

## STUDIES ON HUMAN ANTIBODIES

### V. AMINO ACID COMPOSITION OF ANTIDEXTRANS OF THE SAME AND OF DIFFERING SPECIFICITIES FROM SEVERAL INDIVIDUALS\*

BY MARIANNE M. DORNER, M.D., EMMETT W. BASSETT, PH.D.,  
SAM M. BEISER, PH.D., ELVIN A. KABAT, PH.D., AND  
STUART W. TANENBAUM, PH.D.

(From the Departments of Microbiology and Neurology, College of Physicians and Surgeons of Columbia University, and the Neurological Institute Presbyterian Hospital, New York)

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Recent investigations (1) have revealed striking differences in the amino acid composition of four different human  $\gamma$ G-antibodies from one individual. These antibodies, antilevan, antidextran, antiteichoic acid, and anti-blood group A substance, included an antidextran of  $\alpha$ -(1  $\rightarrow$  6) specificity. In the present study, amino acid analyses were carried out on three additional human antidextrans of  $\alpha$ -(1  $\rightarrow$  6) specificity as washed dextran-antidextran-specific precipitates. In addition, antibody fractions were analyzed from three individuals who had been immunized with a dextran containing two types of antigenic determinants and who had formed some antibodies directed against  $\alpha$ -(1  $\rightarrow$  6)-linked glucosyl units and others specific for  $\alpha$ -(1  $\rightarrow$  2)-linked glucose residues (2). After precipitation of the antidextran of  $\alpha$ -(1  $\rightarrow$  6) specificity with a dextran containing 96% of  $\alpha$ -(1  $\rightarrow$  6) and 4% of  $\alpha$ -(1  $\rightarrow$  3) linkages, the remaining antibody was precipitated from the supernatant fluids with the homologous dextran. Comparison of the amino acid composition of the various antidextrans of  $\alpha$ -(1  $\rightarrow$  6) specificity showed wide variations in certain amino acids. Significant differences were also observed in the antidextran fractions from a given individual produced against the two types of antigenic determinants. The extensive variations found provide further indications of the vast heterogeneity (3) of human antibodies even to well defined antigenic determinants of simple structure.

#### *Materials and Methods*

*Antisera.*—Human antisera from six individuals were studied. Subjects 20 and 30 were injected with clinical Swedish dextrans OP 155 and OP 163 respectively (4), while subject

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Fo received native NRRL B 512 dextran with 96%  $\alpha$ -(1  $\rightarrow$  6)- and 4%  $\alpha$ -(1  $\rightarrow$  3)-linked glucopyranose units (5). The antibodies in these three antisera had been shown to be  $\alpha$ -(1  $\rightarrow$  6) specific (6). Antisera 332 and 333 were produced by injection of dextran NRRL B 1299 S-3, antiserum R.G.M. was obtained by injecting dextran NRRL B 1424. The latter contains 72%  $\alpha$ -(1  $\rightarrow$  6) and 14%  $\alpha$ -(1  $\rightarrow$  2) linkages, and NRRL B 1299 S-3 contains 50%  $\alpha$ -(1  $\rightarrow$  6), 12%  $\alpha$ -(1  $\rightarrow$  4), and 38%  $\alpha$ -(1  $\rightarrow$  2) linkages (5). These three antisera had been shown to contain some  $\alpha$ -(1  $\rightarrow$  6)-specific antibody and some  $\alpha$ -(1  $\rightarrow$  2)-specific antibody (2).

*Isolation of Antibodies.*—Sera were cleared by prolonged centrifugation at 4°C. Amounts of antisera and dextran were chosen to give about 50  $\mu$ g specific precipitate N at the point of maximum precipitation, as determined from quantitative precipitin curves (2, 5). The  $\alpha$ -(1  $\rightarrow$  6) antidextran in sera 332, 333, and R.G.M. were absorbed with a native B 512 dextran N 279. Supernatant fluids were then set up with dextran B 1424 to precipitate antibodies of non- $\alpha$ -(1  $\rightarrow$  6) specificity, which were largely  $\alpha$ -(1  $\rightarrow$  2) specific and are so designated in the tables. All precipitates were washed three times with cold 0.001 M phosphate-buffered saline at pH 7.2. The washed precipitates were used directly for amino acid analyses, and for spectrophotometric determination of tyrosine and tryptophan (7).

*Amino Acid Analyses.*—Samples in 12-ml centrifuge tubes were lyophilized, and dissolved in 0.15 ml constant boiling HCl. The tubes were evacuated, sealed, and refluxed in toluene for 22, 48, and 72 hr respectively. After cooling, the seals were broken and HCl was removed *in vacuo* over NaOH. Residues were dissolved in 0.12 ml starting buffer and applied to the column of the amino acid analyzer. Analyses were carried out by the method of Piez and Morris (8) as described in detail previously (1). For spectrophotometric determination of tyrosine and tryptophan (7), specific precipitates were dissolved in 0.2 ml 0.1 N NaOH and concentrations were adjusted to give *E* values between 0.3 and 0.8 in absorption cells of 10 mm light path. A Gilford photoelectric spectrophotometer with a Beckman DU monochromator was used. Spectra of protein solutions can give varying amounts of irrelevant absorption which contribute to the *E* values at a given wavelength. These decrease with increasing wavelength. Therefore, values varying from 0 to 0.184 for irrelevant absorption were taken into account by readings obtained between 370–340  $m\mu$  plotted and extrapolated to 294.4 and 280  $m\mu$ , as proposed by Goodwin and Morton (7). Additional determinations of tryptophan and phenylalanine were done by the Ba(OH)<sub>2</sub> hydrolysis procedure of Noltmann et al. (9), followed by automatic amino acid analyses of the alkaline hydrolysates.

## RESULTS

Amino acid analyses on the dextran-antidextran-specific precipitates after acid hydrolysis for 22, 48, and 72 hr are presented in Table I, expressed as moles of amino acid residues per mole (160,000) of protein. In those cases in which protein hydrolysis with Ba(OH)<sub>2</sub> was used, the number of phenylalanine residues found agreed closely with data obtained after 22 hr of acid hydrolysis. Spectrophotometric determination of tryptophan averaged 5 residues higher than values after alkaline hydrolysis. The spectrophotometric data for the number of tyrosine residues agreed closely with those obtained by acid hydrolysis, corrected to zero time. The 22-hr hydrolytic sample of subject Fo was unfortunately lost, and the 48 hr values have been used uncorrected.

There are extensive differences in amino acid content among the antidextran studied. For all the specific antibodies in Table I, the differences among the less stable amino acids, aspartic and glutamic acid, are as large as 14 and 16

TABLE I  
Amino Acid Composition of Human Antidexran Antibodies

Hydrolysed, hr...	R.G.M.						333						332						30			20			Fo							
	anti- $\alpha$ -(1-6)		anti- $\alpha$ -(1-2)		anti- $\alpha$ -(1-6)		anti- $\alpha$ -(1-2)		anti- $\alpha$ -(1-6)		anti- $\alpha$ -(1-2)		anti- $\alpha$ -(1-6)		anti- $\alpha$ -(1-2)		anti- $\alpha$ -(1-6)		anti- $\alpha$ -(1-2)		anti- $\alpha$ -(1-6)		anti- $\alpha$ -(1-2)		anti- $\alpha$ -(1-6)		anti- $\alpha$ -(1-2)		anti- $\alpha$ -(1-6)			
	48	72	22	48	72	22	48	72	22	48	72	22	48	72	22	48	72	22	48	72	22	48	72	22	48	72	22	48	72	22	48	72
Aspartic acid	120	119	121	120	114	119	121	118	119	119	120	112	114	112	114	112	122	125	122	119	119	116	116	106	122	102	88.4	104	104	104	104	
Threonine	109	104	112	105	94.8	116	108	81.1	115	103	96.2	108	103	89.5	117	114	87.1	108	108	106	122	102	88.4	104	104	104	104	104	104	104	104	
Serine	125	116	141	126	110	145	133	101	140	120	110	137	121	104	139	120	100	154	124	112	157	134	117	137	114	114	114	114	114	114		
Glutamic acid	151	154	146	143	147	151	150	150	145	143	145	138	137	137	143	140	140	147	145	145	138	140	140	138	140	140	140	140	140	140	140	
Proline	103	107	99.1	98.4	98.2	103	101	100	102	99.0	103	100	99.3	100	105	104	104	100	103	102	99.1	98.4	101	104	102	104	102	104	102	104	102	
Glycine	100	99.7	99.9	111	112	109	105	103	105	102	99.0	103	98.4	98.8	104	101	102	98.2	101	101	104	104	105	104	104	104	104	104	104	104	104	
Alanine	84.2	85.0	83.7	79.5	81.2	80.4	81.0	78.2	89.4	89.0	90.1	86.5	87.1	85.4	81.2	80.5	81.6	82.4	81.5	82.3	88.5	87.1	87.5	88.5	87.1	87.8	87.8	87.8	87.8	87.8		
Valine	131	129	133	123	126	132	130	133	123	125	125	122	121	122	129	129	130	136	138	132	131	134	119	117	117	117	117	117	117	117	117	
1/2 Cys-tine	34.4	36.1	32.2	33.2	33.0	31.6	37.4	35.8	34.2	34.4	34.4	35.3	34.7	34.0	35.0	35.4	35.0	34.5	32.1	35.4	30.2	31.4	34.9	35.2	35.2	35.2	35.2	35.2	35.2	35.2	35.2	
Methionine	12.0	13.6	14.0	13.7	14.5	12.5	14.9	14.1	12.7	14.2	12.2	12.2	—	—	—	—	—	—	13.9	13.9	15.4	14.5	11.5	11.7	11.7	11.7	11.7	11.7	11.7	11.7	11.7	
Isoleucine	35.4	33.9	34.9	36.3	36.7	37.8	36.1	34.5	33.9	36.8	37.0	37.8	35.3	35.3	34.4	34.9	35.0	35.4	30.8	34.7	33.6	31.9	32.0	34.7	34.7	34.7	34.7	34.7	34.7	34.7	34.7	
Leucine (117)	110	110	111	111	110	109	108	105	110	109	108	109	106	109	110	109	111	105	107	107	108	110	108	112	110	110	110	110	110	110	110	
Tyrosine	55.5	52.0	51.5	52.5	51.0	51.2	54.4	50.2	44.0	54.6	54.5	51.0	50.8*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Phenylalanine	52.5	51.1	51.5	49.1	50.0	50.4	51.2	50.4	51.5	51.2	51.6	52.0	52.0	50.8	52.0	50.5	49.6	51.0	—	51.7	52.1	47.4	46.8	46.0	46.0	46.0	46.0	46.0	46.0	46.0	46.0	
Lysine	89.7	87.4	88.6	77.8	81.4	78.6	87.1	88.4	87.6	80.3	78.9	80.2	86.7	85.0	84.8	80.7	81.4	80.8	89.3	90.0	90.1	86.2	84.5	84.4	84.4	84.4	84.4	84.4	84.4	84.4	84.4	
Histidine	32.0	31.1	30.4	28.0	29.6	28.5	29.2	28.0	27.7	27.9	28.0	27.4	26.5	26.6	27.1	27.2	27.8	27.1	25.5	25.4	25.4	27.2	27.0	26.9	26.9	26.9	26.9	26.9	26.9	26.9	26.9	
Arginine	50.1	48.4	49.0	52.6	52.4	53.5	45.0	44.8	44.4	49.0	50.0	50.3	47.3	48.1	47.3	54.1	53.5	43.6	44.0	43.5	44.1	45.2	44.1	47.4	49.0	49.0	49.0	49.0	49.0	49.0	49.0	
Glucosamine	8.1	1.9	—	6.9	—	—	5.6	—	—	6.4	3.6	—	7.9	—	—	7.8	—	4.7	—	—	—	—	—	—	—	—	—	—	—	—	—	
Tryptophan	30.0†	—	—	32.9†	—	—	30.6†	—	—	29.6†	—	—	30.3†	—	—	31.0†	—	30.0†	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Hydroxylysine	—	—	—	2.6	2.4	2.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

\* Ba(OH)<sub>2</sub> hydrolysis.  
† Spectrophotometric determination.

residues respectively. Although the rate of destruction for the acid-labile amino acids threonine and serine varied from one particular antibody sample to the other, the differences in 22-hr values of 14 residues for threonine and 20 residues for serine can be taken as significant. Excluding the value of 117 residues in the R.G.M. 22-hr sample, the range in leucine values was only 7 residues. Since these values then become intrinsically normalized for leucine, results are comparable. Among the more stable amino acids, residue differences as large as 12 lysines, 13 glycines, 10 alanines, 17 valines, 6 histidines, and 10 arginines were

TABLE II  
*Significant Differences in Amino Acid Composition of  $\alpha$ -(1  $\rightarrow$  6) Antidextrans*

	R.G.M.	333	332	30	20	Fo*	1
Gm group.....	a + b -	--	--	a + b -	a - b +	a - b +	a - b -
Inv group.....	a -	--	--	a -	a -	a +	a -
Glycine	100	105	98	98	104	95	100
Valine	131	132	122	136	132	119	134
Alanine	84	80	87	82	89	78	79
Arginine	50	45	47	44	44	47	48
Lysine	90	87	87	89	86	83	88
Hydroxylysine	—	—	—	—	—	—	3.2
Threonine	118	116	108	108	122		109
Serine	147	145	137	154	157		148
Aspartic acid	117	119	112	119	106	120	119
Glutamic acid	154	151	138	147	138	141	149
Proline	105	103	100	100	99	104	101
Tyrosine	56	54	51	54	54	50	56
Leucine	117	109	109	105	108	112	109
Arginine + lysine	140	132	134	133	130	130	136

\* 48-hr values.

found among all the antidextrans studied. Hydroxylysine appeared as a constituent amino acid only in the  $\alpha$ -(1  $\rightarrow$  2)-specific antidextran of R.G.M. Maximum differences of but six residues were found in proline, seven in half-cystine, three in methionine, six in isoleucine, three in tyrosine, and six in phenylalanine.

The most significant differences in amino acid composition of the  $\alpha$ -(1  $\rightarrow$  6)-specific antibodies are summarized in Table II. Values are those obtained at 22 hr, except for subject Fo for whom the 48 hr results are given. The earlier values on subject 1 are included together with information regarding the Gm and Inv groups to which these antibodies belong (10). There is no apparent relationship of these genetic factors to the amino acid composition of antidextrans. This is hardly surprising since the difference between Inv (a) and Inv

(b) involve but a single substitution of leucine for valine on the light chains (11) and perhaps Gm determinants also may involve limited substitutions.

Table III lists the differences in amino acid composition for the  $\alpha$ -(1  $\rightarrow$  6)- and  $\alpha$ -(1  $\rightarrow$  2)-specific populations of antidextran from the three individuals studied. In general, residue differences are smaller among the  $\alpha$ -(1  $\rightarrow$  2) antibodies, than among the  $\alpha$ -(1  $\rightarrow$  6)-specific antidextran in Table II. With all three individuals, the lysine content of the  $\alpha$ -(1  $\rightarrow$  2)-specific antibody was 6–12 residues lower than that of  $\alpha$ -(1  $\rightarrow$  6), but the  $\alpha$ -(1  $\rightarrow$  2)-specific antibodies had a somewhat higher arginine content than those of  $\alpha$ -(1  $\rightarrow$  6) specificity.

TABLE III  
*Significant Differences in Amino Acid Composition of Antidextran  $\alpha$ -(1  $\rightarrow$  6)- and  $\alpha$ -(1  $\rightarrow$  2)-Specific Fractions from the Three Individuals*

	R.G.M.		333		332	
	anti- $\alpha$ -(1 $\rightarrow$ 6)	anti- $\alpha$ -(1 $\rightarrow$ 2)	anti- $\alpha$ -(1 $\rightarrow$ 6)	anti- $\alpha$ -(1 $\rightarrow$ 2)	anti- $\alpha$ -(1 $\rightarrow$ 6)	anti- $\alpha$ -(1 $\rightarrow$ 2)
Glycine	100	111	105	102	98	104
Valine	131	123	132	123	122	129
Alanine	84	80	80	89	87	81
Arginine	50	53	45	49	47	54
Lysine	90	78	87	80	87	81
Hydroxylysine	—	2.6	—	—	—	—
Threonine	118	112	116	115	108	117
Serine	147	141	145	140	137	139
Aspartic acid	117	121	119	119	112	122
Glutamic acid	154	146	151	145	138	143
Proline	105	99	103	102	100	105
Tyrosine	56	53	54	55	51	45
Leucine	117	111	109	110	109	110
Arginine + lysine	140	131	132	129	134	135

With two of the samples, R.G.M. and 333, the valine content of the  $\alpha$ -(1  $\rightarrow$  6) fraction was eight and nine residues higher than that of the  $\alpha$ -(1  $\rightarrow$  2)-specific antibody, but with the third individual 332, the  $\alpha$ -(1  $\rightarrow$  2) antidextran had seven residues more than the  $\alpha$ -(1  $\rightarrow$  6) antidextran. Similar findings may be seen for glutamic acid. The  $\alpha$ -(1  $\rightarrow$  2) fraction of subject 333 was higher in alanine than the  $\alpha$ -(1  $\rightarrow$  6) fraction, but in the other cases values were reversed. The sum of arginine plus lysine ranged from 129 to 140 residues, considerably greater than the spread of 135–141 previously reported for four antibodies from a single individual (1).

#### DISCUSSION

The data presented in this and in an earlier communication (1) provide evidence for variation in the amino acid composition of antibodies formed in man

to carbohydrate-antigenic determinants, since significant differences were found in 13 amino acids of the 18 measured (Table I). The variation in numbers of residues of certain amino acids among antibodies of different specificities formed by a single individual is as great as the variation which can occur in antibodies of the same specificity (antidextran) from different individuals. These amino acids include glycine, lysine, threonine, proline, tyrosine, and leucine. However, the range in valine among the seven antidextrans was only 17 residues (Table II), while that for the four antibodies from one person was 32 residues (Table IV, column seven). With respect to alanine, serine, aspartic acid, and

TABLE IV  
*Maximum Variation in Number of Amino Acid Residues Encountered*

Amino acid	Four antibodies in one person	Total range of antidextran	Seven individuals $\alpha$ -(1 $\rightarrow$ 6) antidextran	Three individuals from $\alpha$ -(1 $\rightarrow$ 6) + $\alpha$ -(1 $\rightarrow$ 2) $\alpha$ -(1 $\rightarrow$ 6)	Three individuals $\alpha$ -(1 $\rightarrow$ 2)	$\alpha$ -(1 $\rightarrow$ 6) versus $\alpha$ -(1 $\rightarrow$ 2) for any individual
Glycine	20	16	10	7	9	11
Valine	32	17	17	10	6	9
Alanine	6	11	11	7	9	9
Arginine	13	9	6	5	5	7
Lysine	15	12	7	3	3	12
Threonine	17	18	14	10	5	9
Serine	10	20	20	10	2	6
Aspartic acid	6	16	14	7	3	10
Glutamic acid	3	16	16	16	3	8
Proline	7	6	6	5	6	6
Tyrosine	10	11	6	5	10	6
Leucine	7	7	7	0	1	1

glutamic acid the different antibodies from one individual showed much less variation than did the antidextrans from different individuals. The ranges for the two groups with respect to glycine and arginine may not be significant. If one compares the range for each amino acid among the antidextrans of  $\alpha$ -(1  $\rightarrow$  6) specificity (Table IV, column four), it is evident that the same relationships hold as for all of the antidextrans, except that the glycine range is but 10 residues as compared with 20 for the four different antibodies.

In columns five and six in Table IV are given the ranges in the  $\alpha$ -(1  $\rightarrow$  6)- and  $\alpha$ -(1  $\rightarrow$  2)-specific antibodies formed in the same three individuals. The valine, threonine, serine, and glutamic acid variation is greater for the  $\alpha$ -(1  $\rightarrow$  6) antidextran, while the range in tyrosine is greater for the  $\alpha$ -(1  $\rightarrow$  2) antibody. Table IV, column seven tabulates the maximum differences for each amino acid between the  $\alpha$ -(1  $\rightarrow$  6) and  $\alpha$ -(1  $\rightarrow$  2) antibodies in a given individual. The

differences are much greater, except for tyrosine, for the  $\alpha$ -(1  $\rightarrow$  6) antibodies than for the  $\alpha$ -(1  $\rightarrow$  2)-specific antibody among the three individuals, especially in lysine, aspartic acid, and glutamic acid. None of these antibody fractions represent homogeneous populations of antibody molecules especially with respect to their antibody-combining sites. They may, however, be relatively more homogeneous than the whole human  $\gamma$ G-immunoglobulin as a consequence of the antigenic stimulus which causes selection of the antibody-forming cells, and hence of  $\gamma$ G-molecules, with respect to the Gm factors and their H and L chains. As in the earlier study, (1), there is no direct basis for attributing the large differences found to the combining sites of these antibodies.

The extensive variations in amino acid composition of the human antibodies is in contradistinction to the relatively small differences reported by Koshland (12) for rabbit antibodies. Two possible interpretations come to mind with respect to these differences. (a) Rabbit antibodies and rabbit immunoglobulins may be intrinsically more homogeneous than human antibodies and  $\gamma$ G-immunoglobulins, although the antibodies studied have also been shown to be heterogeneous with respect to their antibody-combining sites (13; cf. 14). (b) The relatively intensive antigenic stimulation generally employed with animals as compared to the minimal stimulation used in humans may have resulted in the mobilization of most types of cells forming  $\gamma$ G-antibody in the rabbit, thereby giving rise to populations of antibody molecules to the various antigens in which the differences in amino acid composition are averaged.

Studies on human myeloma and Bence Jones proteins show that even these selected populations of molecules and their constituent chains are not homogeneous. Thus, Terry et al. (15) have reported that myeloma proteins give as many as eight light and nine heavy chain bands in disc electrophoresis. Normal  $\gamma$ G-immunoglobulin is even more heterogeneous with respect to the number of heavy and light chain bands which it gives in disc or starch gel electrophoresis (16, 17). Grey and Kunkel (18), and Terry and Fahey (19) have shown that myeloma globulins can be classified into four subgroups based on immunological differences among their heavy chains and that these differences correlate with the Gm genetic factors on the heavy chain (20). The light chains may be of two antigenic varieties, K and L, the specificities of each involving multiple antigenic determinants. In addition, the various purified antibodies as well as myeloma proteins have been shown to possess unique antigenic determinants which endow them with individual specificity (21). All of these differences may be indicative of great variation in amino acid composition and sequence for the myeloma proteins and antibodies. Studies on human antidextran (22) have shown them to be mixtures of molecules with both K and L light chains, to be different in heavy chain subgroups, and also to show individual specificity. There is thus ample evidence based upon these parameters to justify the variations in amino acid composition found.

Sequence determinations on isolated individual Bence Jones proteins (11, 23, 24) have shown that variations are confined to the amino terminal half of the molecule, the carboxy terminal portion being constant except for the leucine-valine substitution (25, 26) on residue 191<sup>1</sup> which is responsible for the Inv(a) and Inv(b) specificity, respectively. Similarly, studies of heavy chains and papain fragments of  $\gamma$ G-immunoglobulin have established that the amino terminal portion of the Fd fragment is also variable in amino acid sequence. Many of the sequence and compositional differences must have no relation to antibody specificity or to the antibody-combining site. Since human antidextran (2, 27) and other antibodies are known to be populations of molecules with combining sites which vary in size and in specificity, these differences too may be responsible for some of the variation in composition observed.

#### SUMMARY

Human dextran-antidextran-specific precipitates from individuals immunized with dextrans containing either one or two antigenic determinants, were analyzed for their content of various amino acids. Large differences in certain amino acids were found among  $\alpha$ -(1  $\rightarrow$  6)-specific antidextrans. Wide variations were also observed in antidextran fractions from a given individual specific for  $\alpha$ -(1  $\rightarrow$  6)- and  $\alpha$ -(1  $\rightarrow$  2)-linked glucose residues.

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<sup>1</sup> Residue 191 is the true residue number and in the numbering scheme of Titani et al. (11) residues were numbered 70a, 70b, and 70c; the desirability of renumbering in sequence was pointed out by Dr. Melvin Cohn.



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