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Research Article

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Human Anti-Phosphorylcholine Antibodies Share Idiotopes and Are Self-Binding

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Abstract

We have previously shown that BALB/c antipneumococcal polysaccharide antibodies with phosphorylcholine (PC) specificity are self-binding, mediated by hypervariable sequence structure of the heavy chain. We extended the observation of self-binding anti-PC antibodies to naturally occurring human anti-PC antibodies. Anti-PC antibodies were purified from normal donor sera and shown to bind to monoclonal antiidiotypic anti-T15 antibodies originally raised against the murine T15 idiotype. These human antibodies are self-binding which is inhibitable by the PC hapten and the murine T15 (50–73)-derived Vh peptide. The anti-PC antibodies were further separated into id-positive and id-negative anti-PC antibodies. Only the T15 id-positive preparation was self-binding. These findings demonstrate an evolutionary, conserved biological property between mouse and man associated with a naturally occurring antibacterial antibody. This conserved biological and structural property may have been selected in evolution because it is part of an important immune defense mechanism against bacterial and other environmental pathogens. (*J. Clin. Invest.* 1991. 88:476–482.) Key words: human • phosphorylcholine • idiotype • antibody

Introduction

Antiphosphorylcholine (PC)[1] antibodies play an important protective role in pneumococcal bacteria infection (1). PC is the immunodominant component found in the cell walls of a variety of pathogenic and nonpathogenic microorganisms, including *Streptococcus pneumoniae,* lactobacillus, certain fungi, and nematodes (2–4). In BALB/c mice anti-PC antibodies are associated with different idiotypes, such as M167, MOPC 603, 511, and T15/H8 (5). The T15+ anti-PC antibodies are dominant in normal BALB/c sera and in the antibodies in mice immunized with C-polysaccharide or PC coupled to keyhole limpet hemocyanin (6). Furthermore, Briles and colleagues (7) have shown that the T15+ anti-PC antibodies are more than 100 times more protective against bacterial challenge than any of the other idiotypes. Because T15 is the most effective protecting antibody, we generated monoclonal anti-T15 anti-ids (8) and used one of them to induce protective immunity via network stimulation (9).

Analogously, the antipneumococcal response in humans involves the PC epitope since it has been observed (10) that the anti-PC titers are increased in children who have recovered from pneumococcal infections. Other investigators have also identified human phosphorylcholine-binding immunoglobulins (11). However, these authors did not identify a dominant idiotype associated with the anti-PC antibodies nor did they demonstrate a mouse–man idiotypic cross-reactivity.

Self-binding antibodies, termed "autobodies," have previously been described (12). These antibodies belong to the group of germline encoded immunoglobulins of the murine S107/TEPC (T15) V1 gene (13). The self-binding site is located near the PC binding site of these antibodies and involves structures of the hypervariable region of the heavy chain. Recently, we have shown that a 24-residue peptide spanning the heavy chain of T15 was nearly as effective as the hapten PC in inhibiting the self-binding complexes (14).

Because anti-PC antibodies are important for protection against bacterial infection mediated by anti-PC antibodies in man, we wanted to investigate the human repertoire of anti-PC antibodies. In particular, we were interested to see if self-binding antibodies occur in human sera and whether such antibodies are PC-specific. We isolated anti-PC antibodies from human sera and could demonstrate that these antibodies self-bind. Using an anti-T15 mAb, F6, we were able to show that human anti-PC self-binding antibodies express the murine T15 idiotype. From these findings, we tested whether the CDR2/FR3 sequence of murine T15 (S107/V1) is critical for self-complexing of human anti-PC antibodies and present evidence that a peptide derived from the CDR2/FR3 region of the heavy chain of murine T15 can inhibit the self-complexing of human anti-PC antibodies. This finding demonstrates that very similar structures of the V_H region in mouse and man are mediating self-binding.

The discovery of a conserved self-binding site between mouse and man anti-PC antibodies is compelling evidence for a functional role of self-binding in protective immune responses.

Methods

Sera. Human sera were purchased from the San Diego Blood Bank, randomly selected. BALB/c sera were obtained from mice in our colony; CBA/N serum was a kind gift from Ann Feeny, Medical Biology Institute, La Jolla, CA.

Antibodies and peptides. Myeloma cell lines T15 and M167 were obtained from Litton Bionetics, Kensington, MD, and purified from ascites fluid by a PC-Sepharose column. The 11E7 hybridoma has been described previously (2). F6, an anti-T15, is described (8). AB1.2 and B39 anti-T15 antibodies were kind gifts of John Kearney, University of Alabama, Birmingham.

The peptides T15H (50–73) (ASRNKANDYTTEYSASVKGRFIVS) (14), 2F10 (50–73) (QIPGDGDTKYNNGKFKGKATLT) (15) used in this study were synthesized as previously described (14).

Purification of human anti-PC antibodies. Ammonium sulfate–precipitated human Ig was separated on Sephacryl 300HR (Sigma

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1. *Abbreviation used in this paper:* PC, phosphorylcholine.

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Chemical Co., St. Louis, MO) and further purified on PC-BSA-Sepharose by dilution with 10^{-3} M PC in borate buffered saline. F6 id-positive Ig was purified on F6-Sepharose by dilution with .2 M glycine pH 2.4. Purity of purified human Ig was determined by SDS-PAGE indicating > 90% of the input to be H and L chains.

ELISA. To determine the isotypes of human anti-PC antibodies, plates were coated with PC-BSA, 2 μg/ml; aliquots from Sephacryl 300HR fractions were added; after washing, goat anti-human IgG or IgM peroxidase was added (1:1,000 diluted; Fisher Scientific Co., Pittsburgh, PA). 2,2'-Azinobis(3-ethylbenzthiazoline sulfonic acid) (ABTS) substrate was added and the color was read on an ELISA reader (Titertek, Elfab Oy, Finland) at 405 nm.

To measure the binding of human Ig to antiidiotype, F6, B39, and AB1.2 plates were coated with 2 mg/ml of respective anti-ids, dilutions of purified human antibodies were added, and after washing, goat anti-human IgG and IgM peroxidase (Fisher) was added (1:1,000 diluted). Color was read as described above.

To measure self-binding of purified human anti-PC antibodies, plates were coated with purified human anti-PC antibodies, 5 μg/ml for 16 h at 4°C. Biotinylated human anti-PC antibodies were added (1:50 diluted) and the plates were incubated for 16 h at 4°C. After washing, peroxidase-conjugated avidin (Fisher) was added (1:1,000 diluted), and color was read as described above.

The inhibition of self-binding of the human anti-PC antibodies was demonstrated by coincubating biotinylated antibodies with increasing amounts of PC or V_H (50–73) derived peptide as described above.

RIA. To quantify the T15 equivalents in human sera with regard to PC and anti-T15 titers a RIA was used. Plates were coated with PC-BSA or F6 (5 μg/ml) for 16 h at 4°C. After washing, dilutions of human sera were coincubated with ^{125}I-labeled T15 (100,000 cpm) for 16 h at 4°C. After extensive washing the bound radioactivity in each well was measured in a gamma counter. T15 equivalents were calculated using as standard the inhibition of these assays by unlabeled T15.

Self-binding was revealed by adding ^{125}I-labeled murine and human antibodies (100,000 cpm/well) to plates coated with 5 μg/ml of 11E7, M167, human purified anti-PC antibodies, and human anti-Tg antibodies for 16 h at 4°C. After extensive washing the bound radioactivity was measured in a gamma counter. For inhibition experiments, ^{125}I-labeled antibodies were coincubated with inhibitors of self-binding, PC, and V_H (50–73) derived peptide at varying concentrations for 16 h at 4°C.

Results

I. Characterization and purification of human anti-PC antibodies

Anti-PC antibodies in human sera. Gray and co-workers (10) have previously reported the presence of anti-PC antibodies in infants who have recovered from streptococcic infections. In addition, Stein and Sigal (16) examined the human anti-PC repertoire in EBV-transformed B cell lines. In neither of these studies was the expression of idiotopes associated with the human anti-PC antibodies examined. Therefore, it was of interest to probe human sera for the expression of idiotopes associated with anti-PC antibodies.

We analyzed randomly selected human normal sera for binding to PC-coated plates in ELISA. The same sera were also tested for binding the F6-coated plates. Sera from BALB/c mice, who show T15 idiotype dominance in anti-PC antibodies (5), were used as a positive control. We used CBA/N mouse sera as a negative control since they lack T15 idiotype dominance on antibodies to PC (17). The data in Fig. 1 represent the T15 equivalent, expressed in micrograms per milliliter serum, determined by inhibition of labeled T15 binding by dilution of human sera (see Methods). These results demonstrate that anti-PC antibodies are detectable in human sera, as well as that

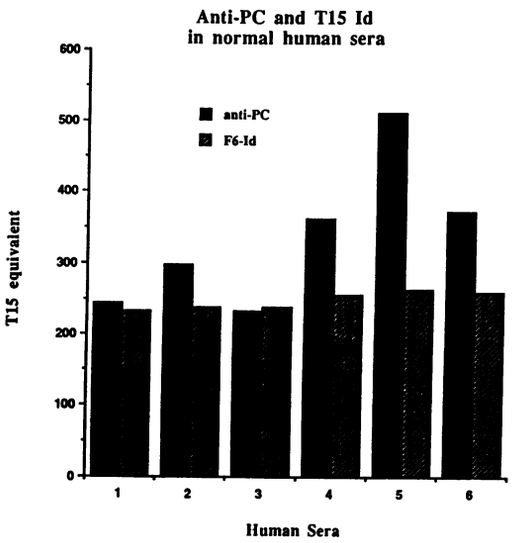

Figure 1. Detection of anti-PC and T15 id in normal human sera by inhibition of binding of ^{125}I-T15 to PC and F6 anti-id. Dilutions of individual sera were coincubated with 100,000 cpm of ^{125}I-T15 on PC-BSA and F6 anti-id coated plates for 16 h at 4°C. Sera from BALB/c and CBA/N mice were included in the assay as positive and negative controls, respectively. After extensive washing the bound reactivity was measured in a gamma counter. The data are represented as T15 equivalent in micrograms, determined by inhibition of labeled T15 binding by dilutions of human sera.

human sera contain antibodies that bind to the anti-T15 F6 monoclonal antibody.

It is noteworthy that several sera contain more anti-PC antibodies than anti-T15 positive antibodies. We also measured the T15 equivalents using other antiidiotype reagents, Ab1.2 and B39. As control for anti-Id binding a murine IgG1, kappa anti-Id, specific for human anti-HIV envelope antibodies (Muller et al., submitted for publication) was used. No binding to 2A11 was observed (Fig. 3 *d*). We found that using these anti-T15 antibodies, human sera contained T15 equivalent values several-fold less than detected by F6 (data not shown).

Purification of human anti-PC antibodies. Anti-PC antibodies in mouse sera are of IgM and IgG subclasses. Therefore, we aimed to isolate human anti-PC antibodies of both isotypes. The ammonium sulfate–precipitated Ig of several human sera were chromatographed on Sephacryl 300HR. Fractions were analyzed for binding to insolubilized PC-BSA. Bound antibodies were assayed with goat peroxidase–conjugated anti–human IgM and IgG antisera. A representative elution profile from a single donor serum is shown in Fig. 2. The data indicate the presence of anti-PC antibodies of IgM and IgG classes eluting at the expected high and low molecular weight positions, respectively. The corresponding fractions were pooled.

The separated IgM and IgG anti-PC pools were then applied to PC-Sepharose immunoabsorbent and binding antibodies were eluted by 10^{-3} M PC according to (18). The purity of IgM and IgG anti-PC antibodies purified from three different sera was analyzed by SDS-PAGE (data not shown). The purity of both preparations was estimated to be > 90%.

Characterization of human anti-PC antibodies. The purified anti-PC antibodies of IgM and IgG isotypes were tested for binding to PC-BSA coated plates. The specificity of binding was demonstrated by using free PC as inhibitor. Both antibody

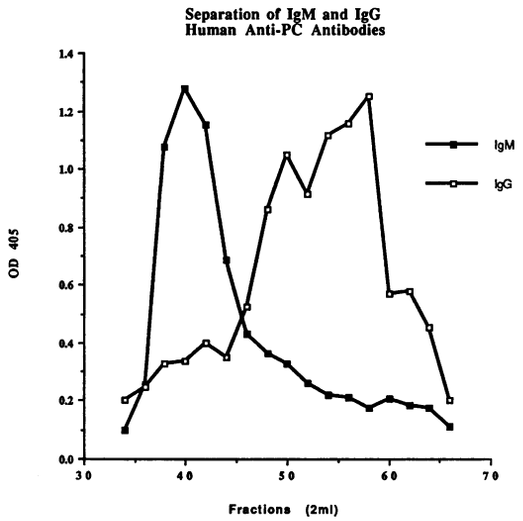

Figure 2. Binding of purified human IgM and IgG antibodies to PC as measured by an ELISA. Ammonium sulfate–precipitated Ig from individual human sera were chromatographed on Sephacryl 300HR. 100 μl from each fraction was added to plates coated with PC-BSA for 1 h. After washing, the plates were incubated with peroxidase-labeled anti–human IgM or IgG. Bound antibodies were revealed by ABTS substrate and the optical density values were measured at 405 nm.

preparations, the IgM and IgG anti-PC antibodies, were completely inhibited by PC (50% inhibition for IgG achieved by 5×10^{-5} M PC, for IgM by 2×10^{-4} M PC). These values are in the range needed for inhibiting the IgM anti-PC hybridoma 11E7 (IgM, kappa) (12).

Next we asked whether anti-PC antibodies bind to anti-idiotype antibodies against the mouse T15 idiotype. Purified anti-PC were added to anti-id coated plates, F6, Ab1.2, or B39. Bound human antibodies were detected by enzyme-labeled goat anti–human IgM and IgG. As representative example the data on anti-PC antibodies from one donor are shown in Fig. 3. Both IgM and IgG human anti-PC antibodies bind all three anti-Ids with different degrees. Individual variations of binding were observed with anti-PC antibodies purified from different donors. This indicates that the three antimurine anti-T15 anti-idiotypic antibodies cross-react with human anti-PC antibodies demonstrating that human and murine anti-PC antibodies share idiotopes.

II. Self-binding of human anti-PC antibodies

Human anti-PC antibodies are self-binding. We have previously shown that murine anti-PC antibodies have the unique property of self-binding (12–14). Here we investigated whether purified total, i.e., not separated into IgG and IgM, human anti-PC antibodies, purified from pooled human serum on PC-Sepharose, have self-binding activity. As seen in Fig. 4 *A*, self-binding is demonstrated by plates coated with purified human anti-PC antibody and incubated with ^{125}I-labeled human anti-PC antibody. As controls are shown: the T15 negative murine anti-PC antibody M167, a purified human antithyroglobulin which does not self-bind, and 11E7 (T15+, IgM,k) which strongly self-binds (12).

Next, the specificity of self-binding was analyzed by hapten inhibition. Plates were coated with mouse or total purified human anti-PC antibody or human antithyroglobulin antibody and incubated with ^{125}I-labeled mouse or human anti-PC antibody or human antithyroglobulin antibody in the presence of various concentrations of PC. As seen in Fig. 4 *B*, PC specifically inhibited the self-binding. Recently (14), we reported the inhibition of murine self-binding anti-PC antibodies by a V_H-derived peptide spanning residues 50–73 in T15 and demonstrated the importance of this region in murine anti-PC self-

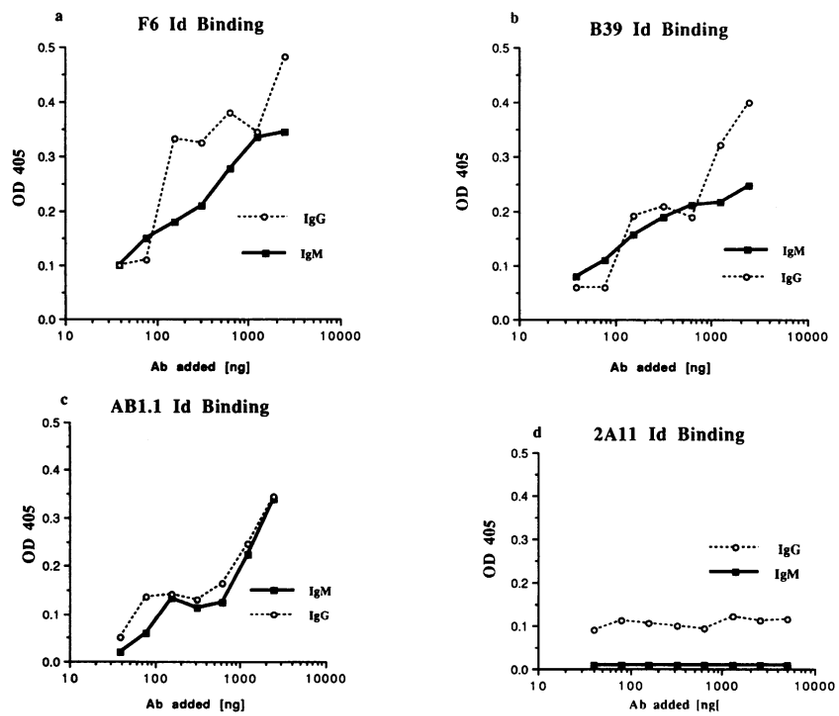

Figure 3. Binding of human IgM and IgG anti-PC antibodies to anti-ids: (*a*) F6; (*b*) B39; (*c*) AB1.2; (*d*) 2A11, as measured by an ELISA. Varying concentrations of human anti-PC antibodies were added to anti-id–coated plates (2 mg/ml) for 2 h at 4°C. After washing, the plates were incubated with peroxidase-labeled goat anti–human IgM or IgG. Bound antibodies were revealed by ABTS substrate and the optical density values were measured at 405 nm.

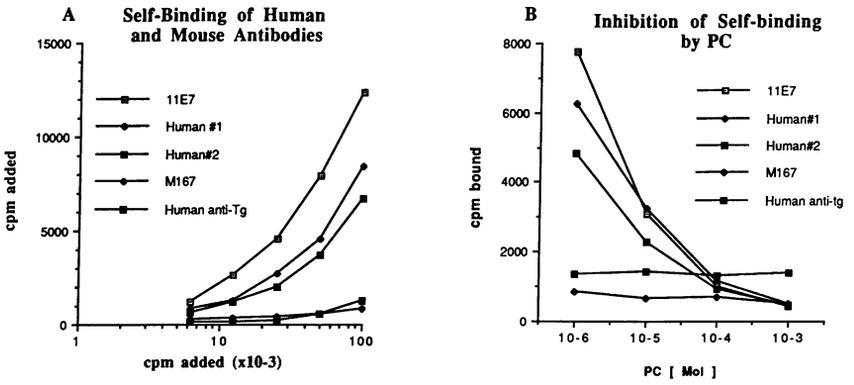

Figure 4. (*A*) Self-binding of human anti-PC antibody in solid phase. Microtiter plates were coated with 5 μg/ml of human anti-PC antibody from two donors (1 and 2), 11E7 (IgM, κ), M167 (IgA, κ), and human antithyroglobulin (anti-Tg) antibody. Decreasing amounts of ^{125}I-labeled human anti-PC antibody, 11E7, M167, and human anti-Tg control were added to the plates and incubated for 18 h. The plates were extensively washed and the bound radioactivity in each well was measured in a gamma counter. (*B*) Inhibition of self-binding of human anti-PC antibody. ^{125}I-labeled antibodies were coincubated with varying concentrations of the hapten PC for 16 h at 4°C. After extensive washing the bound radioactivity was measured in a gamma counter.

binding. In this study we find that this CDR2/FR3 region is also critical in human self-binding. As shown in Fig. 5 *A* this murine peptide is a potent inhibitor of T15+ human anti-PC self-binding. For comparison we show the inhibition of mouse self-binding antibody (11E7, IgM, κ) in Fig. 5 *B*. As a control, the peptide 2F10 V_H(50–73), derived from the heavy chain of an unrelated antibody, had no effect on self-binding.

III. Characterization of human IgG and IgM anti-PC antibodies

The separated IgM and IgG anti-PC antibodies described in Fig. 2 were further characterized with respect to self-binding. Anti-PC antibodies isolated from one donor (FB) by PC affinity chromatography were radiolabeled and biotinylated. Radiolabeled antibodies from donor FB showed clear self-binding (see Fig. 6, *a* and *b*) of labeled IgM anti-PC antibodies. The self-binding is inhibited by PC in a similar degree as the prototype 11E7 (IgM murine anti-PC hybridoma) (12). The self-binding of FB IgM anti-PC antibodies is also inhibited by the murine T15 CDR2/FR3 (50–73) peptide as shown in Fig. 6 *b*. Attempts to demonstrate self-binding of the IgG anti-PC fractions from several donors were unsuccessful. As seen in Fig. 6, *a* and *b*, the human IgG anti-PC antibodies did not self-bind. This failure could be due to the intrinsic low self-binding property of 7S immunoglobulin observed earlier with murine IgG T15+ anti-PC hybridoma (12). The results with purified human anti-PC antibodies demonstrate that these antibodies are indeed self-binding in similar fashion as the murine counterpart.

IV. Further characterization of self-binding antibodies

The finding that human anti-PC antibodies self-bind and express the T15 idiotype led us to ask whether the self-binding property of human anti-PC antibodies is dependent on the expression of the cross-species reactive T15 idiotype. To address this question pooled normal Ig were sequentially purified on PC-Sepharose and F6-Sepharose immunoabsorbents. This scheme produced anti-PC antibodies which are either T15 positive or T15 negative (see Fig. 7).

Ammonium sulfate–purified Ig was affinity purified on an F6-Sepharose immunoabsorbent by elution with acid (data not shown). The eluted material was neutralized, concentrated,

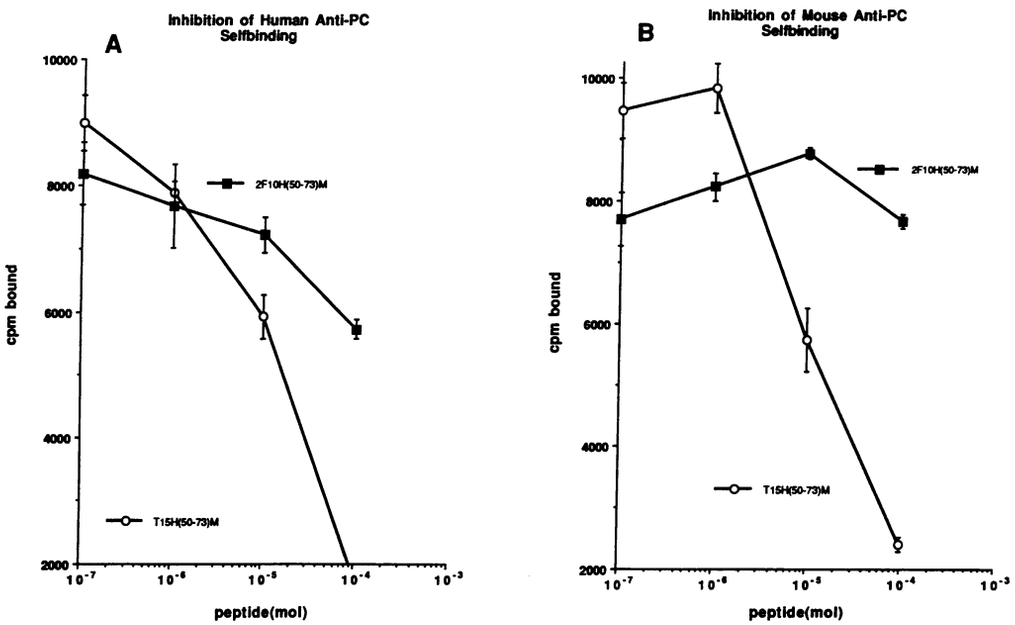

Figure 5. Specific inhibition of self-binding of total human (*A*) and mouse (*B*) anti-PC antibody (11E7, IgM, κ) by the T15 V_H (50–73) peptide. Plates coated with 5 mg/ml of human or mouse anti-PC antibodies were coincubated with 100,000 cpm of ^{125}I-labeled anti-PC antibody and increasing amounts of T15 H (50–73) peptide. The 2F10 VH (50–73) peptide was used as a negative control.

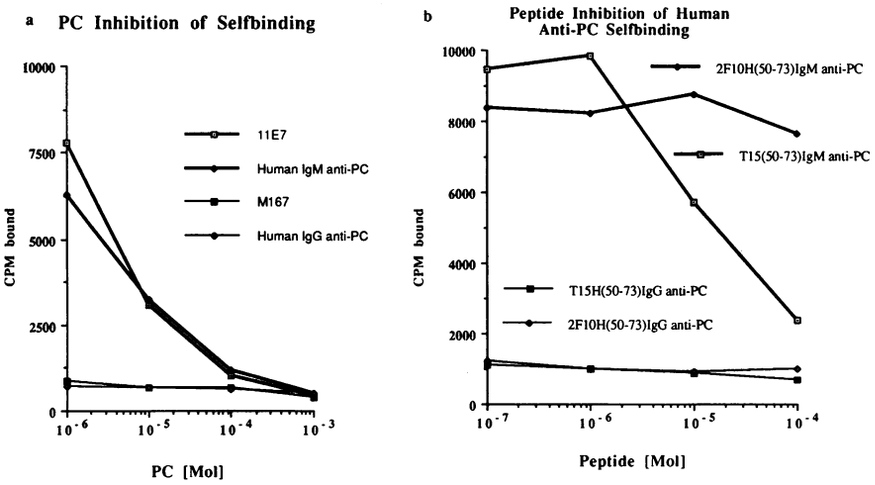

Figure 6. Inhibition of self-binding of ^{125}I-labeled human anti-PC IgM and IgG antibodies by the hapten PC (*A*) and by the T15 V_H (50–73) peptide (*B*) in RIA. Plates coated with 5 μg/ml of purified human anti-PC antibodies were coincubated with varying concentrations of inhibitors and radiolabeled human anti-PC antibodies (IgG and IgM) were added (10^5 cpm/well). 11E7 and M167 were used as positive and negative controls, respectively, (*a*), and the 2F10 H (50–73) peptide was used as a negative control (*b*).

and applied onto a PC-Sepharose column (see right-hand side of Fig. 7). Material binding to PC-Sepharose could be eluted with 10^{-3} M PC. Further analysis of the $F6^+P\,C^+$ material using PAGE in SDS confirmed that it was immunoglobulin (data not shown). The sequentially eluted Ig consists of anti-PC antibodies that express the T15 idiotype. These human anti-PC antibodies were then biotinylated and assayed for self-binding on solid phase. To test for specificity in self-binding increasing concentrations of the T15 CDR2/FR3 peptide (50–73) were added. As seen in Fig. 8 the human $F6^+$ anti-PC antibodies are self-binding and the self-binding is inhibited in the characteristic manner by the T15 peptide and not by the 2F10 peptide.

To confirm that the self-binding of human anti-PC antibodies is associated with the expression of the T15 idiotype we reversed the order of sequential purification on PC and F6-Sepharose immunoabsorbents. Ammonium sulfate–precipitated human pooled Ig was first purified on PC-Sepharose and eluted material was passed over F6-Sepharose (see left-hand side of Fig. 7). Material bound to F6-Sepharose was eluted with acid buffer. The eluted material was neutralized and concentrated. Both the flow through and eluted material from the F6-Sepharose column were biotinylated and assayed for self-binding on solid phase. Fig. 9 shows that the $F6^+PC^+$, but not the $F6^-PC^+$ human Ig, is self-binding and that this self-binding is inhibited by free PC. Fig. 9 also demonstrated that the F6 negative anti-PC (PC^+T15^+) antibodies do not self-bind. Furthermore, we also tested the $F6^+$ anti-PC antibodies for inhibition of self-binding by the T15 CDR2/FR3 peptide and observed strong inhibition (data not shown). Collectively, these results established that the self-binding property of human anti-PC antibodies is dependent on the expression of an idiotope cross-reactive with the murine T15 idiotype.

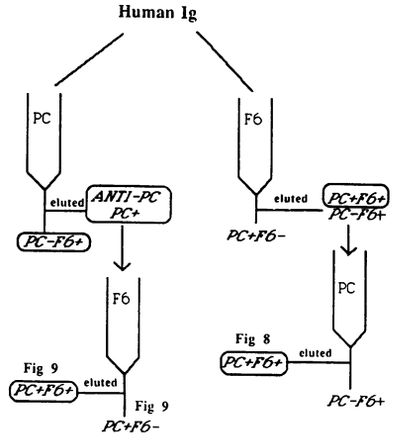

Figure 7. Schematic description of the immunoabsorbent purification steps of human Ig. The circled antibody preparations are self-binding. The superscripted figures indicate the figure number where the data on self-binding are shown.

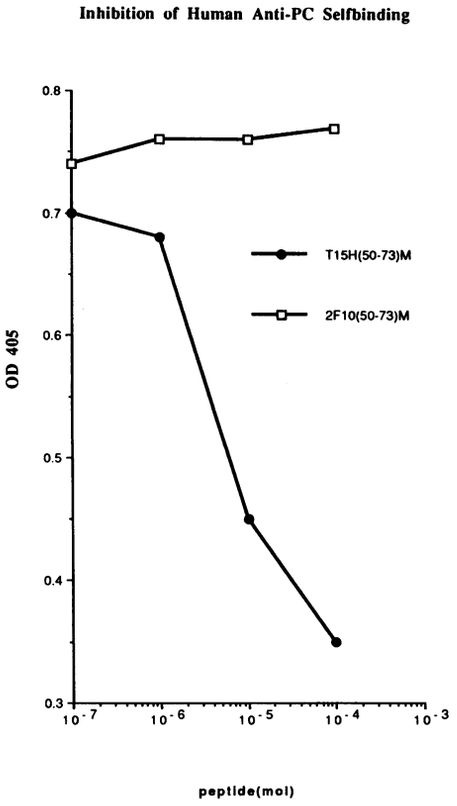

Figure 8. Inhibition of self-binding of human anti-PC antibodies purified on F6 and PC (PC^+, $F6^+$) using CDR2/FR3 derived peptides. Human Ig was first affinity purified on an F6-Sepharose affinity column and subsequently the $F6^+$ fraction was further affinity purified on a PC-Sepharose column. Biotinylated purified anti-PC antibodies were added to plates coated with purified anti-PC antibodies. Inhibition with peptides was done as in Fig. 6 *b*.

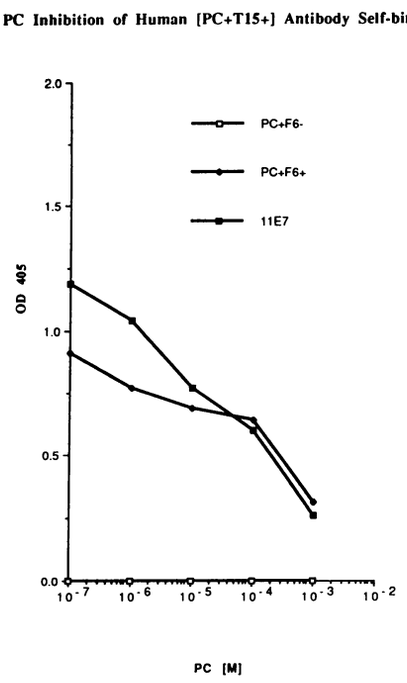

Figure 9. Inhibition of self-binding by PC. Human anti-PC antibodies were first purified on PC-Sepharose, eluted with PC, and further purified on F6-Sepharose. Pass-through material (PC^+, $F6^-$) and eluted material (PC^+, $F6^+$) were biotinylated and added to plates coated with PC^+, $F6^+$ anti-PC antibodies (5 μg/ml). As positive control, self-binding of the murine 11E7 antibody to 11E7 coated plates is shown. Inhibition with PC was done as in Fig. 4 *B*.

Discussion

The key finding in this study is that in normal human sera anti-PC antibodies exist that cross-react with murine anti-PC antibodies of the T15 idiotype. Three monoclonal anti-T15 antibodies, one of which detects an idiotope associated with the PC binding site (8), react with immunoglobulins in sera of normal human donors. Anti-PC antibodies were purified from human sera and tested for hapten specificity and binding to anti-T15 antiidiotypic antibodies. The monoclonal anti-T15 antibodies show binding to affinity purified human anti-PC antibodies of IgM and IgG isotypes. One of the hallmarks of murine anti-PC antibodies of the T15 idiotype is the property of self-binding or specific self-associating (12–14). The purified human anti-PC antibodies share this property with the murine counterpart. Like mouse T15 positive anti-PC antibodies, human anti-PC antibodies show self-binding. The self-binding of human antibody is like the mouse antibody inhibited by the hapten PC, indicating the involvement of the PC binding site in self-binding. Furthermore, a 22-mer peptide derived from the CR2-FR3 region of the mouse T15 heavy chain inhibits human self-binding.

The degree of similarity of self-binding structures of human and murine self-binding antibodies cannot, at this time, be determined because we do not know the sequence of human self-binding antibodies. We searched the sequence data base on human immunoglobulins for homology with the T15 V_H (50–73) region and did not find significant sequence similarities. This would imply that the primary structure responsible for self-binding antibodies can differ significantly and thus, that the conserved self-binding property is maintained in evolution by different primary structures. To further decipher the molecular basis of self-binding, Kaveri (19) has recently demonstrated the specific binding of mouse and human antibodies to the T15 H (50–73) peptide. In addition, the authors were able to isolate T15 H (50–73) peptide-specific antibodies from pooled normal human immunoglobulin and have shown that these peptide specific human antibodies are self-binding (19). This further emphasizes the evolutionary conserved structure of the self-binding locus which must be similar in mouse and man.

An important question is whether self-binding of human antibodies is restricted to the T15 id, like in the BALB/c mouse. To address this question we isolated anti-PC antibodies that were T15 id+ or T15 id−. We found that only the T15 id+ antibodies were self-binding and that the self-binding is inhibited by the hapten PC and the T15 V_H-derived peptide. Since the molarity of PC giving 50% inhibition of murine and human self-binding antibodies is similar the structures involved in self-binding are likely to be similar. Therefore, it is of interest to know to what extent the common biological self-binding potential is based on similar structures.

The finding of a conserved interspecies idiotype that is associated with an antibacterial response suggests that the T15 id may be maintained in evolution because it is fundamental to survival. In the mouse, T15-positive anti-PC antibodies have been shown to be the most effective antibodies for protection against pneumococcal infection (7). Conserved interspecies idiotypes have also been reported for influenza (20) and hepatitis (21) emphasizing the importance of maintaining certain V-region genes in the immune repertoire. In this report we add to a list of functional characteristics of antibodies the property of self-binding as one of the biological functions conserved between mice and human anti-PC antibodies. We speculate that self-binding as part of the protective antibacterial response in two divergent species may have an important role in the regulation of the immune response against infectious agents.

Finally, we do not understand the clonal selection pathways by which B cells producing self-binding antibodies emerge dominant during development of the immune system and whether these processes continue during the ongoing adaptation to environmental and infectious challenges throughout the life of the individual.

Acknowledgments

This manuscript is dedicated to the memory of Roberta Halpern.

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