Journal of Immunological Methods, 62 (1983) 305–314
Elsevier

JIM 02740

A Specific Enzyme-Linked Immunosorbent Assay (ELISA) for the Determination of Human C5a Antigen [1]

Steven L. Kunkel, George L. Manderino *, Wayne Marasco, Kip Kaercher, Arthur A. Hirata * and Peter A. Ward

*Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, and * Laboratory of Immunology, Abbott Laboratories, North Chicago, IL 60064, U.S.A.*

(Received 7 January 1983, accepted 21 March 1983)

An enzyme-linked immunosorbent assay has been developed to detect human C5a antigen. This ELISA methodology has been shown to be a highly sensitive technique capable of detecting C5a antigen concentrations below 10 ng/ml. The microELISA technique used in this study is specific for human C5a and C5a des arg (C5a antigen) but not for human C5. Conditions to establish sensitivity and specificity are outlined in this report.

Key words: *human complement C5a antigen — enzyme-linked immunosorbent assay*

Introduction

Enzymatic cleavage of the third and fifth components of complement by activation of either the classical or alternative pathway yields 2 low molecular weight peptides historically referred to as anaphylatoxins. The potent biological activities of the complement-derived anaphylatoxins, C3a and C5a, have been well documented and include histamine release from mast cells (Johnson et al., 1975), smooth muscle contraction (Cochrane et al., 1968), and increase in capillary permeability (Vallota and Muller-Eberhard, 1973). A further functional attribute of the C5a peptide that is not associated with C3a is its chemotactic activity for polymorphonuclear leukocytes (Fernandez et al., 1978; Hugli, 1979).

Once the complement cascade is activated in vivo, C5a appears to be rapidly cleaved to a des arg 74 derivative (C5a des arg) by a serum enzyme similar to carboxypeptidase B (Bokisch and Muller-Eberhard, 1970). Although C5a des arg is spasmogenically inactive (Fernandez et al., 1978), this peptide has been shown to be

[1] Supported in part by NIH Grants HL 28442, HL 26498, and HL 26809.

chemotactically active especially in the presence of normal human serum or plasma (Perez et al., 1981). In vitro investigations have demonstrated that as little as 15 ng/ml of purified C5a or 150 ng/ml C5a des arg can effectively induce the unidirectional movement of neutrophils (Chenoweth and Hugli, 1980). The potent biological activity of minute concentrations of these chemotactic peptides dictate that very sensitive assays be developed in order to detect trace quantities of the C5a/C5a des arg (C5a antigen) molecules in biological fluids. Therefore, we have developed an enzyme-linked immunosorbent assay (ELISA) capable of detecting nanogram quantities of C5a antigen. This technique, originally developed by Engvall and Perlmann (1971), has proven to be an extremely sensitive immunoassay used to quantitate many antigens of biological importance (Engvall and Perlmann, 1972; Handley et al., 1982). The following C5a ELISA methodology possesses many unique advantages over other quantitative methods: the enzyme conjugates and reaction products are stable for long periods of time, the ELISA method can be automated and conducted in microtiter plates, finally, the technique avoids the problems related to the use of radiolabelled compounds.

Materials and Methods

Isolation of C5a and C5a des arg

Human C5a was isolated employing immunoadsorbent chromatography according to a modification of the procedure of Manderino et al. (1982). Briefly, normal human sera was activated with zymosan in the presence of the serum carboxypeptidase N (SCPN) inhibitor DL-2-mercaptomethyl-3-guandinoethyl-thiopropanoic acid (Calbiochem, La Jolla, CA) (Hugli et al., 1981). After the removal of zymosan by centrifugation, the activated serum was passed over a 200 ml anti-human C5a Sepharose column. The absorbed C5a was eluted with 0.2 M glycine-HCl, pH 2.5, concentrated by ultrafiltration, and further purified by gel filtration on a Bio-Gel P-30 (BioRad Laboratories, Richmond, CA) column (2.5 cm × 90 cm) previously equilibrated with 50 mM ammonium formate, 100 mM NaCl, pH 4.5. The purified C5a was stored in acid conditions at −70°C.

C5a des arg, formed by the activation of serum complement in the absence of the SCPN inhibitor, was purified using the above procedure. In addition, purified C5a was subjected to exopeptidase digestion using pancreatic carboxypeptidase B (Worthington, Freehold, NJ). Digestion was preformed in 1% $NaHCO_3$, pH 7.8, at an enzyme to substrate ratio of 1 : 50 (w/w). After 30 min at 37°C, the incubation was terminated by the addition of 0.05 M sodium acetate buffer.

Isolation of C5

Human C5 was isolated according to the procedure of Kunkel et al. (1980).

Antiserum production

Antibodies used throughout this study were raised in a goat by multisite, intramuscular injections of antigen (Manderino et al., 1982). Fifty micrograms of

C5a des arg emulsified in complete Freund's adjuvant were used as the initial immunogen. The goat was boosted at 4-month intervals and serum collected 10 days after each boost. IgG was purified according to the method of Gray et al. (1969). Goat anti-human C5a des arg was alkaline phosphatase conjugated as previously described (Engvall and Perlmann, 1972).

Immunodiffusion analysis

Ouchterlony immunodiffusion was performed in 1% agarose-PBS. Five micrograms of human C5a or C5a des arg, 15 μg of human C5, or 30 μg anti-human C5a des arg were added to wells (in 15 μl volumes) and incubated overnight at 4°C.

Protein determinations

Protein concentrations were determined by either the method of Lowry et al. (1951), with bovine serum albumin serving as a protein standard, or by ultraviolet absorption at 280 nm.

ELISA procedure

Wells of microtitration plates (Immulon 1, 'u' plates; Dynatech Laboratories, Richmond, VA) were coated by adding 100 μl of a 1 : 3000 dilution of goat anti-human C5a antibody (3.33 μg/well) in phosphate buffered saline (PBS), pH 7.2. The coated microtitre plates were covered with parafilm and incubated overnight at 4°C. Immediately prior to an assay, the coating solution was removed and the wells washed 3 times with PBS, pH 7.2, containing 0.05% Tween 20 and 0.02% sodium azide (PTA). After the final rinse, the microtiter plates were inverted on a dry paper towel and allowed to drain thoroughly. Serial dilutions of either human purified C5a, human C5a des arg, human C5, or activated serum from a number of species were made in PTA and 100 μl of sample were added to each well. The plates were then incubated for 4 h at 4°C. Following 3 additional washes with PTA, 100 μl of the alkaline phosphatase conjugated goat anti-human C5a antibody (diluted 1 : 300 in PTA) was added to each well and incubated for 4 h at 4°C. The plates were again washed 3 times with PTA, and 100 μl of a 1 mg/ml solution of disodium *para*-nitrophenyl phosphate (no. 104; Sigma Chemical Co., St. Louis, MO) in 200 mM carbonate buffer, 4 mM $MgCl_2$, pH 9.8, was added to each well. The microtiter plates were then incubated for a standard period of 100 min in all experiments determining C5a antigen levels. The optical density of the fluid in each well was determined using a Titertek multiscan microtitre plate reader at 405 nm. It was not necessary to stop the enzymatic reaction in each of the 96 wells, because the Titertek multiscan can read and print the results of each plate in less than 1 min. All samples were analyzed in duplicate. Before reading the optical density, the Titertek reader was blanked to a column of wells in a separate plate; each of the wells contained 100 μl of the substrate solution.

Results

Specificity and immunodiffusion analysis of ELISA components

The goat polyclonal antibody used throughout these studies was examined for its ability to immuno-precipitate C5a, C5a des arg, and C5 in Ouchterlony double

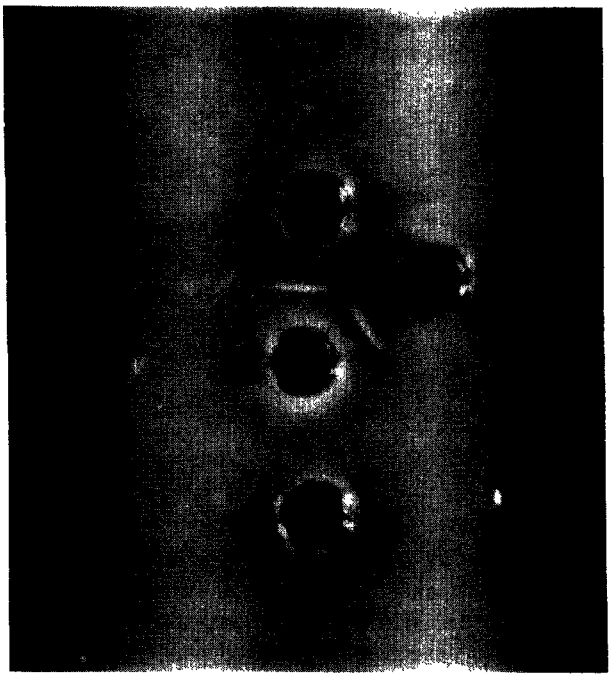

Fig. 1. Ouchterlony analysis of (a) immunoadsorbent purified human C5a prepared according to a modification of Manderino et al. (1982), (b) human C5a des arg, prepared by carboxypeptidase B digestion as described in Materials and Methods, (c) human C5, and (d) goat anti-human C5a antigen.

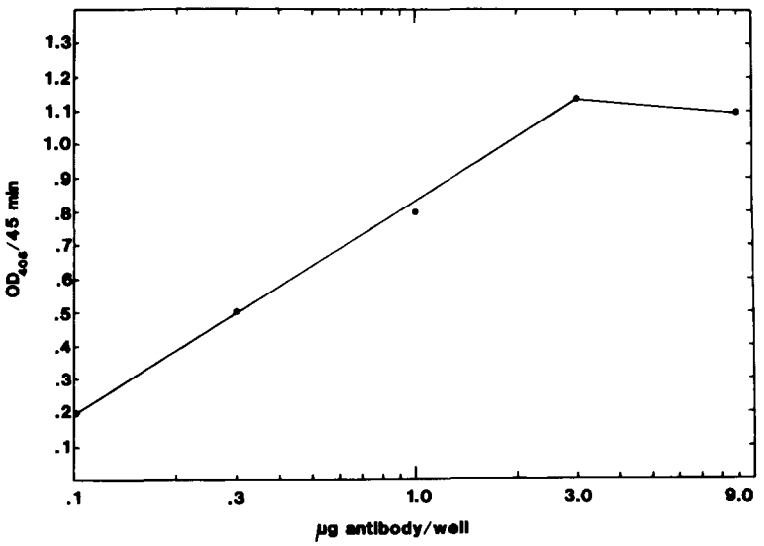

Fig. 2. Determination of optimal goat anti-human C5a concentration for coating the microtitre wells. Wells were coated with various concentrations of antibody, followed by incubation with various dilutions of C5a. The enzyme-anti-IgG conjugate was diluted 1 : 300 in PTA.

immunodiffusion. In the assay, 3 μg of C5a or C5a des arg, 15 μg of C5, and 30 μg of antibody were placed in each of the respective wells. The reactions were then developed at 4°C for 16 h. Immunoadsorbent-purified human C5a des arg showed lines of identity with immunoadsorbent-purified human C5a when tested against the polyclonal antibody (Fig. 1). Human C5 did not react.

Optimal antibody concentration of antibody for coating microtitre wells

To determine the optimal concentration of anti-human C5a needed to coat the wells, 100 μl of varying concentrations of polyclonal antibodies in PBS were added to each well and incubated at 4°C overnight. After washing thoroughly, each well was tested with 100 μl of 300 ng/ml C5a des arg and developed with 100 μl of a 1 : 100 dilution of conjugated antibody. As shown in Fig. 2, 3 μg/well was found to be the optimal concentration for the coating antibody.

Specificity of the ELISA assay

To determine the specificity of this ELISA methodology, purified C5a, C5a des arg, and native C5 were assayed for specificity of detectable antigen. The ELISA assay was shown to be equally specific for either C5a or C5a des arg (Fig. 3). The limit of detection for either antigen was approximately 4 ng/ml. Purified C5 did not significantly cross-react in this ELISA assay. To further examine this observation, 2 μl/ml of purified human C5 was added to 120 ng/ml of purified human C5a; 2-fold serial dilutions of this mixture was then examined in the ELISA. As shown in Fig. 4, the human C5/C5a mixture demonstrated a dose curve that was nearly identical to

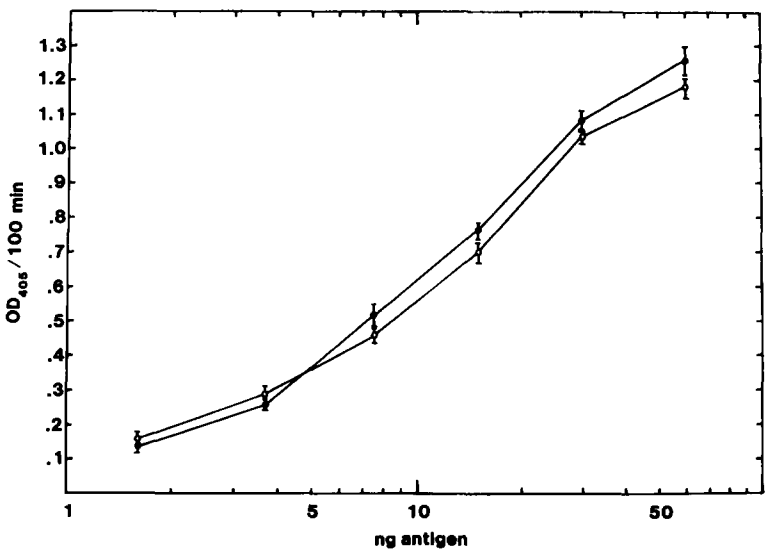

Fig. 3. Determination of the specificity of ELISA methodology for C5a, prepared according to a modification of the procedure of Manderino et al. (1982) (○———○), and C5a des arg prepared by carboxypeptidase B digestion as described in Materials and Methods (●———●).

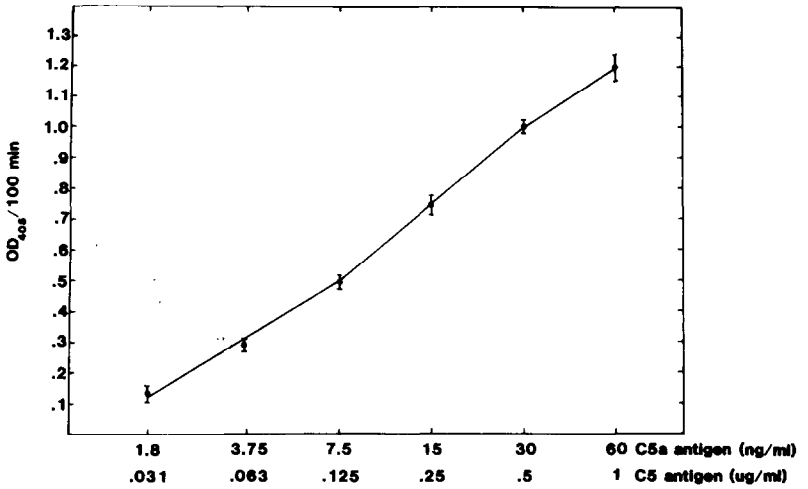

Fig. 4. Determination of the specificity of the ELISA methodology for a mixture of human C5a and human C5. Each point represents the optical density obtained by the additive amount of C5a + C5 found on the abscissa.

the dose-response curve of C5a or C5a des arg alone. Thus, levels of C5 antigen below 1 μg/ml do not alter the ELISA for C5a antigen detection. Both heat-inactivated normal human serum (NHS) and NHS activated with zymosan were examined for C5a antigen (Fig. 5). The zymosan-activated NHS had detectable levels of antigen even when diluted 1/3000. This is in contrast to the heat-in-

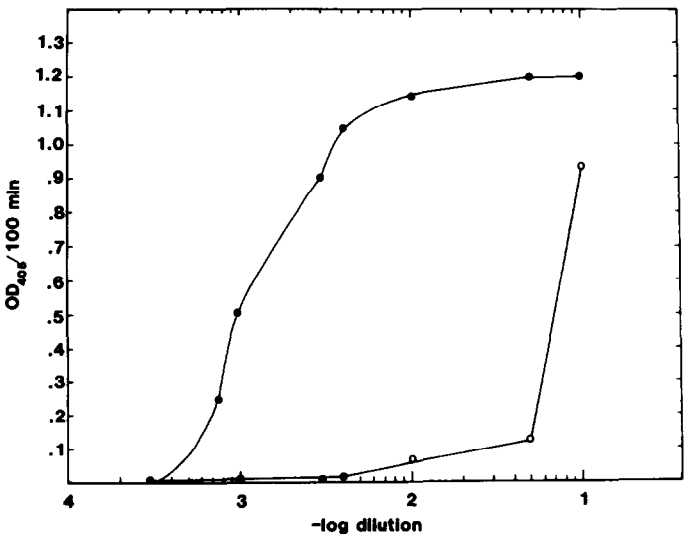

Fig. 5. Titration of C5a antigen found in heat-inactivated normal human serum (○——○) and normal human serum activated with 15 mg/ml zymosan (●——●).

TABLE I

ANALYSIS OF C5a ANTIGEN IN VARIOUS SAMPLES

Sample	ng C5a antigen/ml ± S.E.M.
Normal human plasma	30 ± 5
Normal human serum	112 ± 10
Normal human serum (56°C, 1 h)	158 ± 63
Normal human serum + zymosan (25 mg/ml)	3867 ± 321
Normal human serum (56°C, 1 h) + zymosan (25 mg/ml)	108 ± 7
Normal human serum + EDTA + zymosan	122 ± 6
Human C5-deficient serum	< 5
Human C5-deficient serum + zymosan	< 7
Normal rat serum	< 2
Normal rat serum + zymosan (25 mg/ml)	18 ± 4
Normal mouse serum	50 ± 7
Normal mouse serum + zymosan (25 mg/ml)	320 ± 15
Normal rabbit serum	4 ± 1
Normal rabbit serum + zymosan (25 mg/ml)	15 ± 3

activated NHS, which demonstrated little C5a antigen after a 20-fold dilution. The presence of C5a antigen in NHS may be accounted for by the ability of clotting factors to generate C5a-like antigen from C5 (Wiggins et al., 1981).

The specificity of the ELISA procedure was also examined using C5-deficient human serum, EDTA-treated NHS, and activated serum from various animals. No statistical difference was observed in the C5a antigen levels from NHS, heat-inactivated NHS, heat-inactivated NHS activated with zymosan, or NHS treated with EDTA and then activated (Table I). Zymosan-activated rat or rabbit serum demonstrated no C5a antigen, while activated mouse serum expressed low levels of cross reacting C5a antigen. As a control, zymosan-activated C5 deficient human serum contained no detectable C5a antigen. In contrast, the zymosan-activated NHS was found to possess very high levels of C5a antigen; the levels of antigen exceeded 3.5 μg/ml of activated serum.

Discussion

Proteins of the complement cascade are known to play a significant biological role as mediators of inflammatory processes. Considerable experimental and clinical evidence has accumulated to implicate split products of complement, especially C5a, as the genesis of many pathological consequences (Hammerschmidt et al., 1980). Most of these studies have relied on either bioassays such as polymorphonuclear leukocyte (PMN) aggregation (Craddock et al., 1977a), and chemotaxis (Mayer et al., 1976), diminution of peripheral PMN counts, (Craddock, 1977b), or immunoelectrophoresis (Hammerschmidt, 1980). In the latter technique samples were examined only for specific complement conversion products.

Using PMN aggregation as a bioassay, Hammerschmidt et al. (1980) examined the plasma from a number of patients at risk for Adult Respiratory Distress Syndrome (ARDS). In this study, a significant correlation was observed between the presence in plasma of PMN-aggregating material and the subsequent development of ARDS. The generation of C5a in acute cardiopulmonary complication due to cellophane-membrane hemodialysis has been examined using changes in arterial blood PMN levels and changes in arterial oxygen tension as indices (Craddock, 1977a). During the first 30 min of hemodialysis, patients' neutrophil counts fell precipitously resulting in a transient neutropenia. Arterial oxygen tension also dropped during this time period indicating significant changes in pulmonary functions. Although all of the above studies and assays are important means to determine indirectly C5a or C5a des arg activity, none of these assays directly or specifically quantitate antigen levels of this important C5 product. Recent clinical investigations by Nelson et al. (1983) have examined plasma levels of C5a des arg antigen over various time periods from burn patients. Employing a radioimmunoassay developed by Hugli and Chenoweth (1980), levels of C5a des arg antigen present in burn patients' plasma was examined over a 30-day period. Peak C5a des arg antigen was found at day 9 post-burn injury, at which time more than 60 ng/ml of antigen was found in the plasma. Interestingly, this peak in plasma C5a des arg antigen correlated with a desensitization effect on the patients PMN. In an additional clinical study, radioimmunoassays for both C3a and C5a antigen were used to quantitate levels of these complement products in the plasma of patients undergoing cardiopulmonary bypass (Chenoweth et al., 1981). Although C3a levels were significantly elevated during the bypass operation, C5a levels did not increase significantly. The latter finding was observed even though significant neutropenia was found in the patients during bypass: a phenomenon consistent with pulmonary vascular sequestration induced by C5a-activated PMNs.

In this report, we have described a specific and extremely sensitive microELISA procedure for the detection of human C5a/C5a des arg antigen. As shown in Fig. 3, both C5a and C5a des arg were found to react equally well in the ELISA assay. The C5a des arg used in this analysis was prepared by either purifying C5a des arg from serum in the absence of a SCPN-blocking agent or by purifying C5a and treating this peptide with SCPB. In either case, the polyclonal antibody failed to distinguish between C5a and C5a des arg antigen. The cross-reactivity between C5a and the parent C5 molecule was extremely low (Fig. 4).

In initial studies, we examined the ability of the ELISA to detect C5a/C5a des arg antigen in NHS, normal human plasma, and zymosan-activated serum (Table I). Our findings clearly demonstrate that normal human plasma contains significantly lower levels of antigen than NHS. This observation is in agreement with the radioimmunoassay data generated by Hugli and Chenoweth (1980). In both studies, EDTA-heparinized plasma was shown to contain about one quarter the C5a antigen as compared to NHS. The elevated levels of C5a in serum is consistent with the findings of Wiggins et al. (1981), who demonstrated the ability of specific proteins of the coagulation cascade to generate C5a-like material from rabbit C5. This observation may be important, since it could link activation of the coagulation to the

complement cascade in various inflammatory reactions. In an effort to examine the ability of the human C5a ELISA technique to detect C5a-like antigen from various species, we studied zymosan-activated serum from rats, mice and rabbits. The polyclonal anti-human C5a did not cross react with activated serum from these species (Table I) to any significant extent. Therefore, the human ELISA was neither specific nor sensitive for the other species.

The ability to quantitate levels of C5a in plasma and other biological fluids provides a sensitive assessment of complement activation and C5 cleavage in various disease states. The detection of nanogram levels of C5a by the ELISA methodology permits the quantitation of this biologically active peptide beyond the limits of both bioassays and other alternative methods, an important consideration, since it is well known that this peptide is an extremely potent mediator active at nanomolar concentrations. The appearance of C5a through complement activation appears to correlate well with many phyogistic processes including autoimmune diseases. Although the potential of a specific and sensitive ELISA technique for quantitating human C5a is promising, the applicability of this method to determine C5a/C5a des arg antigen in plasma and other biological fluids will definitely await further studies.

Acknowledgement

The authors are grateful for the secretarial support of Ms. Jeny Breakstone.

References

Bokisch, V.A. and H.J. Müller-Eberhard, 1970, J. Clin. Invest. 49, 2427.
Chenoweth, D.E. and T.E. Hugli, 1980, Mol. Immunol. 17, 151.
Chenoweth, D.E., S.W. Cooper, T.E. Hugli, R.W. Stewart, E.H. Blackstone and J.W. Kirklin, 1981, N. Engl. J. Med. 304, 497.
Cochrane, C.G. and H.J. Müller-Eberhard, 1968, J. Exp. Med. 127, 371.
Craddock, P.R., J. Fehr, K.L. Brigham, R.S. Kronenberg and H.S. Jacob, 1977a, N. Engl. J. Med. 296, 769.
Craddock, P.R., D.E. Hammerschmidt, J.G. White, A.P. Dalmasso and H.S. Jacob, 1977b, J. Clin. Invest. 60, 260.
Engvall, E. and P. Perlmann, 1971, Biochim. Biophys. Acta 251, 427.
Engvall, E. and P. Perlmann, 1972, J. Immunol. 109, 129.
Fernandez, H.N., P. Henson, A. Otani and T.E. Hugli, 1978, J. Immunol. 120, 109.
Gray, G.D., M.M. Mickelson and J.A. Crim, 1969, Immunochemistry 6, 641.
Hammerschmidt, D.E., L.D. Hudson, L.J. Weaver, P.R. Craddock and H.S. Jacob, 1980, Lancet i, 947.
Handley, H.H., M.C. Glassy, P.H. Cleveland and I. Royston, 1982, J. Immunol. Methods 54, 291.
Hugli, T.E., 1979, in: The Chemistry and Physiology of Human Plasma Proteins (Pergamon, New York) p. 225.
Hugli, T.E., and D.E. Chenoweth, 1980, in: Immunoassays: Clinical Laboratory Techniques for the 1980s (Alan R. Liss, New York) p. 443.
Hugli, T.E., C. Gerrard, M. Karvahera, M.E. Sheetz, R. Barton, S. Briggs, G. Koppel and S. Russel, 1981, Mol. Cell. Biochem. 41, 59.
Johnson, A.R., T.E. Hugli and H.J. Müller-Eberhard, 1975, Immunology 28, 1067.
Kunkel, S.L., D.L. Kreutzer, S. Goralnick and P.A. Ward, 1980, J. Immunol. Methods 35, 337.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, J. Biol. Chem. 193, 265.
Manderino, G.L., A.F. Suarey, S.L. Kunkel, P.A. Ward and A.A. Hirata, 1982, J. Immunol. Methods 53, 41.
Mayer, J.E., J. McCullough, B.J. Weiblen, E.L. Kaplan, W.G. Lindsay and D.M. Nicoloff, 1976, Surg. Forum 27, 285.
Nelson, R.D., D.E. Chenoweth, J.S. Solomkin and L.D. Solem, 1983, Agents Action 12 (Suppl.), 274.
Perez, H.D., I.M. Goldstein, D. Chenoff, R.O. Webster and P.M. Henson, 1980, Mol. Immunol. 17, 163.
Perez, H.D., I.M. Goldstein, R.O. Webster and P.M. Henson, 1981, J. Immunol. 126, 800.
Vallota, E.N. and H.J. Müller-Eberhard, 1973, J. Exp. Med. 137, 1109.
Webster, R.O., S.R. Hang, R.B. Johnson and P.M. Henson, 1980, Immunopharmacology 2, 201.
Wiggins, R.C., P.C. Giclas and P.M. Henson, 1981, J. Exp. Med. 153, 1391.