X-linked Inheritance in Females with Chronic Granulomatous Disease

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ABSTRACT Chronic granulomatous disease in males is familial and its transmission is usually clearly x-linked. The mode of inheritance in females with the syndrome is unknown and the carrier state difficult to identify. Defective polymorphonuclear leukocyte bactericidal activity in this disease is associated with an absence of the respiratory burst generated in stimulated phagocytes and may be detected by the chemiluminescence assay. Polymorphonuclear leukocytes from three of four females with chronic granulomatous disease had extremely low chemiluminescence production, their asymptomatic mothers had intermediate values, and their fathers were normal. Polymorphonuclear neutrophils of two affected males in these kinships generated no chemiluminescence, whereas two of seven female relatives had intermediate values, and all nonaffected males had normal values. In the three families in which leukocytes were studied by nitroblue tetrazolium reduction, two populations of neutrophils were demonstrated for the female patients and/or their mothers. The wide phenotypic variability for clinical disease, evidence of two leukocyte populations in the patients or their mothers, and low but detectable leukocyte chemiluminescence in the affected females is consistent with the Lyon hypothesis of x-chromosome inactivation in these families. The findings suggest an x-linked inheritance in these females with chronic granulomatous disease.

INTRODUCTION

The syndrome of chronic granulomatous disease of childhood is characterized by severe recurrent infections of the skin, subcutaneous tissues, and reticuloendothelial system (1, 2). The functional defect has been identified as a severe impairment in polymorphonuclear leukocyte intracellular microbial killing (3, 4). Polymorphonuclear leukocytes in these patients phagocytize bacteria normally but fail to generate the normal respiratory burst associated with phagocytosis of opsonized particles (5, 6) or stimulation by soluble nonparticulate agents (7). Activation of the respiratory burst in normal polymorphonuclear leukocytes is associated with increased oxygen consumption and generation of oxidative intermediates including superoxide anion (8–10), hydrogen peroxide (11), the hydroxyl radical (12, 13), and singlet oxygen (14, 15). Oxidative intermediates are essential for normal microbialicidal activity (4). The depressed intracellular killing of polymorphonuclear leukocytes in chronic granulomatous disease has been attributed to the absence of these intermediates.

Respiratory burst activity in normal polymorphonuclear leukocytes is associated with generation of light energy as chemiluminescence (16–18). This light production is dependent on oxidative intermediates and chemiluminescence can be used as a measure of oxidative metabolism (19–21). The sensitivity of the chemiluminescence assay for detecting oxidative intermediates has been greatly increased by using the cyclic hydrazide, luminol, which reacts as a substrate with these intermediates (22, 23). Stimulated polymorphonuclear leukocytes of patients with chronic granulomatous disease fail to generate oxidative intermediates or produce chemiluminescence (24).

Genetic transmission in the majority of chronic granulomatous diseased males has been established as x-linked recessive inheritance (25). In family studies, the asymptomatic mothers can be identified as heterozygous carriers by intermediate in vitro leukocyte function, the fathers are normal, and some female relatives can be identified as carriers. The male to female ratio in this disease of seven to one (2) also suggests x-linked transmission. On the other hand, chronic granulomatous disease in females (26–35) and in some boys without demonstrable leukocyte defects in either parent (36–38) has suggested another mode of inheritance.
We report here luminol-amplified polymorphonuclear leukocyte chemiluminescence and histochemical nitroblue tetrazolium (NBT) studies in four females with chronic granulomatous disease and their families. The findings support an X-linked transmission in all four families.

METHODS

Patients. Four families, each with one female and a total of two males with chronic granulomatous disease, were studied. These four females represented the total number of females with chronic granulomatous disease in our patient population; none were excluded. The diagnosis of chronic granulomatous disease in both male and female patients has been previously established on the basis of clinical history, markedly reduced polymorphonuclear leukocyte killing of Staphylococcus aureus 502A performed by the method of Quie et al. (3) and failure to reduce NBT dye according to the method of Bakker and Nathan (26) as modified by Windhorst et al. (25).

The polymorphonuclear leukocyte defects of the female patient from family 4 have been previously reported in detail and include significantly diminished oxygen consumption, oxidation of 1-[^14C]glucose during phagocytosis of latex particles (27) and leukocyte glutathione peroxidase activity as compared with the activity of control cells (39).

Family 1. A 15-yr-old white girl was well until 3 yr of age when she developed extensive pulmonary infiltrates with metastatic skin lesions (40). Hyphae of Aspergillus were identified in both the skin and lung biopsies and Aspergillus fumigatus was cultured from the lung biopsy material. The lesions in the lungs and skin cleared on aerosol nystatin therapy. Biopsy-proven pulmonary aspergillosis recurred at 4 and 10 yr of age. She had three additional episodes of clinically similar but unbiopsied pulmonary diseases that were assumed to be due to recurrent aspergillosis. One of these episodes was associated with granulomata of the skin which showed histologic evidence of hyphae. One male sibling and all other family members are healthy. The patient and her mother are products of nonconsanguineous marriages. The pedigree chart (Fig. 1) notes the distribution of the leukocyte abnormalities within the family.

Family 2. A 35-yr-old white woman had recurrent staphylococcal skin abscesses since infancy. In addition, sterile abscesses had been noted at the injection site of each childhood immunization. At 15 yr of age she underwent resection of the upper pole of the left kidney for abscess formation complicating chronic pyelonephritis. At age 19, an infected pilonidal sinus was resected. At age 21, chronic cavitary pulmonary lesions formed to removal of the right middle and right lower lobes of the lung. A Pseudomonas species was cultured from biopsy material. The pathology of the lung tissue showed numerous noncaseating granulomata with many of the macrophages containing distinct, almost glossy, brownish pigment located in the cytoplasm in the vicinity of the nucleus (41). This woman’s son, the product of a nonconsanguineous marriage, had staphylococcal pustules noted at birth. At 15 mo of age, after 3 mo of vomiting, he was found to have a mass lesion obstructing the lumen of the gastric antrum. Biopsy showed many areas of granulomata formation with acute central necrosis and heavy eosinophilic in-

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1 Abbreviations used in this paper: DMSO, dimethyl sulfoxide; HBSS, Hanks’ balanced salt solution; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate.
Preparation of cells. Peripheral blood was drawn by fingerstick into three 65-μl heparinized minimum 3 U. S. Pharmacopeia units ammonium heparin per tube capillary tubes (Dade Div., American Hospital Supply Corp., Miami, Fla.), incubated in 5 ml ice-cold 0.87% NH₄Cl for 10 min at 22°C. Residual leukocytes were washed twice with Hank's balanced salt solution (HBSS) pH 7.4 (without phenol red) to which 0.1% gelatin (wt/vol) was added (gel-Hanks) and the cells resuspended in 1.5 ml gel-Hanks. 75 μl of the cell suspension was carefully mixed with an equal volume of counting solution (0.02% gentian violet in 3% acetic acid) and the cells counted on a hemacytometer. Cells were suspended to 2.5 × 10⁶/ml gel-Hanks.

Preparation of particles. To prepare the zymosan (Schwarz/Mann Div. Becton, Dickinson & Co., Orangeburg, N. Y.) suspension, 500 mg of zymosan was suspended in phosphate-buffered saline (PBS), pH 7.4, incubated in a boiling water bath for 1 h, sedimented at 1,000 g and resuspended in PBS. Zymosan was opsonized by incubating one part zymosan and three parts serum (obtained from three donors) at 37°C for 15 min. The suspension was resuspended at 1,000 g and the zymosan pellet resuspended in PBS to the original four-part volume. Opsonized zymosan suspensions were kept at 4°C in the dark until used.

Luminol (5-amin-2,3-dehydro-1,4-phthalazinedione) (Eastman Kodak Co., Rochester, N. Y.) was prepared as a 275-μM stock solution in dimethyl sulfoxide, luminol was diluted in PBS (pH 7.4) to a final concentration of 1 μM per scintillation vial.

Chemiluminescence assay. The chemiluminescence mixtures were prepared at ambient temperatures in glass scintillation vials by adding 5 mg of opsonized dark-adapted zymosan suspension, 20 μl of stock luminol and enough PBS to give a final volume of 5.5 ml (43). The vials were evenly spaced in a beta counting spectrometer, model LS-100C (Beckman Instruments, Fullerton, Calif.), adjusted out of coincidence, and the reaction mixtures allowed to become dark-adapted over 20 min. Background counts were obtained. Under red illumination, polymorphonuclear leukocytes were added to each vial at zero time, the phagocytic mixture agitated for 30 s and each vial counted for 1 min at ambient temperature. The vials were counted sequentially every 7 min for a total of 60 min. Background counts obtained from unstimulated granulocytes were subtracted from the values obtained from stimulated cells. Preliminary studies had shown that when light emission was measured continuously with readings obtained every 30 s, chemiluminescence consistently peaked between 8 and 10 min. Therefore, peak activity at 9 min was compared for statistical analysis. The results, however, were entirely comparable when expressed as an integral value or as peak activity. All assays were performed in duplicate.

Viability of polymorphonuclear leukocytes was >99% viable when assessed by trypan blue dye exclusion (0.1% trypan blue dye, Gibbco Laboratories, Grand Island Biological Co., Grand Island, N. Y.).

The data was tested for statistical significance by use of Student's t test (P > 0.05, not significant).

Assay of NBT reduction. All chemicals used were reagent grade. Phorbol myristate acetate (PMA, 12-O-tetradecanoylphorbol-13-acetate, mol wt 616, from Consolidated Midland Corp., Chemical Div., Katonah, N. Y.) was dissolved in dimethyl-sulfoxide (DMSO) at a concentration of 1 mg/ml and kept frozen at −70°C between experiments. Before use, stock PMA was thawed and diluted in HBSS to 0.1 μg/ml. NBT (grade III, Sigma Chemical Co., St. Louis, Mo.) was prepared by dissolving 16.8 mg of NBT powder in 200 μl of DMSO at 37°C, and diluting this in 8.85 ml HBSS. 0.5 ml pooled human serum was added to each 0.9 ml of NBT solution to make the NBT serum solution. The PMA and NBT serum solutions were brought to 37°C just before use.

Heparinized venous blood (10 U heparin/ml of blood) was allowed to settle by gravity on lymphocyte separation media (Litton Bionetics, Kensington, Md.) for 40 min at 22°C and theuffy coat removed. 30 μl of buffy coat was placed in a polystyrene 1.5-ml conical vial (Bolab Inc., Derry, N. H.) and 100 μl of PMA (1 μg/ml) was added. The suspension was incubated in a moist chamber for 10 min at 37°C. 150 μl of the cell suspension was placed on a clean glass coverslip and further incubated in a moist chamber for 30 min at 37°C to allow the stimulated cells to adhere to the glass. The coverslips were removed from the chamber and gently washed in sterile normal saline (pH 7.4) to remove all nonadherent cells. 100 μl of the NBT serum solution was placed in the center of a clean, alcohol flamed glass slide and the coverslips inverted over the NBT serum solution, which was incubated in a moist chamber for 20 min at 37°C. The coverslips were carefully removed from the glass slides, washed with normal saline, and air dried at ambient temperature. The coverslips were fixed with absolute methanol for 5 min, rinsed with water, stained with 1% safranin stain for 5 min, refreshed with water and allowed to air dry. The coverslips were then mounted on clean glass slides and read by two independent observers. 400 cells were counted on each coverslip and classified as NBT positive or NBT negative. Cells were judged NBT negative on the basis of two criteria; (a) their nuclear morphology was intact and they could be identified as neutrophils and; (b) their nuclei stained pink and was not associated with any blue precipitate. Cells were judged NBT positive if they were associated with blue precipitates either in their nuclei or cytoplasm; NBT positive cells could have either intact or distorted nuclei. Controls were unstimulated donor leukocytes incubated in HBSS containing 0.01% DMSO instead of PMA (PMA was dissolved in 0.01% DMSO in HBSS). All tests were performed in triplicate.

RESULTS

Chemiluminescence. Polymorphonuclear leukocyte luminol-amplified chemiluminescent response to
opsonized zymosan was measured in the families of four females with chronic granulomatous disease. One female patient (family 4) (27, 39) was not available for chemiluminescence studies.

Kinetic analysis of the chemiluminescent responses of leukocytes from patients, relatives, and controls showed peak response occurred between 8 and 10 min after initiation of the reaction. When leukocyte luminescence was monitored every 7 min as demonstrated for family 1, (Fig. 5), peak values consistently occurred at 9 min. For statistical purposes, peak chemiluminescence values at 9 min were compared for members of all families and their controls (Fig. 6).

The chemiluminescent responses of leukocytes from the three female patients were significantly depressed (11±4 × 10^3 cpm, ±1 SD) as compared with normal (control) leukocytes (422±55 × 10^3 cpm) (P < 0.001; Fig. 6) but were similar to their male relatives with the disease (0 cpm). Leukocytes from the mothers of the four female patients produced chemiluminescent responses intermediate (254±38 × 10^3 cpm) between patient and control values (P < 0.001; Fig. 6). The range of values of the chemiluminescent responses of the mothers’ leukocytes (216–298 × 10^3 cpm) was below the range of values for 70 different controls (325–565 × 10^3 cpm). The chemiluminescent responses of leukocytes from the patients’ fathers (451±81 × 10^3 cpm) and two brothers (528 × 10^3 cpm, and 442 × 10^3 cpm) were within 2 SD of control values, whereas those from the patients’ sisters varied from relatively low values (56 × 10^3 cpm) to normal values (Fig. 6). All assays were performed in duplicate. The patients and their parents were assayed on at least two different occasions and the values shown are a mean of these assays.

**NBT reduction.** According to the Lyon hypothesis (44), there should be two distinct populations of polymorphonuclear neutrophils and no hybrid cells in the neutrophil population of a female who is heterozygous for an x-linked recessive gene phenotypically expressed in neutrophil function (44). To determine whether the Lyon hypothesis was operative in our population of female patients, the peripheral blood leukocytes of three females with chronic granulomatous disease and their mothers were examined histochemically by NBT reduction. Under the experimental conditions

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**FIGURE 5** Chemiluminescent response of polymorphonuclear leukocytes exposed to opsonized bacteria in the presence of 1 μM luminol. The female patients’ cells generated a poor chemiluminescent response to zymosan, the patients’ mother had a response intermediate between the patient and normal (control) cell responses. Cells from the patients’ son had no chemiluminescent response, whereas the patients’ brother and father had responses similar to those of normal control cells. CGD, chronic granulomatous disease.

**FIGURE 6** The mean peak chemiluminescent response of polymorphonuclear leukocytes phagocytizing opsonized zymosan in the presence of 1 μM luminol. The stippled area represents the range of responses from 70 different normal donors. The number of individuals studied are shown in brackets. Cells from the male chronic granulomatous disease patients produced no chemiluminescent response and those from the female patients, very little response. The responses of cells from all of the mothers and two sisters were significantly less than those of 70 normal donors (controls), P < 0.001. Cells from the fathers, brothers, and two sisters were all within the range of the normal donors.
Figure 7 Light micrographs of NBT slide test. Buffy coat leukocytes were incubated (A) with HBSS alone or (B and C) with 1 μM of PMA in HBSS, for 40 min at 37°C before incubation with NBT for 20 min at 37°C as described in Methods. The nuclei of the majority of the unstimulated normal cells in (A) stain bright pink and their characteristic multinucleated or mononuclear morphology is easily identified. The nuclei of the majority of stimulated control cells in (B) are histologically distorted with swollen, deeply stained blue nuclei that contain precipitated reduced NBT. Stimulated leukocytes of a female with chronic granulomatous disease (C) illustrates two leukocyte populations; deeply-stained blue distorted cells containing reduced NBT and bright pink staining nuclei of intact leukocytes (safranin stain, ×640).
whereas in leukocytes had nuclear leukocytes with exceptions, not polymorphonuclear disease. With granulomatous disease had the from trast, excluded as respiratory distributed to the used, the family distinguished from the controls while retained the characteristically swollen, pink, bright nuclei and/or the cytoplasm. These NBT positive cells were generally too distorted to be morphologically identifiable as to cell type and the distortion was attributed to the presence of reduced NBT because control preparations of PMA-stimulated leukocytes without NBT were morphologically intact. Since peripheral blood monocytes (45–57), eosinophils (48, 49), and basophils (50), but not lymphocytes (16), all respond to appropriate stimuli with activation of the respiratory burst, three cell types must all be included as possible NBT positive leukocytes. In contrast, PMA-stimulated leukocytes from a female patient (Fig. 7[C]) were a mixed population of NBT positive and NBT negative leukocytes. The NBT negative leukocytes retained the morphology of intact, unstimulated cells while the NBT positive cells were indistinguishable from the NBT positive cells from controls.

Three female patients and two of their mothers were available for NBT studies (Table 1). PMA-stimulated leukocytes from controls were 99% NBT positive. In family 1 only 10% of the patients' leukocytes were NBT positive. In family 2 and family 3 the patients had <1% NBT positive cells and were comparable to a male patient with chronic granulomatous disease. The mothers of two of the female patients with chronic granulomatous disease had more NBT positive cells than the mother of a male with chronic granulomatous disease.

**DISCUSSION**

Genetic transmission in most females with chronic granulomatous disease has been difficult to identify because polymorphonuclear leukocyte defects could not be demonstrated in either parent. In the few exceptions, leukocyte abnormalities were found in the mother but not the father. In one family, polymorphonuclear leukocytes from both the mother and a brother had intermediate responses for NBT reduction (51) whereas in one other family (52) only the mothers' leukocytes produced intermediate responses in NBT reduction, bactericidal activity, and cytochrome C reduction. In both families, the father had normal leukocyte function. The inability to detect the carrier state in families of females with chronic granulomatous disease has suggested a non-X-linked mode of transmission in these individuals.

The use of insufficiently sensitive assays to measure leukocyte function may in part be responsible for the inability to detect polymorphonuclear leukoeytic defects in mothers of either male or female chronic granulomatous disease patients. Polymorphonuclear leukocyte bactericidal activity in asymptomatic mothers of males with the disease has shown a wide spectrum of activity ranging from normal to levels comparable with that of the patients themselves (38, 43). NBT reduction, a respiratory-burst-dependent chemical reaction between the dye and the O₂ generated during the respiratory burst (53), has also been used as a measure of polymorphonuclear leukocyte oxidative function. This assay, however, is also of limited value in predicting the chronic granulomatous disease heterozygous state. Normal NBT reduction has been observed in four of five mothers of male patients from families in which a clear-cut pattern of X-linked inheritance could be demonstrated (38).

The luminol-dependent chemiluminescence assay used in this study was designed to detect subtle abnormalities in polymorphonuclear leukocyte oxygen metabolism. In this system, respiratory burst activity is maximally stimulated by using an excessive number of ingestable particles. The oxygen intermediates generated during the respiratory burst oxidize available substrates. The zymosan particles employed are themselves oxidizable species but the magnitude of light generated can be greatly augmented by introducing luminol, another oxidizable agent, into the system. When luminol, a cyclic hydrazide, reacts with any of a

**Table 1**

<table>
<thead>
<tr>
<th>Source of leukocytes</th>
<th>NBT positive neutrophils (400 cells counted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>394</td>
</tr>
<tr>
<td>Family 1</td>
<td></td>
</tr>
<tr>
<td>CGD Female</td>
<td>0</td>
</tr>
<tr>
<td>Mother</td>
<td>320</td>
</tr>
<tr>
<td>Family 2</td>
<td></td>
</tr>
<tr>
<td>CGD Female</td>
<td>23</td>
</tr>
<tr>
<td>Mother</td>
<td>ND</td>
</tr>
<tr>
<td>Family 3</td>
<td></td>
</tr>
<tr>
<td>CGD Female</td>
<td>0</td>
</tr>
<tr>
<td>Mother</td>
<td>364</td>
</tr>
<tr>
<td>CGD Male</td>
<td>0</td>
</tr>
<tr>
<td>Mother of CGD Male</td>
<td>281</td>
</tr>
</tbody>
</table>

ND, not done. CGD, chronic granulomatous disease.

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variety of oxidizing agents, the electronically excited aminophthalate ion is generated and relaxes to ground state by photon emission (54). Although light emission by stimulated polymorphonuclear leukocytes remains a nonspecific manifestation of the respiratory burst, and the mechanism of luminol-amplification is not fully understood, this assay has nevertheless proven to be an extremely useful and sensitive measure of neutrophil function (22, 23, 43).

In the present study, respiratory burst activity was measured by polymorphonuclear leukocyte chemiluminescent response during phagocytosis of zymosan particles. Leukocytes from the two male patients had no chemiluminescent response, cells from the three female patients had extremely low leukocyte responses, and those from the mothers had values intermediate between the patients and controls.

The complete absence of respiratory burst activity in leukocytes from the two male patients reported here and five males previously reported (43) is consistent with the hemizygous state of a defective gene. Homozygosity in females for the same gene which specifies the disease in males, would also be expected to result in the complete absence of leukocyte chemiluminescence. The low but detectable light production in the leukocytes from female patients is more suggestive of a mixed population of normal and abnormal cells such as that found in heterozygous females. There is little evidence for homozygosity in these kindreds. Homozygosity on the basis of autosomal recessive inheritance seems unlikely because intermediate leukocyte function has been demonstrated in each of the four mothers and all the fathers have normal leukocyte function. Homozygosity due to spontaneous mutation in the germ line of the father, or postzygotic somatic mutation in the female herself, however, cannot be ruled out. Genetic abnormalities such as Turner's syndrome or testicular feminization is ruled out in the two females who have borne children. The sex of the third female was confirmed by buccal smear (39).

The Lyon hypothesis could best explain how the occasional heterozygous females, such as the four reported here, have the clinical syndrome of chronic granulomatous disease. By the Lyon hypothesis, there is random and permanent inactivation of one x-chromosome in somatic cells early in fetal development. The pattern of inactivation is inherited by all daughter cells (44). This should result in two distinct populations of polymorphonuclear leukocytes but no hybrid cells. Supporting this postulate, two populations of polymorphonuclear leukocytes have been identified in the peripheral blood of heterozygous females by NBT reduction (25) and by autoradiographic studies of bacteria iodination (55). Two populations of polymorphonuclear leukocytes were also identified by NBT reduction in our female patients. Random inactivation of the x-chromosome would be expected to lead to an even distribution of normal and defective cells in heterozygous individuals. However, by chance, the distribution could vary, resulting in a few individuals with preferential inactivation of the defective x-chromosome and phenotypically near normal polymorphonuclear leukocyte function. This mechanism could explain the findings of normal leukocyte function in some mothers of either male and female patients. By the same process of random x-chromosome inactivation, a few individuals with preferential inactivation of the normal x-chromosome would be expected to have markedly abnormal polymorphonuclear neutrophils in which defective respiratory burst activity would be associated with decreased bactericidal activity and clinical disease.

There is evidence from other x-linked diseases, i.e., glucose-6-phosphate dehydrogenase deficiency and hemophilia for nonrandom inactivation of the x-chromosome and clinical disease in heterozygous females (56). The wide variation in clonal population of neutrophils in heterozygotes may possibly be due to selection similar to the situation in Lesch-Nyhan syndrome, another disease with x-linked inheritance. No environmental or genetic factors that influence the proportion of neutrophils that respond to stimulation by NBT reduction has been identified in carrier females. Further evidence against selection is the relatively constant percentage of NBT positive and NBT negative neutrophils in carrier females over time (unpublished observations).

The findings presented here support an x-linked inheritance in females with chronic granulomatous disease and are consistent with the Lyon hypothesis of x-chromosome inactivation. The criteria for x-linked transmission have been met in the four kindreds. The mothers of the affected females have intermediate polymorphonuclear leukocyte function, the fathers and all male relatives without disease have normal function, whereas the maternal history is positive for affected males and heterozygous females. There is no male to male transmission. The Lyon hypothesis explains the variability of expression in the heterozygous females. Thus, females heterozygous for chronic granulomatous disease may have near normal, intermediate, or severely abnormal polymorphonuclear leukocyte function. Depending upon the degree of their leukocyte dysfunction, they may be phenotypically normal individuals, or express mild to severe forms of the clinical syndrome.

The findings of x-linked transmission in both males and females with chronic granulomatous disease does not imply a single gene defect in these individuals. Chronic granulomatous disease may represent a heterogeneous collection of leukocyte respiratory-burst ab-
normalities which are transmitted by genes on the x-chromosome.

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phil chemotactic, metabolic and bactericidal function.