A Comparative Study of Technics for the Detection of Antibodies to Native Deoxyribonucleic Acid

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Davis, Paul, Russell, Anthony S., and Percy, John S.: A comparative study of technics for the detection of antibodies to native deoxyribonucleic acid. Am J Clin Pathol 67: 374-378, 1977. Antibodies to native DNA have been assessed by three separate radioimmunoassay methods: the Millipore filter technic, the Farr ammonium sulfate precipitation test, and a commercially available kit method. Although each method was found to be reliably reproducible, the different units by which results are expressed made comparison difficult because of the technical variations inherent in each test. The correlation between tests was, however, high. To allow for interlaboratory standardization, it is suggested that the assay method used be clearly specified. Results are best expressed in terms of mg of DNA bound per deciliter of test serum. It appears that the centrally standardized radioimmunoassay kit is the most convenient method by which these antibodies can be measured in routine laboratories. (Key words: DNA antibodies; Methods.)

ANTIBODIES to native deoxyribonucleic acid (n-DNA) are known to have a high degree of diagnostic specificity for active systemic lupus erythematosus (SLE).\(^7\,\text{15}\) Fluctuations in levels of n-DNA antibodies have also been shown to correlate well with clinical evidence of disease activity or remission.\(^9\,\text{13}\) These antibodies have an important clinical role in the diagnosis and management of patients who have SLE.

Initial methods of detection were based on hemagglutination and immunodiffusion technics, but are of little practical value due to lack of sensitivity\(^15\) and difficulties in quantitation and standardization.\(^8\) The introduction of sensitive radioimmunoassay technics has allowed the amount of antibody directed against n-DNA to be measured much more accurately.

Several radioimmunoassay methods have been devised, of which the Farr ammonium sulfate test\(^19\) and the Millipore filter technic\(^6\) have proved the most popular. These tests have been shown to be reproducible and sensitive within their laboratories of origin, but to date there are few comparative evaluations of the two technics. The difficulties in standardizing anti n-DNA antibody assays are compounded as the number of centers performing and modifying these technics increases.

We have compared the results of DNA antibody levels obtained by the Farr technic with those obtained by Millipore filter assay. In addition, these results have been compared with those of the recently available commercial n-DNA antibody kit (Amersham/Searle).\(^6\)

Materials and Methods

Eighty-eight sera from patients with lupus erythematosus were tested by each method. Samples were taken from a serum bank composed of specimens from patients with systemic lupus erythematosus in varying stages of disease activity. Sera from 101 patients who had rheumatoid arthritis and from 50 normal controls were also tested. Each specimen was tested by the three methods.

All sera used were inactivated by heating to 56°C for 30 minutes. This step is essential to prevent binding of DNA by Clq.\(^3\)

Farr Technic

A 0.05-ml volume of serum was diluted 1:10 in citrate buffer (pH 8.0). Of this diluted serum, 0.05
ml was mixed with 0.05 ml of $^3$H-DNA solution, 9.2 mg/ml (prepared from human amniotic fibroblast culture) and incubated for 1 hour at 37 C. Samples were left overnight at 4 C and 0.1 ml of saturated ammonium sulfate was then added. After thorough mixing for 1 hour at 4 C, the samples were centrifuged for 45 minutes (1,000 x g at 4 C). A 0.1-ml volume of supernatant was removed and added to 0.9 ml of borate buffer, followed by 10 ml of Bray’s scintillation counting fluid. Similarly, the remaining 0.1 ml containing the precipitate (p) was diluted with 0.9 ml of borate buffer and 10 ml of Bray’s fluid added. Radioactivity in the supernatant (S) and precipitate (p) was measured in counts per minute (cpm) in a liquid scintillation counter. DNA binding was calculated by:

$$\frac{\text{cpm (p)} - \text{cpm (S)}}{\text{cpm (p)} + \text{cpm (S)}}$$

and expressed as a percentage.

This technic was repeated, using $^1$C-DNA of Escherichia coli origin at a concentration of 2 mg/ml (Amersham*).

**Millipore Filter Technic**

This technic is based on the fact that DNA that has reacted with protein develops an affinity for nitrocellulose. A 0.025-ml volume of undiluted test serum was gently mixed with 0.01 ml of $^3$H-DNA solution (9.2 mg/ml). Following incubation for 15 minutes at 37 C, the mixture was passed through a 4.5 µm Millipore filter. The test tube and filter were then washed three times with 5 ml citrate buffer, pH 8.0, and once with 5 ml distilled water. After drying under an infrared lamp, the filters were placed in 10 ml DPO Toluene and counted for 10 minutes in a liquid scintillation counter.

**n-DNA Antibody Assay Kit (Amersham/Searle)**

This kit contains all of the reagents and instructions necessary to allow n-DNA antibody assay to be performed in any laboratory equipped with a gamma counter. The n-DNA used in this kit is labeled with $^{125}$I and is extracted from HeLa cells, cultured in nutrient medium containing iododeoxyuridine.$^{125}$I. The DNA is provided at a concentration of 1 mg DNA/ml.

The antibody activity is assayed by a standard Farr technic and the emissions from the precipitate only are counted.

### Table 1. Percentage Serum Samples with Positive DNA Binding from Normal Subjects and from Patients with Systemic Lupus Erythematosus and Rheumatoid Arthritis

<table>
<thead>
<tr>
<th></th>
<th>Farr Assay</th>
<th>Millipore Assay</th>
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<tbody>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>$^{125}$I (Kit)</td>
</tr>
<tr>
<td>Normal ($n = 50$)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Systemic lupus erythematosus ($n = 88$)</td>
<td>63</td>
<td>64</td>
</tr>
<tr>
<td>Rheumatoid arthritis ($n = 101$)</td>
<td>14</td>
<td>0</td>
</tr>
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* Cut-off values for abnormal binding: Farr assay, $^{14}$C < 305 (<0.66 mg DNA/ml); $^{125}$I < 50 units (<0.36 mg DNA/ml); $^3$H < 65 (<0.36 mg DNA/ml); Millipore assay, $^3$H < 10 (<0.37 mg DNA/ml).

**Characterization of Labeled DNA’s**

$^3$H-DNA. This antigen was extracted from an HAE 70 cell line as previously described. The antigen was passed through a Millipore filter to remove single-stranded DNA (ss-DNA) and the presence of residual ss-DNA breaks or ends determined by reductin in fluorescence with ethidium bromide at increased pH and the rate of incorporation of $^{14}$C-labeled formaldehyde. In addition, absence of contamination by single-stranded components was assured by operational criteria, viz., (1) antigen did not react with antibody raised in rabbits to ss-DNA, (2) cold ss-DNA did not inhibit n-DNA binding using test conditions.

Contamination of this antigen by ss-DNA, single-stranded breaks, or ends was found to be less than 1%. Sedimentation coefficient in a sucrose gradient indicates an average molecular weight of 10⁵.

$^{125}$I-DNA. This DNA is extracted from HeLa cell monolayers. Analysis on a caesium chloride gradient in an ultracentrifuge shows a single peak of DNA activity. Selective adsorption chromatography on hydroxyapatite showed less than 1% ss-DNA. The sedimentation coefficient in a sucrose gradient indicated an average molecular weight of 10⁷. Operational criteria with this DNA were found to be similar to those with $^3$H-DNA.

$^{14}$C-DNA. This DNA was characterized by operational criteria and sucrose density gradient only. Average molecular weight was 10⁵.

**Statistical analysis**

Coefficient of correlation $R$ was calculated using Pearson’s parametric coefficient. The p value was cal-
Results

The overall incidence of DNA antibodies in a variety of test sera using the different technics and antigens is shown in Table 1.

The results obtained using the Millipore filter method and the Farr technics showed high degrees of reproducibility, with an experimental error no greater than 5%.

Data from the results of the Farr technic using $^3$H-DNA and $^{14}$C-DNA showed significant correlation ($R = 0.66$, $p < 0.001$) (Fig. 1) when results were expressed in terms of mg of DNA bound/dl of serum. The poor correlation in percentage binding is due to the different concentrations of $^3$H- and $^{14}$C-DNA used in the same assay. Consequently, a ±5% variation in binding (the limit of reproducibility of the test) (Fig. 4) resulted in greater variation in terms of mg bound per dl when the more concentrated $^3$H-DNA was used.

Data comparing results of the Farr technics and the Millipore filter technic are shown in Figure 2. Despite individual variations obtained in paired results of some sera, overall correlation was good ($R = 0.73$, $p < 0.001$). Sera considered to contain unequivocally normal or high n-DNA binding did so with each technic. Sera with borderline abnormal binding by one method were sometimes found to have normal levels as determined by the alternative method.

Data comparing results with the Millipore technic and the anti-DNA assay kit are shown in Figure 3. The overall correlation between results was again good ($R = 0.77$, $p < 0.001$). The assay kit results are expressed as units of DNA bound using a standardized curve plotted from known standards provided. This kit was found to be a highly sensitive method. Patients with n-DNA binding > 30% by the Millipore method were found to demonstrate maximum binding, i.e. > 140 units of DNA antibody, by the kit method. Many patients who had active systemic lupus erythematosus therefore needed greater serum dilution than recommended in the methods to quantitate their DNA binding capacities precisely.

Discussion

Abnormal binding of n-DNA by serum antibody has proved to be a highly specific test in clinical rheumatology. These antibodies have a higher sensitivity than the lupus erythematosus cell phenomenon and a greater specificity than the fluorescent anti-nuclear antibody test (FANA), both of which are still used in the diagnosis of systemic lupus erythematosus. In addition, quantitation of these antibodies makes it a convenient method of monitoring disease.
progression or remission. For these reasons, the test is rapidly becoming a routine clinical service laboratory procedure. Various technics have been devised to detect and measure antibodies to specific polynucleotides, but few comparative trials exist to determine their relative values for routine use.

In our study the overall correlation between the various technics was good, even though different DNA antigens were used. This was particularly true when direct comparison of results was expressed in milligrams of DNA bound by a standard unit of serum, but not when the serum DNA-binding activity was merely expressed as the percentage of DNA bound. This relates both to differences in the technics and to the specific activity and amount of DNA used in each test mixture. Aarden and colleagues have reported that when using identical reaction conditions the Millipore filtration method gives consistently higher binding than does the Farr assay, and that this is a reflection of the dissociation of low-avidity antibodies by the high salt concentration used in the Farr assay. We have not measured antibody avidity in our study, but we found in general a higher degree of DNA binding with the Farr technic than when the Millipore assay was used. This difference was not constant, but was seen particularly in the lower ranges of DNA binding.

This discrepancy probably occurs because the technics described have been standardized on differing and individually appropriate sigmoidal curves that reflect the optimum antigen/antibody ratios suitable for discriminating between normal and disease states. A further explanation for the discrepancies may be due to the DNA used as the antigen in the methods. It has been shown that binding of DNA in both Farr and Millipore filtration methods will increase in a linear relationship with the molecular weight of the DNA used in the test antigen. The DNA’s used in our methods have been well characterized, and both $^3$H and $^{14}$C are known to have molecular weights $= 10^6$. The finding of a molecular weight of $10^7$ in the $^{125}$I-DNA probably accounts for the consistently higher binding seen in the kit method compared with the others. This variation was, however, constant. Contamination of test antigens by single-stranded DNA (ss-DNA), which could produce falsely high results, has been excluded by characterization of each DNA extract.

In the interests of international standardization, it would seem advantageous to express DNA antibodies...
in terms of amount of DNA bound per deciliter of serum. Variations in technics and antigens used by the various laboratories reporting DNA antibodies have not made this possible at present. DNA antibody results must therefore be interpreted in the light of technic and DNA antigen used. Upper limits of normal can only be expressed in terms of the discriminating ability of these conditions to determine disease states from normal conditions. The technics we have compared are both uncomplicated and reproducible. Providing that purified antigen is obtainable, they can readily be performed in any laboratory with counting facilities. The degree of correlation between the various methods suggests that the method used should not significantly alter the discriminating value of these antibodies. The kit method has been centrally standardized in terms of both DNA used and the preparation of a standard curve with controlled sera, and therefore appears to be a particularly suitable method for routine laboratory application.

References