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Slowly Sedimenting Serum Components Reacting with Anti-IgM Sera

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Summary. Slowly sedimenting components with a sedimentation velocity of about 7S and reacting with specific antisera against IgM were found in six pathological human sera. Four of these came from patients with different forms of reactive macroglobulinaemia of infectious origin. Similar components were produced from purified IgM preparations by bacterial action, and to a lesser extent by spontaneous splitting at room temperature. The conditions for such a splitting were, however, absent for most of the sera investigated in this study. The possibility that slowly sedimenting IgM-like components are artefacts, is discussed.

INTRODUCTION

In the course of investigations of sera from patients with tropical reactive macroglobulinaemias it was noted that some of these sera had small macroglobulin (M) fractions in the ultracentrifuge, while showing strong reactions with anti-IgM sera even in high dilutions (Mattern, Klein, Radema and van Furth, 1967). Comparison of quantitative IgM determinations by an immunological method with total M concentrations derived from the ultracentrifuge diagram showed that in some sera the content of 'immunological' IgM was higher than the total M content. A possible explanation of this phenomenon was sought in the existence of a lower molecular weight component reacting with anti-IgM sera.

Components of this kind have been found by Sandor, Korach and Mattern (1964) in sera of immunized and also of normal horses (see also Sandor and Korach, 1966). Killander (1963) obtained indications for similar components in a normal human serum and Rothfield, Frangione and Franklin (1965) found them in sera from patients with lupus erythematosus. Recently the existence of such components has been reported in some cases of dysgammaglobulinaemia (Gleich, Uhr, Vaughan and Swedlund 1966; Solomon and Kunkel, 1965; Stobo and Tomasi, 1967).

This report deals with a demonstration of the existence of proteins with IgM antigenic determinants in light globulin fractions from pathological human sera. Consideration is also given to the possibility that these components might be formed from 19S IgM by spontaneous splitting or bacterial action.

MATERIALS AND METHODS

Five of the six sera in which the light IgM-like component was found, were collected at the Institut Pasteur in Dakar. The diagnoses were: trypanosomiasis (two cases), syphilitic meningo-encephalitis, multiple filarial infections and lymphatic cryptoleukaemia. A serum from a patient with Waldenström's macroglobulinaemia was obtained from

the Bloodbank of the University Hospital, Leiden. Twenty-three control sera from patients with various forms of reactive macroglobulinaemia were collected at the Institut Pasteur in Dakar.

Immunological determinations of IgM were carried out by the method of Mancini, Carbonara and Heremans (1965) with slight modifications. Calibrations were performed with purified IgM preparations from paraproteinaemic sera. The absolute values thus obtained should be considered with some reserve, since different calibrations sometimes gave rather divergent results. The figures to be discussed here were all obtained from the same calibration curve and, therefore, any experimental deviations from figures obtained by the ultracentrifuge should always be in the same direction. A model experiment with a purified 19S IgM paraprotein showed reasonable agreement between the immunodiffusion and ultracentrifugal analyses.

For ultracentrifugal determinations the Spinco E analytical ultracentrifuge was run at 50,740 rev/min and 20°. Fractions under investigation were dissolved in buffered saline. The ultracentrifuge diagrams were drawn from a projection by a photographic enlarger and the surface under the sedimentation peaks was measured with a planimeter. The base line was not obtained from a double sector cell but extrapolated from the horizontal part of the schlieren curve in front of the fastest peak as it appears at the beginning of sedimentation. Absolute protein contents of fractions were calculated from the measured surfaces with the usual formula (see Pickels, 1952). A value of 0·000188 ml/mg for the refractive increment was calculated from an ultracentrifuge run of a purified 19S IgM preparation, the protein content of which was determined by the Kjeldahl method. It is evident that the total macroglobulin values obtained by this procedure include α_{2M}-globulin as well as IgM.

For zone centrifugation, layers of 30, 20 and 10 per cent sucrose in 0·9 per cent NaCl were made in 13·5-ml tubes of the Spinco L rotor 40. On top of the 10 per cent layer 0·5 ml of a 1 : 2 serum dilution was placed and the tubes spun for 16 hours at 41,000 *g* (25,000 rev/min). The tubes were then slowly emptied by making a small hole in the bottom with a needle, and 2-ml fractions were collected. For analytical ultracentrifugation these fractions were dialysed and concentrated by ultrafiltration to 1 ml.

In some preliminary experiments serum samples of 1 ml diluted twelve times with 0·15 M phosphate buffer of pH 7·5 were centrifuged for 2 hours at 100,000 *g* in a Martin Christ Omega ultracentrifuge. An upper layer of 0·75 cm thickness was drawn off and analysed by immunoelectrophoresis after having been brought back to serum concentration.

Immunoelectrophoresis was carried out on standard microscope slides in agar gel containing barbiturate buffer of pH 8·6. Specific antisera against IgM and α_{2M}-globulin were used.

Sterilization of protein solutions was achieved by filtration through a 450 mμ Millipore filter under a positive pressure of 100 mmHg.

Gel filtration through Sephadex G-200 was performed in a medium containing 0·01 M phosphate buffer of pH 7·8 and 1 M NaCl.

RESULTS

Fig. 1 gives a schematic survey of the analyses of fractions obtained by zone centrifugation of a normal human serum and of a serum from a patient with syphilitic meningoencephalitis. It is seen that in Fraction III of the pathological serum there appears a

new peak in the IgM content as measured by the immunological method while in the ultracentrifuge diagram only the A (= 4S) and G (= 7S) fractions can be found. The reaction with anti IgM serum in the lighter fractions seems to be associated with fractions ⩾7S rather than with the 4S fraction, since it becomes weaker in Fraction IV where A>G and both A and G are increased in comparison to Fraction III.

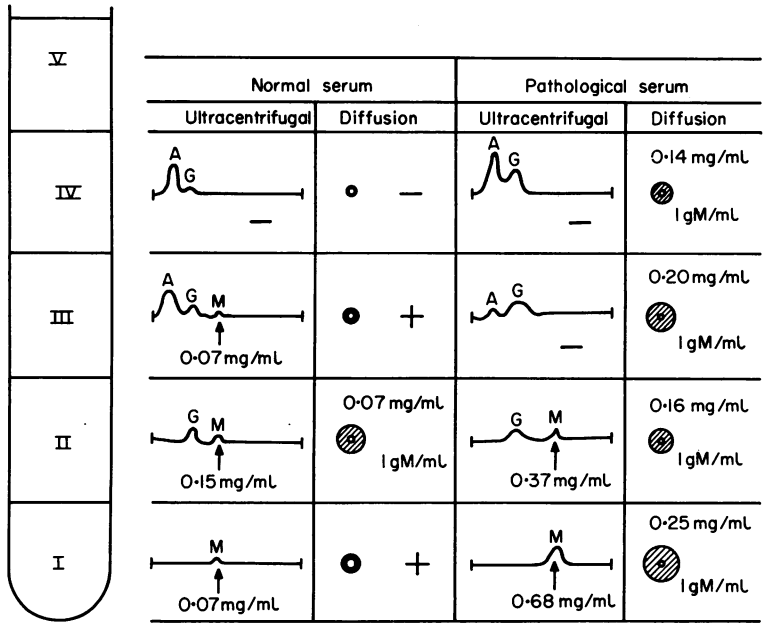

FIG. 1. Schematic survey of the analysis of ultracentrifugal fractions of a normal and a pathological serum.

TABLE 1

ANALYSIS OF ULTRACENTRIFUGAL FRACTIONS OF TWO PATHOLOGICAL SERA

Fraction		Serum I		Serum II	
		Total 19S fraction (mg/ml) (ultracentrifuge determination)	IgM (mg/ml) (immunodiffusion)	Total 19S fraction (mg/ml) (ultracentrifuge determination)	IgM (mg/ml) (immunodiffusion)
Top	V	–	0·04		
	IV	–	0·16	trace	0·03
	III	–	0·24	0·06	0·07
	II	0·80	0·41	0·64	0·33
Bottom	I	0·27	0·16	0·74	0·39

Another example is shown in Table 1 where the analyses of fractions obtained by zone centrifugation of two pathological sera are compared. Serum I was from a patient with multiple filarial infections and had a total immunological IgM content (8·8 mg/ml) which was higher than the total M fraction (6·5 mg/ml) as measured in whole serum. On the other hand, serum II, from a patient with liver cirrhosis, had an immunological IgM content of 7·1 mg/ml and an M fraction of 10 mg/ml. It can be seen that in serum I a

considerable amount of immunological IgM can be found in fractions which contain no measurable M peak. In serum II this is not the case, even though the total M content is higher than in serum I.

It seems improbable that the differences between IgM and total M content are due to experimental errors, since they are rather large and the analysis of the control sera did not show any systematic bias in favour of the immunological IgM determination.

The results obtained with the zone centrifugation were confirmed with two other sera, one from a patient with Waldenström's macroglobulinemia and one from a case of

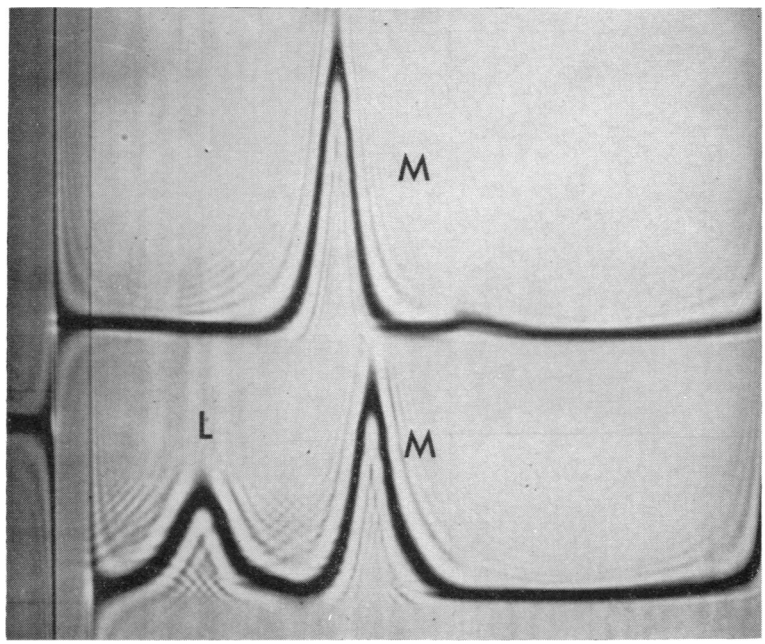

FIG. 2. Ultracentrifuge diagrams of a purified IgM preparation kept at 4°. Direction of sedimentation from left to right. Above: in sterile solution; below: in solution containing *Proteus morganii*. L = light component; M = macroglobulins.

lymphatic cryptoleukaemia. Preliminary experiments with differential centrifugation of two trypanosomiasis sera also indicated the existence of a light IgM-like fraction in these cases. The upper layer after centrifugation showed a strong IgM line in immunoelectrophoresis, while the α_{2M} line had completely disappeared. In twenty-three control experiments with reactive macroglobulinaemias neither the IgM nor the α_{2M} line were found in the upper layer under the same conditions. These two cases were not further analysed by other methods.

In two experiments with electrophoretic fractions indications were obtained that the low molecular weight IgM fraction might be situated in the α_2–β region but the differences were too small to allow definite conclusions.

Efforts to isolate these components were hampered by lack of material, but in one case it could be ascertained that the precipitation at pH 8 and low ionic strength used by Sandor *et al.* (1964) to isolate the light IgM-like fraction from horse serum, did not achieve any concentration in a human serum.

A component resembling those described here was found incidentally in a sample of a purified cryomacroglobulin of IgM paraprotein character, which had been stored for more than a year in the refrigerator at 5°. Ultracentrifuge examination revealed the presence of a fraction with $S_{20} = 7{\cdot}0$ (not corrected for concentration) although immunoelectrophoretic analysis showed that only substances reacting with anti-IgM serum were present. This sample appeared to be contaminated with *Proteus morganii*. Another sample, which had been stored at the same time and which proved to be sterile, had only the usual 19S and 24S components (see Fig. 2).

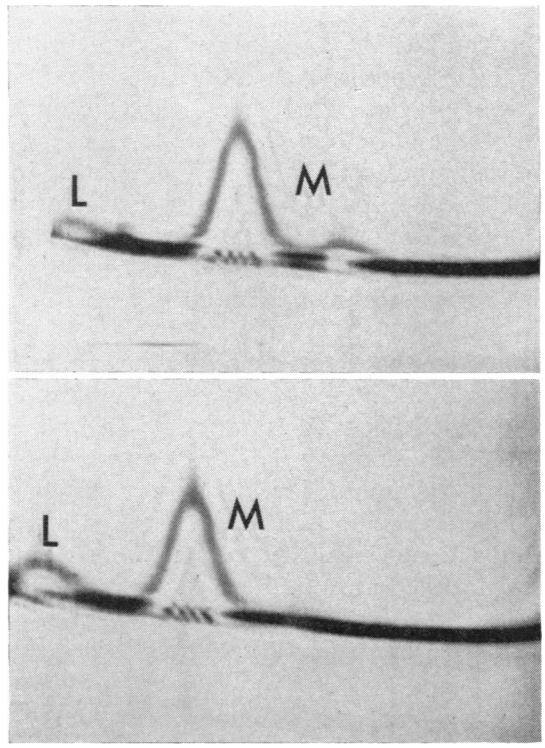

Fig. 3. Ultracentrifuge diagrams of a purified IgM preparation (see Fig. 2, opposite) kept at room temperature. Direction of sedimentation from left to right. Above: in sterile solution. Below: after inoculation with *Proteus morganii*.

It was possible to purify the 7S component, because it had lost the cryoglobulin property. Final purification by gel filtration through Sephadex G-200 gave a product which on immunoelectrophoretic analysis appeared to contain light chains as well as μ chains. Its electrophoretic mobility was still only in the β_2–γ region.

It was attempted to reproduce this cleavage of the IgM molecule by inoculating a sterile solution of the same cryoglobulin in a concentration of 1·5 per cent with a culture of the isolated strain of *Proteus morganii*.

Fig. 3 shows the ultracentrifuge diagrams of the inoculated and the control solution after 51 days standing at room temperature (22°). About 20 per cent of the IgM was converted to a heterogeneous 3·5S component, but in the sterile control sample about 10 per cent of the IgM also appeared as a slower component with $S_{20} = 4{\cdot}7$. Neither the

proteins in the inoculated solution nor those in the control precipitated any longer in the cold at this stage. It was found that Alkalescens–Dispar organisms were also able to split IgM into components of lower molecular weight.

DISCUSSION

The existence of slowly sedimenting serum proteins with antigenic characteristics of IgM has been demonstrated by the experiments described in this article. These components seem to belong to a $\geqslant 7S$ fraction of the serum proteins. Four of the six sera in which the light component was found, belonged to the group of reactive macroglobulinaemias, but even in this condition the phenomenon seems to be rare. It is not known whether any of the light IgM fractions carried antibody activity, as did the fractions of Rothfield *et al.* (1965). Sandor *et al.* (1964) found that the light IgM components had antibody activity in sera of immunized horses.

It is possible that the light IgM components are a naturally occurring variety of the 19S antibodies or an intermediate or byproduct of their biosynthesis. They might be present in very small amounts in normal human sera and increase as a result of a state of hyperimmunization, like the analogous proteins in horse serum. Another possibility is that they are artefacts resulting from the action of serum enzymes or bacterial contamination.

Our experiments demonstrate that lighter components can indeed be formed from 19S IgM by bacterial action but also by cleavage in sterile solution. In one case these fragments had a sedimentation coefficient of 7S and reacted with specific antiserum against IgM. Spontaneous de-polymerization of isolated macroglobulins has been mentioned by Gleich *et al.* (1966), while breakdown of IgG by serum enzymes was demonstrated by Škvařil (1960). In this connection it is of interest to note that Awdeh, Askonas and Williamson (1967) found a homogeneous mouse immune globulin to become electrophoretically heterogeneous in the serum of the animal producing this abnormal protein.

The question arises whether the low molecular IgM-like components in our pathological sera might also be breakdown products of 19S IgM, formed during the storage of the sera. This does not seem very probable since all the sera were stored at low temperatures and proved to be sterile, except the macroglobulinaemia of Waldenström, in which the light component might therefore be an artefact. Contamination during the processing of the fractions could have taken place, but our experiments never showed any observable breakdown by bacteria after a few days incubation at room temperature. Although contamination before, or splitting by serum enzymes during, storage cannot be completely excluded, it seems more probable that the light IgM-like fractions were actually present in the circulation. More work will be required to demonstrate the direct biosynthesis of these components and to rule out the possibility of breakdown in the blood circulation or during storage of the serum samples.

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