Variant Chronic Granulomatous Disease: Modulation of the Neutrophil Defect by Severe Infection


The present studies document the cellular and biochemical processes involved in granulocyte O$_2$\(^{\sim}\) production in three patients from two kindreds with variant chronic granulomatous disease (CGD). Rates of O$_2$\(^{\sim}\) production were 9% to 30% of normal, depending on the individual tested and the stimulus; the two brothers from one family responded to each stimulus with rates very similar to each other. Kinetic analysis of NADPH-dependent O$_2$\(^{\sim}\) production in subcellular fractions revealed all three to have NADPH oxidases with both diminished substrate affinity for NADPH (high K\(_m\)) and decreased maximal velocities of O$_2$\(^{\sim}\) production. Their granulocytes had normal lag times for activation of the respiratory burst but abnormal rates of stimulus-induced membrane depolarization. Cytochrome b was not found in granulocytes or subcellular fractions despite the use of a spectrophotometric assay sensitive enough to detect the cytochrome if its content were proportional to the residual rate of O$_2$\(^{\sim}\) generation. A striking finding in one patient from each kindred was a threefold to tenfold decrease in the rate of O$_2$\(^{\sim}\) production accompanying serious infection. The residual O$_2$\(^{\sim}\)-generating activity of CGD variants helps to explain their relative freedom from the recurrent infections of the classic disease. However, the marked decrease described in the present study indicates the potential for a vicious cycle in which an infection, once established, leads to increasing impairment of host defense.

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CHRONIC GRANULOMATOUS disease (CGD) is a hereditary disorder characterized by recurrent pyogenic infections that may lead to death in childhood. Phagocytes from these patients fail to kill ingested microorganisms because of an inability to produce superoxide (O$_2$\(^{\sim}\)) and related toxic oxygen metabolites. In normal phagocytes, a variety of particulate and soluble stimuli induces a rapid depolarization of the plasma membrane accompanied or followed by activation of a membrane-bound NADPH oxidase that reduces molecular oxygen to O$_2$\(^{\sim}\). Phagocytes from most CGD patients show neither membrane depolarization nor NADPH oxidase activity and hence produce no detectable O$_2$\(^{\sim}\). CGD is inherited in X-linked or, more rarely, autosomal recessive forms; in the former, but not the latter, phagocytes lack cytochrome b$_{245}$, a low midpoint potential cytochrome associated with NADPH oxidase. Alternatively, a flavoprotein component may be absent.

In addition to these classic forms of CGD, several variants have been reported, all with decreased but detectable neutrophil O$_2$\(^{\sim}\) production. Most of these patients presented later in life with milder clinical disease than classic CGD patients. Their neutrophils showed normal or only moderately decreased membrane depolarization responses and altered NADPH oxidase kinetics consisting of an elevated Michaelis constant (K\(_m\)) reflecting decreased affinity for NADPH) with or without a decreased maximum velocity (V\(_{max}\)). In two of the X-linked cases cytochrome b$_{245}$ was undetectable, but in one X-linked case and in the autosomal recessive case, the cytochrome content was normal.

The present study describes two additional kindreds with variant CGD. Both are X-linked, with absent cytochrome b$_{245}$ and near-normal membrane depolarization responses. Two affected patients showed striking, but reversible, decreases in O$_2$\(^{\sim}\) generation, a maladaptive response not previously documented in variant CGD. Neutrophils from two affected, but asymptomatic, siblings in the larger kindred were very similar in their rates of O$_2$\(^{\sim}\) and H$_2$O$_2$ production, depolarization responses, and NADPH oxidase kinetics.

The findings in these patients also raise fundamental questions about the process of O$_2$\(^{\sim}\) generation in normal granulocytes. Their ability to produce some O$_2$\(^{\sim}\) despite an absence of detectable cytochrome b deviates from the direct relationship between cytochrome content and O$_2$\(^{\sim}\) release reported in classic CGD patients and carriers. Furthermore, the defect in membrane potential change in these patients is mild compared with that of respiratory burst activity. These deviations from the relationships previously observed between cytochrome b, membrane depolarization, and O$_2$\(^{\sim}\) generation may require changes in the inferences drawn from classic CGD studies regarding the mechanisms of normal respiratory burst function.

CASE REPORTS

Family J. The propositus, R.J., presented in 1984 at age 15 with respiratory failure resulting from severe Aspergillus pneumonia, probably related to environmental exposure to bark mulch during a summer job on a gardening crew (Fig 1, left panel). He recovered completely after a nearly 2-month hospitalization, with treatment including mechanical ventilation, tracheostomy, amphotericin B, and additional antibiotics (ampicillin and tobramycin) for nosocomial infections with Pseudomonas aeruginosa and Strepto-
Materials and Methods.

coccus faecalis. His past medical history was unremarkable, with no serious or unusual infections. Evaluation of other available family members by nitroblue tetrazolium slide tests revealed CGD in one brother (Re.J.), still entirely asymptomatic at age 9. The mother and one sister proved to be carriers, with slide tests showing mixed populations of normal and deficient cells, the latter identical in appearance to those of the affected brothers (see Results).

Family Q. R.Q. was found to have CGD when his granulocytes were tested for nitroblue tetrazolium reduction shortly after his birth in 1977 (Fig 1, right panel). His brother, previously reported by Orson and Greco,26 had died at the age of 2 months of a disseminated Torulopsis species infection, with the diagnosis of CGD performed by nitroblue tetrazolium reduction testing during the terminal illness. R.Q. had been hospitalized twice for perirectal abscesses (first developed at the age of 18 months) and twice for pneumonia. All infections have responded to prolonged treatment with broad-spectrum antibiotics, including combinations of semisynthetic penicillins (primarily nafcillin and dicloxacillin), aminoglycosides, and trimethoprim/sulfamethoxazole.

MATERIALS AND METHODS

Phorbol myristate acetate (PMA) was purchased from Consolidated Midland Corp, Brewster, NY; Ficoll 400, dextran 500, and Percoll from Sigma Chemical Co, St Louis. All reagents were obtained in the purest form available and used without further purification unless otherwise indicated.

Peripheral venous blood in citrate anticoagulant was obtained from CGD patients and from normal volunteer donors and the neutrophils isolated by dextran sedimentation and Ficoll-Hypaque centrifugation.31 Procedures and consent forms were approved by the University of Massachusetts Medical Center and The Children's Hospital (Boston) Committees on the Protection of Human Subjects in Research.

Superoxide production from pooled cells was measured by a previously described31-33 continuous spectrophotometric assay of superoxide dismutase-inhibitable cytochrome c reduction. Stimuli were PMA (1 μg/mL), arachidonic acid (0.082 mol/L; prepared as in reference 24), and antigen-antibody complexes (prepared from bovine serum albumin and rabbit antiserum as described by Ward and Zvaifler34 and used at a saturating dose of 240 μg/mL). Single-cell analysis for PMA-stimulated nitroblue tetrazolium reduction was performed by nitroblue tetrazolium slide tests as previously described35 using either purified neutrophil suspensions or whole-blood clots as cell sources, with equivalent results.

H2O2 production was assayed by a modification37 of the ferricyanate method of Thurman et al.24 The membrane potential change in response to PMA and antigen-antibody complexes (concentrations and preparation as described for the O2− assay) was measured as the change in emission fluorescence of the dye di-S-C3(5) as previously described.4 The initial linear rate of change (ΔF/min) and the extent of depolarization (ΔF) of the experimental subject’s cells (a measure of the rate of depolarization) were compared with those of a concurrent normal control. The data are expressed as a percentage of normal for the rate of change; virtually identical results were obtained for the extent of depolarization as well, but the data are not presented because of the possible artifact introduced by the differing rates of probe oxidation by the CGD and normal cells.9

NADPH oxidase activity was measured as NADPH-dependent superoxide dismutase-inhibitable cytochrome c reduction at 28 °C in particulate fractions from PMA-stimulated neutrophils.29,30 The NADPH concentration was varied from 0.01 to 4 mmol/L and the data analyzed by the method of Lineweaver and Burk.31 Cell disruption by nitrogen cavitation and fractionation on discontinuous Percoll gradients was performed as previously described.32,33 The resultant three visible bands (α, β, and γ) were separately aspirated, the Percoll removed, and the band contents assayed for cytochrome b, flavoprotein, myeloperoxidase, and vitamin B12-binding protein by previously reported methods.32,34 Cytochrome b content was also measured in whole cells by dithionite difference spectroscopy of cell suspensions in phosphate-buffered saline with 0.2% Triton X-100.34 Intracellular calcium flux was assessed with quin-2 fluorescence by a method of the modification of Lew et al.37 Normal and patient cells (4 × 10⁶) were preincubated in 1 mL of NaCl, 150 mmol/L; KCl, 5 mmol/L; HEPES, 10 mmol/L; CaCl₂, 1.3 mmol/L; and MgCl₂, 1.3 mmol/L (pH 7.4) containing 50 μmol/L quin-2 acetoxymethyl ester for 60 minutes at 37 °C. Cells were then washed and resuspended at 5 × 10⁹/mL in the same buffer containing Ca²⁺ (1 mmol/L) or in calcium-free buffer with 1 mmol/L EDTA. The neutrophils were preincubated in a thermostatted Perkin-Elmer Model 650-105 fluorometer (Perkin-Elmer Corp, Norwalk, Conn) at 37 °C for two minutes and then stimulated from either formylmethionylleucylphenylalanine (10⁻⁶ mmol/L), PMA (150 ng/mL), or arachidonic acid (100 μmol/L) and compared with untreated control cells. The time course of fluorescence change was measured in arbitrary fluorescence units at an excitation wavelength of 339 nm and an emission of 429 nm.

RESULTS

The respiratory burst activities of the variant CGD patients are presented in Table I. The brothers Re.J. and Ra.J. produced similar amounts of O2− and H2O2 in response to a variety of stimuli at different stages of the activation process. Their cells' activity varied from 9% to 30% of normal, depending upon the stimulus. R.Q. produced O2− at a very low rate, 0.5% that of normal controls and well below that of other reported variant CGD patients. Neither patient nor control granulocytes produced any detectable superoxide at rest (data not shown).

During severe fungal or bacterial infection (Aspergillus pneumonia in Ra.J. and perirectal abscess in R.Q.), activity fell dramatically. In Ra.J., granulocytes collected during illness showed 10% to 36% the O2−-generating rate and 20% the H2O2-generating rate of those collected after recovery.
counts on Ra.J during infection and in health showed, during periods of infection. Of NADPH oxidase following stimulation, was normal decreased response in all cells rather than a fall in the proportion. When the total \( \text{O}_2 \) production was rather than the heavy deposits seen in normal neutrophils. Assays of \( \text{O}_2 \) production by normal granulocytes were performed at the same time as the pooled cell \( \text{O}_2 \) measurements, showed the respiratory burst response of the CGD cells to be homogeneously distributed; that is, each cell contained a few grains of formazan rather than the heavy deposits seen in normal neutrophils. When the total \( \text{O}_2 \) production was diminished during infection, the homogeneous pattern persisted, indicating a decreased response in all cells rather than a fall in the proportion of responding cells. The lag time, a measure of the time required for activation of NADPH oxidase following stimulation, was normal (58 to 72 seconds) in PMA-stimulated granulocytes from all three variant CGD patients but lengthened to twice normal during periods of infection. Periventricular blood white cell counts (WBC) and differential counts on Ra.J. during infection and in health showed, respectively, 17 to 29,000 WBC/\( \mu \text{L} \) with 75% to 90% polymorphonuclear and 5% to 7% band forms and 8 to 13,000 WBC/\( \mu \text{L} \) with 50% to 65% polymorphonuclear and no band forms. WBC and differential counts on R.Q. during infection and in health showed, respectively, WBC 15 to 25,000 WBC/\( \mu \text{L} \) with 55% to 62% polymorphonuclear and 0% to 2% band forms and 8 to 16,000 WBC/\( \mu \text{L} \) with 27% to 46% polymorphonuclear and 0% to 2% band forms. Assays of \( \text{O}_2 \) production by normal granulocytes were also performed in the presence of 10% patient’s serum or of patient’s granulocytes in a 1:1 ratio to normal cells. At times of infection, when Ra.J. and R.Q. showed decreased respiratory burst activity, neither their serum nor their cells affected the rate or lag time for \( \text{O}_2 \) by normal cells. Measurements of \( \text{O}_2 \) production and membrane potential change performed on granulocytes from Ra.J. and Re.J. during chicken pox infection showed no change from those obtained when they were healthy.

The membrane depolarization response, one of the earliest events in activation, is presented in Table 2. The rate of change in membrane potential in response to PMA was diminished in all three CGD patients’ granulocytes and fell further during infection in Ra.J. The responses to antigen-antibody complexes were normal in the J. brothers when well but dropped dramatically during infection. R.Q.’s granulocytes showed a diminished response even when he was healthy. All patients’ granulocytes showed normal resting membrane potentials prior to stimulation, indicating that they had not been previously activated.

The rise in intracellular calcium, another early component of granulocyte activation, was normal in Ra.J.’s cells stimulated with formylmethionylleucylphenylalanine, PMA, or arachidonate at the time of infection (data not shown) when \( \text{O}_2 \) production and membrane depolarization were decreased. The assay was calibrated only for the change in calcium concentration, not its absolute value, so the resting calcium level could not be determined.

To further define the defect in cellular \( \text{O}_2 \) production, we examined the kinetics of NADPH-dependent \( \text{O}_2 \) generation in a particulate fraction prepared from granulocytes by sonication and differential centrifugation. As shown in Table 3, all three variant CGD patients had NADPH oxidase with both altered substrate affinity (high \( K_m \)) and decreased maximal velocity. Enzyme preparations from Ra.J.’s granulocytes during health and illness showed no appreciable difference, in contrast to the major changes in whole-cell \( \text{O}_2 \) generation (Table 1). In mixing experiments, combinations of particulate fractions from Ra.J.’s and normal granulocytes showed no significant inhibition of the normal NADPH oxidase activity.

Cytochrome b, a component of the NADPH oxidase that is measurable independently from its enzymatic activity, was undetectable in all three patients’ granulocytes (Table 4). However, analysis of subcellular fractions of Ra.J.’s granulocytes showed a normal distribution of other markers including myeloperoxidase, predominantly in the α-band (primary

### Table 1. Respiratory Burst Function by Variant CGD Granulocytes

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>( \text{O}_2 ) production (Rate (nmol/min/10^6 cells))</td>
<td>PMA</td>
<td>0.79</td>
<td>0.74</td>
<td>0.27</td>
<td>0.28</td>
<td>.046</td>
</tr>
<tr>
<td></td>
<td>Arachidonate</td>
<td>1.17</td>
<td>1.03</td>
<td>&lt;0.1</td>
<td>0.22</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Ag/Ab</td>
<td>0.38</td>
<td>0.75</td>
<td>0.75</td>
<td>ND</td>
<td>3.34 ± 0.86</td>
</tr>
<tr>
<td>Lag time (s)</td>
<td>PMA</td>
<td>69</td>
<td>74</td>
<td>155</td>
<td>67</td>
<td>144</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 ) production (Rate (nmol/min/10^6 cells))</td>
<td>PMA</td>
<td>1.61</td>
<td>1.43</td>
<td>0.28</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Abbreviations:** ND, not determined; Ag/Ab, antigen-antibody complexes.

*Measurements performed during severe infection (see Case Reports).

Data for the patients represent the means of three to six experiments, with ranges less than 20% of the means, and data for normal controls represent the mean ± SD of ten experiments (\( \text{O}_2 \)) and five experiments (\( \text{H}_2\text{O}_2 \)). Unepaired t tests showed \( P < .01 \) for comparisons to normal values for all patient \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) rates and for Ra.J. * and R.Q. * lag times.

### Table 2. Rate of Membrane Depolarization by Variant CGD Granulocytes

<table>
<thead>
<tr>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>38</td>
<td>41</td>
<td>16</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Ag-Ab</td>
<td>91</td>
<td>113</td>
<td>4.6</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

*Measurements performed during severe infection (see Case Reports).

Data represent means of two experiments, each performed in duplicate or triplicate, with less than 20% difference between experiments. Values are the percentages of concurrent normal controls.
granule); and vitamin B₁₂-binding protein, in the β-band. A flavin adenine dinucleotide-containing flavoprotein, another constituent of NADPH oxidase reported to be absent in some CGD patients’ granulocytes, was present in normal quantity and subcellular distribution (Table 4) including the γ-fraction, the site of NADPH oxidase activity.

### DISCUSSION

The present studies document the cellular and biochemical processes involved in granulocyte O₂⁻ production in three patients from two kindreds with variant CGD. All three show decreased, but detectable, O₂⁻ generation by abnormal NADPH oxidases that have both diminished substrate affinity for NADPH (high Km) and decreased maximal velocities of O₂⁻ production. Their granulocytes also have abnormal rates of stimulus-induced membrane depolarization and undetectable cytochrome b.

A striking finding in two patients was the threefold to tenfold decrease in O₂⁻ production accompanying serious infection in Ra.J. (Aspergillus pneumonia) and R.Q. (perirectal abscess). The prolongation of the lag time and decrease in the rate of membrane depolarization during infection along with the lack of change in the kinetics of activated NADPH oxidase suggest an acquired defect in cell activation rather than a direct effect on the O₂⁻-generating enzyme. Transient abnormalities in bactericidal activity have been reported in otherwise normal patients at times of infection, but specific examination of respiratory burst function has shown an increase in activity in most studies and a decrease in others (reviewed in reference 38). This variability may represent the end product of the opposing effects of neutrophil priming deactivation, exhaustion, immaturity, and effects of antibiotics. Thus the change observed in variant CGD cells may represent an exaggeration of the normal granulocyte response to infection or a unique problem related to the described total activation defect in classic CGD. The residual O₂⁻-generating activity of CGD variants helps to explain their relative freedom from the recurrent infections of the classic disease. However, the marked decrease described in the present study indicates the potential for a vicious cycle in which an infection, once established, leads to increasing impairment of host defense.

The two brothers, Ra.J. and Re.J., show very similar granulocyte O₂⁻ production and NADPH oxidase kinetics. Thus, there is no evidence of variability in expression of the variant CGD genetic defect in this family, at least in times of health. In contrast, the patients described by Lew et al and by Styrt and Klempner, although second cousins, showed fivefold differences in PMA-stimulated O₂⁻ production and in the maximal velocity for particulate fraction NADPH oxidase activity. However, the assays were performed by different laboratories at different times.

The absence of cytochrome b from the granulocytes of these patients with X-linked variant CGD is consistent with the genetic pattern observed in classic CGD and in two previously reported variant cases but not in a third apparent X-linked variant. If the cytochrome b content were proportional to the residual rate of O₂⁻ production in CGD variants, it should have been detectable in the J. family. Its absence suggests the possibility of a cytochrome-independent alternative pathway of O₂⁻ production in variant CGD granulocytes; the kinetic data indicate that the pathway might have less affinity for NADPH and lower reaction velocity than the normal oxidase. Alternatively, an amount of cytochrome b below the limit of detection may suffice for the O₂⁻ production observed. In resting granulocytes, most of the cytochrome is found in the secondary granules and only 12% in

### Table 3. Michaelis Constant and Maximal Velocity of NADPH Oxidase from Variant CGD Granulocytes

<table>
<thead>
<tr>
<th>Subj</th>
<th>Re.J</th>
<th>Re.J</th>
<th>R.Q</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km (mmol/L)</td>
<td>0.77</td>
<td>1.44</td>
<td>1.03</td>
<td>8.40</td>
</tr>
<tr>
<td>Vmax (nmol O₂⁻/min/mg protein)</td>
<td>3.70</td>
<td>3.06</td>
<td>2.25</td>
<td>2.23</td>
</tr>
</tbody>
</table>

*Measurements performed during acute infections (see Case Reports).

Results represent the data of Lineweaver-Burk calculations performed on pooled data from three (patient) and eight (control) experiments.

### Table 4. Cytochrome b Content of Granulocytes and Distribution of Markers in Subcellular Fractions

<table>
<thead>
<tr>
<th>Subject</th>
<th>Fraction</th>
<th>Cytochrome b (pmol/mg Protein)</th>
<th>Flavoprotein (pmol/mg Protein)</th>
<th>Myeloperoxidase (U/mg protein)</th>
<th>Vitamin B₁₂⁻ Binding Protein (mg/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re.J.</td>
<td>whole cells</td>
<td>&lt;10</td>
<td>82</td>
<td>4.3</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>&lt;10</td>
<td>82</td>
<td>4.3</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>&lt;10</td>
<td>82</td>
<td>4.3</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>γ</td>
<td>&lt;10</td>
<td>82</td>
<td>4.3</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>S₂</td>
<td>&lt;10</td>
<td>82</td>
<td>4.3</td>
<td>0.02</td>
</tr>
<tr>
<td>R.Q.</td>
<td>whole cells</td>
<td>&lt;10</td>
<td>82</td>
<td>4.3</td>
<td>0.02</td>
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<tr>
<td>Normal</td>
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<td>4.3</td>
<td>0.02</td>
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<tr>
<td></td>
<td>β</td>
<td>442</td>
<td>147</td>
<td>2.3</td>
<td>16.7</td>
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<tr>
<td></td>
<td>γ</td>
<td>225</td>
<td>83</td>
<td>0.4</td>
<td>0.59</td>
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<tr>
<td></td>
<td>S₂</td>
<td>&lt;10</td>
<td>15</td>
<td>0.4</td>
<td>0.59</td>
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</table>

*Bands from discontinuous Percoll gradients as described in Materials and Methods and defined in reference 32.

Results for whole-cell cytochrome b represent data from four experiments (patients) and the mean ± SD for four experiments (normal). Data for cell fractions are presented from one experiment, with results (except for the absence of cytochrome b in RaJ cells) similar to those previously reported from the same laboratory.
the plasma membrane.\textsuperscript{32,33} Possibly, only the latter, the minority, of the total cell content might be essential for \(O_2^\cdot\) respiratory burst activity. In contrast, these and some other CGD patients present a different problem for the theoretic connection between plasma membrane potential change and \(O_2^\cdot\) production. In classic CGD granulocytes, there is no membrane potential response to phagocytic stimuli,\textsuperscript{3,4} whereas in the variant CGD granulocytes, the depolarization response is normal or, if decreased, not nearly in proportion to the defect in \(O_2^\cdot\) generation. That is, the membrane depolarization and respiratory burst defects do not remain proportional in CGD variants, providing an example of a dissociation between the two processes and indicating that normal depolarization is not sufficient to initiate a normal respiratory burst. Thus these cases, like other rare variants of unusual diseases, represent experiments of nature that can guide experiments in the laboratory to the elucidation of normal as well as pathologic physiology.

\textbf{ACKNOWLEDGMENT}

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Variant chronic granulomatous disease: modulation of the neutrophil defect by severe infection

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