CHAIN MODELS OF 6.6S AND 3.9S MOUSE MYELOMA γA IMMUNOGLOBULIN MOLECULES

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The distinctive chemical and antigenic properties of γG, γM, and γA immunoglobulins reside in their heavy chains;[1-3] light chains of either the kappa or the lambda type are both found in association with each type of heavy chain and do not contribute to any of the distinctive class properties.[4, 5] Since most chemical studies have been carried out on proteins of the γG class, more is known of the amino acid sequence and multichain structure of γG immunoglobulin than of the others. A four-chain model of this protein was first proposed by Porter[6] in 1962: a pair of heavy chains are held together by one or more disulfide bonds, and each heavy chain is bonded through a single disulfide bridge to a light chain. Treatment with papain in the presence of cysteine splits the γG molecule into two F_{ab} fragments and an F_c fragment.[7] Each F_{ab} fragment contains an antibody site and is composed of a light chain and the amino-terminal half of a heavy chain, and the F_c fragment is composed of the carboxyl-terminal halves of the two heavy chains held together by one or more disulfide bonds.[8-11] Two intrachain disulfide bridges are present in the light chain and in each half of the heavy chain.[12, 13]

γA immunoglobulin consists of several components of sedimentation constant 6.6*S* and higher, the larger components being polymers of the smallest.[14] The smallest component, which has the same sedimentation constant as γG immunoglobulin, is a four-chain molecular species.[3] A variant of γA immunoglobulin that has a sedimentation constant of 3.9*S* and which is believed to be a two-chain species has been found in a limited number of mouse myelomas.[15] The 6.6*S* γA proteins yield an F_c fragment upon treatment with papain; 3.9*S* γA proteins do not.[16] We have been investigating the structure of two mouse myeloma γA immunoglobulins, one of the 6.6*S* type (Adj. PC-6A) and one of the 3.9*S* type (MOPC 47A).

Materials and Methods.—The immunoglobulins secreted by mouse myeloma tumors Adj. PC-6A and MOPC 47A will be referred to as 6A protein and 47A protein, respectively. The 6A serum protein was purified by chromatography on DEAE-cellulose by eluting with a sodium chloride gradient of up to 0.4 *M* in 0.05 *M* phosphate buffer (pH 8.0) containing 1 *M* urea. The 47A urinary protein was purified by chromatography on DEAE in 0.05 *M* Tris-acetate buffer (pH 5.5). Electrophoresis was carried out on horizontal acrylamide gels by a method adapted from Sogami and Foster.[17] Chromatography on G-100 Sephadex was done in 0.2 *M* NH_4HCO_3 and monitored by the LKB Recychrom System. Alkylation of partially reduced proteins was accomplished with 0.01 *M* dithiothreitol in 0.55 *M* Tris-HCl buffer, pH 8.2, for 2 hr at room temperature, followed by treatment with iodoacetic acid (0.022 *M*) for 1 hr at 4°C. Total alkylation was accomplished in the same medium by incubation in 7 *M* guanidine-HCl for 2 hr. Alkylation without reduction was done in 0.55 *N* Tris-HCl buffer, pH 8.2, by treatment with 0.02 *M* iodoacetic acid containing 5 μc of ^{14}C-iodoacetic acid for 1.5 hr at 4°. Carboxymethylcysteine (CM-cysteine) was determined by radioactivity and by amino acid analysis. Heavy and light chains were separated by gel filtration on a G-100 Sephadex column equilibrated with 8 *M* urea–1 *M* acetic acid (pH 3.6).

Molecular weight determinations were carried out by the gel filtration method. A column (2.5 × 172 cm) of G-150 Sephadex was equilibrated at room temperature (*ca.* 25°) with 0.2 *M* NH_4HCO_3 and used for the native proteins. A column (2.5 × 105 cm) of G-200 Sephadex was equilibrated at room temperature with 5 *M* guanidine-HCl–0.05 *M* Tris-HCl (pH 7.75) and used for the purified heavy chains. Each sample was run two to three times, and an average value for V_e/V_0 was calculated from the void volume (V_0) and the elution volume (V_e) of each sample. To measure the void volume of the columns, blue Dextran 2000, 2 × 10^6 mol wt, was used. Other markers were: horse heart cytochrome *c*, 12,500 mol wt; bovine pepsin 35,000 mol wt; bovine plasma albumin, 67,000 mol wt; mouse γF immunoglobulin, 150,000 mol wt.

Large fragments of partially reduced and carboxymethylated heavy chain were obtained by treatment with cyanogen bromide.[18] The resulting mixture was analyzed on a G-75 Sephadex column (1.25 × 107 cm) equilibrated with 0.2 *M* sodium formate buffer containing 6 *M* urea. Tryptic digests of heavy chain were separated first on a G-25 Sephadex column (0.9 × 140 cm) equilibrated with 0.05 *M* NH_4OH and then on a Dowex 50-X2 column (0.9 × 28 cm) developed with a pyridine-acetic acid buffer with a gradient of from 0.2 *M* and pH 3.1 to 2 *M* and pH 5.0. Carboxyl-terminal amino acids were determined after treatment of heavy chain with carboxypeptidase, and amino-terminal amino acids were determined by the method of Gray and Hartley.[19] Amino acid compositions were determined with a Spinco model 644B analyzer equipped for accelerated sensitive analysis.[20]

Results.—Physical studies: Gel filtration of the purified 6A serum protein on a G-150 Sephadex column in 0.2 *M* NH_4HCO_3 revealed the presence of components with molecular weights larger than 150,000. Acrylamide gel electrophoresis in 8 *M* urea (pH 3.6) yielded a complex pattern. In addition to a diffuse band near the origin, two faster bands having the mobility of 6A light chain were present. Gel filtration of the native protein on G-100 Sephadex column equilibrated with 8 *M* urea–1 *M* acetic acid separated three fractions, heavy chain, dimer of light chain, and free light chain. These results indicate that most of the light chains in this mouse γA protein are not covalently bound to heavy chains. Similar results have been obtained by Abel and Grey[21] with several mouse γA proteins. Gel filtration of purified 47A urinary protein on G-100 Sephadex column in 0.2 *M* NH_4HCO_3 separated three fractions (Fig. 1). Fraction C is a stable component with a sedimentation constant of 3.9*S*. Untreated fraction C did not separate into light and heavy chains on G-100 Sephadex in 8 *M* urea–1 *M* acetic acid or on acrylamide gel electrophoresis in 8 *M* urea (pH 3.6). Thus, unlike the chains of 6A protein, light and heavy chains of 47A protein appear to be covalently bonded to each other. About 80 per cent of fraction B dissociated to fraction C upon refiltration on a Sephadex G-100 column in 8 *M* urea (pH 3.6). The remaining 20 per cent eluted as a single stable peak larger than fraction C.

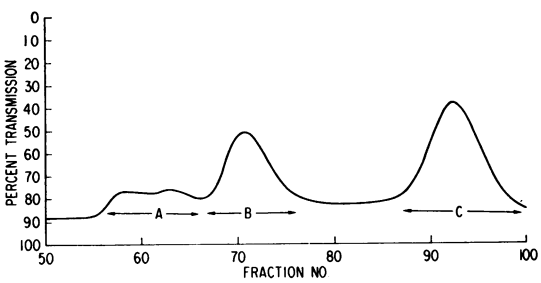

FIG. 1.—G-100 Sephadex gel filtration in 0.2 *M* NH_4HCO_3 of native urinary protein from mouse myeloma MPOC 47A. (*A*) Polymers; (*B*) dimer; (*C*) monomer.

Fraction A has not been examined in detail, but probably consists of aggregates larger than dimers.

Fraction C and the stable fraction B obtained after successive gel filtrations were passed through a G-150 Sephadex column, and these elution patterns were compared with those of several markers (Fig. 2). The markers showed a linear relationship between log molecular weight and the ratio of the elution volume to that of blue Dextran. The calculated molecular weight for fraction C was 61,000 and for fraction B was 140,000, which indicates that fraction B is indeed a dimer of fraction C. Partial reduction and alkylation of fraction C or B and gel filtration on a G-100 Sephadex column equilibrated in 6 *M* urea–1 *M* acetic acid resulted in separation of heavy and light chains (Fig. 3). On acrylamide gel electrophoresis the heavy chain of 47A protein migrated as a diffuse band, and the light chain migrated as two or three sharp bands. The isolated heavy chains of 6A protein and 47A protein were passed through a G-200 Sephadex column equilibrated in 5 *M* guanidine-HCl, and the elution patterns were compared with those of three known markers. A clear difference could be discerned in their elution volumes, which corresponded to molecular weights of 40,000 and 55,000, respectively. Studies in progress on the structure of 47A light chain have shown that it is the same size as previously studied mouse myeloma light chains. With a molecular weight of 23,000 for the light chain, the molecular weight of the two-chain 47A protein is 63,000 and of the four-chain 6A protein is 156,000. The carbohydrate content of 47A protein is 4.3 per cent. Recent determinations of the carbohydrate content of human myeloma γA proteins have averaged 5 per cent,[22, 23] and this value will be assumed for the 6A protein. Since the carbohydrate component of an immunoglobulin is attached to its heavy chain,[1] these values result in net molecular weights of 37,000 and 51,000, respectively, for the polypeptide components of the heavy chain of 47A and 6A proteins.

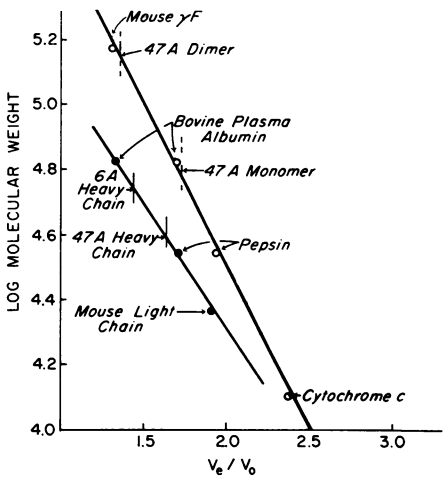

FIG. 2.—Molecular weights by gel filtration. V_e/V_0 is the ratio of elution volume (V_e) of each protein to void volume (V_0) of the column used. The proteins filtered through G-150 Sephadex were native. The proteins filtered through G-200 Sephadex had been completely reduced and carboxymethylated. O, G-150 Sephadex, 0.2 *M* NH_4HCO_3; ●, G-200 Sephadex, 5.0 *M* guanidine-HCl.

Chemical studies: Significant differences were found in the CM-cysteine content of alkylated 6A and 47A heavy chains. Half-cystine residues that were alkylated after partial reduction will be referred to as being labile. Half-cystine residues that are resistant to partial reduction and are alkylated only after total reduction in guanidine hydrochloride will be referred to as being stable. The results are shown in Table 1. The stable half-cystine content cannot be determined directly and is the difference between the CM-cysteine contents of com-

TABLE 1. *Half-cystine contents of 6A and 47A heavy chains.*

	6A heavy chain	6A F_c fragment	47A heavy chain
Total half cystine	15	9	11
Labile half cystine	5	2–3	3
Stable half cystine	10	6–7	8

pletely and partially alkylated chains. Stable and labile half cystines apparently occur in intrachain and interchain disulfide bonds, respectively,[11] and the content of labile residues includes any unoxidized cysteine residues.

Neither the 6A nor the 47A heavy chain revealed a free α-amino group. When a subtilopeptidase digest of totally reduced and alkylated 47A heavy chain was applied to a Dowex 50-X2 (H+ form) column, all peptides were absorbed except for an acidic peptide having the composition Asp (1.1), Ser (1.0), Glu (1.0), Gly (1.2), Val (1.1), Leu (1.0). The peptide was ninhydrin-negative, and its electrophoretic mobility at pH 6.5 indicated a single negative charge at this pH. Presence of glutamic acid in the peptide suggests that absence of a free α-amino group could be due to an amino-terminal pyrrolidonecarboxylic acid residue.

Carboxyl-terminal analysis yielded 0.8 residue of tyrosine per mole of 6A heavy chain and 0.8 residue of glutamine per mole of 47A heavy chain. A tryptic digest of partially alkylated 6A heavy chain was fractionated, and the only peptide with a carboxyl-terminal tyrosine also contained CM-cysteine. On the other hand, the only cyanogen bromide fragment of partially alkylated 47A heavy chain with a carboxyl-terminal glutamine did not contain any cystine or CM-cysteine. This peptide contains 30–35 residues; therefore 47A heavy chain has no half cystine or cysteine within this number of residues of its carboxyl terminus.

In order to test for the presence of free sulfhydryls in 47A protein, fraction C was alkylated without previous reduction. About 0.7 mole of CM-cysteine per heavy chain was found after this treatment. No CM-cysteine was found in the stable dimer of 47A protein after the same treatment. When fraction C was oxidized with performic acid, 0.2–0.3 mole of cysteic acid per heavy chain was released.

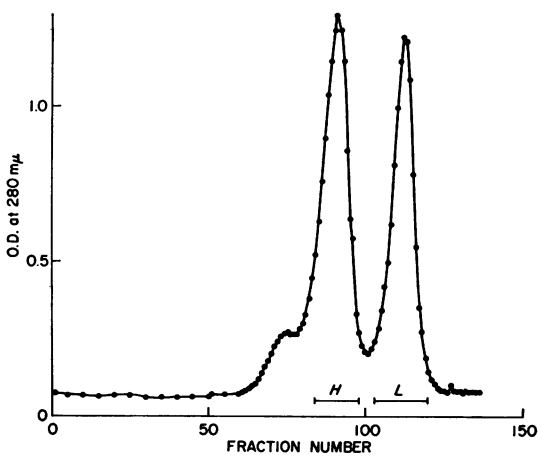

FIG. 3.—G-100 Sephadex gel filtration in 8 *M* urea–1 *M* acetic acid of partially reduced and carboxymethylated monomer of 47A protein (fraction C of Fig. 1).

Discussion.—The 6.6*S* 6A molecule is composed of two light and two heavy chains, the 3.9*S* 47A molecule of one light and one heavy chain. In 6A protein the light chain is able to dimerize but does not form a disulfide bond with a heavy chain;[21] the heavy chain forms stable dimers. In contrast, the light and the heavy chain of 47A protein are cova-

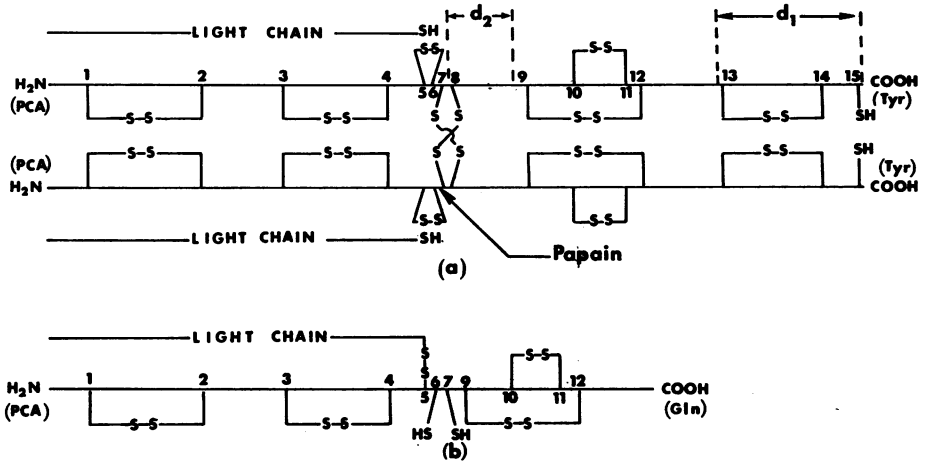

FIG. 4.—Chain models of monomers of (*a*) Adj. PC-6A protein (6.6*S*) and (*b*) MOPC 47A protein (3.9*S*) in unoxidized state. Oxidation of the sulfhydryls of 6A protein may dimerize the light chains[21] and polymerize the protein.[26] Oxidation of 47A protein may stabilize the monomer or produce a stable dimer as shown in Fig. 5.

lently bonded to each other, but stable bonds between heavy chains appear to form slowly and incompletely. The 6*A* and 47A heavy chains differ in molecular weight, content of stable and labile half cystines, and amino acid sequence of carboxyl-terminal region. From these results we have derived chain models of two-chain and four-chain γA immunoglobulins.

Structural and functional similarity of the immunoglobulins suggests a common ancestry for the various classes;[24, 25] a greater structural similarity between the heavy chains of two-chain and four-chain γA immunoglobulins as indicated by their antigenic similarity suggests a more recent divergence in molecular evolution. Therefore, in constructing models of 6A and 47A proteins, we shall postulate maximum homology of 6A protein with γG immunoglobulin on the one hand, and evolution of 47A protein by deletions from 6A protein on the other. The fact that the shortened 47A heavy chain retains γA antigenic specificities argues against nu-

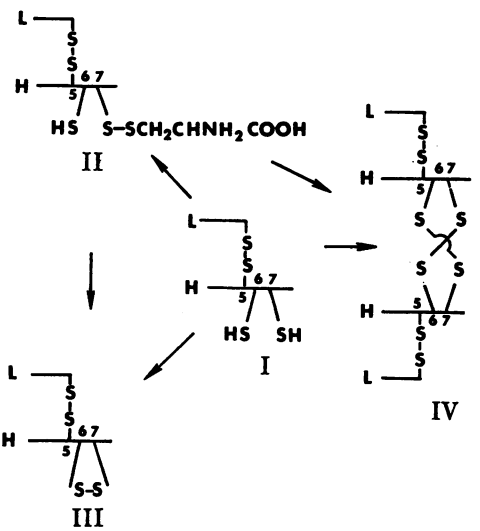

FIG. 5.—Alternative pathways for oxidation of the monomer of 47A protein. Fraction C of Fig. 1 has the properties of a mixture of I, II, and III. Fraction B contains a stable dimer, represented as IV, and a noncovalently bonded dimer. Changes in conformation that result from these reactions as well as from the deletions shown in Fig. 4 may account for the resistance of the central region of 47A heavy chain to papain.[16]

merous small deletions and in favor of a limited number of deletions that would leave unaltered extended segments of the chain. Between 120 and 130 residues must be deleted in order to account for the difference in size of the heavy chains.

The F_c fragment of 6A protein contains nine half-cystine and cysteine residues per chain, of which between two and three are labile. Between six and seven are stable half cystines, so that there are no fewer than three intrachain bridges in the carboxyl-terminal half of 6A heavy chain, in contrast to the two per chain in the F_c fragment of γG immunoglobulin.[13] One of the labile residues is the cysteine near the carboxyl terminus of γA heavy chain,[26] and the remaining two labile half cystines are assumed to take part in interheavy chain bridges. Of the 15 half-cystine and cysteine residues in 6A heavy chain, the ten stable residues are assigned to five intrachain disulfide bridges. Thus, the amino-terminal half of 6A heavy chain contains two intrachain disulfide bonds, the same number as γG heavy chain.[12] Of the five labile residues, one is the cysteine near the carboxyl terminus, and two are the interheavy chain half cystines of the F_c fragment. The other two labile half cystines might also be assumed to be interheavy chain residues, since there is no light-heavy interchain bond. However, the latter assignment of residues, which would require that the half cystine that bonds the γG heavy chain to the light chain is absent in the heavy chain of four-chain γA protein but that it is present in the heavy chain of two-chain γA protein, is contrary to our postulates of homology of the 6A heavy chain with γG heavy chain and of evolution of 47A heavy chain by deletions from 6A heavy chain. We suggest an alternative in which the half cystine of the light-heavy interchain bond is present in 6A heavy chain but is unavailable for bonding to the light chain because it forms a labile intrachain disulfide bond with the last of the labile half cystines.

A schematic model of 6A protein is shown in Figure 4*a*. The numbered half cystines will hereafter be referred to as CyS 1, CyS 2, etc. The lone cysteine residue of 6A heavy chain is CySH 15. Two of the three intrachain disulfide bonds of the F_c region, CyS 9–12 and CyS 13–14, have been placed in positions homologous with those of the heavy chain of γG immunoglobulin, in which CyS 12, 13, and 14 are 126, 80, 22 residue positions, respectively, from the carboxyl terminus.[13, 25] The third intrachain bridge of the F_c region, CyS 10–11, was placed in the vicinity of the amino-terminal disulfide loop of the region for reasons that will become apparent. CyS 5 corresponds to the half cystine that bonds the heavy to the light chain in γG immunoglobulin and in 47A protein, and formation of a labile intrachain disulfide bond, CyS 5–6, is postulated to account for failure of CyS 5 to take part in a light-heavy interchain bond in 6A immunoglobulin.

The 47A protein is a two-chain molecule composed of a normal light and a shortened heavy chain. Three of the 11 half cystines of 47A heavy chain are labile and eight are stable; accordingly, 47A heavy chain probably contains four intrachain disulfide bonds. A deletion of 80–95 residues of the carboxyl terminal end of the chain, indicated as d_1 in Figure 4*a*, would remove the disulfide bridge CyS 13–14 and cysteine CySH 15 and would at the same time leave a half-

cystine free segment of 30–45 residues on the carboxyl side of CyS 12. A second deletion, d_2, would remove CyS 8 and the segment adjacent to it on its carboxyl side but not including CyS 9. The two deletions together would be sufficient to account for the decrease in molecular weight and half-cystine content. Although we have no evidence to favor either the parallel (CyS 7–7, CyS 8–8) or antiparallel (CyS 7–8, CyS 8–7) arrangement of interheavy chain disulfide bonds in the 6A protein model, the antiparallel arrangement was preferred because deletion of only CyS 8 would remove both interchain bonds. CyS 5, CySH 6, and CySH 7 are the three labile half cystines of 47A heavy chain. We propose that the change in conformation that results from these deletions favors the reaction of CyS 5 with the light chain to form a light-heavy interchain bond. A model of 47A protein that incorporates deletions d_1 and d_2 and the consequences of these deletions is shown in Figure 4*b*. Some of the ways in which CySH 6 and CySH 7 might react are shown in Figure 5.

A deletion of 22–50 residues at the carboxyl terminal end of 6A heavy chain would remove CyS 14 and CySH 15 and would leave a segment of 30–58 residues free of half cystine and cysteine carboxyl terminal to CyS 13, which would become an unbonded cysteine residue. However, removal of an additional 60–90 residues (including two labile half cystines) without disruption of the remaining stable disulfide bridges would require deletion of at least two other segments. Such a possibility cannot be ruled out, nor can the possibility that the distribution of disulfide bonds in γA immunoglobulin differs from that in γG immunoglobulin to the extent that a single carboxyl-terminal deletion would suffice to account for our findings.

The 3.9*S* γA protein, unlike 6.6*S* γA protein and other immunoglobulins, is excreted in large amounts into the urine. Dimers of 47A protein in urinary samples were probably formed after excretion, and some of the abnormal γA protein in the plasma of mice with myelomas that secrete the 3.9*S* protein[15] may be molecules that have dimerized in the plasma and are too large to be excreted into the urine. The known differences in structure and properties of 47A protein with respect to γG immunoglobulin are in the central and carboxyl-terminal regions of its heavy chain. If the remainder of the molecule is homologous to the F_{ab} fragment of γG immunoglobulin, 47A monomer is the structural equivalent of an univalent antibody.

The postulate that 47A protein evolved from 6A protein was adopted largely for the purpose of facilitating discussion of the results, since there is no evidence in this case as to the direction of evolution. It has been proposed that genes for the immunoglobulins evolved by successive duplications from a gene for a primordial chain one half the size of light chain and one fourth the size of heavy chain.[24, 25] 47A heavy chain is three times the size of the postulated ancestral chain. The carboxyl-terminal dipeptide sequence, cysteinyl tyrosine, is present in the heavy chains of γM and 6.6*S* γA immunoglobulins.[26, 27] An unequal crossover between a pair of alleles for 6.6*S* γA heavy chain could have resulted in an allele for an ancestral γM heavy chain with a duplicated carboxyl-terminal segment and an allele for a 3.9*S* γA heavy chain with a deleted carboxyl-terminal segment. The amino acid sequence of 3.9*S* γA, 6.6*S* γA, and γM heavy chains

must be determined before we can judge the relevance of our current data to the evolution of an allele.[28]

Summary.—A 6.6*S* type (Adj. PC-6A) and a 3.9*S* type (MOPC 47A) of mouse myeloma γA immunoglobulin were studied. The 6.6*S* type is approximately the same size as γG immunoglobulin but has a different disulfide-bonded structure. The heavy chains of the two mouse γA types differ from each other in molecular weight, disulfide-bonded structure, and carboxyl-terminal primary structure. These results were incorporated into tentative models of Adj. PC-6A and MOPC 47A mouse myeloma γA immunoglobulins.

We thank Dr. Michael Potter for gifts of mouse myeloma tumors Adj. PC-6A and MOPC 47A, and Dr. J. F. Mushinski for a gift of the F_c fragment of Adj. PC-6A protein. We are indebted to Dr. R. Marshall for the carbohydrate determination on MOPC 47A protein.

The nomenclature of immunoglobulins, chains, and fragments is given in a memorandum in *Bull. World Health Org.*, **30**, 447–450 (1964).

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