*Immunology* 1978 **34** 353

# Mediation of cytotoxic functions by classes and subclasses of sheep antibody reactive with cell surface immunoglobulin idiotypic and constant region determinants

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*Received* 23 *June* 1977; *accepted for publication* 4 *July* 1977

**Summary.** Sheep antibodies, reactive with either the idiotypic or constant region antigenic determinants of the immunoglobulin light chain on guinea-pig $L_2C$ leukaemic cells, were separated into IgM and into the two subclasses of IgG, $IgG_1$ and $IgG_2$. Antibody of both IgG subclasses inhibited the migration of $L_2C$ cells along plastic surfaces; IgM was only weakly inhibitory. Antibody of class IgM and of subclass $IgG_1$ mediated complement cytotoxicity against the $L_2C$ cells whereas only that of subclass $IgG_2$ mediated K-cell cytotoxicity; the effector arms were rabbit complement and sheep peripheral leucocytes, respectively.

## INTRODUCTION

The use of idiotypic determinants on the surface immunoglobulin of neoplastic cells as tumour specific antigens has been discussed previously (Stevenson & Stevenson, 1975). In the animal model of a B lymphocytic neoplasm, the $L_2C$ leukaemia of strain 2 guinea-pigs, we have described the urinary excretion of monoclonal light chain, its purification, and its use in raising antibodies in rabbits against the idiotypic (anti-Id) and constant regions (anti-$\lambda$) of the light chain (Stevenson, Elliott & Stevenson, 1977a). Anti-Id was shown to react specifically with the light chain of the surface IgM of the $L_2C$ cell and was able to mediate cytotoxic mechanisms involving both complement and K cells.

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In order to use such antibody for immunotherapy of the tumour it was desirable to immunize a larger animal, and we chose sheep for this purpose. Sheep have the further advantage that the serum IgG can be separated into two subclasses, $IgG_1$ and $IgG_2$. Evidence for some functional separation in the two subclasses of sheep antibody has been presented recently by Grant, Adams & Miller (1975), working with a murine P-815 mastocytoma and an antibody to whole cells reactive against undefined cell surface antigens. These authors found complement-dependent cytotoxic antibody in the IgM and $IgG_1$ fractions of the hyperimmune sheep serum, while K-cell dependent cytotoxic antibody was found in both $IgG_1$ and $IgG_2$ fractions.

We have applied a similar separatory technique to our sheep antiserum and have found complement-activating anti-Id and anti-$\lambda$ in the IgM and $IgG_1$ fractions. Some complement-activating anti-$\lambda$ was found initially in the $IgG_2$ subclass but could be removed by the use of a subclass specific antibody linked to Sepharose-4B (Seph 4B—rabbit anti-sheep $IgG_1$). In contrast to the findings of Grant *et al.* (1975), K-cell-activating anti-Id and anti-$\lambda$ were

restricted to the $IgG_2$ subclass. However, ability to inhibit migration of tumour cells along plastic surfaces was found in both subclasses of IgG, and weakly in IgM.

## MATERIALS AND METHODS

*Preparation of antiserum*

The $\lambda$ light chain was prepared from the urine of strain 2 guinea-pigs in the terminal phase of the $L_2C$ leukaemia by separation on Sephadex G-100 followed by ion exchange chromatography, as described previously (Stevenson, Mole, Raymont & Stevenson, 1975).

Two sheep (Clun crossbreeds) were each immunized with $\lambda$ chain (1 mg) in Freund's complete adjuvant (CFA) (Difco) distributed among 4 intramuscular sites. After 5 weeks a booster injection of the same amount of $\lambda$ chain in CFA was given in the back. The animals were bled at 7, 14 and 21 days after the boost. Sera were prepared after removal of clots by centrifugation and complement was inactivated by heating at 56° for 30 min.

The antisera were very similar as assessed on Ouchterlony immunodiffusion plates using the $\lambda$ chain as antigen, but only one serum was processed.

*Preparation of immunoglobulin fractions*

The method of fractionation of the sheep antiserum into IgM, $IgG_1$ and $IgG_2$ was based on that of Grant *et al.* (1975), which in turn was a modification of the original method of Feinstein & Hobart (1969). Serum (20 ml) was dialyzed into 0·1 M Tris-HCl, pH 8·0, containing 0·2 M NaCl and passed through a column of Ultrogel AcA-34 (LKB, 5 × 70 cm) equilibrated with the same buffer. This procedure separated the 19S and 7S Igs. The 7S fraction was then concentrated to 20 ml by ultrafiltration through an Amicon PM-10 membrane and passed through a column of Sephadex G-150 (5 × 75 cm) equilibrated with 0·02 M Tris-HCl, pH 8·0, containing 0·1 M NaCl and 0·02% sodium azide (Tris-NaCl-azide). This procedure removed traces of material of lower molecular weight.

The 7S IgG fraction (approximately 450 mg) was then dialyzed into 0·01 M sodium phosphate buffer, pH 8·0, and passed through a column of DEAE-cellulose (200 ml) equilibrated with the same buffer. The $IgG_2$ came through the column unretarded, and the $IgG_1$ was eluted by a phosphate gradient up to 0·4 M, pH 8·0.

*Preparation of antibody against the V$\lambda$ region (anti-Id)*

The separation of the IgM, $IgG_1$ and $IgG_2$ preparations into antibody reactive with the $V_\lambda$ region (anti-Id) and antibody reactive with the $C_\lambda$ region (anti-$\lambda$) was essentially as described previously for a rabbit antiserum (Stevenson *et al.*, 1977a). Briefly, the Ig preparation (133 mg) was passed through an immunosorbent column (20 ml) consisting of Sepharose-4B to which guinea-pig immunoglobulin light chains had been coupled. The unretarded fraction contained anti-Id together with normal sheep Ig whereas the absorbed fraction represented anti-$\lambda$ only. The latter was eluted from the immunosorbent by means of 0·5 M $NH_4OH$ followed by dialysis into cold Tris-NaCl-azide. Any immune complexes or other aggregate in this fraction were removed by further chromatography on Ultrogel AcA-34.

The unretarded fraction was then passed through a further column of immunosorbent consisting of Sepharose-4B linked to normal urinary proteins (14 ml). This removed a weak antibody activity presumably directed against a contaminant in the immunogen.

Finally, the various fractions were dialyzed into phosphate-buffered saline (PBS), pH 7·3, concentrated by ultrafiltration through an Amicon PM-10 membrane, and filtered through 0·22 $\mu$m Millipore membranes. Protein concentrations were estimated from absorbancy at 280 nm taking $E_{1\ mg/ml}$ for sheep IgG as 1·35. Preparations were stored frozen.

*Preparation of an immunosorbent specific for the $IgG_1$ subclass*

As it appeared that the $IgG_2$ (anti-$\lambda$) fraction was contaminated with $IgG_1$ it was necessary to prepare an immunosorbent specific for the $IgG_1$ subclass which could then be used to remove $IgG_1$.

Three rabbits (New Zealand White) were each injected with a total of 1 mg of sheep $IgG_1$, prepared by chromatography on DEAE-cellulose as described above. The protein was mixed with CFA and injection was in 4 intramuscular sites. After 5 weeks, the rabbits were given further injections of 0·2 mg of sheep $IgG_1$ in CFA into the rump, and 0·4 mg of an aqueous solution of sheep $IgG_1$ into the back. The animals were bled 1, 2 and 3 weeks after the secondary injection and the third bleed was an

exsanguination from the heart. Serum was prepared and the IgG fraction isolated from it by the method of Stevenson & Dorrington (1970).

In order to render the antibody activity specific for the $IgG_1$ subclass it was necessary to absorb with sheep $IgG_2$. This was done by coupling the $IgG_2$ from the DEAE-cellulose chromatographic separation of sheep IgG to Sepharose 4B by the technique of Axen, Porath & Ernback (1967). A column of coupled $IgG_2$ (3·5 ml, containing 35 mg $IgG_2$) was used to absorb 60 mg of IgG ex rabbit anti-sheep $IgG_1$; the material passing straight through the immunosorbent was found to react with $IgG_1$ but not $IgG_2$ by analysis on Ouchterlony immunodiffusion plates. This antibody was itself coupled to Sepharose 4B to prepare an immunosorbent specific for $IgG_1$. It was found that the column could absorb 2·3 mg out of 5 mg applied sheep $IgG_1$ (46%) whereas it absorbed only 0·8 mg (16%) of 5 mg applied sheep $IgG_2$.

In the definitive absorption of the anti-$\lambda$ $IgG_2$ fraction, 6·4 mg was applied to the same column.

*Assessment of antibody activity*

Inhibition of migration of $L_2C$ cells was assessed as described previously (Stevenson & Stevenson, 1975). The initial concentrations of Ig were 0·6 mg/ml for anti-Id and 0·24 mg/ml for anti-$\lambda$. All dilutions were with Eagle's minimal essential medium (MEM) plus 1% non-essential amino acids, 10% foetal calf serum and 100 i.u./ml of both penicillin and streptomycin (supplemented MEM).

$L_2C$ cells were labelled with $^{51}Cr$ for assays for cytotoxicity, as described previously (Stevenson *et al.*, 1977a). Labelled cells were suspended in cold MEM at $1 \times 10^6$/ml; viability by Trypan Blue exclusion was >95%.

Effector cells for K-cell-mediated cytotoxicity were prepared from normal venous sheep blood as it was found that sheep effector cells were much more efficient in this system than human peripheral blood leucocytes. Briefly, 400 ml blood was taken into 60 ml 3·5% sodium citrate, pH 7·4. After centrifugation at 500 *g* for 20 min, the buffy coat was removed, suspended in isotonic $NH_4Cl$ and incubated at 37° for 10 min to lyse contaminating red blood cells. After the addition of PBS, the leucocyte preparation was centrifuged at 100 *g* for 10 min. After two further washes in PBS and three washes in MEM, the cell pellet was suspended in the supplemented MEM to give $1{\cdot}25 \times 10^7$ cells per ml. Viability at this stage was >96%. The effector to target ratio used was 100 : 1.

For both complement-mediated and K-cell-mediated cytotoxicities, the procedures were as described previously (Stevenson *et al.*, 1977a) except that sheep immunoglobulin was used in all cases. Percentages of specific $^{51}Cr$ release in the cytotoxicity tests were taken as:

$$\frac{\text{Counts released by antibody} - \text{counts released by the corresponding normal Ig} \times 100}{\text{Counts released by detergent} - \text{counts released by the normal Ig}}$$

where detergent lysis was carried out in 1% Nonidet 40. All points on the graphs represent means of duplicate determinations.

## RESULTS

The results of fractionating a 20 ml aliquot of sheep antiserum are shown in Fig. 1. It is clear from this that the majority of the IgG of the serum is in the $IgG_1$ fraction (86% of total recovered IgG). A

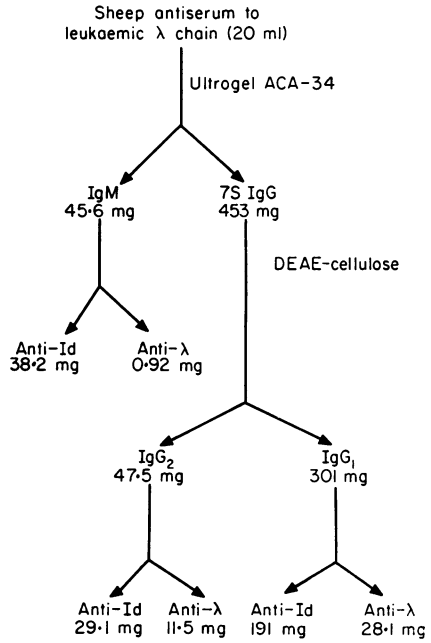


**Figure 1.** Separation of sheep antiserum raised against leukaemic urinary chain into its various classes, subclasses and antibody activities.

**Table 1.** Migration of $L_2C$ cells in presence of various fractions from sheep antiserum raised against leukaemic $\lambda$ chain

| | | Initial concentration (mg IgG/ml) | Relative migration at indicated dilution: Undiluted | 1 : 2 | 1 : 4 | 1 : 8 |
|---|---|---|---|---|---|---|
| Anti-Id | IgM | 0·6 | 0·94 | 0·88 | 0·83 | 0·84 |
| | $IgG_1$ | 0·6 | 0·33 | 0·54 | 0·72 | 0·85 |
| | $IgG_2$ | 0·6 | 0·61 | 0·69 | 0·71 | 0·82 |
| Anti-$\lambda$ | IgM | 0·24 | 0·64 | 0·77 | 0·82 | 0·72 |
| | $IgG_1$ | 0·24 | 0·25 | 0·24 | 0·39 | 0·48 |
| | $IgG_2$ | 0·24 | 0·23 | 0·28 | 0.49 | 0·49 |

Relative migration is defined as the ratio of area covered by cells in the presence of antibody IgG to area covered in the presence of normal IgG at the corresponding concentration.

similar proportion (83%) was recovered from samples of normal sheep serum taken through the same procedure.

Immunoelectrophoresis (Beckman Microzone system) established that the $IgG_1$ and $IgG_2$ formed single arcs in the fast and slow regions of the IgG arc respectively, using a rabbit antiserum raised against either whole sheep serum or sheep IgG. The 19S fraction obtained from the sheep antiserum showed some contamination with other high molecular weight materials, but was not purified further.

## Antibody activity

*Inhibition of migration of $L_2C$ cells.* The effects of the various Ig fractions on migration of $L_2C$ cells are shown in Table 1. The anti-$\lambda$ is purified antibody by virtue of the method of preparation and therefore the initial concentration has been reduced to 0·24 mg/ml. It can be seen that for both anti-Id and anti-$\lambda$, the $IgG_1$ and $IgG_2$ are both inhibitory and that IgM is weakly inhibitory.

## Cytotoxicity

*Complement-mediated cytotoxicity.* Fig. 2 shows the specific cytotoxicity of the anti-Id as measured by release of $^{51}Cr$ from labelled cells in the presence of rabbit complement. It can be seen that the $IgG_2$ fraction is totally ineffective in this system whereas $IgG_1$ is effective up to a high dilution of antibody-containing Ig; IgM also appears to be effective in complement-mediated cytotoxicity.

Fig. 3 demonstrates the activity of the anti-$\lambda$ in the same system. Again $IgG_1$ antibody is very effective at causing lysis in the presence of complement,

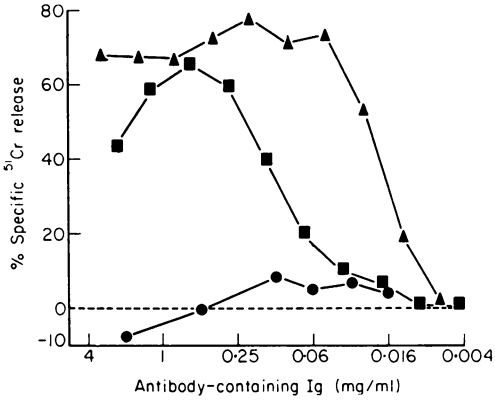


**Figure 2.** Complement-mediated cytotoxicity of $^{51}Cr$ labelled $L_2C$ cells invoked by sheep anti-Id antibodies. (▲) $IgG_1$; (●) $IgG_2$; (■) IgM. Complement was provided by a 1 : 5 dilution of fresh rabbit serum.

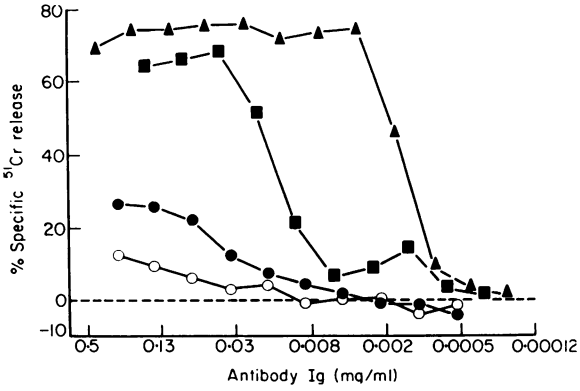


**Figure 3.** Complement-mediated cytotoxicity of $^{51}Cr$ labelled $L_2C$ cells invoked by sheep anti-$\lambda$ antibodies. (▲) $IgG_1$; (●) $IgG_2$; (○) $IgG_2$ after further purification; (■) IgM. Complement was provided by a 1 : 5 dilution of fresh rabbit serum.

but also the $IgG_2$ antibody appears to have some activity, although considerably less than $IgG_1$. In order to find if this was due to contamination, we passed an aliquot of the $IgG_2$ antibody through the immunosorbent which was specific for $IgG_1$, and tested the unretarded material on the $L_2C$ cells. As shown in Fig. 3, this procedure removed a large proportion of the complement-mediated cytotoxicity from the $IgG_2$ fraction.

*K-cell-mediated cytotoxicity.* Fig. 4 shows the specific cytotoxicity of the various fractions of anti-Id in the presence of K cells prepared from sheep peripheral blood. In this system, the $IgG_2$ appears to be the effective antibody, with low specific $^{51}Cr$ release by the $IgG_1$ fraction, and none by IgM. A similar result was obtained for the anti-$\lambda$ activity (Fig. 5).

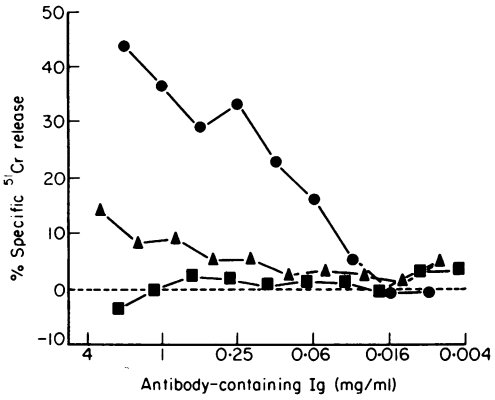


**Figure 4.** K-cell-mediated cytotoxicity of $^{51}Cr$ labelled $L_2C$ cells invoked by sheep anti-Id antibodies. (▲) $IgG_1$; (●) $IgG_2$; (■) IgM. K-cells were provided by leucocytes prepared from fresh sheep blood, at an effector to target ratio of 100 : 1.

## DISCUSSION

In our studies on the $L_2C$ leukaemia we have already shown that administration of whole IgG from our sheep anti-Id antibody will slow the development of this highly malignant tumour (Stevenson, Elliott & Stevenson, 1977b). It would be valuable to find *in vitro* correlates of this retardation by looking at known cytotoxic mechanisms operating *in vitro*. This approach should also help us in designing more effective schedules.

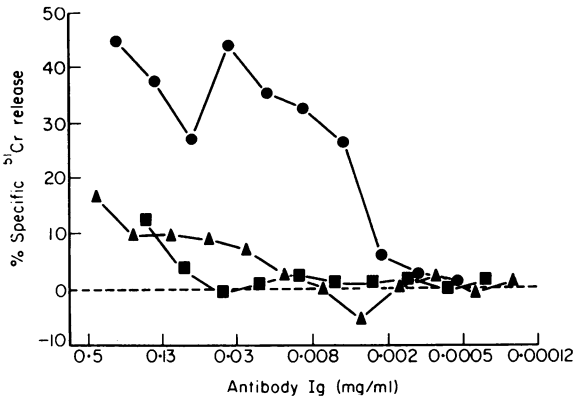


**Figure 5.** K-cell-mediated cytotoxicity of $^{51}Cr$ labelled $L_2C$ cells invoked by sheep anti-$\lambda$ antibodies. (▲) $IgG_1$; (●) $IgG_2$; (■) IgM. K-cells were provided by leucocytes prepared from fresh sheep blood, at an effector to target ratio of 100 : 1.

It is evident from our results that anti-Id activity is found in IgM, $IgG_1$ and $IgG_2$ fractions of the serum. Both the IgG fractions were effective in inhibiting migration of $L_2C$ cells along plastic surfaces. The importance of this ability *in vivo* is not known; apparently the cells are not killed during the inhibition. The two well-described cytotoxic mechanisms were demonstrated each by only one of the IgG subclasses, complement-mediated cytotoxicity by $IgG_1$ and K-cell-mediated cytotoxicity by $IgG_2$.

The anti-$\lambda$ activity isolated from the sheep antiserum is of course not suitable for immunotherapy, but its activity on the $L_2C$ cells enables us to compare the effects of antibody activities against two sets of antigenic determinants on the cell surface IgM molecule. In fact, the two antibodies appear to act synergistically in some of the cytotoxic mechanisms (Elliott, Pindar, Stevenson & Stevenson, in press). It is worth noting that although antibody activities to both the idiotypic and constant antigenic regions of the light chain are found in all the Ig fractions examined, the ratio of the two activities within each fraction is reasonably constant, suggesting that the immune response of the sheep to the two regions is similar.

The anti-$\lambda$ is purified antibody by virtue of the method of preparation and it is strongly active in mediating both complement- and K-cell-dependent cytotoxicity. Again, $IgG_1$ was effective with complement and $IgG_2$ with K cells, but it was found in this

case that the $IgG_2$ fraction was also reacting with complement. The fact that passage through an immunosorbent specific for $IgG_1$ removed a large proportion of the complement-mediated cytotoxicity suggests that there had been contamination of the $IgG_2$ with $IgG_1$. It is possible that during the isolation procedure, a small amount of contaminating $IgG_1$ in the $IgG_2$ fraction has been preferentially bound to the immunosorbent-guinea-pig light chain column, thereby increasing the proportion of $IgG_1$ to higher levels than that found in the anti-Id.

The initial observation that sheep $IgG_1$ fixed complement much more effectively than $IgG_2$ was by Feinstein & Hobart (1969). These authors pointed out that although this applied to guinea-pig and rabbit complement, sheep complement could fix to both subclasses of IgG, $IgG_1$ being only slightly more effective than $IgG_2$ in causing lysis. Tests *in vitro* ideally should reflect conditions *in vivo*, but we found that rabbit complement was much more effective than guinea-pig complement in releasing $^{51}Cr$ from labelled $L_2C$ cells in the presence of sheep antibody. Similarly, in the K-cell-dependent cytotoxic system, effector cells from sheep peripheral blood were much more effective at killing antibody-coated $L_2C$ cells than were those from guinea-pig spleen. These quantitative differences dictated our choices of complement and K cells.

Our results differ in part from those of Grant *et al.* (1975) in the murine P-815 mastocytoma system. These authors detected complement cytotoxicity mediated only by $IgG_1$, but they reported K-cell cytotoxicity mediated by both $IgG_1$ and $IgG_2$. Reasons for this discrepancy are not apparent.

Functional differences in subclasses of xenogeneic anti-Id have been observed by Eichmann & Rajewsky (1975). These authors were using guinea-pig antibody raised against a mouse strain A/J antibody specific for streptococcal Group A carbohydrate. Such anti-Id could be separated into $IgG_1$ and $IgG_2$ subclasses which are not, incidentally, analogous to the sheep subclasses (e.g. Spiegelberg, 1974). It was found that $IgG_1$ sensitized A/J mice against streptococcal Group A carbohydrate whereas $IgG_2$ was suppressive. It remains to be seen whether these interesting effects on the immune response can be reproduced in other systems.

## ACKNOWLEDGMENTS

This work has been supported by Tenovus of Cardiff. We are grateful to Professor G. T. Stevenson for advice and to the staff of Allington Farm, Porton Down, Wiltshire, for animal facilities. Mr M. C. Cleeter and Mr A. Pindar rendered excellent technical assistance.

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