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Antigenic Analysis of Rheumatoid Factor

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Summary. Papain digestion of human γ globulin yields two main antigenic components, of slow (S) and fast (F) electrophoretic mobility. Recent work has shown that β_2M globulin (γ_1 macroglobulin) contains S but not F antigenic groupings. It also contains specific groupings which may be called X. Rabbit antisera have been prepared which are specific for S, F and X.

Sheep red cells, 'sensitized' with rabbit anti-sheep cell serum as for the Rose-Waaler test, were incubated with rheumatoid arthritis sera. Under appropriate conditions these cells failed to agglutinate when resuspended in saline, but could be agglutinated by anti-γ globulin (S+F), anti-S and anti-X, but not by anti-F. Cells incubated with normal serum failed to agglutinate. Thus rheumatoid arthritis sera contain a protein possessing the antigenic characteristics of β_2M globulin which specifically coats sensitized sheep cells. This provides further evidence of the antibody nature of rheumatoid factor.

INTRODUCTION

Despite extensive investigation the significance of rheumatoid factor in the pathogenesis and evolution of rheumatoid arthritis remains obscure (cf. Christian, 1961). Current interest in auto-immune processes has prompted the speculation that rheumatoid factor is an antibody directed against altered γ globulin. The evidence for this is based firstly on observations that repeated injections of altered γ globulin or of other antigenic materials into animals give rise to antibodies which exhibit some of the characteristics of rheumatoid factor (Milgrom and Witebsky, 1960; Abruzzo and Christian, 1961; McClusky, Miller and Benacerraf, 1962); and secondly on the inability of physico-chemical fractionation methods to separate rheumatoid-factor activity from the serum β_2M globulin (19S γ globulin, γ_1 macroglobulin, iota protein), a protein which is known to possess antibody activity (Kunkel, Franklin and Müller-Eberhard, 1959; James, Felix-Davies and Stanworth, 1961). However, it is partly on account of this inability to separate these two materials that a detailed antigenic comparison of rheumatoid factor with other proteins known to possess antibody activity has not previously been undertaken.

Recently the antigenic relationships between two of these proteins, γ globulin and β_2M globulin, have been elucidated. Papain digestion of human γ globulin yields two main antigenic components, labelled Slow (S) and Fast (F) by Edelman, Heremans, Heremans and Kunkel (1960) on the basis of their different electrophoretic mobilities. The β_2M globulin also contains S component, but not F (Franklin and Stanworth, 1961; Rowe, 1962). In addition it contains other specific groupings (Franklin and Kunkel, 1957; Walton, Rowe, Soothill and Stanworth, 1963), which are here termed X. In this work rabbit antisera have been prepared which react specifically with γ globulin (S+F), S, F and X. They have been used to study the antigenic composition of rheumatoid factor.

Rheumatoid factor, free from other globulins of the γ system (Heremans, 1961), has been obtained by the specific reaction between sensitized sheep cells, as used in the Rose-Waaler test, and rheumatoid arthritis sera. It was found that under appropriate conditions suspensions of sensitized sheep cells which had been in contact with rheumatoid arthritis sera could be obtained which did not agglutinate in saline or in normal rabbit serum, but which could be agglutinated by certain of the antisera described above. The results indicate that the human serum protein present on these cells has the antigenic characteristics of 19S γ globulin.

MATERIALS AND METHODS

RHEUMATOID ARTHRITIS SERA. Serum from four patients, A, B, C and H, with typical rheumatoid arthritis was used. Rose-Waaler titres were A — 16,384; B — 128; C — 128; H — 1024. Sera from two normal individuals which gave negative Rose-Waaler tests were used as controls. All the sera were heated to 56° for 20 minutes to inactivate complement, and were absorbed by incubation at 37° for 1 hour with one-quarter the volume of packed sheep cells to remove sheep-cell antibodies.

ANTISERUM TO SHEEP RED CELLS. This was raised in a rabbit by a course of intravenous injections of cells washed in saline. A total of six injections each comprising 1 ml. of packed cells in 1 ml. of saline were given over the course of 15 days, and the rabbit was bled on the 21st day.

ANTISERA TO 7S γ GLOBULIN. Five rabbit antisera (S187, S188, S195, S198 and R335) to human γ globulin were used in this study. The antisera other than R335 were raised in rabbits to γ-globulin preparations obtained by chromatography (Sober, Gutter, Wyckoff and Peterson, 1956) or batch absorption (Stanworth, 1960) of normal serum using diethylaminoethyl cellulose. On immunoelectrophoretic analysis with human serum, S187 produced only the γ-globulin line. S188, S195 and S198 reproduced one or two faint lines in the β-globulin region in addition to the strong γ-globulin line. None of these antisera produced a β_2M-globulin line. R335 was raised to human γ globulin prepared as Cohn Fraction II, 1, 2. Immunoelectrophoretic analysis showed this antiserum to produce β_2M globulin and β-globulin precipitin lines in addition to a strong γ-globulin precipitin line.

Antisera to the S and F migrating components of digested γ globulin were prepared by absorption of anti-γ-globulin sera with F and S components respectively. These had been prepared from a sample of Cohn F II, 1, 2 γ globulin which was shown to be free of β_2M globulin both on double diffusion in agar and after coating tanned cells with the protein, using the antiserum to β_2M globulin.

After digestion of the Cohn fraction with papain by the method of Porter (1959), the S component of the digest was separated by the method of Rowe (1961), and the F component by a method similar to that of Franklin (1960). Antisera to the F component were prepared from the five anti-γ-globulin sera by absorption with approximately 0.5 mg. of S component per ml. of antiserum. The absorbed antisera failed to precipitate S component on double diffusion in agar, but readily precipitated F component and intact γ globulin. Since only small amounts of the F component were available, only one antiserum, S198, was absorbed with it. After absorption this antiserum failed to precipitate F component, but readily precipitated S component and γ globulin, on double diffusion in agar.

ANTISERUM TO β_2M GLOBULIN (S199). This was raised in rabbits to the washed cryo-

globulin of a patient with macroglobulinaemia. It was rendered specific for γ globulin by absorption with the serum of a patient with hypogammaglobulinaemia which was also deficient in the β_2M globulin, and for X component of β_2M globulin by absorption with γ globulin. It will be fully described by Walton *et al.* (1963).

ESTIMATION OF TITRE OF ROSE-WAALER ACTIVITY. A 2 per cent suspension of washed sheep cells in saline was sensitized by incubation at 37° for 1 hour with an equal volume of saline containing one-third of a minimum agglutinating dose (MAD) of the rabbit anti-sheep-cell serum. 0.2 ml. of the cell suspension was then mixed with 0.2 ml. of serial doubling dilutions of the rheumatoid arthritis sera in the depressions, approximately 1.5 cm. in diameter, of a moulded perspex plate. Normal serum and saline were used as controls. The end-point of agglutination was read immediately after incubation for 1 hour at 37°. The titre was recorded as the reciprocal of the highest dilution of the serum which gave positive agglutination.

COATING OF SHEEP CELLS WITH RHEUMATOID FACTOR AND THEIR REACTION WITH ANTI-γ-GLOBULIN SERA. A 2 per cent suspension of sheep red cells was sensitized with one-third MAD of the rabbit anti-sheep-cell serum as for the Rose-Waaler test. One volume of the suspension was then mixed with one volume of rheumatoid arthritis serum diluted with saline so as to contain a known proportion of 1 MAD, as determined by a previous test. After incubation for 1 hour at 37° in a water bath the cells were spun down. The supernatant was removed, and in some experiments the cells were then washed in saline. In others this stage was omitted, and in all experiments the cells were finally resuspended to give a 2 per cent suspension in saline.

Agglutination of these cells was then investigated by mixing 0.2 ml. of the cell suspension with 0.2 ml. of the antiserum diluted in saline in the depression of the moulded perspex plates. Agglutination could be read after incubation for 1 hour at 37°, but was more clear cut, although not quantitatively different, after further standing overnight at room temperature.

SALINE. A solution of sodium chloride, 0.9 g./100 ml., was used.

RESULTS

In initial experiments the sensitized sheep cells were incubated with concentrations of rheumatoid arthritis sera ranging from 4 to 0.25 MAD. After washing twice in saline the cells were then treated with various antisera as shown in Table 1. Even after treatment with more than 1 MAD of the rheumatoid arthritis sera the cells failed to reagglutinate after washing and resuspension in saline. The cells were, however, agglutinated by antisera to γ globulin (S+F), S component, and β_2M globulin (X), but not by antiserum to F component or by normal rabbit serum. Sensitized cells incubated with normal human serum or in saline were not agglutinated by any of the antisera. These results strongly suggested that after incubation with rheumatoid arthritis sera the cells were coated with a protein possessing antigenic components X and S, i.e. β_2M globulin, and that γ globulin (S+F) was not present on them, since anti-F failed to agglutinate.

However, these experiments were not regarded as entirely satisfactory, since positive agglutinations were only obtained on cells which had previously been agglutinated by the doses of rheumatoid arthritis sera employed.

In all subsequent experiments the sensitized cells were incubated with 1, 0.5 and 0.25 MAD of rheumatoid arthritis sera, spun down, and then made up as a 2 per cent

TABLE I

AGGLUTINATION OF RHEUMATOID-FACTOR-COATED CELLS BY VARIOUS ANTISERA. THESE CELLS WERE WASHED TWICE IN SALINE AFTER EXPOSURE TO THE HUMAN SERA

Antiserum (diluted in saline as indicated)		*Sensitized cells incubated with*												
		RA serum A MAD					*RA serum C MAD*					*Normal serum*		*Saline*
		4	*2*	*1*	*0.5*	*0.25*	*4*	*2*	*1*	*0.5*	*0.25*	*1/100*	*1/1000*	
Anti-γ globulin	1/100	+	+	−	−	−	+	+	−	−	−	−	−	−
(S198)	1/1000	+	−	−	−	−	+	+	−	−	−	−	−	−
Anti-F	1/50	−	−	−	−	−	−	−	−	−	−	−	−	−
(S198 absorbed S)	1/100	−	−	−	−	−	−	−	−	−	−	−	−	−
	1/1000	−	−	−	−	−	−	−	−	−	−	−	−	−
Anti-S	1/50	+	+	+	−	−	+	+	+	−	−	−	−	−
(S198 absorbed F)	1/100	+	+	+	−	−	+	+	+	−	−	−	−	−
	1/1000	+	+	−	−	−	−	−	+	−	−	−	−	−
Anti β₂M globulin	1/100	+	+	−	−	−	+	+	−	−	−	−	−	−
(S199) (absorbed γ globulin)	1/1000	+	+	−	−	−	+	+	−	−	−	−	−	−
Normal rabbit serum	1/100	−	−	−	−	−	−	−	−	−	−	−	−	−
	1/1000	−	−	−	−	−	−	−	−	−	−	−	−	−
Saline		−	−	−	−	−	−	−	−	−	−	−	−	−

suspension in saline without intermediate washing. Table 2 shows that positive agglutinations could be achieved, after this procedure, of cells which had been treated with less than 1 MAD of rheumatoid arthritis sera. Similar results to those shown in Table 1 were again obtained. Antisera to γ globulin, S component and β₂M globulin agglutinated the cells, whereas antisera to F component failed to do so. In further experiments using rheumatoid arthritis sera B and H, the same four anti-F sera failed to agglutinate at dilutions of 1 in 10, 1 in 20 and 1 in 40, although the anti-γ-globulin sera again agglutinated at 1 in 100 and 1 in 1000 dilutions.

TABLE 2

AGGLUTINATION OF RHEUMATOID-FACTOR-COATED CELLS BY VARIOUS ANTISERA. THESE CELLS WERE NOT WASHED AFTER EXPOSURE TO THE HUMAN SERA

Antiserum (diluted in saline as indicated)		*Sensitized cells incubated with*								
		RA serum A MAD			*RA serum C MAD*			*Normal serum*		*Saline*
		1	*0.5*	*0.25*	*1*	*0.5*	*0.25*	*1/100*	*1/1000*	
Anti-γ globulin	1/100	+	+	−	+	+	+	−	−	−
(S187)	1/1000	+	+	+	+	+	+	−	−	−
Anti-F	1/100	−	−	−	−	−	−	−	−	−
(S187 absorbed S)	1/1000	−	−	−	−	−	−	−	−	−
Anti-γ globulin	1/100	+	+	−	+	+	+	−	−	−
(S188)	1/1000	+	+	−	+	+	+	−	−	−
Anti-F	1/100	−	−	−	−	−	−	−	−	−
(S188 absorbed S)	1/1000	−	−	−	−	−	−	−	−	−
Anti-γ globulin	1/100	+	+	+	+	+	+	−	−	−
(S195)	1/1000	+	+	+	+	+	+	−	−	−
Anti-F	1/100	−	−	−	−	−	−	−	−	−
(S195 absorbed S)	1/1000	−	−	−	−	−	−	−	−	−
Anti-γ globulin	1/100	+	+	+	+	+	+	−	−	−
(S198)	1/1000	+	+	−	+	−	−	−	−	−
Anti-F	1/100	−	−	−	−	−	−	−	−	−
(S198 absorbed S)	1/1000	−	−	−	−	−	−	−	−	−
Anti-S	1/100	+	+	+	+	+	+	−	−	−
(S198 absorbed F)	1/1000	+	+	−	−	−	−	−	−	−
Anti-β₂M globulin										
(S199)	1/100	+	−	−	+	−	−	−	−	−
(absorbed γ globulin)	1/1000	+	+	−	+	−	−	−	−	−
Saline		−	−	−	−	−	−	−	−	−

To confirm that the failure of the antisera to F component to agglutinate was due to the absence of reacting F antigenic groupings on the cells rather than the absence of agglutinins in the antisera, various antisera were tested with sheep red cells, tanned and coated with γ globulin (Cohn F II, 1, 2) and S component by the method of Heller, Jacobsen, Kolodny and Kammerer (1954).

Table 3 shows that an antiserum to F could readily agglutinate these cells when coated with γ globulin, and also confirms the specificity of the antisera by showing the inability

of the anti-F serum to agglutinate cells coated with S component, and of the anti-β2M-globulin serum (anti-X) to agglutinate cells coated by γ globulin (S+F) or S component.

TABLE 3

AGGLUTINATION OF TANNED SHEEP CELLS COATED WITH γ GLOBULIN AND S COMPONENT BY VARIOUS ANTISERA

Antiserum (diluted in saline as indicated)		*Cells coated with*		
		γ globulin	*S component*	*Saline (control)*
Anti-S (S198 absorbed F)	1/100	+	+	–
	1/1000	+	+	–
	1/10,000	–	–	–
	1/100,000	–	–	–
Anti-F (S198 absorbed S)	1/100	+	–	–
	1/1000	+	–	–
	1/10,000	+	–	–
	1/100,000	–	–	–
Anti-β2M globulin (S199) 1/100, 1/1000, 1/10,000, 1/100,000		–	–	–
Normal rabbit serum 1/100, 1/1000, 1/10,000		–	–	–
Saline		–	–	–

TABLE 4

THE EFFECT OF ABSORPTION OF R335 ANTI-γ GLOBULIN ON ITS AGGLUTINATION OF RHEUMATOID-FACTOR-COATED CELLS

Antiserum (diluted in saline as indicated)		*Sensitized cells incubated with*								
		RA serum A MAD			*RA serum C MAD*			*Normal serum*		*Saline*
		1	*0.5*	*0.25*	*1*	*0.5*	*0.25*	*1/100*	*1/1000*	
R335	1/100	+	+	+	+	+	+	–	–	–
	1/1000	+	+	–	+	–	–	–	–	–
R335 absorbed S	1/50	+	–	–	+	–	–	–	–	–
	1/100	+	+	–	+	–	–	–	–	–
	1/1000	+	+	+	+	–	–	–	–	–
R335 absorbed β2M globulin	1/50	–	–	–	–	–	–	–	–	–
	1/100	–	–	–	–	–	–	–	–	–
	1/1000	–	–	–	–	–	–	–	–	–
Saline		–	–	–	–	–	–	–	–	–

Table 4 shows that the effect of absorption of anti-γ-globulin serum R335 on the agglutination of rheumatoid-factor-coated cells was different from the results previously described. This antiserum was unique in that after absorption by S component it was still capable of agglutination (an experiment using tanned cells coated with S component showed the absorption to be complete). However, immunoelectrophoretic analysis with normal serum also showed that this was the only antiserum to γ globulin which in addition produced the β2M globulin line. This antiserum thus contained antibodies to S, F and X,

and it is entirely in concordance with the previous results that its absorption by S component, still leaving antibodies to F and X, should fail to abolish its agglutinating activity. When the antiserum was absorbed by a purified β_2M globulin preparation it failed to agglutinate the rheumatoid-factor-coated cells. Immunoelectrophoretic analysis showed that the antiserum was still capable of precipitating γ globulin and its F component. These results therefore reinforce the conclusions obtained using the other antisera to γ globulin which did not react with the β_2M globulin.

DISCUSSION

This paper presents an account of the antigenic analysis of a protein, present in rheumatoid arthritis but not in normal sera, which adheres to sheep red cells coated with rabbit antibody. It is reasonable to assume that this protein is in fact rheumatoid factor, although the usual criteria of the activity of rheumatoid factor are based on its properties of agglutination of sensitized cells or particles, or precipitation of altered γ globulin.

Due consideration must be given to the possibility that proteins such as components of complement become attached to the cells during their incubation with rheumatoid arthritis sera and that subsequent agglutination by anti-γ-globulin sera is complicated by the presence in these antisera of antibodies to complement. This possibility exists for all the antisera used, especially for those in which antibodies to proteins other than γ globulins could be demonstrated by immunoelectrophoretic analysis. However, if antibodies to complement were involved it would be necessary to postulate that they could be absorbed by the separated S component of γ globulin, and also by the β_2M globulin preparations used to absorb antiserum R335. It seems unlikely that a combination of circumstances has arisen in which not only were antibodies to complement present in all the antisera used, but also that complement was present in the separated S component and β_2M globulin, but not in separated F component.

Rheumatoid factor may frequently be present in serum as a complex with a 7S protein, probably γ globulin (Franklin, Holman, Müller-Eberhard and Kunkel, 1957). This complex sediments at 22S, and is said to be active in serological tests (Franklin, Kunkel, Müller-Eberhard and Holman, 1957). Rheumatoid serum A used in this work contained this complex in addition to the 19S protein, but it was a striking finding that the antigenic component characteristic of γ globulin could not be detected on the sensitized cells after treatment with any of the four rheumatoid arthritis sera studied. This finding suggests that the γ globulin is dissociated from the complex when the rheumatoid factor becomes attached to the sensitized cell, or that the 7S protein in the 22S complex is not γ globulin, or that the complex with human γ globulin is inactive in this test. Another possibility is that the γ globulin is present on the cell, but that its F component antigenic groupings are shielded from reaction with antibodies to F by rheumatoid factor, since rheumatoid factor probably combines with this portion of the human γ-globulin molecule (Rowe, 1962).

The presence of the characteristic antigenic components of β_2M globulin in rheumatoid factor is a finding which is in accordance with, and amplifies, previous physico-chemical analyses of this protein. Since β_2M globulin is known to possess antibody activity, and since material resembling rheumatoid factor can be produced in animals after appropriate antigenic stimulation, it would now seem reasonable to regard rheumatoid factor as an antibody of the β_2M globulin class.

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