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Characterization of measles virus-specific antibodies in sera from patients with chronic active hepatitis

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SUMMARY

Measles virus-specific antibodies in sera from patients with HBsAg-negative chronic active hepatitis and raised antibody titres against measles virus, have been examined by crossed immunoelectrophoresis. The immunoprecipitates were further analysed by SDS-polyacrylamide gel electrophoresis. Five measles virus-specific precipitation lines were demonstrated using measles virus-infected cells solubilized with Triton X-100. The three major precipitation lines were analysed by SDS-PAGE and contained the virus polypeptides: nucleoprotein, NP (MW ~ 60000); haemoagglutinin, H (MW ~ 80000) and fusion protein F_1 (MW ~ 40000). Considerably higher amounts of antibodies against these three virus polypeptides were demonstrated in the patient sera than in sera from healthy controls. By SDS-PAGE analysis of radiolabelled immune complexes adsorbed to Sepharose-protein A, antibodies against five measles virus polypeptides: NP, H, F_1, P protein (MW ~ 70000) and matrix protein, M (MW ~ 37000) were demonstrated in the patient sera.

INTRODUCTION

The structural components of measles virus have recently been identified and partly characterized (Graves, Silver & Choppin, 1978; Robbins & Bussell, 1979; Stallcup, Wechsler & Fields, 1979; Tyrrell & Norrby, 1978). In addition to RNA the virion contains six major polypeptides which are characterized by their molecular weights (MW) and designated as follows: L, large polypeptide, MW ~ 200000; H, haemagglutinin, a glycoprotein, MW ~ 80000; P, nucleocapsid-associated phosphoprotein, MW ~ 70000; NP, nucleoprotein, MW ~ 60000; F_1, fusion protein, a structural component of the haemolysin, MW ~ 40000, and M, matrix protein, MW ~ 37000. In addition, the virion contains host-cell-derived actin, MW ~ 43000, and probably some other host proteins (Mountcastle & Choppin, 1977; Wechsler & Fields, 1978). Analysis of measles virus-infected cells has revealed some other virus-specific polypeptides. Under non-reducing conditions a polypeptide F_0, MW ~ 62000, has been demonstrated in virus-infected cells. F_0 has been shown to be a glycoprotein and is a precursor protein, being split

into the polypeptides F_1, MW ~ 40000, and F_2, MW ~ 20000 (Graves, Silver & Choppin, 1978; Hardwick & Bussell, 1978; Rima & Martin, 1979). Viral nucleocapsids isolated from the cytoplasm of infected cells contain some additional polypeptides with MW in the range of 45000–55000 (Robbins, Bussell & Rapp, 1980; Vainionpaa, Ziola & Salmi, 1978). These polypeptides probably represent degradation products of the original nucleoprotein, which is easily cleaved by host cell enzymes (Mountcastle & Choppin, 1977; Stallcup, Wechsler & Fields, 1979). The polypeptide P4, MW ~ 55000, usually found by polypeptide analysis of measles virus from infected cells, is assumed to be one of the nucleoprotein degradation products (Mountcastle & Choppin, 1977; Robbins & Bussell, 1979).

We have previously demonstrated high titres of haemagglutination-inhibiting (HI) and complement-fixing (CF) antibodies against measles virus in sera from a majority of patients with chronic active hepatitis (CAH) not caused by hepatitis B virus (Christie & Haukenes, 1979). In some sera, the CF titre was elevated more than a hundred times that of the titre of pooled human serum. Raised antibody titres against measles virus have not been found in sera from patients with CAH caused by hepatitis B virus (Galbraith *et al.* 1976). In serum from patients with HBsAg-negative CAH antibodies against intestinal bacteria and autoantigens have also been demonstrated (Eddleston & Williams, 1976; Triger, 1976). The antigen preparations used in the HI and CF tests are crude and contain several antigens. A cross-reaction might therefore be possible. However, we have found that the major immunoprecipitate obtained by immunoelectrophoresis contains large amounts of measles virus nucleocapsid (Christie & Haukenes, 1979).

The aim of the present study is to examine the specificity of the antibodies involved in the serological measles virus tests, and also to see whether there is an increased antibody response to both external and internal measles virus proteins in patients with HBsAg-negative CAH, compared to healthy controls.

We have used crossed immunoelectrophoresis (CIE) with radiolabelled antigen, and analysed the immunoprecipitate by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autofluorography (Norrild, Bjerrum & Vestergaard, 1977). In addition, radiolabelled immune complexes adsorbed to Sepharose-protein A were analysed by SDS-PAGE. The measles virus-specific antibody patterns of the sera from patients with CAH were compared with those obtained with sera from (*a*) a patient with SSPE; (*b*) patients with acute measles virus infection; (*c*) healthy individuals with and (*d*) without a history of measles.

MATERIALS AND METHODS

Virus

Edmonston strain B measles virus was obtained from Statens Seruminstitut, Copenhagen, and propagated in Vero cells grown in Eagle's Minimum Essential Medium, supplemented with 10% foetal calf serum and 1% glutamine. The cells were infected with virus at an input multiplicity of 0.01 p.f.u./cell and harvested 3–4 days later by scraping the cells into the medium. The infected cell suspension was centrifuged for 15 min at 800 *g*. The cell pellet was stored at −70 °C. The

supernatant was clarified by centrifugation at 10000 *g* for 10 min, and stored at 4 °C for no more than 24 h.

Cell-associated measles virus antigen (cellass-MVA)

A pellet from centrifugation of a suspension of infected cells was treated with 3% Triton X-100 (Packard Instrument Company, Downers Grove, Ilinois) in 0·1 M-NaCl-glycine buffer, pH 8.4, for 1 h at room temperature. The suspended pellet was sonicated for 10 s at 20 kH (MSE Ultrasonic apparatus), with a 3 mm probe, centrifuged for 10 min at 10000 *g* and stored at −70 °C. The supernatant was sonicated for 10 s after thawing before use. Measles virus haemagglutination (HA) and CF tests were used to quantitate the antigen as described previously (Christie & Haukenes, 1979). The HA and CF titres of cellass-MVA were 3200 and 512, respectively. Sham-infected Vero cell antigen was made by the same procedure and served as a control.

Extracellular measles virus antigen (extra-MVA)

The clarified supernatant was centrifuged at 95000 *g* for 60 min. The virus pellet was treated with 3% Triton X-100 in the NaCl-glycine buffer (see above) containing 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF) (Sigma Chemical Company, St Louis, Missouri), 1 mM benzamidine (Sigma) and 100 u. Trasylol/ml (Bayer, Leverkusen, Federal Republic of Germany) for 2 h at 4 °C. The supension was sonicated for 10 s at 20 kH before use. The HA and CF titres of extra-MVA were 1024 and 256, respectively.

Radiolabelling of viral antigen

Forty-eight hours after infection of the cells, 100 μCi ^{35}S-methionine/ml (The Radiochemical Centre, Amersham, England) was added. The infected cells were harvested 24 h later. Radiolabelled cell-associated and extracellular MVA were prepared using Triton X-100 as described above, and stored at −70 °C.

Purified virus

The clarified supernatant was centrifuged for 1 h at 95000 *g*. The supernatant was placed on a 0·5 ml 65% sucrose cushion in 0·05 M Tris-HCl 0·01 M-EDTA-buffer, pH 7·8. Virus collected from the sucrose cushion was sonicated for 20 s and centrifuged first on a discontinuous (30–60%, w/v) sucrose gradient at 95000 *g* for 60 min and thereafter on a linear 15–65% sucrose gradient for 18 h. Purified virus was collected, dialysed against Tris-HCl buffer and treated with 3% Triton X-100. The HA titre of purified extra-MVA was 64.

Test and control sera

Sera were obtained from (*a*) ten patients with HBsAg-negative CAH (CAHS). HBsAg was excluded by radioimmunoassay and CAH verified by examination of liver biopsies giving a histological picture of CAH as defined by Groote (1968); (*b*) from a 10-year-old girl suffering from SSPE (SSPES) and (*c*) from patients with acute measles (human acute serum, HAS). Human convalescent sera (HCS) were

obtained from healthy adults with a history of measles in infancy. Measles virus CF titre of the sera was 1000–8000 for CAHS; 2000 for SSPES; 256 to 512 for HAS and 16–64 for HCS. The total IgG concentration of CAHS was 20–50 mg/ml, the smooth muscle antibody titre varied from < 16 to 256 (examined by the immunoperoxidase test using sections of rat stomach), and the nuclear factor antibody titre was 16–128 (examined by the immunoperoxidase test using sections of mouse liver). Human baby sera (HBS) were obtained from non-vaccinated healthy infants (13–15 months old) with no history of a measles virus infection and without detectable antibodies to measles virus in their sera. HI and CF tests for antibody were performed using heat-inactivated serum as described previously (Christie & Haukenes, 1979).

Immunoelectrophoresis

All experiments were performed with a Shandon Southern U-771-TLE apparatus (Camberley, Surrey, England) equipped with tap water cooling and a Shandon Southern SAE 2766 power supply. The electrophoresis was run at constant voltage in 1 % agarose (Indubiose A 37 L'Industrie Biologique Française, Gennevilliers, France) with 0·05 M veronal buffer, pH 8·6, and 1 % berol, a non-ionic detergent (Berol EMU 043, Berol Kemi AB, Stenungsund, Sweden) added to the gel. MVA was mixed with an equal volume of 2 % agarose and poured into wells or troughs in a 10 × 20 × 0·1 cm gel made on glass plates.

Line immunoelectrophoresis was performed according to Kröll (1973) with MVA in an 8 mm wide trough against an antibody-containing gel. The antibody concentration was adjusted so as to give good separation of the immunoprecipitation lines. The electrophoresis was run at 3 V/cm for 18 h.

Crossed immunoelectrophoresis (CIE) was performed according to Laurell (1965) using labelled or unlabelled, cellass- or extra-MVA in a 6 mm well. The relative concentrations of antigen and antibody were adjusted to give optimal resolution of precipitation lines for each serum. The first dimension run was done at 8 V/cm for 2 h. The second dimension run was done at 3 V/cm for 18 h. The plates were washed, pressed, dried and stained with Coomassie Brilliant Blue as described previously (Christie & Haukenes, 1979). The precipitates were also cut out from the washed gel, suspended in an equal volume of 0·125 M Tris-HCl buffer, pH 6·8, containing 4 % SDS, and analysed by SDS-PAGE.

Sepharose-protein A immunocomplex separation

This was performed according to Kessler (1975) by mixing 100 μl radiolabelled extra-MVA with 400 μl serum diluted in radioimmune precipitation (RIPA) buffer (0·15 M-NaCl, 1 % sodium deoxycholate, 1 % Triton X-100, 0·1 % SDS, 0·01 M-Tris-HCl, pH 7·4, 1 mM-PMSF, 100 u. Trasylol/ml) (modified RIPA buffer from Gilead *et al.* 1976). The mixture was kept at 4 °C for 2 h before precipitating the immune complexes by adding 20 mg of a 1:1 slurry of *Staphylococcus aureus* protein A bound to Sepharose at 4 °C. After 1 h the Sepharose beads were pelleted and washed with 4 × 3 ml RIPA buffer.

SDS-PAGE

This was performed by a modified method originally described by Laemmli (1970). The immunoprecipitates obtained from CIE or fixed to Sepharose-protein A were boiled for 2 min in 0·0125 M Tris-HCl buffer, pH 6·8, containing 2% SDS and 5% 2-mercaptoethanol. The precipitate was analysed on a 7·5–20% continuous gradient gel of polyacrylamide using ^{125}I-labelled IgG and human albumin as standard proteins. The stacking gel contained 4·5% acrylamide and 0·12% NN′-methylene-bisacrylamide. Electrophoresis was performed at a constant 20 mA current until the marker (bromophenol blue) reached the separating gel. The current was then raised to 40 mA. The gel slab was stained overnight with Coomassie Brilliant Blue (100 mg dissolved in 10 ml ethanol and diluted 20 times with 12·5% trichloroacetic acid), and destained with 45% ethanol in 10% acetic acid for 6 h, followed by 12 h in 7·5% acetic acid.

Autofluorography

This was performed according to Bonner & Laskey (1974). The stained gel was equilibrated in dimethylsulfoxide (DMSO), impregnated with 22·8% 2,5-diphenyl-oxazole in DMSO for 2 h and washed for 12 h in tap water. The gel slab was soaked for 10 min at room temperature in distilled water containing 1% glycerol. The gel was dried under vacuum and exposed to Kodak RP-Royal X-omat film for 4–6 days at −70 °C.

RESULTS

Immunoelectrophoresis

Crossed immunoelectrophoresis of cellass-MVA against CAHS showed five precipitation lines. Plate 1*a* and 1*a*′ show a representative line pattern using 5% serum from a patient with HBsAg-negative CAH. Four distinct lines (lines 1, 2, 3 and 4), and a very weak line (line 5) were observed. The same pattern was observed when 1 mM PMSF and 100 u. Trasylol/ml were added to the serum to avoid degradation of the proteins by serum enzymes in the second-dimension immunoelectrophoresis. Three precipitation lines (lines 1, 2 and 3) were observed using 20% HAS (Plate 1*b*). Two precipitation lines (lines 1 and 2) were observed using 50% HCS (not shown). No precipitation line was obtained with HBS. None of the sera precipitated with mock-infected Vero cell antigen on CIE.

Line immunoelectrophoresis of cellass-MVA was performed in order to show the identity of the precipitation lines obtained with different sera. The results showed a reaction of identity of line 1 obtained with CAHS, SSPES, HAS and HCS (Plate 2). Precipitation line 2 from line immunoelectrophoresis of cellass-MVA showed a reaction of identity for CAHS, SSPES and HAS. This line was not observed with HCS when using this method, and no precipitation was observed with HBS.

SDS-PAGE

SDS-PAGE of line 1, obtained by CIE of cellass-MVA against both CAHS and HCS, showed a double polypeptide band with molecular weights about 48000 and

50000 (Plate 3*a*). These polypeptides probably represent degraded nucleoprotein, since SDS-PAGE analysis of the immunoprecipitate of line 1 obtained with extra-MVA prepared in the presence of proteolytic enzyme inhibitors, gave no band between 48000 and 50000, but a major polypeptide band with a MW of 60000 (Plate 3*d*). SDS-PAGE analysis of the immunoprecipitate of line 2, obtained with extra-MVA against both CAHS and PHS, showed a major polypeptide band with a MW of 80000, representing the haemagglutinin (Plate 3*e*). Analysis of the immunoprecipitate of line 3 obtained with extra-MVA against CAHS, showed a major polypeptide band with a MW about 40000, most probably representing the (F_1) fusion protein (Plate 3*f*). In addition to the major band corresponding to haemagglutinin in lane *e* and fusion protein in lane *f*, some very weak bands were discernible in these lanes. This does not mean that individual precipitation lines contain more than one measles virus polypeptide, but most probably this is caused by difficulties in separating closely situated lines when the immunoprecipitates are cut out from the gel.

Sepharose-protein A radio-immunocomplex separation

By this qualitative method, antibodies againt five measles virus polypeptides: H, P, NP, F_1 and M, were demonstrated in CAHS, SSPES, HAS and HCS (Plate 4). None of the measles virus polypeptides was precipitated using HBS. These results, and the results with CIE, show that serum from patients with HBsAg-negative CAH, SSPE, acute measles and healthy individuals with a history of measles in infancy, all contain antibodies against the same five measles virus polypeptides. The results clearly demonstrate the quantitative elevation of the concentration of measles virus antibodies in serum from patients with CAH compared to serum from healthy controls.

DISCUSSION

Enhanced antibody response against measles and/or rubella virus has been associated with many diseases of unknown aetiology: HBsAg-negative CAH (Christie & Haukenes, 1979), multiple sclerosis, MS (Beck & Clausen, 1977; Norrby, Link & Olsson, 1974: Reunanen *et al.* 1976), rheumatoid arthritis (Beck & Clausen, 1977: Laitinen, Vesikari & Vaheri, 1972), systemic lupus erythematosus (Beck & Clausen, 1977; Hollinger *et al.* 1971; Laitinen, Vesikari & Vaheri, 1972) and other autoimmune diseases (Lucas *et al.* 1972). In all these diseases autoantibodies are frequently demonstrated in the patient's sera, and an autoimmune response seems to be involved. In SSPE, a persistent measles virus infection, increased amounts of antibody against both external and internal measles virus proteins have been demonstrated (Salmi, Norrby & Panelius, 1972) and measles virus has been isolated from the brain (Payne, Baublis & Itabashi, 1969). In MS the antibody response against measles virus is only moderately increased and mainly directed against external virus protein, especially the haemolysin (Cendrowski & Niedzielska, 1974; Norrby, Link & Olsson, 1974; Salmi, Norrby & Panelius, 1972). This may suggest a different mechanism for the stimulation of measles virus-specific antibody production in SSPE than in MS.

The aetiology and pathogenesis of most cases of HBsAg-negative CAH remain obscure. The patients suffering from this disease often show raised HI and CF serum antibody titres against both rubella and measles virus (Closs *et al.* 1971; Triger *et al.* 1972). However, the specificity of these antibodies has not been definitively established (Eddleston & Williams, 1976; Lidman *et al.* 1977; Triger, 1976).

By CIE of crude measles virus antigen against serum from patients with HBsAg-negative CAH showing raised antibody titre against measles virus, we have demonstrated five precipitation lines. SDS-PAGE analysis of the immunoprecpitates (Reunanen *et al.* 1976), revealed the specificity of the respective antibodies in three of these lines. Considerably higher amounts of antibody against the measles virus nucleoprotein, haemagglutinin and fusion protein were demonstrated by this semiquantitative method in patients' sera as compared to normal human serum. By Sepharose-protein A radio-immunocomplex separation, antibodies against five measles virus polypeptides, NP, H, F_1, P and M were demonstrated in the patients' sera. The results show that these patients have high concentrations of measles virus-specific antibodies in their sera. Cross-reaction with antibodies against intestinal bacteria or autoantibodies, as an explanation of the raised HI and CF antibody titres, can be excluded.

CIE is obviously less sensitive than separation of radio-immunocomplexes with Sepharose-protein A for the characterization of measles virus-specific antibodies. However, CIE is a semiquantitative method, and another advantage of this method is that the immunoprecipitates can be separated and used for immunization, allowing the production of specific antisera against different measles virus polypeptides (unpublished data).

When CIE was performed with crude cellass-MVA obtained by Triton X-100 treatment of virus-infected cells, the immunoprecipitation line 1 contained two polypeptides with molecular weights of about 48000 and 50000. This observation was made with both patients' and control sera. When virus polypeptides prepared from extracellular virus, free of host cells, were used, an immunoprecipitation line was observed which was weaker than, but otherwise very similar in appearance to, line 1. This immunoprecipitate contained no polypeptide in the range 48000–50000, but one major polypeptide of 60000, corresponding to the nucleoprotein. The explanation of this observation is probably that degradation of native nucleoprotein by host cell enzymes has taken place during the preparation of cellass-MVA. This degradation does not seem to affect the antigenic determinant of the nucleoprotein, since typical nucleocapsid fragments have been identified in the precipitation line 1 obtained with cellass-MVA (Christie & Haukenes, 1979). During the preparation of the immunoprecipitate for SDS-PAGE, solubilization of the nucelocapsid and release of degraded nucleoprotein probably take place. These polypeptides seem to correspond to the novel polypeptides, *y* and *z*, demonstrated by Stallcup, Wechsler & Fields (1979).

The HI and CF antibody titre increase has in many cases been shown to be quite out of proportion to the rise in total IgG, and high titres are seen in sera with normal IgG concentrations (Closs *et al.* 1973). The titres may remain elevated also after normalization of the IgG serum level after treatment with corticosteroids (Closs

et al. 1973). The antibody titres against other common viruses such as Coxsackie B, herpes simplex and parainfluenza 1, have not been found to be increased in these patients (Triger *et al.* 1972). Most of the sera from patients with HBsAg-negative CAH contain autoantibodies againt smooth muscle (SM) antigen,which are shown to be anti-actin antibodies (Gabbiani *et al.* 1973). We have found no correlation between anti-measles virus CF titre and anti-SM titre of sera from these patients, and cross-absorption of the sera with purified measles virus and actin showed that there is no cross-reaction between antibodies against these antigens (unpublished data). These observations, and the findings in the present study that the antibodies are directed against both external and internal measles virus polypeptides, may suggest that the immune response against measles virus in patients with HBsAg-negative CAH may be induced by measles virus and does not represent a non-specific reactivation of immunological 'memory cells'. New information suggests that measles virus normally remains in the body after acute infection (Burns & Allison 1975; Lucas *et al.* 1978), which may explain the lifelong presence of measles virus-specific antibodies. The present results show that the main measles virus-specific antibodies found in sera from patients with HBsAg-negative CAH are directed against the same virus antigens as the antibodies found in sera from patients with acute measles or healthy individuals with a former history of measles. The amounts of these antibodies are, however, markedly raised in CAHS. The extremely high antibody titres often found in serum from these patients are possibly caused by a reactivation of a persisting measles virus. Whether a possible reactivation may play any aetiological or pathogenic role in CAH is uncertain.

Studies are in progress to see if specific antibodies of the IgM class can be demonstrated in serum from these patients. So far measles virus-specific IgM has not been demonstrated by examining fractions after sucrose gradient ultracentrifugation by the CF and HI test.

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EXPLANATION OF PLATES

PLATE 1

Crossed immunoelectrophoresis using measles virus antigen against (*a*) 5% serum from a patient with HBsAg-negative CAH; (*b*) 20% serum from a patient with acute measles. Plate (*a'*) is a drawing to represent the five precipitin lines in Plate *a*: line 1, nucleoprotein; line 2, haemagglutinin; line 3, fusion protein; lines 4 and 5, not identified.

PLATE 2

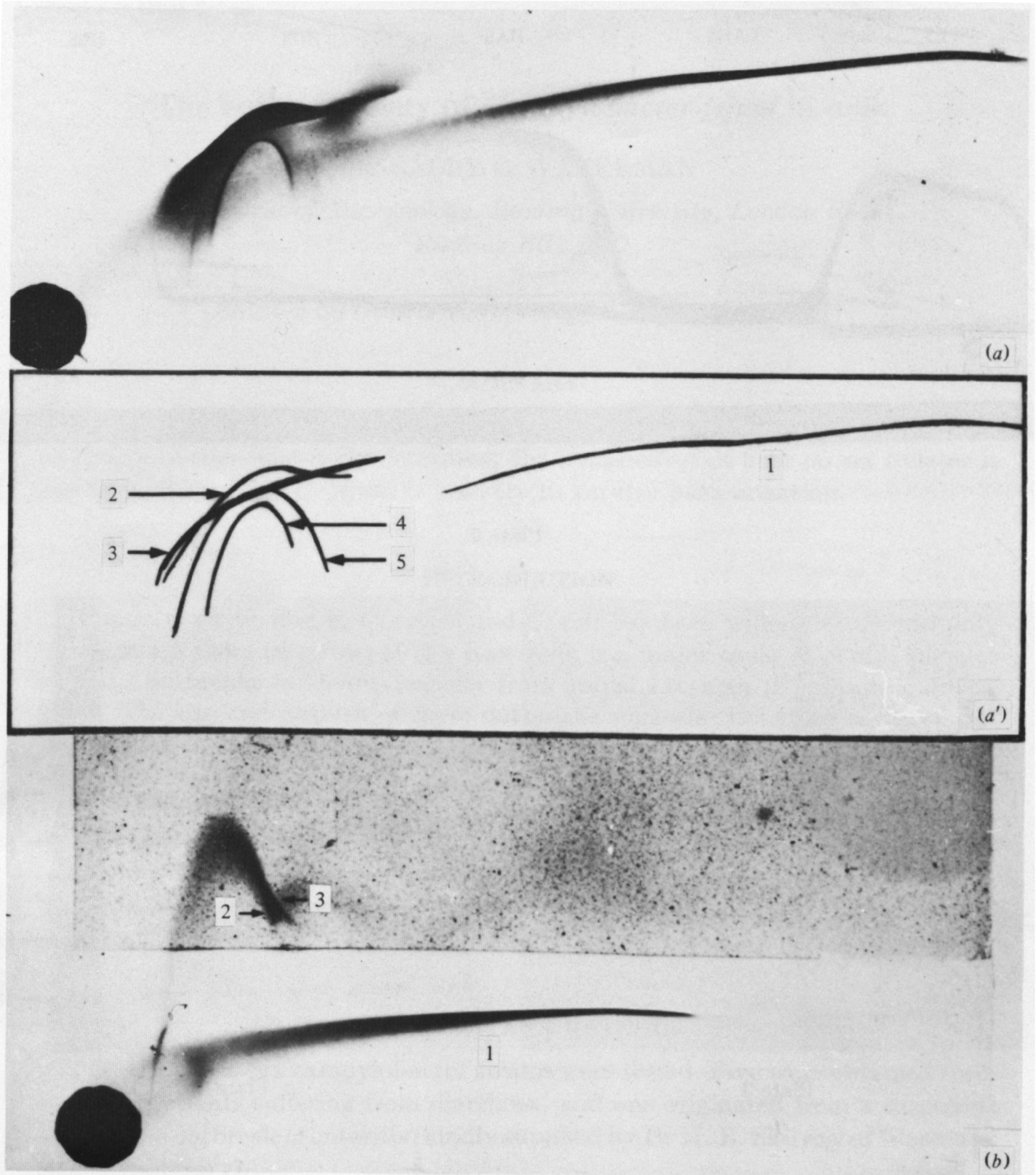
Line immunoelectrophoresis using measles virus antigen against 20% serum from a patient with SSPE (SSPES), 5% serum from a patient with HBsAg-negative CAH (CAHS), 20% serum from a patient with acute measles (HAS), 50% human convalescent (HCS) and baby serum (HBS). Line 1, nucleoprotein; line 2, haemagglutinin.

PLATE 3

Fluorography of SDS-PAGE gels with ^{35}S-methionine-labelled measles virus polypeptides: (*a*) analysis of precipitation line 1 (CIE: intracellular measles virus antigen/5% serum from a patient with CAH); (*b*) analysis of purified measles virus; (*c*) analysis of extracellular measles virus antigen; (*d*, *e*, *f*) analysis of lines 1, 2, 3 (CIE: extracellular measles virus antigen/5% serum from a patient with CAH).

PLATE 4

Fluorography of Sepharose-protein A separated immune complexes of ^{35}S-methionine-labelled extracellular measles virus antigen using: (*a* and *c*) 5% serum from patients with CAH, (*b*) 5% serum from a patient with SSPE, (*d*) 10% serum from a patient with acute measles, (*e*) 25% human convalescent serum, (*f*) extracellular measles virus antigen.



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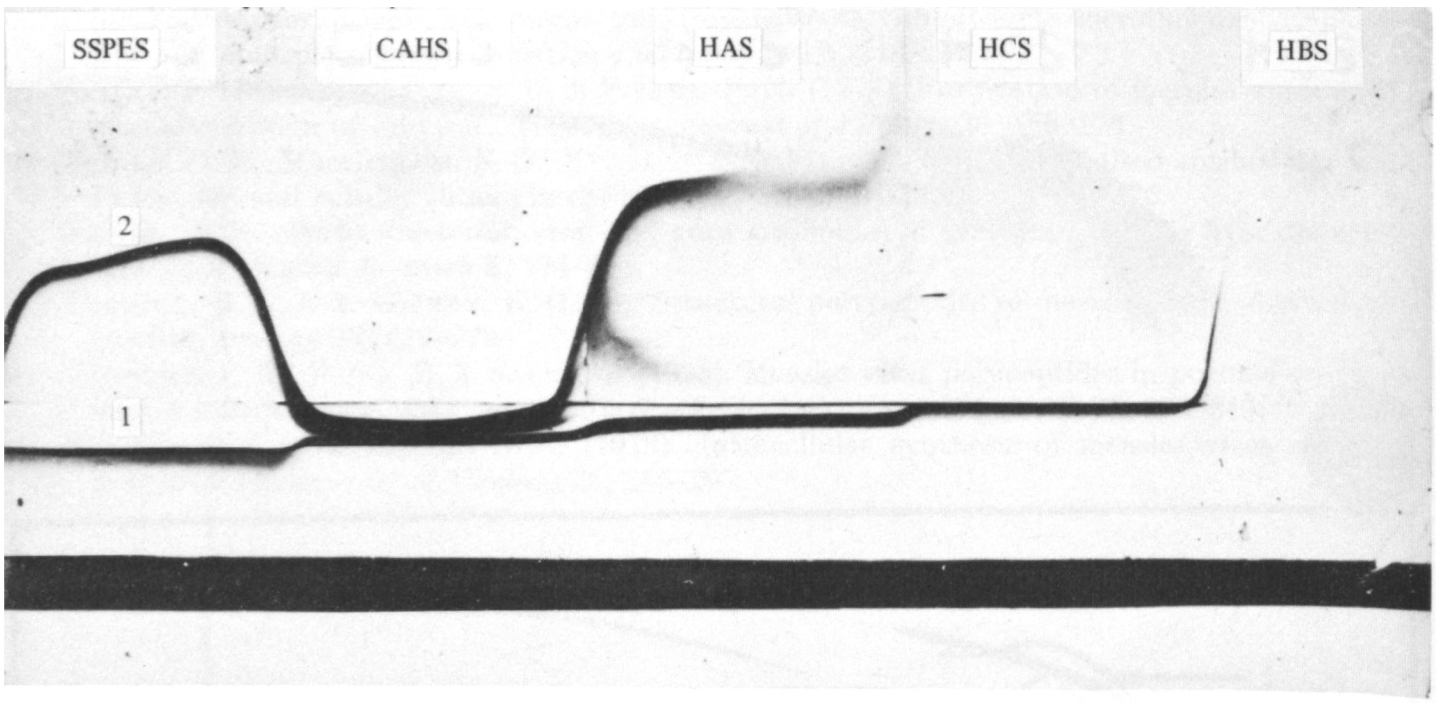


Plate 2

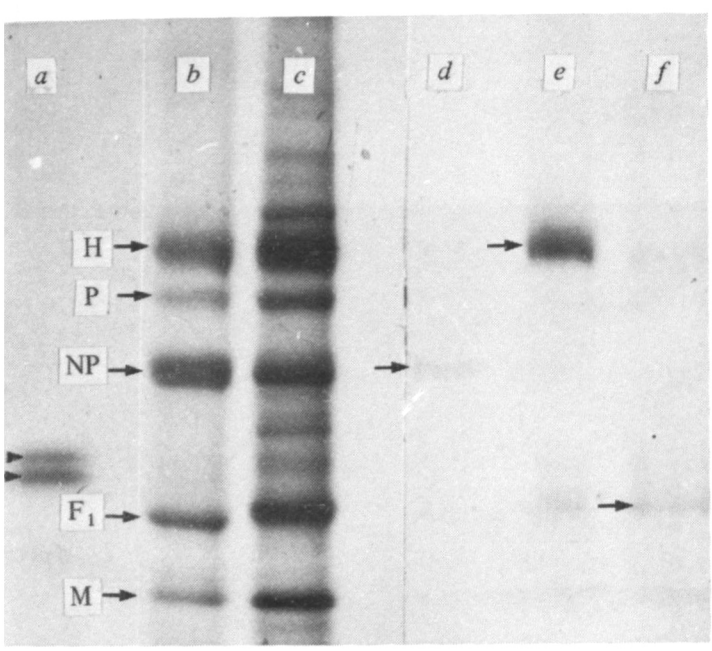
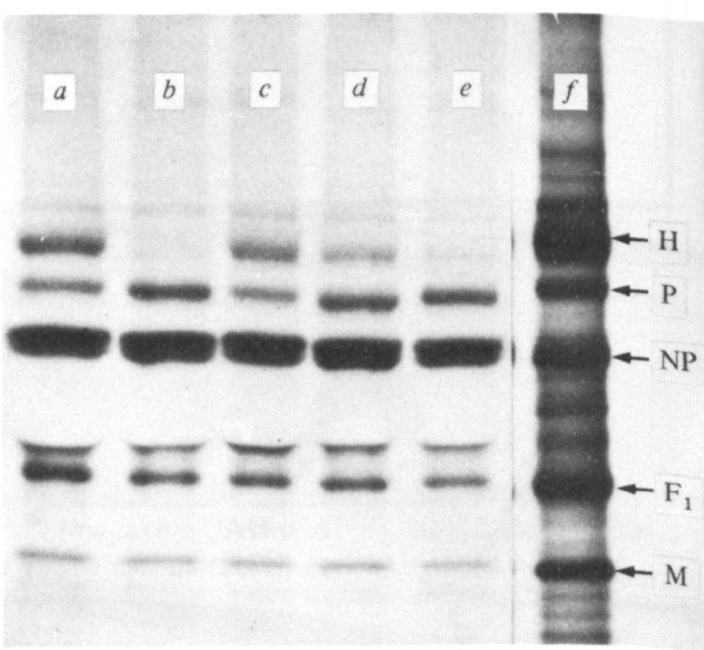


Plate 3



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