Immunology 1985 **54** 389

A key role for fibronectin in the sequential binding of native dsDNA and monoclonal anti-DNA antibodies to components of the extracellular matrix: its possible significance in glomerulonephritis

R. A. LAKE, A. MORGAN, B. HENDERSON* & N. A. STAINES *Chelsea College, University of London, London*

Accepted for publication 25 September 1984

Summary. The interactions of DNA, monoclonal anti-DNA autoantibodies and isolated purified components of the extracellular matrix (ECM) were studied in a solid phase model system. Binding of DNA to each of the components was assessed using monoclonal antibody and enzyme conjugated anti-globulin in direct binding and inhibition assays.

Each of the genetically distinct collagens (Types I–IV), proteoglycan monomer and laminin, bound ssDNA, but dsDNA bound significantly only to fibronectin. When used in an inhibition system, fibronectin linked DNA and anti-DNA antibodies to the collagens; it had a differential effect on the binding of ssDNA and dsDNA to a Type IV collagen matrix, the most striking feature being a 100 fold increase in dsDNA binding to Type IV collagen in the presence of fibronectin.

It is likely that fibronectin binds dsDNA to collagen by separate binding domains for these molecules, and that this may be involved in the deposition of DNA in kidneys in some forms of glomerulonephritis.

* Present address: Dept. Prostaglandin Research, The Wellcome Research Laboratories, Beckenham, Kent BR3 3BS.

Correspondence: Dr N. A. Staines, Dept. Immunology, Chelsea College, University of London, Manresa Road, London SW3 6LX.

INTRODUCTION

Glomerulonephritis is central to the pathology of systemic lupus erythematosus (SLE) in man and in the mouse. Kidney damage is caused by the deposition of material on the glomerular basement membrane (GBM) or within the mesangial matrix. The nature of the deposits is a matter of some conjecture, but DNA and immunoglobulin (Ig) can be identified by immunofluorescence and there is evidence implicating the presence of anti-DNA antibodies. There is further evidence (Tan, 1982) to suggest that antibodies which preferentially react with double-stranded DNA (dsDNA) may be more pathogenic than those reacting solely or preferentially with single-stranded DNA (ssDNA).

The mechanisms whereby such antigen-antibody (immune) complexes deposit and persist long enough to initiate inflammatory damage are not certain, but deposition is probably related to many interacting factors, principally those which affect the size and solubility of the complexes (Theophilopoulos & Dixon, 1979; McCluskey & Bhan, 1981).

Mechanistic models (Couser & Salant, 1980) propose either that preformed immune complexes bind to the glomerulus, or that antigen (DNA) binds first to initiate a nucleus of immune material *in situ*. In either case, it is obvious that the ability of the components of the immune complex to interact with the glomerular

extracellular matrix (ECM) must have a qualitative effect upon the subsequent pathology. The molecular basis of these interactions are hinted at by the findings of Izui, Lambert & Miescher (1976) that DNA can bind to insoluble fragments of GBM and to several of the genetically distinct types of collagen.

We have extended the studies of Izui *et al.* (1976) and, in this paper, report the characteristics of interaction of ssDNA and dsDNA with purified components of the ECM, and identify a key role for serum fibronectin in the initiation of the formation of immune complexes *in situ*.

MATERIALS AND METHODS

Components of the extracellular matrix (ECM)

Type I collagen (CI) and Type IV collagen (CIV) were purchased from Sigma Chemical Co. (Poole, Dorset). Type II collagen (CII) was prepared from bovine nasal septa by pepsin digestion and extraction in 0·45 M NaCl (Staines *et al.*, 1981). Proteoglycan monomer (PPGM) was prepared from pig laryngeal cartilage by extraction with 4 M guanidine hydrochloride and isolated by equilibrium density gradient centrifugation (Hardingham, Ewins & Muir, 1976). Laminin was prepared from mouse EHS sarcoma by extraction with 0·5 M NaCl and isolated by ion exchange chromatography (Timpl *et al.*, 1979). Rat plasma fibronectin was isolated by affinity chromatography on gelatin Sepharose (Vuento & Vaheri, 1979). Glomerular basement membranes were prepared from dispersed rat kidney by fractionation through steel meshes as described by Krakower & Greenspon (1951).

These components were stored at $-30°$ in 100 μl aliquots dissolved in 0·1 M $NaHCO_3$, pH 9·8, containing 0·5 M NaCl.

DNA

Calf thymus DNA (Sigma) was prepared as described by Marmur (1961) and further purified by caesium chloride density gradient centrifugation to eliminate contaminating protein and RNA (Steward, Olsen & Barnett, 1977). This material was sonicated under controlled conditions to achieve an average molecular weight of 4×10^5. DNA at 500 μg/ml in phosphate-buffered saline (PBS) was denatured by boiling for 20 min and cooled rapidly to 0° in an ice/salt bath.

Native and denatured DNA are referred to as dsDNA and ssDNA, respectively. Each physical form of the DNA is known to contain a contamination of the other form of approximately 15% by weight (A. Morgan, R.R.C. Buchanan, A.M. Lew, I. Olsen & N.A. Staines, submitted for publication).

Monoclonal autoantibodies reactive with DNA

The preparation and properties of monoclonal antibodies (MCA) derived from lupus mice have been

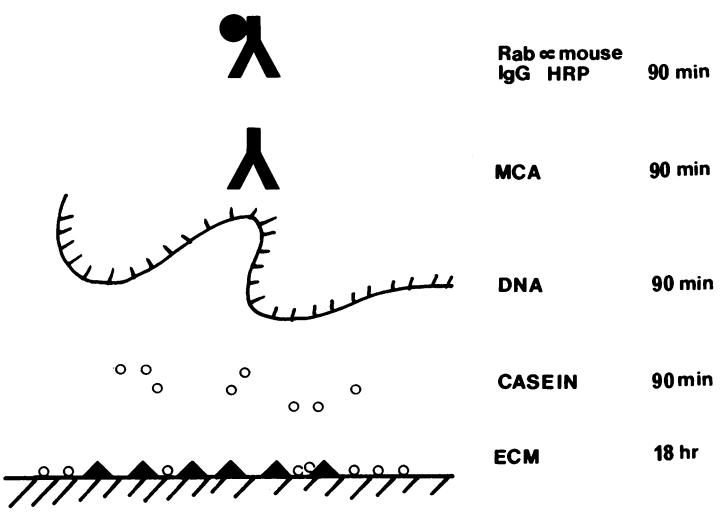

Figure 1. Schematic representation of the system used to analyse the interaction of DNA and ECM components. The assay was conducted at 4° throughout, except for the development of the final enzyme reaction at room temperature. Times of incubation are indicated. Casein (2% in PBS) was used as an inert blocking agent.

described elsewhere (A. Morgan, R.R.C. Buchanan, A.M. Lew, I. Olsen and N.A. Staines, submitted for publication; R.R.C. Buchanan, A. Morgan, P.J.W. Venables, R.N. Maini and N.A. Staines, submitted for publication). Two lines were used in this study: MCA-33, which is specific for dsDNA, and MCA-228, which is specific for ssDNA.

Solid phase model system for identifying interactions between MCA, DNA and components of the ECM

The methodology for the model system was based on the ELISA principle described by Engvall & Perlmann (1972) and modified by Staines *et al.* (1981). The diluent throughout was isotonic phosphate-buffered saline (PBS) containing 1% casein (British Drug Houses, Dagenham, Essex) and 0·05% Tween 20 (Sigma). Further modification of the technique involved the incubation of free DNA with ECM components immobilized in the wells of micro ELISA trays (Nunc Immunoplate II, Gibco, Paisley, Scotland). The binding of the DNA was assessed by sequential exposure to one or other of the MCA and a horseradish peroxidase-conjugated anti-mouse Ig reagent (Miles Laboratories, Slough). The assay was concluded by the addition of H_2O_2 and chromogenic indicator o-phenylenediamine. Extinction coefficients were determined using a Titertek Multiskan spectrophotometer (Flow Laboratories, Irvine, Scotland) at a wavelength of 450 nm. Two assay procedures were adopted to evaluate DNA-ECM component interactions: checkerboard assays were performed by titrating the two interacting components against each other, and inhibition assays were conducted by adding ECM components to the fluid phase to compete for DNA binding with solid phase ECM material.

The model system was standardized for each of the stages as depicted in Fig. 1, such that, in all assays, constant amounts of MCA and the final anti-globulin reagent were used.

RESULTS

The capacity of components of the ECM to bind ssDNA and dsDNA

All the ECM components tested bound ssDNA directly, but dsDNA bound in measurable amounts only to fibronectin.

Checkerboard assays were used to determine the relationship between the concentration of ECM material immobilized in the solid phase of the interacting system and the amount of DNA bound by these components. At each sensitization concentration, an anti-DNA mid-point titre value was derived; this gave a relative measure of the amount of DNA bound to each of the ECM components (Fig. 2). The titre values for dsDNA and ssDNA could not be compared directly because each was derived using a different MCA in the detection system.

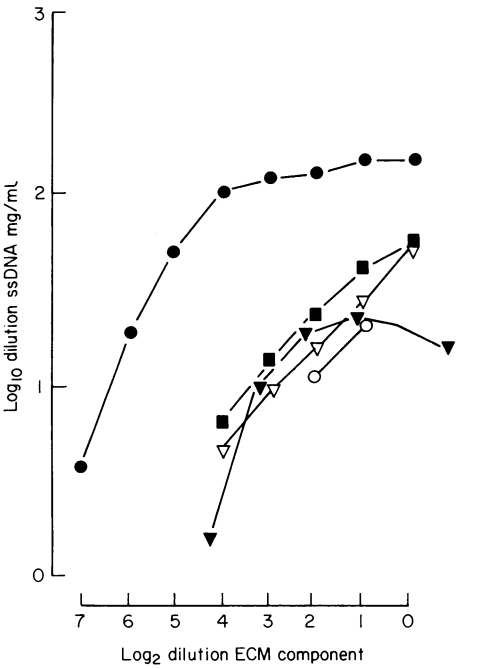

Figure 2. The amount of DNA bound by different ECM components as a function of their concentration. The concentration of free DNA added which produced a given A_{450} value (when exposed to a fixed amount of MCA-228) is plotted (y axis) against the concentration of the ECM component added to the binding system in the first place (x axis) in serial dilutions from 67 μg/ml; (●) fibronectin; (■) CII; (▲) CIV; (△) CI; (○) laminin; (□) porcine proteoglycan monomer.

The characteristics of DNA binding clearly divided the ECM components into two groups (Fig. 2). Previous work (R.A. Lake, unpublished observations) had shown that each of these molecules could maximally sensitize ELISA plates for the binding of specific antibody against each one at concentrations of around 10 μg/ml. In these experiments, fibronectin and CIV behaved similarly in binding DNA, reaching plateaux values at this concentration. CI, CII and laminin, however, did not saturate the solid phase ssDNA binding at sensitization concentrations up to 70 μg/ml.

In addition, the two groups segregated in relation to the gradients of the linear parts of the binding curves. This implies that there is a difference between these groups in the stoichiometry and avidity of DNA binding to the ECM components in them.

The relative avidity of ECM components for DNA assessed by competitive inhibition

A rank order of DNA binding avidity was obtained by adding free components of the ECM to the binding system. The inhibition of the binding of DNA to each ECM component in the solid phase when individual free ECM components were added was used to evaluate their mutual competitive properties. Titre values were obtained by interpolating the concentration of each ECM component required to achieve 50% reduction of the non-inhibited binding controls.

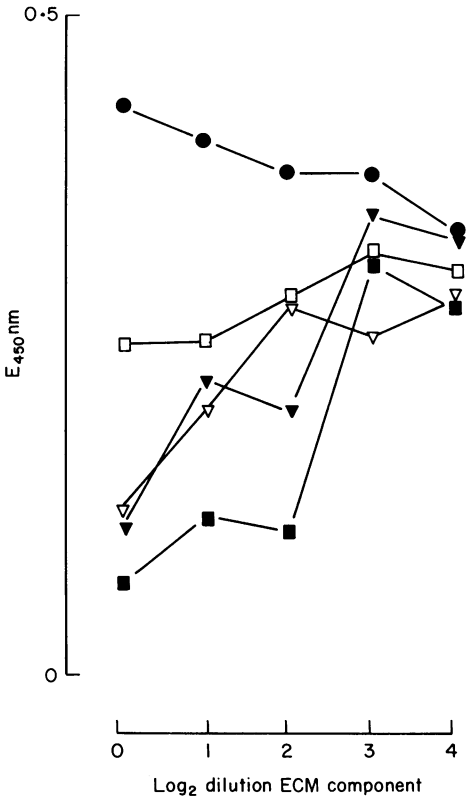

Figure 3. Inhibition of binding of DNA to CIV by free ECM components. DNA binding detected by fixed concentration of MCA-228 (*y* axis); free ECM components in serial dilution from 67 μg/ml (*x* axis). (Symbols as in Fig. 2.)

Figure 3 shows the results of a representative experiment in which each component was titrated against solid phase CIV. The qualitative rank order CII > CIV > CI > PPGM in binding capacity was obtained. The same order was found in experiments using every other ECM component in the solid phase of the binding system.

When fibronectin was added in the free phase, it increased the amount of DNA bound to the immobilized ECM component. The binding of DNA to fibronectin was not inhibited by the addition of any other free ECM component.

The relative ability of fibronectin to augment the binding of ssDNA and dsDNA to components of the ECM

Checkerboard assays were used to investigate the unique augmenting behaviour of fibronectin. Free fibronectin was titrated against free ssDNA and dsDNA. The amount of DNA bound to CIV was markedly increased by fibronectin at concentrations above 100 μg/ml. DNA titre values, obtained as described above, were increased 2½-fold for ssDNA (Fig. 4) in the presence of 1 μg/ml fibronectin, while dsDNA titre values were increased 100-fold (Fig. 5). Thus, fibronectin had a 40-fold greater effect in augmenting dsDNA compared with ssDNA binding to CIV.

DISCUSSION

The persistent retention of immune complexes of antigen and antibody is widely believed to be the critical factor in the initiation of inflammatory damage in systemic autoimmune connective tissue diseases. The basis for this phenomenon in SLE is the demonstrable presence of Ig in the glomerulus, the presence of anti-DNA antibodies in material eluted from diseased kidneys (Krishman & Kaplan, 1967), and the presence of small amounts of specific immune complexes circulating in the blood (Nydegger *et al.*, 1974). Serological findings are the most often quoted in support of this, but are obviously the least reliable, in that they are incapable of identifying whether or not circulating antibodies or complexes have the potential to bind to the target tissues.

The findings here that DNA has a direct affinity for collagens, PPGM and laminin in a model binding system *in vitro* indicate some of the molecular interactions which may be involved in the deposition of immune complexes containing DNA and anti-DNA

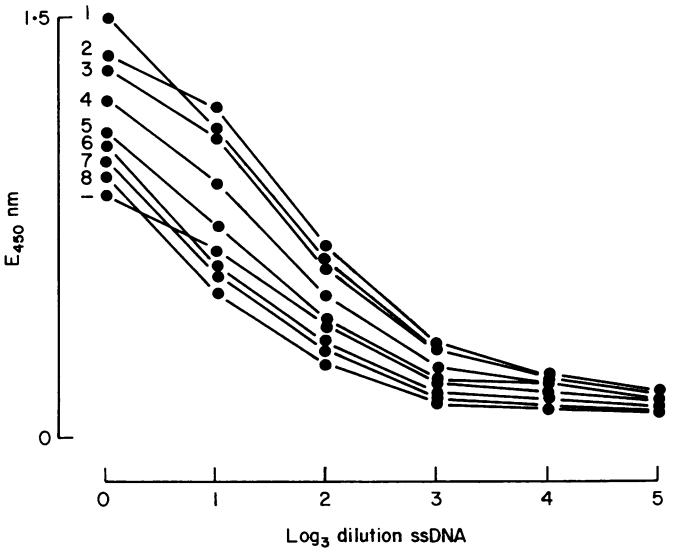

Figure 4. Augmentation of binding of ssDNA to CIV by fibronectin. Titration curves of DNA (detected by MCA-228) diluted from 25 μg/ml in the presence of trebling dilutions of fibronectin from 9 μg/ml (line 1) to 4 ng/ml (line 8).

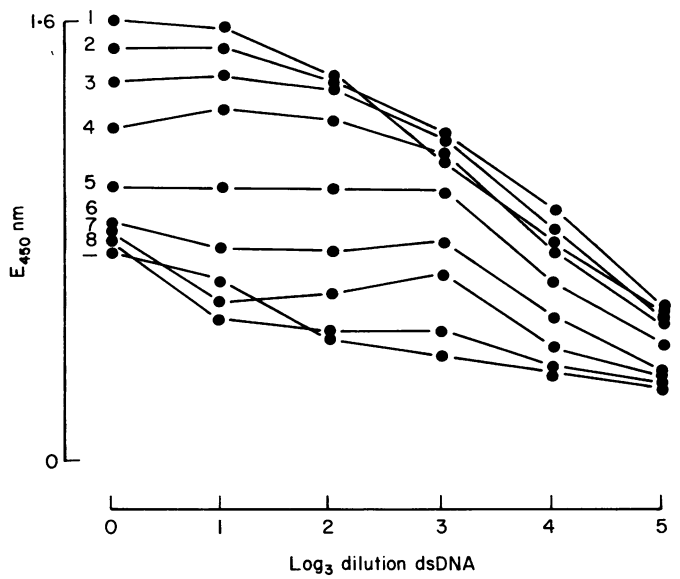

Figure 5. Augmentation of binding of dsDNA to CIV by fibronectin. Details as in Fig. 4, but binding was detected with MCA-33.

antibodies. This was first reported by Izui *et al.*, (1976) and this study confirms and extends their observations.

ssDNA and dsDNA do not bind equally well to isolated components of the ECM in aqueous systems, but the presence of serum fibronectin has an augmenting effect on their binding to CIV and glomerular fragments. This augmentation has a 40-fold greater effect on the binding of dsDNA compared with its effect on the binding of ssDNA. Although there are no direct estimates of the amounts of the two different types of DNA bound in these systems, the amounts of

anti-DNA MCA bound by the immobilized DNA's (detected in turn by the binding of peroxidase-labelled anti-Ig antibodies) are such that it is likely that much more dsDNA than ssDNA will bind to a given amount of CIV in the presence of fibronectin.

The significance of these results for the deposition of DNA in the glomerulus *in vivo* must be interpreted with caution. They do, however, correlate well with the serological findings that severe kidney disease is accompanied by circulating antibodies directed against dsDNA, rather than ssDNA (Holian *et al.*, 1975).

It should be emphasized that both dsDNA and ssDNA, when bound to ECM components, are still immunologically available to MCA reactive with them. Thus, DNA deposited *in vivo* would be expected to be available to bind circulating antibodies, leading to *in situ* formation of nuclei of immune complexes.

The sequential build-up of immune complexes is not the only way in which they become associated with the ECM. Soluble immune complexes of the same antibodies and DNA preincubated together are able to bind to ECM components. It must therefore be the case that both mechanisms could operate to localize complexes in tissues. These experiments do not indicate whether the affinity of these respective associations are different, nor whether they lead to the formation of deposited complexes equally resistent to dissociation.

Fibronectin, which is a normal serum component, is a disulphide-linked heterodimer of two nearly identical polypeptides with a molecular weight of 450,000. One of its main functions is as an adhesive agent for cell interaction with the ECM (Yamada, 1982). It also has binding sites for DNA (Hoch, 1982) and collagen (Engvall, Ruoslahti & Miller, 1978) and, in our experiments, these two functions are coexpressed and lead to the indirect interaction of the two macromolecular species.

Fibronectin is not an integral part of the GBM, but it is presumed to become associated non-covalently with the membrane, possibly in part by trapping, due to the high filtration rate of the glomerulus. Not only is it found on the GBM, but is is also present in high concentration in the glomerular mesangium within the matrix between endothelial and mesangial cells (Dixon & Burns, 1982). Thus, its distribution is consistent with the two major sites of deposition of DNA-anti-DNA immune complexes in the kidney in SLE. Complexes of different physicochemical properties are characteristically deposited in these sites (Lew, Staines & Steward, 1984), therefore we conclude that the augmenting effects of fibronectin on deposition are not restricted to only one type of immune complex.

Laminin, CIV and proteoglycans are normal constituents of the GBM. The other genetically distinct types of collagen have different tissue distributions; CI is found in skin, bone tendon and cornea, CII in articular and related cartilages, and CIII in skin and the cardiovascular system. The affinity of DNA for all collagen types may underlie a universal propensity for DNA (and immune complexes containing DNA) to deposit in other tissues of the body. Certainly in SLE, skin rash is a dominant clinical feature and vasculitis and arthritis are not uncommon. It is unknown whether the same type of immune complexes are responsible for initiating all these lesions, but it would seem likely that the architecture and local physiology of the tissues concerned would influence the type of complexes which can be formed in them. Although collagens exist in a polymerized insoluble form in all tissues, they are available to bind specific antibodies as shown by indirect immunofluorescence assay (Timpl, Wick & Gay, 1977).

The fact that serum fibronectin can associate with DNA when it is, in turn, complexed to specific anti-DNA antibodies suggests that such immune complexes may contain non-specifically associated serum proteins. This might affect the ease with which they can be detected in the circulation and, moreover, may explain the unexpected reactions of anti-DNA antibodies (and rheumatoid factors which could combine with them) with cell membranes.

These studies show that the respective mechanisms of binding of ssDNA and dsDNA to collagens may be different—a conclusion also reached by Izui *et al.* (1976). More recently, Emlen & Mannik (1984) have shown that the clearance kinetics of radiolabelled dsDNA and ssDNA from the circulation are different, emphasizing further that the two physical forms are handled differently *in vivo*. It is possible that both collagens and fibronectin have two binding domains, one for sites on each physical form of the DNA. In consequence, the antigenic content of immune complexes containing DNA will determine where the complexes are cleared or localized.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from The

Arthritis and Rheumatism Council and the Central Research Fund of the University of London, to whom the authors express their gratitude. In addition, the assistance of Miss Hoo Saw Lan in setting up the model binding system is gratefully recognized.

REFERENCES

COUSER W. & SALANT D. (1980) *In situ* immune complex formation and glomerular injury. *Kidney Int.* **17,** 1.

DIXON A.J. & BURNS J. (1982) Ultrastructural localization of fibronectin in mouse kidney. *J. Pathol.* **138**(1), 25.

EMLEN W. & MANNIK M. (1984) Effect of DNA size and strandedness of the *in vivo* clearance and localization of DNA. *Clin. exp. Immunol.* **56,** 185.

ENGVALL E. & PERLMANN P. (1972) Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzynme-labelled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **109,** 129.

ENGVALL E., RUOSLAHTI E. & MILLER E. (1978) Affinity of fibronectin to collagen of different genetic types and fibrinogen. *J. exp. Med.* **147,** 1584.

HARDINHAM T.E., EWINS R.F.J. & MUIR H. (1976) Cartilage proteoglycans. Structure and heterogeneity of the protein core and the effects of specific modifications on the binding to hyaluronate. *Biochem. J.* **157,** 127.

HOCH S. (1982) DNA binding domains of fibronectin probed using western blots. *Biochem. biophys. Res. Comm.* **106,** 1353.

HOLIAN J., GRIFFITHS I., GLASS D.N., MAINI R.N., SCOTT J.T. (1975) *Ann. Rheum. Dis.* **34,** 438.

IZUI S., LAMBERT P.H. & MIESCHER P.A. (1976) *In vitro* demonstration of a particular affinity of glomerular basement membrane and collagen for DNA. A possible basis for a local formation of DNA-anti-DNA complexes in systemic lupus erythematosus. *J. exp. Med.* **144,** 428.

KRAKOWER C. & GREENSPON M. (1951) Localization of the nephrotoxic antigen within the isolated renal glomerulus. *Arch. Pathol.* **51,** 629.

KRISHMAN C.S. & KAPLAN M.H. (1967) Immunopathologic studies of systemic lupus erythematosus. II. Antinuclear reaction of α-globulin eluted from homogenates and Isolated glomeruli of kidneys from patients with lupus nephritis. *J. clin. Invest.* **46,** 569.

LEW A.M., STAINES N.A. & STEWARD M.W. (1984) Glomerulonephritis induced by preformed immune complexes containing monoclonal antibodies of defined affinity and isotype. *Clin. exp. Immunol.* **57,** 413.

MCCLUSKEY R.T. & BHAN A.K. (1981) Immune complexes and renal diseases *Clin. Immunol. Allergy*, **1,** 397.

MARMUR J. (1961) a procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. mol. Biol.* **3,** 208.

NYDEGGER U.E., LAMBERT P.H., GERBER H. & MIESCHER O.A. (1974) Circulating immune complexes in the serum in systemic lupus erythematosus and in carriers of hepatitis B antigen. Quantitation by binding to radiolabelled Clq. *J. clin. Invest.* **54,** 297.

STAINES N.A., HARDINGHAM T., SMITH M. & HENDERSON B. (1981) Collagen-induced arthritis in the rat: modification of immune and arthritic responses by free collagen and immune anti-collagen antiserum. *Immunology*, **44,** 737.

STEWARD M.W., OLSEN I. & BARNETT E.V. (1977) Double isotope method for measuring antibodies to ^{125}I-labelled double stranded and single stranded DNA of defined molecular weight. *Ann. Rheum. Dis.* **36** (Suppl. 1), 119.

TAN E.M. (1982) Autoantibodies to nuclear antigens (ANAN), their immunobiology and medicine. *Adv. Immunol.* **33,** 167.

THEOFILOPOULOS A.N. & DIXON F.J. (1979) The biology and detection of immune complexes. *Adv. Immunol.* **28,** 89.

TIMPL R., ROHDE H., GETHRON ROBEY P., RENNARD S.I. & FOIDART J.M. (1979) Laminin—a glycoprotein from basement membranes. *J. biol. Chem.* **254,** 9933.

TIMPL R., WICK G. & GAY S. (1977) Antibodies to distinct types of collagens and procollagens and their application in immunohistology. *J. Immunol. Meth.* **18,** 165.

VUENTO M. & VAHERI A. (1979) Purification of fibronectin from human plasma by affinity chromatography under non-denaturing conditions. *Biochem. J.* **188,** 331.

YAMADA J. (1982) Isolation of fibronectin from plasma and cells. In: *Immunochemistry of Extracellular Matrix* (ed. H. Furthmays), Vol. 1, p. 111. CRC Press, Boca Raton, Florida.