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Differences between C4A and C4B in the handling of immune complexes: the enhancement of CR1 binding is more important than the inhibition of immunoprecipitation

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SUMMARY

There are two isotypes of C4—C4A and C4B—, encoded within the major histocompatibility complex with quite different properties. In this study we have compared purified C4A and C4B with regard to their ability to prevent immune complex precipitation and to enhance the binding of both preformed and nascent immune complexes to the receptor CR1 on red cells. C4A was modestly more effective than C4B at inhibiting immunoprecipitation, particularly in antibody excess. In the CR1 binding assay C4A was markedly more effective than C4B in enhancing binding to CR1. This difference was seen with both preformed and nascent immune complexes at equivalence and antibody excess. Thus the major differences between C4A and C4B in regard to immune complex handling is at the level of CR1 binding. Given the strong association of C4A* QO alleles with immune complex-mediated diseases like systemic lupus erythematosus, these findings have important pathogenetic implications.

Keywords complement immune complexes C4A C4B CR1 receptor

INTRODUCTION

Human C4 is encoded at two loci within the major histocompatibility complex (MHC); the products of the two loci are named C4A and C4B (Awdeh & Alper, 1980). The two forms differ only in their alpha-chains (Roos *et al.*, 1982), the chain in which the internal thioester bond responsible for the covalent binding of the activation fragment C4b to cell surfaces and soluble proteins is found (Law, Dodds & Porter, 1984). Despite only minor differences in sequence, C4A and C4B have been shown to have quite different properties, with C4B being several-fold more efficient in haemolytic assays (Law *et al.*, 1984). This in turn is related to the ability of C4Bb to preferentially bind covalently with acceptor hydroxyl groups that predominate amongst the carbohydrates of red cell surfaces (Isenman & Young, 1984). In contrast, C4Ab binds preferentially to amino groups such as those found in immune complexes (Law *et al.*, 1984). Indeed, it has been formally demonstrated that three to four times as much C4Ab binds to immunoglobulin as C4Bb (Kishore *et al.*, 1988).

The classical complement pathway has been demonstrated to be involved in the prevention of immune complex precipitation (reviewed by Webb & Whaley, 1986; Schifferli, Ng & Peters, 1986) and in enhancing binding to CR1, predominantly on red blood cells and consequent clearance of immune complexes (Schifferli *et al.*, 1986). This physiological role of the classical complement pathway in preventing the effects of immune complex formation has lead to the concept that a deficiency in the effector mechanisms of the immune response may, somewhat paradoxically, be associated with the development of autoimmune diseases, such as systemic lupus erythematosus (SLE) in which immune complexes are believed to have an important role (Lachmann & Walport, 1987). It has long been known that homozygous deficiency of C4, C2 and C1 predisposes to autoimmune immune complex disease (Lachmann, 1987). More recently, it has been shown clearly in one study involving three different ethnic groups that an excess of C4A* QO alleles was the common genetic factor in the three groups and indeed may account for all the MHC-related genetic risk for SLE (Dunckley *et al.*, 1987).

With this background in mind we have explored the relative efficiency of C4A and C4B in both the prevention of immune complex formation and the enhancement of binding to CR1, both with preformed and nascent immune complexes.

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MATERIALS AND METHODS

Purification of complement components

C1 was prepared from outdated human plasma obtained from the Blood Bank at New Addenbrooke's Hospital, Cambridge, by a modification of the euglobulin precipitation procedure described by Gigli, Porter & Sim (1976). The serine esterase

inhibitor Dip-F (diisopropyl fluorophosphate, Sigma Chemical Co., St Louis, MO) was added to a concentration of 1 mM after the final purification step.

Fresh plasma was obtained from a healthy donor, an individual homozygous for C4B* QO and another individual homozygous for C4A* QO as sources of C4, C4A and C4B, respectively. Ten millimole (10 mM) EDTA and 10 mM benzamidine were added to prevent activation during purification which was carried out as described by Harrison & Lachmann (1986). The final purity of both C1 and C4 were checked by electrophoresis in 10% polyacrylamide gel in buffers containing sodium dodecyl sulphate, C1 under both reducing and non-reducing conditions and C4 under reducing conditions modified from Laemmli (1970).

Measurement of activity of C4

The activity of the various C4 fractions was measured repeatedly by assessing the CH_3NH_2 (methylamine)-max as described by Law, Minich & Levine (1981) and the relative concentrations of C4 used in the assays described below were determined by this (Paul *et al.*, 1988).

Antigen

A solution of five times crystallised ovalbumin (OVA; Koch Light, Haverhill, UK) was radiolabelled with ^{125}I (Amersham Labs, Amersham, UK) using the method of Bolton & Hunter (1973) to a specific activity of 250000 ct/min per mg.

Antibody

IgG anti-OVA was prepared from a pool of serum from rabbits immunized with OVA, by precipitation with 50% saturated ammonium sulphate, ion-exchange chromatography in DEAE-cellulose and affinity purification on an OVA–sepharose CL4B column. After elution with glycine–HCl buffer, pH 2·5, the pooled fractions of antibody were dialysed against PBS containing 10 mM sodium azide.

Immune complex solubility assay

The assay was carried out as follows: ^{125}I–OVA was adjusted to a concentration of 5 mg/ml with CFD (Oxoid, Oxford, UK) containing 0·1% gelatin and 2 mM sodium azide. Likewise the antibody concentration was adjusted to 84 μg/ml. The reagents were held on ice and mixed in a final volume of 50 or 100 μl with the antigen always added last. The mixture was allowed to react for 1 h at 37°C, then the reaction was stopped by addition of CFD buffer at 4°C to a total volume of 200 μl. The tubes were spun for 15 min at 2000 *g* at 4°C, and 100 μl of supernatant were harvested. The amount of radioactivity in both half the supernatant (S) and the pellet (P) was counted (Gamma counter, LKB-Wallac, 80000 series) and the percent precipitation calculated by the formula $[(P-S)/(P+S)] \times 100$. A standard curve was constructed and from this ratios of antigen:antibody ranging from antigen excess through equivalence to antibody excess were used in the inhibition of immune precipitation assays.

Inhibition of immune precipitation assay

Purified C1 and each of C4, C4A and C4B were added to the above reaction mixture. The ratio of C4:C1 was adjusted in a series of preliminary experiments to remove any effect of C1 alone on the immune complex precipitation with a final ratio (w/w) of 1:4 being used. The amount of active C4 (or C4A or C4B) used was 15 μg in a reaction volume of 100 μl. C4 alone never showed any effect on immune complex precipitation. Several time-points were examined and all assays were performed in duplicate.

CR1 binding assay

This was based on the method described by Medof & Oger (1982). In brief, immune complex reactions were carried out as described above in antigen excess, at equivalence and antibody excess. The complexes were pre-incubated for 1 h and then further incubated for 10 min with an equal volume of washed human red blood cells (RBC) 50% volume:volume at 37°C. In parallel experiments, the same volume of human RBC was added to the reaction mixture from the start of the experiment (nascent complexes). Several time-points in both types of experiment were examined.

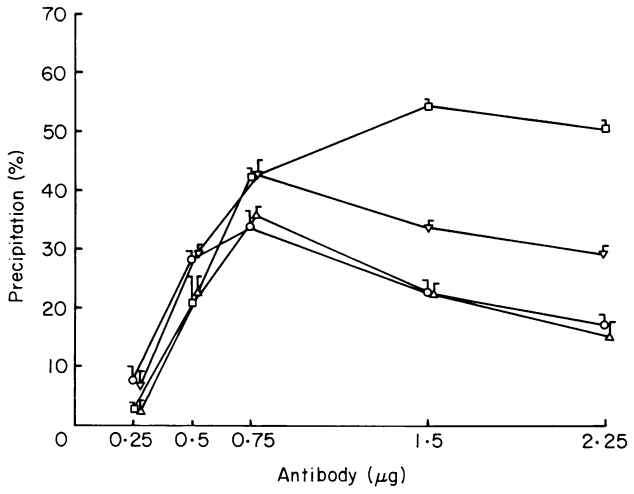

Fig. 1. Inhibition of precipitation of ^{125}I–OVA: anti-OVA by C4 (○); C4A (△); and C4B (▽) when compared with the control (□) in a 1-h incubation assay.

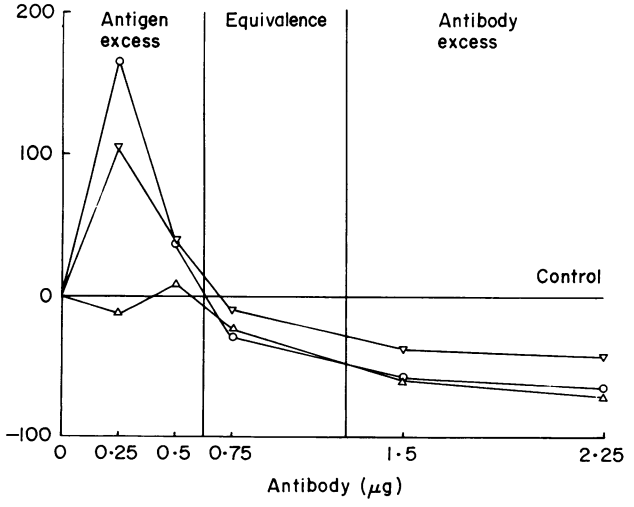

Fig. 2. Change in precipitation of ^{125}I-OVA–anti-OVA immune complexes when incubated with C4 (○); C4A (△); or C4B (▽) at various doses of antibody.

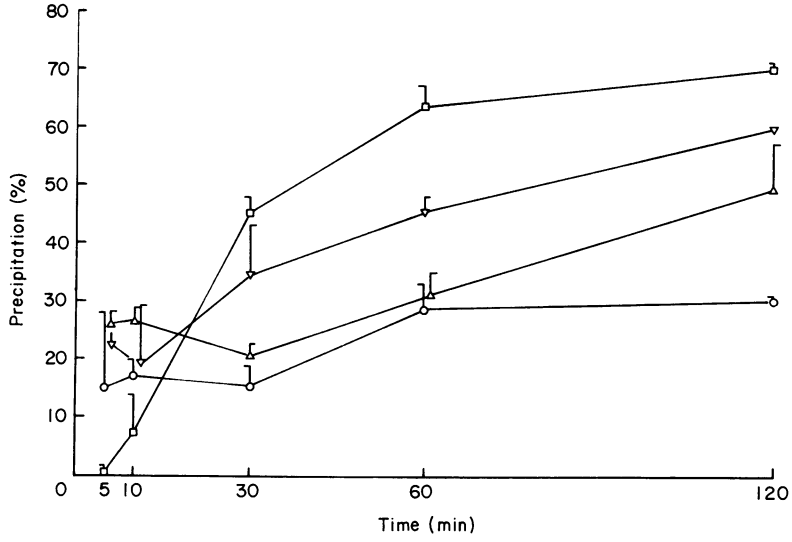

Fig. 3. Effect of time on the inhibition of precipitation of ^{125}I-OVA–anti-OVA complexes formed in antibody excess by C4 (○); C4A (△); and C4B (▽) when compared with the control (□).

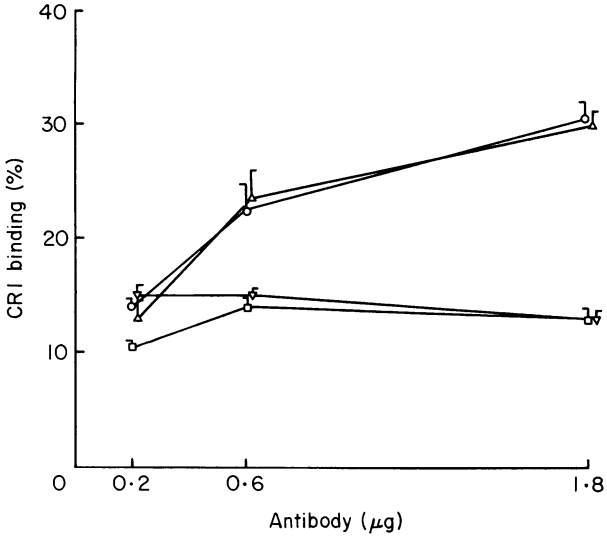

Fig. 4. Binding to CR1 of preformed immune complexes at various ratios of ^{125}I-OVA: anti-OVA for 1 h and incubated for 10 further min with human red blood cells. □, Control; ○, C4; △, C4A; and ▽, C4B.

After incubation with CR1 receptors was complete, duplicate samples of 80 μl of the reaction mixture were layered on to 200 μl of a 4:1 mixture of dibutylphthalate and dinonylphthalate (BDH Chemicals, Poole, UK) in 400 μl polypropylene microfuge tubes. The tubes were centrifuged for 5 min at 8000 *g* (Microhaematocrit Centrifuge, Hawkesly, UK), frozen in dry ice and the tube tip, containing the pellet (P) removed from the supernatant (S) by cutting the tube with wire strippers. Both segments were counted in the gammacounter and % CR1 binding calculated by the formula: $(P/P+S) \times 100$.

RESULTS

Inhibition of immune complex precipitation

The combined results of three separate experiments, each performed in duplicate, are shown in Fig. 1. As can be seen, C4A produces greater inhibition of immunoprecipitation than C4B and this effect although evident at antigen:antibody equivalence is most pronounced in antibody excess. Indeed, C4B too shows some inhibitory effect in antibody excess when compared to the control. Whole C4, as expected, produces an inhibitory effect similar to that of C4A.

There is a suggestion from the data in these experiments that C4B (and whole C4) may enhance immune complex precipitation in antigen excess. When the percent change in immune complex precipitation is plotted against the amount of antibody, this enhancement by C4B becomes more evident in antigen excess. As one would predict the curve for C4 containing both isotypes follows C4B in antigen excess and C4A at equivalence and antibody excess (Fig. 2).

A time course experiment in antibody excess (2·25 μg) clearly shows that the difference between C4A and C4B is not just due to kinetic differences between the two, at least over a range of 120 min (Fig. 3). Similar results were seen at equivalence (data not shown).

Enhancement of CR1 binding

Preformed immune complexes. The relative efficiency of different isotypes of C4 in mediating binding of preformed immune complexes is shown in Fig. 4. Thus both at equivalence and in antibody excess C4A is markedly more effective than C4B. The latter in fact differs little from the control. Whole C4 follows closely the curve of C4A. In these experiments the preformed complexes were allowed to adhere to CR1 for 10 min. In Fig. 5 an experiment done at equivalence (0·6 μg) the time period for adherence has been varied from 5 to 120 min with the difference between C4A and C4B being evident at all points.

Nascent complexes. When immune complexes were allowed to form in the presence of washed human RBC, their binding to CR1 is clearly more efficient in the presence of C4A than C4B (Fig. 6). Indeed, this effect can even be seen to a minor degree in antigen excess and increases through equivalence to antibody excess. A time course experiment in antibody excess (1·8 μg)

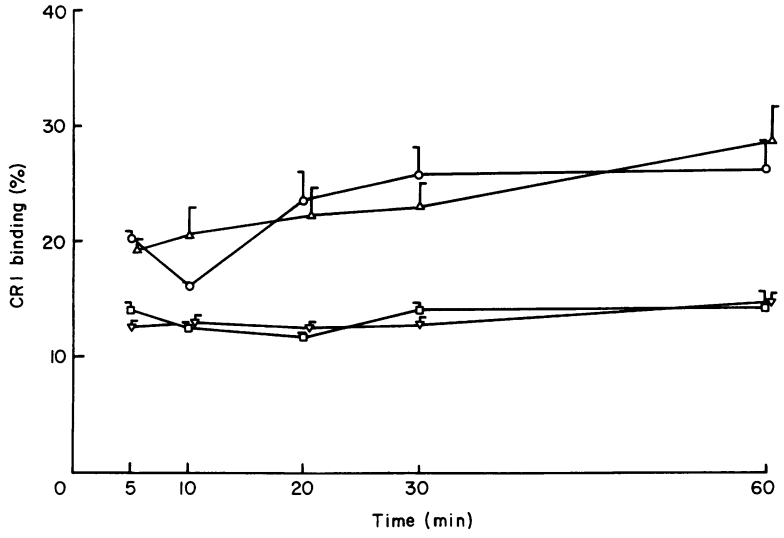

Fig. 5. CR1 binding of preformed ^{125}I-OVA–anti-OVA complexes formed at equivalence in the presence of C4 (○); C4A (△); C4B (▽); or control (□) and incubated for varying times with human red blood cells.

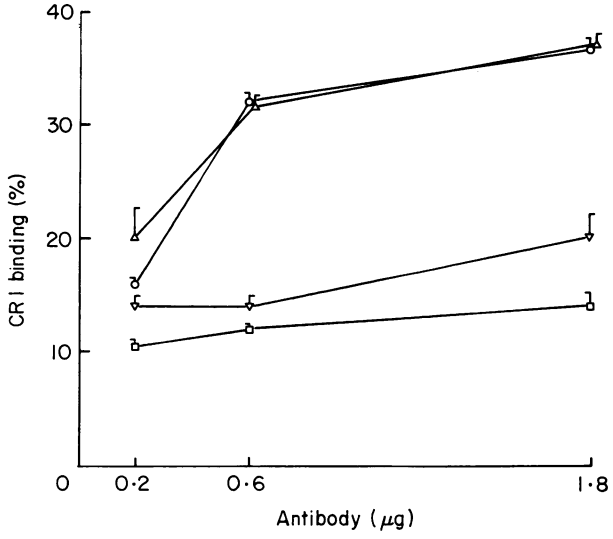

Fig. 6. Binding to CR1 of immune complexes formed at various ratios of ^{125}I-OVA: anti-OVA in the presence of human red blood cells for 1 h. □, Control; ○, C4; △, C4A; and ▽, C4B.

showed some effect of C4B above background at 60 and 120 min, but the difference between the two isotypes was still marked (Fig. 7).

DISCUSSION

The results of the experiments reported here confirm and extend previous observations that C4A is more effective than C4B in inhibiting the precipitation of immune complexes (Schifferli, Bartolotti & Peters, 1986; Paul *et al.*, 1988). As has been demonstrated previously, this action was more apparent with complexes formed in antibody excess (Schifferli *et al.*, 1980). This difference is almost certainly related directly to the greater binding of C4A to amino groups and therefore to proteins (especially the immunoglobulins) in immune complexes (Law *et al.*, 1984; Kishore *et al.*, 1988). The observation that C4B enhances immunoprecipitation in antigen excess is novel, but has not been examined in detail. However, it does serve to emphasize that the *in vivo* pathophysiologic role of C4A and C4B may be very complex, dependent upon antigen:antibody ratios as well as the mol. wt of antigen (Whaley, 1986) and isotype of antibody.

The degree of change in immunoprecipitation when comparing C4A and C4B was far exceeded by the differences between the two C4 isotypes in relation to CR1 binding. Thus, although under the experimental conditions described here C4B still demonstrated some inhibition of immune complex precipitation this was not the case with regard to binding to erythrocyte CR1 where C4B had almost no effect above the control. A brief report which examined only preformed complexes has previously demonstrated the greater efficiency of C4A in this binding (Schifferli, Hauptmann & Paccaud, 1987), although in that study the differences between C4A and C4B were not so marked. The differences reported here are apparent whether preformed or nascent immune complexes are used, although *in vivo* the latter experiments are more likely to be relevant.

The potential *in vivo* role of these findings requires further consideration. While it is clear that C3b is the major ligand for CR1, C4b also demonstrates binding to this receptor *in vitro* (Walport & Lachmann, 1988). The observation that the development of a SLE-like illnesses is more common with C4 deficiency than C2 deficiency (Lachmann & Walport, 1987) argues that C4 plays a role in the handling of immune complexes additional to its prerequisite contribution to generation of the classical pathway C3 convertase. Thus the experimental findings in this study may well have *in vivo* correlates.

Indeed, given the much greater difference between C4A and C4B in CR1 binding assays, especially with nascent complexes when compared with inhibition of immunoprecipitation, it is likely that the differences with regard to CR1 binding are the most important *in vivo* differences between the two isotypes. With a relative deficiency of C4A one may predict that larger complexes would be formed. Such complexes would normally

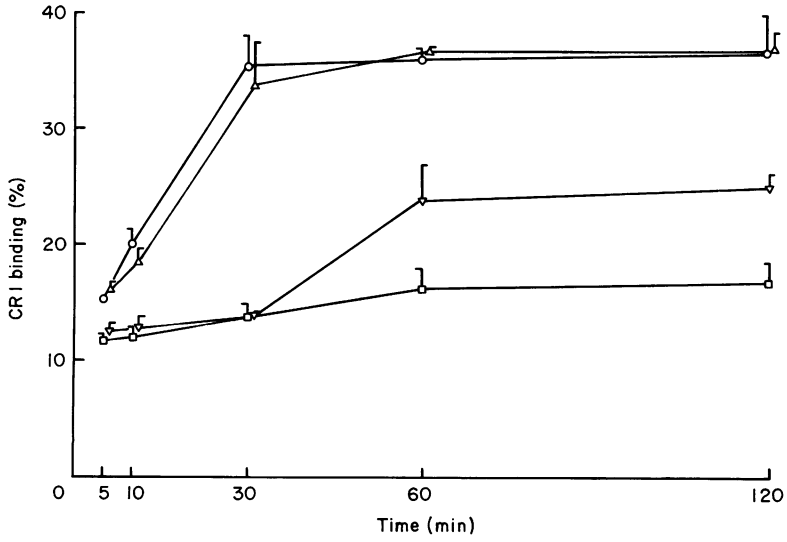

Fig. 7. The effect of time on CR1 binding of ^{125}I-OVA–anti-OVA complexes formed in equivalence in the presence of human red blood cells and C4 (○); C4A (△); C4B (▽); or control (□).

bind more readily to CR1 (Whaley, 1986; Paccaud *et al.*, 1987), but when C4A is limiting, this second protective mechanism may prove ineffective.

The implications of these findings are clear. Previous work done *inter alia* in association with one of us has shown that C4A null genes are the most important MHC encoded genetic risk factors for SLE (Fielder *et al.*, 1983; Dunckley *et al.*, 1987). The experiments reported here provide a pathophysiologic basis for this observation. Essentially, absent or reduced C4A function would encourage the formation and reduce the clearance of immune complexes and thus, as Lachmann & Walport (1987) have previously suggested, SLE may well be an autoimmune disease related to defective effect of function.

The study does, however, raise a number of questions. The role of different immunoglobulin isotypes and antigens remains the subject of on-going experiments. As we have shown, C4A null heterozygotes are also associated with SLE and it has yet to be formally proved that the relatively reduced C4A levels in these individuals leave them sufficiently unprotected to allow the pathological mechanisms outlined here to play a role in disease development.

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REFERENCES

AWDEH, Z.L. & ALPER, C.A. (1980) Inherited structural polymorphism of the fourth component of human complement. *Proc. natl. Acad. Sci. USA*, **77**, 3576.

BOLTON, A.E. & HUNTER, W.M. (1973) The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **133**, 529.

DUNCKLEY, H., GATENBY, P.A., HAWKINS, B., NAITO, S. & SERJEANTSON, S.W. (1987) Deficiency of C4A is a genetic determinant of systemic lupus erythematosus in three ethnic groups. *J. Immunogenet. (Oxf.)* **14**, 209.

FIELDER, A.H.L., WALPORT, M.J., BATCHELOR, J.R., RYNES, R.I., BLACK, C.M., DODI, A.I. & HUGHES, G.R.V. (1983) Family study of the major histocompatibility complex in patients with systemic lupus erythematosus: importance of null alleles of C4A and C4B in determining disease susceptibility. *Br. med. J.* **286**, 425.

GIGLI, I., PORTER, R.R. & SIM, R.B. (1976) The unactivated form of the first component of human complement, C1. *Biochem. J.* **157**, 541.

HARRISON, R.A. & LACHMANN, P.S. (1986) Complement technology. In *Handbook of Experimental Immunology* Vol. I *Immunochemistry* (ed. by D. M. Weir) p. 29.1, Blackwell Scientific Publications, Oxford.

ISENMAN, D.E. & YOUNG, J.R. (1984) The molecular basis for the difference in immune hemolysis activity of Chido and Rodgers isotypes of human complement component C4. *J. Immunol.* **132**, 3019.

KISHORE, N., SHAH, D., SKANES, V.M. & LEVINE, R.P. (1988) The fluid-phase binding of human C4 and its genetic variants, C4A3 and C4B1 to immunoglobulins. *Mol. Immunol.* **25**, 811.

LACHMANN, P.J. (1987) Complement—friend or foe. Heberden Oration 1986. *Br. J. Rheumatol.* **26**, 409.

LACHMANN, P.J. & WALPORT, M.J. (1987) Deficiency of the effector mechanisms of the immune response and autoimmunity. In *Autoimmunity and Autoimmune Disease; Ciba Foundation Symposium 129* (ed. by D. Evered & J. Whelan) p. 149. Churchill-Livingstone.

LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680.

LAW, S.A., DOODS, A.W. & PORTER, R.R. (1984) A comparison of the properties of two classes, C4A and C4B, of the human complement component C4. *EMBO J.* **3**, 1819.

LAW, S.A., MINICH, R.M. & LEVINE, R.P. (1981) Binding reaction between the third human complement protein and small molecules. *Biochemistry*, **20**, 7457.

MEDOF, M.E. & OGER, J.J.-F. (1982) Competition by immune complexes for red cells in human blood. *J. Lab. clin. Immunol.* **7**, 7.

PACCAUD, J.-P., STEIGER, G., SJOHOLM, A.D., SPAETH, P.J. & SCHIFFERLI, J.A. (1987) Tetanus toxoid-anti-tetanus toxoid complexes: a potential model to study the complement transport system for immune complex in humans. *Clin. exp. Immunol.* **69**, 468.

PAUL, L., SKANES, V.M., MAYDEN, J. & LEVINE, R.P. (1988) C4-

mediated inhibition of immune precipitation and differences in inhibitory action of genetic variants, C4A3 and C4B1. *Complement*, **5**, 110.

ROOS, M.H., MOLLENHAUER, E., DEMANT, P. & RITTNER, C. (1982) A molecular basis for the two locus model of human complement component C4. *Nature*, **298**, 584.

SCHIFFERLI, J.A., BARTOLOTTI, S.R. & PETERS, D.K. (1980) Inhibition of immune precipitation by complement. *Clin. exp. Immunol.* **42**, 387.

SCHIFFERLI, J.A., NG, Y.C. & PETERS, D.K. (1986) The role of complement and its receptor in the elimination of immune complexes. *N. Engl. J. Med.* **21**, 488.

SCHIFFERLI, J.A., HAUPTMANN, G. & PACCAUD, J.-P. (1987) Complement-mediated adherence of immune complexes to human erythrocytes. Difference in the requirements for C4A and C4B. *FEBS Lett.* **213**, 415.

SCHIFFERLI, J.A., STEIGER, G., PACCAUD, J.-P. & SJOHOLM, A.G. (1986) Difference in the biological properties of the two forms of the fourth component of human complement (C4). *Clin. exp. Immunol.* **63**, 473.

WALPORT, M.J. & LACHMANN, P.J. (1988) Erythrocyte complement receptor type I, immune complexes and the rheumatic diseases. *Arthritis Rheum.* **31**, 153.

WEBB, J. & WHALEY, K. (1986) Complement and immune complex diseases. *Aust. NZ J. Med.* **16**, 268.

WHALEY, K. (1986) Complement and immune complex diseases. In *Complement in Health and Disease* (ed. by K. Whaley) MTP Press, Lancaster.