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A Comparative Study of Cerebrospinal Fluid and Serum Proteins in Multiple Sclerosis with Special Reference to the Lange Colloidal-Gold Reaction

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Positive colloidal-gold reactions in the cerebrospinal fluid of patients with multiple sclerosis have been reported as occurring in 29–81% of fluids examined by various investigators (Freedman & Merritt, 1949). Coagulation of the colloidal gold has been shown to be associated with the γ -globulin fraction (Kabat, Moore & Landow, 1942). The concentration of γ -globulin in cerebrospinal fluid was determined immunochemically by Yahr, Goldensohn & Kabat (1954), who report an increase in 66.5% of the multiple-sclerosis cases studied. The mechanism of several flocculation tests, including the colloidal gold at pH 7.8 and 6.6, was studied by MacLagan & Bunn (1947), using normal and hepatic serum fractions; they found that albumin and α - and β -globulins protected the colloidal gold and that γ -globulin brought about its coagulation.

Serum diluted to 0.03% protein gives a very strongly positive colloidal-gold reaction (Press, 1955), whereas a normal cerebrospinal fluid, having approximately the same concentration of albumin and globulins, gives a negative colloidal-gold reaction; this is suggestive of a qualitative difference between the protein fractions of cerebrospinal fluid and serum.

In the present study, the proteins of normal and pathological cerebrospinal fluids and normal sera have been compared both quantitatively and qualitatively. Cerebrospinal fluids and sera were analysed electrophoretically, and the percentage of γ -globulin in the cerebrospinal fluids was compared with the type of the colloidal-gold reaction. Albumin and α -, β - and γ -globulins have been isolated from sera and cerebrospinal fluid and the

effect of these fractions on the colloidal-gold reaction studied and their sedimentation constants determined.

Partial fractionations of cerebrospinal-fluid γ - and β -globulins were performed in the ultracentrifuge and the effect on the colloidal-gold reaction of the more slowly sedimenting fraction was examined. The chemical nature of this 'slow' component from both β - and γ -globulin was determined. Cerebrospinal-fluid γ -globulin was also fractionated with 18% sodium sulphate, and the sedimentation constant of the fractions and their effect on the colloidal-gold reaction were determined.

EXPERIMENTAL

Materials

Veronal buffer 1. This buffer (pH 8.6, $I=0.1$), which was used for micro-electrophoresis, contained 0.1M sodium veronal and 0.02M veronal.

Veronal buffer 2. This buffer (pH 8.6, $I=0.0375$), which was used for continuous paper electrophoresis, contained 0.0375M sodium veronal and 0.0075M veronal.

Phosphate buffer 1. This buffer (pH 7.4) was made by mixing 48 ml. of KH_2PO_4 (0.067M) and 202 ml. of Na_2HPO_4 (0.067M).

Phosphate buffer 2. This buffer ($I=0.2$, pH 7.45) contained 0.0142M- Na_2HPO_4 , 0.0025M- KH_2PO_4 and 0.1M-NaCl.

Sodium sulphate (27%, w/v) in phosphate buffer pH 7.8. For this solution 270 g. of anhydrous Na_2SO_4 , 6 g. of K_2HPO_4 and 0.488 g. of KH_2PO_4 were made up to 1 l. at 37°.

Dextran. Initially, a supply of 20% Dextran was available, approximate molecular weight 40 000 (kindly supplied by Dextran Co. Ltd.), but latterly Dextran was prepared by dialysing Intradex (a 6% w/v solution of Dextran in 0.9% NaCl) in cellophane tubing against daily changes of distilled water for 5 days and freeze-drying.

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Cerebrospinal fluid (c.s.f.). Samples (20–30 ml.) were withdrawn by lumbar puncture and replaced by sterile normal saline. C.s.f. was stored at 5° and thiomersal (sodium ethylmercurithiosalicylate) or toluene was used as preservative, but toluene was preferred because it had no effect on the colloidal-Au reaction, whereas thiomersal inhibited coagulation.

Solvents for chromatography. (1) Butanol-acetic acid was made from 60 ml. of *n*-butanol, 15 ml. of glacial acetic acid and 25 ml. of water.

(2) Aqueous phenol consisted of 80% (w/v) phenol in water (200 ml.) to which 1 ml. of 0.880 NH_3 soln. was added before use.

Stabilized citrate colloidal-Au sol. The colloidal-Au sol was prepared as described by Lange & Harris (1948). The turbidity, which falls during the first few hours after preparation, was standardized by comparison with a solution of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (20%, w/v, in 1%, v/v, HCl). The cobalt chloride solution gave a reading of 23 in an EEL portable colorimeter (Evans Electroselenium Ltd.), with a filter of maximum transmission of 7000 Å. The Au sol, which usually gave a reading of 20–25 when first prepared, was allowed to stand until the reading was between 14 and 16; it was then stabilized by adjusting the pH to 6.8 with 1% (w/v) Na_2CO_3 , as described by Lange & Harris (1948). The colloidal Au was stored in the dark at room temperature and was stable for a month.

Methods

Concentration and electrophoresis of c.s.f. Approximately 10–20 ml. of c.s.f. was concentrated to 0.5 ml. by dialysis in cellophane tubing against a 20–30% solution of Dextran in veronal buffer 1, and then dialysed against this buffer for 2 days at 5°. Electrophoresis was performed in an Antweiler micro-apparatus, as described by Geissen, Schuler & Schuster (1950) at protein concentration 1–2% for 20 min. with a current of 1.8 ma and potential difference of 80–90 v. This method of concentrating c.s.f. was considered satisfactory, since serum diluted to 0.03%, and concentrated and dialysed as above, had the same electrophoretic pattern as the original serum, and duplicate analyses on the same c.s.f. were in agreement. Also a sample of c.s.f. concentrated and rediluted had the same colloidal-Au reaction as the original fluid.

Total protein in c.s.f. This was determined by the sulphosalicylic acid turbidimetric method (Kingsbury, Clark, Williams & Post, 1926.)

Electrophoretic analysis of serum. Serum was dialysed against veronal buffer 1 in micro-dialysis buckets, described by Geissen *et al.* (1950), for 2 hr. and diluted to 1–2% protein with veronal buffer and analysed electrophoretically in the micro apparatus described above.

Isolation of protein fractions from c.s.f. and serum. A continuous paper electrophoresis apparatus was used (Durrum, 1951; Hobart & Rose, 1955), with veronal buffer 2, Whatman 3 mm. paper and a voltage of 400. Serum or c.s.f. in veronal buffer 2 was applied at the top of the sheet of filter paper 8 cm. from the negative side, by means of a wick of filter paper 1.5 mm. wide. Only 10–20 ml. of c.s.f. was obtained from each patient; therefore samples of c.s.f. having similar colloidal-Au reactions were bulked and concentrated to yield 1–2 ml. of protein solution 1–2%. So that the comparisons between serum and c.s.f. fractions should be valid, the serum was diluted 50–100 times and concentrated with Dextran, as for the c.s.f.

The protein fractions were collected from fourteen drip points at the bottom of the paper. The distribution of the fractions was determined by drying the paper at 100° and staining with azocarmine solution. The albumin, α -, β - and γ -globulin fractions were concentrated by dialysis against Dextran and dialysed against veronal buffer 1, and the purity of each fraction was established by electrophoresis in the micro apparatus described above. The protein fractions were then dialysed against phosphate buffer 2 and the concentration of protein was determined by the ultraviolet absorption of the solutions.

Determination of protein concentration by ultraviolet absorption. The absorption at 2770 Å of protein fractions in phosphate buffer 2, pH 7.45, was determined in a Hilger Uvispek photoelectric spectrophotometer H700/302 in 1 mm. silica cells, at suitable dilution to give an optical density of between 0.1 and 1.0. To correct for light absorption due to haze of the solution (Beaven & Holiday, 1952) the optical density at 3200 and 3600 Å was read, and a line through these points projected back to 2770 Å to serve as a base-line. The ultraviolet absorption was referred to protein concentration calculated on the usual convention of 16% of nitrogen in all the protein fractions, and the conversion factors were determined on pure fractions from c.s.f. and serum, prepared by the method of continuous paper electrophoresis described above (Table 1). Protein nitrogen was determined by the micro-Kjeldahl method of Ma & Zuazaga (1942) with the distillation apparatus described by Markham (1942).

Colloidal-Au test. The method of Mellanby & Anwyl-Davies (1923) was used for the results reported in Tables 2 and 3, this test being carried out regularly in the Bland Sutton Institute, Middlesex Hospital Medical School.

For the comparisons of the effects of various protein fractions on the colloidal-Au test, the qualitative method of Lange, Miller & Harris (1950), using the stabilized citrate colloidal-gold sol, was used by the author. Serial dilutions of the c.s.f. were performed with phosphate buffer 1, as described by Lange (1939), and the series read after 6 hr., the degree of coagulation being represented by numbers 0–5. The degree of coagulation is judged subjectively by the colour of the supernatant, which varies from pink when no coagulation occurs (0), through shades of pinky mauve (1), mauve (2), blue (3), blue-grey (4) to colourless when coagulation is complete (5). For normal c.s.f. the reading is 0001110000. A control solution was used, consisting of normal sera diluted 1:100 with 50% aqueous glycerol, the reading for this solution being 2355543211.

Table 1. Relationship between ultraviolet absorption at 2770 Å and protein concentration

Protein	$E_{1\text{ cm. at } 2770 \text{ Å}}$	Protein concn. calc. from total N (g./100 ml.)	Conversion factor
Serum albumin	2.7	0.49	0.181
C.s.f. albumin	3.3	0.65	0.191
Serum α -globulin	4.6	0.38	0.083
C.s.f. α -globulin	2.6	0.23	0.089
Serum β -globulin	2.7	0.21	0.078
C.s.f. β -globulin	3.0	0.25	0.083
Serum γ -globulin	10.4	0.79	0.076
C.s.f. γ -globulin	4.9	0.37	0.076

Table 2. γ -Globulin concentration in cerebrospinal fluid and serum in cases of multiple sclerosis

Type of patient	Cerebrospinal fluid			Serum		
	No. of cases	γ -Globulin as percentage of total protein		No. of cases	γ -Globulin as percentage of total protein	
		Mean	S.D.		Mean	S.D.
Negative Lange Au test; protein 20–80 mg./100 ml. in c.s.f.	18	17.7	2.4	15	17.1	2.9
Positive Lange Au test; protein 20–140 mg./100 ml. in c.s.f.	17	26.2	7.3	14	16.6	2.8

Effect of albumin, α -, β - and γ -globulins on the colloidal-Au reaction. γ -Globulin from c.s.f. and sera in phosphate buffer 2 were added to normal c.s.f., and albumin, α - and β -globulins added to c.s.f. having positive colloidal-Au reaction, and the colloidal-Au test was carried out. Control experiments were always done by adding the same volume of buffer to the c.s.f., and all comparisons performed on the same day, with identical reagents.

Ultracentrifuge studies. Sedimentation constants were determined for albumin, α -, β - and γ -globulins of c.s.f. and serum in the Spinco ultracentrifuge, by the technique described by Charlwood (1952*a, b*). Four photographs were taken at 16 min. intervals and sedimentation constants, in Svedberg units corrected to water at 20°, calculated from the following formula, in which x is the distance of the sedimenting boundary from the centre of rotation, t is the time and ω is the angular velocity:

$$S = \frac{\Delta \log_e x}{\Delta t \omega^2}.$$

The value for partial specific volume, $\bar{V}$, used in the temperature correction for all fractions was 0.735, since even though the true value of $\bar{V}$ may differ considerably from this value, the magnitude of the temperature correction is little affected.

Fractionation of β - and γ -globulin in the ultracentrifuge. The slower component of these c.s.f. fractions was separated from the faster one in the partition cell of the ultracentrifuge. This cell is similar to that used for analytical experiments but the centre-piece is divided into two sections by means of a perforated partition of Dural, held in place and supported by slots in the walls of the sector, approximately two-thirds down the cell. Supported by and adjacent to the partition is a section of filter paper, Whatman no. 50 (Fig. 1). The sedimentation boundaries were observed on the screen and centrifuging was terminated 20 min. after the faster-moving component had reached the partition. The solution in the upper part of the cell was removed and its effect on the colloidal Au determined by adding to a c.s.f. having a positive colloidal-Au reaction. Control experiments were performed with serum fractions prepared in the same way, but no protein was detected in the upper part of the cell.

Fractionation of c.s.f. and serum γ -globulin with 18% sodium sulphate. Sodium sulphate (27%) in phosphate buffer (2 vol.) was added to 1 vol. of γ -globulin in phosphate buffer 2 at 37°, and left overnight at this temperature and then centrifuged at approximately 3000 rev./min. The supernatant solution was dialysed against saline and concentrated with Dextran solution, and finally dialysed against phosphate buffer 2. The precipitate was dissolved in phosphate buffer 2 and dialysed against the same buffer. Sedimentation constants of the supernatant solution and pre-

Fig. 1. Centre-piece of partition cell of Spinco ultracentrifuge.

cipitate were determined. The effect of these fractions on the colloidal Au was studied by adding to a c.s.f. having a positive colloidal-Au reaction and carrying out the colloidal-Au test on the resulting solutions.

Chromatographic and phosphorus analysis of c.s.f. fractions. The chemical nature of the 'slow' components in β - and γ -globulins was investigated. Approximately 200 μ g. of material in 5.7 N-HCl in a sealed tube was heated to 100° for 16 hr. Two-way chromatography, by the ascending method, was performed on 10 in. $\times$ 10 in. Whatman no. 1 paper, in butanol-acetic acid and aqueous phenol, and amino acids were located by colouring with ninhydrin. The carbohydrate content of the components was determined by the chromatographic method of Partridge (1949), after hydrolysis in 0.5 N-HCl for 4 hr. at 100°. Phosphorus analysis was performed by a modification of the method of Martland & Robison (1926).

RESULTS

Electrophoretic analysis of serum and c.s.f. and the relation between the colloidal-gold test and percentage of γ -globulin in the c.s.f.

The mean concentrations and standard deviations found for serum and c.s.f. γ -globulin are given in Table 2. The results have been divided into two groups, according to the type of colloidal-gold reaction in the c.s.f. The mean concentrations of serum γ -globulin in the two groups are not significantly different ($P=0.5$), but the difference between the mean values of the c.s.f. γ -globulin is highly significant ($P<0.001$), thus showing strong correlation with the colloidal-gold reaction. The

Table 3. *Mean concentrations of albumin and α - and β -globulins in c.s.f. having negative and positive colloidal-gold reactions*

Type of patient	No. of cases	Albumin as percentage of total protein	α - and β -Globulin as percentage of total protein
Negative Lange colloidal-Au reaction	18	61.8	20.5
Positive Lange colloidal-Au reaction	17	56.7	17.1

Table 4. *Effect on the colloidal-gold reaction of adding γ -globulin to a normal c.s.f.*

For an explanation of the figures relating to the colloidal-Au reaction here and in Tables 5 and 6, see text.

γ -Globulin added (mg./100 ml. c.s.f.)	Source of γ -globulin	Colloidal-Au reaction
None	—	0001111000
16	Normal serum	0134433221
16	C.s.f. having negative colloidal-Au reaction	0001232211
16	C.s.f. having positive colloidal-Au reaction	0012333211

Table 5. *Effect on the colloidal-gold reaction of adding serum and c.s.f. α -globulin to a c.s.f. having a positive colloidal-gold reaction*

Source of α -globulin added	Amount of α -globulin added (mg./100 ml. c.s.f.)	Colloidal-Au reaction
	None	5555432211
C.s.f.	7	1234332110
Serum	12	1234432110

mean concentration of albumin, α - and β -globulins (Table 3) do not differ sufficiently to account for the colloidal-gold reactions.

Effect of albumin and globulin fractions on the colloidal-gold test. The coagulating effect of γ -globulin derived from serum and c.s.f. on the colloidal-gold sol is given in Table 4. Albumin and α - and β -globulins from normal serum were found to protect the colloidal gold and, as found by MacLagan & Bunn (1947), the protective power of α - and β -globulins was ten times that of albumin. Albumin and β -globulins from both normal and pathological c.s.f. had the same quantitative effect on the colloidal-gold test as the serum fractions. C.s.f. α -globulin, however, was twice as effective in protecting the colloidal gold as was serum α -globulin (Table 5), but no difference was found between α -globulins derived from c.s.f. having positive and negative colloidal-gold reactions.

Ultracentrifuge studies

C.s.f. γ -globulin contained a slow component, 'S', in addition to the normal serum γ -globulin component of $S=6.5$ (Fig. 2). The sedimentation constant of component 'S' could not be calculated accurately, because of its low concentration, but it

Fig. 2. Ultracentrifuge pattern of γ -globulin in phosphate buffer 2. Sedimentation is from left to right. (A) Serum γ -globulin (0.21%), after 20 min. at top speed. (B) γ -Globulin (0.18%), prepared from c.s.f. with negative colloidal-gold reaction, after 26 min. at top speed. (C) γ -Globulin (0.30%), prepared from c.s.f. with positive colloidal-gold reaction, after 20 min. at top speed.

Fig. 3. Ultracentrifuge pattern of β -globulin in phosphate buffer 2. Sedimentation from left to right. (A) Serum β -globulin (0.50%), after 49 min. at top speed. (B) C.s.f. β -globulin (0.25%), after 54 min. at top speed.

was of the order of 2.0 Svedberg units. This component was present in all preparations of c.s.f. γ -globulin, but the relative concentration varied, being greatest in γ -globulin derived from normal c.s.f. It was not detected in serum γ -globulin. C.s.f. β -globulin also had a slow-moving component, $S=2.1$, which could not be detected in

serum β -globulin (Fig. 3). The concentration of this component, relative to the normal component, $S=5.1$, was approximately the same in all the c.s.f. β -globulin preparations. Serum α -globulin separated into four components (Fig. 4). The sedimentation constant of the first component was 17.1; the concentration of the second component was too low to permit calculation. The other two components had sedimentation constants of 7.5 and 2.8. C.s.f. α -globulin sedimented as one broad peak (Fig. 4) probably representing several very similar components of average sedimentation constant 2.9. This difference between c.s.f. and serum α -globulin is not due to the difference in protein concentration, since four components are detectable even at 0.15%. No difference was detected in the sedimentation behaviour of albumin derived from c.s.f. and from serum.

Slow component of β - and γ -globulin, as separated in the ultracentrifuge. This component was found to protect the colloidal-gold sol (Table 6). Chromatographic analysis indicated that the same amino acids were present as in serum γ -globulin, and that the carbohydrate content was less than 4%. Phosphorus analysis of the trichloroacetic acid precipitate showed that less than 0.5% was present. It is assumed therefore that component 'S' is a protein.

Fig. 4. Ultracentrifuge pattern of α -globulin in phosphate buffer 2. Sedimentation from left to right. (A) Serum α -globulin (0.29%), after 19 min. at top speed. (B) C.s.f. α -globulin (0.14%), after 21 min. at top speed.

Table 6. Effect on the colloidal-gold reaction of adding component 'S' of β - and γ -globulin, separated in the ultracentrifuge, to a c.s.f. having a positive colloidal-gold reaction

Nature of protein fraction added	Amount of protein fraction added (mg./100 ml. c.s.f.)	Colloidal-Au reaction
None	—	5554322100
β -Component 'S'	10.5	0112222100
None	—	3333221100
γ -Component 'S'	3.0	2222211000

Fractionation of γ -globulin with 18% (w/v) sodium sulphate

In the presence of 18% (w/v) sodium sulphate, 95% of serum γ -globulin was precipitated, but only 75–85% of c.s.f. γ -globulin. The fraction which was soluble in 18% sodium sulphate was found to have a protective effect on the colloidal gold and had a sedimentation constant of the same order as component 'S'. The fraction insoluble in sodium sulphate coagulated the gold sol and had a sedimentation constant of 6.8. The protein nature of these fractions was established by chromatography.

DISCUSSION

The components in c.s.f. responsible for the colloidal-gold reaction were shown to be non-dialysable, by comparing the colloidal-gold test on a c.s.f. which had been dialysed against phosphate buffer 1, with the original c.s.f. This non-dialysable material is a mixture of proteins having the electrophoretic mobilities of albumin, α -, β - and γ -globulins.

The difference in the colloidal-gold reaction on diluted serum and normal c.s.f. is due, in part, to a difference in the α -globulin and also to the presence of the slow component 'S' found in the γ -globulin fraction of c.s.f., which protects the colloidal gold.

The coagulating power of serum and c.s.f. γ -globulins on the colloidal-gold sol (Table 4) depends on the varying proportions of component 'S' in these preparations (Fig. 2), and the difference between c.s.f. having positive and negative colloidal-gold reactions is due to a difference in the concentration of the γ -globulin component with $S=6.5$, relative to component 'S'. The fraction of c.s.f. γ -globulin soluble in 18% sodium sulphate is probably identical with component 'S'. The slow component of c.s.f. β -globulin may be the same protein: its sedimentation constant is of the same order.

Although the mean concentration of γ -globulin in c.s.f. having positive colloidal-gold reactions was significantly increased, the serum γ -globulin concentration was not raised. This has been noted by other workers (Field, 1954; Kabat, Freedman, Murray & Knaub, 1950), who assumed that the increased γ -globulin is produced in the central nervous system, since the concentrations of the other protein fractions in the c.s.f. are not similarly raised. However, the increased γ -globulin could be derived from the blood, if there is an increased selective permeability to γ -globulin. Instances of selective permeability to a particular protein are known in the case of certain antibodies, e.g. it has been shown that antibody protein passes from the

blood to the milk in the rabbit and into colostrum in the goat without degradation and resynthesis, whereas other plasma proteins are not found in the milk (Askonas, Campbell, Humphrey & Work, 1954). In an attempt to discover whether the increase in c.s.f. γ -globulin is due to its production in the central nervous system, the antistreptolysin 0 titre (A.S.O.) in the sera and c.s.f. of twelve multiple sclerosis cases was determined. In 75 % of the samples of c.s.f. the titre was 0.2–0.4 unit and in 25 % it was less than 0.2 unit. In all cases it was only a tenth to a half of the expected titre, calculated from the ratio of the γ -globulin concentrations in serum and c.s.f. However, there was no correlation between the A.S.O. titre and the concentration of γ -globulin in the c.s.f., which supports, but does not prove, the theory that γ -globulin is produced in the central nervous system.

SUMMARY

1. The concentration of γ -globulin in the cerebrospinal fluid (c.s.f.) showed strong correlation with the Lange colloidal-gold reaction. In those cases where c.s.f. γ -globulin concentration is increased, the concentration in the serum is normal, but it is not known whether the increased γ -globulin in the c.s.f. originates from the blood, by increased selective permeability of the blood/c.s.f. barrier to γ -globulin, or whether γ -globulin is produced in the central nervous system.

2. Albumin, α - and β -globulins from serum and c.s.f. inhibit coagulation of the colloidal-gold sol and γ -globulin coagulates the sol.

3. C.s.f. proteins are similar to serum proteins, except that the more rapidly sedimenting components of serum α -globulin are not detected in the c.s.f.; also, c.s.f. α -globulin inhibits coagulation of the colloidal-gold sol more than does serum α -globulin. An additional protein is found in the c.s.f. in the β - and γ -globulin fractions, component 'S', which has a very low sedimentation constant and inhibits the colloidal-gold reaction. This component, together with the difference in the α -globulins, is responsible for the difference between the colloidal-gold reaction of normal c.s.f. and that of diluted serum.

4. The proportion of component 'S' in the c.s.f. γ -globulin is greater in normal c.s.f. than in c.s.f. having a positive colloidal-gold reaction. The

positive colloidal-gold reaction, given by c.s.f. from some multiple sclerosis patients, is due to the increased concentration of the γ -globulin component with sedimentation constant $S=6.5$, relative to component 'S'.

5. Component 'S' is a protein and is not precipitated by 18 % sodium sulphate.

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