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Antigenic modulation of lymphocytic surface immunoglobulin yielding resistance to complement-mediated lysis

I. CHARACTERIZATION WITH SYNGENEIC AND XENOGENEIC COMPLEMENTS

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Summary. Following previous authors, the term antigenic modulation is used to describe the induction, by antibody, of resistance to lysis by antibody plus complement. A report is given of the rapid antigenic modulation *in vitro* of surface immunoglobulin (Ig) on guinea-pig L_2C leukaemic lymphocytes: incubation of the cells for 2 min or longer at 37° with anti-Ig diminished or removed completely the lysis occurring during subsequent incubation with anti-Ig plus complement. The modulation was effective for both xenogeneic (rabbit) and syngeneic (guinea-pig strain 2) complements, but more rapid for the latter. It appeared simply to require the action of antibody on a metabolically active cell: no requirement could be demonstrated for any serum component other than antibody, and there was a need to raise the temperature to 37° after attachment of the antibody. There was molecular specificity inasmuch as modulation with anti-Ig failed to confer any resistance to lysis by another antibody (anti-Ia) plus complement.

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INTRODUCTION

Neoplastic cells subjected to environmental pressures sometimes show diminished expression of certain surface antigens. Examples include losses of viral, foetal and histocompatibility antigens, and probably reflect a variety of mechanisms (Ioachim, Keller, Dorsett & Pearse, 1974; Ortaldo, Ting & Herberman, 1974; Cikes, 1975; Doig & Chesebro, 1978).

The term 'antigenic modulation' was applied by Boyse and colleagues (Boyse, Old & Luell, 1973; Boyse & Old, 1969) to an apparent antigenic loss induced by exposure to antibody, reversible upon removal of the antibody, and probably facilitating cellular survival *in vivo*. Murine leukaemias positive for the thymus-leukaemia (TL) antigen could be grown in hosts which had been immunized with TL to the extent of exhibiting specific cytotoxic antibody. Cells recovered from these animals were TL-negative as judged by tests for cytotoxicity in the presence of anti-TL and complement, but regained positivity if grown in unimmunized hosts. Similar modulation was achievable *in vitro* (Old, Stockert, Boyse & Kim, 1968): cells incubated at 37° with anti-TL alone were not lysed upon subsequent exposure to anti-TL plus complement.

Later work (Stackpole, Jacobson & Lardis, 1974; Esmon & Little, 1976) showed that the antigenic modulation of TL was not accompanied by complete

clearing of antigen and modulating antibody from the cell surface. Instead antigen-antibody complexes persisted in a form or distribution which failed to invoke lysis by homologous (murine) or guinea-pig complement, although susceptibility to rabbit complement remained (Stackpole, Jacobson & Lardis, 1974). Simple redistribution of antigen due to linkage by bivalent antibody does not seem a sufficient explanation, as monovalent Fab fragments of antibody have achieved modulation (Stackpole, Jacobson & Lardis, 1974; Lamm, Boyse, Old, Lisowska-Bernstein & Stockert, 1968) and there is some evidence of complement itself being implicated in the modulating process (Stackpole, Jacobson & Galuska, 1978; Stackpole, 1979). Despite these problems about the underlying mechanism, the term 'antigenic modulation' remains a convenient one for describing an important phenomenon—antibody-induced resistance to lysis by antibody plus complement—and it will be used in this sense in the present paper.

B-lymphocytic leukaemias in man and guinea-pig have been studied with particular regard to exploiting idiotypic determinants on cell surface immunoglobulin (Ig) as tumour-specific therapeutic targets (Hough, Eady, Hamblin, Stevenson & Stevenson, 1976; Stevenson, Elliott & Stevenson, 1977). Antigenic modulation in such a system would clearly be of practical importance. Apparent antigenic modulation for surface Ig on mouse myeloma cells has been reported by Takahashi (1971). The present paper reports detailed observations on the antigenic modulation, *in vitro*, of surface Ig on guinea-pig L_2C leukaemic lymphocytes. Our findings suggest that modulation in this system relies simply on interaction of the cell with antibody at 37° with no requirement for complement or any other non-antibody serum component.

MATERIALS AND METHODS

Cells

The LE line of L_2C leukaemic B lymphocytes (Nadel, 1977) was obtained in 1972 from Drs I. Green and E. Shevach of the National Institutes of Health and has since been maintained in this laboratory by continuous passage in the syngeneic host, strain 2 guinea-pigs. Blood from animals near death from the leukaemia was drawn by cardiac puncture into 0·2 volume 120 mM sodium citrate, pH 7·4. The cells were separated and washed as described previously (Stevenson, Eady, Hough, Jurd & Stevenson, 1975). Such preparations contain L_2C cells of >95% viability as judged by exclusion of trypan blue, and <2% contaminating cells. The cells possess surface monomeric IgM, of light chain class λ, at a density of approximately 50,000 per cell (Hough, Chapple, Stevenson & Stevenson, 1978).

Antibodies

Two antibodies directed against L_2C surface IgM were used: anti-Ig, specific for the constant region of the λ chain; and anti-Id, specific for the idiotypic determinants. Anti-Ig was in the form of purified antibody and was obtained from sheep anti-guinea-pig Fab γ serum (G. T. Stevenson *et al.*, 1975). The serum was passed over an immunosorbent consisting of L_2C λ chains (F. K. Stevenson, Mole, Raymont & Stevenson, 1975) linked to Sepharose 4B-CL (Pharmacia) (Porath, Axen & Ernback, 1967); the bound antibody was eluted with 0·5 M NH_3 and dialysed immediately into cold neutral buffer. Anti-Id serum was prepared in sheep as described previously (Stevenson *et al.*, 1977); antibodies directed to Ig constant regions were removed on an immunosorbent bearing guinea-pig globulins, and anti-Id-containing IgG was then prepared from the serum by sequential precipitation with 1·6 M $(NH_4)_2SO_4$ passage through DEAE-cellulose (Whatman DE52) equilibrated with 0·06 M phosphate, pH 7·3, and gel filtration on Sephadex G-150.

Anti-Ia serum, directed towards the histocompatibility antigens Ia(2,4), was raised in strain 13 guinea-pigs by immunization with normal strain 2 splenic and nodal lymphocytes (Schwartz, Kask, Paul & Shevach, 1976). Antiserum to the guinea-pig complement component C3 was not available at the time of our study. Instead we used rabbit antiserum to human C3 (batch 41 RP-8, Organon Teknika, Huntingdon, Cambs., England), which was found to cross-react with the guinea-pig protein.

Complement-mediated cytotoxicity

L_2C cells were labelled with ^{51}Cr for cytotoxicity assays. 0·1 μCi $Na_2{}^{51}CrO_4$ (4–7·7 ng Cr/ml; Radiochemical Centre, Amersham) was added to 9×10^7 cells in 3 ml Eagle's minimal essential medium (MEM), and the mixture was incubated for 30 min at 37°. After four washes at 0° the cells were made up to 1×10^6/ml in cold MEM. At this stage viability as judged by exclusion of trypan blue was >95%.

0·1 ml suspension containing 1×10^5 labelled cells was added to 0·1 ml of PBS containing dilutions of IgG from antibody preparations or from a normal

serum control in 2·5 ml perspex tubes. The tubes were incubated at 0° for 15 min to allow maximal binding of antibody; further incubation depended on the experiment concerned. 0·8 ml of complement, provided by a 1:4 dilution of either fresh syngeneic (strain 2 guinea-pig) serum or fresh rabbit serum in MEM, was added at 0° and after mixing the tubes were transferred immediately to a 37° water bath. After 30 min the tubes were returned to 0° and following centrifugation the supernatant was assayed for released ^{51}Cr in a gamma scintillation counter (Wallac).

Percentage cytotoxicity in the assays was taken as:

$$\frac{\text{counts released with antibody} - \text{counts released with normal IgG}}{\text{counts released with detergent} - \text{counts released with normal IgG}} \times 100$$

where detergent lysis was carried out in 1% Nonidet P40. All points on graphs are means of duplicate determinations which were always within 10% and usually within 5% of each other.

Modulation

In experiments where modulation was sought, tubes containing ^{51}Cr-labelled cells and IgG from antibody preparations or from normal serum controls were incubated initially at 0° for 15 min as described for cytotoxicity assays. Tubes were then transferred to a 37° waterbath for varying times in the absence of serum. After returning to 0° for a further 15 min to allow standardization of the assay complement was added to the tubes and cytotoxicity at 37° assessed as previously described. Percentage modulation was taken as:

$$\frac{\%C \text{ following pre-incubation at } 0° - \%C \text{ following pre-incubation at } 37°}{\%C \text{ following pre-incubation at } 0°} \times 100$$

where %C is percentage cytotoxicity.

RESULTS

Lysis by anti-Ig plus guinea-pig or rabbit complement

The ability of anti-Ig to induce the lysis of L_2C cells by guinea-pig or rabbit complement is shown in Fig. 1. Rabbit complement was the more effective in requiring less antibody to initiate lysis, and in achieving a higher plateau.

Staining by trypan blue indicated that at the plateau

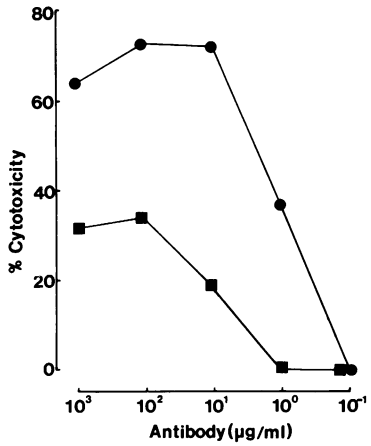

Figure 1. Complement-mediated lysis of L_2C cells, invoked by anti-Ig. ●, Rabbit complement; ■, guinea-pig complement.

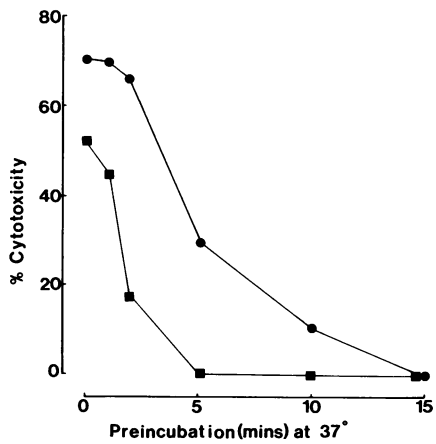

Figure 2. Time course of modulation by anti-Ig. Suspensions of cells at 5×10^5/ml were made up in anti-Ig, 100 μg/ml, at 0° and then transferred to a water bath at 37° for the times indicated. The suspensions were then chilled, complement added, and ^{51}Cr-release measured after a further incubation at 37° for 30 min. ●, Rabbit complement; ■, guinea-pig complement.

of ^{51}Cr-release by guinea-pig complement only about one third of the cells were lysed, so that partial release of ^{51}Cr did not reflect uniform partial damage to the cells. Neither increasing the time of incubation at 37° nor decreasing the cell input raised the extent of lysis. Fractionations of the L_2C population by volume (Miller & Phillips, 1969) or density (Pertoft, Back & Lindahl-Kiessling, 1968) failed to yield subpopulations differing significantly in susceptibility to lysis. Variability in the extent of lysis obtainable with

guinea-pig complement was, however, noted between experiments, without obvious cause; the range for experiments quoted in this and the following paper was 19–53%.

Characteristics of modulation

In Fig. 2 the effects are shown of pre-incubations at 37° with anti-Ig at 100 μg/ml for varying times before adding complement. After only 2 min, the susceptibility to guinea-pig complement had diminished appreciably and by 5 min there was complete resistance. Resistance to rabbit complement required longer times but after 15 min complete resistance was again attained.

Dependence on antibody concentration is shown in Figs 3 and 4. In the experiment depicted in Fig. 3, complement was added after pre-incubation with antibody at 37° for 15 min, and degrees of lysis are compared with those obtained after otherwise identical pre-incubations at 0°. For rabbit complement partial modulation was apparent at about 2 μg/ml and had proceeded to complete modulation by about 10 μg/ml. For guinea-pig complement all concentrations of antibody capable of inducing lysis were able to modulate completely when allowed a pre-incubation with the cells at 37°. In an attempt to establish whether antibody concentrations below those effective for lysis were able to modulate for guinea-pig complement, we pre-incubated with varying concentrations at 37° for 15 min, chilled, and at 0° added further antibody to increase the concentration by 100 μg/ml; this gave in all tubes an antibody concentration capable of inducing maximum lysis. Complement was then added and the temperature raised to 37°. The results in Fig. 4 show that under such conditions modulation was apparent after preincubation at 1 μg/ml, and was complete after concentrations above 6 μg/ml.

In all the above experiments modulation was achieved by pre-incubation with anti-Ig directed towards Cλ determinants on the surface molecules. Anti-Id, directed towards idiotypic determinants on V regions of the surface Ig (Stevenson *et al.*, 1977), yielded modulation under the same conditions and with apparently equal efficiency for syngeneic complement.

Modulation of the surface Ig did not confer non-specific cellular resistance to complement-mediated lysis. Figure 5 compares the susceptibility of the cells to lysis by anti-Ia plus guinea-pig complement after pre-incubations with either anti-Id or normal sheep IgG: no difference was detected, and both curves are in fact indistinguishable from that given by anti-Ia plus complement without a pre-incubation. (Modulation was carried out with anti-Id rather than anti-Ig to avoid the complication of the latter reacting with Cλ determinants on the guinea-pig anti-Ia.)

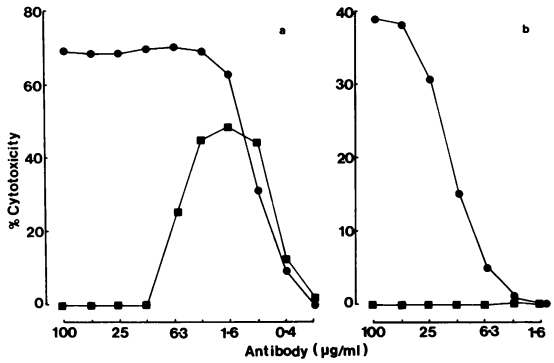

Figure 3. Modulation and complement-mediated lysis at varying concentrations of anti-Ig. (a) Cells at 5×10^5/ml were pre-incubated for 15 min with the indicated concentrations of anti-Ig. They were then chilled, rabbit complement added, and the temperature raised to 37° to allow lysis to proceed • control, pre-incubated at 0° ■ test, pre-incubated at 37°. (b) The same experiment, but using guinea-pig complement.

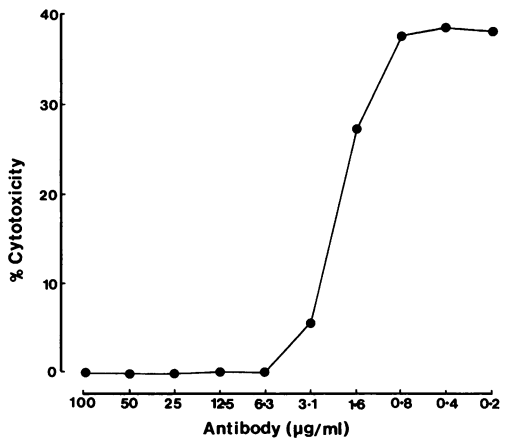

Figure 4. Modulation at varying concentrations of anti-Ig, followed by complement-mediated lysis in the presence of surplus antibody. After preincubation for 15 min at 37° with the indicated concentrations of anti-Ig, the cell suspensions were chilled and further antibody was added to increase the concentration by 100 μg/ml in all tubes. Guinea-pig complement was then added in standard amount (4 volumes of 25% fresh serum) and the temperature was raised to 37° for 30 min to allow lysis to proceed.

Role of non-Ig serum factors in modulation

Although modulation in our experiments was carried

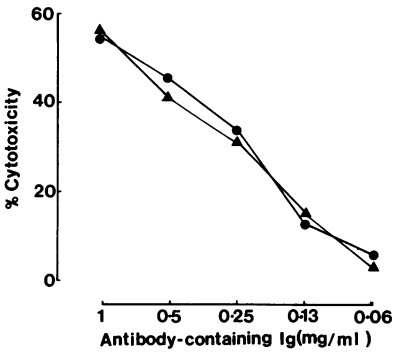

Figure 5. Effect of modulating surface Ig on subsequent lysis mediated by syngeneic complement and anti-Ia. Cells at 5×10^5/ml were pre-incubated at 37° for 15 min with anti-Id (•) or normal sheep IgG (■) at 1 mg/ml, chilled, and then incubated with the indicated concentrations of anti-Ia at 0° for 15 min. Finally guinea-pig complement was added and the temperature raised to 37° to allow lysis to proceed.

Table 1. Role of non-Ig serum factors in modulation

Complement used in cytotoxicity assay	Cells	% Modulation following pre-incubation with anti-Ig* for (min): 2	5	15
Guinea-pig	Citrate-collected	57	100	100
	EDTA-collected	60	100	100
	EDTA-collected/serum†	60	100	100
Rabbit	Citrate-collected	14	53	100
	EDTA-collected	11	57	100
	EDTA-collected/serum	12	53	100

* Present at 100 μg/ml.

† Cells prepared from blood collected into EDTA and subsequently incubated with 50% fresh guinea-pig serum for 30 min at 37°.

out by incubating washed lymphocytes with purified antibody in the absence of serum, a role for non-Ig serum factors remains a possibility in the light of reports that complement components can coat lymphocytes prepared from blood, in amounts varying with the nature and concentration of anticoagulant used (Wilson, Lachmann & Coombs, 1979; Gutierrez, Vega & Kreisler, 1979). We found that L_2C cells from blood which had been collected into sodium citrate, final concentration 20 mM, had variable amounts of C3 on their surfaces as judged by two tests: rabbit anti-human C3 (which cross-reacts with guinea-pig C3) reacted with the cells as shown by indirect immunofluorescence; and rosetting with red cells coated with antibody and complement, seen normally with L_2C cells due to their C3b receptor (Shevach, Ellman, Davie & Green, 1972), was inhibited. However, cells from blood collected into an equal volume of 10 mM Na_2EDTA in PBS as anticoagulant were negative by both these tests. Positivity was conferred by a subsequent incubation of the cells with fresh guinea-pig serum for 30 min at 37°.

Modulation as defined in our system was exactly the same for citrate-collected cells, EDTA-collected cells, and EDTA-collected cells coated with C3 by incubation with serum (Table 1).

DISCUSSION

Throughout the experiments syngeneic (guinea-pig strain 2) complement yielded lower cytotoxicities than xenogeneic (rabbit) complement, and was more susceptible to antigenic modulation. Although widely employed because of their efficacy, xenogeneic sera as sources of complement introduce uncertainty because they might also contain natural antibodies against the target cells (Ferrone, 1977; Gossett, Naeim, Zeller & Johns, 1978). The sera are usually at a dilution which gives no cytotoxicity in control preparations (containing complement but no known antibody), but this does not guarantee absence of synergism between the natural and added antibodies in test preparations. We regard the results obtained with guinea-pig complement as more reliable indications of what is invoked by anti-Ig as the sole antibody, and as clearly more relevant to the killing of these cells *in vivo*.

Surface Ig appears particularly susceptible to modulation. In our system, complete modulation required 5 and 15 min at 37° for syngeneic and xenogeneic complements, respectively, compared with 1 and 2 h for xenogeneic complement in the TL system (Old *et al.*, 1968; Stackpole *et al.*, 1974). This could be related to the unusually rapid redistribution induced in surface Ig by antibody (Schreiner & Unanue, 1976; Braun, Fujiwara, Pollard & Unanue, 1978), a possibility we examine in the following paper (Gordon & Stevenson, 1980).

We have noted that purified antibody in the absence of other serum components effects rapid modulation, and that the irregular presence of C3 on washed target cells prior to exposure to antibody is irrelevant. These findings contrast with evidence from Stackpole *et al.* (1978) that mouse C3 is required for modulation of TL

antigen, and with a report Ioachim & Sabbath (1979) that a heat-labile serum factor promotes the antigenic modulation of Gross murine leukaemia virus antigens on cell surfaces. It must therefore remain a possibility that non-antibody factors can exhibit a helping role with antibodies directed against antigens which are modulated less readily than immunoglobulin.

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