Immunology 1981 **42** 13

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

II. RELATIONSHIP TO REDISTRIBUTION OF THE ANTIGEN

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Accepted for publication 30 July 1980

Summary. Experiments were carried out on guinea-pig L_2C leukaemic lymphocytes to investigate the mechanism of antigenic modulation of their surface immunoglobulin (Ig) defined as the conferring by anti-Ig of resistance to lysis by anti-Ig plus complement. The phenomenon reflects, and is probably a consequence of, redistribution of the Ig molecules by bivalent antibody. Fab fragments of the antibody were completely ineffective. Parallel studies by indirect immunofluorescence of the movement of the surface antigen–antibody complexes revealed that modulation for syngeneic complement was apparent when the complexes were minimally aggregated: capping and extensive endocytosis were not necessary. Modulation for xenogeneic (rabbit) complement required more extensive movement but was still appreciable while complexes persisted on the surface. Sodium azide at 10 mM, which inhibits antibody-induced redistribution of surface molecules, diminished modulation. In experiments omitting pre-incubation with antibody alone, the presence of azide during incubations with anti-Ig plus syngeneic complement increased lysis from a low and variable to a consistently high level; there was no effect on the already high level of lysis occurring with the non-modulating anti-Ia plus syngeneic complement. This effect of azide provides further evidence that antigenic modulation can be a major factor determining a cell's survival when it is confronted simultaneously by antibody and complement.

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0019-2805/81/0100-0013$02.00

INTRODUCTION

In a previous paper (Gordon, Robinson & Stevenson, 1980) the characteristics were noted of antigenic modulation of lymphocytic surface Ig as exemplified by lysis with either syngeneic or xenogeneic complement. The only apparent requirements for the phenomenon were anti-Ig molecules and a metabolically active cell.

Here, the relationship of modulation to redistribution of the surface antigen–antibody complexes is studied. It is shown that redistribution is an essential requirement, although it need only be of minimal degree to confer resistance to syngeneic complement. Evidence is also presented that modulation can confer significant protection against complement lysis when the cell is confronted simultaneously by antibody and complement.

MATERIALS AND METHODS

L_2C leukaemic lymphocytes, antibodies directed against their surface Ig (anti-Ig, specific for the constant region of the λ chain; and anti-Id, specific for the idiotypic determinants), and antibodies directed

against their surface Ia were all prepared as described previously (Gordon *et al.*, 1980). Assays of complement-mediated cytotoxicity and of modulation were also carried out as described previously.

The Fab′γ fragment of anti-Ig was prepared from the purified antibody by peptic digestion followed by reduction and alkylation (Stevenson, Eady, Hough, Jurd & Stevenson, 1975).

Immunofluorescence

The indirect method was used. Viable cells in suspension were allowed to react with antibody or control preparations under varying conditions as described in Results. The cells were then chilled rapidly and washed three times in cold phosphate-buffered saline (PBS) containing 10 mM sodium azide. To the final cell pellet was added fluorescein-conjugated anti-antibody: the anti-antibody was affinity-purified rabbit anti-sheep IgG or anti-guinea-pig Fcγ, in each case absorbed to avoid direct reaction with the L_2C surface IgM. After 15 min at 0°, the cells were washed free of unbound conjugate and resuspended in glycerol-PBS (1:1) for examination with a Zeiss microscope fitted with a mercury vapour lamp and epi-illumination.

RESULTS

Requirement for antibody bivalency

To establish whether monovalent antibody (the Fab′γ fragment of antibody) is capable of modulating the surface Ig, we preincubated the cells with Fab′γ and then exposed to whole antibody in presence of complement. This basic design encounters the problem that non-complement-fixing Fab′γ might block access of whole antibody to antigenic sites during the incubation with complement. We therefore used antibodies directed towards different determinants on the surface Ig for the two stages: for the pre-incubation, Fab′γ from anti-Ig; and for the incubation with complement, whole anti-Id. In Table 1 we see that Fab′γ had no detectable modulating activity, whereas whole anti-Ig modulated quite effectively against subsequent induction of lysis by anti-Id.

Table 1. Modulating activity of monovalent anti-Ig

Modulating antibody	Pre-incubation at 37° (min)	Cytotoxicity in presence of anti-Id + complement* (% specific ^{51}Cr release)
Anti-Ig, Fab′γ fragments†	0	18
	15	15
	30	24
	60	25
Anti-Ig, IgG molecules‡	15	0

* Following pre-incubation with anti-Ig, cells at 5×10^5/ml were chilled and incubated with an equal volume of anti-Id (1 mg/ml) for 15 min at 0°; cytotoxicity was then assessed in the presence of guinea-pig complement as described for Fig. 1.

† Present at 70 μg/ml.

‡ Present at 100 μg/ml.

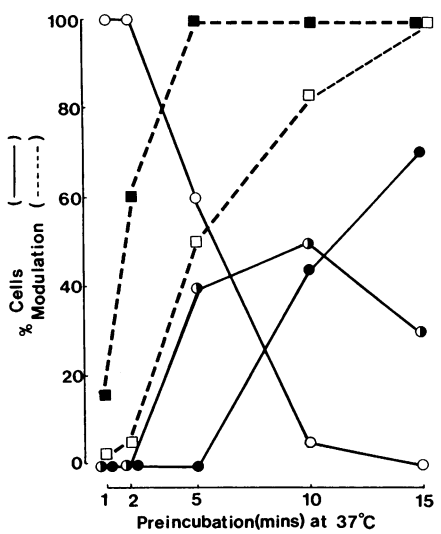

Figure 1. Distribution of anti-Ig on modulated cells. Duplicate lots of cells at 5×10^5/ml were incubated with anti-Ig at 100 mg/ml for the indicated times at 37°; one lot was chilled and stained with fluorescent anti-antibody at 0° while the matching lots were assessed for modulation with ■ guinea-pig complement, or □ rabbit complement as described in the text. ●, Cells negative for stain; ◐, cells exhibiting caps; ○, cells exhibiting uniform circumferential stain.

Distribution of antibody on modulated cells

Duplicate lots of cells were incubated with anti-Ig at 37°, and at varying times one lot was chilled and its distribution of antibody visualized by staining with fluorescent anti-antibody at 0°, while the matching lot was assessed for modulation in the usual way. Keeping antibody and cells at 0° revealed a strong uniform ring of stain on all cells. Between 1 and 2 min at 37° a slight speckling of this stain was observed. With increasing length of incubation at 37° the patching became more

gross and cells began to exhibit a polar staining pattern so that at 5 min, 40% of cells had redistributed the antibody into caps (Fig. 1). After 10 min at 37°, an approximately equal number of cells either exhibited caps or were negative for anti-Ig.

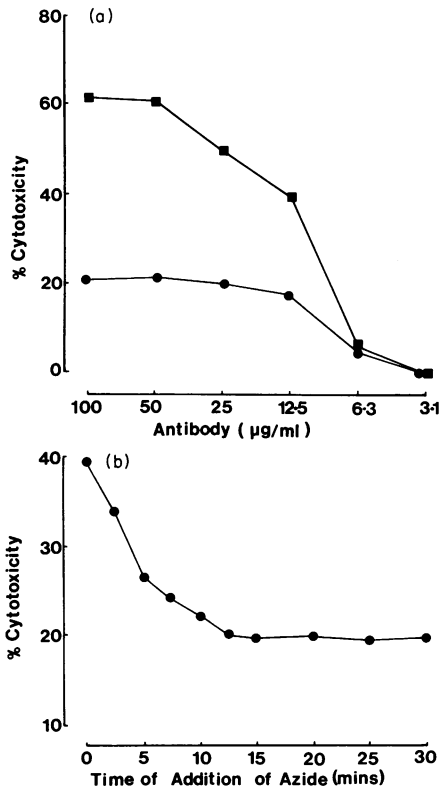

Figure 2. Complement mediated lysis induced by anti-Ig in the presence of azide. (a) Cells at 5×10^5/ml were incubated with the indicated concentrations of anti-Ig for 15 min at 0°. Four volumes of 25% guinea-pig serum were then added either with ■ or without ● sodium azide giving a final concentration of 10 mM, and the temperature was raised to 37° to allow lysis to proceed. (b) Cells at 5×10^5/ml were incubated with anti-Ig, 100 μg/ml, for 15 min at 0°. Four volumes of 25% guinea-pig serum were then added, the temperature was raised to 37°, and sodium azide was added to a final concentration of 10 mM at the times indicated during the 30 min incubation.

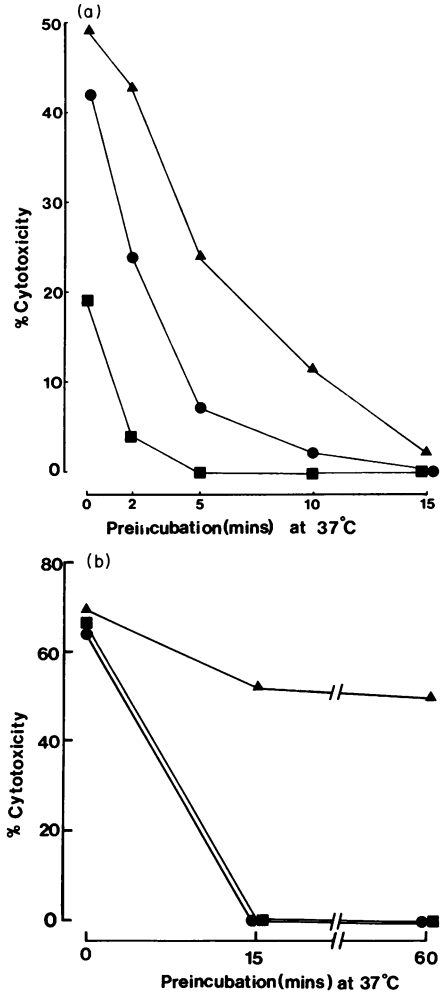

Figure 3. Effect of azide on the rate of antigenic modulation. Cells at 5×10^5/ml were mixed with anti-Ig, 100 μg/ml, at 0°, and then transferred to a 37° water bath for the times indicated. The cells were then chilled, complement added, and the temperature raised to 37° for 30 min to allow lysis to proceed. Sodium azide, 10 mM, was present during the following stages: ▲, both the pre-incubation with antibody and the incubation with antibody plus complement; ●, only the incubation with antibody plus complement; ■, absent at all stages. (a) Guinea-pig complement, (b) rabbit complement.

Loss of susceptibility to lysis with rabbit complement closely paralleled the appearance of caps but was not complete until a majority of cells stained negatively for modulating antibody (Fig. 1). In contrast, modulation determined by cytotoxicity with guinea-pig complement was apparent well before the appearance of caps: even at 2 min, when only a slight speckling of the stain was observed, modulation had proceeded to greater than 50%. Fab'γ from anti-Ig, which we have shown above to be non-modulating, gave a uniform ring of stain around the circumference of the cell regardless of the time of pre-incubation at 37°.

It was of interest to investigate the distribution of an antigen–antibody system which is non-modulating.

Whole Ig from guinea-pig anti-Ia serum was found to give no modulation whatever when incubated with cells at 37° prior to the addition of complement. Staining of the cells with fluorescein-conjugated sheep anti-guinea-pig IgG subsequent to the pre-incubation procedure revealed that the anti-Ia was present on the cells as a uniform ring.

Effects of azide

We investigated the effect of sodium azide on modulation because this agent at 10 mM is known to retard antibody-induced redistribution of surface molecules (Taylor & Duffus, 1971). It was not feasible to have azide present only during pre-incubation with antibody as its removal from culture fluid prior to the addition of complement still leaves a problematical intracellular residue. We therefore investigated first the effects of azide when present during simple incubations with antibody and complement.

Sodium azide at 10 mM enhanced the degree of lysis induced by anti-Ig and guinea-pig complement (Fig. 2a). Not only was the plateau achieved higher, but its level was far more consistent between experiments than in the absence of azide. Azide did not increase the background release of ^{51}Cr observed in the presence of sheep normal IgG, nor did it increase the already high levels of lysis achievable either by anti-Ig plus rabbit complement or by anti-Ia plus guinea-pig complement. In Fig. 2b we demonstrate the effects of adding azide at progressively later times during the incubation: the increment in lysis which can be achieved falls with time and disappears after 15 min, suggesting that azide exerts its effect by inhibiting some reaction which is otherwise complete at 15 min.

The effect of adding azide during a pre-incubation with antibody is demonstrated in Fig. 3. If azide was absent during pre-incubation but present during exposure to antibody plus guinea-pig complement, modulation appeared to follow a normal course but with the extent of lysis set at a higher level throughout the curve. If azide was present during pre-incubation as well as during exposure to complement, modulation was significantly retarded, although still attaining completion by 15 min (Fig. 3a). When the same experiment was carried out using rabbit instead of guinea pig complement modulation was almost ablated (Fig. 3b).

DISCUSSION

Antigenic modulation of surface Ig has required that the antibody be bivalent, and has been diminished by azide. These findings suggest the need for some degree of redistribution of the surface antigen–antibody complexes. The failure of the non-modulating anti-Ia to redistribute its target suggests further that redistribution is a requirement common to any modulating system, but at first sight this is contradicted by reports that Fab fragments of antibody can modulate mouse TL antigens (Stackpole, Jacobson & Lardis, 1974; Lamm, Boyse, Old, Lisowska-Bernstein & Stockert, 1968). However, interpretation of the latter experiments is complicated by the possible blocking of antigenic sites by persistent Fab, and by the possibility that Fab can itself induce redistribution and endocytosis given sufficient time (Esmon & Little, 1976; de Petris & Raff, 1973).

Redistribution of surface Ig by antibody has two stages: simple tethering of the target molecules, and subsequent metabolism-dependent gathering into large patches and caps with accompanying endocytosis (Schreiner & Unanue, 1976). Modulation in our experiments required metabolism, shown by the need to raise the temperature to 37° and by the inhibitory effect of azide. But it is clear from Fig. 1 that modulation for syngeneic complement is well advanced before there is significant capping or endocytosis: so if modulation is linked to redistribution one can conclude that only a minimal degree of metabolism-dependent redistribution is required.

If the connection with redistribution is accepted one can ask whether modulation is a direct consequence of the redistribution, or a reflection of some metabolic change induced by cross-linking the surface Ig. We know already some clear metabolic consequences of cross-linking the surface Ig in L_2C cells: there is a surge in the level of intracellular cyclic AMP peaking within 1 min (Virji & Stevenson, 1979), and a shut-down in delivery of Ig molecules to the plasma membrane (Glennie, Stevenson, Stevenson & Virji, 1979). But one would anticipate that any metabolic change conferring resistance to cytolysis, say by facilitating membrane repair, would be effective against antibody-complement attack on any surface molecule. We saw no evidence of heightened resistance to attack on surface Ia following modulation of surface Ig (Gordon *et al.*, 1980). So we are left with the tentative conclusion that redistribution is directly responsible. Perhaps the new steric relationship of the antigen–antibody complexes is unfavourable for complete activation of complement. Alternatively the membrane attack complexes C5b-9, which are believed to act by insertion into the

lipid bilayer (Mayer, 1972) might have their access to the bilayer impeded when they are formed on impenetrable rafts of antigen–antibody complexes; the proximity of the attack complexes to each other could also promote self-aggregation through those postulated hydrophobic areas which are designed for bonding to membrane lipid.

A cell *in vivo* will confront antibody and complement simultaneously, and its survival will be favoured if modulation can occur suffieiently rapidly to avoid significant complement damage to its membrane. Because of the complexity of the events it is difficult to conduct experiments *in vitro* to assess this situation. However, the effects we have observed with azide—whereby it diminishes modulation in sequential exposures to anti-Ig and complement, and increases killing in a simultaneous exposure to anti-Ig and syngeneic complement—are entirely consistent with modulation occurring with the required rapidity. The variability in lysis observed with anti-Ig and syngeneic complement in the absence of azide could well reflect competition between modulation and the effective activation of lytic complement.

The original observations on mouse TL (Boyse, Old & Luell, 1963; Boyse & Old, 1969) indicated that antigenic modulation is probably an important means whereby vertebrate cells elude killing by antibody and complement. It might often have a useful role, as in lessening the consequences of autoantibody formation. When it prevents the killing of tumour cells by antibody it becomes disadvantageous, and one aim of the therapist must be to elude modulation in its turn. To this end surface Ig, which is well understood and against which purified antibodies are available, could be of great value in elucidating mechanisms.

ACKNOWLEDGMENTS

We are indebted to Valerie Anderson for her expert assistance and wish to thank Daniele Robinson for confirming some of the experiments where the effects of sodium azide on modulation were investigated.

This work was supported by Tenovus of Cardiff, the Wessex Regional Health Authority and the Cancer Research Campaign.

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