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A COMPARISON OF THE IN VITRO AND IN VIVO ACTIVITIES OF CONJUGATES OF ANTI-MOUSE LYMPHOCYTE GLOBULIN AND ABRIN

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Anti-mouse lymphocyte globulin and normal immunoglobulin have been conjugated to abrin using two procedures, one involving linkage through an amide bond and a piperazine ring and the other the introduction of two amide bonds flanking a disulphide bridge. The four conjugates produced were equipotent as inhibitors of protein synthesis in rabbit reticulocyte lysates. Each antibody-containing conjugate was a more effective inhibitor of protein synthesis in cultured cells than the equivalent normal immunoglobulin-containing conjugate. In addition the conjugates with disulphide linkage groups were ten times more potent than their counterparts. The disulphide conjugates were also twice as toxic to mice in an acute toxicity test but when used to suppress their immune responses to sheep red blood cells it was the non-disulphide-linked conjugates that were superior. In all instances antibody-containing conjugates were more powerful immunosuppressants than those containing normal IgG. The results are taken to indicate a relative lack of stability of the disulphide conjugates in the tissues.

Introduction

Recently, chemical conjugates of antibodies and certain toxins or their component A-chains have been employed in attempts to produce a new generation of putative chemotherapeutic agents [1–6]. For the most part the emphasis has been upon the introduction of a disulphide bridge between the component parts and for this various methods have been devised [7–9]. Studies from our laboratory [2,10] have described the preparation of conjugates by the use of a derivative of chlorambucil which results in conjugation through a bond not susceptible to reduction or to cleavage by sulphydryl exchange.

In the present work the properties of conjugates formed by the use of a disulphide bridge have been compared with those of conjugates not involving such a linkage group. Effects on cell-free extracts, cells in tissue culture and living animals have been measured and susceptibility to cleavage by dithiothreitol studied.

Materials and Methods

Immunopurified horse anti-mouse lymphocyte globulin (AMLG), normal horse immunoglobulin (nIgG) and abrin were obtained as described previously [11,12].

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Abbreviations: AMLG, immunopurified horse anti-mouse lymphocyte globulin; nIgG, normal horse immunoglobulin; SPDP, N-succinimidyl 3-(2-pyridyl-dithio)-propanate; PFC, plaque-forming cells.

Disulphide bridge conjugates. *N*-Succinimidyl 3-(2-pyridyl-dithio)-propionate (SPDP) was obtained from Pharmacia (U.K.) Ltd., Hounslow, Middlesex and used in accordance with the makers instructions to prepare AMLG-S-S-abrin and nIgG-S-S-abrin which were isolated by chromatography on Sephacryl 300 and analyzed as previously described [10]. The general structure and abrin to globulin ratios are given in Table I.

Chlorambucil (CB 1348) bridge conjugates. AMLG-1348-abrin and nIgG-1348-abrin were prepared, isolated and analyzed as described previously [10]. The structures and abrin to globulin ratios are given in Table I.

Electrophoresis in sodium dodecyl sulphate-containing gel. Samples of protein were made up in a solution of 2% (w/v) SDS, 80 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol and 0.002% (w/v) bromophenol blue. A second set of samples were prepared in the same solvent with the addition of 2.5 mM dithiothreitol. All samples were heated at 60°C for 15 min prior to electrophoresis which was carried out as described by Laemmli [13]. A 3% stacking gel and a 10% running gel were used and the gel was stained with Coomassie brilliant blue and destained in an aqueous mixture of 10% (w/v) methanol and 10% (v/v) acetic acid.

Tests in vitro and in vivo. Details of the cell-free protein synthesis inhibition assay, of [^{3}H]leucine uptake inhibition in tissue culture, acute toxicity (LD_{50}) in mice and of suppression of the immune response of the mouse to an injection of sheep red blood cells have all been given elsewhere [10,12,14].

Serum haemagglutinating titres were measured by the method described by Takatsky [15]. The values shown are the means of the reciprocals of the dilutions of serum used.

Results

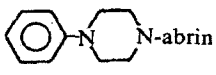
Analysis of SDS-polyacrylamide gel electrophoresis before and after treatment with dithiothreitol. The abrin: IgG ratios shown in Table I support the view that the major constituent molecular species is a conjugate with immunoglobulin and abrin in a 1:1 molecular combination and the presumptive structures of the conjugates are shown.

The different sensitivities of the two types of conjugate to reduction by 2.5 mM dithiothreitol are illustrated in Fig. 1. Lanes 1–4 show the conjugates, their constituent globulin and abrin to be relatively stable to SDS in the absence of reducing agent. Lanes 5–8 show the materials run on the same gel but after pretreatment with the reducing agent. Comparison of lanes 1 and 5 shows nIgG-S-S-abrin to be largely dissociated by dithiothreitol giving products corresponding to those given by reduced abrin and globulin (lanes 7 and 8). nIgG-1348-abrin (lanes 2 and 6) is relatively unaffected giving only small amounts of breakdown products which correspond to those from free abrin (lane 7). Comparison with lanes 4 and 8 which demonstrate the breakdown of immunoglobulin by the reducing agent suggests that in the nIgG-1348-abrin conjugate the immunoglobulin is stabilized presumably by the formation of internal cross-links formed when the chloro-ethyl groups are activated.

Inhibition of protein synthesis in rabbit reticulocyte lysate. The four conjugates were used as inhibitors of protein synthesis in a lysate of rabbit

TABLE I

STRUCTURES AND ANALYSES OF CONJUGATES OF IMMUNOGLOBULINS AND TOXINS PRODUCED BY TWO DIFFERENT METHODS

Conjugation method	Structural formula	Conjugate	Ratio abrin/IgG
Chlorambucil [10]	IgG-NHCO(CH_2)$_3$  N-abrin	AMLG-1348-abrin	0.81
		nIgG-1348-abrin	0.83
SPDP [8]	IgG-NHCO(CH_2)$_2$-S-S-(CH_2)$_2$-CONH-abrin	AMLG-S-S-abrin	0.84
		nIgG-S-S-abrin	0.78

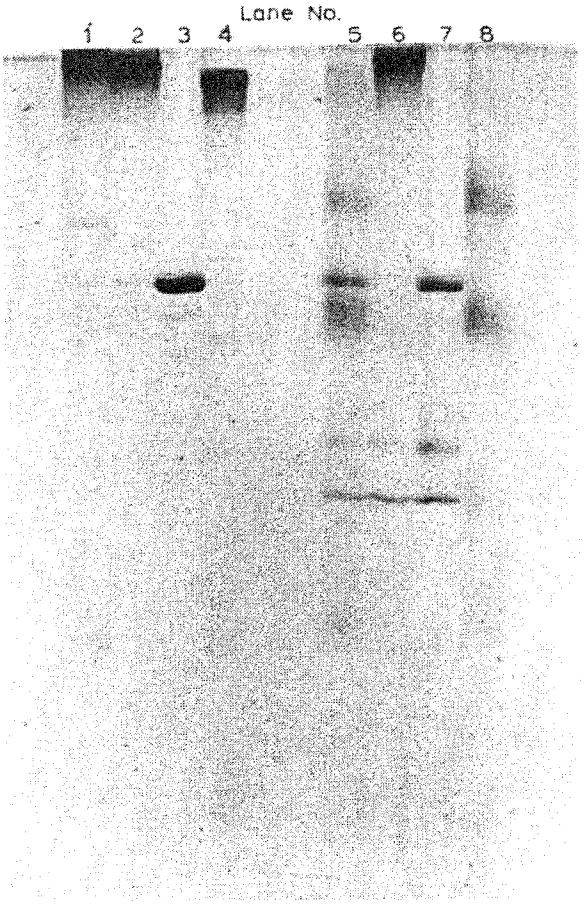


Fig. 1. Elecrophoresis of abrin, nIgG, nIgG-S-S-abrin and nIgG-1348-abrin in gels containing SDS with and without dithiothreitol. Lanes 1 and 5, $7.7 \cdot 10^{-11}$ mols nIgG-S-S-abrin; 2 and 6, $7.6 \cdot 10^{-11}$ mols nIgG-1348-abrin; 3 and 7, $6.5 \cdot 10^{-11}$ mols abrin; 4 and 8, $5.6 \cdot 10^{-11}$ mols nIgG. Lanes 5–8 were run in the presence of 2.5 mM dithiothreitol.

reticulocytes and the results shown in Fig. 2 were obtained. From the figure it is clear that the levels of inhibition are indistinguishable from each other indicating A-chain activity to be identical in each preparation. Thus, differences in A-chain activity can be eliminated as a source of any other variation in biological effect of the conjugates.

Cytotoxicity in tissue culture. Both AMLG-S-S-abrin and AMLG-1348-abrin were about ten times more effective at inhibiting protein synthesis in mouse spleen lymphocytes in tissue culture than the corresponding conjugates with normal immunoglobulin (Fig. 3). The superiority of the antibody-based conjugates on this occasion exceeded

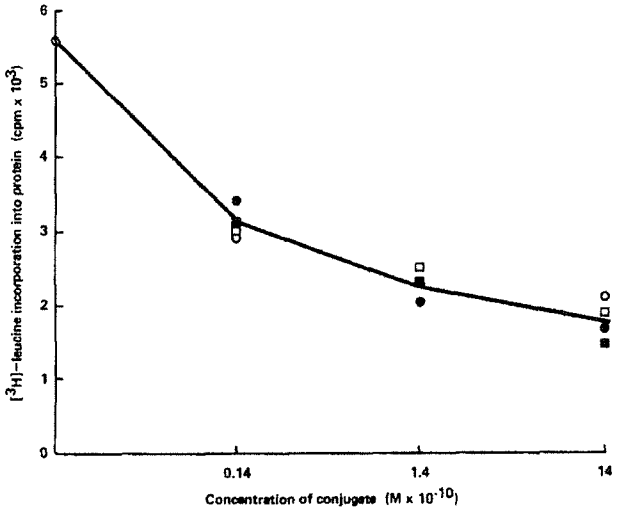


Fig. 2. The effect of antibody-abrin conjugates on cell-free protein synthesis. ○, AMLG-1348-abrin; □, nIgG-1348-abrin; ●, AMLG-S-S-abrin; ■, nIgG-S-S-abrin.

that reported previously [12]. The two disulphide linked conjugates were each about twice as cytotoxic as the corresponding non-reducible conjugate.

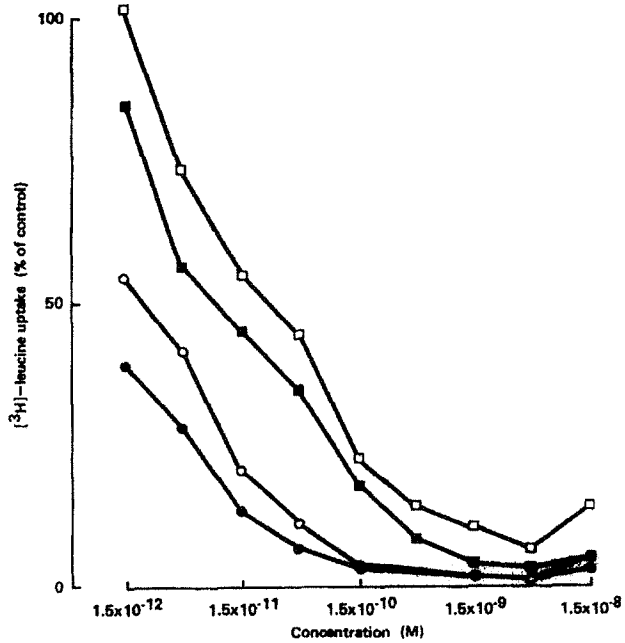


Fig. 3. The effect of antibody-abrin conjugates on protein synthesis of mouse spleen cells in tissue culture stimulated with concanavalin A. ○——○, AMLG-1348-abrin; □——□, nIgG-1348-abrin; ●——●, AMLG-S-S-abrin; ■——■, nIgG-S-S-abrin.

TABLE II

EFFECT OF CONJUGATES ON THE ABILITY OF MICE TO RESPOND IMMUNOLOGICALLY TO AN INJECTION (INTRAPERITONEAL) OF SHEEP RED BLOOD CELLS

A total of $5 \cdot 10^8$ sheep red blood cells were injected in all treatments except for the blank control.

Treatment (intraperitoneal)	Dose (mol× 10^{-13})	PFC/10^6 spleen cells			PFC/spleen			Serum haemag-glutinating titre,
		Geo-metric mean	×S.D. ÷	*P*	Geo-metric mean (×10^2)	×S.D. ÷	*P*	
(Control) none	0	79	1.39		66	1.29		0
None	0	1561	1.07		1905	1.17		223
AMLG-1348-abrin	3.8	669	1.18	<0.001	646	1.26	<0.001	74
	7.5	617	1.39	<0.001	468	1.44	<0.001	23
	15.0	449	1.46	<0.001	398	1.48	<0.001	18
nIgG-1348-abrin	3.8	1158	1.35	N.S.	1148	1.58	N.S.	223
	7.5	681	1.47	<0.01	617	1.55	<0.001	97
	15.0	819	1.10	<0.001	851	1.20	<0.001	74
AMLG-S-S-abrin	3.8	1429	1.23	n.s.	1380	1.23	<0.05	111
	7.5	1479	1.29	n.s.	912	1.05	<0.001	111
	15.0	1549	1.12	n.s.	759	1.45	<0.001	97
nIgG-S-S-abrin	3.8	1279	1.36	n.s.	1288	1.86	n.s.	169
	7.5	1186	1.19	<0.05	1380	1.45	n.s.	215
	15.0	1685	1.33	n.s.	1047	1.99	n.s.	128

Acute toxicity to mice. AMLG-1348-abrin and nIgG-1348-abrin were equipotent, the LD_{50} values being $77 \cdot 10^{-13}$ and $80 \cdot 10^{-13}$ mol, respectively. AMLG-S-S-abrin and nIgG-S-S-abrin were also equipotent but with LD_{50} values of $35 \cdot 10^{-13}$ and $40 \cdot 10^{-13}$ mol, respectively, were twice as toxic to mice as the chlorambucil-linked materials.

Immunosuppressive activities. Table II shows the effects of the conjugates on the immune response of the mouse to an injection of sheep erythrocytes. It is clear from the number of plaque-forming cells (PFC) produced per 10^6 spleen cells that AMLG-1348-abrin has reduced the immune response and that it is approximately twice as effective as nIgG-1348-abrin. AMLG-S-S-abrin and NIgG-S-S-abrin on the other hand are without discernable effect. To take into account changes in the sizes of the spleens of the mice results are also expressed on a PFC per total spleen cells number basis. Again the superiority of AMLG-1348-abrin over nIgG-1348-abrin is demonstrated but now in addition AMLG-S-S-abrin but not nIgG-S-S-abrin is seen to be immunosuppressive. The potency of AMLG-S-S-abrin appears to be of similar magnitude to that of nIgG-1348-abrin and is clearly less than that of AMLG-1348-abrin. Confirmation of these findings is given by the circulating anti-sheep erythrocyte antibody titres.

Discussion

Covalent linkages between immunoglobulins and the toxic protein, abrin, have been introduced by two methods. In the first the two proteins were joined through the formation of an amide and probably a piperazine ring (Table I). The second method involved the introduction of a 3-3′-dithiobis(propionyl) group linking an amino group on each protein. In both procedures the attachment to the protein is thought to be through ε-amino groups of lysine residues. The main distinguishing feature between the two types of linkage is the susceptibility of the disulphide bridge to reduction and sulphydryl exchange. This is clearly illustrated by the break-down of the conjugate occasioned by the use of dithiothreitol.

Toxins like abrin owe their ability to kill cells to an inhibitory effect of their constituent A-chains

on protein synthesis in the cytosol [16]. In the present experiments, A-chain activities were indistuinguishable in all four conjugates, suggesting that the linkages between the immunoglobulins and the toxin are similarly distributed between the A- and B-chains. The superior cytotoxicity of the disulphide-linked conjugates in vitro could be explained by the fact that both antibody-S-S-A-chain · B-chain and antibody-S-S-B-chain · A-chain would be able to release A-chain or a derivative will only a small increase in molecular weight within the cell, whereas only the antibody-1348-B-chain · A chain derivatives would do so. This would also probably account for the increased acute toxicity of the disulphide-linked conjugates in vivo.

The relative failure of AMLG-S-S-abrin as an immunosuppressive agent may reflect a susceptibility to reduction or sulphydryl exchange in the tissues. Albumin in particular is known to exist with a proportion of sulphydryl groups and would be available for exchange, as could other sulphydryl containing molecules [17]. The possibility of unprotected disulphide bridges exchanging with other -S-S- compounds such as the widely distributed glutathione must also be recognised. nIgG-S-S-abrin may likewise be subject to dissociation in the body fluids. This may explain its inefficacy relative to nIgG-1348-abrin which would retain its binding capacity for cells via its abrin B-chain.

In previous studies abrin has been shown to be a potent immunosuppressive agent in its own right [10,18] and it is important to note that any abrin released by reduction of the -S-S- bond would be a derivative of the form abrin-$(HN \cdot CO \cdot (CH_2)_2\text{-}SH)_n$ where $n = 1$ or 2. This substitution number may be critical since in a study in which abrin was reacted with succinic anhydride [19] to give three succinyl groups per abrin molecule, the product, although retaining 80% of the A-chain activity of the original toxin, was 13% as toxic for mice. As with SPDP the reaction involved the formation of amide bonds.

Since abrin itself comprises two polypeptide chains linked by a single disulphide bridge we must also enquire why it too does not dissociate in plasma. In a study on the recombination of purified A- and B-chains it has been claimed that the preferrred combination is A-S-S-B and that the toxicity of the product suggests that virtually all the chains reassociate in this fashion [20]. If this is so it argues strongly for a preferrred orientation probably independent of the sulphydryl groups. This view is reinforced by a study on a closely related plant toxin, ricin in which it was shown that the molecule remained intact even when the disulphide bridge was fully reduced [21]. Also, native protein required 50-fold more mercaptoethanol for reduction of the bond than did denatured ricin. Thus, the evidence seems to favour the view that the disulphide bridge in ricin is protected within the molecule and from the postulated homologies, abrin may be presumed to be similar. Artificially introduced reducible bonds would be unlikely to benefit from such stabilization, either through favourable orientation or folding within the molecule, or both, and this is believed to be reflected in the results presented in this paper. It is concluded that in all cases in which disulphide bridges are used to form new molecular pairings it will be important to establish their stability in vivo.

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