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The paraproteins in systemic capillary leak syndrome

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SUMMARY

Systemic capillary leak syndrome (SCLS) is a rare disease characterized by episodes of collapse due to rapid transfer of considerable volumes of plasma from the intravascular to the extravascular compartment. The pathogenesis of this disease is unknown. The diagnosis is made largely on clinical grounds, and investigations are unhelpful. The only consistent abnormality is that an IgG paraprotein is found in most patients, raising the possibility that the paraprotein may be involved in the pathogenesis of the disease. Reduction of the paraprotein level in our patient was associated with remission. Blood samples from three SCLS patients and one probable SCLS have been studied. All patients had monoclonal IgG paraproteins. The purified paraproteins were all of IgG1 subclass and had κ light chains. However, they differed in size and charge. Antibodies against each of the paraproteins were raised in rabbits. Affinity-purified anti-idiotypic antibodies were tested for cross-reactivity against the other paraproteins using immunoblotting and Ouchterlony assay. These assays showed that the anti-idiotypic antibodies reacted only with the immunizing paraprotein and not with any of the other paraproteins, i.e. that the paraproteins do not share a common idiotype. Paraproteins did not bind to cultured endothelial cells, either unactivated or following activation with interferon-gamma (IFN-γ), IL-2 or IL-6. In addition, we were unable to demonstrate any cytotoxicity towards cultured human endothelial cells by paraprotein alone, or in the presence of neutrophils (pronounced neutrophilia being a feature of attacks). The relationship between the paraproteins and the disease remains unclear. It is likely that additional, as yet unidentified, factors are required for the paraprotein to lead to capillary leak.

Keywords systemic capillary leak syndrome paraprotein

INTRODUCTION

Systemic capillary leak syndrome (SCLS) is a rare disease, first described in 1960 [1]. Since then, only 30 cases have been reported [2–6]. SCLS is characterized by episodes of sudden movement of large amounts of plasma, including water, electrolytes and proteins of mol. wt up to at least 200 kD [7], from the intravascular to the extravascular space, which results in hypovolaemic shock, haemoconcentration, and sometimes diffuse swelling. The episodes may be as short as a few hours or as long as several days, and symptoms subside when the plasma re-enters the intravascular space. Between attacks, patients are well. The frequency of attacks differs in different patients, but the trend is for frequency to increase with time. Some patients have died during attacks, although the cause of death often appears to be due to pulmonary oedema, because of fluid overload when rapid intravenous infusions are continued after the capillary leak has stopped.

The pathogenesis of this disease is unknown. Although various abnormalities have been described, they occurred in only one or two patients. The only laboratory abnormality consistently found in most patients is that they have monoclonal IgG paraproteins. The concentration of paraprotein varies in different patients, but is constant in the same patient during attack and remission. Patients' serum, plasma or partially purified paraprotein, taken in attack or in remission, in no case gave an increase in capillary permeability when tested by injection into guinea pig skin [7]. However, one of our patients has been treated with plasma exchange and later by extracorporeal removal of immunoglobulin on a protein A column. After both treatments, a period of remission occurred when the level of the paraprotein was reduced, but attacks recurred when the paraprotein returned to its pretreatment level [8]. This finding suggests that the paraprotein might be involved in the pathogenesis of the disease.

In this study, we report on the properties of the paraproteins.

MATERIALS AND METHODS

Sera and plasmas

Blood samples were obtained from three SCLS patients (patients 1, 2 and 3) and one (patient 4) with suspected SCLS. Serum and heparinized plasma were obtained from patient 2 at

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different times: at the onset of an attack, during an attack and at the end of an attack. Single samples were obtained from the other patients. Normal human serum (NHS) or plasma was obtained from healthy donors.

Preparation of anti-idiotypic antibody

The paraprotein from the patient's serum was first purified through a DEAE-Sephacel (Pharmacia, Milton Keynes, UK) column eluted with 10 mM Tris/HCl, pH 8·0, and then through Mono S column (Pharmacia) by fast protein liquid chromatography (FPLC), eluted with 50 mM 2-(N-morpholino) ethanesulphonic acid (MES), pH 6·0. Antibodies against each paraprotein were raised in rabbits (New Zealand white rabbit, male, 2 years old). The anti-idiotypic antibody was purified by affinity chromatography first through a human IgG-coupled Sepharose CL-4B column, and then through a paraprotein-coupled Sepharose CL-4B column (Pharmacia) [9]. Anti-idiotypic antibody bound to the paraprotein column was eluted by 4 M magnesium chloride.

Immunoblotting

The agarose gel electrophoresis was run with veronal buffer at pH 8·8. The protein was transferred onto nitrocellulose paper (Schleicher & Schuell, Dassel, Germany) and probed with ^{125}I-labelled rabbit anti-idiotypic antibodies.

SDS–PAGE

SDS–PAGE was performed according to the method of Laemmli [10].

Assay for immune complexes

A conglutinin binding polyethylene glycol (PEG) precipitation assay was used for the detection of immune complexes [11]. Briefly, 20 μl ^{125}I-bovine conglutinin (50 000 ct/min) were added to 100 μl serum diluted in 300 μl borate buffered saline (BBS) containing 10 mM calcium chloride. After 1 h incubation at 4°C, 420 μl 4% PEG 6000 (Merck Ltd, Lutterworth, UK) containing 10 mM calcium chloride were added and incubated for 90 min at 4°C. After centrifugation, the radioactivity in the precipitate was counted (1277 Gammamaster gamma counter, Pharmacia). Positive controls were prepared by adding soluble heat-aggregated human IgG to each serum sample.

Paraprotein concentration in patients' sera

The concentration of paraprotein in patients' sera was tested by sandwich ELISA. A 96-well plate (Dynatech, Billingshurst, UK) was coated with affinity-purified rabbit anti-human IgG antibody or rabbit anti-idiotypic antibody in 50 mM carbonate-bicarbonate buffer at 4°C overnight. The plate was washed with PBS/0·5% Tween 20 and blocked with 200 μl 2% bovine serum albumin (BSA)/PBS/0·5% Tween 20. Test sample (100 μl) was then added to each well and incubated for 1 h at room temperature. After washing, 100 μl alkaline phosphatase-conjugated rabbit anti-human IgG antibody or anti-idiotypic antibody were added and incubated for 1 h at room temperature. After washing, 100 μl (1 mg/ml) *p*-nitrophenyl phosphate (Sigma, Poole, UK) were added. The optical density (OD) was read by a microplate reader (Bio-Rad, model 3550) at 405 nm.

Endothelial cell ELISA

Endothelial cells isolated from human umbilical cord veins were grown on fibronectin (Sigma)-coated tissue culture flasks (Falcon, Oxford, UK) in medium 199 (Flow Labs, High Wycombe, UK) containing 83 μg/ml heparin (Sigma), 2·5 ng/ml acidic fibroblast growth factor (Sigma), 17% heat-inactivated fetal calf serum (FCS), penicillin and streptomycin. The cells were passaged by trypsinization and seeded into 96-well plates (Life Technologies, Paisley, UK) precoated with fibronectin at a density of 3×10^4 cells/well and grown until they were confluent. The cells were used after one or two passages.

Endothelial cells in 96-well plates were used inactivated or after activation with 100 U/ml recombinant human interferon-gamma (IFN-γ; Biogen Ltd, London, UK) for 35 h, 10 U/ml recombinant human IL-2 (Cetus Diagnostics, Emeryville, CA) for 20 h or 10 ng/ml recombinant human IL-6 (British Biotechnology Ltd., Oxford, UK) for 20 h. The cells were then fixed with 0·025% glutaraldehyde at room temperature for 10 min and treated with 0·2% Triton X-100 (BDH) for 5 min. After blocking with 2% BSA-PBS-0·5% Tween 20, paraproteins were added and incubated at 4°C overnight. The positive controls were rabbit anti-human von Willebrand factor (Factor VIII-related antigen) (Sigma) and anti-CD59 antibody, produced in our own laboratory. The negative control was the IgG fraction from AB positive sera from healthy donors. Protein bound to endothelial cells was detected by alkaline phosphatase-conjugated rabbit anti-human IgG antibody or goat anti-rabbit IgG antibody (Sigma).

Endothelial cell cytotoxicity assay

Neutrophils (PMN) were isolated from buffy coat from healthy donors by Ficoll–Paque (Pharmacia) density gradient centrifugation and dextran 110 (Pharmacia) sedimentation. The purity of separated neutrophils was usually more than 98%. Neutrophils were primed with 0·5 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) [12] and 100 U/ml recombinant human IFN-γ (Biogen) [13–16] for 23 h at 37°C. After priming, more than 99% neutrophils were viable when tested by trypan blue exclusion.

Cytotoxicity was measured by a ^{51}Cr-release assay. Confluent endothelial cells were incubated with 4 μCi of $Na_2{}^{51}CrO_4$ per well (Amersham, Amersham, UK) at 37°C for 5 h, then washed with assay medium (RPMI 1640 medium containing 5% FCS, 25 mM HEPES at pH 7·2, and penicillin and streptomycin). Rabbit anti-CD59 (10 μg) was added as positive control. After incubation for 1 h at 37°C, the wells containing anti-CD59 antibody were washed twice with assay medium. Then, 10 μg paraproteins and 2×10^6 primed neutrophils were added to each well in a final volume of 250 μl. Each sample was tested in quintuplicate. Normal human IgG (Sigma) was used as a negative control. After incubation at 37°C for 5 h, supernatant was removed from each well and counted. The total release was obtained by solubilizing the remaining cells with 50% formic acid. Spontaneous release was calculated from the wells containing medium alone. Spontaneous release was about 15% or less of total release. The percentage of cytotoxicity was calculated as follows: per cent cytotoxicity = (experimental release − spontaneous release)/(total release − spontaneous release).

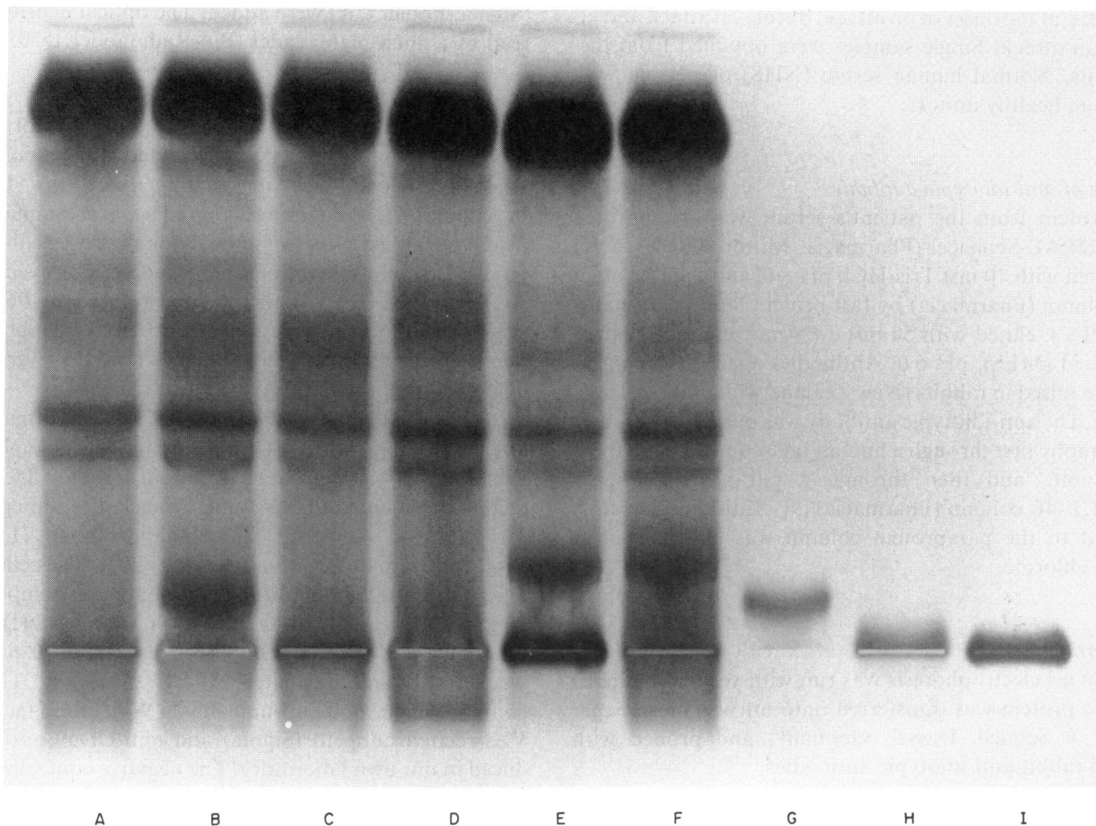

Fig. 1. Agarose gel electrophoresis of serum or plasma samples. A, Normal human serum; B, patient 2 serum; C, patient 1 serum; D, patient 4 serum; E, patient 3 plasma; F, normal human plasma; G, purified paraprotein from patient 2; H, purified paraprotein from patient 1; I, purified paraprotein from patient 3.

RESULTS

Existence of paraproteins

All SCLS patients had a single band in the IgG region on agarose gel, indicating the presence of a monoclonal IgG paraprotein. Patient 4, who was only suspected of having SCLS, also had a single band at the edge of IgG region, but this was less intense (Fig. 1). The serum from patient 2 at different time points covering an attack (at the onset, during and at the end of an attack) showed a constant concentration of paraprotein by ELISA (Fig. 2). The total concentration of paraprotein in patient 2 was 9·2 g/*l*, and total IgG was 16·7 g/*l*.

The purified paraproteins showed a single band on agarose gel (Fig. 1). All were of IgG1 subclass and had κ light chains (data not shown). However, they differed in size and charge. Patient 2's paraprotein migrated towards the anode on agarose gel at pH 8·8, while the paraproteins of patients 1 and 3 stayed at the origin, and patient 4's paraprotein moved towards the cathode (Fig. 1). The size of the paraproteins of patients 2 and 3 were the same as normal IgG on SDS–PAGE, but patient 1's paraprotein had a slightly smaller heavy chain (about 48 kD), and a larger light chain (about 32 kD) (Fig. 3).

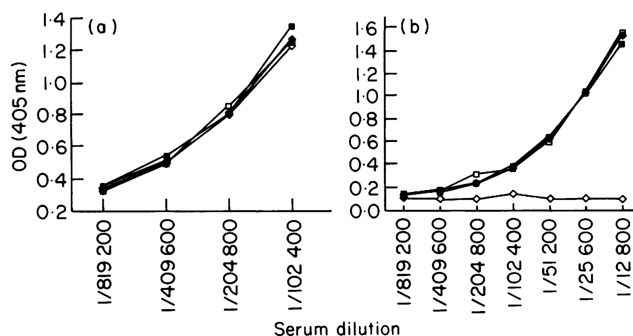

Fig. 2. Concentration of IgG and paraprotein from patient 2 serum measured by ELISA. (a) The plate was coated with anti-human IgG, and IgG bound from serum was detected by alkaline phosphatase-conjugated anti-human IgG. (b) The plate was coated with anti-idiotypic antibody, and paraprotein bound from serum was detected by alkaline phosphatase-conjugated anti-idiotypic antibody. □, At the onset of an attack; ■, during an attack; ◆, at the end of an attack; ◇, normal human serum (NHS).

Cross-reactivity of paraproteins

Antibodies were raised in rabbits against the purified paraproteins. The anti-idiotypic antibodies were affinity purified and tested for cross-reactivity against the other paraproteins using immunoblotting and Ouchterlony assay. These assays showed that the anti-idiotypic antibody reacted only with the immunizing paraprotein, but not with any of the other paraproteins (Figs 4 and 5). These paraproteins therefore showed no cross-reactivity, and thus do not appear to share any common idiotypes.

Existence of immune complexes

Only patient 2's serum was tested, and immune complexes were not detected (Fig. 6).

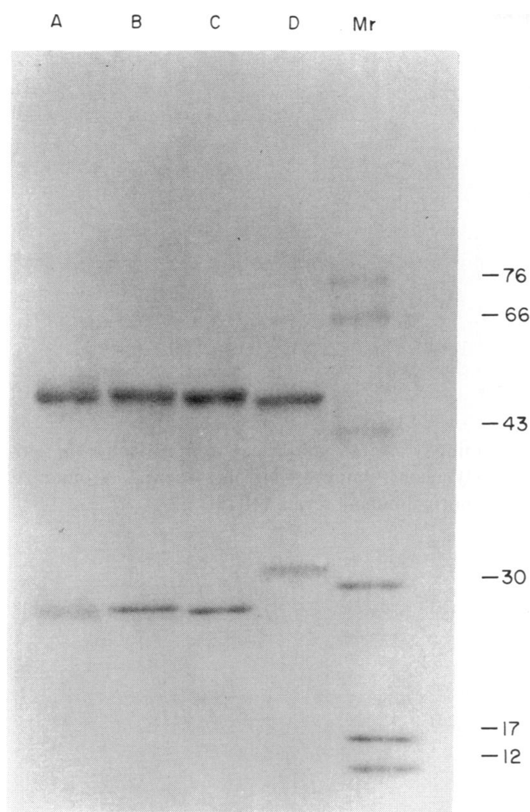

Fig. 3. SDS–PAGE of paraprotein (7–17% gradient gel, reduced). A, normal human IgG; B, paraprotein from patient 2; C, paraprotein from patient 3; D, paraprotein from patient 1; Mr, molecular weight markers (kD).

Binding of paraproteins to endothelial cells

The binding of paraproteins to human endothelial cells was tested by ELISA. None of the paraproteins purified from patients 1, 2 and 3 showed any binding to endothelial cells compared with normal human IgG as negative control, the OD at 405 nm was less than 0·25 for all paraproteins, at concentrations up to 20 μg/ml, tested on unactivated or IFN-γ- or IL-2-activated endothelial cells. For IL-6-activated endothelial cells, they had OD less than 0·41. In contrast, however, the ELISA showed marked binding of rabbit anti-human von Willebrand factor with OD > 1·2 and anti-CD59 with OD > 0·7 to endothelial cells when the antibody concentration was 5 μg/ml (positive control) (Fig. 7).

Cytotoxicity

Paraproteins and rabbit anti-CD59 antibody at a concentration of 40 μg/ml had no toxic effect when incubated with endothelial cells. In addition, there was no cytotoxicity when paraproteins were incubated with both endothelial cells and neutrophils (less than 6% specific release of ^{51}Cr). However, more than 23% specific release of ^{51}Cr was reached when anti-CD59 antibody was incubated with both endothelial cells and neutrophils (Fig. 8).

DISCUSSION

The presence of a paraprotein is the commonest abnormality in SCLS. Thirty cases, most between the ages of 30 and 50 years, have been reported in the literature. Twenty-five of these were found to have a paraprotein, whereas only two did not have a paraprotein and in three cases paraproteins were not reported [2–5]. All paraproteins were IgG, although they differed in subclass and light chains. In this paper, we have studied three SCLS patients and one patient suspected of having SCLS, all of whom had IgG paraproteins. The high incidence of paraprotein in SCLS, compared with the expected incidence of only 0·1% in a 30–50 year population, raises the possibility that the paraprotein may be involved in the pathogenesis of the disease. This hypothesis is supported by the observation that in one of our patients (patient 2), reduction in the paraprotein level was associated with remission [8].

If paraproteins are involved in the pathogenesis, one possible mechanism is that they act like antibodies, since all have IgG structure. They could bind to an unidentified endothelial or other antigen, leading to capillary leak. However, we were unable to identify, either in the fluid phase or by studies of binding to endothelial cells, a possible target for the paraproteins. This however does not exclude binding to a vascular antigen, and it is possible that some preactivation of endothelium is required for binding of the paraprotein to occur. The fact that attacks occur intermittently is in favour of this type of explanation. Immunohistochemistry, reported by others, has failed to show any immunoglobulin deposition in tissues [5,7,17,18]. Muscle cells and connective tissue appeared normal. One paper noted thickening of the capillary basement membrane, but the significance of this is not clear [5].

Although we demonstrated that the paraproteins do not share a common idiotype, it is still possible that the paraproteins bind to the same antigen, e.g. the paraproteins may bind to different antigenic sites on the same antigen. It has been reported that some myeloma proteins do not share a common idiotype, although they all bind to a small hapten [19].

It seems unlikely that the paraproteins alone cause the blood vessel leakage, and other factors are possibly involved. A common feature of SCLS is that patients have neutrophilia or leucocytosis. For example, patient 2 rapidly developed a pronounced neutrophilia (in the range $30–60 \times 10^9/l$) in the hours just before the onset of an attack. Thirteen of 19 SCLS patients from the English literature have been reported to have a neutrophilia or leucocytosis, and where values were stated leucocyte counts varied from 14 to $59 \times 10^9/l$. This raises the possibility that the leakage of blood vessels could be due to neutrophil-mediated tissue damage. We therefore cultured endothelial cells in the presence of both neutrophils and paraprotein and tested cytotoxicity by ^{51}Cr release assay. No cytotoxicity was detected, however.

So far, there is little evidence to show endothelial cell damage in SCLS [5,18], and it has been proposed that leakage occurs through widened gap junctions between endothelial cells [2,4]. If endothelial cell damage occurs during an attack, the plasma level of von Willebrand factor should rise [20], since this factor is produced only by endothelial cells and megakaryocytes [21]. We measured von Willebrand factor levels in patients' plasmas by ELISA. Only the suspected SCLS patient had a raised level of von Willebrand factor, the other patients having normal levels either during an attack or in remission (data not shown).

It is also possible that paraproteins do not have a direct relationship to the capillary leakage, but are an epiphenomenon of the disease. However, no other mechanism for producing

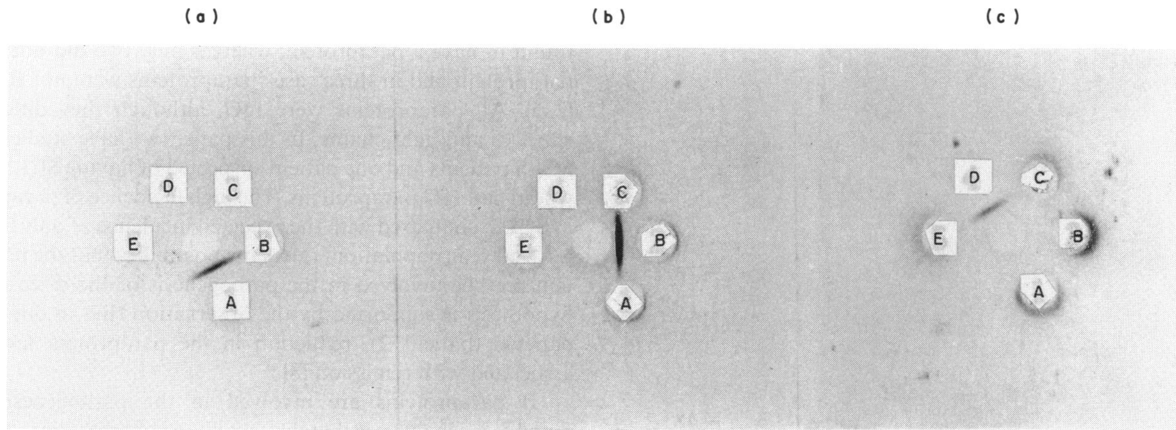

Fig. 4. Ouchterlony assay of patient blood samples. Central: (a) anti-idiotypic antibody against paraprotein from patient 1; (b) anti-idiotypic antibody against paraprotein from patient 2; (c) anti-idiotypic antibody against paraprotein from patient 3. Around: A, patient 1 serum; B, patient 2 serum; C, patient 4 serum; D, patient 3 plasma; E, normal human serum (NHS).

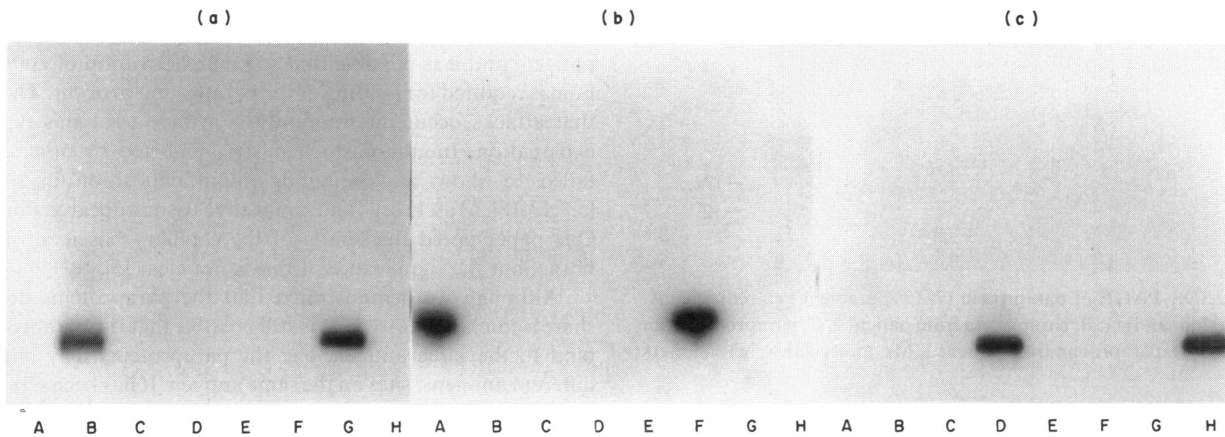

Fig. 5. Immunoblotting of patient serum or plasma samples. (a) Probed by ^{125}I-anti-idiotypic antibody against paraprotein from patient 1. (b) Probed by ^{125}I-anti-idiotypic antibody against paraprotein from patient 2. (c) Probed by ^{125}I-anti-idiotypic antibody against paraprotein from patient 3. A, patient 2 serum; B, patient 1 serum; C, patient 4 serum; D, patient 3 plasma; E, normal human serum; F, paraprotein from patient 2; G, paraprotein from patient 1; H, paraprotein from patient 3.

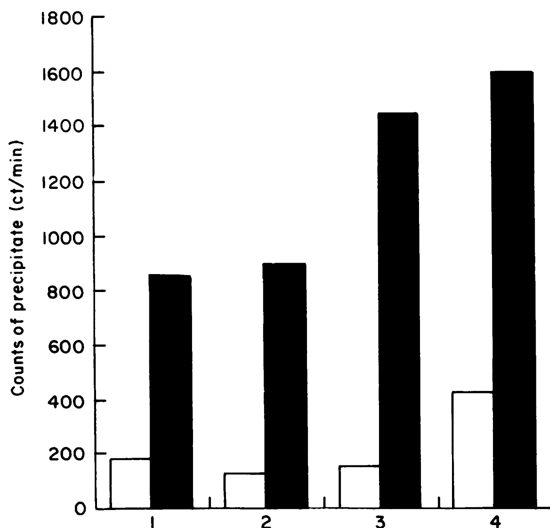

Fig. 6. Detection of immune complexes in patient 2 serum by conglutinin binding test. 1, At the onset of an attack; 2, during an attack; 3, at the end of an attack; 4, normal human serum (NHS). □, Serum; ■, 5 μg heat-aggregated IgG/ml serum.

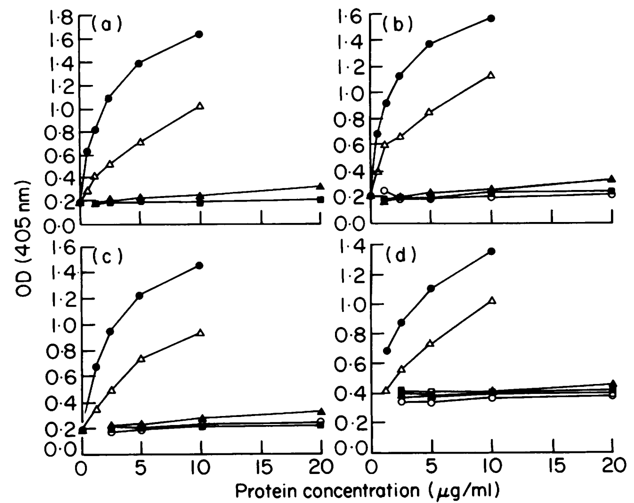

Fig. 7. Binding assay of paraproteins to endothelial cells by ELISA. (a) Endothelial cells were unactivated; endothelial cells were activated by (b) IFN-γ, (c) IL-2, and (d) IL-6. ■, Paraprotein from patient 1; □, paraprotein from patient 2; ○, paraprotein from patient 3; ●, rabbit anti-human von Willebrand factor; △, anti-CD59; ▲, normal human IgG.

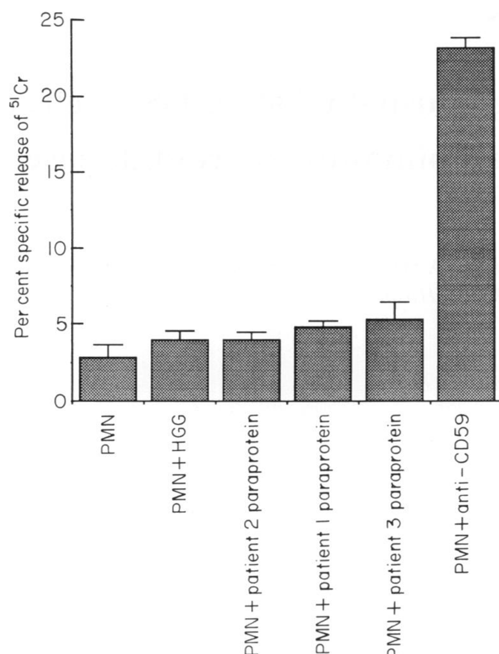

Fig. 8. Antibody-dependent cell-mediated cytotoxicity (ADCC) test on endothelial cells in the presence of paraproteins and neutrophils; $n=5$.

capillary leakage has so far come to light, and another explanation would then be needed for the clinical remission associated with removal of the paraprotein in our patient.

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