and incubated at room temperature overnight. Plates were developed using 100 µl/well biotinylated rat anti-mouse \(\kappa\) antibody (clone ATCC HB 58; ATCC, Rockville, MD) followed by SAAAP and substrate as described above. The mM concentration of hapten yielding 50% inhibition \((I_{50})\) was determined based on the OD of the uninhibited antibody.

\section*{Determination of association constants \((K_{d})\)}

The affinity of the hybridoma anti-PC antibodies for PC–hapten was measured by fluorescence quenching as described by Glaudemans and Jolly (25).

\subsection*{Flow cytometric assay for antibody binding to bacteria}

WU-2, a virulent type-3 strain of \textit{S. pneumoniae}, was obtained from Dr David Briles (University of Alabama, Birmingham,
AL). Bacteria were prepared, stained, and analyzed by flow cytometry as previously described (16). The *S. pneumoniae* binding index for purified anti-PC antibodies was determined by staining 2×10^6 bacteria with 1 µg of anti-PC antibody or of an isotype-matched non-PC-binding control. Bacteria were washed twice with 1.5 ml of HBSS containing 0.1% BSA and then stained with 1 µg of FITC-conjugated goat anti-mouse Ig (Fisher Biotech, Silver Spring, MD). The fluorescence intensity of the isotype control was adjusted so that <1% of the cells were above the 10^1 fluorescence level. The binding index was then calculated as the ratio of the mean fluorescence intensity (MFI) of anti-PC antibody over the MFI of the isotype control.

**Passive protection assay**

Affinity purified antibody (100 µg) was injected i.p. along with *S. pneumoniae* (10^1 to 10^4 colony forming units) into naive C.CBA/N recipients. Survival curves were plotted and LD<sub>50</sub> values calculated to determine protective efficacy of the antibody treatment.

**Isolation of RNA**

Total cellular RNA was isolated from hybridomas by a single-step method (26). In brief, cells were lysed with RNA STAT-60 (TEL-TEST B, Friendswood, TX) and cell lysates were centrifuged after treatment with chloroform. RNA present in the aqueous phase was precipitated with isopropanol, washed, dried, dissolved in RNase-free water and then quantified by spectrophotometry at 260 nm.

**cDNA preparation and RT-PCR**

First-strand cDNA synthesis was performed using 1.0 µg RNA in a final volume of 20 µl. The reaction mixture contained 2.5 µM oligo (dT) primer, 50 units of Moloney's mouse leukemia virus reverse transcriptase (Perkin Elmer, Branchburg, NJ), 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 5 mM MgCl<sub>2</sub>, 20 U RNase inhibitor, and 1.0 mM each of dGTP, dCTP, dATP and dTTP (dNTPs). Reactions were carried out for 30 min at 42°C and then for 10 min at 65°C. The resulting cDNAs were used as templates for amplification of rearranged heavy or light chain genes. PCR reactions contained 0.2 mM dNTPs, 2.5 U Taq polymerase (Perkin Elmer), 0.25 µM specific primers and 20 µl of cDNA templates in a final volume of 100 µl. For amplification of V<sub>H</sub> genes, the reactions were subjected to 30 cycles consisting of: 94°C for 30 s, 56°C for 45 s and 72°C for 50 s. For the amplification of V<sub>L</sub> genes, the reactions were subjected to 30 cycles consisting of: 94°C for 45 s, 65°C for 45 s and 72°C for 50 s. All PCR reactions were subjected to denaturation at 94°C for 2 min prior to the first cycle and to a final extension at 72°C for 7 min following the last cycle.

The oligonucleotides used to amplify H and L chain genes from cDNA were as follows. For the rearranged V<sub>H</sub>1 gene, 5'-ATGAGATTTGGTTAAAAGCTGGG-3' was used as the 5'-sense primer in combination with one of the following 3'-antisense primers, 5'-CTGGCTCAGGGAAATATCCCTTGACCA-3'(C<sub>1</sub>), 5'-AGGGGGCCAGTGGGATAGACCAGT-3'(C<sub>2a</sub>), or 5'-AACAGTTGTATCTACCCCACCCAGG-3'(C<sub>2b</sub>). The V<sub>L</sub> sense primers were: (i) V<sub>L</sub>1-IC, 5'-CTTTAGAGTGGCCTGTAGGCT-3'; (ii) V<sub>L</sub>22, 5'-GTATATATGTTTGTGTTATTTCAGGTTCTTGTTCTG-3'; and (iv) V<sub>L</sub>24, 5'-TCTCACGGTTCTTGCTAGGTC-3' and the antisense primer 5'-GAAGTTGTCAAGGAACAGCAG-CTGAGGC-3', originated in C.<sub>L</sub>

**Subcloning of PCR products**

The PCR amplified fragments were size-purified from agarose gels using spin columns (Promega, Madison, WI) and then purified using a plasmid DNA purification kit (Promega, Madison, WI). Plasmids containing PCR inserts were isolated and purified using a plasmid DNA purification kit (Promega), according to the manufacturer's specifications.

**Sequence analysis**

DNA sequences were determined in both directions from either purified PCR fragments or pCRII inserts by the dideoxy chain termination method (27). To minimize possible ambiguities in sequence determination on gels or misincorporations introduced by PCR, three PCR templates from three different cDNA preparations of each hybridoma were sequenced in separate experiments and each sequence repeated at least twice. The sequences were aligned with germ-line H or L chain genes using the program manual for the GCG package, version 8, September 1994 (Genetics Computer Group, 575 Scientific Drive, Madison, WI 53711). Amino acid sequences predicted from the nucleic acid sequences were numbered according to Kabat (28).

**Analysis of mutations**

The binomial probability model described by Shlomchik et al. (29) was used to determine whether the number of replacement (R) mutations in the CDR was significantly higher than the number expected randomly. For the V<sub>H</sub> gene, only CDR 1 and 2 were considered using a P value of 0.19 for the expected mutations in these regions. For the L chains, both V and J segments were considered, but junctional changes in 95L were not counted. A P value of 0.21 was used for L chain analysis based on 0.28 as the size of the CDR and 0.75 as the fraction of R-type mutations. In codons containing two or more mutations, where the order of the mutations can not be determined, some mutations could be either R or silent (S). In these situations i.e. the L chains of 24-1B10, 24-1B11 and 32-6-2, two sets of statistical analysis were performed.

**Results**

**Isotype and idiotype analysis of hybridoma anti-PC antibodies from C.CBA/N mice**

Twelve hybridomas from EPC–KLH-immune C.CBA/N mice were characterized by ELISA for H and L chain isotype, H chain and H + L combinatorial idiotypes. The data in Table 1 show that all the Xid anti-PC hybridomas produced IgG<sub>x</sub> antibodies with equal numbers of γ1, γ2a and γ2b being represented. All hybridoma antibodies expressed the V<sub>H</sub>1 idiotype detected by clone T68.3 (23), which recognizes the T15 (V1) H chain. However, only a single hybridoma (27-CD3) expressed the combinatorial T15 idiotype produced.
by association of the V_H1 H chain and a κ22 L chain. This T15 idiotype+ hybridoma was unusual in that secretion of the antibody could not be detected in an ELISpot assay of logarithmically growing cells, but antibody was present in low levels in supernatant from aged cultures exhibiting low viability. This suggests that this hybridoma is a non- and low viability. This suggests that this hybridoma is a non- and 2. A third hybridoma, 31-23-1, utilized a V_H1 gene segment. These predictions were confirmed in the sequence analysis shown in Figs 1 and 2. A third hybridoma, 31-23-1, utilized a V_H1 H chain and a κ22 L chain. This T15 idiotype+ hybridoma was unusual in that secretion of the antibody could not be detected in an ELISpot assay of logarithmically growing cells, but antibody was present in low levels in supernatant from aged cultures exhibiting low viability. This suggests that this hybridoma is a non-secretor and that antibody is released into the supernatant only when cells die. A second hybridoma, 31-34-2, expressed the V_H1x24-dependent combinatorial M167 idiotype detected by the binding-site-specific 28-5-15 antibody and the cross-reactive κ24-dependent idiotype detected by antibody 28-6-20 (14). Inasmuch as the remainder of the Xid PC-specific hybridomas were negative for both the T15 idiotype and M167 idiotypes, their H and L chain genes were sequenced in order to elucidate how their structure differed from the prototypic anti-PC antibodies induced in normal mice.

Variable region gene usage in hybridoma antibodies from Xid mice

To determine the variant form of the V_H1 H chain gene used in each of the Xid hybridomas and the L chain gene associated with these H chains, total RNA was extracted from each hybridoma, the H and L chain mRNA was then converted to cDNA, amplified by PCR and sequenced. Table 2 summarizes the data on gene segment utilization in the Xid hybridomas. The idiotype analysis described above predicted that all the EPC–KLH-induced anti-PC hybridomas from Xid mice would use the V_H1 gene segment and that two of the hybridomas, 27–7C3 and 31-34-2, would utilize κ22 and κ24 L chain V genes respectively. These predictions

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Isotype H/L</th>
<th>Idiotype</th>
<th>Passive protection(\frac{LD_{50}}{K_d})</th>
<th>Antigen binding and inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VH1</td>
<td>T15</td>
<td>M167</td>
<td>M167X</td>
</tr>
<tr>
<td>27-7C3</td>
<td>γ2b/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>31-23-1</td>
<td>γ1/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>31-34-2</td>
<td>γ1/Cκ</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>32-11-1</td>
<td>γ1/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>24.1B10</td>
<td>γ2a/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>24.1B11</td>
<td>γ2b/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>24.2B2.4</td>
<td>γ2b/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1B8E5</td>
<td>γ2a/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>31-4-2</td>
<td>γ2a/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>32-6-2</td>
<td>γ1/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>32-15-1</td>
<td>γ2a/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>32-17-1</td>
<td>γ2a/Cκ</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^{1}\)Isotype, idiotype, antigen binding and passive protection assays were described in Methods.

\(^{2}\)LD\(_{50}\), number of bacteria injected along with 100 µg antibody, for which 50% of mice survived. The LD\(_{50}\) for the positive and negative control antibodies shown in Fig. 3 is >10\(^4\) and <10\(^3\) bacteria respectively.

\(^{3}\)The binding index for S. Pneumoniae was measured as described in Methods.

\(^{4}\)K\(_d\), measured by fluorescence quenching as described by Glaudemans and Jolly (25). The T15 idiotype+ antibody from the H8 myeloma, which was used as a positive control, exhibited a K\(_d\) of 8×10\(^{-5}\).

\(^{5}\)Hapten concentration in mM. Control T15 idiotype γ1 (HPCG14) exhibited I\(_{50}\) values of 0.0011 and 0.028 for PC and NPPC respectively, while the γ2a (PCG2a-2) antibody was 0.0015 and 0.03 respectively. Thus, I\(_{50}\) ratios were 20 or greater for both these T15 idiotype antibodies.

\(^{6}\)ND, not determined.

\(^{7}\)NM, not measurable by fluorescence quenching.
M167 myelomas respectively (32), whereas, Jκ5 is used in only three of the Xid hybridomas (Fig. 2 and Table 2).

Comparison of H chain DNA and amino acid sequences

Using X-ray diffraction analysis, Padlan et al. (33,34) identified the contact residues for PC in the M603 antibody. In the H chain, Tyr33, Glu35, Ala50, Arg52, Lys52b, Glu58, Asn95 and Trp100b are directly involved in hapten binding or in maintaining the structural integrity of the binding pocket. In addition, Chen et al. (35) have recently demonstrated critical roles for Ser51 and Asn52a of the H chain. In the prototypic L chains of PC-binding myelomas, Leu96 is the major hapten contact residue with residues 91L to 95L playing an important role in maintaining the choline binding pocket. In the D16 antibody, which utilizes the VH1 and κ1C L chain, this choline binding pocket is lost resulting in a shift in the location of PC binding site (35).

In the H chain of the hybridomas from EPC–KLH-immune Xid mice, there are only a few mutations in the contact residues for PC, indicating that these residues are critical for maintaining PC binding. In hybridomas 31-4-2, 32-15-1 and 32-17-1, contact residues Glu35H and Glu58H have been mutated to His and Asp respectively (Fig. 1). In 31-4-2, Ser51H is mutated to Arg and in 31-34-2, Trp100b is mutated to Gly. In addition to these mutations, shared mutations are also found at positions 1, 53, 55, 61, 91 and 93 of the H chains of these three hybridomas. The fact that hybridoma 31-4-2 is from a different fusion than hybridomas 32-15-1 and 32-17-1, and yet shares over half (nine of 17) of its mutations with these clones may indicate that these shared mutations are being selected for during the hypermutation process. Hybridoma 31-34-2 makes the only other antibody with an H chain exhibiting a mutation in a contact residue with a Trp → Gly at 100b (Fig. 1). This residue is not critical for PC binding and has been lost in other PC-specific antibodies (32). In the three Xid hybridomas using the prototypic κ22, κ8 and κ24 L chains, hybridoma 31-34-2 is the only one exhibiting a change in the amino acids important for forming the choline binding pocket. In this κ24 L chain, the choline contact residue Leu96 has been changed to a Phe due to Vκ24 joining contact residue with residues 91L to 95L playing an important role in maintaining the choline binding pocket. In the D16 antibody, which utilizes the VH1 and κ1C L chain, this choline binding pocket is lost resulting in a shift in the location of PC binding site (35).

Previous studies from our laboratory (15,18) have shown that the normally dominant T15 idiotype+/anti-PC antibodies are rarely produced in Xid mice. However, a single clone produced in fusion 27, hybridoma 27-7C3, was T15 idiotype−, expressed a κ22 L chain with no replacement mutations (Fig. 2) and had two replacement mutations (44H Arg → Gly and 104H Gly → Arg) in the H chain framework regions (Fig. 1), which may have caused this hybridoma to be a non-secretor. The substitutions at 14H and 16H (Pro → Ser and Gly → Arg) (Fig. 1) are allotypic changes found in the CBA/N background which had not yet been completely removed via back crossing when this fusion was performed.

### Table 2. V region gene segments in PC-specific antibodies from Xid mice

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>VH gene</th>
<th>VH–D junction</th>
<th>VL gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>27-7C3</td>
<td>V1</td>
<td>DFL16.1</td>
<td>JH1,1</td>
</tr>
<tr>
<td>31-23-1</td>
<td>V1</td>
<td>DSP2.6</td>
<td>JH1,1</td>
</tr>
<tr>
<td>31-34-2</td>
<td>V1</td>
<td>DSP2.2</td>
<td>JH1,1</td>
</tr>
<tr>
<td>32-11-1</td>
<td>V1</td>
<td>DFL16.1</td>
<td>JH1,1</td>
</tr>
<tr>
<td>24.1B10</td>
<td>V1</td>
<td>DSP2.6</td>
<td>JH1,1</td>
</tr>
<tr>
<td>24.1B11</td>
<td>V1</td>
<td>DSP2.6</td>
<td>JH1,1</td>
</tr>
<tr>
<td>24.2B2.4</td>
<td>V1</td>
<td>DSP2.6</td>
<td>JH1,1</td>
</tr>
<tr>
<td>1B8E5</td>
<td>V1</td>
<td>Ni</td>
<td>JH1,4</td>
</tr>
<tr>
<td>31-4-2</td>
<td>V1</td>
<td>Ni</td>
<td>JH1,4</td>
</tr>
<tr>
<td>32-6-2</td>
<td>V1</td>
<td>Ni</td>
<td>JH1,4</td>
</tr>
<tr>
<td>32-15-1</td>
<td>V1</td>
<td>Ni</td>
<td>JH1,4</td>
</tr>
<tr>
<td>32-17-1</td>
<td>V1</td>
<td>Ni</td>
<td>JH1,4</td>
</tr>
</tbody>
</table>

V gene usage was assigned following alignment with germ-line genes using the program manual for the GCG package, version 8, September 1994.

bNI, not identifiable.

---

V–D junction determines binding to S. pneumoniae
V–D junction determines binding to S. pneumoniae
Statistical analysis suggests that changes within the CDR do not reflect antigen-driven selection

Antigen-driven affinity maturation for PC itself has not been seen in anti-PC antibodies from normal mice. In fact, the highly protective germline T15 idotype+ antibodies, which dominate the immune response to PC in normal mice (6, 7, 36), exhibit a decrease in affinity upon somatic mutation (35, 37). Higher affinity antibodies appear to develop via mutations that generate increased affinity for the chemical moieties linking PC to the carrier molecule (38–40). To elucidate whether the EPC-induced anti-PC antibodies from Xid mice were undergoing affinity maturation, we used the binomial probability model described by Shlomchik et al. (29) to determine whether the number of R mutations in the CDR was significantly higher than the number expected randomly. The number of mutations observed in the H and L genes, their location, and whether they were R or S is listed in Table 3. The observed R/S ratios of 3.1/1 and 2.8/1 for all H and L chains respectively is approximately equal to the value of 2.9/1 expected for random mutations (29). However, analysis of both the H and L chain CDR suggests that antigen-driven selection may be occurring since the R/S ratio in these regions is 4.3/1 and 8.8/1 respectively. Antigen-driven selection within the Gly95H variants is also indicated by the fact that seven of the eight proteins have an Asn to Gln or Tyr mutation at hypervariable position 53H, an Ala to Thr or Glu at hypervariable position 61H, and five of these proteins have an Arg to Val mutation at positions 83H in the third framework region. However, our statistical analysis demonstrated that only the κ8 L chain of hybridoma 31-23-1 and the κ1C L chain of 32-11-1 exhibited $P < 0.05$ (0.044 and 0.031 respectively). With only four or less observed total mutations in either of these two Vg genes, it is difficult to make a case for antigen-driven affinity maturation on the basis of these statistical data. In the H chain CDRs of hybridomas 31-4-2, 32-15-1 and 32-17-1 and the L chain CDRs of 24.1B10 and 32-6-2, there is a large number of R mutations which suggests that these antibodies are undergoing antigen-driven selection; however, in every case except that of 32-6-2, there is an equivalent or higher number of R mutations in the FRMWK regions. Although the CDR and FRMWK mutations could be structurally linked in adapting the binding site to the linker or carrier epitopes, the high frequency of R-type mutations in the FRMWK regions statistically negate the potential significance of the R-type mutations in the CDRs.

Passive protection to challenge with S. pneumoniae

We have previously demonstrated that Xid mice can be protected from a lethal challenge of S. pneumoniae following immunization with EPC–KLH, which induces predominantly T15 idiotype–anti-PC antibodies, but not with DPPC–KLH (15, 16), which induces antibodies having high affinity for PPC (39). It was of interest to determine which of the variant forms of the Xid anti-PC hybridomas sequenced above was capable of protecting Xid mice in order to understand how changes in the structure of the binding site correlate with protection. Five to 10 mice per group were injected i.p. with 100 µg of individual Xid anti-PC antibody and S. pneumoniae, strain WU-2, at concentrations ranging from 10^1 to 10^4. Representative survival curves are shown in Fig. 3 and the LD_{50} for each hybridoma is given in Table 3.

Table 3. Somatic mutation in PC-specific antibodies from Xid mice

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Mutations</th>
<th>R/S a</th>
<th>CDR R/S</th>
<th>FRMWK R/S</th>
<th>V genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_H^b$</td>
<td>$V_L^b$</td>
<td>$V_H^b$</td>
<td>$V_L^b$</td>
<td></td>
</tr>
<tr>
<td>27-7C3</td>
<td>2 (3)</td>
<td>2 (3)</td>
<td>1/1</td>
<td>0/2</td>
<td>0/0</td>
</tr>
<tr>
<td>31-23-1</td>
<td>5 (1)</td>
<td>2</td>
<td>4/1</td>
<td>2/0</td>
<td>1/0</td>
</tr>
<tr>
<td>31-34-2</td>
<td>7 (2)</td>
<td>3</td>
<td>5/2</td>
<td>1/2</td>
<td>0/1</td>
</tr>
<tr>
<td>32-11-1</td>
<td>7 (9)</td>
<td>4 (5)</td>
<td>5/2</td>
<td>3/1</td>
<td>1/1</td>
</tr>
<tr>
<td>24-1B10</td>
<td>10 (11)</td>
<td>15</td>
<td>7/3</td>
<td>14/1</td>
<td>3/1</td>
</tr>
<tr>
<td>24-1B11</td>
<td>10 (11)</td>
<td>9</td>
<td>7/3</td>
<td>7/2</td>
<td>3/1</td>
</tr>
<tr>
<td>24-2B2.4</td>
<td>8 (9)</td>
<td>3 (4)</td>
<td>6/2</td>
<td>3/0</td>
<td>3/0</td>
</tr>
<tr>
<td>1B9E5</td>
<td>5 (6)</td>
<td>9</td>
<td>1/4</td>
<td>7/2</td>
<td>0/1</td>
</tr>
<tr>
<td>31-1-4</td>
<td>17 (13)</td>
<td>9 (10)</td>
<td>15/2</td>
<td>6/3</td>
<td>7/0</td>
</tr>
<tr>
<td>32-6-2</td>
<td>17 (18)</td>
<td>18 (19)</td>
<td>12/5</td>
<td>11/7</td>
<td>4/3</td>
</tr>
<tr>
<td>32-15-1</td>
<td>16 (20)</td>
<td>4 (5)</td>
<td>14/2</td>
<td>3/1</td>
<td>6/0</td>
</tr>
<tr>
<td>32-17-1</td>
<td>15 (17)</td>
<td>5 (6)</td>
<td>13/2</td>
<td>4/1</td>
<td>6/0</td>
</tr>
<tr>
<td>Ratio b</td>
<td>3.1/1</td>
<td>2.8/1</td>
<td>4.3/1</td>
<td>8.8/1</td>
<td>2.7/1</td>
</tr>
</tbody>
</table>

For the $V_H$ region, mutations in $D_H$ and $J_H$ were not included in the statistical calculations but are indicated in the parentheses for those sequences where germ line D regions could be determined.

For $V_L$, mutations were counted from codon 1 to 108 excluding position 95; total differences including junctional region are given in parentheses.

R versus S mutations in the H and L chains respectively.

Ratio: mean value of R/S.
antibody is shown in Table 1. The single T15 idiotype+ hybridoma 27-7C3 (Asp95H) was a non-producer, and therefore, could not be tested for protection. However, T15 idiotype+ PC-specific antibodies are known to be highly protective against infection with *S. pneumoniae* (41) but are not readily induced in Xid mice (18). Hybridoma 31-23-1 (Asn95H) had an LD50 of >10^4 (16) (Table 1) and was as effective as the T15 idiotype γ1 control antibody, PC2-γ1 (42) (Fig. 3A versus E). Hybridoma 31-34-2 (Asp95H–Ala96H) provided at least 70% protection against challenge with >1000 bacteria (Fig. 3B), whereas, the dominant Gly95H:k1C variants exhibited either no protection or <50% protection against as few as 250 bacteria.

**Affinity analysis of Xid anti-PC antibodies**

To determine whether changes in affinity for PC were responsible for the lack of protection against *S. pneumoniae* seen in the Gly95H variants and the lower protection seen with the Asp95H–Ala96H variant (clone 31-34-2), we attempted to measure affinity constants for each of the Xid anti-PC hybridomas using the hapten-induced fluorescence quenching method of Glaudemans and Jolly (25). Only the two protective antibodies, 31-23-1 and 31-34-2, exhibited sufficient changes in fluorescence to permit affinity determinations. The remainder of the proteins either had Ks too low to measure or did not exhibit either fluorescence quenching or enhancement upon hapten binding. The Ks of antibody 31-23-1 was 6.5×10^5 M^-1 and antibody 31-34-2 had a Ks of 1.1×10^5 M^-1 as compared to 8×10^5 M^-1 for the T15 idiotype positive antibody, H8 (Table 1). The value we obtained for H8 was 2-fold higher than previously published Ks for this anti-PC antibody (32).

Inasmuch as all the Xid antibodies sequenced above bind to EPC–BSA coated plates, we directly evaluated their relative affinity for PC by determining the concentration of PC-hapten required to give 50% inhibition (I50) of binding. The T15 idiotype γ1 and γ2a antibodies from hybridomas HPGC-14 (43) and PCG2a-2, which were used as controls, exhibited I50 values of 1.1 and 1.5×10^-6 M respectively. As shown in Table 1, all the anti-PC hybridomas from Xid mice required

---

**Fig. 2.** Nucleotide sequence of L chains and their predicted amino acid sequence of anti-PC hybridomas from Xid mice. Nucleotide sequences were determined as described in Methods. The nucleotide sequences are aligned with Vκ1–C, Vκ8, Vκ22 and Vκ24 respectively. A dash is introduced to indicate homology. Amino acid changes due to a replacement mutation are given above the nucleotide sequence. The J regions are aligned with either Jκ1 or Jκ3.
from $2 \times 10^{-6}$ to $>10^{-2}$ M PC to obtain 50% inhibition. Thus, the best of these antibodies, 1B8E5, is at least 2-fold lower in relative affinity for PC than the prototypic T15 antibodies and many of these Xid antibodies are almost not inhibitable by free PC in spite of the fact that they were isolated from NPPC–Sepharose by elution with $10^{-2}$ M PC. It was possible that the EPC–KLH, which has no phenyl ring in the hapten–protein linker, could induce antibodies that are heteroclitic with respect to NPPC. This possibility was tested by inhibiting the binding of the anti-PC antibodies to EPC–BSA with NPPC and calculating the ratio of $I_{50}$, NPPC/PC. The data in Table 1 show that all the antibodies containing the 95H Gly variant of the $\kappa_1$H chain except 1B8E5 were indeed heteroclitic; thus, their binding to EPC–BSA was inhibited with a lower concentration of NPPC than PC. Only three antibodies (31-23-1, 31-34-2 and 1B8E5) exhibited an equal or higher relative affinity for PC compared to NPPC. The T15 idotype* controls also exhibited at least 20-fold higher relative affinity for PC than NPPC (legend Table 1). Interestingly, 1B8E5, which was non-protective, appears to have a 7-fold higher relative affinity for PC than the two protective antibodies 31-23-1 and 31-34-2. This would suggest that relative affinity for PC does not directly correlate with protection. Chen et al. (35) have recently shown that the orientation of PC in the binding site of the Gly95H: $\kappa_1$C antibody D16 is altered when compared to prototypic T15 and M603 anti-PC antibodies. Thus, the fact that none of these $\kappa_1$C anti-PC antibodies provided efficient protection against S. pneumoniae could be due to the failure of these antibodies to bind the form of PC present on the C-polysaccharide of S. pneumoniae.

Protection correlates with antibody binding to virulent bacteria
In an attempt to correlate protection against S. pneumoniae with binding to the bacterial PC, we measured the binding ability of each of the Xid anti-PC hybridoma antibodies to S. pneumoniae in the presence and absence of $10^{-2}$ M PC. The binding index for each of the Xid hybridoma antibodies to the virulent WU-2 strain of bacteria is given in Table 1. These data show that the two hybridoma antibodies that provide good passive protection, 31-23-1 and 31-34-2, are the only

---

**Fig. 2** (cont.). Nucleotide sequence of L chains and their predicted amino acid sequence of anti-PC hybridomas from Xid mice.

---
V–D junction determines binding to *S. pneumoniae*

Fig. 3. Passive protection studies using anti-PC antibodies from Xid mice. C.BA/N mice were injected i.p. with $10^7$ to $10^4$ viable WU-2 and 100 µg of purified antibody. LD$_{50}$ data are given in Table 1. (C) Data from clone 32-11-1 and (D) data from clone 24-1B10. The T15 idiotype$^+$ γ1 antibody, PC2γ1 (35), was used as a positive control (E). Negative controls received only Wu-2 (F).

Discussion

The data presented in this paper demonstrate the importance of V–D junctional mutations in determining which anti-PC antibodies will bind to the form of PC expressed on *S. pneumoniae* and protect against infection with this bacteria. Immunization with EPC–KLH induces a variety of PC-specific antibodies in Xid mice that include both prototypic T15, M603-like and M167-like antibodies, and low-affinity anti-PC antibodies that exhibit heteroclitic binding for NPPC. The antibodies showing a high intensity of binding to *S. pneumoniae*. Thus, these two antibodies exhibit a binding index that is >50-fold higher than the isotype-matched, non-binding control, whereas, hybridoma antibodies such as 1B8E5 or 32-15-1 that give low or no protection exhibited binding indexes of <20. The binding of all proteins with an index of >10 was PC-inhibitable (data not shown).
to the D16 hybridoma recently described by Chen et al. (35). Thus, all these antibodies utilize the same V\(\kappa\)1 gene as the prototypic PC-specific antibodies, but an Asp→Gly mutation is found at 95H in eight of the 11 T15 idioype-\(\kappa\)1 hybridomas and this mutation appears to be associated with the use of the \(\kappa\)1 L chain to achieve optimum PC binding. Antibodies expressing \(V_{\kappa}1\) Gly95H-\(\kappa\)1C exhibit a binding site in which the bound choline moiety of PC is shifted toward \(V_{\kappa}1\) CDR3, more shallowly located than in \(V_{\kappa}1\) antibodies, and no longer electrostatically stabilized (35). These alterations change the orientation of PC in the binding site of \(\kappa\)1C antibodies so that the PC on \(S.\ pneumoniae\) is no longer efficiently recognized and little or no protection is provided. In the \(\kappa\) negative B cells derived from T15i knockin mice by Taki et al. not active. The N region mutations seen in most of the \(\kappa\)1C L chain usage in anti-PC antibodies, but may help to generate a higher affinity antibody. This is similar to the situation with the Asp→Asn mutation at 95H, which dictates the use of \(\kappa\)8 L chain for optimum PC binding (24,30), although Asp95H and \(\kappa\)8 will also produce a low-affinity PC-specific antibody (24,43). Because we were unable to measure the actual affinity for PC of any of the \(\kappa\)1C antibodies, we cannot say how 32-11-1 compares to the other \(\kappa\)1C antibodies; however, its relative binding based on inhibition with PC and NPPC is not greatly different (Table 1). It would be of interest to know whether the Gly at 95H is generated by N region diversification or by hypermutation in the germinal centers. We favor the idea that this Asp→Gly change at 95H represents an N region mutation since the three \(\kappa\) Gly95H variants from fusion 24 appear to use the DSP 2.6 D region and the Gly codon or the GT nucleotides required to produce Gly95 are not present in the required 5' position of this D region sequence. Furthermore, there were no Gly95H variants found in the 40 \(V_{\kappa}1\) sequences from PC–KLH-induced \(\mu\) negative B cells derived from T15i knockin mice by Taki et al. (45). Having Gly at 95H seems to result in a different set of antigen-driven, common mutations than those seen in the 32-11-1 \(\kappa\)1C antibody, which expresses Asp rather than Gly at 95H. Thus, seven of the eight Gly95H variants, from three different fusions, exhibit an Asn to Gin or Tyr mutation at hypervariable position 53H, an Ala to Thr or Glu at hypervariable position 61H and five of these proteins have a Arg to Val mutation at positions 83H in the third framework region. The Asn to Gin or Tyr mutations at 53H and the Ala to Thr mutation at 61H have also been seen in the \(V_{\kappa}1\) : V\(\kappa\)1-C, NPPC-specific antibodies described by Stenzel-Poore and Rittenberg (46).

The analysis of EPC–KLH-induced hybridomas demonstrates that the response to this PC antigen is much more limited in terms of H and L chain usage than the memory response to NPPC-conjugated KLH (39). Seven different H chains from five \(V_{\mu}\) gene families are utilized in the response to EPC–KLH, whereas all the antibodies from EPC–KLH-immune mice, including those with heteroclitic binding for NPPC, use a single H chain, \(V_{\mu}1\). The contact residues in this H chain are vital for PC binding and few mutations are tolerated even in the D16 antibody, which Chen et al. (35) have shown undergoes affinity maturation to PCC. It is therefore of interest that three of the Xid hybridomas (31-4-2, 32-15-1 and 32-17-1) have mutations in four of these contact residues without loss of binding to EPC–BSA. Group II NPPC-specific antibodies also use both \(\lambda\)1 and \(\lambda\)3 L chains in addition to \(\kappa\)1A, \(\kappa\)1C and \(\kappa\)24 L chains (39,46). The only reported group I, PC-specific antibody expressing a \(\lambda\) L chain came from a wild mouse (47) and this antibody used the V11 gene of the S107 family rather than the V1 gene.

The data presented above are consistent with the hypothesis that PC-specific B cells must be rescued from the bone marrow of Xid mice via cognate interactions with T helper cells. Klinman and Stone (48) have demonstrated that PC-specific B cells were absent in the spleens of Xid mice but could be rescued from the bone marrow by transferring slg\(^\kappa\) bone marrow cells into carrier primed recipients. In Xid \(\mu\) anti-PC transgenic mice, PC-specific B cells develop normally in the bone marrow (13) where they presumably encounter an autoantigen or environmental antigen and are clonally deleted. These PC-specific bone marrow cells can be rescued by over expression of bcl-2, which appears to prevent this receptor-mediated apoptosis (49). Using SP6 H+L transgenic mice crossed onto the background of the Rag-2T knockout, Andersson et al. (50) have also shown that autoreactive immature bone marrow B cells, which recognize double-stranded DNA, can be rescued from clonal deletion by immunization with the cross-reactive T1-2 antigen, TNP-Ficoll. The T cell-mediated rescue of the PC response following EPC–KLH immunization of Xid mice is clearly a situation where the T15 idioype does not dominate the immune response to PC as it does in virtually every strain of normal mouse.

The ontogeny of the anti-PC response in BALB/c mice (51) indicates that T15 establishes its clonal dominance in early neonatal development via antigen-driven expansion into the long-lived self renewing CDS\(^++\) B-cell subset (52). The V-D and D–J junctions of T15 are germline encoded, which suggests that these early progenitors may be of fetal liver origin where terminal deoxynucleotide transferase (TdT) is not active. The N region mutations seen in most of the hybridomas from Xid mice clearly indicate that these clones are derived from bone marrow progenitors where TdT is active during H chain rearrangement. Once TdT is activated and the junctions altered, the T15 Asp95H variant will no longer be the predominant H chain generated. In the Xid mice where PC-specific B cells are either clonally deleted or at least not antigen-selected into the periphery, T15 has no obvious advantage over other PC-specific antibodies. Furthermore, T15 may be at a disadvantage once hypermutation starts in the germinal centers in that many mutations result in the loss of PC binding (35,37) and in prevention of antibody secretion from the B cell (53). Thus, mutations in the second CDR of T15H were shown to result in loss of secretion (54) and the 27-7C3 antibody is an example where a mutation in the framework region may also result in loss of secretion.

The fact that the heteroclitic Gly95H variants dominate the response to PC in Xid mice might indicate that this set of low-affinity heteroclitic clones may not be clonally deleted in the bone marrow as the prototypic anti-PC B cells appear to be. None of these low-affinity \(\kappa\)1C anti-PC antibodies were able to provide high levels of protection against \(S. pneumoniae\) in a passive protection assay. The failure to protect correlates with the fact that the \(\kappa\)1C antibodies bind PC very poorly in the context of the bacterial C-carbohydrate, even though

\[ V-D \text{ junction determines binding to } S. pneumoniae \]
many of them can bind EPC–Dextran (unpublished data). On the other hand, the protective Xid hybridomas 31-23-1 and 32-34-2 bind very well to the surface of virulent \textit{S. pneumoniae}. This direct binding to bacteria appears to be the best predictor of protection for anti-PC antibodies.

In spite of a paucity of T15 antibody, the data in this paper, along with our previous work (15,16), demonstrate that EPC–KLH is a useful immunogen for the induction of PC-specific antibodies capable of providing long lasting protection to \textit{S. pneumoniae}. It appears to do so by expanding rare B cell clones rescued from clonal deletion and by not inducing the high-affinity non-protective PPC-specific antibodies induced by immunization with DPPC–KLH. Studies are in progress to determine whether this antigen will induce protective immunity in humans.

**Acknowledgements**

The authors would like to acknowledge the technical assistance of Erin Latimer and Gretchen Guelde in the production of the Xid hybridomas, and to thank Dr Neil Glaudemans for his assistance in carrying out affinity analysis. We also thank Mr Charles Riggs and Dr Chris Morrell for performing the statistical analysis on mutation distributions in our DNA sequences. We acknowledge the National Cancer Institute for allocation of computing time and staff support at the Frederick Biomedical Supercomputer Center of the Frederick Cancer Research and Development Center.

**Note**

This work was supported in part by the Naval Medical Research and Development Command Research Task 61102A001,01,45X:1277. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human services, nor does mention of trade names, commercial products or organizations imply endorsement by the US Government. The experiments reported herein were conducted according to the principles set forth in the \textit{Guide for the Care and Use of Laboratory Animals}, Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services Publication (National Institutes of Health) 86–23, (1985).

**GenBank accession numbers for sequences in Figs 1 and 2:**

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>(V_H) accession no.</th>
<th>(V_L) accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>27-7C3</td>
<td>U29138</td>
<td>U29423</td>
</tr>
<tr>
<td>31-23-1</td>
<td>U29600</td>
<td>U29424</td>
</tr>
<tr>
<td>31-34-2</td>
<td>U29601</td>
<td>U29425</td>
</tr>
<tr>
<td>32-11-1</td>
<td>U29602</td>
<td>U29428</td>
</tr>
<tr>
<td>24-1B10</td>
<td>U29603</td>
<td>U29429</td>
</tr>
<tr>
<td>24-1B11</td>
<td>U29202</td>
<td>U29430</td>
</tr>
<tr>
<td>24-2B2.4</td>
<td>U29203</td>
<td>U29431</td>
</tr>
<tr>
<td>188E5</td>
<td>U31539</td>
<td>U29426</td>
</tr>
<tr>
<td>31-4-2</td>
<td>U29237</td>
<td>U29427</td>
</tr>
<tr>
<td>32-6-2</td>
<td>U29238</td>
<td>U29266</td>
</tr>
<tr>
<td>32-15-1</td>
<td>U29239</td>
<td>U29268</td>
</tr>
<tr>
<td>32-17-1</td>
<td>U29236</td>
<td>U29267</td>
</tr>
</tbody>
</table>

**Abbreviations**

- **BBS**: borate-buffered saline
- **btk**: Bruton’s tyrosine kinase
- **DPPC**: 6-(O-phosphocholine)hydroxyhexanoate
- **EPC**: keyhole limpet hemocyanin
- **KLH**: keyhole limpet hemocyanin
- **EPC–Dextran**: 6-(O-phosphocholine)hydroxyhexanoate–Dextran

**References**


20 Methods in Carbohydrate Chem. 8:145.


