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# IMMUNOELECTROPHORETIC STUDIES ON $\beta_{1E}$ -GLOBULIN IN HUMAN SERUM<sup>1</sup>

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**S**UMMARY Highly purified human fourth component of complement (C'4) which showed a single precipitin line against horse antihuman serum in the  $\beta_1$ -globulin region was used for identification of the precipitin line of  $\beta_{1E}$ -globulin ( $\beta_{1E}$ -line) in the immunoelectrophoretic pattern of fresh human serum. Identification was achieved by a combination of immunoelectrophoresis and Ouchterlony's double diffusion technique. Differentiation of the  $\beta_{1E}$ -line from other lines in the  $\beta_1$ -globulin region has been facilitated by the use of anti  $\beta_{1C/1A}$  serum and specific absorbed horse antihuman serum.

In the immunoelectrophoretic pattern of normal human serum, the  $\beta_{1E}$ -line ran parallel with the  $\beta_{1C/1A}$ -globulin line ( $\beta_{1C/1A}$ -line) from the antigen well to the  $\beta_1$ -globulin region and crossing of the two lines was never seen.

It was also shown that the  $\beta_{1E}$ -line of normal human serum changed in appearance, and crossing of the  $\beta_{1E}$ -line and  $\beta_{1C/1A}$ -line was observed after hydrazine treatment or addition of immune precipitate.

A close relationship between the serum C'4 level and the appearance of the  $\beta_{1E}$ -line in the serum was demonstrated. It is interesting to note that the  $\beta_{1E}$ -line was indistinct or absent when the C'4 level of the serum was definitely low.

## INTRODUCTION

In the previous paper (INAI *et al.*, 1967a), it was reported that the serum complement level was decreased in some cases in various

diseases, and that a markedly decreased or zero C'4 level was also found in these conditions.

In 1963, Müller-Eberhard and Biro reported the isolation of a new protein fraction as the result of purification of C'4 from human serum. This was identified as  $\beta_{1E}$ -globulin by immunoelectrophoretic analysis. However, in their report, the differentiation of the  $\beta_{1E}$ -line

<sup>1</sup> Parts of this work were reported at the Third Symposium on Complement in August 1966 at Hakone, Japan, and at the 16th Annual Meeting of the Japanese Society of Allergy in October 1966 at Tokyo, Japan.

from other precipitin lines in the  $\beta_1$ -globulin region was not clear from the photograph of the immunoelectrophoretic pattern of normal human serum. Moreover, it is still uncertain whether C'4 actually corresponds with  $\beta_{1E}$ -globulin. There have been numerous reports of immunoelectrophoretic analyses of sera of patients with various diseases, but no reports on the relationship between the serum C'4 level and the  $\beta_{1E}$ -line as far as we know. FOSTIROPORLOS *et al.* (1965) investigated the synovial fluid in patients with various joint diseases and reported on the alteration of  $\beta_{1E}$ -globulin, but they did not mention the relation between the C'4 level and  $\beta_{1E}$ -globulin.

In the previous paper (INAI *et al.*, 1967b), we reported a new method for purifying C'4 which combined several methods that were effective in eliminating the contaminating C'1 inactivator. Immunoelectrophoretic analysis of purified C'4 gave a single precipitin line and this line corresponded to the line of  $\beta_{1E}$ -globulin which was obtained with horse antihuman serum.

The present study was to determine the situation of the precipitin line of purified C'4 and to distinguish it from other lines of normal human serum by immunoelectrophoresis. The relation between the decrease of C'4 activity in patients' sera and the alteration of the precipitin line of  $\beta_{1E}$ -globulin was also investigated.

## MATERIALS AND METHODS

### 1. Purification of C'4

The procedure for purification of C'4 from fresh human serum was described in the previous paper (INAI *et al.*, 1967 a).

### 2. Sera

As a control in immunoelectrophoresis, sera were obtained from many healthy people, they were pooled and stored at  $-20^\circ\text{C}$  until use. In the clinical investigation, sera of randomly selected persons with rheumatoid arthritis, cirrhosis of liver and multiple myeloma were obtained and stored at  $-20^\circ\text{C}$  until use. These serum specimens were not stored for

more than 3 weeks.

### 3. Antisera

#### 1) Horse antihuman serum

This was prepared at the Osaka Microbial Diseases Research Foundation (Lot No. 2), and was used for immunoelectrophoresis and Ouchterlony's double diffusion technique in this study.

#### 2) Partially purified horse antihuman serum

As horse antihuman serum has remarkable anti-complementary activity, elimination of this activity was attempted.

To 4.7 ml of this antiserum was added an equal volume of saturated ammonium sulphate. After four hours at room temperature, the precipitate was separated by centrifugation at 8,000 rpm at  $0^\circ\text{C}$  for 15 minutes and dissolved in deionized water. This solution was dialysed overnight against tapping water and for 24 hours against deionized water. Then, this material was dialysed overnight against Tris-phosphoric acid buffer of pH 8.4, conductance  $1.2 \times 10^3$  micromhos. The dialysate was centrifuged at 4,000 rpm at  $0^\circ\text{C}$ . The supernatant was applied to a  $1.5 \times 18$  cm column of DEAE cellulose previously equilibrated with Tris-phosphoric acid buffer of pH 8.4, conductance  $1.2 \times 10^3$  micromhos. The column was thoroughly washed with this buffer of  $1.2 \times 10^3$  micromhos conductance, then with buffer of  $1.95 \times 10^3$  micromhos, and then with buffer of  $3.3 \times 10^3$  micromhos conductance. The fraction which was eluted with Tris-phosphoric acid buffer of  $3.3 \times 10^3$  micromhos conductance was collected and concentrated to its original volume by ultrafiltration. This fraction corresponded to T-globulin and exhibited the same precipitin lines with normal human serum as the original antiserum on immunoelectrophoresis, and its anticomplementary activity had been almost completely eliminated.

#### 3) Absorbed horse antihuman serum

As described in the previous paper (INAI *et al.*, 1967 b), horse antihuman serum was absorbed with the fractions of human serum which did not include C'4 and  $\beta_{1C}$ -globulin.

#### 4) Rabbit antihuman C'4 serum

Five ml of purified C'4 (total protein content, 3.26 mg) in complete Freund's adjuvant (containing 1 mg dried tubercle bacilli, 4.25 ml of Drackeol No. 6 and 0.75 ml of Aracel A) was divided into three equal portions and each was injected into the foot pad and intracutan of the back of 3 rabbits. Animals were bled one month after the injection.

The sera of these animals were frozen at  $-20^{\circ}\text{C}$  without inactivation. These sera gave a single precipitin line not only with isolated C'4 but also with normal human serum upon immunoelectrophoresis and by Ouchterlony's double diffusion technique.

5) Goat antihuman  $\beta_{1\text{C}/1\text{A}}$ -globulin serum

This antiserum produced by Hyland Laboratory was used for differentiation of the  $\beta_{1\text{E}}$ -line from the  $\beta_{1\text{C}/1\text{A}}$ -line.

4. *Materials used for immune hemolysis in gels*

All diluents and preparation of sheep erythrocytes (E), amboceptor (A), sensitized sheep erythrocytes (EA), guinea pig C'1 (C'1 gp), guinea pig C'2 (C'2 gp), EDTA treated guinea pig complement (C'-EDTA) and EA with C'1 gp activity (EAC'1 gp) were as reported previously (NAGAKI *et al.*, 1967 a). Titration of C'H50, C'1 and C'4 was done as described in the previous report (INAI *et al.*, 1967 a).

5. *Immunolectrophoresis*

Ten ml of 1 per cent Oxoid Ionagar No. 2 in veronal-HCl buffer ( $\mu=0.05$ , pH 8.6) was layered over a photographic glass slide ( $7.5 \times 12$  cm) and then allowed to gelatinize. An antigen well (diameter 2 mm) was punched out at a point 3 cm from the cathodal end. This well was filled with pooled human serum or purified human C'4. The electrode reservoirs contained veronal-HCl buffer ( $\mu=0.05$ , pH 8.6). Electrophoresis was carried out at  $4^{\circ}\text{C}$  for 1.5 hours at 6 v/cm. Under these conditions, the spot indicating the albumin fraction moved to the anodal side about 2.5 cm from the antigen well, while the  $\beta_1$ -globulin fraction did not move.

After electrophoresis, an antibody trough (2 mm width) was made with a razor blade. The distance between the edge of the trough and the well was usually 4 mm. Horse antihuman serum was put into the trough, and the slide was kept in a moist chamber at room temperature overnight.

After this procedure, precipitin lines were visualized, and the plate was washed for one day in a large volume of physiological saline solution, and then dried at  $37^{\circ}\text{C}$ , and stained with 0.1 per cent Amido black 10 B solution.

## RESULTS

### 1. *Hemolytic activity of C'4 after electrophoresis on agar plate*

The situation of purified C'4 on the agar plate after electrophoresis was studied.

In this experiment, C'4 with an activity of about  $20 \times 10^{12}$  effective molecules (eff. mol.) per ml was applied in a volume of 0.03 ml. The electrophoresis of C'4 on agar gel was performed as follows; a glass slide ( $7.5 \times 2.5$  cm) was coated with a layer of 1 per cent Difco Special Agar Noble in veronal HCl buffer ( $\mu=0.05$ , pH 8.6) which was allowed to gelatinize. Then, a well (diameter 2 mm) was punched out in the center of this slide, and the C'4 was put into this well. Electrophoresis was carried out at  $4^{\circ}\text{C}$  for 1.5 hours, at 6 v/cm.

To detect the hemolytic activity of C'4 to EAC'1 on the agar gel, 4 ml of 1 per cent Difco Special Agar Noble, 4 ml of EAC'1 cells ( $7.5 \times 10^8$  cells per ml), 4 ml of C'2 (600 eff. mol. per cell) and 0.2 ml of 1 per cent DEAE-Dextran were mixed at  $50^{\circ}\text{C}$ . The C'4 on the agar was subjected to electrophoresis and then the above mixture was spread thinly on this agar plate. The plate was incubated at  $37^{\circ}\text{C}$  for 20 minutes and then it was treated with 12.5 fold of C'EDTA for 2 hours at  $37^{\circ}\text{C}$ .

As shown in Fig. 1, the hemolytic zone was visualized as an oval shaped spot on the anodal side from the original point. Namely, the position of the hemolytic zone which was produced by electrophoresis of hemolytically active C'4 was in the  $\beta_1$ -globulin region.

### 2. *Immunolectrophoretic pattern of purified C'4*

To investigate the purity of the purified C'4, immunolectrophoretic analysis was performed with horse antihuman serum.

Volumes of 0.03 ml of whole human serum and purified C'4 ( $48.5 \times 10^{12}$  eff. mol. per ml, protein content 2 mg per ml) were filled in the wells. After electrophoresis, horse antihuman serum was put into the troughs. As shown

in Fig. 2, a single precipitin line between C'4 and antiserum was seen in the  $\beta_{1E}$ -globulin region. This single line of C'4 might correspond to  $\beta_{1E}$ -globulin, as reported by MÜLLER-EBERHARD and BIRO (1963).

On the other hand, as shown in Fig. 3, when rabbit antihuman C'4 serum was used on immunoelectrophoresis of C'4 and normal human

serum, this antiserum reacted and a single precipitin line developed with both antigens. This antiserum against human C'4 had high neutralizing activity for the hemolytic activity of C'4 and high agglutinating activity for the EAC'1, 4 cells (NAGAKI *et al.*, 1967b). These results showed that the C'4 used in the following experiments was pure.

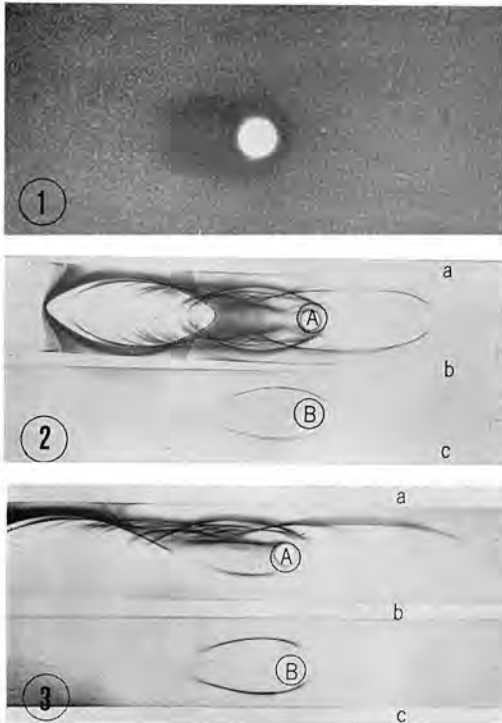


FIGURE 1 Hemolytic activity of C'4 after electrophoresis on agar plate. C'4 was subjected to electrophoresis on an agar plate, and then the hemolytic reaction was carried out on this plate.

FIGURE 2 Immunoelectrophoretic pattern of purified C'4.

A: normal human serum B: purified C'4  
a, b and c: horse antihuman serum

FIGURE 3 Immunoelectrophoretic pattern of normal human serum and purified C'4 against rabbit anti C'4 serum.

A: normal human serum B: purified C'4  
a and c: horse antihuman serum  
b: rabbit antihuman C'4 serum

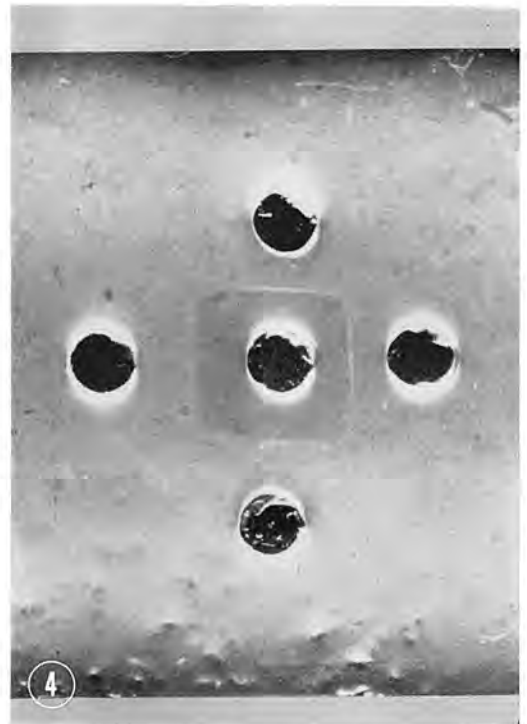


FIGURE 4 Lysis of EAC'1 cells by purified C'4 in diffusion plate.

The central well was filled with purified C'4 and the four peripheral wells were filled with horse anti-human serum. The distances from the central well to the peripheral wells were 2 mm, 3 mm, 4 mm and 5 mm respectively. The hemolytic zone is limited by a fused precipitin line.

### 3. Lysis of EAC'1 cells by purified C'4 in the diffusion plate

The following experiments were done to test whether so-called  $\beta_{1E}$ -globulin actually corresponds to C'4 itself.

Four ml of 2.5 per cent Oxoid Ionagar No. 2, 4 ml of  $1.5 \times 10^8$  EAC'1 cells and 4 ml of C'2 (to supply 600 eff. mol. per cell) were mixed at  $50^\circ\text{C}$ , and 0.2 ml of a 1 per cent solution of DEAE-Dextran was added to this mixture. This was poured on the glass slide ( $7.5 \times 2.5$  cm) and gelatinized. The distances between the central well and peripheral wells were 2 mm, 3 mm, 4 mm and 5 mm respectively. The central well was filled with  $5.5 \times 10^{12}$  eff. mol. per ml of C'4 (total protein content, 0.22 mg per ml) and the peripheral wells were filled with partially purified horse antihuman serum. As described in MATERIALS AND METHODS, the anticomplementary activity of horse serum had been almost completely eliminated from

this purified horse antihuman serum.

After a faint precipitin line had appeared on incubation for 8 hours at room temperature, a 12.5 fold of C'EDTA was poured onto the plate. Hemolysis of EAC'1 cells and precipitin lines were observed after incubation at  $37^\circ\text{C}$  for 2 hours and then at  $30^\circ\text{C}$  overnight.

As shown in Fig. 4, the precipitin lines formed between C'4 and horse antihuman serum in the peripheral wells fused with each other. A hemolytic zone was formed from the edge of the central well to the precipitin lines. The spread of this hemolytic zone was completely stopped by the precipitin line. It is conceivable that anti- $\beta_{1E}$  antibody in anti-serum reacted with C'4 ( $\beta_{1E}$ -globulin), and

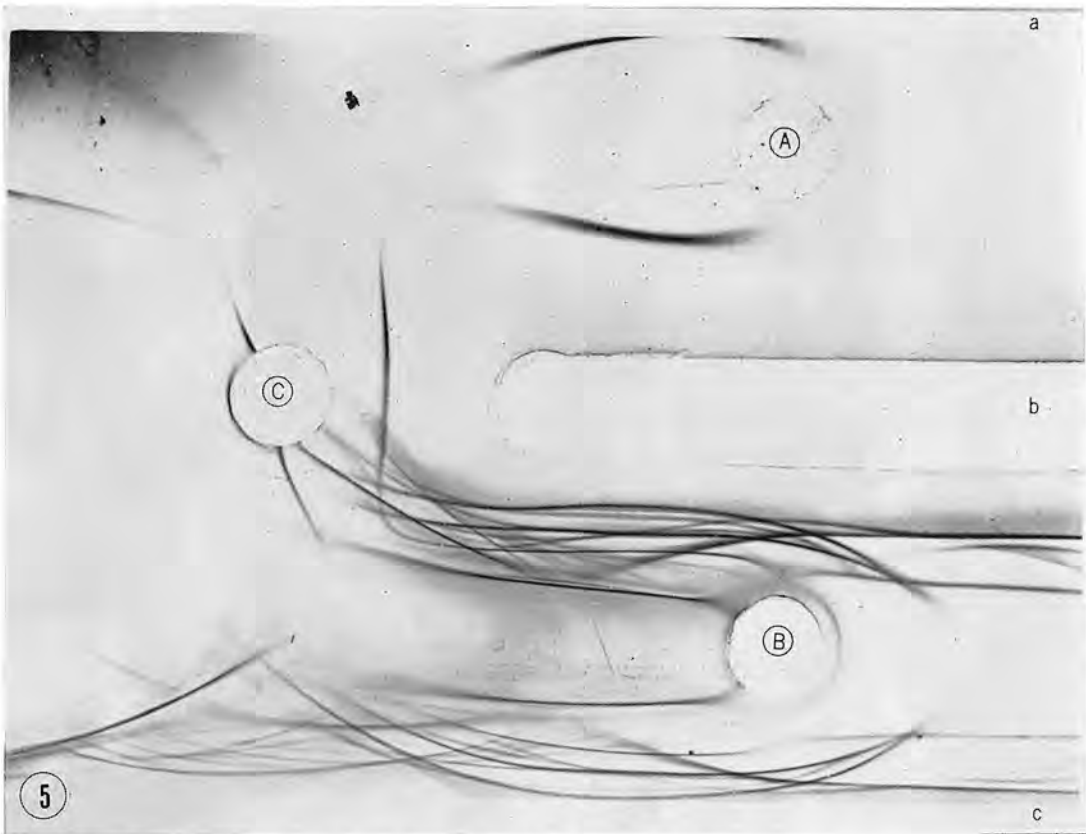


FIGURE 5 Immunoelectrophoretic situation of  $\beta_{1E}$ -globulin in normal human serum.

C'4 and normal human serum were put into wells A and B. After electrophoresis, C'4 was put into well C and horse antihuman serum into troughs a, b and c.

the hemolytic activity of C'4 was neutralized.

#### 4. Immunelectrophoretic situation of the precipitin line of $\beta_{1E}$ -globulin in immunelectrophoresis of normal human serum

To examine the precipitin line of C'4 in normal human serum, Ouchterlony's double diffusion technique was combined with immunelectrophoresis.

Normal human serum and  $7.5 \times 10^{13}$  eff. mol. per ml of C'4 (total protein content, 0.35 mg per ml) were subjected to electrophoresis as described above, and allowed to react with horse antihuman serum. At the same time C'4 was allowed to diffuse in the plate. As shown in Fig. 5, the precipitin line formed between C'4 and antiserum fused with the precipitin line in the  $\beta_1$ -globulin region. This precipitin line seems to correspond to the line of so-called  $\beta_{1E}$ -globulin.

#### 5. Immunelectrophoresis of normal human serum by use of absorbed horse antihuman serum

It is difficult to distinguish the  $\beta_{1E}$ -line from other lines in the  $\beta_1$ -globulin region, and it is particularly difficult to distinguish the  $\beta_{1E}$ -line from the  $\beta_{1C/1A}$ -line. Therefore, absorbed horse antihuman serum and goat antihuman  $\beta_{1C/1A}$ -globulin serum were applied in the same experiments. C'4 and normal human serum were subjected to electrophoresis and allowed to react with these antisera.

As shown in Fig. 6, when this absorbed antiserum was used on immunelectrophoresis of normal human serum, the lines of transferrin,  $\beta_{2A}$ -globulin and a part of the slowly migrating  $\alpha_2$ -globulin disappeared. Then the lines in the  $\beta_1$ -globulin region became clear.

As shown in Fig. 7, the precipitin lines of  $\beta_{1E}$ -globulin and  $\beta_{1C/1A}$ -globulin were more easily identified. Crossing of the  $\beta_{1E}$ -line and  $\beta_{1C/1A}$ -line was never seen in normal human serum.

#### 6. Effect of Hydrazine on the $\beta_{1E}$ -line

Using immunelectrophoresis, MÜLLER-EBERHARD and BIRO (1963) showed that

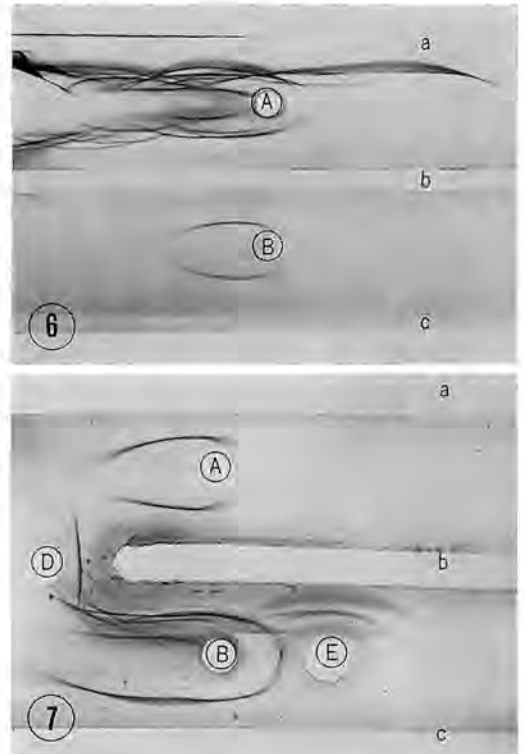


FIGURE 6 Immunelectrophoresis of normal human serum using absorbed horse antihuman serum.

A: normal human serum B: purified C'4  
a and c: unabsorbed horse antihuman serum  
b: absorbed horse antihuman serum

FIGURE 7 Differentiation of the  $\beta_{1E}$ -line from the  $\beta_{1C/1A}$ -line using absorbed horse antihuman serum.

Purified C'4 in well A and normal human serum in well B were subjected to electrophoresis. After electrophoresis, horse antihuman serum was put into troughs a and b, goat antihuman  $\beta_{1C/1A}$ -globulin serum was put into trough c and well E, and purified C'4 was put into well D.

hydrazine treatment of C'4 first resulted in an increased electrophoretic mobility of the  $\beta_{1E}$ -line and then after a few minutes caused a marked decrease in its mobility.

In this experiment, alteration of the  $\beta_{1E}$ -globulin was observed using normal human serum instead of purified C'4.

Five ml of normal human serum was mixed with 1.25 ml of 0.075 M hydrazine, and this mixture was incubated at 37°C. At suitable

intervals (10, 20, 30 and 60 minutes), 1.25 ml of this mixture was withdrawn and neutralized with 0.25 ml of 0.075 M HCl. In these experiments, scarcely any C'4 activity was detected even after 10 minutes. These samples and nontreated serum were subjected to electrophoresis. After electrophoresis anti-serum was put into the troughs.

As shown in Fig. 8, on treatment with hydrazine, the  $\beta_{1E}$ -line gradually extended to the cathodal side with time and crossed the  $\beta_{1C/1A}$ -line. No change was found on the anodal side, as C'4 activity had been lost after 30 minutes treatment.

#### 7. Effect of immune precipitate on the $\beta_{1E}$ -line

Peetoom and Pondman (1963) reported that the immunoelectrophoretic pattern of  $\beta_{1E}$ -globulin separated into  $\beta_{1E1}$  and  $\beta_{1E2}$  when normal human serum was treated with immune precipitate (Ea/anti-Ea). An experiment was performed to confirm this.

As the immune precipitate, egg albumin and anti-egg albumin specific precipitate were used. Volumes of 7.5 ml, 6.0 ml, 3.0 ml and 1.5 ml of suspension of immune precipitate were introduced into 4 tubes and centrifuged and the supernatants were discarded. To these tubes, 1.5 ml of pooled human sera were added and tubes were incubated for 30 minutes at 30°C and centrifuged. Then the C', C'1 and C'4 in each supernatant were measured. As a control, a tube was set up containing 1.5 ml of pooled human serum without added immune precipitate.

The C'H50, C'1H63 and C'4H63 in these treated sera and in control serum are shown

in Table 1. Sample No. 3 contained the least C'1H63 and C'4H63. The causes of the discrepancy between the total N in the immune precipitate used for treatment of serum and the decreases in C'1H63 and C'4H63 is uncertain.

These samples and control sera were used for immunoelectrophoretic analysis. As shown in Fig. 9, with treated serum the  $\beta_{1E}$ -line crosses the  $\beta_{1C/1A}$ -line, and the  $\beta_{1C}$ -line is converted to the  $\beta_{1E}$ -line. However, transfer of the  $\beta_{1E}$ -line to the cathodal or anodal side and formation of lines  $\beta_{1E1}$  and  $\beta_{1E2}$  were not observed.

#### 8. Immunoelectrophoretic pattern of the $\beta_{1E}$ -line in the sera of various patients

The relationship between the activity of C'4 and the  $\beta_{1E}$ -line of the serum in various diseases was investigated.

Sera were subjected to electrophoresis on an agar plate, and allowed to react with horse antihuman serum, absorbed horse antihuman serum or rabbit antihuman C'4 serum.

Results of these experiments were summarized in Table 2.

These results show a close relationship between the C'4 levels and the appearance of the  $\beta_{1E}$ -line in the serum in many cases. Thus, the  $\beta_{1E}$ -line appeared normal when the C'4 level was normal, and was not present when the C'4 level was low or apparently zero. In a few cases, as in case 3 on Aug. 10, 1966 (Fig. 13) and case 4 (Fig. 15), the  $\beta_{1E}$ -line was barely formed, and as in case 3 on Dec. 29, 1966 (Fig. 14), the C'4 level was apparently zero but the  $\beta_{1E}$ -line was only slightly decreased.

TABLE 1 C' and its components in serum specimens treated with immune precipitate

Sample	Total N in immune precipitate used ( $\gamma$ )	C'H50	C'1H63	C'4H63
1	1,500	3.4	35,500	21,500
2	1,200	4.2	10,000	14,000
3	600	6.3	7,800	7,600
4	300	16.4	88,000	63,000
Control	0	66.3	165,000	155,000



FIGURE 8 Effect of Hydrazine on the  $\beta_{1E}$ -line.

The immunoelectrophoretic patterns of normal human serum treated with hydrazine are shown. Control serum (untreated serum in well A) and treated sera (treated for 10, 20, 30 and 60 minutes in wells B, C, D and E, respectively) were subjected to electrophoresis. After electrophoresis, absorbed horse antiserum and unabsorbed horse antiserum were put into the troughs from a to f alternatively. The  $\beta_{1E}$ -line extended to the cathodal side with time and crossed the  $\beta_{1C/1A}$ -line.

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FIGURE 9 Effect of immune precipitate on the  $\beta_{1E}$ -line.

The immunoelectrophoretic patterns of normal human serum treated with immune precipitate are shown.

The  $\beta_{1E}$ -line crossed the  $\beta_{1C/1A}$ -line, and the conversion of  $\beta_{1C}$ -line to  $\beta_{1A}$ -line was observed.

C: control serum

1: sample No. 1

2: sample No. 2

3: sample No. 3

4: sample No. 4

troughs: absorbed horse antihuman serum

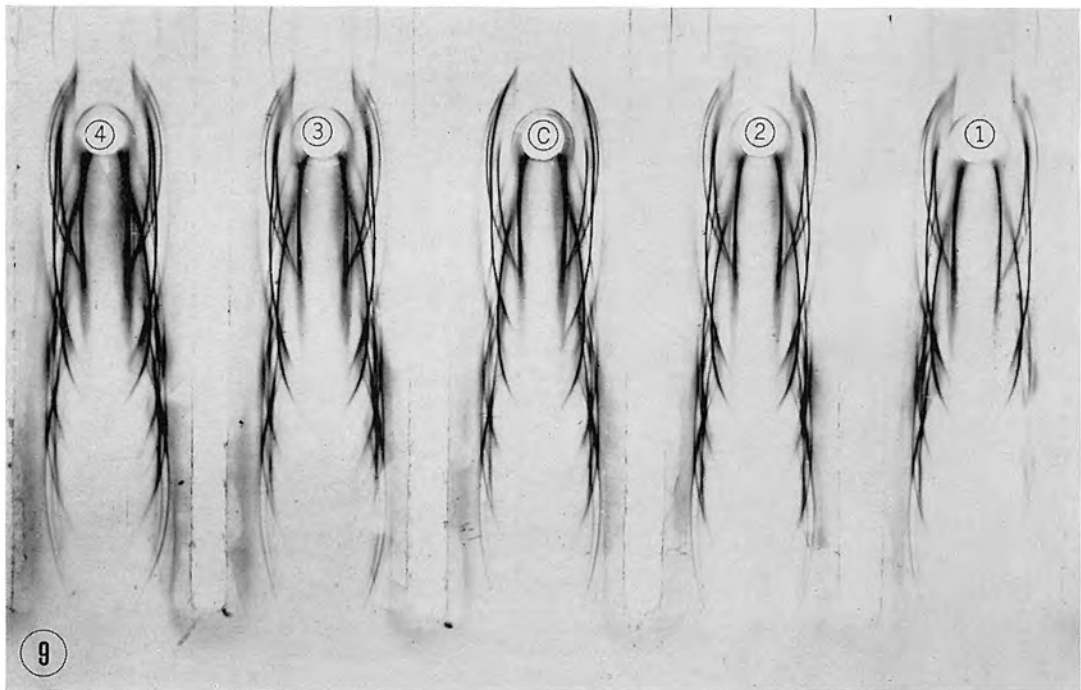
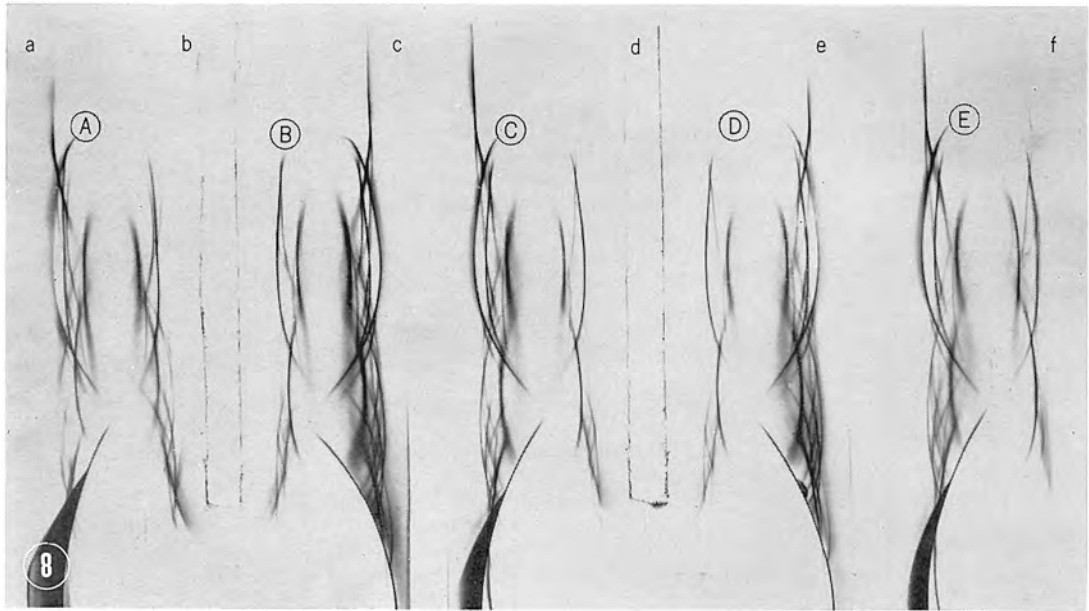


FIGURE 10 Immuno-electrophoretic pattern of the  $\beta_{1E}$ -line in case 1.  
The  $\beta_{1E}$ -line was normal.

P: serum of case 1  
a: horse antihuman serum  
b: absorbed horse antihuman serum

FIGURE 11 Immuno-electrophoretic pattern of the  $\beta_{1E}$ -line in case 2.  
No  $\beta_{1E}$ -line was observed.

P: serum of case 2  
a: absorbed horse antihuman serum  
b: horse antihuman serum

FIGURE 12 Immuno-electrophoretic pattern of the  $\beta_{1E}$ -line in case 2.  
No  $\beta_{1E}$ -line was observed.

N: normal human serum  
P: serum of case 2  
a and c: horse antihuman serum  
b: rabbit antihuman C'4 serum

FIGURE 13 Immuno-electrophoretic pattern of the  $\beta_{1E}$ -line in case 3.  
The strength of the  $\beta_{1E}$ -line was slightly decreased.

N: normal human serum  
P: serum of case 3  
a and c: horse antihuman serum  
b: rabbit antihuman C'4 serum

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FIGURE 14 Immuno-electrophoretic pattern of the  $\beta_{1E}$ -line in case 3.  
The strength of the  $\beta_{1E}$ -line was slightly decreased.

N: normal human serum  
P: serum of case 3  
a and c: horse antihuman serum  
b: rabbit antihuman C'4 serum

FIGURE 15 Immuno-electrophoretic pattern of the  $\beta_{1E}$ -line in case 4.  
The  $\beta_{1E}$ -line was very faint.

N: normal human serum  
P: serum of case 4  
a and c: horse antihuman serum  
b: rabbit antihuman C'4 serum

FIGURE 16 Immuno-electrophoretic pattern of the  $\beta_{1E}$ -line in case 5.  
No  $\beta_{1E}$ -line was observed.

N: normal human serum  
P: serum of case 5  
a and c: horse antihuman serum  
b: rabbit antihuman C'4 serum

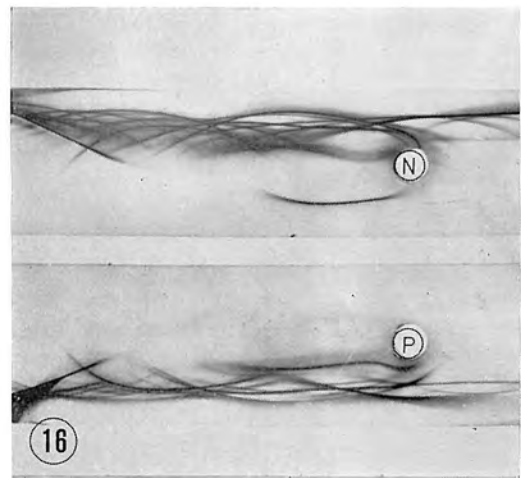
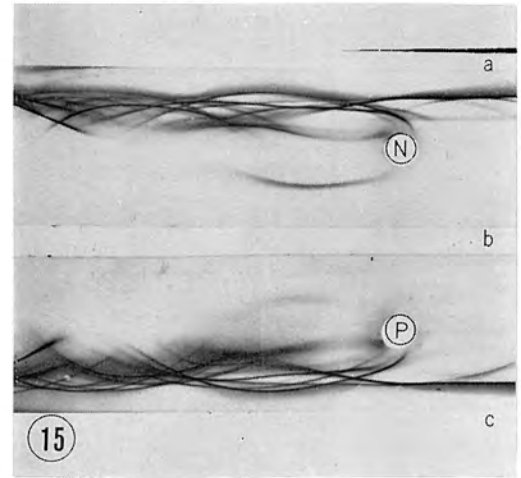
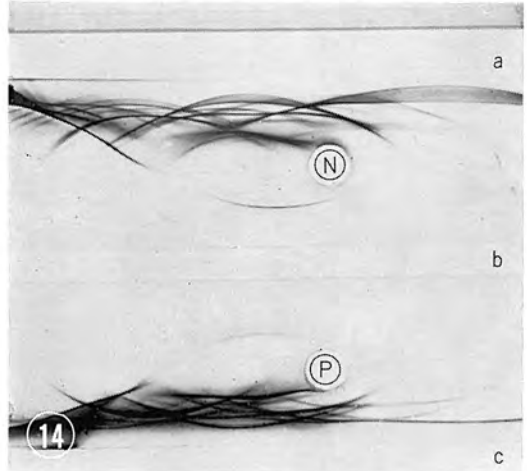
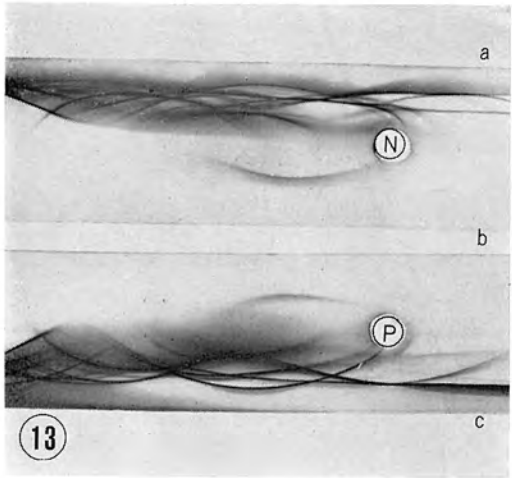
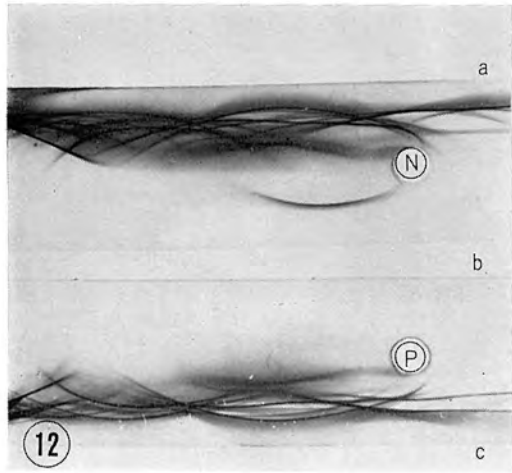
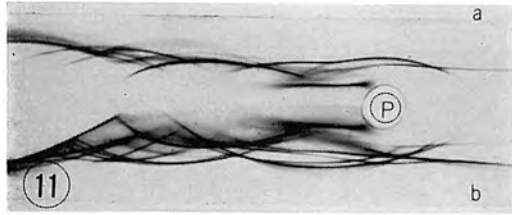
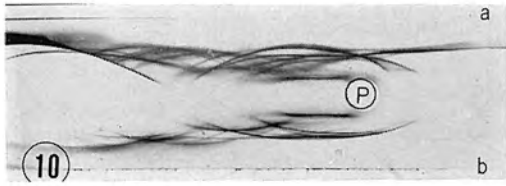


TABLE 2 *Immunoelectrophoretic pattern of the  $\beta_{1E}$ -line, and the C'H50 and C'4 levels in the sera of various patients*

Case No.	Dignosis	C'H50	C'4 level $\times 10$ eff. mol.	Appearance of $\beta_{1E}$ -line	Fig. No.
1	Rheumatoid arthritis	166.0	540.0	normal	10
2	Cirrhosis of liver	3.6	not detected	not observed	11
		5.0	0.45	not observed	12
3	Cirrhosis of liver	12.5	82.5	slightly decreased	13
		15.2	not detected	slightly decreased	14
4	Cancer of stomach	10.0	not detected	hardly observed	15
5	Cirrhosis of liver	10.0	0.27	not observed	16
6	Suspected multiple myeloma	16.7	42.0	not observed	17
7	Bence-Jones type multiple myeloma	92.3	6400.0	elongated and crossed $\beta_{1C/1A}$ -line	18

Case 7 (Fig. 18), which showed a persistently elevated serum C'4 level, as reported in the previous paper, exhibited an interesting immunoelectrophoretic pattern. The  $\beta_{1E}$ -line obtained from a sample of this patient was enlarged and crossed the  $\beta_{1C/1A}$ -line on the cathodal side, in parallel with the high C'4 activity. This  $\beta_{1E}$ -line was hardly identified and appeared to be absent in the precipitin lines formed between the patient's serum and antihuman serum. However, when rabbit antihuman C'4 serum was used, the  $\beta_{1E}$ -line was clearly seen. Moreover, as the horse antihuman serum used in this experiment did not contain antibody to  $\kappa$ -type Bence-Jones protein, rabbit antiserum against  $\kappa$ -type Bence-Jones protein was used for determination of the type of myeloma.

This suggests that the appearance of the  $\beta_{1E}$ -line is closely related with the C'4 level, but a few exceptions were seen when the  $\beta_{1E}$ -line was visible though the C'4 level was apparently zero. However, no case was found where C'4 was detected but no  $\beta_{1E}$ -line was formed.

#### DISCUSSION

As reported in the previous paper (INAI, *et al.*, 1967b), attempts were made to separate human C'4 from C'1 inactivator by various procedures, including DEAE cellulose column chromatography, CM-Sephadex column chromatography and Pevikon block electrophoresis.

The purified human C'4 so obtained had potent C'4 activity with a negligible amount of C'1 inactivator, and showed a single precipitin line in the  $\beta_1$ -globulin region by immunoelectrophoresis. It was considered that this precipitin line might correspond to the precipitin line of  $\beta_{1E}$ -globulin reported by MÜLLER-EBERHARD and BIRO (1963).

To see whether C'4 is identical with  $\beta_{1E}$ -globulin or whether C'4 activity is simply carried by molecules which are completely unrelated to, although physicochemically similar to  $\beta_{1E}$ -globulin, several experiments were performed by MÜLLER-EBERHARD and BIRO (1963). They concluded that  $\beta_{1E}$ -globulin might be identical with C'4. In this experiment, it was clearly demonstrated that the hemolytic zone of purified C'4 on an agar plate was completely limited by a single precipitin line between purified C'4 and purified

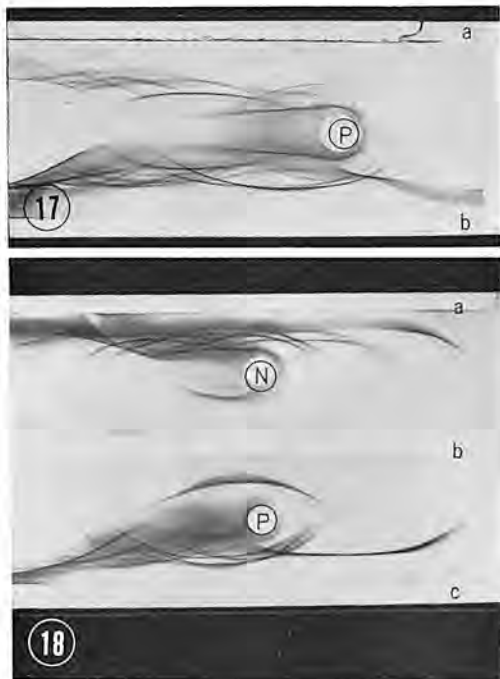


FIGURE 17 Immunoelectrophoretic pattern of the  $\beta_{1E}$ -line in case 6.

No  $\beta_{1E}$ -line or  $\beta_{1C/1A}$ -line was observed.

P: serum of case 6

a: absorbed horse antihuman serum

b: horse antihuman serum

FIGURE 18 Immunoelectrophoretic pattern of the  $\beta_{1E}$ -line in case 7.

The  $\beta_{1E}$ -line was elongated and crossed the  $\beta_{1C/1A}$ -line.

N: normal human serum

P: serum of case 7

a and c: horse antihuman serum

b: rabbit antihuman C'4 serum

horse antihuman serum on the same agar plate. The antihuman C'4 serum produced in rabbits showed a single precipitin line of  $\beta_{1E}$ -globulin against fresh human serum by immunoelectrophoresis. As reported in the following paper this anti C'4 serum had a remarkable capacity to neutralize the hemolytic activity of human C'4.

These results support the conclusion that human C'4 may be identical with  $\beta_{1E}$ -globulin.

If this conclusion is correct, the parallel between the C'4 levels in the sera of patient and

the precipitin line of its  $\beta_{1E}$ -globulin could be explained. To investigate this problem, the  $\beta_{1E}$ -line in the immunoelectrophoretic pattern of fresh human serum had to be identified. There are many precipitin lines in the  $\beta_1$ -globulin region in the immunoelectrophoretic pattern of normal human serum, namely those of transferrin, hemopexin,  $\beta_1$ -glycoprotein,  $\beta_{1C/1A}$ -globulin,  $\beta_{1B}$ -globulin, fibrinogen and  $\beta_1$ -lipoprotein and part of both  $\alpha_2$ -globulin and  $\gamma$ -globulin in this region.

To identify the  $\beta_{1E}$ -globulin line, immunoelectrophoresis and Ouchterlony's double diffusion technique were employed together. To differentiate the  $\beta_{1E}$ -line from the  $\beta_{1C/1A}$ -line, goat antihuman  $\beta_{1C/1A}$  serum was also used in this experiment.

Absorbed antiserum which did not contain the antibody to C'4 and  $\beta_{1C/1A}$ -globulin was used to reduce the number of precipitin lines in the  $\beta_1$ -globulin region in the immunoelectrophoretic pattern of whole human serum. In this way, the identification of the  $\beta_{1E}$ -line in the immunoelectrophoretic pattern of whole human serum became easier. Thus, the  $\beta_{1E}$ -line runs parallel with the  $\beta_{1C/1A}$ -line from the antigen well to the  $\beta_1$ -globulin region and crossing of the two lines was never found in normal human serum.

To analyse the changes in the  $\beta_{1E}$ -line in the sera of patients, the changes in this line on chemical or immunological treatment of normal human serum were studied.

On treatment of purified human C'4 with hydrazine, MÜLLER-EBERHARD and BIRO (1963) reported that the electrophoretic mobility of  $\beta_{1E}$ -globulin increased immediately after hydrazine treatment, but the subsequently its mobility gradually decreased. PEETOOM and PONDMAN (1963) stated that treatment of normal human serum with hydrazine or immune precipitate caused a cleavage of the  $\beta_{1E}$ -line into two lines,  $\beta_{1E1}$  and  $\beta_{1E2}$ , using anti C' serum. In this experiment, hydrazine treatment of normal human serum resulted in the extension of the  $\beta_{1E}$ -line to the anodal side and crossing of the  $\beta_{1E}$ -line and  $\beta_{1C/1A}$ -line.

Treatment of normal human serum with immune precipitate also resulted in the crossing of the two lines, though C'4 activity in treated serum was moderately decreased. However no cleavage of the  $\beta_{1E}$ -line into two lines was observed.

From these results, it is clear that the  $\beta_{1E}$ -line in human serum was changed but did not disappear when the hemolytic activity of C'4 was reduced or completely destroyed by these treatment.

It was found that there was a close relationship between the C'4 level in the sera of patients and the appearance of the  $\beta_{1E}$ -line in their sera. In particular, scarcely any of the sera with a markedly decreased C'4 level showed a  $\beta_{1E}$ -line by immunoelectrophoresis. However, in a few cases, the  $\beta_{1E}$ -line was found, though the C'4 level was apparently zero. However, in sera with a normal or high C'4 level, absence of a  $\beta_{1E}$ -line has never been seen.

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The discrepancies between the serum C'4 level and  $\beta_{1E}$ -line observed in a few cases suggest that the appearance of a  $\beta_{1E}$ -line in the immunoelectrophoretic pattern does not always imply the existence of C'4 activity in the sample.

From the present results it seem likely that the site of hemolytic activity of C'4 on molecules of  $\beta_{1E}$ -globulin might differ from that of the antigen determinant for the precipitin reaction of  $\beta_{1E}$ -globulin. Accordingly, the existence of  $\beta_{1E}$ -globulin which lacks the site of hemolytic activity of C'4 is conceivable.

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