To the Editor:

Smith and Curnutte give an excellent survey about the molecular basis of chronic granulomatous disease (CGD). Unfortunately, in a short clinical section the nitroblue tetrazolium (NBT) reduction assay is advised as the general method to diagnose this disease. However, "variant" CGD (with retained ability to produce some O₂) can be missed by the semiquantitative NBT slide test with potentially hazardous consequences for the patient. We recently experienced such a patient who was not diagnosed correctly by the NBT test. Moreover, his mother could not be identified as a carrier by this test in three different labs. A continuum of slightly different purple-stained neutrophils was seen rather than two clear-cut subpopulations. However, the typical two
populations could be identified by staining with dihydrorhodamine 123 and flow cytometry, and also by staining with the monoclonal antibody 7D5.

Similar difficulties in the diagnosis of "variant" CGD can occur using, eg, luminol-enhanced chemiluminescence. Because such missing of "variant" CGD is not unique (personal communication, May 1990), methods enabling sufficient and objective quantification of the production of reactive oxygen intermediates like cytochrome c-reduction or flow cytometry should be advised.

Roesler and Emmendorfer raise an important issue in their letter regarding which assays should be used to diagnose those variant forms of chronic granulomatous disease (CGD) in which phagocytes have "leaky mutations" and produce low levels of oxygen radicals. We certainly agree that it is important to diagnose variant CGD because these patients can suffer from many of the same catastrophic infections that plague the typical CGD patient whose phagocytes fail completely to generate oxygen radicals. Of the nearly 60 patients we follow at Scripps Clinic, we have seen six cases of variant CGD from four different kindreds. In four of these six cases, the patients have had episodes of life-threatening infections.

Two factors should be considered when choosing the assay(s) to diagnose variant CGD. The first is whether the assay is of sufficient sensitivity and specificity, especially at the low end of the measured response. In our experience with the six variant patients followed in our clinic, both the cytochrome c assay for superoxide and the nitroblue tetrazolium (NBT) slide test have proven reliable, sensitive, and specific. Typical results are shown in our review article in Fig 1 (CGD 2) and in Fig 4 (panel E). To detect the weak activity present in the variant CGD neutrophils, it is necessary that the unstimulated controls have low background activity (eg, panel A in Fig 4) and that the positive controls (normal fresh neutrophils stimulated with an optimal dose of a strong respiratory burst stimulus such as phorbol myristate acetate) have a sufficiently strong response. We, too, have seen one case of variant CGD where the diagnosis was missed because the weakly positive cells on the NBT test were construed as normal. In reviewing that case, it was apparent that the problem was due to suboptimal stimulation of the corresponding control neutrophils. In our experience, a slightly modified version of the NBT slide test described by Ochs and Igo and by Malawista is quite sensitive. We allow the neutrophils and monocytes from a fresh drop of blood to adhere to a clean, uncoated microscope slide and then stimulate the cells for 15 minutes with an NBT buffer supplemented with phorbol myristate acetate (200 ng/mL). Under these conditions, virtually all of the control neutrophils and monocytes stain strongly positive, while those from typical CGD patients fail to show any staining whatsoever. Variant CGD phagocytes have clearly demonstrable weak staining that is substantially less intense than that seen in control cells. The same assay has been quite successful in identifying the carrier state in X-linked CGD, although the interpretation of the results is complicated by other issues, including the degree of inactivation of the abnormal X chromosome and whether the CGD arose from a spontaneous mutation in a parental cell line. We have also evaluated several of our variant patients with the dichlorofluorescen assay described by Bass et al. This assay is also able to discern weakly positive cells, although our preliminary studies suggest that the fluorescent signal is stronger than might be expected from the low level of superoxide production. We would not recommend this assay as the sole diagnostic procedure because substantial levels of background fluorescence are seen which increase with time in both unstimulated normal and classic CGD neutrophils. The biochemical reactions responsible for this nonoxidase fluorescence have yet to be characterized. Used in conjunction with other respiratory burst assays, however, the dichlorofluorescin assay has many attractive features, including quantitative analysis of thousands of cells at a time. We have not had an opportunity to test the related dihydrorhodamine 123 assay mentioned by Roesler and Emmendorfer. Similarly, we have not evaluated the flow cytometric assay using the monoclonal antibody 7D5 to label surface-expressed cytochrome b. Provided that this latter assay has sufficient sensitivity and specificity (and the antibody becomes commercially available), it will only be useful for evaluating patients with quantitative defects involving cytochrome b and is unlikely to distinguish individuals with normal levels of dysfunctional cytochrome or individuals with defects in cytosolic oxidase components. As with the NBT slide test and the superoxide assay, the experience of a given laboratory in performing the flow cytometric assays will also influence the sensitivity and specificity of these assays.

The second factor is closely related to the first in that the choice of the diagnostic assay should depend on the nature of the laboratory. For most clinical laboratories, a simple and reliable screening method is preferable because experience working with neutrophils may be limited and/or a flow cytometer may not be 

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RESPONSE

Roesler and Emmendorfer raise an important issue in their letter regarding which assays should be used to diagnose those variant forms of chronic granulomatous disease (CGD) in which phagocytes have "leaky mutations" and produce low levels of oxygen radicals. We certainly agree that it is important to diagnose variant CGD because these patients can suffer from many of the same catastrophic infections that plague the typical CGD patient whose phagocytes fail completely to generate oxygen radicals. Of the nearly 60 patients we follow at Scripps Clinic, we have seen six cases of variant CGD from four different kindreds. In four of these six cases, the patients have had episodes of life-threatening infections.

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The second factor is closely related to the first in that the choice of the diagnostic assay should depend on the nature of the laboratory. For most clinical laboratories, a simple and reliable screening method is preferable because experience working with neutrophils may be limited and/or a flow cytometer may not be
available. In this context, we feel that the NBT slide test is the method of choice provided that the appropriate controls are satisfactorily performed. Given the fact that most clinical labs performing this assay will not have access to known CGD patients who can periodically serve as negative controls, it may be appropriate to have CGD screening done by reference laboratories on 24-hour blood samples (eg, references 3 and 5) or by specialized laboratories familiar with phagocyte/CGD biochemistry. In the latter case, we would recommend that several respiratory burst assays be used to establish firmly the diagnosis of both typical and variant CGD. In our own laboratory, we use the following assays: NBT slide test, superoxide assay, dichlorofluorescin assay, cytochrome b spectroscopy, immunoblot analyses of oxidase components, and oxidase gene sequencing. With this type of comprehensive analysis, the diagnosis of CGD can be confirmed and variant forms reliably identified. Given the current technology that allows the genetic subtype of most CGD cases to be determined accurately, it would seem advisable to have all CGD patients analyzed in a major center at least once.

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REFERENCES

Diagnosis of chronic granulomatous disease [letter; comment]

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