STUDIES ON HUMAN ANTIBODIES

III. Amino Acid Composition of Four Antibodies from One Individual*

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The amino acid composition of pooled human γ-globulin was studied comprehensively by Brand and coworkers (1). More recent studies of 6.5S human globulins, now called γG-globulin (2), using automatic amino acid analytical techniques (3), have been reported for example, by Hsaio and Putnam (4), and by Crumpton and Wilkinson (5). In contrast to the situation with amino acid analyses of specific antibodies from the rabbit (5–10), the residue composition of human antibody γ-globulins to well defined antigens, especially from individual subjects, has yet to be investigated.

This paper is concerned with a study of the amino acid composition of four purified γG-antibodies, namely antilevan, antidextran, antiteichoic acid, and antiblood group substance A, from the serum of one individual. These four antibody preparations were previously examined for their allotypic specificities (11) and for the electrophoretic patterns of their H and L chains in starch gel (12). As has previously been reported by Koshland *et al.* for rabbit antibodies to several antigens (7–9), analytically significant differences in amino acid composition among these purified human antibodies have also been observed. Thus, there is wide variation in the number of glycine, valine, leucine, proline, threonine, tyrosine, and arginine residues in these γG-antibodies, as well as smaller differences in other amino acid residues. Furthermore, hydroxylysine is present in antidextran and in antiteichoic acid whereas it cannot be detected in antilevan, anti-A, or in the normal non-specific γG-globulin from this subject.

Materials and Methods

Preparation of γG.—Serum from subject 1 (11, 12) was dialyzed against several changes of 0.01 M potassium phosphate buffer, pH 7.2. It was then subjected to chromatography (13) on a DEAE-cellulose column (75 × 2.5 cm) which had previously been equilibrated with this buffer. The γG-fraction was eluted as the first protein peak in the effluent, and was dialyzed

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for 48 hours against tap water. The sample was then lyophilized. A sample of human serum (Cohn fraction II), kindly supplied by Dr. B. F. Erlanger, was purified in a similar manner. This preparation was demonstrated to contain four moles of C-terminal amino acid per mole (10).

Antibodies.—Serum from subject 1 which contained antidextran, antilevan, antiteichoic acid (14), (antigen from *Staphylococcus aureus*) (15), and anti-A was used for these experiments. In previous studies, these antibodies were demonstrated to be of the γG-variety with the following exceptions: anti-A was estimated to contain 10 to 20 per cent γM- and a trace of γA-immunoglobulin, and antiteichoic acid (14) was estimated to contain 5 to 15 per cent γM- and a trace of γA-globulin (11).

Two samples of 12 ml each (I and II) were absorbed in sequence with dextran (I_1), levan (I_2), teichoic acid (I_3 and II_3), and blood group A substance (I_4 and II_4), using amounts of antigen calculated to precipitate all of the corresponding antibody. To rule out possible differences in amino acid content of specific antibodies due to sequence of isolation, one sample was absorbed with dextran (I_1) followed by levan (I_2) and the other by levan (II_1) followed by dextran (II_2). The specific precipitates were washed six times in the cold with saline, and prepared for hydrolysis as given below. Another preparation of purified antilevan previously obtained from subject 1 (II) and two additional samples of anti-A specific antibody which were prepared by direct precipitation from the antiserum (Nos. 205 and 206), were also analyzed for their amino acid composition. Since contamination of anti-A antibodies by antigen can amount to as much as 6 to 7 per cent, three of the anti-A specific precipitates, I_4, II_4, and 206, were each extracted twice with 5 per cent trichloracetic acid followed by ether (16, 17). For purposes of comparison, the fourth, sample 205, was hydrolyzed and analyzed directly.

A sample of pooled γM-globulin was kindly provided by Dr. E. C. Franklin of New York University. This sample was shown to be at least 90 per cent pure by immunoelectrophoresis.

Sample Preparation.—Solutions containing approximately 0.30 mg of antibody or of γG were transferred to 12 ml thick walled centrifuge tubes. The contents were frozen and lyophilized at 0.01 mm pressure. These dried specimens were then dissolved in 0.15 ml of twice distilled, constant boiling HCl. The tubes were frozen, evacuated, sealed, and then heated under reflux in toluene. After cooling, the seals were broken, and the HCl was removed in a desiccator containing KOH. The residues were dissolved in 0.12 ml starting buffer, pH 2.855 (radiometer pH meter), 0.1 μmole norleucine was added as internal standard, and 0.10 ml of each was applied to the column of the amino acid analyzer.

Amino Acid Analyses.—The amino acids were analyzed using a technicon auto analyzer which embodies a single ion exchange column and a buffer gradient system as outlined by Piez and Morris (18). Preparation of ninhydrin and determination of color constants followed the procedure of Spackman *et al.* (3). The analyzer was calibrated with twenty determinations on a standard amino acid mixture (Beckman Instruments, Inc., Palo Alto, California) to which had been added 0.1 μmoles each of L-cysteic acid, DL-hydroxylysine, and methionine sulfoxide. This standard mixture was employed as a check on performance of the analyzer with each new lot of buffer or of ninhydrin. Hydroxylysine isomers appeared as twin peaks at 501 and 504 ml of column eluant, relative to the lysine ninhydrin-positive peak at 552 ml, both in standard mixtures and in those hydrolysates of antibodies which contained this residue. It had previously been shown by Hamilton and Anderson (19) that inversion of L-hydroxylysine occurs following reflux in 6 N HCl, and that separation of the isomers by chromatography on Dowex 50 takes place (5, 18-20).

RESULTS

The amino acid analyses of specific antibodies and of a γG from subject 1 are presented in Table I. Also included are analyses of purified pooled γG de-

rived from Cohn fraction II and of a pooled human serum γM-globulin. γM-immunoglobulin was not obtained from subject 1. The results of these determinations are compared with data presented in recent publications dealing with amino acid composition of human γG- (4) and human γM-globulins (21). These data are expressed as moles of amino acid residues per mole (160,000) of protein, based upon the assumptions that the samples examined contained 2.6 per cent tryptophan (5), and 2.0 and 10.0 per cent non-nitrogenous sugar in γG and γM, respectively (17). Half-cystine and methionine determinations include the sum of their respective oxidation products. The values used were those at 22 hours and no zero time corrections for destruction of labile hydroxy-amino acids have been applied. Time hydrolysis curves were carried out with antilevan and with whole γG-globulin and show amounts and rates of destruction of labile amino acids comparable to those found in the literature (22).

It can be seen from the data assembled in Table I, that certain conspicuous differences in amino acid composition were observed among specific γG-antibodies isolated from this individual. However, two sets of figures, obtained by conversion of the experimental data from μmoles to residues, appear to be anomalous. These are the inordinately high values found for glucosamine which resulted from analysis of anti-A preparations 205 and 206, and the low tyrosines found in samples I_4 and II_4. In the case of the amino sugar determinations, steps taken for the removal of precipitating antigen (*i.e.* washing with trichloracetic acid-ether) from sample 206, did not appreciably diminish the amount of glucosamine. It was also observed only in analyses 205 and 206 that higher than trace quantities of galactosamine were present. The reasons for the relatively high concentrations of amino sugars in these two anti-A samples, as opposed to the near-average values found in preparations I_4 and II_4 are not known. For purposes of transforming the experimentally observed data into residues of amino acids per mole of anti-A γG in experiments 205 and 206, the average value for glucosamine obtained from analysis of the other specific immunoglobulins (Table I) was used. The tyrosine values found with anti-A samples II_4 and I_4, were extremely low and none of the expected peaks corresponding to chlorotyrosine were found on the chromatograms. In this instance, in order to calculate the amino acid composition of the proteins, average values obtained for tyrosine in the non-specific γG (experiments 146, 147, and 148) were used. Amino acid compositions for runs 205, 206, II_4 and I_4 are considered reliable, since corrections for the anomalous pairs of determinations as shown above, gave values which essentially were normalized with respect to aspartic acid.

Since the anti-A preparations contain, as an upper limit, *ca.* 10 to 20 per cent γM (11), the absolute amino acid composition of these particular antibodies is somewhat less reliable than results reported in the Table for antilevan and anti-dextran. This reservation holds for antiteichoic acid as well. In spite of these uncertainties, the analytical values for a number of amino acids are extremely

TABLE I

Amino Acid Composition of Antibodies and Normal γ-Globulin From One Individual

	Antilevan					Antidextran		Antiteichoic acid		Anti "A"				γG-Globulin			Pooled γG		Pooled γG(4)	Pooled γM		Pooled γM (21)*
Designation.....	II_1	I_2	II	II	II	I_1	II_2	I_3	II_3	205	206	II_4	I_4	146	147	148	140	141		187	188	
Hrs. hydrolysed..	22	22	22	48	72	22	22	22	22	22	22	22	22	22	48	72	22	22	22	22	22	20
Aspartic acid	122	123	123	122	—	119	118	122	125	122	121	122	120	123	123	120	118	117	117	111	112	127
Threonine	113	113	114	106	—	108	110	109	111	126	128	120	124	111	105	99	113	113	115	116	116	140
Serine	146	144	146	124	—	148	147	150	151	156	155	153	150	146	126	106	156	153	171	141	141	173
Glutamic acid	152	155	152	152	151	149	149	149	150	148	150	147	145	146	143	143	145	142	143	139	136	151
Proline	103	99.2	99.5	102	102	102	99.4	108	108	109	112	104	108	107	108	108	115	115	112	97.1	97.1	106
Glycine	102	102	100	102	101	100	99.2	119	121	108	109	108	110	107	104	108	106	107	98.9	105	105	112
Alanine	86.2	84.7	84.5	85.7	85.4	79.2	79.0	82.4	84.0	84.4	81.5	83.0	84.2	81.4	79.9	79.9	81.2	80.8	75.4	85.4	82.6	97.9
Valine	131	132	132	131	131	133	135	102	102	116	114	116	113	139	136	139	127	129	126	106	104	124
Half-cystine‡	37.1	34.9	39.9	32.2	31.9	39.1	37.2	34.0	34.5	31.2	30.4	30.4	32.0	35.1	35.5	35.9	33.6	35.2	30.4	35.1	36.4	29.3
Methionine‡	14.4	13.7	14.6	14.3	13.3	14.8	16.5	16.4	16.7	13.4	13.7	14.0	14.9	13.7	13.1	13.9	12.0	12.2	7.5	13.3	12.7	17.8
Isoleucine	33.8	32.0	34.9	32.2	31.9	31.0	31.0	33.6	34.4	33.4	34.0	33.2	34.1	34.4	33.7	34.6	26.6	28.7	33.7	42.9	42.3	38.7
Leucine	116	115	116	118	116	108	110	109	111	116	114	113	110	116	116	115	113	113	108	98.0	98.0	114
Tyrosine	45.3	44.7	46.5	45.6	45.8	55.9	56.9	53.6	53.0	52.4	54.1	(9)§	(15)§	55.9	57.0	57.0	58.2	57.9	61.2	40.5	41.6	43.4
Phenylalanine	54.9	52.8	54.3	54.3	53.7	54.1	56.3	54.5	54.7	48.4	47.4	48.8	49.0	52.0	52.1	50.7	47.2	46.6	46.7	47.8	46.4	52.8
Lysine	93.4	94.0	93.0	93.9	91.8	87.0	88.0	80.8	83.0	80.1	81.2	78.6	76.5	93.3	92.9	93.2	89.9	89.5	90.9	64.7	63.4	78.0
Histidine	28.6	27.2	27.4	27.8	28.5	25.9	26.3	29.1	28.3	28.3	29.0	26.2	26.9	30.1	30.6	29.6	26.9	27.2	27.1	26.0	25.3	27.0
Arginine	46.5	46.1	46.5	47.1	46.5	47.8	48.6	58.1	59.1	56.2	56.0	53.8	55.0	47.8	49.5	47.5	45.1	45.2	42.5	55.0	56.1	62.2
Hydroxylysine	‖	‖	‖	‖	‖	3.2	3.2	3.7	3.6	‖	‖	‖	‖	‖	‖	‖	‖	‖	—	4.3	3.6	—
Glucosamine	5.0	6.1	7.2	5.0	¶	6.4	5.5	5.5	6.2	(17.8)§	(14.7)§	6.4	5.8	2.4	¶	¶	7.5	8.7	—	12.9	12.3	—

* The data of Heimer *et al.* (21) were converted into residues per 160,000 gm of γM, based upon the premise that the original percentages were given as gm anhydroamino acid per 100 gm of protein. With the exception of isoleucine and half-cystine, these amino acid residue values are consistently higher than found here for γM. On the other hand, the data of Chaplin *et al.*, given in reference 46, when calculated into residues as above, provide for a lower residual amino acid composition of γM, except for tyrosine.

‡ Sum includes oxidation products.

§ Values enclosed in parentheses are subject to the reservations discussed in the text.

‖ None detected in the protein hydrolysate.

¶ Trace quantity (less than 0.1 residue) found.

uniform for all of the antibodies. Thus, constancy in the number of isoleucine, methionine, aspartic, and glutamic acid residues give confidence to the significance of very appreciable differences which can be noted in the relative amounts of certain other amino acid residues.

The first salient finding in comparing the amino acid compositions of these specific γG-immunoglobulins was of a qualitative nature. It was noted (Table I) that antidextran and antiteichoic acid contain, to the nearest integer, 3 and 4 moles of hydroxylysine, respectively, and that γM contains 4 moles of this residue. This amino acid was absent from the antilevans (II_1, I_1, and II_2), from

TABLE II

*Significant Differences in Average Amino Acid Composition Among 6.5S Antibodies from One Individual**

	Antilevans	Anti-dextrans	Anti-teichoic acid	Anti-"A"	Normal γG	Pooled γM
Gm group	a−b−	a−b−	a−b−	a+b−	a+b+	
Glycine	101	99.5	120	108	107	105
Valine	132	134	102	115	139	105
Leucine	116	109	110	115	116	98.0
Tyrosine	45.5	56.0	53.3	53.2	55.9	41.0
Arginine	46.4	48.2	58.6	56.1	47.8	55.5
Lysine	93.5	87.5	81.9	79.1	93.2	64.0
Hydroxylysine	0	3.2	3.7	0	0	4.0
Threonine	113	109	110	127	111	116
Proline	101	101	108	108	107	97.1
[Arginine + lysine]	140	136	141	135	141	120

* All values taken at 22 hours hydrolysis. Residues per mol wt 160,000, with corrections as described in the text.

the anti-A samples (205, 206, II_4 and I_4), from the non-specific γG of subject 1 (experiments 146, 147, and 148), and from pooled human γG (experiments 140, 141).

The largest quantitative differences in average amino acid composition among the γG- and γM-proteins are assembled in Table II. Also listed are the Gm groups to which these immunoglobulins belong (11). Residue differences as large as 20 for glycine, 32 for valine, 10 for tyrosine, 12 for arginine, 7 for proline, 13 for lysine, and 14 for threonine, exist among the γG- specific antibodies. Even with the antiteichoic and anti-A antibodies, the direction or magnitude of some of these differences obviates reservations regarding the possible contamination with γM. It is a coincidence worth noting that the sum of lysine and arginine residues in all these proteins (with the exception of γM) is almost constant. In all four antibodies and in the γG- globulin there appears to be an

inverse relationship between the number of valine and of glycine residues. There is no apparent relationship of the pattern of these large amino acid differences to the allotype specificity of these immunoglobulins.

Many other less pronounced differences in amino acid content among these antibodies can also be seen on inspection of Table I. Examples of these are the lower average number of serine residues in antilevan and antidextran *versus* the quantity found in antiteichoic acid and anti-A; the converse holds true for the half-cystine composition of these antibodies.

Comparison of the analyses of pooled γG and of pooled γM in this study, with those published from other laboratories (21, 4), also reveals some noteworthy differences. While the amino acid content of pooled γG is remarkably similar to that given by Hsaio and Putnam (4) with respect to several amino acids, large discrepancies in the number of serine, glycine, half-cystine, methionine, tyrosine, and arginine residues can be observed (Table I). These analytical discrepancies may reflect the relative heterogeneity of the samples selected for amino acid determinations, since the range of variation in numbers of residues is no greater than the differences reported here among residues contained in the individual specific antibodies.

DISCUSSION

Previous reports have indicated that amino acid differences may be found between antibodies of different specificities isolated from single rabbits (7, 9). The amino acid composition of these antibodies did not vary according to the allotype of animal (9). It was inferred (7) that these residue differences were complementary in terms of numbers of acidic and basic amino acids, to the basic and acidic haptenic antigens which were used for antibody induction. Subsequently (9), a third antibody, directed against an uncharged antigen was found to differ from the other two in several amino acid residues. Antibodies isolated from individual rabbits specific for the β-D-glucosylphenylazo- and β-D-galactosylphenylazo-determinants, have also been demonstrated to differ in amino acid composition. In this study, the antiglucoside specific antibody was consistently found to contain 6 more tyrosine residues than the antigalactoside (10).

The experiments reported here present even more striking amino acid differences among purified antibodies from a single individual, in this case a human. Antibodies with four different specificities, as well as γG-globulin were analyzed. Differences of from 7 to 32 residues were found from one antibody to another for the amino acids listed in Table II, as compared with maximum variation of eight amino acids found with the three different rabbit antibodies (7, 9, 10). The Gm Groups of these human antibodies are also known and although the γG from the individual studied was Gm (a + b +), three of the antibodies were Gm (a − b −) and one, (anti-A) was Gm (a + b −). Since

the Gm (a+) titer of the anti-A was much lower in the purified antibody than in the original serum, the anti-A probably represents a mixture of Gm (a + b −) and Gm (a − b −) molecules. The amino acid composition of pooled γG- globulin was also determined but, as can be seen from Table II, neither contamination with γM nor the differences in Gm allotype appear to account for the variations found. There is no reason to ascribe the extensive divergences noted in amino acid composition of the human antibodies to differences in specificity. However, the existence of such significant differences among purified antibodies from a single individual, as well as the earlier data from single rabbits (7–10), appear to make untenable the hypothesis (23); (for a recent review see reference 24) that antibody specificity results exclusively from the folding of identical peptide chains. It is not known whether these differences are randomly distributed throughout the antibody molecules, or whether they are restricted to particular fragments. Koshland (25) has reported that some of the amino acid differences in rabbit antibody were associated with the H and others with the L chains. Determination of the extent to which the amino acid variations are associated with H or L chains, or with fragments obtained following enzymatic digestion, should help determine whether or not the variations are indeed associated with specificity. In a parallel investigation (10), amino acid differences between antigalactoside and antiglucoside antibodies isolated from individual rabbits were not reflected in their Porter I fragments.

The extensive heterogeneity of human γG-immunoglobulins has been demonstrated repeatedly. The evidence for this rests upon (*a*) the genetically determined (26–28) Gm and InV factors, (*b*) the large number of L chains observed (29) after starch gel electrophoresis at pH 8.8 following reduction and alkylation (30), (*c*) the finding that human myeloma globulins (31) and antibodies (32, 12) represent selected populations with respect to L chains, (*d*) the division of human γG- globulins into four or more subgroups with respect to their H chains (33, 34), (*e*) the existence of K and L (type I and II) determinants on the L chains revealed by use of antisera to Bence Jones or myeloma proteins (35, 36), and (*f*) the existence of antibodies with individual antigenic specificity (37). In combination, these indications of 6.5S antibody heterogeneity provide reasonable *a priori* explanations for the observed variations in amino acid composition among the different antibodies.

Furthermore, the antibodies studied here were isolated from the serum of an individual who had been immunized with a minimal dose of the corresponding purified antigens. If the response to different antigens results from the chance stimulation and proliferation of certain cell lines to produce selected populations of antibody molecules whose properties correspond to the type of γG which they synthesize, differences in composition among the antibodies would also be expected.

The antidextrans produced in different individuals varied in the Gm and

InV groups (11), and in the mobilities of their L chains on starch gel electrophoresis (12). It is probable that other antibodies also differ in these genetic properties from individual to individual. For example, by analysis of purified antidextran of differing Gm and InV group specificity, differences in amino acid composition might be found. However, it is possible that some of the amino acids listed in Table II will exhibit residual constancy and that this might be associated with antibody or other specificities. The extensive differences in amino acid composition between the types K and L Bence Jones Proteins (47, 48) and among individual Bence Jones proteins of type K (see reference 49) could be responsible for a good part of the differences reported for the purified antibodies.

The large dissimilarities in amino acid composition of the human antibodies as compared to those in rabbit antibodies would tend to indicate that the latter are relatively less heterogeneous. This has been evident for some time since rabbit antibody and γ-globulin gave one N-terminal alanine per mole and contain the sequence H_2N-ala-leu-val-asp-glu (38, 39), while γG-globulins of other species showed the presence of several N-terminal amino acids in less than molar ratios (40–42). It is interesting that a corresponding situation was not found among the C-terminal amino acids, since, for both human and rabbit γ-globulin, 4 moles of C-terminal amino acid were found per mole globulin (10).

The inverse ratios of glycine to valine and of arginine to lysine among the specific antibodies (Table II), may reflect an amino acid substitution mechanism involving single nucleotide alterations which could be involved in the determination of primary sequence which in turn endows specificity. It has already been demonstrated, at least in the case of one particular enzyme, that such a mechanism is involved (43). Additional speculation on a template theory for antibody formation which embodies RNA coding has been discussed by Haurowitz (24).

The presence of hydroxylysine in γM and in two of the four specific γG-antibodies, and its absence in two others, may explain conflicting reports in the literature. Waldschmidt-Leitz *et al.* (44) reported that this amino acid was present in human γ-globulin, but Crumpton and Wilkinson (5) were unable to confirm this finding. In addition Askonas and coworkers (45) detected hydroxylysine in certain electrophoretically separated rabbit globulins, whereas experiments in this laboratory which have sought the presence of this amino acid in highly purified specific rabbit γG- antibodies isolated from individual rabbits were negative (10). These discrepancies may be readily accounted for since hydroxylysine, which is present only in selected γG- immunoglobulins in extremely low quantity, would undoubtedly be missed in an analysis of pooled γG. In fact it may be noted that hydroxylysine was not detected in the γ-globulin of subject 1, despite its presence in two of his antibodies. It is significant that the antidextran which is free of γM contains as much hydroxylysine as the antiteichoic acid which may have a small γM- contamination (11).

SUMMARY

Antilevan, antidextran, antiteichoic acid, anti-"A" and normal γG, all isolated from a single human, as well as pooled γG and γM, were analyzed for amino acid content. Differences ranging from 7 to 32 residues were found among these antibodies in glycine, valine, leucine, tyrosine, arginine, lysine, and threonine content. These conspicuous differences in amino acid composition were not correlated with the Gm type of these antibodies.

Hydroxylysine was found in two of the four antibodies, antidextran and antiteichoic acid, and in γM- immunoglobulin. No hydroxylysine was found in γG- globulins. These findings probably account for discrepancies in the literature concerning the occurrence of this residue in antibody protein.

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