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Isoelectric Focus Analysis of Rat Anti-phosphocholine Antibodies†

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ABSTRACT: Anti-phosphocholine (PC) antibodies in sera from four strains of rats were examined before and after immunization with either *Streptococcus pneumoniae* R36A, which contains PC as a cell wall component, or with PC-coupled keyhole limpet hemocyanin (PC-KLH). PC-specific protein was purified from pooled immune sera and shown by a combination of isoelectric focus (IEF) in acrylamide and crossed immunoelectrophoresis, as well as by molecular weight determination in $NaDodSO_4$-acrylamide, to be immunoglobulin. An additional, small molecular weight, nonimmunoglobulin protein (pI = 7.1-7.3) was present in sera from normal and germ-free rats which had the ability to bind the C-carbohydrate of *S. pneumoniae* R36A, but without specificity for PC. The IEF profile of normal and immune sera showed marked sharing of bands of anti-PC antibody between individual rats as well as between strains. In addition, other anti-PC antibodies which focused between pH 8.5 and 9.5 were less regularly shared. The uniformity of IEF profile of the bulk of anti-PC antibodies in rats is most consistent with their being the products of germ line genes.

Detailed studies of antibodies with similar antigen binding specificities have revealed striking structural similarities. In particular, the analysis of mouse anti-phosphocholine (PC)[1] immunoglobulins by antigenic (Claflin and Davie, 1974a), functional (Claflin and Davie, 1974b), and structural means (Claflin and Rudikoff, 1977) has shown that all mice regardless of genetic background produce similar antibodies. This conservation of variable region structure is consistent with the existence of a germ line gene for anti-PC antibodies in mice.

In addition, preliminary examination of the fine specificity of anti-PC antibodies raised in different species of rodents showed that each species developed distinctive binding patterns for PC and its analogues and that within a species, indistinguishable patterns were found in all individuals examined (Claflin and Davie, 1974b). These studies were based on the ability of soluble haptens to inhibit plaque formation by antibody secreting cells. The present paper extends these studies in the rat by examining the isoelectric focusing pattern of 7S anti-PC antibodies in normal and immune rat serum. While minor differences in these spectra exist, extensive sharing of antibody patterns is seen regardless of the genetic background of the animal.

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[1] Abbreviations used are: PC, phosphocholine; KLH, keyhole limpet hemocyanin; IEF, isoelectric focusing; PBS, phosphate-buffered saline; BGG, bovine γ-globulin; BSA, bovine serum albumin; CFA and IFA, complete and incomplete Freund's adjuvant, respectively; PnC, C polysaccharide.

Materials and Methods

Animals. Young adult female Wistar/Furth (W/Fu) and Fischer 344 rats were obtained from National Animal Laboratory Co., O'Fallon, Mo.; Lewis/maif (Lew) and Brown Norway/maif (BN) rats were purchased from Microbiological Associates, Walkersville, Md.; axenic F344 weanling rats were obtained from ARS/Sprague Dawley, Madison, Wis.

Immunization. Animals were immunized intraperitoneally (ip) with either 1×10^8 heat-killed (56 °C, 30 min) *Streptococcus pneumoniae* strain R36A (ATCC 27336) bacteria, which contain PC as a cell wall component (Tomasz, 1967), or with 250 μg of PC-coupled keyhole limpet hemocyanin (PC-KLH) in complete Freund's adjuvant (CFA) and bled at weekly intervals. PC-KLH, prepared as previously described (Claflin et al., 1974), contained 19 PC groups per 10^5 daltons of protein. The same dose of antigen and route were used for secondary immunizations with either vaccine without adjuvant or PC-KLH in incomplete Freund's adjuvant (IFA).

Measurement of Antibody Response. Antigen binding capacity was determined by a double isotope Farr assay (Gotschlich, 1971) using ^{22}NaCl as a volume marker and ^{125}I-labeled C polysaccharide (PnC) from *S. pneumoniae* R36A (Liu and Gotschlich, 1963). [^{125}I]PnC was prepared by chloramine-T iodination (Greenwood et al., 1963) of tyraminyl PnC (Keck, 1972). Twenty microliters of serial threefold dilutions of test sera was mixed with an equal volume of diluent (1% BSA, 0.01% BGG) containing 2×10^4 cpm of ^{22}NaCl and 2×10^4 cpm of [^{125}I]PnC (sp act. 19 μCi/μg). Immunoglobulins were precipitated by addition of 50 μL of 85% saturated ammonium sulfate; the amount of [^{125}I]PnC precipitated was measured in a γ counter, and the dilution of serum needed to bind 33% of the ligand was determined (ABC_{33}).

In some cases, sera were separated by sucrose density ultracentrifugation into 7S and 19S fractions as described by Perlmutter et al. (1977) prior to determination of ABC_{33}.

Purification of Anti-PC Antibodies. Rat sera were collected and pooled 7 days following secondary immunization with 250 μg of PC-KLH in saline. Seventeen milliliters of pooled immune serum was passed through 3 mL of PC-Sepharose. The beads were washed extensively with phosphate-buffered saline (PBS, 0.056 M Na_2HPO_4, 0.016 M KH_2PO_4, 0.077 M NaCl, pH 7.4) until the absorbance at 280 nm returned to baseline. A 10^{-2} M PC solution in PBS was then applied. The eluate (20 mL) was concentrated on Amicon filters (exclusion limit 25 000) and dialyzed against PBS. The yield of antibody was 500 μg/mL of serum, which corresponds well with values obtained by precipitation of immune sera with PnC. Antibodies were also purified from normal serum by similar techniques.

Isoelectric Focusing. Isoelectric focusing in vertical slab polyacrylamide gels was performed as described by Briles and Davie (1975). The gel was 5% acrylamide (Bio-Rad Laboratories, Richmond, Calif.), 2% ampholytes, pH 5 to 9.5 (LKB, Chicago, Ill.), and 3 M urea (Schwarz-Mann Ultra Pure, Orangeburg, N.Y.). The anode solution (upper trough) was 1.5% phosphoric acid and the cathode solution (lower trough) was 2% monoethanolamine. Whole serum samples or purified antibodies were focused for 15 h, 4 °C, 2 mA constant current, and then for an additional 0.5 h at 800 V.

Detection of Focused Antibody. Anti-PC antibodies in the focused gels were detected by autoradiography (Briles and Davie, 1975) or by immunoelectrophoresis in a second dimension (Pierce et al., 1976).

In the case of autoradiography, the gel was incubated sequentially in two changes of 18% sodium sulfate (2–4 h), in 0.01% glutaraldehyde in 18% sodium sulfate (1 h), and in 0.001% sodium borohydride in borate buffer (0.2 M borate, 0.15 M NaCl, pH 8.2) overnight. This procedure precipitates and cross-links immunoglobulins without affecting antigen binding capacity. The gel was then incubated with 30 μCi of [^{125}I]PnC in 13 mL of 1% BSA for 24 h. After several sequential washes in PBS, water, and 40% ethanol, the gel was allowed to air dry. The dried gel was autoradiographed using Kodak single-coated SB-54 x-ray film for 1 to 3 days.

In the case of immunoelectrophoresis in a second dimension, a strip of the isoelectric focused gel (one well in width) was removed and overlaid onto a horizontal 1% agarose (Sigma, St. Louis, Mo.) gel plate in 0.025 M sodium barbital (pH 8.6) containing goat anti-rat 7S Ig (64 μL/mL agarose; Gateway Immunosera Co., Cahokia, Ill.). Electrophoresis was then used to move the focused proteins into the agarose gel. The direction of electrophoresis was perpendicular to the long axis of the IEF strip in the plane of the agarose gel. The electrode running buffer was 0.1 M sodium barbital (pH 8.6). After electrophoresis for 30 min at 4 °C, 34 mA constant current (~10 V/cm), the polyacrylamide strip was removed and the run continued for an additional 5 h.

Preparative IEF and Sodium Dodecyl Sulfate Gel Electrophoresis. About 8.7 mg of purified anti-PC antibodies which were dialyzed overnight against 2 M urea was fractionated by liquid isoelectric focusing on the LKB 8101 (110 mL) column (LKB, Chicago, Ill.). The liquid system was stabilized against convection by means of a sucrose gradient (0–40%). Carrier ampholytes, in the pH 6–8 range, were added to the sample at loading time (2% final concentration). The focusing was run in 3 M urea at 2 or 15 W for 2 days at 10 °C. At the end of the run 1-mL fractions were collected and individually focused in an analytical gel and the similar fractions were pooled. The pools (fraction a to g) were concentrated on Amicon filters (exclusion limit 25 000) and dialyzed against PBS. Aliquots of the fractions were labeled with ^{125}I (Greenwood et al., 1963) and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Perlmutter et al. (1977). Reference proteins included lysozyme, bovine albumin, and bovine IgG.

Results

Humoral Anti-PC Response. Rats were shown previously to respond to pneumococcal vaccine with the production of direct (IgM) plaque-forming cells (Claflin and Davie, 1974b). The serum antibody response was measured in the present studies in response to either 1×10^8 pneumococci given ip in saline or to 250 μg of PC-KLH in CFA. Table I summarizes the PnC-binding capacity of normal and immune sera from four strains of rats as measured by a Farr assay. It can be seen that all strains except Brown Norway have detectable PnC-binding activity in normal serum. Immunization with either pneumococcus or PC-KLH augments the binding capacity in all strains except Wistar which has high levels of antibody even before immunization. To determine whether both 7S and 19S antibodies were present, sera were fractionated by sucrose density ultracentrifugation before assaying for antibody. Primary immune sera of the 3 strains tested had the bulk of the binding activity in the 7S fractions. Surprisingly, even normal sera had substantial activity in the 7S fractions. The specificity of the PnC-binding activity from both normal and immune sera was directed to PC determinants since adsorption of the sera with PC-Sepharose removed detectable PnC-binding activity. While differences exist between strains in the magnitude of the response and in the antigenic preference, the significance of

TABLE I: PC-Binding Capacity in Normal and Immune Rat Antisera.[a]

	Normal serum		Vaccine immune			PC-KLH immune		
			Primary		Secondary	Primary		Secondary
	ABC_{33}	% 7S	ABC_{33}	% 7S	ABC_{33}	ABC_{33}	% 7S	ABC_{33}
Fischer	7.7 (1.2)		26.9 (1.1)		60.8 (1.6)	8.0 (1.4)		13.6 (1.2)
Brown/Norway	<5		6.5 (3.1)	95	64.6 (1.9)	9.1 (2.7)	94	20.2 (1.8)
Wistar	13.2 (1.6)	78	7.9 (1.6)	72	8.7 (1.4)	10.0 (1.1)	93	16.3
Lewis	6.4 (1.1)	37	33.6 (1.6)	82	46.6 (1.8)	79.1 (2.0)	94	52.9 (3.5)

[a] Rats in groups of 4–6 were immunized with 1×10^8 *S. pneumoniae* R36a (vaccine) ip in saline or 250 μg of PC-KLH in CFA. Four weeks later, the animals received the same dose of antigen; PC-KLH was now given in IFA. Animals were bled 7 days after each injection and anti-PC titers determined. Shown are the geometric means and, in parentheses, standard errors of the reciprocal of the dilutions of sera needed to bind 33% of [^{125}I]PnC (50 ng/mL), ABC_{33}. In addition, 2 sera from several groups were separated by sucrose density ultracentrifugation into 19S and 7S fractions before antigen binding assay. Shown are the averages of the determinations expressed as the percent of total binding which appears in the 7S fractions.

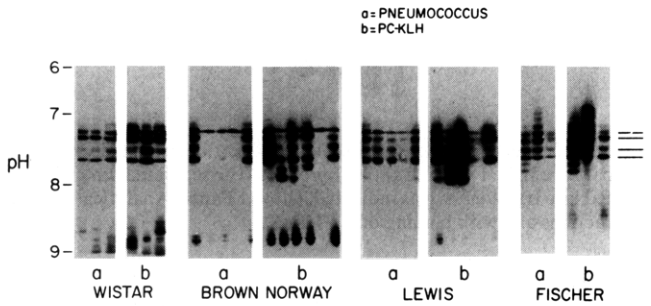

FIGURE 1: Comparison of anti-PC spectrotypes seen after immunization of Wistar, Brown Norway, Lewis, and Fischer rats with either (a) 1×10^8 *Streptococcus pneumoniae* strain R36A in saline or (b) 250 μg of PC-KLH in CFA. Immune sera (5 μL) from individual animals were isoelectric focused in acrylamide gels and PC-binding focused proteins were detected by incubation with [^{125}I]PnC and autoradiography (2–3 day exposure). Shown at the right is the position of frequently shared bands in the neutral region.

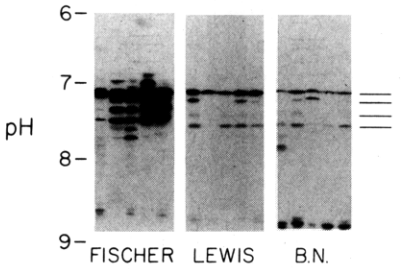

FIGURE 2: Comparison of anti-PC spectrotypes present in unimmunized Fischer, Lewis, and Brown Norway rats. Whole sera from individual animals (30 μL) were isoelectric focused, incubated with [^{125}I]PnC, and autoradiographed. Shown at the right is the position of shared bands in Figure 1.

these differences is not clear. However, as will be shown below, the difference in strains is quantitative rather than qualitative.

Isoelectric Focusing of Immune Sera. The 7S anti-PC antibodies were examined for heterogeneity by isoelectric focusing. Figure 1 is an autoradiograph of the isoelectric focusing pattern of immune sera from 33 individual rats from 4 strains immunized with either vaccine or PC-KLH. It can be seen that there is [^{125}I]PnC-binding activity in two regions of the gel, a cluster of bands between pH 7 and 8 and a few bands from pH 8.5 to 9. It should be noted that, under these conditions, IgM does not enter the gel. The most striking features of the anti-PC response in rats as shown by these focused patterns of immune sera are the high degree of restriction and the ubiquity of the prominent bands between pH 7 and 8 both in individual rats within the same strain and among different strains. This uniformity between strains is particularly unexpected since Fischer rats have a different light chain allotype than the other strains (Gutman and Weissman, 1971). The position of these prominent, shared bands in the neutral region is marked at the side of all figures by drawn lines for reference.

Isoelectric Focusing of Normal Sera. Figure 2 shows that the prominent bands in the gel from pH 7 to 8 are also present to varying degrees in unimmunized animals. Autoradiography of isoelectric focused gels exposed to [^{125}I]PnC proves to be a very sensitive technique for detecting rat anti-PC antibodies. As shown in Table I, unimmunized rats of all strains except Brown Norway appear to have [^{125}I]PnC binding activity as detected by Farr assay. By focusing large amounts of normal sera (6 times more sera are focused in Figure 2 than in Figure 1), significant anti-PC binding activity can be detected which is indistinguishable by IEF from the anti-PC antibodies in immune sera. Thus, it appears that immunization of rats with vaccine or PC-KLH simply induces an increase in concentration of anti-PC immunoglobulins already present before immunization.

Isoelectric Focusing of Germ-Free Rat Sera. Since it was clear that unimmunized rats could not be considered "non-immune" with regard to phosphocholine, sera from weanling germ-free Fischer rats were isoelectric focused (Figure 3). It is evident that the sera from germ-free rats contain PnC-binding activity which is similar to that found in the neutral pH region of normal and immune focused sera, in spite of the fact that these same animals have appropriately low levels of γ-globulins as shown by immunoelectrophoresis and by the absence of protein bands in the γ region (Figure 3A). When these rats were housed in conventional animal facilities, their sera at 2 and 3 weeks showed a gradual decline in the PnC-binding content (Figure 3B) only to return, by 8 weeks, to the IEF patterns of unimmunized conventional rats. It appears likely that the germ-free rats had acquired maternal anti-PC antibodies from colostrum and that while these rats and their mothers lived in a germ-free environment, it probably was not antigen-free.

Immunoglobulin Nature of PnC-Binding Focused Bands. A characteristic of even homogeneous immunoglobulins is charge microheterogeneity detected in IEF as a cluster of 2–4 bands (Awdeh et al., 1970). While the majority of the PnC-binding bands are consistent with immunoglobulin, a single band which focuses at pH 7.1 is clearly atypical. This atypical band, seen particularly clearly in normal and germ-free sera, does not show charge heterogeneity and, in fact, is the major protein in this region in germ-free sera (Figure 3A). Thus, it

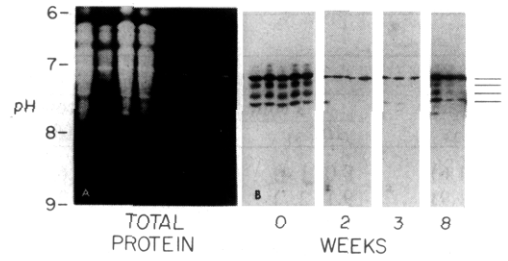

FIGURE 3: PC-binding proteins present at various times after conventionalization. (A) Whole sera (30 μL) from 4 adult (left) and 4 germ-free (right) Fischer rats were isoelectric focused and precipitated with 18% Na_2SO_4 to detect total protein. (B) Germ-free Fischer rats were bled at 0, 2, 3, and 8 weeks of housing in conventional animal facilities and their sera focused. Shown is the autoradiograph of [^{125}I]PnC binding to these sera. At the right is the position of shared bands in Figure 1.

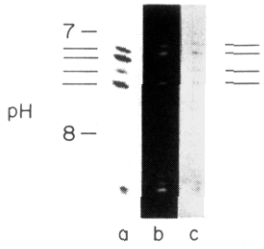

FIGURE 4: IEF of PC-binding proteins eluted from PC-Sepharose immunoadsorbent with 10^{-2} M PC. Purified PC-binding proteins were focused in acrylamide gels. Identical gels were exposed to (a) [^{125}I]PnC to disclose PC-binding ativity, (b) goat anti-rat 7S Ig antisera (after immunoelectrophoresis in a second dimension) to detect immunoglobulin determinants, and (c) Coomassie blue stain to detect protein. The slight misalignment of bands is probably due to the variability in the pH gradient between different gels or parts of the same gel. It is thought that each band is detected by each technique. On the right and left borders are marked the positions of shared bands in Figure 1.

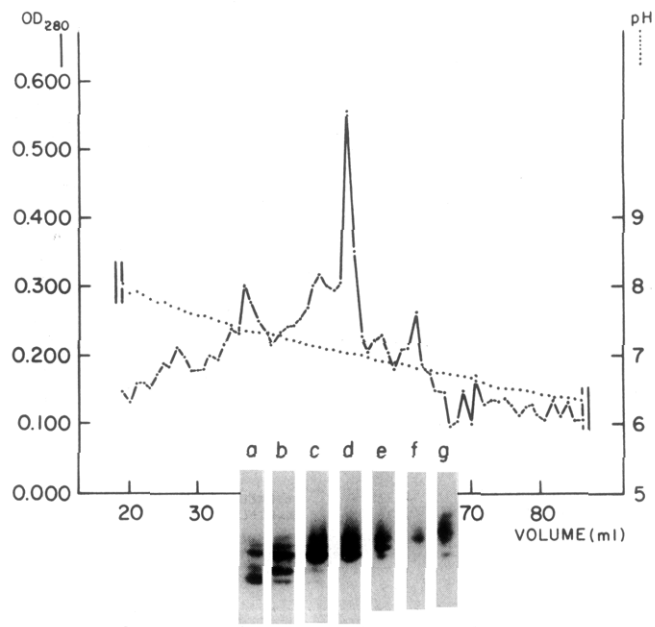

FIGURE 5: Separation of purified anti-PC antibodies. (Upper Panel) Preparative IEF of purified anti-PC proteins. The separated proteins were pooled into fractions a–g as indicated. (Lower Panel) Analytical IEF gel of fractions a–g with binding of [^{125}I]PnC.

was suspected that this protein was not immunoglobulin.

We have taken several approaches to determine whether some or all of the bands of protein with PnC-binding capacity are immunoglobulin. The first and most direct was to purify the PnC-binding proteins on a PC-immunoadsorbent column. Normal Wistar serum (150 mL) was passed over a 1-mL PC-Sepharose immunoadsorbent. The Farr assay disclosed no [^{125}I]PnC-binding activity in the fall-through fraction. However, when this material was fractionated by isoelectric focusing and exposed to [^{125}I]PnC, it was seen to contain the single prominent PnC-binding band at pH 7.1–7.3. Since that protein did not bind to PC-Sepharose, it must have low affinity for PC. In addition, this protein eluted from Sephadex G-200 with albumin and is therefore clearly not immunoglobulin. About 6 mg of the protein which bound to the PC-Sepharose was eluted with 10^{-2} M PC and isoelectric focused. It can be seen in Figure 4 that the PC-eluted material contained both neutral and alkaline populations of PnC-binding activity as detected by [^{125}I]PnC binding (Figure 4A), and that this material constituted the major protein components in this region (Figure 4C). To determine which of these proteins was immunoglobulin the technique of crossed immunoelectrophoresis was used. A strip of the isoelectric focused gel containing the focused bands of the 10^{-2} M PC eluate was overlaid on a plate of 1% agarose containing goat anti-rat 7S Ig antisera and the focused bands were electrophoresed into the agarose gel. It can be seen (Figure 4B) that all bands formed precipitates with the goat anti-rat 7S Ig antibodies; if normal goat serum is incorporated into the agarose gel, no precipitates are formed. Thus, all of the major bands of protein contain immunoglobulin. However, since this technique is not quantitative we cannot determine what fraction of a band is immunoglobulin.

Therefore, about 8.7 mg of purified anti-PC immunoglobulins from 17 mL of PC-KLH immune Wistar sera was separated by preparative isoelectric focusing (Figure 5). The upper part of Figure 5 shows the protein distribution and the insert shows the PC-binding activity of the major protein peaks as measured by analytical IEF. After radiolabeling protein in fractions a–e with ^{125}I, molecular weights of these proteins were estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis before and after reduction. In each instance the major protein was indistinguishable from immunoglobulin. Thus, more than 80% of each fraction comigrated with BGG on 10% polyacrylamide gels and, after reduction with mercaptoethanol, these molecules dissociated into heavy and light chains.

In the present studies a particularly important feature was the distinction between 7S anti-PC immunoglobulin and C reactive protein. Both molecules are about the same size (Gotschlich and Edelman, 1967), bind PC (Volanakis and Kaplan, 1971), and fix complement (Osmand et al., 1975; V. Braciale, unpublished results). In addition, immunization with heat-killed bacteria or antigen in CFA could provide the inflammatory stimulus for the increase in C reactive protein. Immunization of rats with another heat-killed bacterial vaccine (group A streptococci), however, did not cause an increase in the PC binding capacity (data not shown). More directly, as shown here the purified PC-binding molecules reacted with goat anti-rat immunoglobulin and dissociated into heavy and light chains; C reactive protein is composed of 5 or 6 subunits each of about 22 000 mol wt (Gotschlich and Edelman, 1967). Thus, it is clear that most of the focused bands are immunoglobulin.

Discussion

It is clear that sera from normal rats of several strains possess PnC-binding activity, most, but not all of which seems to be due to PC-specific immunoglobulin. Normal or natural antibody to PC has also been found in mice (Lieberman et al.,

1974) and guinea pigs (Schroer and Davie, 1977) and probably represents a response to environmental antigens, since germ-free rats and mice lack these antibodies. In all three species, the characteristics of the antibodies in normal sera were indistinguishable from those in immune sera. The preliminary characterization of rat anti-PC antibodies reported in the present study indicates that some of the IgG antibodies in both normal and immune sera are strikingly similar in their isoelectric focus profile and most likely are derived from a small number of clones of antibody secreting cells. Immunization with either pneumococcal vaccine or PC-KLH results in an increase of the 7S anti-PC antibody concentration without a clear change in the heterogeneity of the antibody when compared to that found in normal sera. This is consistent with a previous study from this laboratory which showed no significant individual variation or time-dependent changes in antibody affinity or specificity of IgM anti-PC in rats (Claflin and Davie, 1974b). The limited heterogeneity of rat anti-PC antibodies is consistent with studies of anti-PC antibodies raised in other rodents. A more detailed study of murine and guinea pig anti-PC antibodies by a combination of functional, antigenic, and structural means, including limited amino acid sequences of mouse antibodies, has supported the limited heterogeneity of these populations as well. In the mouse, it appears likely that at least four distinct clones of anti-PC antibody secreting cells exist and that most mouse strains possess all four clones, but to varying degrees (Claflin, 1976). In the guinea pig, it is clear that even among outbred animals, significant structural similarity exists among most guinea pig anti-PC immunoglobulin since most anti-PC antibodies from both outbred and inbred animals react identically with antibodies raised in rabbits to the variable regions of highly restricted IgG_2 antibodies purified from a single outbred animal (Schroer and Davie, 1977). The antigenic similarity in these antibodies was supported by isoelectric focus and functional studies.

The rationale for studying antibodies of limited heterogeneity is the opportunity which they offer to probe mechanisms of antibody diversity. If all members of a set of genetically identical animals produce identical antibodies to an antigenic stimulus, it is likely that those antibodies arise from genes which are not modified from those in the germ line. On the other hand, if no two animals in this set produce identical antibodies, one of several possible explanations is that mutation has occurred among a small number of inherited immunoglobulin genes during the differentiation of each animal to generate a larger number of noninheritable genes which in turn produce families of similar but different antibodies.

There have been a variety of approaches used to distinguish between somatic and germ-line theories of antibody diversification including structural studies of immunoglobulin molecules (Hood and Prahl, 1971; Capra and Kehoe, 1975; Kindt, 1975), studies of the inheritance of variable region antigenic determinants (Williamson, 1976), and attempts to enumerate immunoglobulin genes (Tonegawa, 1976; Leder et al., 1974). A relatively direct approach taken by several groups has been to search among induced antibodies for structural or antigenic similarities to homogeneous "proband" antibodies, either myeloma proteins or homogeneous antibodies. The idea here is that if the proband molecule is the product of a germ-line gene, all animals may express an antibody identical with the proband; if the proband is derived from a somatically modified gene, only a rare animal will express it. In fact, it appears both results have been found. Immunization of A/J mice with *p*-azophenyl arsonate (Ar)–KLH induces some anti-Ar antibodies that appear in the sera of all immune A/J mice (Keuttner et al., 1972) which Nisonoff has termed public idiotypes, and others which are detectable only in an occasional animal (private idiotype) (Ju et al., 1977). Similarly, BALB/c anti-PC antibodies seem to be composed of uniformly shared idiotypes (i.e., T-15) (Claflin and Rudikoff, 1977) and rare idiotypes (Gearhart et al., 1977). Tentatively these results have been interpreted as being consistent with expression of both germ-line genes and their somatic variants. The crucial issue in this type of experiment is whether the failure to detect an idiotypic determinant is because of insensitive assays or because of the absence of the structural gene. Along these lines, studies from this laboratory of murine anti-$\alpha(1\rightarrow3)$-dextran antibodies have shown a considerable range in concentration and expression of different idiotypic determinants (Hansburg et al., 1977) so that the generalization that common determinants may reflect germ-line genes and that rare determinants may reflect somatically modified genes could be overly simplified. A second problem with studies that rely solely on idiotypic determinants is the uncertainty of specificity of idiotypic antisera (see, for example, Perlmutter and Davie, 1977) and the likelihood that both framework and hypervariable regions may contribute to such determinants (Michaelsen et al., 1977). Therefore, structural studies of idiotypically uniform antibodies are required.

A different approach to compare antibodies from different individuals for structural relatedness is isoelectric focusing. Obviously structurally identical molecules should cofocus; what is not clear is the discriminatory capacity of the technique for small changes in immunoglobulin structure. Perlmutter (unpublished observations) has demonstrated that light chains which differ by three amino acids, only one of which is charged, can be distinguished by IEF, but whether intact IgG molecules differing by a single charge can be distinguished has not been tested. Nonetheless, the results of the present study, which show extensive sharing of IEF bands among neutral anti-PC antibodies, are consistent with the existence of a germ-line gene(s) in rats for anti-PC antibody. It is not possible to say whether or not somatic modification of this gene also takes place.

While the study of antibodies with limited heterogeneity continues to be an important model to explore mechanisms of antibody diversification, the possibility that evolutionary relationships between species may be approached through antibodies with similar specificity is only beginning to be examined. If, in fact, the shared, neutral bands in rat anti-PC sera, the TEPC-15 group of mouse myeloma proteins, and possibly the induced anti-PC antibodies in guinea pigs are all products of germ line genes, it should be possible to determine whether these rodent anti-PC germ-line genes share a common origin through structural analysis of the proteins produced. Along these lines, Riesen et al. (1976) have recently shown that a human PC-binding macroglobulin differs by only 4 out of 36 amino acids in the amino-terminal sequence of the heavy chains when compared to 5 mouse IgA myeloma proteins with similar binding specificity. This remarkable similarity between human and mouse proteins may either reflect strong structural constraints upon any immunoglobulin in order to maintain PC specificity or it may reflect a common evolutionary origin.

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Inhibition of Deoxyribonucleic Acid Chain Initiation: A New Mode of Action for 1-β-D-Arabinofuranosylcytosine in Human Lymphoblasts†

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ABSTRACT: A novel biochemical effect of 1-β-D-arabinofuranosylcytosine (*ara*-C) on DNA replication in cultured human lymphoblasts is described. By incubating the cells with [^{3}H]thymidine for 5 min and analyzing the nascent DNA by velocity sedimentation in alkaline sucrose gradients, it was possible to discern the initial effect of a very low concentration of drug (5 nM) on DNA replication. During the first 30 min of incubation, *ara*-C inhibited the initiation of new replicating units of DNA but did not affect the elongation of previously initiated units. A later effect was the reduction of the rate of DNA chain elongation. A model, based on the incorporation of *ara*-C into nascent DNA, is presented to account for a differential effect of the drug on DNA chain initiation and elongation in mammalian cells.

1-β-D-Arabinofuranosylcytosine (*ara*-C; generic name, cytarabine)[1] is a potent inhibitor of DNA replication in a variety of cell types, bacterial (Cohen, 1966) as well as mammalian (Roy-Burman, 1970), and in DNA viruses (Ch'ien et al., 1973). In clinical trials the drug has been quite effective in the treatment of acute granulocytic leukemia (Gee et al., 1969; Fleming et al., 1974). Although *ara*-C has been actively studied for over a decade, its mode of action within the cell nucleus remains controversial. Two alternative hypotheses for its inhibitory effect on DNA synthesis rest with the replicative DNA polymerase. In the first model the 5′-triphosphate of

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[1] Abbreviations used: *ara*-C, 1-β-D-arabinofuranosylcytosine; *ara*-CTP, the 5′-triphosphate of *ara*-C; SSC, 0.15 M NaCl–15 mM sodium citrate (pH 7.4); BrdUrd, bromodeoxyuridine; FdUrd, fluorodeoxyuridine.