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CHROMATOGRAPHIC AND ELECTROPHORETIC STUDIES OF IMMUNE COMPLEXES IN NON-A, NON-B HEPATITIS

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SUMMARY

Immune complexes isolated from two patients with chronic non-A, non-B hepatitis, one patient with acute non-A, non-B hepatitis and one patient with juvenile rheumatoid arthritis were examined by means of a combined chromatographic and electrophoretic method. Both analyses showed the presence of complexes consisting of IgG, IgM, complement c1q factor and albumin; no antigen constituents were detected. The IgG-to-IgM ratio varied from 1:1 to 4:1, suggesting that one could be dealing with complexes of both IgG–IgM and IgG–IgG types Moreover, the detectable presence of c1q factor might indicate that such complexes were capable of activating complement.

INTRODUCTION

Chronic active hepatitis may be a disease of viral origin that can take place after a B, non-A, non-B or CMV infection, in some instances preceded by an acute phase symptomatology. The disease is often associated with immunological alterations which, on the humoral immunity side, consist of increased plasma immunoglobulin (Ig) levels, mostly of the IgG class, the presence of anti-nuclear and anti-smooth muscle antibodies and augmented amounts of circulating immune complexes (CICs). It is well known that CICs in both neoplastic [1] and infectious diseases [2] might play an important role in modulating immune behaviour, especially for a decreased response to antigens [3,4]. It has also been suggested that anti-idiotypic antibodies participate in this kind of inhibitory im-

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mune regulation [5] and that they may be constituents of immune complexes [6].

From the analytical point of view, studies of CICs in type B hepatitis have been performed with methods specific for hepatitis B surface antigen in both the acute and chronic periods of the disease, such as immunochemical detection with enzyme-linked immuno-sorbent assay (ELISA) [7,8]. When the antigen constituents of immune complexes are unknown, the most commonly applied method for antigen detection is Western blot analysis. This method has been used in many pathological situations, such as rheumatoid arthritis [9], leukaemia and systemic lupus erythematosus [10]. In recent years, size-exclusion high-performance liquid chromatography (SE-HPLC) had been widely used to analyse immunoglobulins [11], their subunits [12] and their complexes with other molecules [13]. We have analysed CICs from two patients with chronic active hepatitis of non-A, non-B origin, one patient with acute non-A, non-B hepatitis and one patient with juvenile rheumatoid arthritis (JRA). CICs were isolated by means of polyethylene glycol (PEG) precipitation and purified using protein A-Sepharose chromatography. Subsequent analyses were effected using SE-HPLC and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Our aim was to identify the constituents of CICs and to verify their immunological activity.

EXPERIMENTAL

Serum samples (5–15 ml) were obtained from three patients with non-A, non-B hepatitis in the acute (patient A) or chronic phase (patients B and C) of the disease and from one patient with JRA (patient D). All samples were negative for rheumatoid factor. Sera were tested for anti-nuclear, anti-smooth muscle and anti-mitochondrial antibodies with indirect immunofluorescence. IgG, IgA and IgM in sera and samples obtained from the different steps of the analysis (see below) were measured both with an ICS nephelometric device (Beckman Instruments, Fullerton, CA, U.S.A.) and with radial immunodiffusion (LC-Partigen plates) (Behring Institute, Marburg, F.R.G.).

Isolation of immune complexes

CICs were isolated by PEG precipitation according to Chia et al. [14]. The PEG precipitates of samples from patients C and D behaved like cryoglobulins. The final precipitate was resuspended in 0.1 *M* phosphate buffer (pH 7.4). The percentages of Ig in the precipitates were: IgG 2–10%, IgA 1–6% and IgM 25–35%.

Purification of immune complexes

Protein A–Sepharose affinity chromatography of precipitated CICs was performed following the method of Chenais et al. [15]. The material was eluted with 1 *M* acetic acid and subsequently dialysed overnight at 4°C in 0.1 *M* phosphate buffer (pH 7.4). No differences were observed in the subsequent SE-HPLC elution profiles and SDS-PAGE patterns when the material was eluted with 0.1 *M* glycine hydrochloride (pH 3.2) and immediately buffered. The percentages of Ig

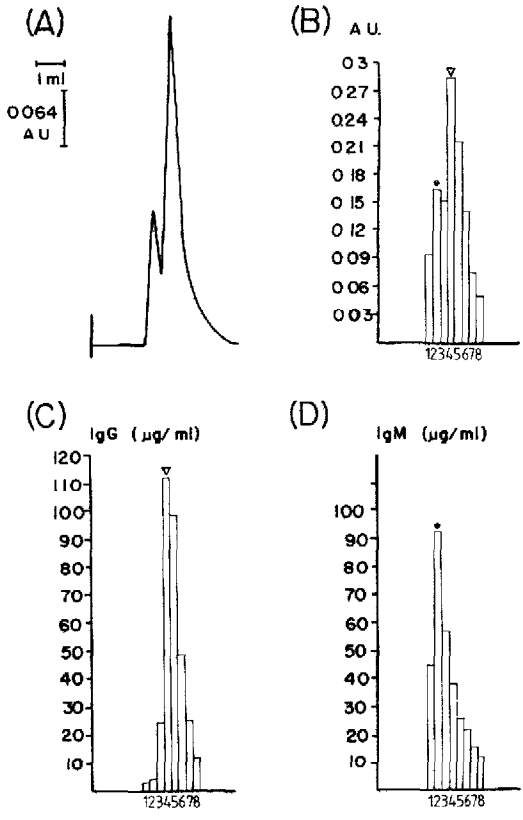

Fig. 1 Patient A: (A) SE-HPLC elution profile, (B) the corresponding eight 250-µl fractions and the immunochemical determination of (C) IgG (µg/ml) and (D) IgM (µg/ml) amounts. The triangle and the circle indicate the main IgG- and IgM-containing fractions, respectively.

in affinity-purified CICs were: IgG 50–70%, IgA 0% and IgM 10–20% (with respect to the amount of each Ig class in the PEG precipitate). The purified CICs from each patient were lyophilized after overnight dialysis with water at 4°C. The lyophilized material was resuspended for chromatographic analyses in 200 µl of 0.1 *M* phosphate buffer (pH 7.4). Concentration by means of Minicon B-15 (Amicon, Danvers, MA, U.S.A.) was followed by a loss of about 50% of the material, with no differences in the SE-HPLC elution profiles and SDS-PAGE patterns.

SE-HPLC studies

The HPLC system used consisted of a Clar pump (Violet, Rome, Italy), a Synchropac GPC 300 column (250 mm×4.6 mm I.D.) and a 30 mm×4.6 mm I.D. guard column (Synchrom, Linden, IN, U.S.A.). Isocratic elutions were performed with 0.1 *M* phosphate buffer (pH 5.8) at a flow-rate of 0.5 ml/min; 20-µl samples were injected. The absorbance was monitored at 280 nm with an LC 75 spectrophotometric detector (Perkin-Elmer, Norwalk, CT, U.S.A.). A calibration graph was prepared with high-molecular-weight (HMW) and low-molecular-weight (LMW) gel permeation calibration kits from Pharmacia (Uppsala,

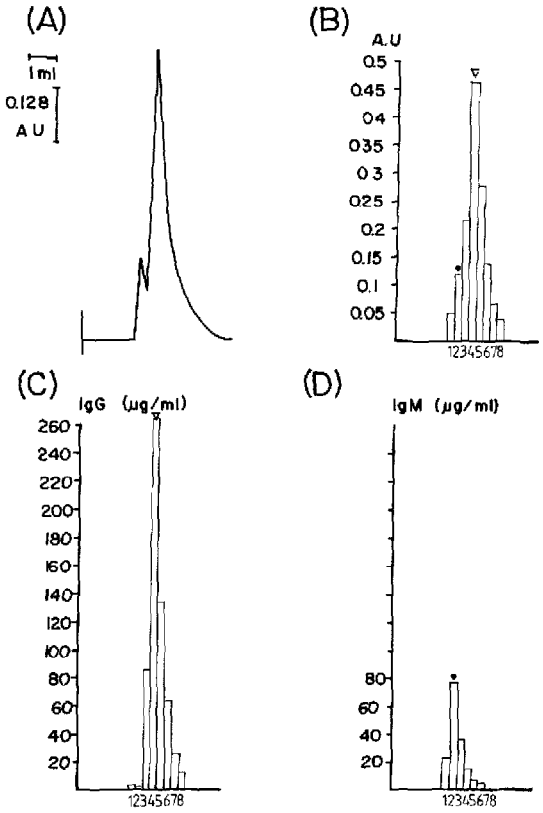

Fig. 2. As Fig. 1, for patient B.

Sweden) and human IgG (Sigma, St. Louis, MO, U.S.A.). Fractions of 250 μl were collected from SE-HPLC and the absorbance of the fractions was monitored at 280 nm (Beckman Model 25 spectrophotometer). Fractions were dialysed overnight at 4°C in 0.1 *M* phosphate buffer (pH 7.4).

SDS-PAGE studies

SDS-PAGE was performed by the method of Laemmli [16] in 10% polyacrylamide gel for samples from patients A and B and for their main IgG- and IgM-containing fractions obtained from SE-HPLC and in 15% polyacrylamide gel for samples from patients C and D. Protein standards for SDS gel electrophoresis from Bio-Rad Labs. (Richmond, CA, U.S.A.) were used. The gels were stained with Coomassie Blue.

Immunochemical studies

The main IgG- and IgM-containing fractions (see Figs. 1–3) of samples from the three hepatitis patients were tested in indirect immunofluorescence using an F(ab')2 fluoresceinated anti-human Ig serum (Delta Biologicals, Rome, Italy). The main IgG-containing fractions were reacted against the main IgM-contain-

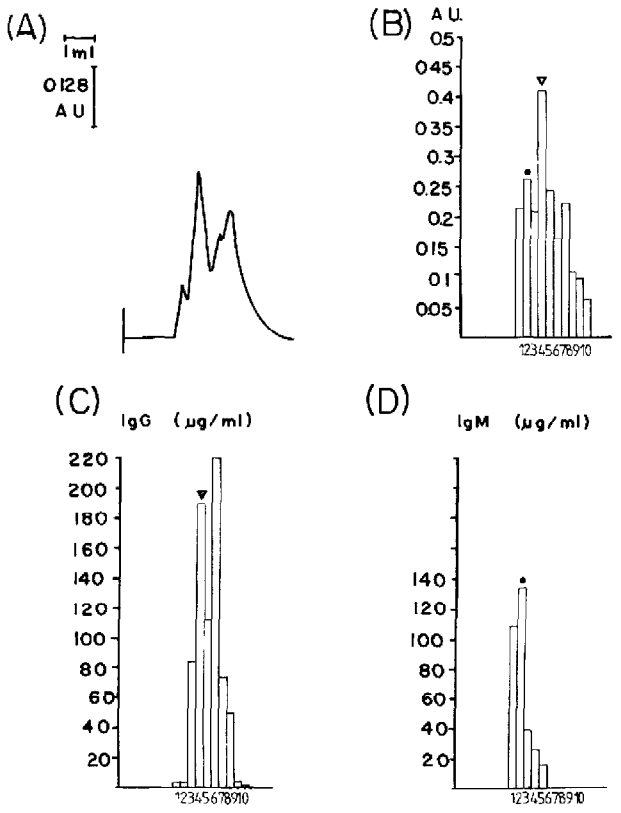

Fig. 3. As Fig. 1, for patient C.

ing fraction from the same patient, and from each of the other patients by means of double diffusion according to Ouchterloney [17]. Detection of c1q factor and albumin in the fractions was also performed by means of double diffusion using monospecific antisera (Behring Institute).

RESULTS

SE-HPLC studies

Our results show two different elution patterns: a two-peak profile for samples from patients A and B and a four-peak profile for those from patients C and D. The immunochemical characterization of the two-peak profiles showed a striking similarity: the peaks corresponded to IgM and IgG, respectively, with an IgG-to-IgM ratio of 1 for patient A (Fig. 1) and 4 for patient B (Fig. 2). The main IgG- and IgM-containing fractions were subsequently submitted to SDS-PAGE (see below).

The immunochemical characterization of the four-peak profiles showed the presence of IgM, IgG and two other molecular species of lower relative molecular masses (20 000 and less than 6000); at least part of this lighter material should be of IgG nature, as shown by the nephelometric determination (see Fig. 3C). In

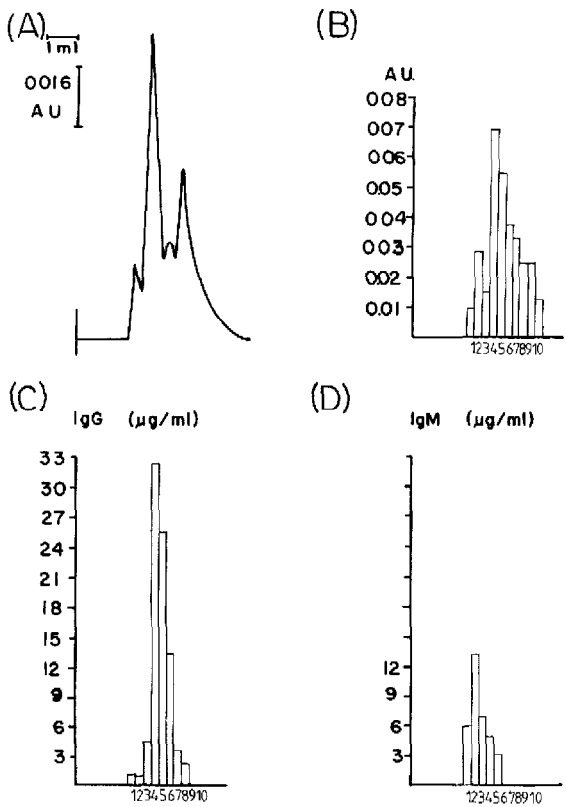

Fig. 4. As Fig. 1, for patient D.

these two instances also the elution profiles showed similarities; the IgG-to-IgM ratios were 1.4 for patient C (Fig. 3) and 3 for patient D (Fig. 4).

SDS-PAGE studies

SDS-PAGE was performed on samples from all the patients, utilizing 10% polyacrylamide gel for patients A and B and 15% polyacrylamide gel for patients C and D; in these two instances 15% gel was used for the presence of peaks indicating molecular masses lower than Ig light chains in SE-HPLC profiles.

Fig. 5 shows the electrophoretic patterns of protein A–Sepharose-purified CICs in samples from patient A (track 2) and patient B (track 7); tracks 3 and 6 show the main IgM-containing fractions in samples from patients A and B, respectively; tracks 4 and 5 show the main IgG-containing fractions in samples from patients A and B, respectively. Plotting from the reference electrophoretic pattern (tracks 1 and 8), IgG, IgM (H+L chains), albumin and c1q factor could be detected.

Fig. 6 shows the protein A–Sepharose-purified CICs in samples from patients C and D. The same molecular species as for patients A and B were observed; many other bands representing traces of other molecular species could also be seen. In

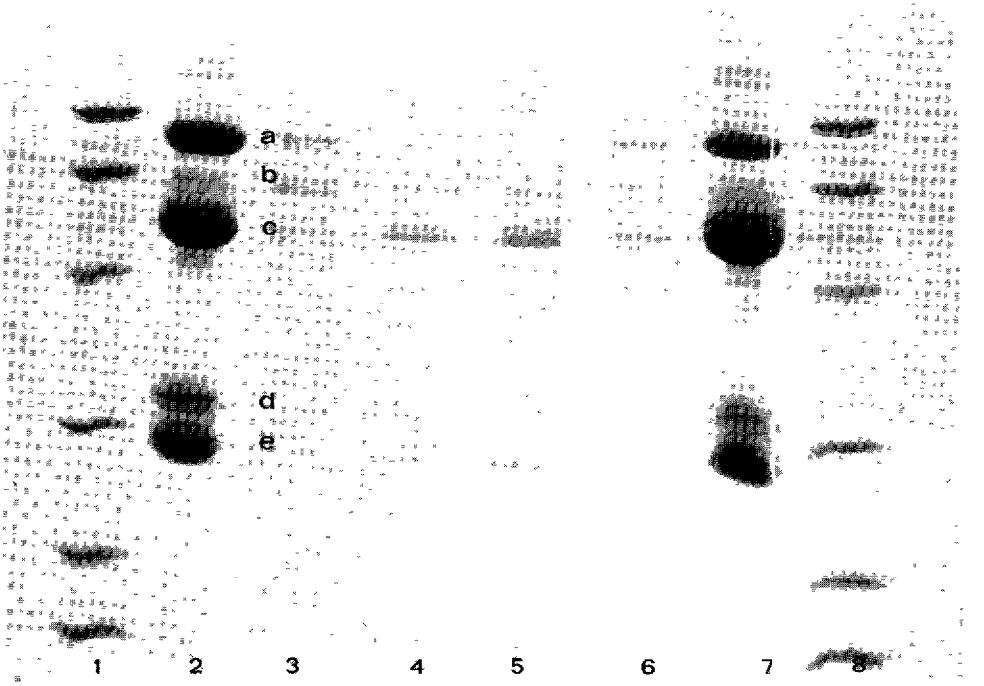

Fig. 5. SDS-PAGE patterns of protein A-Sepharose-purified CICs in samples from patients A and B (tracks 2 and 7). On the right side of track 2 the molecular constituents are indicated: (a) = mi heavy chain; (b) = albumin; (c) = gamma heavy chain; (d) = c1q factor; (e) = light chains. Tracks 3 and 6, main IgM-containing fractions from patients A and B; tracks 4 and 5, main IgG-containing fractions from patients A and B; tracks 1 and 8, reference MW (92 500, 66 200, 45 000, 31 000, 21 500, 14 400).

contrast with the SE-HPLC profile, no molecular masses lower than these of the light chains of Ig could be detected.

Immunochemical studies

Table I shows the anti-smooth muscle reactivity of sera from patients A, B and C and their main IgG- and IgM-containing fractions; it should be observed that the IgM fractions are not involved as autoantibodies, whereas the IgG fractions showed positivity in sera from patients A and B. No anti-nuclear or anti-mitochondrial positivity was observed in any of the fractions tested. Double diffusion performed with the main IgM-containing fractions reacted against the main IgG-containing fractions of CICs from the same patient and of CICs from each of the other patients showed no positivity.

Double diffusion performed on the SE-HPLC main IgM and IgG fractions revealed the presence of c1q factor and albumin in both groups of fractions.

DISCUSSION

The analysis of CICs in samples from the patients with non-A, non-B hepatitis (acute and chronic) and the patient with JRA gave substantially identical results

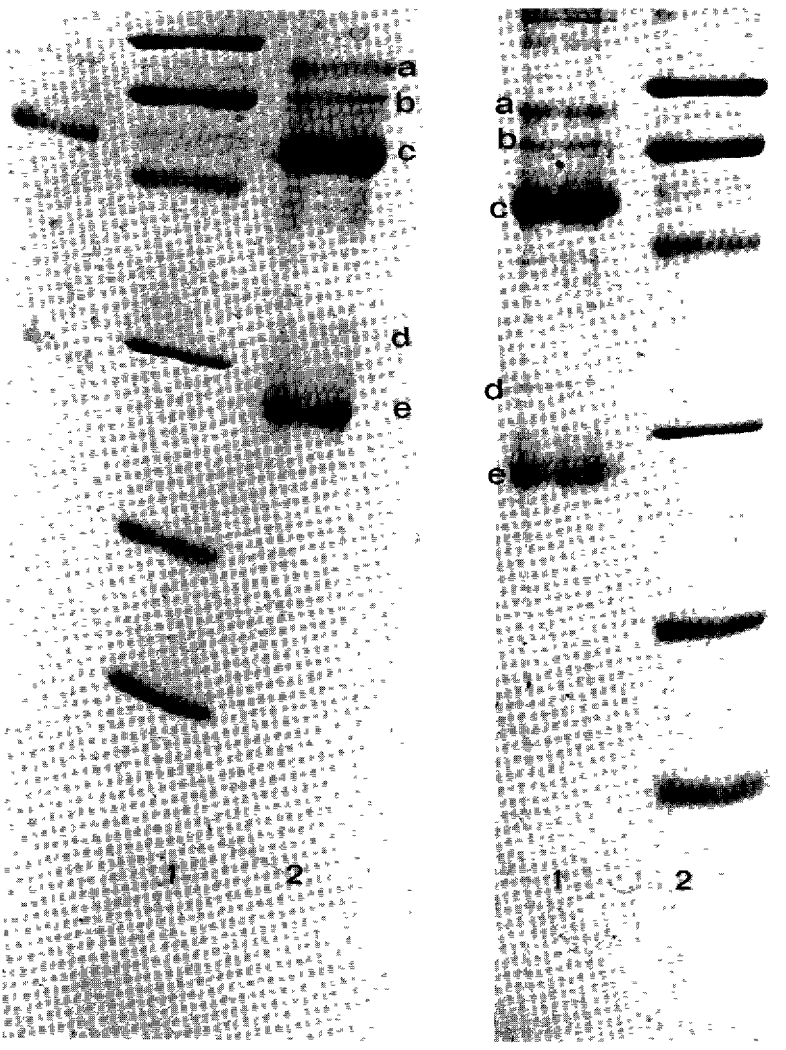

Fig. 6. (Right) SDS-PAGE pattern of protein A–Sepharose-purified CICs in sample from patient C. Track 1, patient; track 2, reference MW (Left) SDS-PAGE pattern of protein A–Sepharose-purified CICs in sample from patient D. Track 1, reference MW; track 2, patient. a–e As in Fig. 5.

TABLE I

SCHEME OF THE ANTI-SMOOTH MUSCLE REACTIVITY IN SERA AND MAIN IgM AND IgG FRACTIONS OF PATIENTS A, B AND C

Patient	Serum reactivity	IgM fraction reactivity	IgG fraction reactivity
A	1:400	—	1:40
B	1:40	—	1:20
C	—	—	—

in all four cases. SE-HPLC studies of protein A–Sepharose-purified complexes demonstrated that these consist of Ig of the IgG and IgM classes; moreover, the IgG-to-IgM ratios found (ranging from 1:1 to 4:1) indicate the presence of both IgG–IgM and IgG–IgG complexes, which might suggest the possibility of idiotypic–anti-idiotypic CICs, at least for patients A and B. However, when the IgG and IgM fractions were tested by double diffusion in a reciprocal way for the same patient and in a crossed way among the three patients A, B and C, no precipitation bands were observed.

SDS-PAGE studies confirmed that the main constituents of CICs were Ig of the IgM and IgG classes.

Albumin and c1q factor, not detectable as peaks in the chromatographic profiles, were revealed in the eluted material with double diffusion. Albumin was detected in CICs in patients positive for rheumatoid factor and also in CICs in normal subjects [9]. In the patient with JRA who we studied albumin was also found; c1q factor was not observed as a chromatographic peak, because its binding to Ig Fc involves covalent bonds [18]. Its presence demonstrates the complement fixing activity of such complexes.

No molecular species attributable to putative antigen, as far as non-A, non-B hepatitis is concerned, could be detected, especially when considering that the chromatographic analysis was performed on CICs treated with 1 *M* acetic acid. It has been reported that treatment with 1 *M* acetic acid determines a great loss of antibody affinity in CICs [19], and in our cases such treatment was able to separate the complexed IgG and IgM components.

Anti-smooth muscle activity was found in the IgG components of CICs from patients A and B, whereas autoantibody behaviour for any of the IgM components or for the IgG component in samples from patient C was found. Bearing in mind the acidic treatment to which the CICs were submitted, and the lower IgG concentration in the examined fractions with respect to serum (about ten-fold), we can affirm that the IgG with such autoantibody specificity represents a significant part of the CICs.

It is of interest that in patients A (acute hepatitis) and B (chronic hepatitis), with the same SE-HPLC elution profiles, SDS-PAGE patterns and IgG specificity, the IgG-to-IgM ratios differ significantly (1:1 and 4:1, respectively), suggesting that an IgG-mediated anti-idiotypic modulation may be effective in the early phases of the disease.

REFERENCES

1 U.E. Nydegger, in R H. Adrian, E. Helmreich, H. Holzer, R. Jung, O. Krayer, R J. Linden, F. Lynen, P.A. Miescher, J. Püper, M. Rasmussen, A.E. Renold, U. Trendelemburg, K. Ullrich, W. Vogt and A. Weber (Editors), Reviews of Physiology, Biochemistry and Pharmacology, Vol. 85, Springer-Verlag, Berlin, Heidelberg, New York, 1979, p. 63.
2 R.C. Williams, Jr., Annu. Rev. Med., 32 (1981) 13.
3 I. Hellstrom, K.E. Hellstrom, G.E. Pierce and J.P.S. Yang, Nature (London), 220 (1968) 1352.
4 F. Uher, M.C. Lamers and H B. Dicker, Cell. Immunol., 95 (1985) 368.
5 G.R. Olds and T.F. Kresina, J. Clin. Invest., 76 (1985) 2338.
6 L.M. Rose, M. Goldman and P.H. Lambert, J. Immunol., 128 (1982) 2126.
7 F. Careoda, R. De Franchis, A. D'Arminio Monforte, M. Vecchi, E. Rossi, M. Primignani, M. Palla and N. Dioguardi, Lancet, ii (1982) 358.

8 P. Coursaget, J.L. Barres, E. Tortey, P. Cotti, T. Diop, B. Yvonnet, M.T. Sow, S. Mboup, B. Diop, J.E. Bocande, L. Pourcelot, I. Diop-Mar and J. Chiron, Cancer Res., 46 (1986) 1492.
9 R.D. Melsom, P.R. Smith and R.N. Maini, Ann. Rheum. Dis., 46 (1987) 104.
10 M.A. Maire, M. Barnet, N Carpentier, P.A Miescher and P.H. Lambert, Clin. Exp. Immunol., 51 (1983) 215.
11 M.J.E. Schmerr, K.R. Goodwin, H.D. Lehmkuhl and R.C. Cutlip, J. Chromatogr., 326 (1985) 225.
12 M.T. Short, F.A. Westholm, M. Schiffer and F.J. Stevens, J. Chromatogr., 323 (1985) 418.
13 E.H Cooper, E.A. Johns, Y. Itoh and J.R. Webb, J. Chromatogr., 327 (1985) 179.
14 D. Chia, V. Barnett, J. Yamagata, D. Knudson, C. Restivo and D. Furst, Clin. Exp. Immunol., 37 (1979) 399.
15 F. Chenais, G. Virella, C.C. Patrick and H.H. Fudenberg, J. Immunol. Methods, 18 (1977) 183.
16 U.K. Laemmli, Nature (London), 227 (1970) 680.
17 O. Ouchterlony, Prog. Allergy, 5 (1958) 1.
18 F. Vondervizst, J. Torok, S. Lakatos, F. Kilar and P. Zavodszky, Biochem. J., 243 (1987) 449.
19 D.N. Hogben, S.E. Brown, C.R. Howard and M.W. Steward, J. Immunol. Methods, 93 (1986) 29.