Rabbit Immunoglobulin A Allotypic Specificities. Localization to Two Papain Fragments, $Fab_{2\alpha}$ and $Fc_{2\alpha}$, of Secretory Immunoglobulin A

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ABSTRACT: Rabbit secretory IgA was digested with papain and three fractions were isolated by gel filtration. These fractions were identified as undigested sIgA, $Fc_{2\alpha}$ and $Fab_{2\alpha}$ by their sedimentation and antigenic properties. The fragments were analyzed for the presence of allotypic specificities unique to IgA by quantitative precipitation of the ^{125}I-labeled fragments with anti-allotype antisera. The IgA allotypic specificities were found on both the $Fc_{2\alpha}$ and the $Fab_{2\alpha}$ fragment indicating that the IgA allotypic specificities reside on the constant portion of the α heavy chain. Thus, genetic markers are now available for the constant as well as the variable region of rabbit α chains, and this facilitates further studies of the complex Ig heavy-chain chromosomal region.

Immunoglobulins are classified as IgG, IgA, IgM, IgD or IgE[1] according to whether they have γ, α, μ, δ, or ϵ heavy polypeptide chains (chains with mol wt 50,000–75,000) (Edelman and Gall, 1969). Immunoglobulins are further subdivided into κ and λ types according to whether they have κ or λ light polypeptide chains (chains with mol wt 23,000). The basic "7S" unit (or subunit) of immunoglobulins is composed of two identical light chains and two identical heavy chains. The IgG, IgA, IgD, and IgE of serum have this four-chain structure (Edelman and Gall, 1969). However, serum IgM is a pentamer composed of five "7S" subunits (Miller and Metzger, 1965) plus a J chain (mol wt approximately 25,000) (Mestecky *et al.*, 1971b). Also, IgA of secretions (colostral, respiratory, intestinal, salivary, etc.), *i.e.*, sIgA, has been characterized as a dimer composed of two "7S" subunits plus a J chain and a secretory component (mol wt approximately 70,000) (Halpern and Koshland, 1970; Tomasi and Bienenstock, 1968). In both the light and heavy chains, the N-terminal amino acids (approximately 100) constitute variable regions, *i.e.*, differing in sequence for antibodies of different specificity. The remaining amino acids of the light chain and of the heavy chain constitute constant regions, *i.e.*, the amino acid sequence is independent of the antibody specificity and is the same for all chains of a given heavy-chain subclass or light-chain type except for genetic variants known as allotypes (Edelman and Gall, 1969).

Allotypes as genetic variants of immunoglobulins have been most extensively investigated for man, rabbit, and mouse (Steinberg, 1969; Kelus and Gell, 1967; Herzenberg *et al.*, 1968). Allotypes of rabbit immunoglobulins can result from amino acid differences in the polypeptide chains of different individuals and are identified by their antigenic properties, *i.e.*, the allotypic specificity (Koshland *et al.*, 1968; Reisfeld *et al.*, 1965; Prahl *et al.*, 1969; Appella *et al.*, 1971). The specific antibodies to identify these allotypic specificities are obtained from rabbits immunized with immunoglobulins of other rabbits. Each anti-allotype antiserum reacts with the immunoglobulins of some but not all rabbits (Dray *et al.*, 1963).

From the study of rabbit immunoglobulin allotypes, at least three independent chromosomal regions controlling the synthesis of immunoglobulins have been identified: one region controls the κ chains; another, the λ chains; and a third, the heavy chains. The *b* locus has four alleles which result in allotypic specificities b4, b5, b6, or b9 present on κ chains[1] (Dray *et al.*, 1963; Dubiski and Muller, 1967). The *c* locus has at least two alleles which result in allotypic specificities c7 or c21 (Gilman-Sachs *et al.*, 1969) on λ chains (Appella *et al.*, 1968). The *a* locus has three alleles which result in allotypic specificities a1, a2, or a3 on the variable part of the heavy chains. The *a*, *b*, and *c* genes assort independently (Dray *et al.*, 1963; Gilman-Sachs *et al.*, 1969).

The *a*, *b*, or *c* locus allotypic specificities may appear in all classes (IgG, IgA, IgM, and IgE) of immunoglobulins (Lichter, 1966; Todd, 1963; Kindt and Todd, 1969). The presence of the *a* locus allotypic specificities on the variable part (Koshland, 1967; Mole *et al.*, 1971) of the γ, α, and μ chains as well as the finding of a variable and a constant region (by amino acid sequence) led to the concept that separate V_H and C_H genes control the variable and constant parts of the amino acid sequence (Lennox and Cohn, 1967). Thus, the V_Ha gene[2] may associate with either the C_γ, C_α, or C_μ gene before directing the synthesis of the γ, α, or μ heavy chains. Recently, allotypic specificities x32 and y33 have been identified on immunoglobulin subgroups lacking *a* locus specificities and these appear to be markers for two additional V_H genes, V_Hx and V_Hy, which are closely linked to the V_Ha gene (Kim and Dray, 1973). Two sets of allotypic specificities, d11 and d12 (Prahl *et al.*, 1969) as well as e14 and e15 (Dubiski, 1969)

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[1] The nomenclature for immunoglobulins is that recommended in *Bull. W. H. O.* (*30*, 447 (1964); *41*, 975 (1969); and in *Biochemistry 11*, 3311 (1972). For allotypes the notation is that of Dray *et al.* (1962) except that the allotypic specificities Aa1, Aa2, Aa3, Ab4, Ab5, Ab6, etc., as well as the corresponding genes designated in italics, are abbreviated by omitting the capital "A".

[2] Since the *a* locus controls allotypic specificities on the variable portion of the heavy chains, the gene is referred to as the V_Ha gene.

have been localized to different positions in the constant part of the γ chain and these are markers for the $C_\gamma de$ gene[3] which is also closely linked to the $V_H a$ gene. Recently, the n81 and n82 allotypic specificities have been found to be unique to the IgM class and closely linked in their inheritance to the *a* locus allotypes suggesting that these specificities are markers for a $C_\mu n$ gene (Gilman-Sachs and Dray, 1973). Also, two sets of allotypic specificities, f71, f72, and f73 as well as f74 and f75 (each set controlled by allelic genes), had been found to be unique to the IgA class of immunoglobulins (Conway *et al.*, 1969a,b). Since f71, f72, and f73 are associated with one subclass of IgA, and f74 and f75 are associated with another subclass of IgA (Lichter *et al.*, 1970), we at this time prefer to change the designation of the latter to g74 and g75 to indicate that the two sets of allotypic specificities probably represent the product of genes at different loci, *f* and *g*. The close linkage of the *f* and *g* loci to the *a* locus (Hanly *et al.*, 1972) suggests that the allotypic specificities of the *f* and *g* loci are markers for the heavy chains of IgA. Moreover, the presence of *f* and *g* allotypic specificities in serum IgA which lacks the secretory component also suggests that the heavy chains have these allotypic specificities.

The purpose of this investigation was to localize the allotypic specificities of the *f* and *g* loci in the IgA molecule. For this purpose, sIgA was digested with papain and the fragments obtained were characterized as $Fc_{2\alpha}$ and $Fab_{2\alpha}$ by their antigenic and sedimentation properties. Although enzymatic digestion of sIgA has been reported (Steward, 1971; Shuster, 1971; Cederblad *et al.*, 1966), the degradation products have not been well characterized. Lawton (1969) succeeded in isolating small quantities of $Fc_{2\alpha}$ and $Fab_{2\alpha}$ following papain digestion of sIgA. In the experiments presented here, we extend the characterization of the fragments obtained from papain-treated sIgA and provide evidence that the *f* and/or *g* allotypic determinants occur on the constant portion of the α heavy chains and thus serve as markers for the C_α genes.

Materials and Methods

Purification of sIgA. Secretory IgA was isolated from colostrum of individual rabbits essentially by the method described previously (Cebra and Robbins, 1966). Daily samples of colostrum (no distinction is made between colostrum and milk) were obtained from rabbits beginning 24-hr postpartum for 2-3 weeks. Following centrifugation of the colostrum for 2 hr at 4° and 10,000*g*, the middle layer (whey) was removed and centrifuged (4°) for 2 hr at 100,000*g*. Approximately 30 ml of this clarified whey was placed on a 150 × 5 cm Sephadex G-200 column in borate-saline buffer (0.2 M borate–0.15 M NaCl, pH 8.0). The eluate was collected in fractions of approximately 10 ml and monitored at 280 mμ. The sIgA comprises most of the protein in the first peak eluted from the column. By immunoelectrophoresis using goat anti-(rabbit) whey, the ascending portion of the first peak (peak 1A) was found to contain some high molecular weight proteins other than sIgA, *e.g.*, α-macroglobulins, low-density lipoprotein, and IgM; thus, this fraction was discarded. The material in the descending portion of peak 1 (peak 1B) usually appeared to be free of contaminating protein and this fraction was pooled and concentrated by vacuum ultrafiltration. The concentrated protein was examined for contaminating proteins by immunoelectrophoresis using goat anti-whey.

Papain Digestion. The purified sIgA was treated with papain essentially as described by Lawton (1969). Papain (papaya latex, Schwarz-Mann) was activated with 0.01 M cysteine in 0.1 M phosphate–0.002 M EDTA (pH 7.1) for 30 min at 37° after which the cysteine was removed by dialysis on a column of Sephadex G-25 fine. The absence of cysteine in the eluted papain was confirmed by use of the Ellman sulfhydryl reagent (Ellman, 1959). Activated papain was added to sIgA (10-20 mg/ml) in a ratio of approximately 1:30 (w/w) and the mixture was incubated for 16 hr at 37° in 0.1 M phosphate–0.002 M EDTA (pH 7.1). Immediately after digestion, the sample was placed on a Bio-Gel P-200 column (150 × 2.5 cm) in borate–saline buffer (pH 8.0). The eluate was monitored at 280 mμ and three well-separated peaks were observed. The fractions from each peak were pooled and concentrated by vacuum ultrafiltration. In one experiment, the papain digestion was done in the presence of 0.01 M cysteine and following chromatography on Bio-Gel P-200, approximately seven poorly separated peaks were eluted; this profile was very different from the three well-separated peaks obtained when the digestion was done in the absence of cysteine.

Antisera. Goat anti-(rabbit) sIgA was prepared by immunization of a goat with approximately 1 mg of sIgA purified especially for immunization purposes. The sIgA obtained from Sephadex G-200 chromatography of whey, as described above, was further purified by DEAE-cellulose chromatography and by disc electrophoresis. Following disc electrophoresis, the section of the gels containing sIgA was homogenized, mixed with Freund's complete adjuvant (15% Aracel A, 85% Bayol F and *Mycobacterium butyricum*, 0.4 mg/ml) and injected intramuscularly into a goat. An additional injection of 0.2 mg was given 1 month later in Freund's incomplete adjuvant and the goat was bled at weekly intervals. The resulting antiserum (anti-sIgA) was made specific for IgA (anti-α) by passing the antiserum through an immunosorbant in which Fab fragment of rabbit IgG was coupled to Sepharose (Axen *et al.*, 1967; Wofsy and Burr, 1969); this procedure removed antibody activity to the light chains and to the variable portion of the heavy chains. When this antiserum was tested with sIgA and serum IgA, dense precipitin lines which coalesced in a reaction of identity were observed. Goat anti-(rabbit) secretory component was prepared by the injection of secretory component isolated after its dissociation from sIgA in guanidine-HCl (Cebra and Small, 1967). The secretory component (1 mg) was mixed with Freund's complete adjuvant and injected intramuscularly into a goat. The goat was bled 4 weeks later and the antiserum obtained was absorbed with purified rabbit IgG (0.3 mg of IgG/ml of antiserum).

Goat anti-(rabbit) Fc_γ was prepared by the injection of a goat with 1 mg of twice-recrystallized Fc_γ fragment (Porter, 1959) in Freund's complete adjuvant. A second injection with 0.2 mg was given 1 month later in Freund's incomplete adjuvant and the goat was bled weekly. Small amounts of contaminating anti-Fab_γ activity were removed by passage of the antiserum through an immunosorbant in which rabbit Fab_γ was coupled to Sepharose (Axen *et al.*, 1967; Wofsy and Burr, 1969). Anti-allotype antisera, anti-a1, anti-a2, anti-a3, anti-b4, anti-b5, anti-f73,g74, anti-f72,g74, and anti-f71,g75 were prepared as previously described (Dray *et al.*, 1963; Conway *et al.*, 1969b). Goat anti-(rabbit) light chain was a gift of Dr. A. Nisonoff, goat anti-(rabbit) J chain was

[3] Since *d* and *e* control allotypic specificities on the constant portion of the same γ chain, the gene for the constant part of the γ chain is designated $C_\gamma de$.

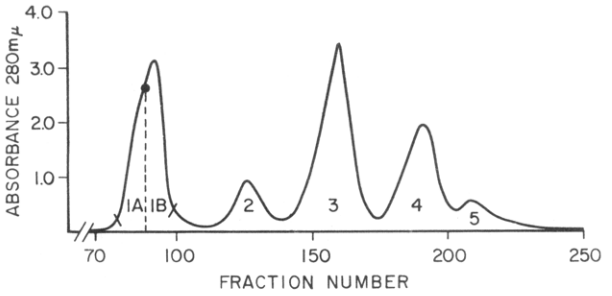

FIGURE 1: Elution pattern of rabbit whey from Sephadex G-200 (5.0 × 150 cm) in borate–saline buffer (pH 8.0).

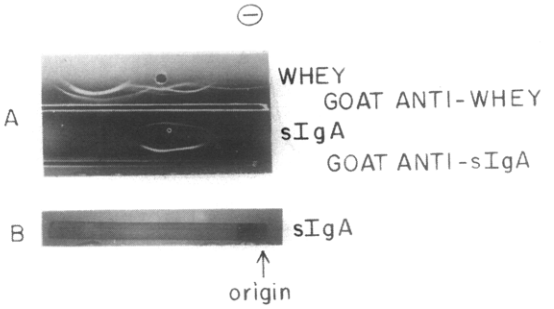

FIGURE 2: Immunoelectrophoresis and disc electrophoresis of purified sIgA. (A) Immunoelectrophoresis in 1.5% noble agar showing the precipitin reaction of purified sIgA with goat anti-whey and with goat anti-sIgA. (B) Disc electrophoresis in 7.5% polyacrylamide gel of purified sIgA.

a gift of Dr. M. Koshland and guinea pig anti-goat IgG was a gift of Dr. S. Bratcher.

Radioprecipitation Analyses. The intact sIgA or papain fragments thereof were iodinated with ^{125}I essentially by the method of McFarlane (1958). The samples were iodinated with two to four atoms of iodine per molecule of protein and following dialysis, the proteins generally had a specific activity of approximately 3×10^{-2} Ci/g. Over 99% of the radioactivity was precipitated by 6% trichloroacetic acid in all samples except for the $Fab_{2\alpha}$. In the $Fab_{2\alpha}$ samples, approximately 50–60% of the radioactivity was precipitated by 6% Cl_3CCOOH. In an effort to remove "trapped" ^{125}I, the labeled $Fab_{2\alpha}$ was dialyzed against acetate buffer (pH 5.0). However, this procedure did not increase the percentage of radioactivity precipitated by 6% Cl_3CCOOH. Since the reaction of labeled $Fab_{2\alpha}$ with various antibodies resulted in the precipitation of more than 90% of the radioactivity, the low percentage of radioactivity precipitated by 6% Cl_3-CCOOH was not investigated further.

Radioprecipitations were done with labeled intact sIgA and labeled papain fragments. Assuming an extinction coefficient, $E_{1\,cm}$, at 280 mμ of 13.0, 0.2 μg of each ^{125}I-labeled antigen was incubated with an excess (5 or 10 μl) of anti-allotype antiserum at 37° for 1 hr (Spring *et al.*, 1970). Subsequently, goat anti-Fc_γ was added in slight antibody excess to precipitate the complexes formed between the labeled antigen and the rabbit anti-allotype antibody which is of the IgG class (Gilman *et al.*, 1964). After 1 hr at 37°, the tubes were placed at 4° overnight. After centrifugation, the precipitates were washed three times and dissolved in 0.5 ml of 0.1 M NaOH. The radioactivity in the supernatant fluids and in the precipitates was determined in a well-type gamma scintillation counter. The per cent of total radioactivity precipitated was calculated for each sample. The data reported are averages of duplicate values which varied less than 3% from each other.

For radioprecipitations with goat anti-IgA or goat anti-secretory component, the ^{125}I-labeled antigen was incubated with an excess of the antiserum at 37°. After 1 hr, an appropriate amount of unlabeled rabbit sIgA (60–80 μg) was added to bring the antigen–antibody reaction mixture to equivalence. The tubes were incubated at 37° for 1 hr, then at 4° overnight and the precipitates were washed and treated as described above for the reactions with anti-allotype antisera.

Analytical Methods. Immunoelectrophoresis was performed in 1.5% Noble agar in 0.05 M sodium barbital buffer (pH 8.6) (Grabar and Williams, 1955). Sedimentation velocity was determined using schlieren optics in the Spinco Model E ultracentrifuge using the An-D rotor with 12-mm double-sector cells. Ultracentrifugation was performed at 20° in borate–saline (pH 8.0) or 0.1 M phosphate (pH 7.1) at 60,000 rpm. The sedimentation coefficients reported are not corrected to zero concentration. Sedimentation equilibrium studies were done in a Spinco Model E ultracentrifuge with the use of a multiplex and ultraviolet scanner (Lamers *et al.*, 1963). The samples were centrifuged in borate–saline (pH 8.0) at 6 or 9°. All samples were run at 12,000 and 18,000 rpm. For the molecular weight calculations, a $\bar{v}$ of 0.741 was used for $Fab_{2\alpha}$ (Miller and Metzger, 1966) and a $\bar{v}$ of 0.723 was used for $Fc_{2\alpha}$. The $\bar{v}$ of 0.723 was calculated from the amino acid composition of rabbit α chains (O'Daly and Cebra, 1971) and is the same value reported by Tomasi and Bienenstock (1968) for human sIgA. Disc electrophoresis was done at pH 8.9 in 7.5% polyacrylamide gels by the method of Williams and Reisfeld (1964). Disc gels were stained for protein with Coomassie Blue (Weber and Osborn, 1969), or for carbohydrate with the periodic acid–Schiff reagent (Zacharius *et al.*, 1969). Ouchterlony analyses were performed in 1.5% Noble agar in borate–saline (pH 8.0) or acetate buffer (pH 5.0) as described previously (Dray *et al.*, 1963).

Results

Isolation of sIgA. A representative elution pattern of whey proteins from a Sephadex G-200 column is shown in Figure 1. The sIgA obtained from peak 1B was found to be free of contaminating proteins by ultracentrifugation, immunoelectrophoresis, and disc electrophoresis. By analytical ultracentrifugation, a single symmetrical peak with a sedimentation coefficient of 9.2 S was observed. By immunoelectrophoresis, only a single precipitin band developed with goat anti-whey (Figure 2); by disc electrophoresis, a characteristic pattern for sIgA was obtained (Conway *et al.*, 1969a), *i.e.*, a major band near the top of the gel and a smaller band slightly more anodal (Figure 2).

Characterization of the Papain Digest of sIgA. The unfractionated papain digest of sIgA and the three fractions (pools 1, 2, and 3) separated on Bio-Gel P-200 (Figure 3) were examined by immunoelectrophoresis (Figure 4) with anti-sIgA, anti-light chain, and anti-α chain. The unfractionated papain digest exhibited three precipitin arcs with anti-sIgA comparable to those identified by Lawton (1969) as Fc fragment, Fab fragment, and undigested sIgA. One of these arcs, cathodal to the origin, represents the reaction with the Fab fragment of sIgA, and corresponds in position to (1) the precipitin arc formed by the reaction of unfractionated papain digest of sIgA with anti-light chain and (2) the major band

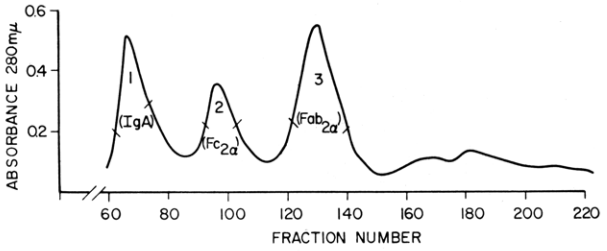

FIGURE 3: Elution pattern of the papain digest of sIgA from Bio-Gel P-200 (2.5 × 150 cm) in borate–saline buffer (pH 8.0). The sIgA is from rabbit H51-2 with allotypes a1, b5, f73, and g74.

observed when pool 3 reacted with anti-sIgA. A second precipitin arc, anodal to the origin, represents the reaction with the Fc fragment of sIgA and corresponds to the position of the bands observed when pool 2 reacted with anti-sIgA or with anti-α chain. As expected, pool 2 (Fc fragment) did not react with anti-light chain. The third precipitin arc represents the reaction of undigested sIgA since it corresponds to the position of the precipitin arc observed when intact sIgA or pool 1 was reacted with anti-sIgA or anti-α chain. As seen below, the identification of the proteins in the three pools was consistent with the data obtained by analytical ultracentrifugation and disc electrophoresis.

Pools 2 and 3, when subjected to analytical ultracentrifugation, each appeared as a single symmetrical peak with sedimentation coefficients of 4.6 and 4.5 S, respectively (Figure 5). These values are as expected for the $Fc_{2\alpha}$ and $Fab_{2\alpha}$ fragments and are essentially in agreement with the value of 5.2 S calculated for one of the two peaks observed during ultracentrifugation of the unfractionated papain digest (Figure 5). This 5.2S peak apparently contained a mixture of the $Fc_{2\alpha}$ and $Fab_{2\alpha}$ fragments. The approximate molecular weights of $Fc_{2\alpha}$ and $Fab_{2\alpha}$ are 130,000 and 100,000, respectively. The 10.0S peak obtained with the unfractionated papain digest represents the undigested sIgA (Figure 5). A time-course experiment of the digestion of sIgA with papain revealed that the reaction was essentially complete at 1 hr as determined by the similarity of the ultracentrifugation patterns obtained after 1, 3, 5, 8, 12, and 16 hr of reaction (Figure 6).

Sedimentation equilibrium studies were done on three different preparations of pool 2 ($Fc_{2\alpha}$) and on two different preparations of pool 3 ($Fab_{2\alpha}$). Using a $\bar{v}$ of 0.723 for $Fc_{2\alpha}$, the molecular weights as determined at 12,000 rpm were

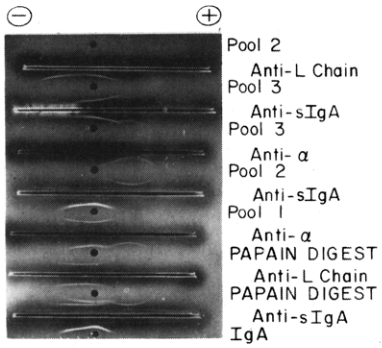

FIGURE 4: Immunoelectrophoresis in 1.5% noble agar showing the precipitin reactions of the papain fragments of sIgA (rabbit H51-2) with goat anti-light chain, goat anti-sIgA, and goat anti-α chain.

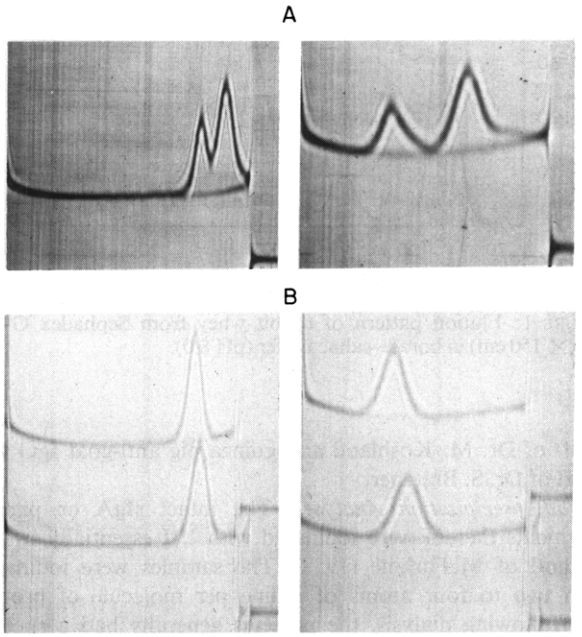

FIGURE 5: Ultracentrifugal patterns of papain digested sIgA and of pools 2 and 3 of papain digested sIgA. The centrifuge was run at 60,000 rpm and 20°. Sedimentation is from right to left. (A) Papain digested sIgA (10 mg/ml). Photographs were taken at 60° bar angle at 8 min (left) and 40 min (right) after attaining speed. (B) Pool 2 ($Fc_{2\alpha}$, 6 mg/ml) (top) and pool 3 ($Fab_{2\alpha}$, 6 mg/ml) (bottom) of the papain digest of sIgA. The photographs were taken at 24 min (left, 60° bar angle) and 80 min (right, 55° bar angle) after attaining speed.

117,000, 134,000, and 142,000. The variation in the three values may be partly related to the presence or absence of secretory component and/or to the fact that the sIgA was obtained from rabbits of different *f* and *g* allotypes. The molecular weights of the $Fab_{2\alpha}$ samples using a $\bar{v}$ of 0.741 (Miller

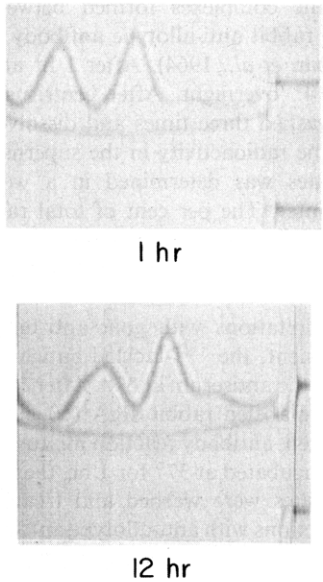

FIGURE 6: Ultracentrifugal patterns of sIgA, after digestion with papain for 1 and 12 hr. The centrifuge was run at 60,000 rpm and 20°. After attaining speed, photographs of the 1- and 12-hr digests were taken at a 60° bar angle at 56 and 48 min, respectively. Sedimentation is from right to left.

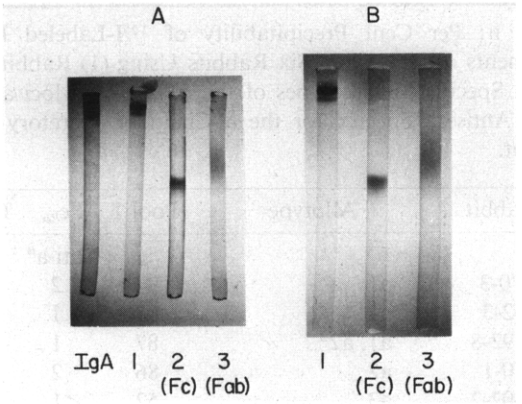

FIGURE 7: Disc electrophoresis in 7.5% polyacylamide gel of the papain fragments of sIgA. The disc gels in A were stained with Coomassie Blue and the gels in B were stained with the periodic acid–Schiff reagent.

and Metzger, 1966) as determined at 12,000 rpm were 97,000 and 104,000. The molecular weights of each Fab and each Fc sample obtained at 18,000 rpm were within 6% of the values obtained at 12,000 rpm.

When pools 1, 2, and 3 were assessed by disc electrophoresis (Figure 7), the following was observed: pool 1 exhibited a major and a minor band near the top of the gel similar to that observed with intact sIgA; pool 2 exhibited one discrete, intensely staining band characteristic of the molecular homogeneity of the Fc fragment and two very faint bands slightly anodal to the major band (not visible in Figure 7); pool 3 exhibited a very diffuse band (characteristic of the molecular heterogeneity of Fab fragments) with a mobility slightly slower than that of the major band of pool 2. The bands of all three pools contained carbohydrate since they all reacted with the periodic acid–Schiff reagent.

Pools 1, 2, and 3 of the papain digest were further assessed for their antigenic relationships by Ouchterlony analysis (Figure 8). Pool 1 (undigested sIgA) gave reactions of identity with intact sIgA when tested with anti-light chain (goat anti-light chain or rabbit anti-b5 light chain) and antibodies (anti-a1) directed toward the variable part of the α heavy chain. However, pool 1 (undigested sIgA) gave reactions of partial identity with intact sIgA when tested with anti-secretory component or with anti-f73,g74. Moreover, pool 1 gave only one band compared to two for intact sIgA when tested with anti-sIgA. Thus, the undigested sIgA (pool 1) is not identical in all respects to the intact sIgA.

As expected, pool 3 ($Fab_{2\alpha}$) gave a reaction of identity with sIgA when tested with anti-light chain and anti-a1; furthermore, pool 2 ($Fc_{2\alpha}$) reacted with neither anti-light chain nor anti-a1. The anti-sIgA gave two precipitin bands with pool 2 and with pool 3. The dense band with pool 3 (closer to the antibody well) represents the reaction with $Fab_{2\alpha}$ and this band coalesces with the faint band of pool 2 (closer to the antigen well) indicating that pool 2 is slightly contaminated with $Fab_{2\alpha}$. Similarly, the dense band with pool 2 (closer to the antibody well) represents the reaction with $Fc_{2\alpha}$ and this band coalesces with the faint band of pool 3 (closer to the antigen well), indicating that pool 3 is slightly contaminated with $Fc_{2\alpha}$. The Fab band crosses the Fc band. When the anti-sIgA is absorbed with IgG (Fab_γ) to obtain anti-α, the Fab band no longer developed from pools 2 and 3. Moreover, the anti-α also reveals the slight contamination of pool 3 with $Fc_{2\alpha}$ as indicated by the reaction of identity. Pool 2 ($Fc_{2\alpha}$) but not

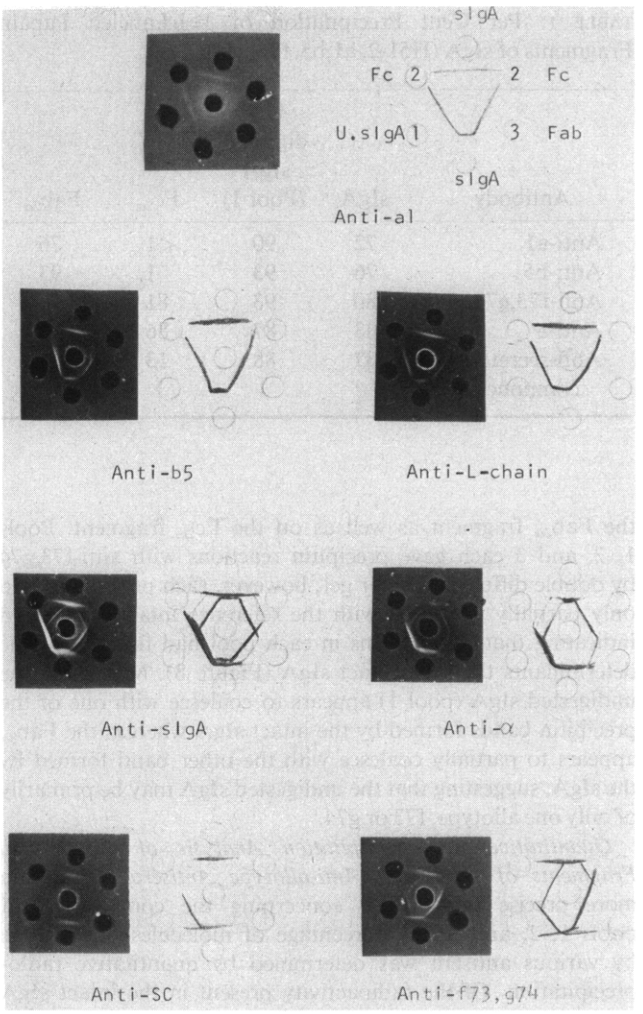

FIGURE 8: Double-diffusion experiment showing the precipitin reactions of the papain fragments of sIgA (from rabbit No. H51-2 with allotypes a1, b5, f73, g74) with anti-a1, anti-b5, anti-f73,g74, anti-light chain (anti-L), anti-secretory component (anti-SC), anti-sIgA, anti-α and anti-f73,g74.

pool 3 ($Fab_{2\alpha}$) reacted with anti-secretory component to give a faint precipitin band indicating that pool 2 also contained small amounts of secretory component.

Immunodiffusion Reactions of the Anti-f73,g74 Antiserum with the Papain Fragments of sIgA. By immunoelectrophoresis, the anti-f73,g74 gave precipitin reactions with pools 1, 2, and 3 and the precipitin arcs observed were similar in position to those developed with anti-sIgA (Figure 9). This suggested that the f73 and/or g74 allotypic specificities are located on

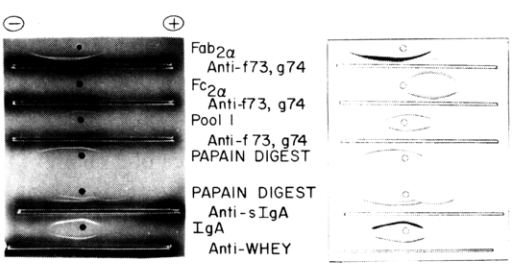

FIGURE 9: Immunoelectrophoresis in 1.5% Noble agar showing the precipitin reactions of the papain fragments of sIgA (from rabbit H51-2 having allotypes f73,g74) with anti-f73,g74.

TABLE I: Per Cent Precipitation of ^{125}I-Labeled Papain Fragments of sIgA (H51-2, a1,b5, f73,g74).

Antibody	sIgA	Undigested sIgA (Pool 1)	$Fc_{2\alpha}$	$Fab_{2\alpha}$
Anti-a1	72	90	<1	76
Anti-b5	76	93	1	93
Anti-f73,g74	80	93	81	97
Anti-α	83	87	86	4
Anti-secretory component	37	88	13	<1

the $Fab_{2\alpha}$ fragment as well as on the $Fc_{2\alpha}$ fragment. Pools 1, 2, and 3 each gave precipitin reactions with anti-f73,g74 by double diffusion in agar gel; however, each precipitin band only partially coalesced with the reference intact sIgA band indicating that the proteins in each pool had fewer allotypic determinants than the intact sIgA (Figure 8). Moreover, the undigested sIgA (pool 1) appears to coalesce with one of the precipitin bands formed by the intact sIgA whereas the $Fab_{2\alpha}$ appears to partially coalesce with the other band formed by the sIgA, suggesting that the undigested sIgA may be primarily of only one allotype, f73 or g74.

Quantitative Radioprecipitation Analysis of the Papain Fragments of sIgA with Anti-allotype Antisera. To obtain more precise information concerning the composition of pools 1, 2, and 3, the percentage of molecules precipitable by various antisera was determined by quantitative radioprecipitation. Of the radioactivity present in the intact sIgA or undigested sIgA (pool 1), 72–83 or 87–93%, respectively, was precipitated by each of the various antisera; anti-a1, anti-b5, anti-f73,g74, and anti-α (Table I). Less than 1% of the radioactivity in pool 2 ($Fc_{2\alpha}$) was precipitated by anti-a1 or anti-b5 indicating the contamination with Fab fragments shown by immunodiffusion was less than 1%. However, 13% of the radioactivity of pool 2 ($Fc_{2\alpha}$) was precipitated by anti-secretory component indicating that pool 2 does contain a significant amount of secretory component. Since 81% of the radioactivity in pool 2 ($Fc_{2\alpha}$) is precipitated by anti-f73,g74 and 86% by anti-α, the allotypic specificities of the *f* and/or *g* locus must reside on the Fc part of the α chain (Table I).

Anti-b5 precipitated 93% and anti-a1 precipitated 76% of the radioactivity in pool 3 ($Fab_{2\alpha}$), indicating that the maximum contamination of pool 3 by $Fc_{2\alpha}$, observed by immunodiffusion, could be 7%. In fact, the contamination was approximately 4%, *i.e.*, the radioactivity precipitated by anti-α. Little or no secretory component (less than 1%) was found in pool 3 ($Fab_{2\alpha}$). Since anti-f73,g74 precipitated 97% of the radioactivity, the allotypic specificities of the *f* and/or *g* locus must also reside on the Fab part of the molecule (Table I).

With the limited amount of goat anti-J chain available it was possible to analyze for the presence of J chain in the papain fragments of one of the sIgA preparations (rabbit H52-3, see Table II) by an indirect radioprecipitin method using guinea pig anti-goat IgG to precipitate the goat antibody bound to iodinated fragments. By this procedure, the anti-J chain precipitated 37% of the radioactivity in pool 1 (undigested sIgA), 26% of that in pool 2 ($Fc_{2\alpha}$) and less than 1% of that in pool 3 ($Fab_{2\alpha}$). This indicates that J chain may be attached to the Fc portion of the molecule as previously suggested (Meinke and Spiegelberg, 1971; Mestecky *et al.*, 1971a). However, since 81% of the radioactivity in pool 2 ($Fc_{2\alpha}$) and 97% in pool 3 ($Fab_{2\alpha}$) is precipitated by anti-f73,g74, the J chain cannot account for the *f* and/or *g* allotypic specificities in the $Fc_{2\alpha}$ or $Fab_{2\alpha}$ pools.

TABLE II: Per Cent Precipitability of ^{125}I-Labeled Papain Fragments of sIgA from Six Rabbits Using (1) Rabbit Antiserum Specific for Allotypes of the *a*, *b*, *f* or *g* loci and (2) Goat Antisera Specific for the α Chain or Secretory Component.

Rabbit	Allotype	Pool 1	$Fc_{2\alpha}$	$Fab_{2\alpha}$
			Anti-a[a]	
J170-3	a1	94	2	88
H52-3	a1	91	1	80
H192-8	a1, a2[b]	87	1	82
H70-1	a2	86	2	78
G102-2	a3	52	<1	49
G208-1	a2	90	1	70
			Anti-b	
J170-3	b4	95	2	96
H52-3	b4,5[c]	95	2	93
H192-8	b4	70	1	73
H70-1	b4	87	2	90
G102-2	b4	64	1	56
G208-1	b4	92	1	95
			Anti-fg	
J170-3	f72,g74	91	78	98
H52-3	f73,g74	93	83	96
H192-8	f71,72 g74,75[d]	91	80	94
H70-1	f71,g75	89	83	92
G102-2	f71,g75	88	78	92
G208-1	f71,g75	94	83	95
			Anti-α	
J170-3		94	94	1
H52-3		92	88	1
H192-8		90	—	—
H70-1		81	81	27
G102-2		93	89	<1
G208-1		88	88	24
			Anti-secretory component	
J170-3		90	12	<1
H52-3		86	12	<1
H192-8		77	14	<1
H70-1		76	35	1
G102-2		84	16	<1
G208-1		84	20	<1

[a] Anti-*a*, anti-*b*, and anti-*fg* refer to antibodies specific for allotypes controlled by the *a*, *b*, *f*, and *g* loci. [b] Values represent the sum of the per cent radioactivity precipitated by anti-a1 and anti-a2. [c] Values represent the sum of the per cent radioactivity precipitated by anti-b4 and by anti-b5. [d] Values represent the sum of the per cent radioactivity precipitated by anti-f71,g75 and by anti-f72g,74.

The $Fab_{2\alpha}$ and $Fc_{2\alpha}$ fragments isolated from the papain digests of sIgA from each of six additional rabbits were analyzed by quantitative radioprecipitation using anti-f73,g74, anti-f72,g74, or anti-f71,g75 antisera (Table II). In each experiment, the *f* and/or *g* allotypic specificities were found on nearly all the molecules of both the $Fc_{2\alpha}$ and the $Fab_{2\alpha}$ pools. As expected, the *a* and *b* allotypic specificities were present on molecules in the $Fab_{2\alpha}$ pools and absent on those in the $Fc_{2\alpha}$ pools. No secretory component was found in the $Fab_{2\alpha}$ pool but a small amount was present in the $Fc_{2\alpha}$ pool (Table II). Anti-α precipitated approximately 90% of the radioactivity in the $Fc_{2\alpha}$ pool and in three out of five experiments did not react with the Fab fragments. In the other two experiments, anti-α precipitated 24 and 27% of the radioactivity in the $Fab_{2\alpha}$ pool. This does not appear to be due to contamination with Fc fragments since (1) by immunoelectrophoresis, no contamination by Fc was observed, and (2) anti-b4 precipitated 95 and 96% of the radioactivity. The explanation for the variable results obtained with the $Fab_{2\alpha}$ pool with anti-α is not clear; possibly this reflects differences in the site of papain cleavage as occurs with IgG (Smyth and Utsumi, 1967; Hill *et al.*, 1967).

Discussion

The five allotypic specificities of rabbit IgA (f71, f72, f73, g74, and g75) are controlled by two closely linked genes, *f* and *g*. The *f* and *g* genes are also closely linked to the *a* gene controlling the variable part of Ig heavy chains and the *a* gene is closely linked to the *de* gene controlling the constant region of the γ chain. Thus, the close linkage of *a* with *f* and *g* suggested that the allotypic specificities controlled by the *f* and *g* genes might reside on the α heavy chain of IgA. As it is difficult to obtain pure α chains without the use of strong denaturing agents such as 8 M urea or 6 M guanidine-HCl, we digested the sIgA with papain to obtain the Fc and Fab fragments of sIgA. Quantitative radioprecipitation analyses of these Fc and Fab fragments showed that specificities controlled by the *f* and/or *g* loci do indeed reside on the α chain; some of the determinants are on the Fc portion of the heavy chain and some are on the Fab portion of the molecule, presumably on the constant part of the Fd region.

Proteolytic digestion of sIgA with papain in the absence of cysteine, followed by chromatography on Bio-Gel P-200 resulted in three major peaks identified as undigested sIgA (pool 1), $Fc_{2\alpha}$ (pool 2), and $Fab_{2\alpha}$ (pool 3). That pool 2 is primarily $Fc_{2\alpha}$ was indicated by the following characteristics: (1) a sedimentation coefficient of approximately 5 S; (2) a molecular weight of 117,000–142,000; (3) absence of light-chain determinants as evidenced by the lack of reactions with goat anti-light chain or with anti-b5 allotype reagents; (4) lack of allotypic determinants, characteristic of the variable part of the heavy chain, which are controlled by the *a* locus; and (5) presence of α-chain determinants as evidenced by its reaction with goat anti-α chain. Pool 2 was further characterized as follows: (1) only some (approximately 15%) of the molecules reacted with anti-secretory component thus suggesting that some contamination with secretory component occurs; (2) disc electrophoresis of the fraction revealed a well-defined band, consistent with results obtained from Fc of IgG; and (3) immunoelectrophoresis of the fraction revealed a single precipitin band in the anodal region.

Since 80% of the molecules in the $Fc_{2\alpha}$ fraction reacted with antibodies specific for allotypes of the *f* and *g* loci (anti-*fg*), the allotypic specificities controlled by the *f* and/or *g* locus reside on the α chain. The presence of secretory component as a contaminant of the $Fc_{2\alpha}$ fraction to the extent of approximately 15% cannot account for the reactivity with anti-*fg* reagents. Also, nearly all (>92%) of the $Fab_{2\alpha}$ molecules (pool 3) reacted with the anti-*fg* reagents. The $Fab_{2\alpha}$ molecules are composed of light chains and the Fd region of α chains. Since the light chains and the variable part of the Fd region are identical in all Ig classes, the reaction of the $Fab_{2\alpha}$ with anti-*fg* must result from allotypic determinants in the constant region of the Fd fragment of α chain. Thus, the *f* and/or *g* allotypic specificities appear to reside on the constant portion of the Fd part of the α chain as well as on the Fc part of the α chain. The antigenic determinants on the Fc and Fd parts of the α chain are not likely to be identical. In fact, Ouchterlony analysis revealed that the precipitin band formed by the reaction of anti-f73,g74 with $Fc_{2\alpha}$ partially coalesced with the precipitin band formed with intact sIgA. This indicated that $Fc_{2\alpha}$ lacks some of the determinants which characterize the f73 and/or g74 allotypic specificities and which apparently reside in the constant region of the Fd part of the heavy chain. Moreover, by Ouchterlony analysis, the precipitin band formed by the reaction of anti-f73,g74 and $Fab_{2\alpha}$ partially coalesced with the precipitin band formed with intact sIgA. This indicated that the $Fab_{2\alpha}$ lacks some of the allotypic determinants which characterize the f73 and/or g74 allotypic specificities and which apparently are on $Fc_{2\alpha}$.

Since the anti-f73,g74 reagent has specificities directed against both f73 and g74, it is not clear if the reagent is reacting with f73 or g74 or both of these specificities in the three pools and consequently we have referred to the specificities present in these pools as *f* and/or *g* specificities. When monospecific anti-f73 and anti-g74 antisera become available, it will be possible to determine if one or both of the specificities are present in each of the pools.

Masuda *et al.* (1969) prepared an antibody specific for a rabbit IgA allotype and their antibody did not precipitate the Fab fragents obtained by papain digestion of sIgA. They therefore suggested that the specificity was on the Fc fragments. However, since no Fc fragment was recovered, the localization of the specificity to the Fc fragment was not explicit. Also, since their assay was qualitative and an indirect radioprecipitin assay was not used, the possibility of the specificity occurring on secretory component, J chain, or even the Fab fragment cannot be excluded (Gilman *et al.*, 1964).

Radioprecipitation analysis of the original undigested sIgA with anti-α or anti-*fg* reagents suggested that the sIgA was only 80–83% pure; however, no evidence of contaminating protein was observed by analytical ultracentrifugation, disc electrophoresis, or immunoelectrophoresis. Moreover, nearly all of the molecules in each of the papain fragments obtained from these sIgA preparations reacted with the antibodies specific for IgA, *i.e.*, anti-α, anti-*fg*, or anti-secretory component. The apparent 15% of "nonprecipitable" material in the intact sIgA may be due to denaturation of the intact sIgA during iodination at pH 8.7 resulting in alteration of some of the antigenic determinants. Thus the intact sIgA was probably of much higher purity than indicated by the radioprecipitation data. However, we cannot exclude the possibility that a protein contaminant may have been present in the original preparation but is lost during the isolation of the papain fragments.

Following papain digestion, most of the secretory component appears to be associated with the undigested sIgA. Based on the absorbance at 280 mμ of each fraction and as-

suming that the extinction coefficients of each fraction are similar, the undigested sIgA was calculated to represent 35% of the protein eluted from the Bio-Gel P-200 column. Since 37% of the intact sIgA had secretory component attached and since most (88%) of the undigested sIgA molecules (pool 1) had secretory component, it appears that most of the secretory component was attached to sIgA molecules which were resistant to papain cleavage. However, some molecules containing secretory component were digested by papain since approximately 15% of the radioactivity in the $Fc_{2\alpha}$ fraction was precipitated by anti-secretory component. The relationship between the presence or absence of secretory component and sensitivity to proteolytic digestion has been noted by Steward (1971) and needs further investigation. That only 37% of the intact sIgA molecules had secretory component attached differs from the results of Cebra and O'Daly (1969) who reported that nearly all the molecules had secretory component. However, we have found thus far that with two of our rabbits, nearly all the sIgA molecules have secretory component, but that with three other rabbits less than half of the molecules have secretory component and that this might be related to the *f* and/or *g* genotype (unpublished observations). This point requires still further investigation since it is conceivable that the particular goat anti-secretory component which we used may not have reacted with all secretory component molecules in each rabbit.

That the *f* and *g* allotypic specificities are not present on J chain is indicated by the fact that (1) the anti-J chain precipitated only 26% of the radioactivity in the $Fc_{2\alpha}$ fraction and less than 1% in the $Fab_{2\alpha}$ fraction, and (2) the anti-J chain does not precipitate serum IgA (Morrison and Koshland, 1972) whereas the anti-*fg* antisera do precipitate serum IgA (Conway *et al.*, 1969a).

The chemical nature of the *f* and *g* allotypic specificities is undetermined. However, amino acid differences have been correlated with allotypic specificities for the *a*, *b*, *d*, and *e* allotypic systems (Koshland *et al.*, 1968; Reisfeld *et al.*, 1965; Prahl *et al.*, 1969; Appella *et al.*, 1971). The d11 and d12 reflect a Met–Thr interchange in the hinge region of the γ chain (Prahl *et al.*, 1969) and the e14 and e15 allotypic specificities reflect a Thr–Ala interchange at position 309 of the γ chain (Appella *et al.*, 1971). The *a* allotypic specificities reflect several amino acid interchanges in the variable region of heavy chains (Koshland, 1967; Mole *et al.*, 1971). Since *f* and *g* are closely linked to *a*, *d*, and *e*, it is also likely that the *f* and *g* allotypic specificities reflect amino acid interchanges. However, carbohydrate is present in both the $Fc_{2\alpha}$ and $Fab_{2\alpha}$ fragments, and thus we cannot rule out the possibility that the allotypic determinants involve carbohydrate moieties.

The heavy-chain chromosomal region of rabbit includes at least seven closely linked genes for which genetic markers are known: V_Ha, V_Hx, V_Hy, $C_\gamma de$, $C_\alpha f$, $C_\alpha g$, and $C_\mu n$. The three V genes[2] control allotypic specificities of the variable region and the four C genes, control the allotypic specificities of the constant regions of the heavy chains. The large number of known genetic markers facilitates the search for genetic recombinants in order to map the heavy-chain chromosomal region (Kindt and Mandy, 1972; Mage *et al.*, 1971).

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Mouse Immunoglobulin Chains. A Survey of the Amino-Terminal Sequences of κ Chains†

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ABSTRACT: Thirty-one immunoglobulin κ chains from the highly inbred BALB/c mouse have been examined by automatic sequence analysis and compared with thirteen additional proteins from the literature. These 44 proteins represent at least 32 different sequences over their amino-terminal 23 residues. Although there are nine cases in which two or more κ chains have identical sequences, a majority of the proteins clearly differ from one another by multiple nucleotide substitutions. There appears to be a great deal more sequence diversity in the myeloma κ chains of the BALB/c mouse than in the myeloma κ chains of man. A genealogic tree has been constructed from certain of these sequences to illustrate the genetic events required for any mechanism of antibody diversity. This analysis suggests that the mouse κ chains are quite distinct from their human counterparts and that a large number of germ line variable genes are present in the κ family of the BALB/c genome.

The immune system appears to represent an ideal model system for studying the genetic and evolutionary mechanisms of information storage in higher organisms (Hood and Prahl, 1971). The basic structure of immunoglobulins is now largely understood due to the sequence analysis of homogeneous proteins derived from plasma cell tumors (Potter, 1967). The basic subunit for immunoglobulin molecules is composed of two identical light and two identical heavy polypeptide chains generally covalently linked with disulfide bonds (Edelman and Gall, 1969). Each polypeptide chain is made of an amino-terminal variable (V) region, and a carboxy-terminal constant (C) region (Cohen and Milstein, 1967; Smith *et al.*, 1971).[1] The V regions exhibit an enormous diversity of amino acid sequences presumably related to antibody specificity, while each C region has a single sequence for chains of a given class except for genetic polymorphisms. Structural and genetic studies indicate that the synthesis of immunoglobulin chains is regulated by at least three unlinked families of genes containing κ, λ, and heavy chains (see Gally and Edelman, 1971). Since different V region sequences can be associated with the same C region sequence, it seems that two genes (a V and a C) may exist for each immunoglobulin polypeptide chain. A special joining mechanism to unite V and C genes at the DNA level has been postulated (see Hood, 1972).

The availability of large numbers of myeloma proteins has permitted us to ask questions about the genetic and evolu-

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[1] The abbreviations used for immunoglobulin chains are those proposed by the World Health Organization: see *Bull. W. H. O. 41*, 975 (1969).