

Lack of specificity for antibodies to double stranded DNA found in four commercial kits

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Abstract

Four commercially available kits (three enzyme linked immunosorbent assays and one modified Farr radioimmunoassay) were compared for their ability to detect specifically autoantibodies to double-stranded DNA (dsDNA) using 66 patient sera. This was assessed by comparing the results of the kits with those from an ELISA specifically measuring antibodies against highly purified dsDNA, single-stranded DNA (ssDNA), native DNA and histones. The RIA and two of the ELISAs seemed equally efficient at detecting antibodies to dsDNA, but all three also detected anti-ssDNA (the RIA being particularly bad for this). The need for highly purified dsDNA was clearly shown. The results obtained with one ELISA did not correlate with any variable investigated in this study. A total of 220 sera were assayed with the IDS RIA, of which 130 were recorded as positive. Of these, 50 sera seemed to contain no identifiable autoantibodies. This very high false positive rate may be due, at least in part, to precipitation of non-specifically bound labelled DNA.

The immunopathology of systemic lupus erythematosus (SLE) includes polyclonal activation of B lymphocytes. In addition to hypergammaglobulinaemia, antibodies to many nuclear antigens are produced.¹ Among these, antibody to double-stranded DNA (dsDNA) is historically the most important, and its presence constitutes one of the 11 criteria commensurate with a diagnosis of SLE according to the revised criteria of the American Rheumatism Association.² The reported prevalence of antibodies to dsDNA in SLE varies from 40%³ to 88%.⁴ Although this variability results to some extent from the choice of patient group, much is likely to be due to the choice of assay method. These antibodies were originally measured using a Farr ammonium sulphate precipitation method⁵ but have since been measured by double diffusion,³ passive haemagglutination,⁶ *Criethidia luciliae* staining,⁷ millipore filter assay⁸ and a great number of radioimmunoassays (RIAs) and enzyme linked immunosorbent assays (ELISAs). These methods differ considerably in their physicochemistry and so it is not surprising that reported comparisons have described them as giving very different

results.⁹⁻¹¹ For a large laboratory with a heavy service commitment, however, cost effectiveness and efficiency dictate the use of only one available method, usually RIA or ELISA, for routine diagnosis.

It was therefore decided to compare a modified Farr RIA (Immunodiagnosics Ltd) with three commercially available ELISAs (IDS, Bioscot, and BioHyTech). The aim was not to prove yet again that different kits produce different results, but to determine what these kits, which all purport to detect exclusively antibodies to dsDNA, were actually measuring. To investigate the specificity of these kits further, a highly specific ELISA, using highly purified target antigen and detecting only IgG antibodies to double-stranded DNA, with a low non-specific binding, was developed. This assay was also used to measure IgG antibodies to single-stranded DNA and histones as well as IgM antibodies to native DNA.

Methods

Antibodies to DNA were measured using four commercially available kits according to the manufacturers' instructions. The kits used were as follows: (1) Gamma-B (Immunodiagnosics (IDS), Washington, Tyne & Wear); (2) Diamedix (IDS, Washington, Tyne & Wear); (3) Autostat (Bioscot, Livingston, Scotland); and (4) BioHyTech (Ramat Gan, Israel). The IDS RIA uses a circular double-stranded DNA of unspecified source; all three commercial ELISAs (Diamedix, Autostat, and BioHyTech) use calf thymus DNA. The conjugates used in these three ELISAs bind to IgG and IgM (IDS), IgG (Bioscot), and IgG, IgM, and IgA (BioHyTech). The upper limit of normal for the IDS RIA, determined in our laboratory using 100 donated sera, was 5 mg/l. The upper limit of normal for the three ELISAs were those recommended by the kit manufacturers, these being 33.3 IU/ml (IDS), 50 IU/ml (Bioscot), and 305 AU/ml (BioHyTech).

SPECIFIC ELISA

Antibodies to native DNA (nDNA), double-stranded DNA (dsDNA), and single-stranded DNA (ssDNA) were measured in the following ELISA: 96-well polystyrene microtitre plates (Nunc-Immuno Plates, Gibco Ltd, Paisley, Scotland) were pre-coated with methylated BSA (Sigma, Poole, Dorset)—200 μ l at 10 μ g/ml for 2 hours at 4°C. After washing in PBS, 200 μ l of DNA at 20 μ g/ml

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was added and incubated for two hours at 4°C. The wells were washed with PBS and 200 µl of serum (1 in 100) was incubated for 60 minutes at 30°C. After washing in PBS/Tween 20, 200 µl of horseradish peroxidase-conjugated sheep anti-human IgG (Scottish Antibody Production Unit—SAPU, Law Hospital, Carlisle) was added and incubated for 60 minutes at 30°C. After a further PBS/Tween 20 wash, 200 µl of 2,2' Azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) (0.1 g% in 0.1 M phosphate/citrate buffer (pH 4.6) containing 0.15% hydrogen peroxide) was added and incubated for 45 minutes at 30°C. The reaction was stopped with 50 µl of 1.25% sodium fluoride and the plate read at 405 nm. For antibodies to nDNA and dsDNA the results were expressed as IU/ml by comparison with the Wo80 standard.¹² The sensitivities of these two assays (expressed as mean + 2 SD of the zero standards were 3.73 and 6.61 IU/ml, respectively). Antibodies to ssDNA were expressed in arbitrary units. In one experiment calf thymus histone (Sigma type IIS) was used as antigen and in another, horseradish peroxidase-conjugated sheep anti-human IgM (SAPU) was used in conjunction with nDNA as antigen. In both these cases the results were expressed as arbitrary units. In all cases a normal range was established using 44 control sera. The upper limit of normal was taken as the mean plus 2 standard deviations and was 18.8 IU/ml (nDNA), 25.3 IU/ml (dsDNA), 0.31 (ssDNA), 0.31 (histone) and 5.65 (IgM). Inhibition experiments were performed by incubating diluted sera with various concentrations of either ds- or ssDNA for one hour at 37°C before their use in the ELISA.

DNA PREPARATION

The source of nDNA used in this study was calf thymus DNA (Sigma). dsDNA was purified from this as follows: 50 mg calf thymus DNA was dissolved in buffer A (10 mM TRIS/HCl, pH 7.4, 1 mM EDTA). Sodium dodecyl sulphate (SDS) was added to a concentration of 1% and to this was added 2 mg proteinase K (Sigma) in buffer A containing 1% SDS, the mixture being incubated overnight at 37°C. An equal volume of ice-cold chloroform/isoamylalcohol (24:1) equilibrated with buffer A was added, shaken vigorously, and centrifuged for five minutes at 5000 × g. The lower phase and interphase were discarded and the partition repeated. To the resultant aqueous phase was added 0.25 volumes of 3M sodium acetate (pH 5.0) and this was mixed. To this was added 2 volumes of absolute ethanol pre-cooled to -20°C. After vigorous mixing the precipitated DNA was removed by spooling, washed twice with ethanol/buffer A (2:1), redissolved in 20 ml buffer A and dialysed overnight against 30 mM sodium acetate (pH 4.4), 100 mM NaCl, 5 mM ZnCl₂. S1 nuclease was added (100 U/mg DNA) and the mixture was incubated for three hours at 37°C before being dialysed overnight against buffer A. The DNA concentration was determined at 260 nm

(OD₂₆₀ of 1.07 is equivalent to a DNA concentration of 50 µg/ml). ssDNA was prepared by boiling dsDNA for 15 minutes and cooling rapidly on ice. Protein was measured by the method of Maddy and Spooner.¹³ The strandedness of the DNA preparations was assessed by benzoylated naphthoylated DEAE cellulose (BNDC) chromatography.¹⁴ Briefly, DNA was added to a BNDC column equilibrated with 0.3M NaCl buffer. dsDNA is eluted with 1.0 M NaCl buffer and ssDNA and/or dsDNA with ss regions is eluted with 1.0 M NaCl/50% formamide buffer.

SERA

The 66 sera used in the commercial kit comparison were chosen randomly from patients with suspected or definite connective tissue disease for which a dsDNA autoantibody response had been requested. These sera were numbered 1 to 66 and it is these numbers which are used to identify the various sera in the text and tables. The choice of sera was not made on diagnostic criteria but to provide sera with a broad spectrum of negative and positive results as determined by the IDS RIA. An additional 154 sera screened only with the IDS RIA and the in-house ELISA were chosen in a similar manner to investigate the role of non-DNA, non-IgG antibodies in the production of false positive results. Of the 220 patients investigated, 56 had definite SLE; nine patients had definite or suspected variants of SLE such as discoid lupus or subacute lupus erythematosus; one had drug induced lupus; and five patients had a definite connective tissue disease other than SLE. Sera from the remaining 125 patients had been sent for analysis to confirm or preclude possible connective tissue disease because of their clinical presentation. These were renal (n = 34), haematological (n = 14), dermatological (n = 11), neurological (n = 7), vasculitic (n = 5), pulmonary (n = 4), cardiac (n = 4), rheumatoid (n = 9) and miscellaneous (n = 37).

Results

Table 1 shows that the positivity of the 66 samples using commercial kits varied from 36 (55%) with the IDS RIA to five (7.6%) with the BioHyTech ELISA. Antigen specific analysis showed that nine sera (14%) were positive for antibodies to dsDNA and that 18 (27%) were

Table 1 Comparison of results obtained in all four commercial assays with in-house ELISA

	Positive	Negative	% positive
<i>Commercial</i>			
IDS RIA	36	30	55
IDS ELISA	24	42	36
Bioscot	18	48	27
BioHyTech	5	61	7.6
<i>In-house Elisa</i>			
dsDNA	9	57	14
ssDNA	18	48	27
Histone	9	57	14
IgM	8	58	12
dsDNA or ssDNA	20	46	30

Table 2 Comparison of IDS RIA with commercial ELISAs

IDS RIA (mg/ml)	Commercial ELISA		
	Total	Negative	Positive
Negative (< 5.00)	30	24	6
Intermediate (5.00–6.99)	11	7	4
Positive (> 7.00)	25	6	19

Sera scored as positive by ELISA were positive in one more of the three ELISAs

positive for antibodies to ssDNA. In all, 20 sera (30%) were positive for ds or ssDNA, or both.

Nine sera (14%) also had antibodies to histones and eight (12%) had IgM antibodies to nDNA. Table 2 divides the 66 sera on the basis of their IDS RIA score that is, negative (less than 5.00 mg/ml), intermediate (5.00–6.99 mg/ml), and positive (more than 7.00 mg/ml). All those sera scoring positive in one or more of the commercial ELISAs (table 2) are analysed in greater detail in table 3. Of the 29 sera found to be positive in one or more of the ELISAs (and therefore presumed to contain antibodies to dsDNA), only eight were found to contain antibodies to highly purified dsDNA. These eight sera, plus one other (No 12) which also had measurable anti-dsDNA, are shown in table 4. Seven of these were detected by the IDS RIA, IDS ELISA, and Bioscot ELISA (although not the same seven) and two using the BioHyTech ELISA. Table 5 shows a further 12 sera that were negative for anti-dsDNA but positive for anti-ssDNA. All but one were recorded as positive by the IDS RIA, nine by the IDS ELISA, five with the Bioscot ELISA, and none by the BioHyTech ELISA.

Table 3 Analysis of sera found to be positive in one or more of commercial ELISAs

Serum No	IDS	Bioscot	BioHyTech	dsDNA	ssDNA	Histone	IgM
<i>IDS RIA negative/kit ELISA positive sera</i>							
2	+	+	+	–	–	–	–
13	–	–	+	–	–	–	–
14	–	+	+	+	–	–	–
29	+	+	–	+	+	–	–
32	–	–	+	–	–	–	–
53	+	–	–	–	–	–	–
<i>IDS RIA intermediate/kit ELISA positive sera</i>							
3	+	–	–	–	–	–	–
24	+	–	–	–	–	–	–
51	+	–	–	–	+	–	–
59	–	+	–	–	–	–	–
<i>IDS RIA positive/kit ELISA positive</i>							
4	+	+	–	–	–	–	–
5	+	+	–	+	+	–	+
7	+	–	–	+	+	–	–
18	+	+	–	–	+	–	–
20	+	–	–	–	–	–	–
23	+	+	–	–	+	–	–
25	+	–	–	–	–	–	–
28	+	–	–	–	+	–	–
39	+	+	–	+	+	+	–
40	+	+	–	–	+	+	–
41	+	+	–	–	+	+	–
47	+	+	–	+	+	–	+
49	+	+	–	–	+	–	–
50	+	–	–	–	+	+	+
57	–	+	–	–	–	–	–
60	+	+	–	–	+	+	+
61	+	+	–	–	–	–	–
62	+	+	+	+	+	+	+
66	+	+	–	+	+	–	+

The sera are divided into three groups depending on their IDS RIA score—that is, negative (n = 6), intermediate (n = 4), and positive (n = 19), as defined in table 2.

Thus 18 IDS RIA positive sera, eight IDS ELISA positive sera, six Bioscot ELISA positive sera and three BioHyTech positive sera had no detectable antibodies to either dsDNA or even ssDNA. Nine sera had detectable anti-histone antibodies and eight had IgM antibodies to nDNA (table 1). All of these, however, were found either in association with IgG antibodies to DNA or in sera which were negative in all four commercial assays.

Table 6 shows the breakdown of results from all 220 sera used in this study and compares them with the results obtained from sera from patients with definite SLE. Of all 220 sera, 130 were positive using the IDS RIA but only 48 had measurable antibodies to dsDNA. The percentage incidence in all antibody categories is much higher in the SLE group but the relative incidences within each group are similar. Thus in both groups the incidence of antibodies to ssDNA is about 50% greater than that of antibodies to dsDNA. With only one exception, all sera (from both groups) with antibodies to dsDNA or ssDNA were recorded as positive using the IDS RIA.

The IDS RIA results were compared with those obtained from IgG- anti-dsDNA, IgG-anti-ssDNA, and IgM-anti-nDNA using the paired Student's *t* test. All associations were significant ($p < 0.05$) and the correlations (r) were 0.476, 0.680, and 0.613, respectively. The binding of anti-ssDNA antibodies to ssDNA was completely abolished by prior incubation with ssDNA at a concentration of 300 $\mu\text{g/ml}$; dsDNA up to a concentration of 550 $\mu\text{g/ml}$ had no effect whatsoever.

Analysis of Sigma calf thymus nDNA showed that it contained 2.3% protein (w/w). After digestion with proteinase K and phase separation this had decreased to less than 0.5%. Measurement of the strandedness of the DNA preparations showed that all the purified dsDNA was eluted from the BNDC column by the 1.0 M NaCl buffer (indicating 100% dsDNA) whereas none of the ssDNA was eluted with his buffer, all being eluted with the 1.0 M NaCl/50% formamide buffer (indicating 100% ssDNA). Using this system, untreated Sigma calf thymus nDNA was found to contain 22.1% dsDNA.

Discussion

In the first part of this investigation 66 sera were assayed for autoantibodies to double-stranded DNA using four commercially available kits. To assess what these kits were actually measuring, comparison was made with an in-house ELISA which allowed for the measurement of IgG antibodies to dsDNA, ssDNA, and histones as well as IgM antibodies to nDNA.

Some variability in the results produced by the ELISAs was expected as the three chosen used different conjugates. Thus one was specific for IgG (Bioscot), one detected IgG and IgM (IDS), and one detected IgG, IgM, and IgA (BioHyTech). Consequently the BioHyTech ELISA might have been expected to yield the greatest number of positive results, but only five were recorded (table 1). The IDS ELISA did

Table 4 Analysis of nine sera found by in-house ELISA to have antibodies to pure dsDNA

	dsDNA positive sera			
	IDS RIA	IDS ELISA	Bioscot	BioHyTech
5*	+	+	+	-
7*	+	+	-	-
12	+	-	-	-
14	-	-	+	+
29*	-	+	+	-
39*	+	+	+	-
47*	+	+	+	-
62*	+	+	+	+
66*	+	+	+	-
Total	7	7	7	2
Percentage of all positive results	19	29	39	40

*These sera were also found to have antibodies to ssDNA.

yield more positive results than the Bioscot ELISA, but table 3 shows that of the nine sera positive using IDS and negative using Bioscot, only one had IgM antibodies. Therefore these differences among the ELISAs do not seem to result from the differing specificities of the conjugates used.

Of the 30 sera reported as negative with the IDS RIA, six were positive by one or more of the ELISAs. Such a discrepancy is not surprising, but the finding that four of the five BioHyTech positive results were in this group is indeed surprising. Clearly the latter kit does not share the same consensus result with the other three kits. Eleven sera gave an IDS RIA score of between 5.00 and 6.99 mg/l and these were assigned into an intermediate group. None was found to contain antibodies to dsDNA and most of these sera (n = 7) were also negative in all three ELISAs. Nevertheless, three were recorded as positive by the IDS ELISA and one with the Bioscot ELISA.

Twenty five sera were reported as strongly positive using the IDS RIA (more than 7.00 mg/l). Even here, however, six sera were recorded as negative by all three ELISAs. One of these six had dsDNA antibodies while the rest had no measurable antibodies whatsoever (results not shown). Of the 19 also reported as positive by one or more of the ELISAs, 18 were detected by the IDS kit, 15 by the Bioscot kit, and only one by the BioHyTech kit. Table 3, however, shows that only six of these 19 sera had measurable antibodies to dsDNA whereas most (n = 4) had antibodies to ssDNA. This

Table 5 Analysis of 12 sera found by in-house ELISA to have antibodies to ssDNA in absence of antibodies to dsDNA

Serum No	ssDNA positive dsDNA negative sera			
	IDS RIA	IDS ELISA	Bioscot	BioHyTech
18	+	+	+	-
24	+	+	-	-
28	+	+	-	-
35	+	-	-	-
40	+	+	+	-
41	+	+	+	-
43	+	-	-	-
48	-	-	-	-
49	+	+	+	-
50	+	+	-	-
51	+	+	-	-
60	+	+	+	-
Total	11	9	5	0
Percentage of all positive results	31	38	28	0

Table 6 Comparison of results obtained from all 220 sera with definite SLE sera (n = 56)

	All sera (SLE sera)		
	Positive	Negative	% positive
IDS RIA	130 (48)	90 (18)	59 (86)
dsDNA	48 (26)*	172 (30)	22 (46)
ssDNA	68 (39)†	152 (17)	31 (70)
Histone	22 (11)‡	198 (45)	10 (20)
IgM	39 (18)‡	181 (38)	18 (32)
dsDNA or ssDNA	81 (41)	139 (15)	37 (73)

*43 of all sera were reported as positive using the IDS RIA (25 of the SLE sera were similarly positive).

†29 of the total sera, all of them IDS RIA positive, had no associated dsDNA antibodies (14 of the SLE sera, all but one of them IDS RIA positive, had no associated dsDNA antibodies).

‡Nine of the total sera (two in the SLE group) were negative for IgG antibodies to DNA but positive using the IDS RIA.

message is emphasised further in tables 4 and 5. In the former the nine sera found to contain antibodies to dsDNA have been listed. Seven of these sera (although not the same seven) were detected by the IDS RIA, IDS ELISA, and Bioscot ELISA, while the BioHyTech ELISA detected only two. Table 5 lists the 12 sera which contained antibodies to ssDNA in the absence of anti-dsDNA. Most were reported as positive by the IDS RIA (n = 11) and the IDS ELISA (n = 9). Five were reported as positive using the Bioscot ELISA and none with the BioHyTech ELISA.

The IDS RIA, IDS ELISA, and Bioscot ELISA are equally efficient at detecting antibodies to dsDNA (seven out of nine) whereas the BioHyTech ELISA detected only two out of nine dsDNA antibody positive results. The IDS RIA, however, also records as positive virtually all sera with antibodies to ssDNA (eleven out of twelve). The Bioscot ELISA is much less sensitive to ssDNA antibodies than the IDS ELISA (five out of 12 compared with nine out of 12). Thus these kits clearly detect antibodies to ssDNA, in some cases more frequently than antibodies to dsDNA, and the most likely cause is contamination of the dsDNA with ss regions, as has been suggested previously.¹⁵ The care taken in the work reported here to remove protein contaminants as well as ss regions from the commercially supplied DNA has yielded material which was 100% pure dsDNA, as measured by BNDC chromatography, and this has consequently resulted in a much more specific assay. In support of this, seven sera were found which were positive using unpurified nDNA but which were subsequently found to be negative using the purified dsDNA (results not shown). Of these seven, five were positive for antibodies to ssDNA. Notably, all seven were recorded as positive using the IDS RIA, the ELISA positivity being six (IDS), five (Bioscot), and 0 (BioHyTech). The Sigma nDNA used here was found to contain only 22.1% pure dsDNA, the rest being either pure ssDNA or more likely dsDNA with several segments of ssDNA. The detection of anti-ssDNA using nDNA as target antigen is therefore easily explained and one can only guess whether such a phenomenon may explain some of the poor specificity observed here with the commercial kits.

The possible role of non-anti-DNA, non-IgG antibodies in the production of false positive results was investigated by extending the study to 220 sera. All were assayed using the IDS RIA as well as the in-house ELISA. The results are given in table 6 which compares all 220 sera with the 56 sera from patients with definite SLE. In both groups anti-ssDNA is more prevalent than anti-dsDNA, and more importantly all those sera which had ssDNA antibodies exclusively (with one exception) were recorded as positive using the IDS RIA. In total, nine IDS RIA positive sera were found which were negative for antibodies to ds- or ssDNA but had IgG antibodies to histones or IgM antibodies to nDNA. Thus in 50 sera that were positive in the IDS RIA no specific antibodies could be found. Such a non-specific ammonium sulphate precipitation method is easily influenced by the total serum Ig, and in this particular patient group where hypergammaglobulinaemia is very common it is very likely that there is significant coprecipitation of non-specifically adsorbed labelled DNA. This lack of specificity for anti-dsDNA is further confirmed by the finding that the IDS RIA results correlate much better with IgG-anti-ssDNA and IgM-anti-nDNA than they do with IgG-anti-dsDNA.

In conclusion, it is difficult to decide what the BioHyTech ELISA is measuring, although it did detect two out of nine of the anti-dsDNA sera. The other three kits were equally efficient at detecting anti-dsDNA antibodies (seven out of nine) but, to varying degrees, produced false positive results. A major fault was the detection of antibodies to ssDNA, this being particularly noticeable in the IDS RIA and IDS ELISA, and this was thought to be due to the use of impure DNA as target antigen. Other false positive results were recorded which may have been due to other nuclear components or IgM

antibodies. The very high false positivity for the IDS RIA was felt to be due, in large part, to the non-specific coprecipitation of labelled DNA. The in-house method used here clearly shows that specificity requires a combination of highly purified target antigen, the detection of IgG antibodies alone and low non-specific binding.

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