Antinuclear antibodies are almost always found in sera of patients with systemic lupus erythematosus. To differentiate antinuclear antibodies from antibodies to DNA in the recently described *Crithidia luciliae* assay, we developed an immunoperoxidase technique for detecting antibodies to native, double-stranded DNA and compared results by it with those by the Farr assay. Smears of cultured *Crithidia luciliae* were incubated with human sera, peroxidase-labeled anti-human IgG serum, and diamobenzidine. The peroxidase stain was examined by conventional light microscopy, which facilitated differentiation between the kinetoplast and the nuclear stainings. The *Crithidia* assay appeared to be specific for double-stranded DNA antibodies, seemed to be more sensitive than the Farr assay, and allowed us to determine the immunoglobulin classes of antibodies to native DNA. Some patients with systemic lupus erythematosus had only IgM or IgA antibodies to DNA.

**Additional Keyphrases:** lupus erythematosus - "kt" methods - proposed screening test - intermethod comparison

It has long been known that antibodies to nuclear components are present in sera of patients with systemic lupus erythematosus (SLE)\(^2\) (1, 2). Among these antibodies, only antibodies to DNA are considered to be specific for SLE (3, 4). Various techniques were used to detect DNA antibodies, such as double diffusion in agar (4), complement fixation (5), hemagglutination (6), precipitation of radiolabeled DNA-anti-DNA immune complexes with ammonium sulfate (Farr assay, 7), counterimmunoelectrophoresis (8), immunofluorescence (9), radioimmunoassay (10), fluorescent assay (11), and enzyme immunoassay (12).

Production of DNA antibodies seemed important, not only for the diagnosis of SLE but also for evaluating its prognosis and the efficacy of treatment (7). However, reports on the presence of DNA antibodies in patients with various other diseases (13, 14) raised doubts about the specificity of such antibodies for SLE. Only antibodies to native, double-stranded DNA (dsDNA) and not to single-stranded DNA (ssDNA) were considered specific for SLE. The finding of dsDNA antibodies in sera of patients with diseases other than SLE (15-17) was thought to be due to pitfalls in the techniques for measuring antibodies to dsDNA. Virtually all preparations of mammalian DNA used for the detection of dsDNA antibodies were contaminated by ssDNA (18, 19). To circumvent this problem, a test was recently devised in which *Crithidia luciliae*, a hemoflagellate that has a kinetoplast composed of mitochondrial dsDNA, is used (20). In this assay, smears of *C. luciliae* are incubated with sera to be tested and fluorescein-labeled anti-human immunoglobulins serum; fluorescence of the kinetoplast indicates the presence of antibodies to DNA. Sera of SLE patients almost always have antibodies reacting with cell nuclei, i.e., anti-nuclear antibodies (ANA); therefore, a nuclear fluorescence commonly occurs in the *C. luciliae* assay. The nuclear fluorescence is often difficult to differentiate from the kinetoplast fluorescence, especially when sera are diluted for titration purposes.

To obviate this problem, we developed an immunoperoxidase technique for detecting antibodies to dsDNA that is similar to the immunofluorescent technique. We compared the sensitivity and specificity of the immunoperoxidase assay to both the immunofluorescent (20) and the Farr assays. We determined the immunoglobulin classes of dsDNA antibodies and investigated the clinical significance of antibodies to dsDNA for the diagnosis of SLE and its complications.

**Materials and Methods**

**Sampling.** Blood from patients with SLE (as defined by the American Rheumatology Association provisional criteria, ref. 21) or other diseases, as well as blood from normal subjects was collected in sterile evacuated glass tubes (Vacutainer Tubes; Becton & Dickinson, Rutherford, NJ 07070). The sera were heated for 30 min at 56 °C and stored frozen at −20 °C for various time intervals until tested.

**ANA assay.** Mouse-liver sections, 4–6 μm thick, were cut with a cryostat (Ames, Elkhart, IN 46514) and kept at −20 °C for as long as two weeks. The sections were incubated for 30 min at room temperature with sera previously heated for 30 min at 56 °C and diluted 10-fold in 10 mmol/L phosphate-buffered isotonic saline, pH 7.2 (Difco Laboratories, Detroit, MI 48222), rinsed for 30 min with phosphate-buffered isotonic saline, incubated for 30 min with fluorescein-labeled goat anti-human IgG (Meloy Laboratories, Inc., Springfield, VA 22151; lot no. 89361; antibody concentration, 0.8 g/L; total protein concentration, 11.2 g/L; fluorescein to protein ratio, 2.9) or with polyvalent, fluorescein-labeled rabbit anti-human immunoglobulins serum (IgG + IgA + IgM; Behring Diagnostics, Somerville, NJ 08876). Before use, the fluo-
rescein-labeled sera were centrifuged for 20 min at 3000 rpm in a GLC-1 centrifuge (Sorvall, Newton, CT 06470). The fluorescein-labeled sera were checked against the WHO reference serum SBL SH 480010 and were used after appropriate dilutions. The labeled antisera were also checked by immunoelectrophoresis and tested on monoclonal human bone marrow cells. Known ANA-positive and -negative sera were included as controls. Slides were read for fluorescence with an incident-light microscope (vertical fluorescence, Model 2070; American Optical, Buffalo, NY 14215).

C. luciliae immunofluorescent assay (IFA). The test was performed essentially as originally described by Aarden et al. (20). The organisms, obtained from the American Type Culture Collection, Rockville, MD 20852, were cultured in trypticase-peptone medium (BBL, Cockeysville, MD 21030) containing, per liter, 100,000 USP units of penicillin and 100 mg of streptomycin, at 25 °C for 48 h (22), harvested during the logarithmic phase of growth, washed three times with isotonic saline, and suspended in distilled water at a concentration of 2 × 10⁸ organisms per milliliter. Drops of the suspension were applied to glass slides, air dried, fixed in 95% ethanol for 10 min, and stored at −20 °C for no longer than one month. Ethidium bromide (1 g/L in phosphate-buffered saline; Calbiochem, LaJolla, CA 92112) and Giemsa stain (Harleco, Gibbstown, NJ 08027) were used to check the morphology of the organisms. The slides were incubated for 30 min at room temperature with unknown and control sera diluted 10-fold and rinsed with phosphate-buffered isotonic saline, and incubated with each fluorescein-labeled antisera to human IgG, IgA, IgM, IgD, or IgE (Meloy Laboratories, Inc. and Behring Diagnostics) as described above for the ANA assay. The slides were examined for kinetoplast fluorescence under an incident-light fluorescence microscope. Positive sera were serially diluted in phosphate-buffered saline and the highest dilution showing fluorescence was recorded as the titer of anti-dsDNA antibodies. Dilutions of positive sera were tested against dilutions of the fluorescein-conjugated antisera and the optimal dilutions of the conjugate were obtained from this chessboard technique. A constant titer of anti-dsDNA antibodies was obtained over a range of dilutions of the conjugate.

C. luciliae immunoperoxidase assay (IPA). Substrate slides prepared as described above were incubated for 30 min at room temperature, in a moist chamber, with sera diluted 10-fold, rinsed for 30 min in phosphate-buffered isotonic saline, and then layered with peroxidase-labeled rabbit anti-human IgG serum (Cappel Laboratory, Downingtown, PA 19335) for 30 min. After incubation, they were washed with phosphate-buffered isotonic saline for 30 min, briefly rinsed with distilled water, and immersed for 10 min in the dark, at room temperature, in a freshly prepared solution of 3,3′-diaminobenzidine tetrahydrochloride (35 g/L of phosphate-buffered saline; Sigma Chemical Co., St. Louis, MO 63178) with hydrogen peroxide (15 μL of a 300 g/L solution in 100 mL of phosphate-buffered isotonic saline) as previously described (23). The slides were washed with phosphate-buffered saline, dehydrated twice for 1 min, and mounted with Coverbond (Harleco). They were examined under oil-immersion objective with a Microstar Series 10 microscope (American Optical).

Titration of positive sera was done as described above.

Farr assay. A commercial kit (Amer sham Corp., Arlington Heights, IL 60005) was used to do this assay according to the manufacturer. The kit supplied DNA at a concentration of 10 g/L, extracted from a HeLa cell monolayer cultured in medium containing 125I-labeled iododeoxyuridine. The DNA preparation was analyzed for purity and found to be composed of daDNA (24). The results were expressed as units of radio-labeled-DNA bound, and read on a curve plotted from standards provided, which contained bovine serum albumin (10 g/L). Values higher than 25 units/mL were considered positive.

Specificity testing. To ascertain the specificity of the IPA for dsDNA antibodies, we incubated sera from SLE patients that were strongly positive in this assay for 60 min at 37 °C with various amounts of daDNA and ssDNA, centrifuged at 3000 rpm, and tested for inhibition of the peroxidase staining. A DNA solution (1 g/L in phosphate-buffered isotonic saline) was freshly prepared from native DNA extracted from calf thymus (Worthington Biochemical Corp., Freehold, NJ 07728) and passed through a Millipore filter, type HAWP, to remove single-stranded DNA. A portion of this solution was heated in a boiling water bath for 10 min, followed by sudden cooling in iced water; another portion was sonicated for 10 min (Branson Sonifier, cell disruptor, Model 350; Branson Sonic Power Co., Danbury, CT 06810). The optical density of the DNA solutions was checked at 230, 260, and 280 nm with a digital spectrophotometer (Model 191; Hitachi Ltd., Tokyo, Japan).

The specificity of the IPA for daDNA antibodies was also ascertained by digestion of the Crithidia substrate smears. Slides with fixed organisms were pre-incubated with DNase (EC 3.1.4.5, 100 g/L in phosphate-buffered isotonic saline containing 3 mmol of magnesium ion per liter, Worthington) or with phosphate-buffered isotonic saline for 2 h at 37 °C and were layered thereafter with known positive sera, followed by peroxidase-labeled antisera, as described above.

Results

We tested 114 persons for ANA and antibodies to daDNA: 16 were normal controls, 55 had SLE, and the rest had various other diseases.

Specificity of test. Slides with Crithidia incubated only with peroxidase-labeled anti-human IgG antisera did not show endogenous peroxidase staining after treatment with dianaminobenzidine. Ethidium bromide and Giemsa staining showed that the morphology of the organisms was not altered in culture or during preparation of the slides. After pre-incubation with DNase there was no staining of the kineto-plast with both ethidium bromide and peroxidase-labeled anti-IgG; however, the nuclear staining persisted. The amount of dsDNA added to two sera, positive by IPA, to produce disappearance of the peroxidase stain was three to four times greater than the amount of dsDNA producing the same phenomenon (Table 1).

Sensitivity of test. The IPA as described above was as sensitive in our hands as the IFA just described; 24 patients had daDNA antibodies with IPA and 23 with IPA when labeled anti-human IgG was used (Figure 1). Moreover, when sera were tested after serial dilutions, IPA seemed more sensitive than IFA; six patients showed a higher titer with the IPA than with the IFA assay (Figure 1). Assays of the same sera performed on various occasions revealed similar titers with IPA. The highest titer found with the IPA was 800.

The Farr assay, done with use of the Amer sham kit, did not

<table>
<thead>
<tr>
<th>Table 1. Absorption of dsDNA Antibodies in IPA a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
</tbody>
</table>

a Immunoperoxidase Crithidia assay.

b Serum samples (50 μL) were incubated for 60 min at 37 °C with various amounts of DNA preparations before testing in IPA. The figures represent the minimum amount of DNA that inhibited peroxidase staining.
appear more sensitive than the IPA when the cut-off value of 25 units/mL was chosen to indicate positivity for antibodies to dsDNA (Figure 2). Several patients with SLE had low binding values and we considered the results of the test to be negative (Figure 3). One SLE patient had antibodies to dsDNA demonstrable only with the Farr assay.

Immunoglobulin class. Although most patients with SLE had IgG anti-dsDNA antibodies detected with the IPA and IFA, seven patients showed only IgM, one only IgA, and two had IgA and IgM anti-dsDNA antibodies with the IFA (Table 2). The Farr assay was positive in five and negative in two SLE patients who had only IgM anti-dsDNA antibodies. There were no patients who had only IgG class of antibodies to dsDNA, and the IgM anti-dsDNA seemed to be the most common antibodies in our SLE patients. However, in seven SLE patients there was only a very weak kinetoplast fluorescence with sera diluted 10-fold; when these sera were assayed undiluted, the fluorescence was definitely stronger. No antibodies to dsDNA of IgD or IgE classes were found in sera diluted 10-fold. Sera from seven patients who had high titers of IgM, IgG, and (or) IgA anti-dsDNA antibodies were tested for IgD and IgE dsDNA antibodies without dilution; all were negative. Several sera from patients who did not show dsDNA antibodies at 10-fold dilution were assayed undiluted and were found to be negative. Sera which were positive in IFA when either one of fluorescein-labeled anti-IgG, anti-IgM, or anti-IgA was used, were also positive with the polyvalent, fluorescein-labeled anti-human immunoglobulins (IgG + IgA + IgM).

Clinical correlation. There was not a good correlation between the ANA titers and the titers of antibodies to dsDNA or between the clinical renal involvement of SLE and the presence and (or) the titer of dsDNA antibodies as measured by IPA. There was also no correlation between the immunoglobulin classes of dsDNA antibodies and the clinical manifestations of SLE.

Discussion

The IPA is simple to perform and has not shown false-positive results attributable to a possible endogenous peroxidase activity of the *C. luciliae*. Taking into account the fact that we used two different commercial sera, one conjugated with fluorescein and one with peroxidase, we found the IFA to be at least as sensitive as IPA; in fact, one SLE patient had IgG anti-dsDNA antibodies demonstrable only with IPA and not with IFA. The advantages of the IFA over the IFA include the use of conventional light microscopy, the possibility of permanently mounting and storing the slides, and particularly the ease with which one can differentiate the nuclear staining from kinetoplast staining. This differentiation is sometimes difficult (24, 26) and requires experience. Moreover, an additional fluorescence, not localized in the kinetoplast or the nucleus, was recently described (26). In the IPA the morphology of the organisms can be estimated after counter-

![Fig. 1. Comparison between the titers of IgG anti-dsDNA antibodies determined with the *C. luciliae* immunofluorescent and immunoperoxidase assays in SLE patients.](image1)

![Fig. 2. Comparison between the Farr assay (Amersham kit) and the *C. luciliae* immunoperoxidase assay for anti-dsDNA antibodies.](image2)

![Fig. 3. Prevalence of DNA-binding activity in patients with various diseases.](image3)

<table>
<thead>
<tr>
<th>SLE</th>
<th>COLLAGEN DISSEASES</th>
<th>OTHER DISSEASES</th>
<th>NORMALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>IgA</td>
<td>IgM</td>
<td>IgD</td>
</tr>
<tr>
<td>23</td>
<td>7(13)</td>
<td>29(53)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Table 2. Immunoglobulin Classes of Anti-dsDNA Antibodies Detected by IFA* in 55 Patients with SLE

*Immunofluorescent *C. luciliae* assay.

*No. of positive sera; in parentheses, percentages.
staining with Giemsa stain (Figure 4) and inappropriate substrate can be discarded.

It has been shown that the ssDNA can react with some anti-dsDNA antibodies (27); however, the Crithidia assay seemed more sensitive for anti-dsDNA than anti-ssDNA antibodies because, as previously shown (30), dsDNA was more effective on a quantitative basis than ssDNA in absorbing the kinetoplast-binding antibodies. Sonicated DNA was more effective than ssDNA in absorbing antibody to dsDNA (Table 1), perhaps because sonicated DNA contains more dsDNA determinants (28).

The Crithidia assay appeared to be positive mainly in patients with SLE. One patient with rheumatoid arthritis and another patient with arrhythmia, being treated with procainamide, had both IgG and IgM anti-dsDNA antibodies with the IFA but not with the Farr assay. Antibodies to dsDNA detectable with the IFA were reported in patients with drug-induced SLE (29). In a report on IFA, five of 66 individuals with Sjögren’s disease, rheumatoid arthritis, polyarteritis, or Felty’s syndrome showed positive results (30). A positive result with IFA was also found in a patient with myasthenia gravis (31).

The kit used in this study for the Farr assay included highly purified radiolabeled-dsDNA and was shown to be sensitive and reliable for determining dsDNA antibodies (24, 32). In the Farr assay it is not necessary for the proteins binding DNA to be immunoglobulins; any protein which can nonspecifically bind DNA and precipitates with ammonium sulfate will give positive results. Such non-immunoglobulin proteins were found in human serum and spinal fluid (33, 34), which might explain our finding of five patients without SLE whose sera were positive with the Farr assay and negative with the IFA and IFA. In the present study, the Farr assay was not more sensitive than the Crithidia assay, in contrast to previously reported results (30). Furthermore, the binding values obtained in our SLE patients were lower than those shown by others (24). Perhaps the cut-off value arbitrarily chosen in our Farr assay accounted for the lower number of positive results.

As reported by Aarden et al. (20), we also found several SLE sera that showed dsDNA antibodies only with the Crithidia assays. It was suggested that some DNA antibodies are extremely pH dependent and are undetected by the Farr assay (20, 35). The Crithidia assay was shown to detect low-activity antibody, in contrast to the Farr assay (36).

The sensitivity of the various tests for the detection of antibodies to dsDNA could not be properly evaluated in a heterogeneous group of SLE patients because the presence of antibodies was related to the clinical course of the disease (37). It is possible that some SLE sera had a low concentration of dsDNA antibodies, undetectable after the 10-fold dilution of sera (26). However, we used this initial dilution to avoid false-positive results caused by nonspecific attachment of immunoglobulins to the kinetoplast.

The Farr assay measures directly the binding of DNA to anti-DNA antibodies and permits their quantitation. Although the IFA and IFA do not permit a true quantitation of these antibodies, they allow the titration of the positive sera. We could not find a good correlation between the titer of dsDNA antibodies and the severity of SLE. A correlation between DNA antibodies and renal involvement in SLE was reported with use of the Farr assay (7) but not the IFA (29–31). This discrepancy between the results obtained with the two different tests cannot be explained.

The Farr assay does not allow a direct determination of the immunoglobulin classes of dsDNA, in contrast with the Crithidia assays. It has been suggested that immunoglobulin classes and subclasses of ANA could have diagnostic and prognostic significance (38–40). Recently, it was reported that a small percentage of SLE patients exhibited a limited anti-

DNA antibody response, characterized by the presence of chiefly IgM antibodies and mild disease, whereas patients with mainly IgG dsDNA antibodies tended to have a more severe disease. The dsDNA antibodies were detected by the Farr assay after chromatographic fractionation of the sera (41). In our study, SLE patients who only had IgM or IgA anti-dsDNA antibodies did not differ in the severity of the disease from patients who also had IgG antibodies. The rheumatoid factor can confuse the picture, because it combines preferentially with antigen-bound IgG. However, we have seen sera showing kinetoplast fluorescence only with conjugated anti-IgM, i.e., they did not have IgG anti-dsDNA antibodies. If nonspecific attachment of rheumatoid factor were the case, a generalized fluorescence would be expected. Others also reported a lack of nonspecific rheumatoid-factor-dependent anti-IgM fluorescence in the absence of specific IgG (42). Moreover, several sera with IgG and IgM anti-dsDNA antibodies (with and without rheumatoid factor as detected by latex particle agglutination) were incubated overnight at 4 °C with aggregated human IgG (Cohn fraction II, heated to 73 °C for 10 min) to remove rheumatoid factor. There was no disappearance of the specific kinetoplast fluorescence with anti-IgM serum. The relationship between the immunoglobulin classes of anti-dsDNA antibodies and the clinical subsets of activity of SLE should be investigated in further prospective studies. Regardless of the possible clinical significance of the immunoglobulin classes of dsDNA antibodies, it is noteworthy that some SLE patients have only IgM and (or) IgA dsDNA antibodies; therefore, it is important to use labeled antihuman immunoglobulin sera or antisera specific for the three major immunoglobulin classes.

CLINICAL CHEMISTRY, Vol. 25, No. 3, 1979 369
The IPA described here is simple and reproducible, and could be used as a screening test for all patients suspected of SLE.

We thank Mrs. Jacqueline Teitz for preparing the manuscript. This work was supported in part by a grant from the Junior Board of The Buffalo General Hospital.

References