Three IgA Myeloma Immunoglobulins from the BALB/c Mouse: Precipitation with Pneumococcal C Polysaccharide

Abstract. *Three of 64 IgA immunoglobulins, derived from plasma cell tumors induced by mineral oil in BALB/c mice, precipitated with species-specific pneumococcus C polysaccharide. A related antigen was also found in group O and some group H streptococci. A difference in ability to precipitate a C polysaccharide from a pneumococcus type XIV was demonstrated between protein 603 which did precipitate and protein 167 which did not precipitate this polysaccharide. Studies of the 167 and 603 proteins showed differences in electrophoretic mobility and polypeptide chains. The antigen-combining site of the 167 and 603 proteins resided on the papain-digestion Fab fragment.*

We have found 11 of 64 immunoglobulin A (IgA) (*1*, *2*) and one of three IgM (*3*) myeloma proteins of mice that specifically precipitate with chemically defined antigens and haptens. These proteins extend the list of myeloma proteins in man (*4*, *5*) and mouse (*6*) that combine with specific antigens. In our collection we have three myeloma proteins that react with dinitrophenyl (DNP) and two that react with trinitrophenyl (TNP) ligands (*1*); three that react with different salmonella lipopolysaccharides (*2*) and one that reacts with dextrans (*3*). Three IgA myeloma proteins that precipitate with pneumococcal polysaccharides are described in this report.

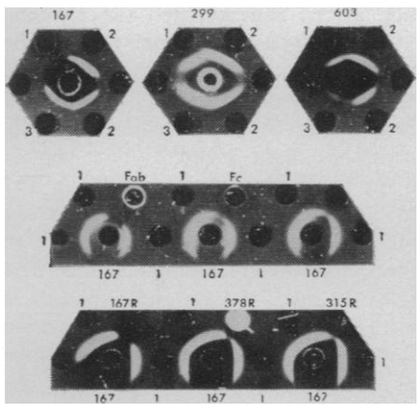

Fig. 1. Ouchterlony studies of IgA myeloma proteins. (Top) Reaction of 167, 299, and 603 proteins with pneumococcus type XIV preparation (wells marked 1), PnC (wells marked 2), and streptococcus group O extract (wells marked 3). These studies were done with serums from mice bearing the tumors. Similar reactions were obtained with 2 percent solutions of the isolated myeloma proteins. (Middle) Inhibition of precipitation by Fab fragment. Wells marked 1 contain PnC. The precipitin lines fail to join in the region near the Fab, but control and Fc reactions join. (Bottom) Inhibition of precipitation of 167 by 167R reduced, *S*-carboxymethylated (R) subunit. Wells marked 1 contain PnC. Two other reduced IgA proteins 378R (reacts with TNP) (*1*) and 315R (reacts with DNP) (*1*) do not inhibit precipitation.

The myeloma proteins were produced by plasma cell tumors induced by mineral oil in the inbred BALB/c strain of mice (*7*) or backcross BALB/c-2 mice (*8*). We tested 64 IgA myeloma proteins in our collection for precipitation with species- or group-specific pneumococcus C polysaccharide (PnC) (*9*). Three IgA proteins—167, 299, and 603 (*10*)—precipitated with PnC (Fig. 1, top).

Streptococcal polysaccharides A, C, and C variant and 32 acid extracts from different groups and strains of streptococci were also tested (*11*). All three proteins precipitated only with extracts of two group O strains (B361, B357) and one group H strain, K208 (*11*) (Fig. 1, top).

The 167, 299, and 603 proteins did not precipitate with any of 40 lipopolysaccharides from salmonella and *Escherichia coli* (*12*), with DNP-, TNP-, or lactose-substituted proteins (*1*), or with a series of dextrans, levans (*13*), mannans (*14*), teichoic acids (*15*), oyster glycogen, or hemicelluloses (*16*), or with human blood group A and B substances and type III specific pneumococcus capsular polysaccharide (*17*).

Many type-specific pneumococcal polysaccharide fractions contain the group C polysaccharide as a contaminant. Pneumococcal polysaccharide preparations from types I, II, VI, and X (*17*) were precipitated by the three myeloma proteins. A type XIV preparation was precipitated by 603, but not by 167 (Fig. 1, top). Chromatography of these pneumococcus polysaccharides on diethylaminoethyl-(DEAE) cellulose separates the polysaccharides into two fractions: an orcinol-positive fraction that contains the type-specific capsular polysaccharide and an orcinol-negative fraction containing the C polysaccharide. We separated preparations of types I, II, VI, X, and XIV on DEAE-cellulose, and none of the type-specific fractions was precipitated. The orcinol-negative polysaccharide fraction obtained from the type XIV preparation precipitated only with the 603 protein (Table 1).

Pneumococcal polysaccharide C has been fractionated into two polymeric components, a mucopeptide polymer and a polysaccharide that contains a high content of *N*-acetylgalactosamine phosphate; only the latter was precipitated by the myeloma proteins (*9*).

To demonstrate that the precipitation of PnC by the myeloma immunoglobulins was an antigen-antibody interaction, it was necessary to show that the Fab portions of these myeloma proteins contained the antigen-combining site. The 167 and 603 proteins were isolated from diluted serum or ascites fluid by addition of ammonium sulfate to 40 to 50 percent saturation at *p*H 7.4 at 5°C; the precipitate dissolved in the minimum volume of water was reprecipitated by prolonged dialysis against water. The 167 and 603 proteins were split by papain into Fab and Fc fragments; the fragments were separated on A-50 DEAE-Sephadex (*1*). The Fab fragments of 167 or 603 did not

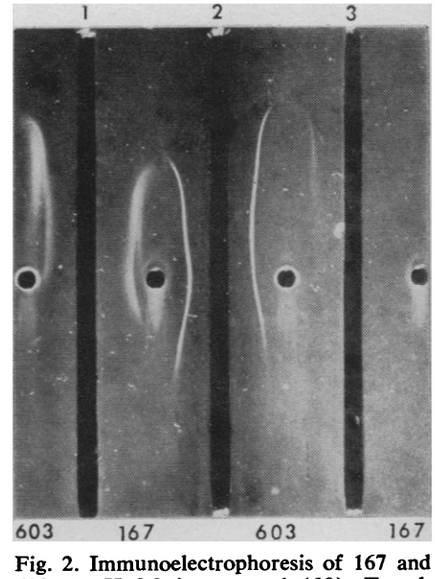

Fig. 2. Immunoelectrophoresis of 167 and 603 at *p*H 8.2 in agar gel (*19*). Trench marked 1 contains type VI pneumococcus polysaccharides, including capsular and PnC. The trench marked 2 contains a specific rabbit antiserum to mouse IgA, and the trench marked 3 contains the pneumococcus XIV polysaccharides. Note the difference in distribution of the IgA + arcs in 167 and 603; the cathodic portions react more strongly with PnC. The cathodic migrating proteins are the higher polymeric forms and therefore most likely to precipitate.

Table 1. Precipitin reactions of myeloma proteins with pneumococcal polysaccharides and streptococcal extracts. Results indicate precipitation (+) or no precipitation (−) with the fractions indicated.

Myeloma protein	I, II, VI, X		XIV		PnC (9)		Streptococcal extracts	
	Orcinol pos.	Neg.	Orcinol pos.	Neg.	Muco-peptide	Polysac-charide	Group O (B361, B357)	Group H (K208)
167	−	+	−	−	−	+	+	+
299	−	+	−	+	ND	ND	+	+
603	−	+	−	+	−	+	+	+

precipitate with C polysaccharide, but did react with it to form soluble complexes that inhibited precipitation of the native protein with PnC (Fig. 1, center).

The 167 and 603 proteins were reduced with 0.03*M* DTT (dithiothreitol) (*p*H 8.2) for 2 hours at 37°C and alkylated with 0.066*M* iodoacetamide. This procedure converts the mixture of IgA monomers and polymers normally found in serum and ascites to the monomeric subunit which has a sedimentation coefficient of approximately 6.6*S* (*1*, *18*). This subunit does not precipitate PnC but specifically combines with it, as judged by its ability to inhibit precipitation of PnC with the polydisperse serum IgA (Fig. 1, bottom). This result suggests that the monomer forms soluble complexes with PnC, and is monovalent with respect to reaction with PnC. Another mouse IgA myeloma protein has been shown to be monovalent in the monomer form (*1*).

In addition to the differences in ability to precipitate with type XIV, differences in structure between the two proteins were found. Electrophoretically, the 167 protein distributes both anodally and cathodally in agar-gel electrophoresis at *p*H 8.2, while the 603 protein distributes from the origin over a broad cathodal portion of gel; this difference is demonstrated by immunoelectrophoresis with both an antibody, a specific rabbit antiserum to mouse IgA, and the antigen (PnC) to develop the precipitin arcs (Fig. 2).

Structural differences in the light (kappa type) and heavy (alpha type) polypeptide chains of 167 and 603 were demonstrated by tryptic peptide maps. Complete reduction of 167 and 603 proteins was carried out in 7*M* guanidine and 0.01*M* DTT at *p*H 8.2 for 1 hour at 37°C; the reduction was followed by alkylation at 5°C with 0.022*M* iodoacetamide (recrystallized six times). The chains were separated on a G-100 Sephadex column (100 by 2.54 cm) equilibrated with 6*M* urea, 0.01*M* acetic acid, and 0.0037*M* β-mercaptoethanol. Tryptic peptide maps of each chain and composite maps containing mixtures of each chain type of the proteins were then compared. (Fig. 3). The map of each heavy chain contained approximately 50 peptides. Only one peptide clearly differentiated the 167 and 603 heavy chains. Analyses of the amino acid sequences will be required to demonstrate further differences between these two heavy chains. By contrast, the light chain tryptic peptide maps were clearly different. The 167 map contained at least 23 peptides, the 603 map contained 26 peptides, and the composite map contained 29 peptides. In the 167 light chain map there were four distinguishing peptides not found in 603, and in the 603 map there were six distinguishing peptides not found in 167.

The IgA myeloma proteins 167 and 603 reacting with PnC polysaccharide differ from each other in primary structure. These differences attain particular immunological significance if they can be shown to be related to the combining site in the Fab portion of the molecule. Indeed, the most striking differences between 167 and 603 were found in the distinguishing kappa light chain peptides that are part of the Fab fragment. Thus the possibility exists that in 167 and 603 quite different primary sequences are arranged to form sites reactive with a common antigen (PnC polysaccharide). The two proteins may recognize different chemical structures in PnC or they

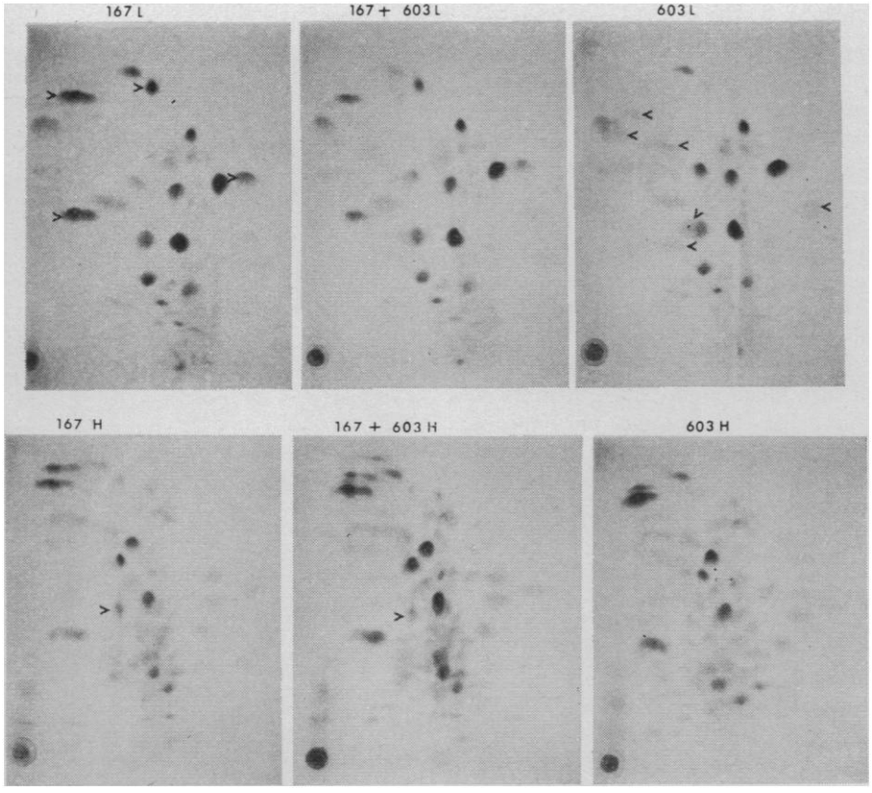

Fig. 3. Tryptic peptide maps of kappa light (L) and alpha heavy (H) chains of 167 and 603 and appropriate composite maps (*19*). Descending chromatography was done first (left to right) in butanol, acetic acid, and water; the chromatographed products were subjected to electrophoresis at *p*H 3.6. Cathode is at the top of each map. Two milligrams of light chain were used in individual maps, and the composite map contained 1.5 mg of each. Four milligrams were used for each individual heavy chain map, and 3 mg of each for the composite; arrows indicate distinguishing peptides in the individual maps.

may recognize different multiples of the same chemical determinant; either of these possibilities is supported by the fact that an antigen in a preparation of pneumococcal type XIV polysaccharide is precipitated by 603 but not 167 (see above).

Finally, it is of considerable biologic interest that three proteins in our series of 64 and one in Cohn's series of 53 IgA myelomas (*6*) identify the same antigen. In addition, a relatively high and unexpected frequency of tumors producing myeloma proteins that react with DNP has also been found (*1*). The method of production of tumors used in these studies may selectively stimulate proliferation of clones of cells producing a restricted range of antibodies.

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References and Notes

1. H. N. Eisen, E. S. Simms, M. Potter, in preparation.
2. M. A. Leon and M. Potter, unpublished observations.
3. M. A. Leon, K. R. McIntire, M. Potter, in preparation.
4. H. N. Eisen, J. R. Little, C. K. Osterland, E. S. Simms, *Cold Spring Harbor Symp. Quant. Biol.* **32**, 75 (1967).
5. H. Metzger, *Proc. Nat. Acad. Sci. U.S.* **57**, 1490 (1967).
6. M. Cohn, *Cold Spring Harbor Symp. Quant. Biol.* **32**, 211 (1967).
7. M. Potter and C. R. Boyce, *Nature* **193**, 1086 (1962).
8. M. Potter and R. Lieberman, *Cold Spring Harbor Symp. Quant. Biol.* **32**, 187 (1967).
9. E. C. Gotschlich and T.-Y. Liu, *J. Biol. Chem.* **242**, 463 (1967). We thank Dr. Gotschlich for this C polysaccharide and for testing the precipitability with the mucopeptide and polysaccharide fractions.
10. Tumor MOPC 167 was induced in a BALB/c mouse given three injections of 0.5 ml of Bayol F intraperitoneally when the mouse was 2, 4, and 6 months of age. This mouse was immunized with ovalbumin at the same time. Tumor Mc 603 was induced by Dr. K. R. McIntire in an ex-germfree, nonimmunized BALB/c mouse by the same method as above. Tumor MOPC 299 was similarly induced in a BALB/c mouse. This mouse was immunized concomitantly with red blood cells from sheep, pig, horse, cow, goat, and mule. Further characterization of 299 was omitted because this transplant line began producing little or no myeloma protein.
11. Streptococcal culture extracts and polysaccharides prepared by Dr. R. Lancefield, Rockefeller University.
12. *Salmonella* and *E. coli* lipopolysaccharides given by Dr. O. Lüderitz, Max-Plank-Institut.
13. Dextrans and levans given by Dr. A. Jeanes, Northern Regional Research Laboratory, USDA, Peoria, Illinois.
14. Mannans were given by Dr. M. Slodki, Northern Regional Research Laboratory, USDA, Peoria, Illinois.
15. Teichoic acids were provided by Dr. D. Tipper, Department of Pharmacology, University of Wisconsin.
16. Hemicelluloses were provided by Dr. R. L. Whistler, Purdue University.
17. Type-specific pneumococcal polysaccharides were supplied by Dr. M. Heidelberger, New York University, and Dr. R. Brown, New York State Department of Health Laboratories.
18. J. I. Fahey, *J. Exp. Med.* **114**, 399 (1961); M. Potter and E. L. Kuff, *J. Mol. Biol.* **9**, 537 (1964).
19. M. Potter, in *Meth. Cancer Res.* **2**, 105 (1967).
20. We thank Miss R. Lieberman for discussions and suggestions in the preparation of the manuscript.

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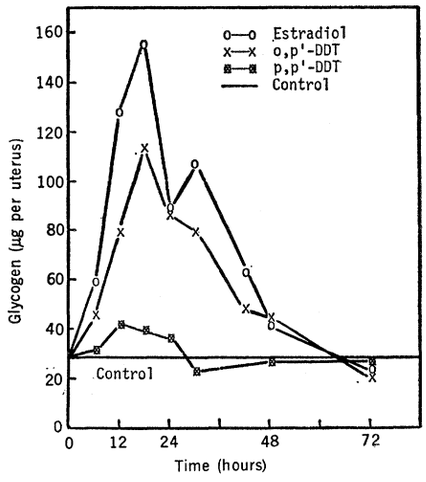

Fig. 1. Glycogenic response of immature rat uterus to DDT isomers and 17β-estradiol. Four milligrams of *o,p'*-DDT or *p,p'*-DDT, or 0.4 μg of 17β-estradiol, was injected subcutaneously at 0 time. Each point consists of determinations of 4 to 14 uteri of 22- to 25-day-old rats; the control level was established by use of 70 rat uteri. Glycogen was determined by the anthrone procedure (*12*).

Estrogenic Activity of o,p'-DDT in the Mammalian Uterus and Avian Oviduct

Abstract. *When rats and birds were treated with* o,p'*-DDT, their reproductive tissues exhibited the same response as when they were treated with estrogen. Weight, water content, glycogen, and RNA increased in uteri and oviducts of rats, chickens, and quail receiving* o,p'*-DDT;* p,p'*-DDT produced little if any response. The* o,p'*-DDT did not accumulate in the reproductive or adipose tissues to a greater extent than* p,p'*-DDT.*

The similarity in the configuration of DDT (*1*) to the synthetic estrogen, diethylstilbestrol, has prompted investigation of the estrogenic activity of DDT in mammals and birds (*2*). These studies have yielded conflicting results and both positive (*2*) and negative (*3*) evidence of estrogenicity was obtained. Recently, Levin, Welch, and Conney (*4*) reported that *o,p'*-DDT is estrogenic, while *p,p'*-DDT is only weakly active. Since commercial technical DDT contains approximately 80 percent *p,p'*-DDT and 15 to 20 percent *o,p'*-DDT, this finding offers a possible explanation for inconsistent reports in the literature.

We investigated the estrogenic activity of *o,p'*-DDT and *p,p'*-DDT, comparing the time course of effects of these DDT isomers with 17β-estradiol on several biochemical constituents of the immature rat uterus. Glycogen, an extremely sensitive indicator of estrogen action (*5*), increased after *o,p'*-DDT administration in a manner very similar to 17β-estradiol (Fig. 1), while *p,p'*-DDT exhibited only slight activity. The *o,p'*-DDT stimulated characteristic estrogenic responses in the uterus—increases in wet weight, water content, and RNA content 24 hours after administration being similar to those elicited by estradiol.

Woodwell (*6*) and Wurster and Wingate (*7*) have determined DDT residues in a number of species of bird and have implicated contamination by insecticides as a probable major cause of the decline in reproduction in several species. In view of the estrogenicity of *o,p'*-DDT in the uterus of a mammalian species, it was of interest to determine whether sublethal concentrations of this isomer of DDT would have an effect upon the oviduct, the reproductive tract of avian species. Five-week-old chickens (Cornish, weighing 700 to 900 g) and 25-day-old Japanese quail (Coturnix quail, weighing 70 to 90 g) received three daily intraperitoneal injections of *o,p'*-DDT, *p,p'*-DDT, or 17β-estradiol in olive oil. Twenty-four hours after the last injection (72 hours after the initial injection), the birds were killed and oviducts were removed for analysis. The results (Table 1) demonstrate that *o,p'*-DDT produced the same effects as estradiol in the oviducts of chickens and quail. A 100 percent increase in oviduct weight and 150 to 175 percent increases in glycogen content occurred. Little if any estrogenic activity was shown by *p,p'*-DDT.

In order to determine whether *o,p'*-