Hydralazine binds covalently to complement component C4

Different reactivity of C4A and C4B gene products

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Long-term treatment with hydralazine is sometimes associated with deposition of immune complexes and development of systemic lupus erythematosus (SLE) as an adverse side-effect. Hydralazine inhibits the covalent binding reaction of the complement protein C4. We show that when hydralazine inhibits C4, it becomes covalently bound to the polypeptide chain containing the active site thiol ester. C4 is encoded at 2 adjacent polymorphic loci, C4A and C4B, within the major histocompatibility complex. We show that hydralazine binds more efficiently to the C4A than to the C4B gene product and suggest that C4 type may predispose patients to hydralazine-induced SLE.

Drug toxicity *Immune complex disease* *MHC*

1. INTRODUCTION

Some patients who are treated with hydralazine, an anti-hypertensive drug, develop symptoms resembling systemic lupus erythematosus [1] with deposition of immune complexes predominantly in the vascular epithelium but also in other tissues [2]. The complement system is closely involved in immune complex handling, stimulating phagocytosis [3] and solubilization [4] through covalent binding of C3. Solubilization of preformed immune complexes [5] and inhibition of precipitation of immune complexes [6] occur more effectively via the classical complement pathway. So inhibition of the early components, C1, C2 or C4, would be likely to increase the size of immune complexes. Since individuals who lack the early complement proteins are at increased risk of developing idiopathic SLE [7], it does seem as if the association between an inhibited complement pathway and immune complex deposition is important in the pathology of SLE.

When the classical pathway is activated by immune complexes, C4 is cleaved by the protease $\overline{C1s}$. Activated C4 binds covalently to the immune complex [8] (or other activating surface) as C4b which then serves as part of the complex enzyme which activates C3 and causes it to bind covalently as C3b by the same mechanism [9,10]. It has been suggested that hydralazine induces SLE in susceptible individuals by inhibiting the covalent binding reaction of C4 [11]. Hydralazine is a substituted hydrazine (fig.1), and it has been shown that when another substituted hydrazine, phenylhydrazine, inhibits covalent binding of activated C3, the compound becomes covalently bound to the protein [12]. To substantiate the proposed molecular basis of hydralazine inhibition of C4, we therefore investigated whether hydralazine becomes covalently bound to activated C4.

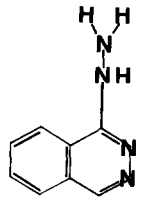

Fig.1. Hydralazine.

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With doses of hydralazine of less than 200 mg/day the proportion of patients who develop adverse effects may be less than 5% [13] although 11% of female patients have been reported to show the lupus syndrome [14]. These incidence figures suggest that the genetic make-up of those who develop hydralazine lupus is important. Hydralazine is metabolised by a polymorphic *N*-acetyltransferase and acetylator phenotype [13] is partly responsible since the syndrome develops almost exclusively in patients of the slow acetylator phenotype. Women are also at increased risk [13,14]. C4 is a class III MHC antigen and both the C4A and the C4B loci are highly polymorphic [15]. C4A corresponds to the Rodgers blood group antigen and C4B to Chido [16]. It has previously been demonstrated that C4A is more reactive with amino nucleophiles than C4B [17,18]. To test whether C4 polymorphism could provide another factor in determining predisposition to hydralazine-induced lupus, we have looked at C4A and C4B to determine if they differ in susceptibility to inhibition by hydralazine.

2. MATERIALS AND METHODS

2.1. *Proteins*

$\overline{C1s}$ was prepared from human serum and C4 from pooled human plasma as described [11]. C4A was prepared from Rodgers positive/Chido negative plasma and C4B from Chido positive/Rodgers negative plasma as described [18]. Chido or Rodgers typed plasmas were confirmed to be C4B1 or C4A3, respectively, by Ms A. Fielder, Department of Immunology, Royal Post Graduate Medical School, London.

2.2. *Binding of hydralazine to C4b*

To demonstrate the covalent binding of hydralazine to C4b, C4 (168 μg) was incubated (20 min, 37°C) with or without $\overline{C1s}$ (6 μg) in a total volume of 250 μl of 10 mM sodium phosphate, 145 mM NaCl, pH 7.5 (PBS), containing up to 5 mM [^{14}C]hydralazine (spec. act. 19.8 mCi/mmol; Amersham International, England). Protein was precipitated at 4°C (16 h) with 10% (w/v) trichloroacetic acid. Pellets were washed twice with 1 ml of ice-cold trichloroacetic acid (10%, w/v) containing 10 mM hydralazine, then twice with 1 ml of ice-cold acetone. Pellets were resuspended in 80 μl of 100 mM Tris-HCl, pH 6.8, 4 M urea, 1% (w/v) SDS containing 3 mg/ml of dithiothreitol. After reduction and alkylation, samples were electrophoresed on 7.5% polyacrylamide gels containing SDS. Gels were stained for protein as before [11]. The positions of radioactive bands were determined by fluorography [19] using Fuji RX X-ray film. Alternatively, the C4 α and α' region was cut out of the gel, solubilised with NCS tissue solubiliser and radioactivity was determined in an LKB-Wallac Ultrabeta scintillation counter.

2.3. *Binding of C4 to Sepharose-$\overline{C1s}$*

Pooled C4, C4A and C4B were also labelled with ^{125}I using Iodobeads (Pierce) and inhibition of binding of ^{125}I-C4 to Sepharose-$\overline{C1s}$ was

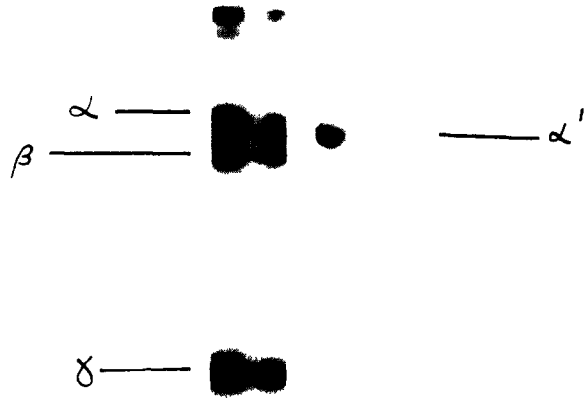

Fig.2. Binding of [^{14}C]hydralazine to C4 on activation by $\overline{C1s}$. Track 1, ^{125}I-C4 + PBS (control); track 2, ^{125}I-C4 + $\overline{C1s}$ (control); track 3, C4 + $\overline{C1s}$ + [^{14}C]hydralazine (5 mM); track 4, C4 + PBS + [^{4}C]hydralazine (5 mM). Incubations were performed as described in section 2 except that for control samples 20000 cpm of ^{125}I-labelled C4 was added.

measured as described [11]. The rate of cleavage of ^{125}I-C4A and ^{125}I-C4B by $\overline{C1s}$ was the same as determined by the rate of appearance of the α' chain on SDS-polyacrylamide gel electrophoresis. The positions of iodinated protein bands were determined by autoradiography as in [11].

3. RESULTS

When C4 prepared from pooled human plasma is activated by $\overline{C1s}$ in the presence of [^{14}C]hydralazine, the drug becomes covalently bound to the α' chain of C4b (fig.2, track 3). With no C1s present (fig.2, track 4), [^{14}C]hydralazine is not incorporated. If preformed C4b is incubated with [^{14}C]hydralazine, then no radioactivity is bound (not shown).

The binding of ^{125}I-C4A and ^{125}I-C4B to Sepharose-$\overline{C1s}$ has been measured as a function of time (fig.3). The ^{125}I-C4B protein binds more rapidly and to a greater extent than ^{125}I-C4A.

The binding of hydralazine to C4A and C4B has been measured and it is clear that more hydralazine binds to C4A than to C4B (table 1).

The inhibition of binding of ^{125}I-C4A or ^{125}I-C4B to Sepharose-$\overline{C1s}$ by hydralazine shows that the C4A type is more readily inhibited by hydralazine than C4B (fig.4). 50% inhibition of C4A binding occurs at 550 μM whereas 50% of C4B binding is inhibited at 2 mM hydralazine. These results are in good agreement with the value of 50% inhibition of C4 from pooled human plasma which is 840 μM [11].

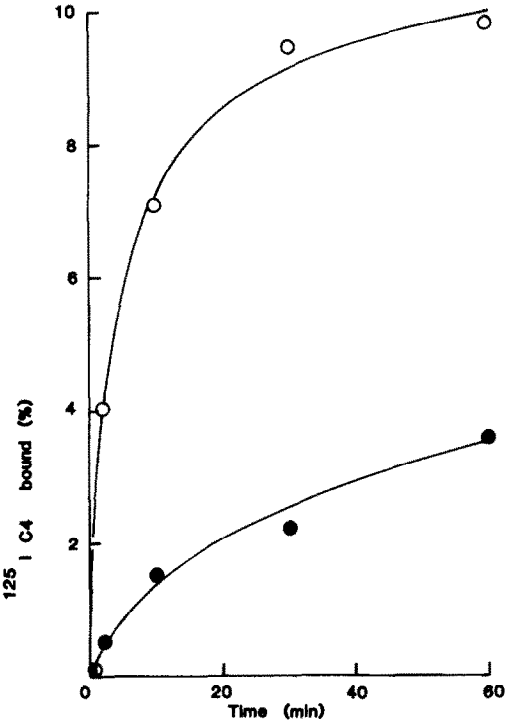

Fig.3. Time course of covalent binding of ^{125}I-C4A and ^{125}I-C4B to Sepharose-C1s. Incubation mixtures contained 10^6 cpm of ^{125}I-C4A or ^{125}I-C4B in 200 μl of 25% (v/v) Sepharose-$\overline{C1s}$ in PBS. After various times at 37°C, the reaction was stopped and radioactivity associated with the pellet was measured as before [11]. Non-covalently bound radioactivity associated with the pellet, which was less than 1% of the radioactivity supplied, was measured in the presence of 20 mM NH_2OH [11]. (●) C4A, (○) C4B.

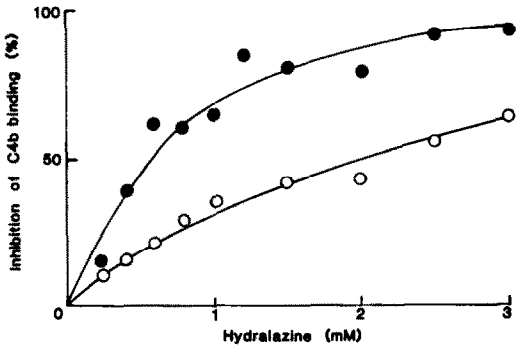

Fig.4. Hydralazine inhibition of binding of ^{125}I-C4A and ^{125}I-C4B to Sepharose-$\overline{C1s}$. ^{125}I-C4A (●) or ^{125}I-C4B (○) (10^6 cpm) was incubated with Sepharose-$\overline{C1s}$ (25%, v/v) for 30 min at 37°C with or without hydralazine in a total volume of 200 μl PBS. The radioactivity associated with the pellets and the percentage inhibition was determined as described before [11].

Table 1

Binding of [^{14}C]hydralazine to C4A and C4B

[^{14}C]Hydralazine (mM)	cpm in α/α' region of SDS-polyacrylamide gel	
	C4A	C4B
0.07	139	33
0.60	448	270
2.13	1780	962

4. DISCUSSION

C4, like C3, contains an intrachain thiol ester bond which on proteolytic activation becomes exposed and available for reaction for a very short time (probably less than 1 ms) [12] with nucleophilic compounds, including putrescine [20] and phenylhydrazine [12]. Putrescine has been shown to become bound to C4 by a trans-acylation reaction to glutamate in the thiol ester site. [^{14}C]Hydralazine, which is also nucleophilic (fig.1), is found covalently bound to the α' polypeptide chain of C4b only when the drug is present during activation of C4 by $\overline{C1s}$. Since the thiol ester site is in the α' chain, it is likely that hydralazine also binds to this group in C4. These results provide a chemical basis for the earlier observation that hydralazine will inhibit the covalent binding of C4 to an activating surface [11]. A possible outcome of the binding of hydralazine to activated C4 is that the drug may then act as a hapten and there have been reports of antihydralazine antibodies in adverse reactions to hydralazine [21,22].

It has been shown previously that C4B reacts preferentially with oxygen nucleophiles [17,18]. This is demonstrated here also in that C4B binding to Sepharose-$\overline{C1s}$ is more rapid than C4A binding (fig.3). More than twice as much C4B is bound to Sepharose-$\overline{C1s}$ after 30 min which is in good agreement with the 2–3-fold greater haemolytic activity of C4B [18]. The higher haemolytic activity of C4B has been shown to depend on more C4B binding to sheep erythrocytes coated with antibody [18]. In contrast, more C4A binds to immune complexes in which the antigen is bovine serum albumin [18]. It may be that the different types of C4 have different physiological roles.

Within the major histocompatibility complex, the C4 type C4A3BQ0 (i.e., with a null allele at the C4B locus) is in linkage disequilibrium with HLADR4 [23]. It has been reported that the DR4 allele is associated with hydralazine lupus [24] although this has been disputed [25]. Nevertheless, we suggest that individuals with the C4A3BQ0 phenotype would be more susceptible to inhibition by hydralazine of C4 binding to immune complexes with an increased risk of SLE. To test this hypothesis, C4 typing of drug-induced SLE patients is required and these studies are now in progress.

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