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Abrogation of the non-specific toxicity of abrin conjugated to anti-lymphocyte globulin

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SUMMARY

A covalent conjugate of abrin and anti-human lymphocyte globulin (AHLG) was prepared in an endeavour to create a cytotoxic agent with specificity for human lymphoid cells. The AHLG–abrin conjugate was found to be around 10-fold better able to inhibit ^{3}H-leucine uptake by the human lymphoblastoid cell line, Daudi, in tissue culture than was the control conjugate comprising abrin and normal IgG (nIgG). Both materials were less potent than native abrin. Galactose, which is known competitively to antagonize the binding of abrin to cells, strongly inhibited the toxicities of abrin and the nIgG–abrin conjugate whereas that of ALG–abrin was unimpaired. Thus, at least for Daudi cells in tissue culture, abrin can be made selectively toxic, by linkage to AHLG, towards cells bearing antigens to which the antibody moiety of the conjugate can attach.

INTRODUCTION

Attempts have been made in several laboratories to prepare specific cytotoxic agents by attaching to antibodies highly potent and non-selective toxins of bacterial and plant origin (Moolten, Capparell & Cooperband, 1972; Philpott, Bower & Parker, 1973; Moolten *et al.*, 1975; Moolten, Zajdel & Cooperband, 1976).

Our own previous work demonstrated that diphtheria toxin when covalently linked to AHLG was far more toxic to human lymphoblastoid cells in tissue culture than was the free toxin (Thorpe *et al.*, 1978; Ross *et al.*, 1980). Control experiments, which showed, for example, that normal immunoglobulin (nIgG) was relatively ineffective as the carrier molecule and that a mixture of antibody and toxin lacked synergistic effect, contributed to the interpretation that the binding of the toxin to the lymphoid cells had been facilitated by the antibody.

In the present study, abrin, a toxin derived from the seeds of *Abrus precatorius*, has been covalently linked to anti-human lymphocytic globulin (AHLG). It is demonstrated that the antibody–abrin conjugate is around 10 times more toxic to human lymphoblastoid cells than a conjugate containing nIgG. Furthermore, in the presence of excess galactose which competitively antagonizes the binding of abrin to the cell surface (Olsnes, Refsnes & Pihl, 1974) the toxicity of abrin and nIgG–abrin is abrogated whereas that of AHLG–abrin is unaltered. Thus, at least for human lymphoblastoid cells in tissue culture, abrin can be rendered more selective in its cytotoxic action by covalent attachment to an antibody.

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MATERIALS AND METHODS

Materials. Seeds of *Abrus precatorius* were gifts from Dr Stuart (Society of Herbalists, London, UK) and Professor G. C. Clarke (Department of Botany, University of Ghana). Chlorambucil, i.e. 4-[di-(2-chloroethyl)amino] benzene butanoic acid, was a gift from Burroughs Wellcome and Co. (London, UK). Horse anti-human lymphocytic globulin (AHLG) was obtained from Mr J. S. Courtenay (Department of Protein Chemistry, Wellcome Research Laboratories, Beckenham, Kent). Other materials were purchased as follows: normal horse IgG (Miles Research Products, UK); Bolton and Hunter reagent (IM 861) and ^{3}H-leucine (TRK 170) (Radiochemical Centre, Amersham, UK); Sephadex and Sepharose (Pharmacia Ltd, UK); Hy-Flo Super Gel (Mansville Ltd, UK); RPMI 1640 tissue culture medium and foetal calf serum (GIBCO-BIOCULT Ltd, Scotland, UK); D(+)galactose (Sigma Ltd, USA).

Abrin. Abrin was extracted from the seeds of *Abrus precatorius* by a modification of the method of Olsnes & Pihl (1973). The cotyledons from 40 g of seeds were extracted twice in a Waring blender with 5% v/v acetic acid. The extracts were pooled, filtered under vacuum through Hy-Flo Super Gel and the filtrate dialysed three times overnight against 10 litres of 10 mM Tris–HCl buffer, pH 7·7, containing 30 mM NaCl (Tris/NaCl buffer). The retentate was applied to a column (2·4 × 50 cm) of Sepharose 4B which had been pretreated with 1 M propionic acid overnight at room temperature to improve the ability of the column to bind the abrin (Professor J. H. Humphrey, personal communication) and then equilibrated with Tris/NaCl buffer. When the u.v. absorbance of the effluent from the column matched that of the Tris/NaCl running buffer, a linear gradient of D-galactose (0 to 0·01 M) over a total volume of 1 litre was applied. Two protein peaks emerged, the first containing abrin and the second Abrus agglutinin. The abrin was dialysed against Tris/NaCl buffer and was rechromatographed. Recovery was typically 70–80 mg abrin. The product was homogeneous by electrophoresis in sodium dodecyl sulphate and displayed a molecular weight of approximately 65,000.

Immediately before conjugation to the immunoglobulin 2 ml of abrin at 25 mg/ml was mixed with 100 μl of ^{125}I-radiolabelled abrin (40 μg/ml, 2·5 μCi/mg), that had been prepared by the method of Bolton & Hunter (1973), and was filtered on a column (2·2 × 90 cm) of Sephadex G-100, superfine grade, to remove dimers and larger aggregates.

Immunoglobulins. The anti-human lymphocyte globulin, batch RCL4, had been raised by injection of cells of the human lymphoblastoid line WRL7 into a horse and the IgG fraction purified from the serum by the method of Woiwod *et al.* (1970).

Both AHLG and normal horse IgG were rechromatographed on Sephadex G-150 immediately before conjugation to remove aggregates.

Preparation of conjugates. Abrin was conjugated to immunoglobulin using a mixed anhydride derivative of chlorambucil as the coupling agent in the manner described previously for conjugates containing diphtheria toxin (Thorpe *et al.*, 1978).

The fractions of conjugates selected for biological testing were those having a molecular weight of around 210,000 as indicated by gel filtration on Sephadex G-200 and by electrophoresis on polyacrylamide gels in sodium dodecyl sulphate. The molar ratios of IgG to abrin, obtained from measurements of radioactivity and optical density at 261 and 279 nm, were 1·0:0·91 for the AHLG–abrin conjugate and 1·0:1·2 for the nIgG–abrin conjugate. The approximate molecular weight and molar ratios of abrin to IgG suggested that the conjugates comprised, as the predominant species, one molecule of abrin linked to one molecule of immunoglobulin.

Toxicity testing. Daudi, a non-adherent human lymphoblastoid cell line (that had been shown previously to possess a high capacity to bind AHLG and no detectable ability to bind nIgG [Ross *et al.*, 1980]) was chosen as the cell type for determining the relative toxicity of the conjugates.

A suspension of 10^6 Daudi cells/ml in RPMI 1640 medium supplemented with 10% v/v heat-inactivated foetal calf serum, 200 u/ml penicillin and 100 μg/ml streptomycin was dispensed in 200-μl volumes into 96-well flat-bottomed microplates. Test solutions (22 μl) were added and the plates incubated at 37°C for 24 hr in an atmosphere of humidified air and 5% CO_2.

In other experiments Daudi cells were suspended at 10^6 cells/ml in medium containing 100 mM glalactose (prepared by mixing the medium with an isotonic, 270 mM, solution of galactose in

double-glass distilled water). The suspension was distributed into the microplates and 22 μl of the test solutions in 100 mM galactose added. The cultures were incubated for 1 hr at 37°C before being washed five times in 100 mM galactose-containing medium, twice in medium without galactose and, finally, resuspended into fresh medium for a further 23-hr period of incubation. Experiments were also performed in which cells in medium containing 30 mM galactose were incubated with the test solutions continuously for 24 hr at 37°C.

All cultures received 1 μCi of ^{3}H-leucine 24 hr after addition of the test solutions and the radioactivity incorporated into trichloroacetic acid-precipitable material during a subsequent incubation period of 16 hr was determined (Thorpe & Knight, 1974).

RESULTS

Toxic effects of abrin, AHLG–abrin and nIgG–abrin upon Daudi cells

Abrin in non-conjugated form was extremely toxic to Daudi cells and reduced their ^{3}H-leucine incorporation by half at a concentration of 0·1 ng/ml. When covalently linked to immunoglobulin, abrin was substantialy less potent, a concentration of abrin of 30 ng/ml in the nIgG–abrin conjugate and of 3 ng/ml in the AHLG–abrin conjugate being needed to reduce the leucine uptake by 50%. The conjugate comprising abrin and AHLG was thus about 10 times more toxic to the cells than was the conjugate containing normal immunoglobulin (Fig. 1).

When abrin and AHLG were added separately to the cultures in proportions matching those in the conjugate preparation, no increase in toxicity over that of abrin alone was observed. AHLG alone, at concentrations of 2·5 μg/ml or less, was without effect upon leucine uptake.

Inhibition of toxicity of abrin and nIgG–abrin by galactose

Daudi cells exposed either to abrin or to nIgG–abrin in the presence of 100 mM galactose for 1 hr (before washing to remove toxin not irreversibly associated with the cell surface) subsequently displayed no reduction in protein synthesis even with the highest concentration of abrin used of 1 μg/ml (Fig. 2). By contrast, AHLG–abrin was as toxic when exposed to the cells in the presence of galactose as it was in control cultures without galactose (controls not shown).

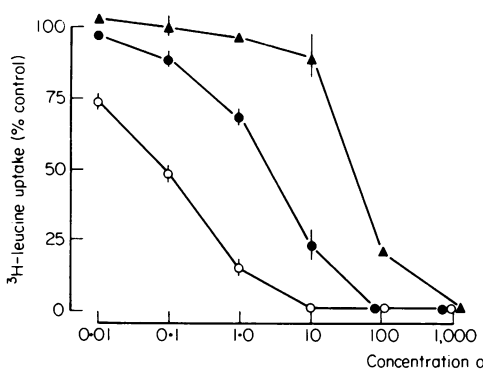

Fig. 1

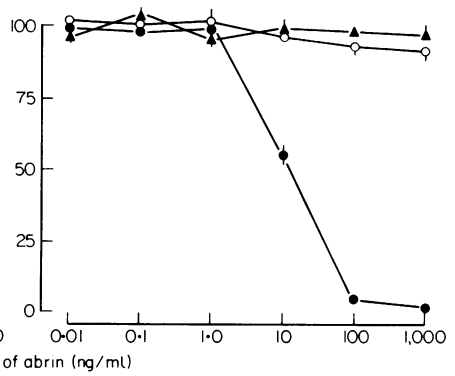

Fig. 2

Fig. 1. The effect of abrin alone (o) and of conjugates with anti-human lymphocyte globulin (•) or normal IgG (▲) upon the ^{3}H-leucine incorporation by Daudi lymphoblastoid cells. The ^{3}H-leucine incorporation is expressed as a percentage of the uptake by untreated cultures. Each point in the figure was calculated from the geometric mean of triplicate determinations, the standard deviations of which are represented by vertical lines unless smaller than the points as plotted.

Fig. 2. The effect of treatment with abrin (o), nIgG–abrin (▲) or AHLG–abrin (•) for 1 hr in the presence of 100 mM galactose upon the ^{3}H-leucine incorporation by Daudi cells. (Controls without galactose are not shown.) Other details as in the legend to Fig. 1.

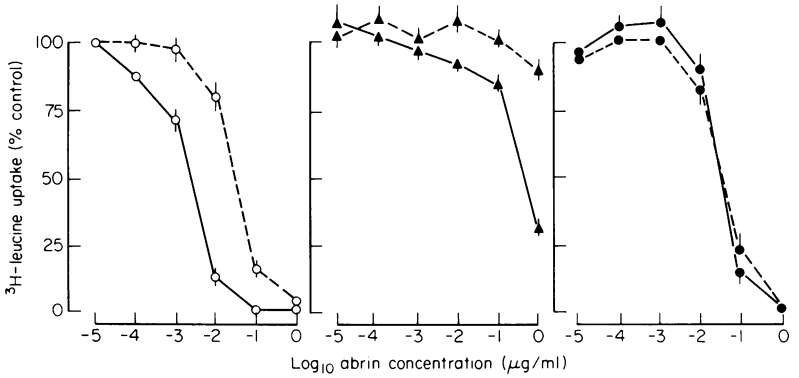

Fig. 3. Inhibition by D-lactose of the toxic effects upon Daudi cells of abrin (○) and nIgG–abrin (▲) but not of AHLG–abrin (●). The cells were incubated with abrin or conjugates in the presence (– – –) or absence (——) of 30 mM galactose throughout the experiment. Other details as in the legend to Fig. 1.

Treatment of the cells with medium containing 100 mM galactose alone for 1 hr was without deleterious effect.

In other experiments cells were incubated with the conjugates in the presence of D-galactose throughout the entire culture period. For these the galactose concentration was 30 mM which was the maximal level permissible to avoid suppression of cell growth. Both abrin and nIgG–abrin were less toxic to Daudi cells in the presence of the galactose, the reduction for abrin alone being around 10-fold. The ability of the AHLG–abrin to inhibit leucine uptake by the cells was unaltered by the galactose (Fig. 3).

DISCUSSION

Abrin is a potent cytotoxic agent comprising two polypeptide chains, A and B, joined by a disulphide bridge (Olsnes *et al.*, 1974). The initial step in the cytotoxic process of abrin is that it binds via a recognition site on the B chain to a galactose-containing glycoprotein on the cell surface (Olsnes *et al.*, 1976). There then follows an ill-understood process whereby the A chain penetrates the cell membrane and within the cytosol terminates protein synthesis by inactivating ribosomes, most probably by an enzymic mechanism (reviewed by Olsnes & Pihl, 1977).

The objective of the present study was to alter the specificity of binding of abrin to cells so that it attached to and killed designated target cells. In principle this necessitates two modifications to the abrin molecule; the first is to add a target-specific means of attaching to cells and the second is to negate any pre-existing binding properties of the abrin molecule. The proviso must be met that these manipulations neither impair the putative enzymic properties of the A chain nor the mechanism by which it penetrates the cell. To some extent, these modifications are both achieved when abrin is covalently linked to an antibody using the present methodology [*vide infra*].

Abrin, when conjugated to normal immunoglobulin was observed to be around 300 times less toxic to Daudi cells than the native toxin. The drop in toxicity cannot be explained entirely by irreversible inactivation of the abrin by its linkage to the immunoglobulin since abrin retained much of its potency when linked to AHLG, which, in common with most conventionally raised antibodies, could be expected to contain only a minority of molecules able to attach to lymphoid cells (Woodruff, Reid & James, 1967). In addition the 2-chloroethyl-amino groups of the coupling agent are most likely to react with the amino groups of lysine residues under the conditions used for the conjugation reaction (Ross, 1949, 1958) and Sandvig, Olsnes & Pihl (1978) have shown that 20 new methyl groups can be introduced into the abrin molecule by reductive methylation of free amino groups with relatively little loss in toxicity. More likely, the bulky immunoglobulin molecule, lacking the means to attach to Daudi, hinders the freedom with which the abrin moiety can interact with receptors on the cell surface.

The AHLG–abrin conjugate was around 10 times more toxic to Daudi cells than the conjugate with normal immunoglobulin and it is likely that the antibody-combining site had facilitated the binding of the abrin moiety to antigens on the cell surface. It is of interest that the AHLG–abrin conjugate possessed similar potency to a conjugate of AHLG and diphtheria toxin described previously (Thorpe *et al.*, 1978; Ross *et al.*, 1980).

The differential between the toxic effects of AHLG–diphtheria toxin and nIgG–diphtheria toxin upon Daudi cells was found in our earlier work to be 1,000-fold, a result now seen as a consequence of the relative insensitivity of Daudi and other lymphoblastoid cell lines to diphtheria toxin (Ross *et al.*, 1980). This insensitivity is not observed with abrin and this contrast is mirrored in the greater toxicity of nIgG–abrin over that of nIgG–diphtheria toxin.

Daudi cells could be completely protected by the addition of excess galactose to the culture medium from short periods of exposure to abrin or nIgG–abrin. This derives from the ability of galactose, like that of lactose, competitively to antagonize the association of the abrin molecule with the cell surface (Olsnes *et al.*, 1976; Sandvig, Olsnes & Pihl, 1976). AHLG–abrin, by virtue of its antigen-binding sites, was fully toxic. This procedure could be of importance in situations *in vitro* in which it is desired selectively to kill one cell type in the presence of others that differ qualitatively in their antigenic expression but which may have comparable capacities to bind the toxin.

When AHLG–abrin was incubated with Daudi cells in the continued presence of galactose throughout the entire culture period, no reduction in its toxicity was observed. This result accords with that of Youle, Murray & Neville (1979) who coupled ricin, another plant toxin with galactose specificity, to monophosphopentamannose and showed that the toxicity of the conjugate for fibroblasts was insensitive to lactose. It may merely be that the antibody moiety of the present conjugate, when attached to antigens on the cell surface, holds the abrin molecule in close proximity to its receptor with the result that galactose can no longer so easily discourage their interaction. Alternatively the initial event in the toxic process of free abrin, namely its binding to a galactose-containing glycoprotein on the cell surface, can entirely be replaced in a conjugate by the binding properties of the antibody moiety and it may be that other properties of the B chain then come into play to transport the A chain across the cell membrane.

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