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Classification of Blood-Group Antibodies as β_2M or Gamma Globulin

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Summary. Thirty selected blood-group antibodies (excluding anti-A and anti-B) have been classified as β_2M (19S γ) globulin, γ (7S γ) globulin or mixtures, using the following three methods: fractionation on a DEAE-cellulose column; indirect anti-globulin tests, using specific anti-β_2M-globulin and anti-γ-globulin sera; and treatment with 2-mercapto-ethanol. With only minor exceptions, results obtained with the three methods were in agreement.

Most blood-group antibodies within the Le, MNSs and P systems appear to be 'naturally occurring' and these were found to be β_2M globulin. Blood-group antibodies within the Rh, K and Jk systems, which had arisen after an antigenic stimulus, were usually γ globulin but were occasionally β_2M globulin.

Antibodies composed of β_2M globulin usually behave as agglutinins but may behave as incomplete antibodies (e.g. some examples of anti-Jk[a]); conversely, antibodies composed of γ globulin usually behave as incomplete antibodies but may behave as agglutinins (e.g. an example of anti-M).

The ability to bind complement seems to be related more to the blood-group specificity of the particular antibody than to its molecular size. For example, anti-Jk[a], when composed either of γ or β_2M globulin, seems invariably to bind complement, whereas potent anti-M or anti-Rh, whether composed of γ or β_2M globulin, do not bind complement.

INTRODUCTION

Several different terms are in use to describe the various proteins of which antibodies are composed. In the present paper the terminology of Grabar and Burtin (1960) has been followed. Thus the protein of which most antibodies are composed is described as γ globulin, rather that 7S γ globulin and the macroglobulin (19S γ globulin) of which some antibodies are composed is described as β_2M globulin.

The use of an ion-exchange column to separate plasma proteins (Peterson and Sober, 1956; Sober, Gutter, Wyckoff and Peterson, 1956) has proved particularly valuable in separating γ globulin from β_2M globulin. Thus, if a fraction containing γ and some β globulin is first separated from whole serum by an electrophoretic technique and is subsequently subfractionated by anion-exchange DEAE-cellulose chromatography using phosphate buffer pH 8 of increasing molarity (from 0.02 to 0.2M), a large part of γ globulin is recovered in the early fractions while the β_2M globulin is present only in the later fractions (Fahey and Horbett, 1959). Whole serum can be fractionated in the same way to discover whether particular blood-group antibodies are associated with γ or

β_2M globulin (Abelson and Rawson, 1959; Kochwa, Rosenfield, Tallal and Wasserman, 1961).

'Incomplete' blood-group antibodies can also be classified as γ globulin or β_2M globulin by using the indirect antiglobulin test and employing specific anti-γ and anti-β_2M globulin sera (Stratton, 1961; Polley, Mollison and Soothill, 1962).

A further useful aid to classification is to treat antibody-containing sera with a sulphydryl compound, which results in the dissociation of macroglobulins and thus inactivates β_2M globulin antibodies (Deutsch and Morton, 1957; Grubb and Swahn, 1958).

The object of the present work was to apply these three different methods to human sera containing various blood-group antibodies and thus to classify the antibodies as γ or β_2M globulin and to relate these findings to the serological characteristics of the particular antibodies.

MATERIALS AND METHODS

DONORS. Thirty donors were selected to provide a variety of blood-group antibodies. For convenience the sera have been divided into two main classes: those containing blood-group antibodies belonging to the Le, MNSs and P systems, together with isolated examples of antibodies of the ABO and Ii systems. These various blood-group antibodies are considered together because in most cases they appear to be 'naturally occurring'; that is to say, they are found in subjects who have received no recognizable antigenic stimulus. The results of studying examples of anti-A and anti-B are not included in the present paper but will be reported separately. Antibodies belonging to the Rh, K and Jk systems have been considered together because they are usually found in subjects who have received a blood transfusion or have given birth to an infant affected with haemolytic disease of the newborn and have in one or other of these two ways received an appropriate antigenic stimulus. A single example of serum from a patient with syphilitic paroxysmal cold haemoglobinuria was also studied.

Antibodies within the Le, MNSs and P Systems (with Single Examples of Anti-O(H) and Anti-I)

Anti-Le^a. Nine examples of anti-Le^a were studied (cases 1–9). Of these, eight were found in subjects who had not previously been transfused; the remaining subject (case No. 9) was a man who within the previous few months had received at least two transfusions of Le(a+) blood and several test injections of Le(a+) red cells; other details about this patient were reported by Cutbush, Giblett and Mollison (1957). Of the eight subjects who had not previously been transfused, seven were women, all but one (case No. 4) of whom had had one or more pregnancies.

The male subject (case No. 8) who had not been transfused at the time when anti-Le^a was first detected in his serum, was given a test injection of Le(a+) red cells preceding a cardiac operation, during which he was transfused with blood from many Le(a+) donors. A sample of serum taken 2 weeks after the injection of Le(a+) red cells, at a time when the titre of anti-Le^a had risen from 4 to 64, was used in the present investigation.

All the examples of anti-Le^a agglutinated Le(a+) red cells in saline at 20°, although the reactions of some samples, particularly from case No. 9, were very weak. All the sera gave a positive indirect antiglobulin test with Le(a+) red cells at 37°.

Anti-M. Two examples of anti-M were tested; one came from a woman (case No. 10) who had not previously been transfused but 28–36 years previously had undergone four normal pregnancies. This patient's serum agglutinated red cells strongly at 20° but only

very weakly at 34° and not at all at 37°. The serum did not sensitize red cells to an antiglobulin serum. Other details of this case were published by Cutbush and Mollison (1958).

The other example of anti-M came from a woman (case No. 11) who had not been transfused but had had three pregnancies, the last of which resulted in the birth of twins affected with haemolytic disease of the newborn due to anti-M. Details of the case were published by Stone and Marsh (1959) who found that the patient's serum agglutinated M-positive cells just as strongly at 37° as at lower temperatures.

Anti-S. Of the two examples of anti-S, the first came from a woman (case No. 12) who had had seven previous pregnancies and had had blood transfusions on three occasions. The anti-S was found only after the third blood transfusion and was not present in the earlier samples of serum.

The second example of anti-S (case No. 13) came from a patient who had had a hysterectomy and who had been deliberately immunized with S-positive red cells. The serum agglutinated red cells suspended in saline.

Anti-s. The example of anti-s came from a patient described by O'Riordan and Cann (1959). The patient (case No. 14) had not been transfused and the antibody was discovered during her third pregnancy, which resulted in the birth of a normal infant. The patient's serum agglutinated red cells suspended in saline. The reactions were as strong at 37° as at 19°. The reactions were not significantly enhanced by using enzyme-treated cells, or by suspending the cells in albumin.

Anti-P_1. Of the two samples of anti-P_1, the first came from a woman (case No. 15) who had never been pregnant and had not been transfused. The other (case No. 16) came from a man who had not previously been transfused. Both sera agglutinated P_1-positive red cells suspended in saline at temperatures up to about 30°. At 37° serum from case No. 15 sensitized P_1-positive red cells to an antiglobulin serum. Serum from case No. 16 originally sensitized red cells to an antiglobulin serum but the sample available in the present work has been stored for some years and was virtually unreactive at 37° in all tests.

Anti-$P+P_1$. Of the two samples of anti-$P+P_1$ (anti-Tj^a), the first (case No. 17) came from a woman who was pregnant for the first time. She had never been transfused with red cells but 5 years previously she had received a transfusion of plasma, some of whch presumably came from P_1-positive donors. This patient gave birth to an infant of genotype P_2p. The mother's serum agglutinated red cells suspended in saline and in the presence of complement produced brisk haemolysis.

The serum obtained from her infant agglutinated P_1-positive red cells suspended in saline but it did not agglutinate P-negative red cells suspended in saline. However, it did react weakly at 4° with P-negative red cells which had been enzyme-treated (personal communication from Drs. R. R. Race and Ruth Sanger).

The other example of anti-$P+P_1$ came from a woman (case No. 18) who had never been transfused and had not been pregnant. The characteristics of this antibody were very similar to those of case No. 17.

Anti-O(H). The only antibody of the ABO system investigated in the present work was an example of anti-O(H) found in a woman (case No. 19) who had had four normal pregnancies and had never been transfused. The serum agglutinated group O red cells strongly at temperatures up to about 30° and was not inhibited by H substance; at 37° the serum sensitized group O red cells to an antiglobulin serum; other details have been published previously (Mollison, 1959).

Anti-I. The single example of anti-I (case No. 20) came from the normal donor described by Jenkins, Marsh, Noades, Tippett, Sanger and Race (1960). The serum strongly agglutinated red cells at temperatures up to 30°, but in tests carried out strictly at 37° with cells and serum warmed to this temperature before being mixed together, the serum did not agglutinate cells but sensitized them to agglutination by an antiglobulin serum.

Antibodies within the Rh, K and Jk Systems (with a Single Example of Serum from a Patient with Syphilitic Cold Haemoglobinuria)

Anti-D. Three examples were tested; all came from women who had been immunized by pregnancy alone and had given birth to infants affected with haemolytic disease of the newborn. One of the sera (case No. 21) agglutinated cells suspended in saline and when diluted to a point at which it would no longer produce agglutination, it gave a positive indirect antiglobulin test. The other two sera (cases Nos. 22 and 23) failed to agglutinate Rh-positive cells suspended in saline but strongly sensitized them to agglutination by an antiglobulin serum. Serum from case No. 23 was selected because it was the only one, out of about twenty examples of incomplete anti-Rh, the activity of which was diminished by treatment with 2-mercapto-ethanol.

Anti-c. Of the two examples tested, one came from a woman (case No. 24) who had originally been immunized by transfusion and had subsequently given birth to infants affected with haemolytic disease of the newborn. The other example came from a woman (case No. 25) who had previously been pregnant and had also received many blood transfusions. Both examples of anti-c were 'incomplete'.

Anti-E. The single example tested came from a patient (case No. 26) with chronic lymphatic leukaemia who had received many blood transfusions. The serum agglutinated red cells suspended in saline and no incomplete antibody could be demonstrated.

Anti-Jk^a. One example came from case No. 26 referred to above and the other from a subject (case No. 27) who had received many injections of red cells.

Anti-K. Two examples were tested: one came from a woman (case No. 28) who had never been pregnant but had received a large number of blood transfusions and the other came from a woman (case No. 29) who had been pregnant with one K-positive infant after receiving a transfusion of K-positive blood.

Serum from a Patient with Syphilitic Cold Haemoglobinuria. Serum from a patient with syphilitic paroxysmal cold haemoglobinuria (case No. 30) was tested. When fresh serum from this patient was mixed with normal group O red cells at temperatures in the range 0° to 18°, antibody was bound as shown by the fact that the red cells, after washing, were agglutinated by an antiglobulin serum. When the mixtures were left at 18°, the red cells were only very weakly sensitized but at 0° the cells were very strongly sensitized and on subsequent incubation at 37°, haemolysis occurred. The method of titrating the serum is described below.

ION-EXCHANGE CHROMATOGRAPHY. The method used was a minor modification of that described by Levy and Sober (1960). The diethylaminoethyl-cellulose anion exchanger used was Whatman's powder DE 50.

The material was treated with N NaOH, then washed with a large volume of distilled water followed by 0.02 M NaH_2PO_4 solution. The column was then packed and equilibrated with 0.02 M phosphate buffer pH 8.1. About 2 g. of DEAE-cellulose was used to prepare a column for 0.5–1.0 ml. of serum.

The column was mounted above a fraction-collector and separate 5 ml. amounts were

collected in tubes. Only two buffers were used, the first at 0.02 M (pH 8.1) and the second at 0.2 M (pH 8.1). In most cases the second phosphate buffer contained in addition 1 M NaCl. About 75 ml. of the first buffer was used followed by a similar volume of the second. The effluent fractions of each tube were examined in a Unicam spectrophotometer at 280 mμ. From each fraction (0.02 M and 0.2 M) tubes containing significant amounts of protein were pooled and the pool was concentrated by dialysis against polyethylene glycol ('Carbowax') M.W. 20,000 for 36–48 hours at 4° and then dialysed against 1 per cent saline buffered to a pH of 7.4 for 24 hours at 4°.

In order to study the distribution of antibody in one particular serum in more detail, step-wise elution using buffers ranging from 0.02 M to 0.2 M was used. Selected portions of effluent were collected, concentrated with 'Carbowax' and then tested for their content of γ globulin and β_2M globulin and of antibody.

Assay of γ and β_2M Globulin in Fractions from the DEAE-Cellulose Column

The concentration of γ and β_2M globulin in fractions obtained from the anion-exchange column was estimated in every case, using the inhibition of antiglobulin method. The γ globulin concentration was estimated by inhibiting the reaction between anti-γ-globulin and Rh-sensitized red cells, following the technique of Mollison (1956) (see also Kekwick, Vallet, Cutbush, Mollison, Thomas, Gell and Soothill, 1961); β_2M globulin was estimated by inhibiting the reaction between anti-β_2M-globulin and Le^a-sensitized red cells, using the technique described by Polley *et al.* (1962).

SEROLOGICAL TESTS

Agglutination of Red Cells Suspended in Saline

Serial, doubling, dilutions of the serum were made in saline and to each was added an equal volume of a 2 per cent suspension of red cells in saline. In titrating Rh antibodies the mixtures were left at 37°, but in all other cases were left at room temperature (approx. 20°). After 2 hours the deposited red cells were examined microscopically. The degree of agglutination was scored, using the system recommended by Race and Sanger (1956).

Indirect Antiglobulin Tests

The two-stage antiglobulin test described by Polley and Mollison (1961) was used; that is 1 volume of a 20 per cent suspension of red cells was first incubated at 37° for 90 minutes with 4 volumes of EDTA-treated antibody-containing serum; the cells were then washed three times and incubated at 37° for 15 minutes with 2 volumes of fresh normal serum, as a source of complement. Finally the cells were washed and tested with specific anti-γ-, anti-β_2M- and anti-β_1- (complement) globulin sera. (The preparation of the anti-γ and anti-β_2M sera used in the present work is described by Polley *et al.* (1962).)

The fact that the reactions were due respectively to anti-γ and anti-β_2M was confirmed by showing that the reactions were abolished by adding to the diluted antiglobulin serum an equal volume of 0.01 per cent γ globulin or of 0.005 per cent β_2M globulin. The fact that the reactions were due to anti-β_1-(complement) globulin was confirmed by showing that the addition to the antiglobulin serum of a 1 in 40 dilution of serum from a patient with hypogammaglobulinaemia, inhibited the reactions but that the addition of γ and β_2M globulin (as above) did not; further confirmation was obtained by incubating the red cells with EDTA-treated serum alone and showing that no reaction was then obtained with an anti-β_1-globulin serum.

The score of each antibody-containing serum was determined by preparing a series of doubling dilutions of the serum and testing each one as above. The degree of agglutination, with the antiglobulin serum, of each dilution was scored and the sum of the scores was recorded as the antibody score.

When sera agglutinated red cells suspended in saline at 37° the indirect antiglobulin test could not be applied (see Tables 2 and 3).

When testing serum from the patient with syphilitic cold haemoglobinuria (case No. 30) by the indirect antiglobulin test, a two-stage test was not used because, although some antibody was adsorbed on red cells in the absence of complement, more was adsorbed when complement was present at the same time. Antibody-containing serum and fresh normal serum were absorbed with Zymosan (3 mg./ml.) to prevent haemolysis, and 4 volumes of both were mixed with 1 volume of a 20 per cent suspension of normal group O red cells and allowed to stand at 0° for 90 minutes. The mixtures were then transferred to a water bath at 37° and after 15 minutes' incubation the cells were washed and tested with antiglobulin sera.

Haemolysis

Four volumes of each of a series of doubling dilutions of the antibody-containing sera were incubated at 37° for 90 minutes with 4 volumes of fresh normal serum and 1 volume of a 5 per cent suspension of appropriate red cells. The tubes were then spun and examined for haemolysis and the results were scored, using an arbitrary scale.

When testing samples of serum from the patient with syphilitic cold haemoglobinuria, the mixture of the patient's serum, fresh normal serum and red cells was first allowed to stand at 0° for 90 minutes; then, without washing, the mixture was transferred to a water bath at 37° and incubated for 30 minutes before being examined for haemolysis.

Comparison of Results on Serum and on Fractions

In comparing results obtained on fractions, dilution had to be taken into account. The method of doing this was as follows: supposing the fraction was diluted 1 in 2, then in considering the score the reaction of the undiluted serum was excluded and the first reaction counted was that for the 1 in 2 dilution. This is illustrated in Table 1.

TABLE 1

METHOD OF CORRECTING THE 'SCORE' OBTAINED WITH WHOLE SERUM FOR COMPARISON WITH THE SCORE OBTAINED WITH A DILUTED FRACTION

	Dilutions of serum (reciprocals)					
	1	*2*	*4*	*8*	*16*	*32*
Observed reaction	+++	+++	++	+	(+)	–
Score*	10	10	8	5	3	0

*See Race and Sanger (1956, p. 246).

In this example the observed score is 36; if the reaction with undiluted serum is omitted, the 'corrected score' becomes 26.

TREATMENT WITH 2-MERCAPTO-ETHANOL. One volume of undiluted serum was mixed with an equal volume of 0.1 M 2-mercapto-ethanol in phosphate buffer pH 7.4 and left at 37° for 2 hours; the mixtures were then dialysed overnight against buffered saline at 4°. As a control, 1 volume of undiluted serum was treated with buffer alone in the same way and examined at the same time as the treated serum.

RESULTS

Amount of γ Globulin and β_2M Globulin in Fractions from the DEAE-Cellulose Column

Estimates of the γ globulin recovered in the 0.02 M fraction ranged from 80 to 130 per cent; a small amount of γ globulin could always be detected in the 0.2 M fraction but the amount never exceeded 10 per cent of the total. The recovery of β_2M globulin in the 0.2 M fraction ranged between 80 and 130 per cent; no β_2M globulin could be detected in the 0.02 M fraction.

Antibodies of the Le, MNSs and P Blood-Group Systems (with Single Examples of Anti-O(H) and Anti-I)

Results of examining antibodies belonging to these various blood-group systems are shown in Table 2. As will be seen, with only three exceptions, the antibodies appeared to be exclusively β_2M globulin. Thus, apart from these three exceptions, serological activity was found only in the 0.2 M fraction from the DEAE-cellulose column; in those cases in which a positive indirect antiglobulin test was obtained, the cells reacted with an anti-β_2M-globulin but not with an anti-γ-globulin serum and, finally, all these antibodies were inactivated by treatment with 2-mercapto-ethanol.

The three exceptions were examples of anti-Le^a (case No. 9), anti-M (case No. 11), and anti-$P+P_1$ (case No. 17):

The anti-Le^a from case No. 9 was apparently composed partly of γ globulin because some serological activity was found in the 0.02 M fraction from the DEAE-cellulose column. The 0.02 M fraction, in the presence of complement, sensitized Le(a+) red cells to agglutination by an anti-β_1-globulin serum but this fraction, with or without complement, did not sensitize the red cells to agglutination by an anti-γ-globulin serum. These findings are referred to again in the Discussion.

The anti-$P+P_1$ from case No. 17 also appeared to be composed to a small extent of γ globulin; thus the 0.02 M fraction from the column agglutinated enzyme-treated P_1-positive red cells but not P_1-negative red cells (according to the terminology of Sanger (1955)).

The anti-M serum from case No. 11 was apparently composed exclusively of γ globulin, being present only in the 0.02 M fraction from the column, but treatment of the whole serum with 2-mercapto-ethanol slightly reduced the titre of the antibody.

Antibodies belonging to the Rh, K and Jk Systems (with a Single Example of the 'Donath-Landsteiner' Antibody

Of the blood-group antibodies listed in Table 3, only two — the anti-D from case No. 21 and the anti-E from case No. 26 — were capable of agglutinating red cells suspended in saline. In both cases only the 0.2 M fraction eluted from a DEAE-cellulose column displayed agglutinating activity; moreover, in both cases the agglutinating activity of whole serum was abolished after treatment with 2-mercapto-ethanol. Evidently, then, these agglutinins were β_2M globulin. The 0.02 M fraction prepared from the serum of case No. 21 behaved as an incomplete antibody, demonstrating that this serum contained a mixture of γ globulin (incomplete) and β_2M globulin (agglutinating) antibody molecules.

Of the remaining incomplete antibodies, almost all appeared to be composed solely of γ globulin. Thus they were found wholly or predominantly in the 0.02 M fraction eluted from a DEAE-cellulose column, they sensitized red cells to an anti-γ-globulin serum, and

TABLE 2

TESTS ON SERA CONTAINING ANTIBODIES OF THE Le, MNSs AND P SYSTEMS (WITH SINGLE EXAMPLES FROM THE ABO AND Ii SYSTEMS)

Case No.	Specificity of antibody	Test	Corrected score (see text) Whole serum	0.02 M fraction*	0.2 M fraction*	Reactions with antiglobulin sera Anti-γ	Anti-β1	Anti-β2M	Serological activity after SH treatment†
1	Anti-Le^a	Antiglob.	46	0	40	−	+	+	Nil
2	Anti-Le^a	Antiglob.	36	0	30	−	+	+	Nil
3	Anti-Le^a	Antiglob.	22	0	16	−	+	+	Nil
4	Anti-Le^a	Antiglob.	10	0	7	−	+	+	...
5	Anti-Le^a	Antiglob.	24	0	26	−	+	+	Nil
6	Anti-Le^a	Antiglob.	10	0	10	−	+	+	Nil
7	Anti-Le^a	Antiglob.	27	0	29	−	+	+	Nil
8	Anti-Le^a	Antiglob.	41	0	28	−	+	+	...
9	Anti-Le^a	Antiglob.	31	7	20	−	+	+	Nil
10	Anti-M	Agglut. (20°)	26	0	23	...	...	...	Nil
		Antiglob.	0	0	0	−	−	−	Nil
11	Anti-M	Agglut.	24	25	0	...	...	...	Diminished (16)
12	Anti-S	Agglut.	23	0	23	...	...	...	Nil
13	Anti-S	Agglut.	26	0	24	...	...	...	Nil
14	Anti-s	Agglut.	38	0	44	...	...	...	Nil
15	Anti-P_1	Agglut. (20°)	31	0	34	...	...	...	Nil
		Antiglob.	37	0	39	−	+	+	Nil
16	Anti-P_1	Agglut. (20°)	24	0	20	...	...	...	Nil
		Antiglob.	0	0	0	−	−	−	Nil
17	Anti-P+P_1	Agglut.	53	0	58	...	...	...	Diminished (17)
		Haemol.	20	0	23	...	...	...	Diminished (4)
18	Anti-P+P_1	Agglut.	40	0	44	...	...	...	Nil
		Haemol.	53	0	47	...	...	...	Nil
19	Anti-O(H)	Agglut. (20°)	15	0	12	...	...	...	Nil
		Antiglob.	18	0	18	−	+	+	Nil
20	Anti-I	Agglut. (20°)	36	0	22	...	...	...	Nil
		Antiglob.	26	0	18	−	+	+	Nil

Antiglob. = antiglobulin test after incubating red cells with antibody-containing serum and complement at 37°. When the antibody-containing serum agglutinated red cells at 20° but not at 37° the antiglobulin test could be carried out at 37° (cases 10, 15, 16, 19 and 20). When the antibody-containing serum agglutinated red cells at 37° the antiglobulin test could not be carried out on whole serum but those fractions which contained no agglutinating activity could be tested (for example the 0.02 M fraction in cases 12, 13, 14, 17 and 18 and the 0.2 M fraction in case 11). Negative antiglobulin tests were obtained in all these cases although the 0.02 M fraction from case 17 weakly agglutinated papain-treated red cells.

Agglut. = agglutination of saline-suspended red cells (at 37° unless otherwise specified).

Haemol. = haemolysis of red cells suspended in fresh serum at 37°.

... = not tested.

*Fractions obtained from a DEAE-cellulose column.

†Serological activity after treatment of the serum with a sulphydryl compound (2-mercapto-ethanol). When the activity was diminished, but not abolished, the figure in parentheses refers to the 'score' of the treated serum.

TABLE 3

TESTS ON SERA CONTAINING ANTIBODIES OF THE Rh, K AND Jk SYSTEMS (ALSO SERUM FROM A PATIENT WITH SYPHILITIC COLD HAEMOGLOBINURIA)

Case No.	*Specificity of antibody*	*Test*	*Corrected score (see text)* *Whole serum*	*0.02 M fraction**	*0.2 M fraction**	*Reactions with antiglobulin sera* *Anti-γ*	*Anti-β1*	*Anti-β2M*	*Serological activity after SH treatment†*
21	Anti-D	Agglut.	70	0	57	...	...	...	Nil
		Antiglob.	...	69	...	+	−	−	Unchanged
22	Anti-D	Antiglob.	101	121	56	+	−	−	Unchanged
23	Anti-D	Antiglob.	71	71	22	+	−	−	Diminished (53)
24	Anti-c	Antiglob.	105	87	46	+	−	−	Unchanged
25	Anti-c	Antiglob.	25	20	0	+	−	−	Unchanged
26	Anti-E	Agglut.	31	0	23	...	...	...	Nil
	Anti-Jk^a	Antiglob.	59	49	0	+	+	−	Unchanged
27	Anti-Jk^a	Antiglob.	49	0	44	−	+	+	...
28	Anti-K	Antiglob.	37	38	5	+	−	−	Unchanged
29	Anti-K	Antiglob.	18	16	8	+	+	+	Diminished (12)
30	Syphilitic cold haemoglobin-uria	Haemol.	28	21	0	...	...	...	Unchanged
		Antiglob.	26	10	0	+	+	−	Unchanged

Except in case No. 30 (see text), tests were carried out at 37°.

Antiglob. = antiglobulin test, after incubation of cells with antibody-containing serum and complement. In cases 21 and 26 (anti-E) the antiglobulin test could not be carried out on whole serum or on the 0.2 M fraction because these produced agglutination at 37°. The 0.02 M fraction could be tested and the results obtained in case 21 are shown. The results in case 26 were negative and are not shown.

Agglut. = agglutination of saline-suspended red cells.

Haemol. = haemolysis of red cells in fresh serum.

... = not tested.

*Fractions obtained from a DEAE-cellulose column.

†Serological activity after treatment of the serum with a sulphydryl compound (2-mercapto-ethanol). When the activity was diminished, but not abolished, the figure in parentheses refers to the 'score' of the treated serum.

they were not affected by treatment with 2-mercapto-ethanol. The only exceptions were one example of anti-Jk[a] (case No. 27) which appeared to be entirely β2M globulin; one example of anti-K (case No. 29) which appeared to be partly β2M globulin and one example of anti-D (case No. 23) which behaved in an anomalous way.

The anti-D from case No. 23 behaved as an incomplete antibody but after treatment with 2-mercapto-ethanol the titre was reduced, suggesting that the antibody might be composed partly of β2M globulin; on the other hand red cells sensitized with the antibody were agglutinated only by anti-γ globulin and not by anti-β2M globulin and the reaction with anti-γ globulin was completely inhibited by the addition of a preparation (G4) of γ globulin. On fractionating the serum on a DEAE-cellulose column, most of the antibody was found in the 0.02 M fraction. In order to study the distribution more completely fractionation was repeated with a much larger number of steps than usual. As Fig. 1 and Table 4 show, the activity was distributed between several fractions but none was found in the fraction containing the greatest amount of β2M globulin.

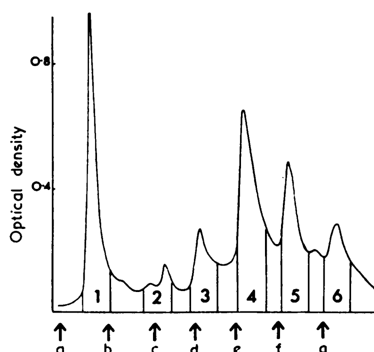

FIG. 1. Fractionation of serum from case No. 23. Elution was carried out from a DEAE-cellulose column using a series of phosphate buffers, pH 8, of increasing molarity, thus: a, 0.02 M; b, 0.04 M; c, 0.05 M; d, 0.06 M; e, 0.12 M; f, 0.16 M; g, 0.2 M. Successive 5 ml. samples were collected and the optical density of each determined. Samples were pooled as shown ('1', '2' etc.); serological tests on the samples are shown in Table 4.

TABLE 4

TESTS ON SEVERAL FRACTIONS OBTAINED FROM SERUM OF CASE 23

	Content of γ *globulin* (*as a percentage of amount in whole serum*)	*Content of* β2*M globulin* (*as a percentage of amount in whole serum*)	*Antibody 'score'* *Before SH* treatment*	*Antibody 'score'* *After SH* treatment*	*Reactions with antiglobulin serum*†
Whole serum	100	100	42	31	+
Fraction 1	40	<2	19	8	+
Fraction 2	4	<2	5	2	+
Fraction 3	6	<2	14	0	+
Fraction 4	<2	5	0	0	–
Fraction 5	<2	25	0	0	–
Fraction 6	<2	25	0	0	–

The fractions are those illustrated in Fig. 1.

*Treatment with a sulphydryl compound (see text).

†The reactions were inhibited completely by adding to the antiglobulin serum an equal volume of 0.01 per cent γ globulin. Reactions with an anti-β2M-globulin serum were all negative.

DISCUSSION

AGREEMENT BETWEEN METHODS

Fahey and Horbett (1959) used continuous-gradient elution from a DEAE-cellulose column with phosphate buffer pH 8 from 0.02 M to 0.30 M and divided the protein-containing effluent into five consecutive fractions of equal volume. On analysis in the ultracentrifuge, γ globulin only was detected in the first four fractions and most of this (about 75 per cent) was found in the first fraction. The fifth fraction contained predominantly β_2M globulin.

In the present work, elution was carried out with only two buffer solutions, the first of 0.02 M, pH 8.1 and the second of 0.2 M, pH 8.1. After concentrating each effluent fraction as described, the concentration of γ globulin in each fraction was determined by the inhibition of antiglobulin serum method. This method has been shown to be at least as sensitive as any other in estimating γ globulin; thus, it is possible to estimate concentrations considerably below 1 mg./100 ml. (Kekwick *et al.*, 1961). The method used here has a coefficient of variation of the order of ± 20 per cent.

The results confirm that the 0.02 M fraction eluted from the column contains most of the γ globulin present in the original serum. These findings agree broadly with the findings of Levy and Sober (1960) that 50–100 per cent γ globulin could be recovered from the column and with Fahey and Horbett's estimate of 90–100 per cent recovery.

No β_2M globulin could be detected in the 0.02 M fraction; to be more precise, this fraction, when added undiluted to diluted anti-β_2M-globulin serum, failed to inhibit the reaction with Le^a-sensitized red cells, indicating that the concentration of β_2M globulin was less than 0.3 mg./100 ml. in a solution containing more than 500 mg./100 ml. of γ globulin.

The 0.2 M fraction appeared to contain about as much β_2M globulin as was present in the original serum and between 3 and 10 per cent of the γ globulin.

Antibody composed solely of β_2M globulin is expected to be found exclusively in the 0.2 M fraction. The finding of any blood-group antibody activity whatever in the 0.02 M fraction indicates that the antibody is composed at least partly of γ globulin.

Antibody composed solely of γ globulin would be expected to be present mainly in the 0.02 M fraction. Accordingly, the results observed in cases 22, 23 and 24 call for comment. In these cases the antibody appeared to be composed solely of γ globulin, as judged by the fact that the sera sensitized red cells only to an anti-γ-globulin serum and not to an anti-β_2M-globulin serum. Nevertheless, instead of finding about one-tenth of the serological activity in the 0.2 M fraction obtained from the DEAE-cellulose column there appeared to be about one-third of the total, as judged by antibody scores. This is, in all probability, an artefact because the method of scoring overestimates the amount of antibody in relatively dilute samples.

It has been shown that in the antiglobulin test, γ globulin and β_2M globulin do not cross-react to any appreciable extent. Thus, red cells sensitized with incomplete anti-Rh are agglutinated by anti-γ-globulin but not by anti-β_2M-globulin sera and red cells sensitized with incomplete anti-Le^a are agglutinated by anti-β_2M-globulin but not by anti-γ-globulin sera (Polley *et al.*, 1962). In the present work, none of the antibodies classified as β_2M globulin on the fractionation results sensitized red cells to an anti-γ globulin and none of the antibodies classified as γ globulin on the fractionation results sensitized red cells to an anti-β_2M-globulin serum.

There was one unexpected negative result. Thus the anti-Le^a from case No. 9, a small

amount of which was recovered in the 0.02 M fraction from the column, failed to sensitize red cells to an anti-γ-globulin serum. The fact that this particular example of anti-Le^a was composed partly of γ (7*S* γ) globulin was previously reported (case No. 8) by Kekwick and Mollison (1961) as a result of tests on ether-fractionated samples.

Results following treatment with 2-mercapto-ethanol were in general those expected on the assumption that such treatment dissociates β_2M globulin but not γ globulin. Thus in all cases in which antibody appeared to be composed solely of β_2M globulin, treatment with 2-mercapto-ethanol resulted in loss of all serological activity. On the other hand, in a few cases in which an antibody appeared to be composed partly or solely of γ globulin there was some loss of activity after treatment with 2-mercapto-ethanol.

The anti-Le^a from case No. 9 which, as described above, appeared to consist predominantly of β_2M globulin but partly of γ globulin, was completely inactivated by treatment with 2-mercapto-ethanol, an observation which was repeatedly confirmed. The example of anti-M from case No. 11, which appeared to be composed solely of γ globulin, was partially inactivated by treatment with 2-mercapto-ethanol. Finally, the example of incomplete anti-D from case No. 23 which sensitized cells to an anti-γ-globulin serum but not to an anti-β_2M-globulin serum was partially inactivated by treatment with 2-mercapto-ethanol. This last result calls for some discussion, particularly in view of the fact that Kekwick and Mollison (1961) also concluded that this particular example of anti-D (case S.T. in that paper) was composed partly of β_2M globulin. As described in the Results, the antibody was subjected to a more detailed fractionation than that used for the remaining sera and it was found that all the antibody activity was in the fraction containing γ globulin and none in the fraction containing β_2M globulin. There was some evidence that the antibody in fraction 3 was particularly susceptible to damage by 2-mercapto-ethanol (see Table 4).

It must be concluded that the anti-D in case No. 23 was composed of protein which was antigenically identical with normal γ globulin, as shown by the fact that all fractions from the serum sensitized red cells to agglutination by an anti-γ-globulin serum. The unusual susceptibility of this antibody to treatment with 2-mercapto-ethanol cannot at present be explained.

The conclusion of Kekwick and Mollison (1961) that this incomplete anti-D was composed to some extent of β_2M globulin was based partly on the finding that the serological activity was reduced by 2-mercapto-ethanol and partly on the finding that a considerable part of the serological activity was recovered in the 'G 2/2' fraction obtained by the ether-fractionation process. It is now apparent that results with 2-mercapto-ethanol are not decisive and it has become apparent that separation of γ and β_2M globulin using the ether-fractionation method is sometimes rather unsatisfactory (unpublished findings based on estimates made by the inhibition of antiglobulin serum method).

In conclusion, the idea that incomplete anti-Rh may sometimes be composed of β_2M globulin is now seen to be unsupported by any really satisfactory findings.

Relation between Known Antigenic Stimuli and Molecular Size of Antibodies

In this paper antibodies have been divided into those belonging to blood-group systems in which the antibodies are usually 'naturally-occurring' and those in which the antibodies are usually 'immune'. Antibodies of the Le, MNSs and P systems are usually found in subjects who have not received a recognized antigenic stimulus. In the present work, eight out of nine examples of anti-Le^a were composed of β_2M globulin; the ninth was apparently

composed partly of γ globulin but this subject had received several transfusions of Le(a+) blood before his serum was examined for anti-Le^a.

One example of anti-M, two examples of anti-S and one of anti-s were all β_2M globulin; one example of anti-M was a γ globulin but this came from a woman who had been recently delivered of M-positive twins and she may have been immunized by pregnancy.

Two examples of anti-P_1 and one of anti-P+P_1 were composed solely of β_2M globulin; one other example of anti-P+P_1 was composed partly of β_2M globulin but this came from a woman who had been transfused with plasma which may well have contained P_1 substance; moreover, she had been pregnant with an infant of the genotype P_2p. The fact that some of this antibody was γ globulin had been expected from the fact that anti-P_1 had been found in the cord serum of her last infant.

A single example of anti-O(H) from a pregnant woman and one of anti-I from a normal donor described by Jenkins *et al.* (1960) were also found to be β_2M globulin.

Observations on case No. 8 showed that an increase in the titre of anti-Le^a following an antigenic stimulus could be due solely to the production of β_2M antibody. Similarly persons of group B when injected with human A substance, if they have an immune response, produce mainly β_2M anti-A (unpublished observations; see also Rawson and Abelson, 1960).

Antibodies of the Rh, K and Jk systems are usually γ globulin but may be β_2M globulin. For instance, out of fifteen examples of anti-Jk^a tested by Polley *et al.* (1962) twelve were γ globulin and three were β_2M globulin.

In the Rh system, β_2M-globulin antibodies behave as agglutinins; see case No. 21, and the anti-E in case No. 26; see also Campbell, Sturgeon and Vinograd (1955), Fudenberg, Kunkel and Franklin (1959) and Kekwick and Mollison (1961). However, in other blood-group systems β_2M globulin antibodies may behave as incomplete antibodies; see the example of anti-Jk^a in case No. 27.

Complement-Binding by Different Blood-Group Antibodies

The ability of particular blood-group antibodies to bind complement seems to be related more to their blood-group specificity than to their molecular size. Thus in the ABO, Le, P and Jk systems all antibodies active at 37° bind complement. Some of these antibodies, for example antibodies of the Le system, are almost always β_2M globulin but on the other hand most examples of anti-Jk^a are γ globulin.

Conversely, antibodies of the Rh, MNSs and Lu systems do not as a rule bind complement, the only exceptions being some examples of anti-S. Antibodies of the K and Fy systems bind complement in about 50 per cent of cases (unpublished observations).

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