A Staphylococcal Slide Test for Detection of Antineutrophil Antibodies

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We describe a simple test for direct or indirect detection of antineutrophil antibodies. Sensitized leukocytes adherent to glass slides and fixed with pafarormaldehyde can be stored in buffer for at least 3 wk. Killed Cowan I staphylococci, containing protein A, bind to sensitized but not control cells, and binding is ascertainable by light microscopy. Indirect tests were positive for 39/41 patients suspected of having immune neutropenia and found to have antineutrophil antibodies by an indirect radiochemical opsonic method. Fifty-four control sera from healthy persons, patients with bone marrow failure, or with immune complex diseases without neutropenia, gave negative indirect tests. Direct tests for cell-bound antibody could be done even during severe neutropenia by reacting fixed autologous cells with staphylococci in the absence of added serum. In some patients only the direct test was positive.

ANTIBODIES directed against neutrophils are of importance for the pathogenesis of neonatal and acquired neutropenia and are a limiting factor in the efficacy of transfusions. Neutrophil antigens, the targets of such antibodies, are also important in understanding neutrophil surface membrane turnover, and possibly the basis of certain neutrophil function disorders. The attempt to find a sensitive yet specific test has led to the development of many techniques for detecting antineutrophil antibodies. All of these methods require fresh neutrophils, and some require equipment not easily adapted for routine use. Taking advantage of the observation that a gentle fixation with paraformaldehyde does not destroy the antigenicity of neutrophils, we have developed a simple slide test to detect antineutrophil antibodies. Whole staphylococci containing protein A, which binds the Fc portion of IgG, label immunoglobulin on the surface of sensitized neutrophils. This new method is easy and inexpensive to perform without special equipment and allows direct visualization of the cells of interest in both direct and indirect tests. Cells can be stored for at least 3 wk before testing. In this report we describe the staphylococcal slide method and document its sensitivity and specificity.

MATERIALS AND METHODS

Isolation Fixation and Storage of Neutrophils

Four milliliters of each subject's blood was collected in a 12-ml plastic syringe containing 50 U of preservative-free heparin in 1 ml of 0.15 M NaCl. Then, 10 ml of Dextran T-500 (Pharmacia, Piscataway, N.J.), 10 mg/ml in 0.15 M NaCl, was drawn into the syringe and mixed with the blood. The syringe was placed on the hub of the plunger for 10-15 min, during which time the red cells sedimented. Five milliliters of the leukocyte-rich supernate was expressed into a 50-ml Falcon plastic tube (Fisher Scientific Co., Pittsburgh, Pa.) and mixed with 45 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4 (Buffer A). The mixture was centrifuged for 5 min at 62 g at room temperature. Forty-five milliliters of the resulting supernate was aspirated and resuspended in the remaining 5 ml of fluid. An additional 45 ml of Buffer A was added and the centrifugation repeated. The cell pellet was resuspended in 5 ml of fluid (or in only 1 ml if the total neutrophil count was less than 300/μl). Two drops of this cell suspension were placed onto each of 24 9 x 9 mm glass slides (Bellco Glass, Inc., Vineland, N.J.) with a plastic pipette and incubated for 20-30 min at room temperature on a rack in a covered plastic washtub containing a cup of water for moisture. Each slide was rinsed free of nonadherent cells with 2 ml of Buffer A and transferred to a covered slide holder filled with 1% paraformaldehyde for use was prepared every 2-3 wk by diluting the stock solution with 3 vol of Buffer A. Each slide was rinsed free of nonadherent cells with 2 ml of Buffer A and transferred to a covered slide holder filled with 1% paraformaldehyde fixative in Buffer A. A 4% stock fixative solution was prepared by heating 4 g of paraformaldehyde in 100 ml of Buffer A to 70°C and adding dropwise 1 N NaOH until the solution became clear. After cooling, the pH was readjusted to 7.4 with 1 N HCl. This stock solution was kept for 2-3 mo in the dark at 4°C. Fresh fixative for use was prepared every 2-3 wk by diluting the stock solution with 3 vol of Buffer A. The cells were fixed for 1-2 hr at room temperature and then rinsed with Buffer A. If not processed further immediately, the slides were stored at 4°C in sodium azide, 0.2 mg/ml in Buffer A, or in 0.1 M cacodylate buffer, pH 7.4.

Sensitization of Neutrophils

Slides with fixed cells were overlaid with 50 μl of test or control serum and incubated in the covered washtub for 30 min at room temperature. Excess serum was then rinsed off by dipping the slides in Buffer A containing bovine serum albumin, 40 mg/ml (Sigma Chemical Co., St. Louis, Mo.). In each assay, two known positive and two known negative control sera were evaluated in triplicate in parallel with the test sera.

Preparation of the Staphylococcal Suspension

S. aureus strain Cowan I (a generous gift of Arne Forsgren, Department of Clinical Bacteriology, Malmö General Hospital, Sweden) was grown onbrain heart infusion (BHI) agar at 37°C for 24 hr. The culture was suspended in 0.15 M NaCl, and the concentration was adjusted to 10 × 10⁶ organisms/ml. In each test, two known positive and two known negative control sera were used in parallel with the test sera.

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incubation in M NaSO₃ in 0.15 M NaCl at 4°C. When ready for use, the bacteria were washed by suspending 100 µl of the stock preparation in 2 ml of Buffer B (0.05% [v/v] Triton X-100, 0.15 M NaCl, 5 mM Na₂EDTA, 0.2 mg/ml sodium azide, pH 7.4, and centrifuging at 12,800 g for 30 sec. To the resultant pellet of 50 µl of packed staphylococci, 1 ml of Buffer B containing bovine serum albumin, 200 mg/ml, was added and swirled with a vortex mixer to form a suspension.

Detection of Surface IgG

Slides with fixed, sensitized cells were overlaid with 50 µl of the staphylococcal suspension and incubated for 30 min at room temperature in the covered tub. The slides were then immersed sequentially in bovine albumin solutions of decreasing concentration in Buffer A: 40 mg/ml for 2 min, 20 mg/ml for 2 min, and finally in Buffer A without albumin for 2 min. Each 9 × 9 mm slide was then placed upon a larger 2.5 × 7.5 cm microscope slide. A drop of crystal violet solution, 0.1 mg/ml in 0.15 M NaCl, was added to each small slide and a coverslip applied. The slides were examined in the microscope under oil immersion with 1000× magnification. By focusing in and above the plane of adherent cells, the percentage of neutrophils on the upper surface of which 3 or more staphylococci were adherent was determined. Each test was done in triplicate, at least 100 cells were observed, and the average percentage of labeled neutrophils was recorded. A test was considered positive for antineutrophil antibodies if more than 20% of the neutrophils had 3 or more staphylococci adherent, and negative if fewer than 5% of neutrophils were labeled. Tests in the borderline zone of 5%–20% were repeated. The results of any assay were considered uninterpretable if any of the controls failed to give the expected results.

The quantity of antineutrophil antibody in selected sera was estimated by serially diluting those sera in Krebs-Ringer phosphate buffer, pH 7.4, and recording the last dilution at which a positive test was obtained.

Patient and Control Sera and Cells

Normal control blood was obtained from 16 consenting laboratory personnel. None of the controls had been pregnant or received blood transfusions. Serum was prepared by allowing blood to clot in glass tubes for 2 hr at room temperature then centrifuging at 1000 g for 10 min, and storing at −20°C. Sera were referred to this laboratory from 67 neutropenic patients suspected of having antineutrophil antibodies associated with neonatal isoimmune neutropenia, lymphoma, Felty’s syndrome, systemic lupus erythematosus, Graves’ disease, drugs, or idiopathic disease. Fresh blood was also available in 10 cases. Serum containing anti-NA antineutrophil antibodies was kindly supplied by F.W.A. Verheugt (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Additional control blood was obtained from 20 healthy persons, 12 patients with nonimmune neutropenia due to folate deficiency, chloramphenicol, cyclophosphamide, radiation, aplastic anemia, or acute leukemia, and 6 patients with immune complex diseases without leukopenia, including systemic lupus erythematosus, rheumatoid arthritis, Sjogren’s syndrome, and cryoglobulinemia. For special studies, IgG was purified from serum by ammonium sulfate precipitation followed by ion-exchange chromatography on a DEAE-cellulose column. (Fab')² fragments were prepared by pepsin digestion of IgG,²⁶ followed by gel filtration on a Sephadex G-100 column. Purity was assessed by immunodiffusion.

Opsonic Antineutrophil Antibody Test

A radiochemical test for antineutrophil antibodies was performed with gradient-purified neutrophils or mixed leukocytes as previously described.² This test is based on the capacity of normal neutrophils to recognize and respond to sensitized (opsonized) autologous neutrophils by increasing their glucose oxidation.

RESULTS

Rationale for the Conditions Adopted for the Standard Staphylococcal Slide Test

Adequate numbers of neutrophils were easily obtained by simply allowing neutrophils from freshly drawn blood to adhere to glass slides and later rinsing other elements away with Buffer A. However, fibrin and platelets also adhered to glass, and trapped staphylococci, thereby interfering with the assessment of staphylococcal binding to fixed neutrophils. Although ACD and EDTA anticoagulants eliminated fibrin deposition, they also inhibited neutrophil adherence, as did the preservatives in most commercial heparin preparations. Therefore, it was essential to dilute these solutions or to use preservative-free heparin. The dextran sedimentation followed by two slow centrifugations also eliminated platelets. Under standard conditions, blood from normal subjects yielded 200–400 neutrophils per slide. Similar yields were obtained from neutropenic patients by resuspending and concentrating their cells in less buffer.

Unfixed cells did not maintain normal morphology reliably, and they occasionally ingested the staphylococci. Fixation with ethanol, methanol, or acetone under various conditions preserved the neutrophils' morphology but resulted in equal binding of staphylococci to antineutrophil antibody-treated and control cells. Fixation with glutaraldehyde or periodate lysine paraformaldehyde reproducibly promoted heavier labeling of antibody-treated than of control cells, but background labeling of control cells remained unacceptably high. This labeling of control cells was minimized by using the 1% paraformaldehyde fixative for 5–10 min. Thereafter, whether left in fixative or in Buffer A, cells maintained good morphology and reactivity for 4–5 days at room temperature. After more than a week, they lost their morphology if left in buffer or their reactivity if left in fixative. However, if fixed for 2 hr and then kept at 4°C in either Buffer A with azide or in cacodylate buffer, neutrophils retained their morphology and reactivity for at least 3 wk.

For convenience and to permit comparison with the opsonic method, all antisera were applied to cells at room temperature for 30 min. After this sensitization, excess serum was rinsed off by dipping slides in Buffer A. More vigorous rinsing was unnecessary and sometimes diminished subsequent labeling with staphylo-
cocci. To minimize clumping, staphylococci were washed and suspended in the detergent Triton X-100 just before use. Albumin reduced background adherence of staphylococci to the glass slides, but because it promoted clumping of staphylococci, decreasing concentrations were used in successive washes.

**Establishment of Criteria for a Positive Test**

Fourteen sera from neutropenic patients shown by the opsonic method to contain antineutrophil antibodies were used as positive controls and 14 sera from normal subjects as negative controls. With these sera, the slide test for antineutrophil antibodies was performed following the steps outlined above, up to and including treatment of sensitized cells with staphylococcal suspension. The slides were then washed and examined after each wash until all of the negative controls showed fewer than 5% of neutrophils with 3 or more adherent staphylococci. Three washes were found to yield this arbitrary endpoint. Under these conditions, the positive controls showed 20%-80% of neutrophils with 3 or more adherent staphylococci. Accordingly, a positive test was defined as one in which 20% or more of the neutrophils had 3 or more staphylococci adherent after 3 washes, and a negative test as one in which less than 5% of neutrophils were so labeled. Figure 1A shows an oil immersion field from a positive test and Fig. 1B, a field from a negative one.

**Validation of the Staphylococcal Slide Test**

To demonstrate that the adherence of staphylococci to neutrophils depended on a protein A interaction with the Fc portion of IgG, a protein-A-deficient strain of *S. aureus*, Wood 46 (a generous gift of Daniel Lew, Hôpital Cantonal, Geneva, Switzerland) was substituted for and run in parallel with the protein-A-rich Cowan I strain. When the Cowan I strain was used, there were 3 sera that resulted in greater than 80% of neutrophils with 10 or more adherent staphylococci. However, when the protein-A-deficient Wood 46 strain was used with these same sera, no neutrophil had more than 2 adherent staphylococci. In other studies with the Cowan I strain, a positive test was obtained with IgG by itself, when purified from a positive but not from a negative control serum. However, in either case, (Fab')2 fragments used alone gave a negative test. In fact, subsequent sensitization by the positive control serum was blocked when cells were first incubated with the (Fab')2 fragments from a positive but not from a negative control serum.

The 14 sera used to define a positive test were used as controls a total of 380 times and failed to meet the criteria for a positive test only 5 times, 3 times giving borderline labeling of 5%-20%, and twice giving less than 5%. The 14 sera used to define a negative test were also used as controls a total of 380 times, and none gave a false-positive test. Three times, borderline labeling was seen. Thus, 8 of 190 assays were considered uninterpretable because of failed controls.

The opsonic test for antineutrophil antibodies served as our standard of reference. In most cases, sera were evaluated by the staphylococcal method blindly, that is, without knowledge of the findings of the opsonic method or of the subject's clinical condition. As shown in Table I, 95 sera have been evaluated by both methods with concordant results in 89. Sera from 67 patients suspected of having immune neutropenia were tested. Of these, 39 were positive and 22 were negative by both methods. Four sera were positive in the staphylococcal slide test but negative in the opsonic, while 2 other sera gave the opposite pattern. Negative tests were seen with both methods with the sera of 28 controls, including 16 normal subjects, 6 patients with nonimmune neutropenia, and 6 with immune complex diseases without leukopenia. Negative tests were also obtained by the staphylococcal slide method on 26 additional sera from healthy persons or patients with bone marrow failure of various etiologies. With 3 sera tested, the staphylococcal slide method was more sensitive than the opsonic with final titers of 1:5 versus 1:2, 1:18 versus 1:10, and 1:32...
versus 1:4. It was incidentally noted that the average number of staphylococci per cell and the percentage of neutrophils with 3 or more adherent staphylococci were greater with sera that titered out further, both decreasing with each dilution.

**Direct Test for Antibodies on Cells**

Staphylococcal slide tests have been done directly on neutrophils from 27 subjects, 10 of whom were suspected of having immune neutropenia. In this series, the fixed cells were treated for binding of staphylococci without the intervening sensitization and washing steps. As seen in Table 2, of 6 patients who gave positive tests in any system, 4 had positive findings when their sera were tested by either the opsonic or staphylococcal methods and when their cells were tested directly. Two had positive tests only with the direct staphylococcal slide test done on their cells. None of the 17 control subjects had positive tests.

**Other Cell Types**

On all slides, neutrophils were present in greatest abundance. Of the lymphocytes present, most had no adherent staphylococci. However, about 5%–10% were heavily coated with 10 or more staphylococci per cell whether exposed to antisera or not. Neutrophil bands, monocytes, eosinophils, and basophils were also seen, but their scarcity hampered quantitation of staphylococci adherent to them. Nevertheless, it was clear that these cells and leukemic blast cells did not label as heavily as mature neutrophils when sensitized with anti-NA, and most other antineutrophil antisera. As mentioned previously, numerous staphylococci adhered to platelets. In some cases, red cells that had not been rinsed away were coated with adherent staphylococci. It could be shown that this labeling of red cells occurred when type O serum was used on type A but not type O cells, and did not occur when type A serum was used on either type of cells. Neutrophils from individuals with type A red cells did not bind staphylococci when reacted with type O serum or anti A serum.

**DISCUSSION**

It has been shown that the radiochemical opsonic test is a sensitive method for detecting antibodies of etiologic significance in the development of immune neutropenia. We used the opsonic test as a standard of reference for the simple slide method that we have developed. Comparison of results obtained by both methods on a large number of sera shows that the new method detects antibodies in more samples than the opsonic one. These techniques appear quite specific for physiologically important antineutrophil antibodies, giving positive results only in cases in which antineutrophil antibodies are suspected on clinical grounds. Recently, a test for antineutrophil antibody detection with soluble staphylococcal protein A has been has been described. Still, there are patients in whom immune neutropenia is strongly suspected but cannot be demonstrated by any of these methods. Since staphylococcal protein A reportedly binds primarily to IgG of subclasses 1, 2, and 4, this method may miss cases due to IgG type 3, IgA, IgM, complement, or cell-mediated bone marrow suppression. In fact, one might surmise from the concordance between the opsonic and staphylococcal tests that most antineutrophil antibodies detected by the opsonic test include at least some IgG of type 1, 2 or 4.

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<td>Nonimmune neutropenia</td>
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Another possible cause of false negatives in the staphylococcal slide method might be failure of preservation of antigens. However, Na, and the other antigens against which our patients' antibodies were directed were still reactive for at least 3 wk when fixed as described. Preliminary work suggests that preservation may be possible for months for most neutrophil antigens. The development of a storable panel of neutrophil antigens would provide a way to rapidly identify the specificity of different antisera. It is interesting that with this technique we detected activity against A antigens on red cells but not on neutrophils, since claims have been made for and against their presence on granulocytes. 

In addition to its simplicity, the staphylococcal slide test offers a theoretical advantage. Any test that measures immunoglobulin on the surface of neutrophils may detect nonspecific antibodies bound to surface Fc receptors in addition to detecting true antineutrophil antibodies with their Fc portions free and their Fab regions bound to neutrophil antigens. Protein A would label only those antibodies with accessible Fc portions, perhaps avoiding a source of false positives. Since phagocytes recognize only the Fc region of IgG, the opsonic assay has the same specificity. However, since neutrophils can bind immune complexes, it is always a concern whether IgG detected on their surfaces arrived there by this route rather than by being specifically directed against neutrophils. Sera from several patients with rheumatoid arthritis and other diseases in which immune complexes were detected by latex fixation or Raji cell assay did not sensitize neutrophils as detected by the staphylococcal slide test. This does not rule out that in certain cases such complexes might be bound and detected.

Another advantage of the slide technique is that it allows direct visualization of the cells of interest, revealing the distribution of antigens among subpopulations of cells and over the surfaces of individual cells. This feature permits the study of antigen expression in marrow precursors, leukemic cells and other cells difficult to purify. Study of marrow precursors may be especially relevant because some have suggested that the "immune neutropenia" and "maturation arrest" of Felty's syndrome result from a lack of production of cells, rather than peripheral destruction. Very few cells are required, so that a patient's neutrophils can be studied even during severe neutropenia.

The simplicity of the staphylococcal slide tests make them ideal for clinical application for detection of antineutrophil antibodies and elucidation of their significance in the pathophysiology of autoimmune diseases. The potential for developing a storable panel of neutrophil antigens invites use of the tests in a granulocyte crossmatching technique and in genetic studies.

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A staphylococcal slide test for detection of antineutrophil antibodies

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