Clin. exp. Immunol. (1982) **50**, 603–610.

Measurement of low avidity anti-dsDNA by the *Crithidia luciliae* test and the PEG assay

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(Accepted for publication 30 April 1982)

SUMMARY

With the immunofluorescence technique (IFT) using *Crithidia luciliae* as a substrate, 14,417 sera sent to our laboratory for routine anti-dsDNA determination, were screened for the presence of antibodies to dsDNA. The 1,260 sera that were found IFT positive were then assayed with the Farr radioimmunoassay, in which ^{3}H-labelled PM2-DNA is used as antigen. Only 470 sera (37%) were found to be Farr positive. This discrepancy is, at least partially, caused by the fact that the Farr assay does not detect anti-DNA of low avidity, whereas the Crithidia-IFT does. Sixty-eight percent of the IFT-positive/Farr negative sera were found positive with the PEG assay, a radioimmunoassay that also employs double stranded PM2-DNA as antigen, and that also detects anti-dsDNA of low avidity. The IFT performed on IFT positive/Farr negative sera was found to be rather irreproducible. It was shown that this was due to local increases of the salt concentration resulting from the way the assay was performed. The problem could be overcome by careful control of the assay conditions, i.e. never letting Crithidia slides dry up after washing with PBS. In the PEG assay, these sera sometimes showed a DNA binding that decreased with time. It could be shown that this is caused by a parallel increase in pH during the incubation as a result of CO_2 evaporation from the serum.

INTRODUCTION

Antibodies against DNA are found in the serum of patients with systemic lupus erythematosus (SLE) (Stollar, 1973). Not only do they serve as markers of the disease, but also they are implicated in the pathogenesis of the disorder. The marker function is illustrated by the fact that anti-DNA is reported to be quite specific for SLE, which makes detection of these antibodies of diagnostical importance (Griffiths & Carson Dick, 1979; Deegan, 1980). The clinical impact of anti-DNA is underlined by the finding that an exacerbation of the disease is generally preceded by a marked rise of the anti-DNA level and coincides with a sharp drop of this level (Swaak *et al.*, 1979). Disease manifestations are thought to be brought about by deposition of immune complexes, in particular DNA–anti-DNA complexes, in different kinds of tissue, thereby giving rise to distinct symptoms

Abbreviations: dsDNA = double stranded deoxyribonucleic acid; SLE = systemic lupus erythematosus; PEG = polyethylene glycol; DXS = dextran sulphate; NHS = normal human serum; PBS = phosphate-buffered saline; IgG/IgM = immunoglobulin of class G or M, respectively; HGG = human gammaglobulin; IFT = immunofluorescence test; FITC = fluorescein isothiocyanate.

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(Koffler *et al.*, 1971; Winfield, Koffler & Kunkel, 1975; Bruneau & Benveniste, 1979). It is therefore apparent that quantitative as well as qualitative differences within the population of antibodies to DNA may influence the pattern of the disease (Gershwin & Steinberg, 1974; Ballou & Kushner, 1979; Ebling & Hahn, 1980).

Testing panels of serum samples from selected patients with well defined clinical manifestations by the Farr radioimmunoassay using ^{3}H-labelled PM2-DNA as antigen and by the immunofluorescence technique with *Crithidia luciliae* as substrate, we found both assays to be very specific for SLE (Swaak *et al.*, 1981). Furthermore, there was no significant difference in sensitivity of the assays (Aarden *et al.*, 1975a). However, upon screening of patients' sera sent to our laboratory for routine anti-DNA determination, we noticed a clear discrepancy between the Farr assay and the IFT: only half of the IFT positive sera were found to be Farr positive (Swaak *et al.*, 1981). This discrepancy might be due to the fact that anti-DNA of low avidity is not measured in the Farr assay (Aarden *et al.*, 1975a; Leon *et al.*, 1977). The dissociating reaction conditions of the Farr assay, which prohibit detection of low avidity anti-DNA, are avoided in the PEG assay in which polyethylene glycol precipitation replaces ammonium sulphate precipitation (Riley, McGrath & Taylor, 1979; Smeenk & Aarden, 1980). The PEG assay has indeed been shown to detect antibodies to DNA of low avidity (Riley, Addis & Taylor, 1980; Smeenk, Lelij & Aarden, 1982a). Notwithstanding this, the PEG assay was still found to be fairly SLE-specific (Smeenk *et al.*, 1982b).

We now report that antibodies to dsDNA that are missed by the Farr assay upon routine screening may be detected by the PEG assay. During this study, we noticed that measurements of low avidity anti-dsDNA may easily lead to irreproducible or false negative results. The cause of this has also been studied. Finally, we tried to correlate the IFT and the PEG assay in a quantitative way using sera only containing low avidity anti-dsDNA.

MATERIALS AND METHODS

Sera. The sera of 14,417 patients, sent to our laboratory for routine anti-dsDNA determination, were used in this study. Two hundred normal human sera (NHS) were obtained from healthy blood donors. All sera were stored at -20°C.

DNA. ^{3}H-labelled dsDNA was isolated from PM2 bacteriophages cultured in the presence of ^{3}H-thymidine and purified as described by Espejo & Canelo (1968). The specific activity was 40–60×10^3 d.p.m./μg ^{3}H-DNA.

Immunofluorescence test. Details of this IFT have been published (Aarden, De Groot & Feltkamp, 1975b; Aarden & Smeenk, 1981). *Crithidia luciliae* were grown, harvested and used as described. Sera are diluted 1 : 10 in phosphate-buffered saline (PBS; 0·14 M NaCl, 0·01 M phosphate, pH 7·4); 30 μl of this dilution was incubated for 30 min at room temperature with a spot of *C. luciliae*, contained on an eight spotted slide. After washing the slide for 30 min in PBS, 0·5 ml of FITC conjugated anti-immunoglobulin (this institute, batch No. SH 17-1-F9) is layered over the slide, followed by an incubation of 30 min at room temperature. The slide is washed with PBS and mounted, using a solution of 65% (w/v) sucrose in PBS, pH 8·2, in which 0·5 μg/ml propidium iodide (Calbiochem) is dissolved. Fluorescence of kinetoplasts indicates anti-dsDNA activity. The propidium iodide acts as a counterstain to facilitate kinetoplast localization. A KP-560 barrier filter is used to separate propidium iodide fluorescence from FITC fluorescence. Serial dilutions of sera in PBS were tested in the IFT if a titre was needed.

Farr assay. The Farr assay was performed as described by Wold *et al.* (1968) and modified by Aarden, Lakmaker & Feltkamp (1976a) and Aarden & Smeenk (1981). To 100 μl of the appropriate serum dilution, 50 μl of a solution of 16 mg/ml normal human gammaglobulin (HGG) and 50 μl of a solution of 2 μg/ml ^{3}H-PM2-DNA were added. All components were dissolved or diluted in PBS. After an incubation of 1 hr at 37°C, 5 ml of a cold 50% saturated ammonium sulphate solution was added. The precipitate was allowed to form for 30 min at 4°C, after which it was centrifuged for 15 min at 3000 ***g***. The precipitate was washed twice with semi-saturated ammonium sulphate, and dissolved in 1 ml of Soluene-100 (Packard). After addition of 10 ml scintillation fluid (Instafluor II, Packard), radioactivity was measured. The mean binding of 150 normal controls was 5 ± 3%. To

express anti-dsDNA activity in Farr units/ml, the definition of Aarden, Lakmaker & De Groot (1976b) is used: a sample contains one Farr unit of anti-DNA if it binds 30% of 100 ng PM2-DNA under the conditions described above.

PEG assay. Details of this method have been described (Smeenk & Aarden, 1980). To 50 μl of serum, 50 μl of a solution of 1·6 mg/ml HGG, 50 μl of a solution of 0·2 mg/ml dextran sulphate (Pharmacia Fine Chemicals AB, Sweden; lot No. 5259) and 50 μl of a solution of 2 μg/ml ^{3}H-PM2-DNA were added, together forming an incubation volume of 200 μl. An equal volume of 7% (w/v) polyethylene glycol (PEG 6000, Koch-Light Laboratories, Colnbrook, UK) was added to the mixture. All components were dissolved or diluted in PBS. The mixture was incubated for 1 hr at 37°C, then for 2 hr at 4°C. After centrifugation for 15 min at 3000 *g*, 200 μl of the supernatant were taken, dissolved in 8 ml of NE-260$_{sp}$ (New England Nuclear Corp.) and counted for radioactivity. A '0% binding' control, in which serum was omitted, was included in each experiment. Mean binding of 200 normal control sera was 6 ± 2%. To express anti-dsDNA activity in PEG units/ml, the same definition as in the Farr assay was used.

Note. Due to differences in reaction mechanisms, a Farr unit does not comprise the same amount of anti-dsDNA as a PEG unit.

RESULTS

Discrepancy between IFT and Farr assay

Analysis of sera from patients with well defined clinical syndromes in the Farr assay and the IFT, demonstrated a clear cut specificity of both methods for SLE (Swaak *et al.*, 1981). Notwithstanding the fact that the sensitivity of IFT and Farr assay has been found to be in the same order of magnitude, we noticed a huge discrepancy between the IFT and the Farr assay upon screening of 14,417 sera sent to our laboratory for routine anti-dsDNA determination: only 37% of the IFT positive sera were found to be Farr positive (Fig. 1).

To explain this discrepancy, we studied whether the antibodies to dsDNA, measured with the IFT but missed by the Farr assay, are of relatively low avidity. It has been reported that the Farr assay, due to the dissociating conditions of the ammonium sulphate precipitation, does not detect anti-DNA of low avidity (Aarden *et al.*, 1975; Leon *et al.*, 1977), whereas the PEG assay does (Smeenk *et al.*, 1982a). When we used the PEG assay on the 790 IFT positive/Farr negative sera, we found 535 of these to be positive (Figs 1 & 2). Of the 200 tested normal human sera, all but one were found negative with the PEG assay (Fig. 2).

We conclude that the deviating sera contain anti-dsDNA of low avidity, which is measured with the IFT and the PEG assay, but not with the Farr assay.

Reproducibility of the IFT on C. luciliae

While studying the sera that were found IFT positive but Farr negative, sera were frequently re-tested in the immunofluorescence test. It was noticed that sera initially found to be IFT positive

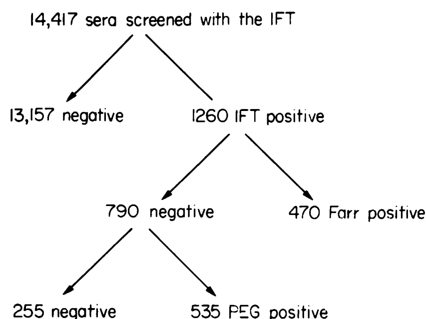

Fig. 1. Screening of 14,417 patients' sera on the presence of antibodies to dsDNA.

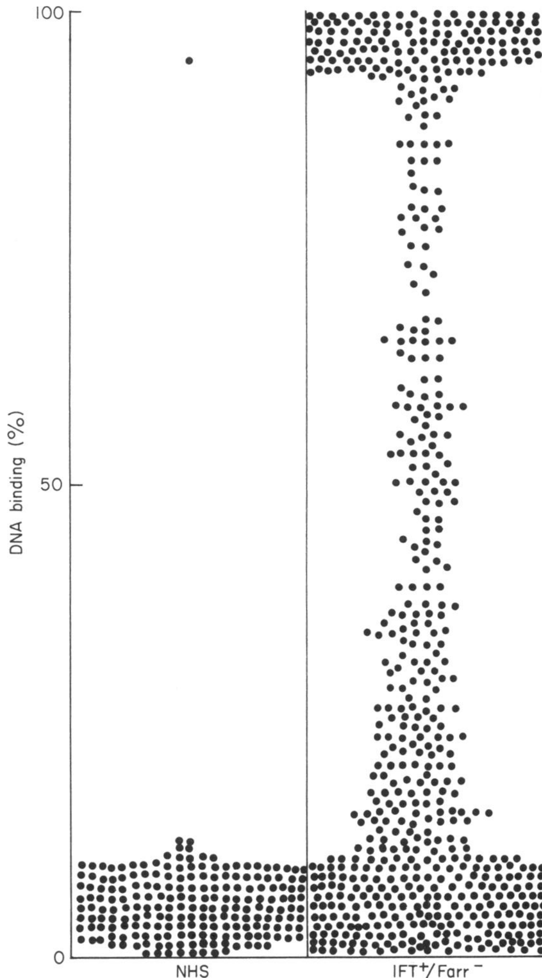

Fig. 2. dsDNA binding measured with the PEG assay of 790 IFT positive/Farr negative sera and of 200 normal human control sera.

then often reacted negatively. To explain this, a number of assay variables were studied. Performing the IFT at different temperatures (4°C, room temperature or 37°C) did not influence the results. Also variation of the incubation periods or washing periods did not affect the outcome of the test. However, changing the molarity of the washing buffer did make a difference.

In a particular experiment, 14 sera were tested; after incubation with serum, the slides were washed, either in PBS containing 0·14 M NaCl, or in PBS containing 0·10 M NaCl. We observed a significant difference: after washing with the 0·14 M NaCl buffer, 29% of the sera were found IFT positive; after washing with the 0·10 M NaCl buffer, this figure became 64%. Normal control sera remained negative, if measured this way. The notion that the salt concentration of the washing buffer is of marked importance was strengthened by the fact that the interaction of IFT positive/Farr negative sera was found to be extremely salt labile (Smeenk *et al.*, 1982a).

It was realized then that, after washing the Crithidia slides with PBS, they were usually left on the table for a while, before the addition of the FITC conjugated antiserum. During this period —of maximally 10 min—slides dried up, resulting in (locally) increased salt concentrations which gave rise to dissociation of DNA–anti-DNA interactions. Prevention of this drying up, by taking the slides out of the washing buffer one by one, followed by immediate addition of conjugate, completely eliminated the reported irreproducibility.

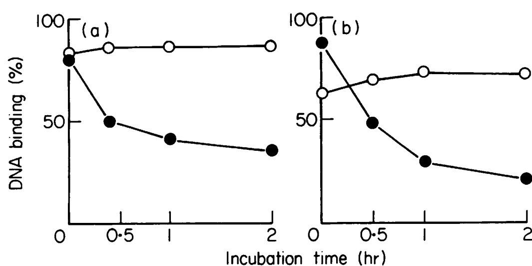

Fig. 3. Variation of the incubation time in the PEG assay. (a) serum VV; before (●——●) and after (○——○) dialysis against PBS (50 μl serum tested). (b) serum HV; with (●——●) or without (○——○) 50 μl NHS (9·1 μl H.V. tested).

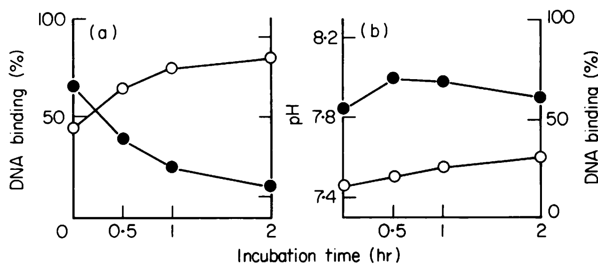

Fig. 4. Relation between DNA binding and pH of the incubation mixture. Serum HV (8 μl) was tested in the presence of 50 μl NHS in the PEG assay. (a), using PBS as diluting buffer; (b), using 50 mM PBS (pH 7·4) as diluting buffer. (●——●) = DNA binding; (○——○) = pH of the incubation mixture.

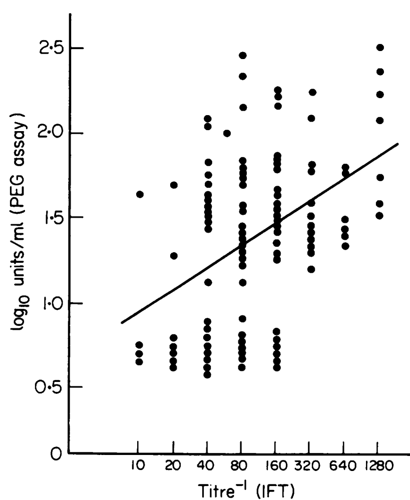

Fig. 5. Correlation between IFT and PEG assay, obtained by titration of 106 IFT positive/Farr negative sera in both assays.

Kinetics of the PEG assay

In the PEG assay, measurement of low avidity anti-dsDNA is also subject to potential artefacts. Normally, patients' sera and DNA are incubated for 1 hr at 37°C, because DNA binding was found to have reached a plateau there. However, when we performed kinetic experiments using sera that contained relatively little anti-dsDNA of low avidity only, i.e. sera of which 50 μl were tested, we found the DNA binding to decrease in time. After dialysis of the sera against PBS, this effect had completely disappeared (Fig. 3a). Sera of which 10 μl or less were tested did not show this phenomenon (Fig. 3b). At first, it appeared that low avidity anti-dsDNA sera contained an inhibitory factor. However, the same effect could be obtained if we added 50 μl of NHS to such a serum (Fig. 3b). So, the factor is contained in every serum.

Studying the influence of pH on the PEG assay we observed the decrease in DNA binding to be completely reflected in an increase of the pH during the incubation (Fig. 4a). Proper pH control, by using a buffer with more buffering capacity than PBS, i.e. 50 mM PBS (pH 7·4), instead of 10 mM, completely eliminated the phenomenon of in-time decreasing DNA binding (Fig. 4b).

Correlation between IFT and PEG assay

One hundred and six sera, randomly chosen from the 790 available IFT positive/Farr negative sera, were titrated in both the IFT on *C. luciliae* and the PEG assay. Results of the PEG assay were, for each serum individually, plotted against the IFT titre (Fig. 5).

The correlation between results obtained with the PEG assay and the IFT was found to be rather high ($r=0{\cdot}44$, $P<0{\cdot}001$). However, it must be kept in mind that the sera used were selected on the basis of IFT positivity. So, a number of IFT positive/PEG negative sera is incorporated in this figure, but IFT negative/PEG positive sera, which indeed also exist (Smeenk *et al.*, 1982b), are missed. (Note. We find inter-assay variations, both in PEG assay as well as in IFT, to be very small.)

DISCUSSION

Since its introduction in 1975, the IFT using *C. luciliae* as a substrate to detect antibodies to dsDNA has been the subject of extensive investigation. Not only its specificity for SLE but also the correlation between IFT and Farr radioimmunoassay have repeatedly been confirmed (Stingl *et al.*, 1976; Slater, Cameron & Lessof, 1976; Crowe & Kushner, 1977; Sontheimer & Gilliam, 1978; Monier *et al.*, 1978; Ballou & Kushner, 1979). Out of 127 SLE sera, Slater *et al.* (1976) found 50% to be positive in both tests, whereas 25% was negative in both tests; 5% of the sera were only IFT positive and 20% only Farr positive, the latter probably because their *E. coli* DNA contained single stranded regions. Monier *et al.* (1978) tested a total of 565 sera and found 56 sera to be positive and 488 sera to be negative in both tests. Fifteen sera were only found positive with the Farr assay, six sera only with the IFT. DNA binding by the latter six sera could be absorbed by nDNA, which proved its specificity. Three out of four of these sera became negative if the slides were washed in hypertonic PBS after incubation with serum. From this, they concluded these sera to contain anti-DNA of low avidity. With respect to the 15 sera found positive with the Farr assay only, they showed that in nine of these, DNA binding was not caused by immunoglobulin.

When we tested sera from patients with definite SLE, we too found a good correlation between Farr assay and IFT. However, when we tested sera referred to us for routine diagnostic anti-dsDNA determination, the correlation was completely lost (Swaak *et al.*, 1981). Furthermore, anti-dsDNA was often detected by the IFT in patients who did not fulfil the preliminary ARA criteria for SLE, and were not diagnosed to have SLE. It was hypothesized that these patients would develop SLE in the future. On the other hand, anti-dsDNA found in these patients differed from anti-dsDNA in SLE patients, as was suggested by the low correlation between Farr assay and IFT found with these sera.

In the study presented here, 14,417 sera were screened for the presence of anti-dsDNA by the IFT on *C. luciliae*. Of the 1,260 sera found positive with the IFT, 63% could not be detected with the Farr assay. The finding that 68% of these IFT positive/Farr negative sera were positive with the PEG assay indicates that the antibodies they contain are of low avidity. This leaves us with a

number of sera that react positively in the IFT, but negatively in both Farr assay and PEG assay. This may be a matter of sensitivity: complexes must be large to be precipitated in the PEG assay (Smeenk & Aarden, 1980), which reduces the sensitivity of the PEG assay compared to the IFT. Furthermore, the mechanisms of the PEG assay and the IFT are also very different: in the former, the antigen is soluble; in the latter, it is fixed; in the former, there is no check on the Ig nature of DNA binding substances, whereas in the latter there is. Also, the observed relation between pH and DNA binding in the PEG assay might lead to false negative results: upon screening, 50 μl serum is tested in the PEG assay; this leads to an increased pH of the incubation mixture, because PBS has not enough buffering capacity to control the pH-increasing effect of CO_2 evaporation from the serum. Use of a stronger buffer prohibited both the pH increase and the DNA binding decrease.

With the Farr assay, we demonstrated before that an increase in pH is the optimal way of dissociating DNA–anti-DNA interactions (De Groot *et al.*, 1980). So, with sera containing relatively little anti-dsDNA of low avidity, the pH increase might very well lead to negative results. However, pH control at pH 7·4 often leads to DNA binding by NHS. Therefore, we here presented only results obtained using PBS as diluting buffer, although we would probably get a better correlation between PEG assay and IFT if we could exclude false negative results of the PEG assay.

During this study, we found the IFT on *C. luciliae* to be rather irreproducible when testing IFT positive/Farr negative sera. From avidity studies using these sera, we knew DNA–anti-DNA interactions of low avidity to be extremely salt labile (Smeenk *et al.*, 1982a). It became apparent that it was of utmost importance to prevent the slides from drying up during the execution of the assay: partial drying up might result in local increases of the salt concentration on the slide, resulting in dissociation of DNA–anti-DNA interactions. Indeed, we found that proper control of these conditions resulted in reproducible results of the IFT.

The authors thank Dr R.C. Aalberse, Prof Dr T.E.W. Feltkamp, Dr C.A.M. v.d. Schaal and Dr A.J.G. Swaak for their critical reading of the manuscript and their stimulating discussions. The assistance of Ms Jetty Gerritsen in preparing the typescript is highly appreciated.

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