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Complement-component-C3-opsonized immunoglobulin G anti-DNA antibodies do not bind effectively to red blood cells unless aggregated on a high-M_r DNA matrix

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Large, soluble ds (double-stranded) DNA–IgG (immunoglobulin G) anti-dsDNA immune complexes ($\geqslant$200S) that were previously opsonized with complement were digested with DNAase. The small complement-component-C3-fragment-labelled IgG (11–14S) that was then isolated did not bind effectively to complement receptor type 1 on human red blood cells. However, when this IgG was immune-complexed with ^{3}H-labelled PM2 (bacteriophage directed against a marine *Pseudomonas*) dsDNA ($M_r \sim 6 \times 10^6$), substantial binding of both the DNA antigen and IgG to the erythrocytes was demonstrable.

The recognition of complement-fixing immune complexes by RBCs may be a key event in the normal clearance of these complexes from the circulation (Hebert *et al.*, 1978; Miyakawa *et al.*, 1981; Iida *et al.*, 1982; Medof & Oger, 1982; Medof *et al.*, 1983; Wilson *et al.*, 1982; Inada *et al.*, 1982; Cornacoff *et al.*, 1983; Taylor *et al.*, 1983*a*; Walport & Lachmann, 1984). Several lines of evidence indicate that the complexes bind to CR1 on the RBCs (C3b–C4b receptor) when the IgG on these complexes (and perhaps the antigen as well) are labelled with C3b (Sim *et al.*, 1981; Gadd & Reid, 1981; Medof *et al.*, 1982; Taylor *et al.*, 1983*b*; Takata *et al.*, 1984).

As it is now known that even monomeric IgG can be labelled with C3 fragments in an 'innocent-bystander' mechanism (Jacobs & Reichlin, 1983), it is important to understand under what conditions C3b- or iC3b-bearing IgG (Ross & Lambris, 1982) will bind to RBCs. Our previous studies with the soluble-DNA–anti-DNA immune-complex system have suggested that this binding event requires a multivalent interaction between the C3b-labelled immune complex and a number of CR1 receptors on the RBC (Horgan & Taylor, 1984; Taylor *et al.*, 1983*a*). We here present direct evidence that aggregation of C3b (or iC3b)-labelled anti-DNA IgG on a dsDNA matrix is a necessary requirement for binding to RBCs.

Abbreviations used: C3, C3b, iC3b, C3d and C3d,g, the third component of complement and breakdown products thereof; C′-DA, complement-opsonized DNA–IgG (immunoglobulin G) anti-DNA complexes: C′-A, complement-opsonized IgG anti-DNA antibodies; CR1, complement receptor type 1; Con A, concanavalin A; dsDNA, double-stranded deoxyribonucleic acid; DNAase, deoxyribonuclease; GVB^{++} buffer, gelatin/Veronal buffer containing 0.5mM-$MgCl_2$ and 0.15mM-$CaCl_2$; IgM, immunoglobulin M; Ma, SLE (systemic lupus erythematosus) patient with high-titre anti-dsDNA antibody plasma; NHS, normal human serum; phosphate-buffered saline 0.15M-NaCl/0.01M-phosphate, pH7.4; PM2, bacteriophage directed against a marine bacterium of the genus *Pseudomonas*; RBC(s), human red blood cell(s); RBC-CF assay, the red-blood-cell-linked complement-fixation assay.

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Materials and methods

Preparation of C′-DA

C′-DA were prepared exactly as previously described (Horgan & Taylor, 1984). Briefly, dsDNA–IgG anti-dsDNA complexes were prepared with PM2 dsDNA (6MDa) and SLE plasma Ma. The complexes were opsonized with normal human serum complement and isolated by sucrose-density-gradient ultracentrifugation.

Evidence that the complexes contain IgG anti-dsDNA antibodies

The DNA-binding activity of plasma Ma has previously been shown to be exclusively associated with the IgG fraction, as more than 90% of the C′-DA were precipitated with anti-IgG-coated immunobeads and less than 10% were precipitated with anti-IgM-coated immunobeads (Horgan &

Taylor, 1984). In addition, antibody class can be determined by the interaction of the immunoglobulin with Protein A and Con A (Langone *et al.*, 1978). Protein A selectively binds IgG, and Con A binds IgM. By using a procedure identical with that described by Borsos *et al.* (1981), plasma Ma was preabsorbed with Protein A–Sepharose, and the DNA-binding activity was abolished, whereas plasma Ma preabsorbed with Con A–Sepharose retained more than 90% of its DNA-binding capacity.

Preparation of C′-A

C′-DA were incubated with an equal volume of DNAase (Worthington) (0.15 mg/ml in phosphate-buffered saline containing 0.01 M-$MgCl_2$) for 1 h at 37°C in order to destroy the DNA antigen. EDTA was then added to a final concentration of 0.01 M, and the mixture was centrifuged at 25000 rev./min on sucrose density gradients that were isokinetic for protein (Johns & Stanworth, 1976) for 20 h at 4°C. The fractions that corresponded to 6–22S were collected, and these C′-A fractions were used in the binding assays.

DNA-binding assays

The Farr assay and modified RBC-CF assay (binding in the absence of complement) were performed as described for the C′-DA binding studies (Horgan & Taylor, 1984). Briefly, equal volumes of the ^3H-labelled PM2 dsDNA and the antibodies to be tested were mixed and incubated for 1 h at 37°C. In the modified RBC-CF assay (complement omitted), aliquots of these samples were then mixed with equal volumes of 25% (v/v) suspensions of human RBCs, and after a 20 min incubation at 37°C the samples were centrifuged and the percentage of ^3H bound was determined by counting the supernatant for ^3H. When the standard RBC-CF assay was used (Pedersen *et al.*, 1980) the final incubation mixture contained immune complex, RBCs, and a 20-fold dilution of NHS as a complement source.

Determination of human IgG bound to RBCs

The RBC-CF assay was adapted to determine the amount of IgG in a given test sample that bound to RBCs, through the use of ^{125}I-labelled purified rabbit anti-human IgG (Taylor & Horgan, 1984); 100 μl of the test sample was incubated with 100 μl of 25% RBCs for 20 min at 37°C, and then the RBCs were pelleted by centrifugation at 8000*g* for 5 min. Where appropriate, the percentage of [^3H]DNA bound to the RBCs was measured by counting portions of the supernatant for ^3H. Subsequently, the RBC pellet was washed three times and then incubated with 200 μl of affinity purified ^{125}I-labelled rabbit anti-human IgG for 1 h at 37°C. Then the RBC pellet was again washed three times and counted for ^{125}I radioactivity. Calibration curves were established by measuring the uptake of the ^{125}I label by RBCs containing known amounts of heat aggregated human IgG that had been bound to the human RBCs after opsonization with complement. In order to minimize non-specific interactions with the RBCs, the C′-A were first pre-absorbed against sheep RBCs (which lack CR1).

Preabsorption of C′-A with monoclonal antibodies to C3d(C3b)

C′-A were preabsorbed with mouse monoclonal anti-C3d antibodies (which also bound C3b) (Ortho Diagnostics) as follows: 300 μl of a solution of C′-A were mixed with 50 μl of a 1% bovine serum albumin solution and 50 μl of a 12-fold dilution of the mouse monoclonal antibody to human C3d and incubated for 45 min at 37°C. Next, 50 μl of affinity purified goat anti-mouse IgG (Boehringer) were added and the mixture was incubated for 45 min at 37°C. Next, 50 μl of affinity purified goat anti-mouse IgG (Boehringer) were added and the mixture was incubated for 45 min at 37°C, followed by 20 min at 4°C. The samples were then centrifuged for 10 min at 8000*g* at 4°C, and the supernatants were used as the preabsorbed C′-A fractions and then tested for dsDNA-binding activity. Controls were included in which the same protocol was followed, except that buffer was substituted for either the anti-C3d antibodies or for both the anti-C3d and the affinity-purified anti-mouse IgG antibodies as well.

Results

C′-A were prepared by treating C′-DA with DNAase (in order to digest and release the DNA antigen), and then the subfractions that corresponded to 6–22S were isolated by sucrose-density-gradient ultracentrifugation. Substantial DNA-binding activity in both the Farr and modified RBC-CF assays was demonstrated in samples with *s*-values in the range of 11–16S (Table 1).

Two protocols were used in order to determine the requirements for [^3H]DNA binding by C′-A in the modified RBC-CF assay. When the C′-A were mixed directly with the DNA (Table 1), considerable binding occurred, and less than 10% of the bound [^3H]DNA was released when the RBC pellets were washed twice with GVB^{++} buffer. Alternatively, the modified RBC-CF assay was performed with C′-A fractions which were not previously incubated with ^3H-labelled dsDNA. In this case the C′-A were incubated directly with the RBCs, and after the RBCs had been centrifuged and washed twice, the washed cells were incubated

with the [^{3}H]DNA for 30min at 37°C. Under these conditions, no [^{3}H]DNA binding (above a background of about 10%) was demonstrable (results not shown).

Table 1. *^{3}H-labelled PM2-dsDNA-binding activity in sucrose-density-gradient fractions of C′-A*
The input of ^{3}H-labelled dsDNA was 2.5ng/assay. Spontaneous precipitation was less than 10%. Binding to human RBCs was determined in the absence of complement in the modified RBC-CF assay.

		[^{3}H]DNA bound (%)	
Fraction	$s_{20,w}$ (S)	Farr assay	Modified RBC-CF
3	6.3	100	0
4	8.8	100	29
5	11.3	97	94
6	13.8	96	87
7	16.3	84	65
8	18.8	85	31
9	21.3	66	12

Next, the binding of C′-A to RBCs was monitored by using ^{125}I-labelled rabbit anti-human IgG in order to detect RBC-bound IgG. When the C′-A were first incubated with PM2 dsDNA and allowed to bind to RBCs, the amount of ^{125}I-anti-IgG subsequently bound to the cells was considerably greater than when the C′-A alone were added to the RBCs (Table 2). When the assay was performed with C′-A that had been previously incubated with sonicated dsDNA (500kDa), the net ^{125}I-anti-IgG bound was not significantly higher than that of the buffer control (results not shown).

Finally, the C′-A fractions were first treated with monoclonal antibodies to C3d (which also bound C3b) and the residual DNA-binding activity of these treated fractions was determined in the RBC-CF assay (binding in the presence of complement) and the modified RBC-CF asay (binding in the absence of complement) (Table 3). Treatment of the C′-A with mouse anti-C3d (anti-C3b) antibody + affinity-purified goat anti-mouse IgG completely abolished the DNA binding activity in

Table 2. *Incubation of C′-A fractions with ^{3}H-labelled PM2 dsDNA allows both antibody (IgG) and DNA to bind to human RBCs: detection of bound IgG with ^{125}I-anti-human IgG*
C′-A fractions were preabsorbed against sheep RBCs (which lack CR1) to decrease the non-specific binding of the IgG to surface components other than CR1. A total of 94000c.p.m. of ^{125}I were used per assay. Non-specific binding of this label to human RBCs was 1760c.p.m., and this number was subtracted from all data to give the net values reported. The equivalent amount of IgG bound to the RBCs was calculated on the basis of the standards of aggregated human IgG in the calibration described by Taylor & Horgan (1984). When the C′-A fractions were previously incubated with sonicated dsDNA, the net ^{125}I-anti-IgG bound was not significantly different from that of the buffer control (result not shown).

		+ ^{3}H-labelled PM2 dsDNA			Buffer control (no DNA added)	
Fraction	$s_{20,w}$ (S)	[^{3}H]DNA bound (%)	Net ^{125}I-anti-IgG bound (c.p.m.)	Equivalent amount of IgG bound (ng)	Net ^{125}I-anti-IgG bound (c.p.m.)	Equivalent amount of IgG bound (ng)
5	11.3	81	4340	30	810	6
6	13.8	63	1470	8	250	1

Table 3. *Preabsorption of C′-A fractions with anti-C3d (anti-C3b) antibodies: determination of residual DNA activity*
In the RBC-CF assay, binding was determined in the presence of complement. Complement was omitted in the modified RBC-CF assay. A different C′-A preparation than that used in Table 1 was used in this experiment.

		[^{3}H]DNA bound (%)					
Preabsorption procedure	...	Monoclonal mouse anti-C3d(anti-C3b) + affinity-purified goat anti-mouse IgG		Buffer + affinity-purified goat anti-mouse IgG		Buffer alone	
Fraction		RBC-CF	Modified RBC-CF	RBC-CF	Modified RBC-CF	RBC-CF	Modified RBC-CF
6		30	<5	87	82	82	93
7		16	<5	71	85	67	93

the absence of complement. When these treated C′-A samples were examined in the presence of complement, there was also a marked reduction in dsDNA binding activity, but it was not completely abolished. As expected, samples that were simply incubated with buffer alone or with the affinity purified goat anti-IgG retained DNA binding activity in the RBC-CF assay in both the presence and absence of complement (Table 3).

Discussion

Requirements for binding of C′-A to RBCs

These experiments demonstrate that relatively small complement-opsonized IgG molecules (which probably include dimers or trimers in the region of 11–16S) do not bind effectively to human RBCs unless they are aggregated, for example as immune-complexed IgG. In this study, ^{3}H-labelled PM2 dsDNA ($M_r \sim 6$MDa) provided a matrix for the aggregation of C′-A, and the complex of ^{3}H-dsDNA and C′-A clearly bound to the RBCs (Table 1). When sonicated dsDNA was used ($M_r \sim 500$kDa), it apparently did not bind sufficient antibody to achieve as effective an aggregation, as binding to RBCs did not occur (see the Results section).

The ^{125}I-anti-IgG assay revealed a small amount of IgG bound to the RBCs when the C′-A were added to the RBCs in the absence of DNA (Table 2). However, when the experiment was performed in the presence of dsPM2 DNA, considerably more IgG was bound to the RBCs. It is likely that the binding in the absence of DNA was due to a small amount of non-specific absorption, and the higher protein content of fraction 5 (~11.4S) over fraction 6 (~13.8S) (Waller *et al.*, 1981) probably led to a net greater amount of non-specific binding in that fraction (Table 2). However, even if a small amount of C′-A did bind specifically to the CR1 of the RBCs, there was not sufficient RBC-bound IgG anti-DNA to then bind additional ^{3}H-DNA (see the Results section).

Proof that C′-A contain C3 fragments

The C′-A fractions were indeed opsonized with C3b (and probably iC3b as well), since they could bind dsDNA and then bind to RBCs in the absence of complement. Additional evidence that most of the IgG anti-DNA antibodies in the fractions were complement-opsonized stems from our observation that there was no residual DNA binding activity in the RBC-CF assay (in the absence of complement), after preabsorption of the fractions with anti-C3d antibodies (which also bound C3b) (Table 3). The fact that some binding was detectable in the presence of complement after the preabsorption step would suggest that some of the IgG anti-dsDNA antibodies in the fractions were not sufficiently well labelled with C3b (and iC3b) that they could be absorbed out by the anti-C3d (also anti-C3b) antibodies.

Although we do not know the relative proportions of C3b and iC3b in these complexes, they were opsonized briefly with small amounts of complement in order to try and minimize conversion of C3b into iC3b (Taylor *et al.*, 1984). However, it is likely that under the conditions of these experiments both C3b *and* iC3b binding may be important.

Attempts to quantify C3 binding to IgG

Previous attempts to directly demonstrate the amount of C3b (or iC3b) per IgG in C′-A proved exceedingly difficult because the amount of specific anti-DNA, even in plasma Ma, one of our highest titre anti-DNA plasmas, is only 0.2–0.3% of the total IgG (Taylor & Horgan, 1984), and thus use of radiolabelled IgG would involve a very low 'signal-to-noise' ratio. Also, use of radiolabelled C3 proved non-productive for probably a number of reasons. It has been reported by others (Gadd & Reid, 1981) that only 2–3% of the C3b generated during complement activation by insoluble immune complexes is incorporated into the complexes. In addition, the DNA–anti-DNA immune complexes are soluble and therefore they cannot be washed to remove the non-incorporated C3b (probably 97% or more of the radiolabel), whereas complement-opsonized immune precipitates can be washed. Finally, under the conditions of the assays, the concentrations of the proteins that had reacted, IgG and C3b, are extremely low (i.e. nanogram quantities) and techniques much more sensitive than those described here (for example, the use of specific monoclonal antibodies to the complement fragments) will be needed for accurate quantification of C3b/IgG stoichiometries for the antibody–dsDNA immune-complex system. However, on the basis of previous studies by other workers (Sim *et al.*, 1981; Takata *et al.*, 1984), it is unlikely there is more than one C3 fragment incorporated per IgG molecule in most immune complexes, including C′-DA.

In conclusion, although we do not have direct information on the C3b/IgG stoichiometries, the following point should be emphasized. The original C′-DA were prepared under conditions which led to maximum binding of the complexes to RBCs (Horgan & Taylor, 1984). Presumably the complexes had incorporated optimal amounts of C3b (and perhaps iC3b as well). However, under these conditions, the isolated C′-A fractions did not bind effectively to the RBCs unless they were allowed to form a complex with dsDNA. We therefore conclude that the effective binding of C3b-labelled

IgG to RBCs requires a multivalent interaction. Relatively small IgG C'-A (9–14S) apparently do not contain sufficient C3b or iC3b that they can bind to RBCs alone. However, when these molecules are immune-complexed on a dsDNA matrix, the complex does bind quite effectively. Additional evidence that aggregation of C3b enhances binding to RBCs has recently been reported by Arnaout *et al.* (1983). They reported that even at 0°C (where binding should presumably be stronger) monomeric C3b or monomeric C3b-IgG did not bind effectively to RBCs. However, they found that when the C3b was aggregated into dimers and trimers, the binding to RBCs was enhanced considerably. Their findings and the results reported in the present work may have significance with respect to the general question of the RBC-mediated clearance of immune complexes from the circulation (Cornacoff *et al.*, 1983).

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