

## Dysfunction of Natural Killer Activity in a Family with Chronic Fatigue Syndrome

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A family was identified with 5 of 6 siblings and 3 other immediate family members who had developed chronic fatigue syndrome (CFS) as adults. All 8 met criteria for the CFS case definition as recommended by the Centers for Disease Control and Prevention. Sixty-eight blood samples were obtained over a period of 2 years from 20 family members (8 affected, 12 unaffected) and 8 normal controls. All blood samples were tested for NK activity in 4-h <sup>51</sup>Cr-release assays and for the number of circulating CD3-CD56<sup>+</sup> and CD3-CD16<sup>+</sup> by flow cytometry. NK activity of the affected immediate family members (cases, *n* = 8) was significantly lower (*P* = 0.006, two-sided) than that of the concurrently tested normal controls. The results for unaffected family members were intermediate between these two groups, and the pairwise comparison of unaffected family members to either cases or controls showed no statistically significant difference (*P* = 0.29, two-sided). No differences were seen between the groups in the absolute number of CD3-CD56<sup>+</sup> or CD3-CD16<sup>+</sup> lymphocytes in the peripheral blood. Familial CFS was associated with *persistently* low NK activity, which was documented in 6/8 cases and in 4/12 unaffected family members. In the family with 5 of 6 siblings who had documented CFS, 2 of their offspring had pediatric malignancies. Low NK activity in this family may be a result of a genetically determined immunologic abnormality predisposing to CFS and cancer.

**Key Words:** chronic fatigue syndrome; natural killer cells; NK activity; familial CFS.

### INTRODUCTION

Chronic fatigue syndrome (CFS) has been defined as an illness of unknown etiology associated with severe debilitating fatigue lasting more than 6 months and a constellation of signs and symptoms often including

myalgias and migratory arthralgias (1, 2). Cases have occurred as a part of outbreaks (3–5) and multiple-case families have been reported (5, 6), but the majority of cases apparently occurs sporadically. Despite many studies of immune cells and cytokines in CFS over the past 10 years, the contribution of immunologic disturbance to the pathogenesis of CFS remains unknown.

Low natural killer (NK) syndrome (LNKS), first described by Aoki *et al.* in 1987 (7), is often associated with many of the signs and symptoms of chronic fatigue syndrome (8). LNKS may define a subset of patients with CFS who have a metabolic defect (low serum L-cystine level) which may or may not be related to depressed NK cell function (8). Low levels of NK activity in patients with CFS have been frequently reported in the literature (9–11), and it remains undetermined to what extent NK cell abnormalities contribute to the pathogenesis of CFS. We have recently identified a family in which several family members had CFS, and we undertook laboratory evaluations to determine whether a correlation could be established between clinical signs and symptoms of CFS and the level of NK activity or absolute numbers of NK cells in the circulation.

### MATERIALS AND METHODS

#### *Subjects*

Family members entered into this study were evaluated by one of us (P.H.L.) for comparability to the case diagnosis for chronic fatigue syndrome (1, 2) by means of personal interview, evaluation of medical records, and, in selected cases, more detailed evaluation by consulting neurologists and rheumatologists at the National Institutes of Health. The presence of features relevant to the diagnosis of chronic fatigue syndrome is noted in Table 1. All patients have undergone extensive medical evaluation by multiple physicians to rule out other causes of fatigue. An alternative diagnosis was suggested for only one patient (CFS 17) who was seen in 1986 at the University of Pennsylvania by a

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rheumatologist for associated arthralgia, myalgia, episodic photosensitivity or "butterfly" rash associated with flu-like symptoms, headaches, hair loss, and cognitive dysfunction. Blood studies, including antinuclear antibody, rheumatoid factor, thyroid and complement studies, and Lyme antibodies, were unrevealing and the patient has been tentatively considered as having "seronegative lupus erythematosus." The patients (index cases) had been living apart in different environments for more than a decade at the time they developed symptoms of CFS. None of the patients were under specific therapy known to alter NK activity. At the time of blood draws for this study, none had overt inflammatory signs. A pedigree of the family, which designates the affected and unaffected family members, is presented in Fig. 1.

### Samples

Peripheral venous blood was collected from family members and normal volunteers into heparinized tubes in the morning. Altogether, 28 individuals contributed 68 blood samples collected over a period of 2 years. Serial samples were obtained from cases, who donated four or five times, except for case 8 who donated twice, and from noncases, who donated as few as one or as many as nine times. Immediately following blood drawing, the specimens were placed in shipping containers, stored at ambient temperature, and shipped by an overnight air service to the Immunologic Monitoring Laboratory at the University of Pittsburgh Cancer Institute. The specimens were always received and processed within 24 h of blood draws. The laboratory routinely receives blood samples for NK cell assays from distant sites, and it has established an assay in which all patient and control specimens alike are incubated overnight and tested the next day. This procedure has been implemented to "standardize" the time period between blood collection and the assay. Comparisons in paired assays (with fresh vs stored PBMC) indicated that the procedure is acceptable and necessary to eliminate the variability resulting from various incubation periods prior to the assay. The normal range of NK activity used in the laboratory was established using this same procedure.

Blood draws were obtained from subjects participating in this study on 17 occasions and in every case were assayed as described below. Upon arrival, blood samples were separated on Ficoll-Hypaque gradients and mononuclear cells (MNC) were recovered, washed with RPMI 1640 medium (Gibco, Long Island, NY), and resuspended in sterile RPMI medium supplemented with 2 mM glutamine, antibiotics, and 2% (v/v) fetal calf serum (all from Gibco). The cells were washed, counted in a trypan blue dye to determine their viability, and assayed for NK activity in a 4-h  $^{51}\text{Cr}$ -release

assay exactly as described by us earlier (12) and for surface markers by flow cytometry (12).

### Natural Killer Cell Assay

Briefly, for NK cell assay, K562 targets were labeled with 100  $\mu\text{Ci}$  of  $^{51}\text{Cr}$  (sp act, 5 mCi/ml; NEN, Boston, MA) for 1 h at 37°C. Target cells were washed  $\times 3$  in tissue culture medium, resuspended in fresh medium, counted, and aliquoted at  $5 \times 10^3$  targets/well into 96-well U-bottom plates. For measurement of maximal release, 100  $\mu\text{l}$  of 5% Triton X-100 was added to triplicate wells containing target cells. Spontaneous release wells contained only target cells and medium. Effector cells (fresh peripheral blood mononuclear cells obtained by separation of heparinized blood on Ficoll-Hypaque) were added to test wells to achieve E:T ratios of 50:1, 25:1, 12:1, and 6:1. Plates were centrifuged at 208g for 3 min and incubated in 5%  $\text{CO}_2$  in air at 37°C for 4 h. Medium containing  $^{51}\text{Cr}$  was harvested from each well, using Skatron supernatant harvesting apparatus. All determinations were done in triplicate. Percentage specific lysis was determined according to the formula

$$\frac{\text{Experimental mean cpm} - \text{spontaneous release mean cpm}}{\text{Maximal mean cpm} - \text{spontaneous release mean cpm}} \times 100.$$

Results are reported in lytic units (LU), calculated according to the formula of Pross *et al.* (13). One LU is defined as the number of effector cells needed to lyse 20% of the targets ( $5 \times 10^3$ ) and calculated per  $10^7$  effector cells.

The daily variability of the NK cell assay was monitored by using cells from three selected normal individuals whose peripheral blood mononuclear cells were cryopreserved. Effector cells with low, medium, and high levels of NK activity were cryopreserved in quantities sufficient for 3 to 4 months when used in daily NK cell assays. Aliquots of these cells were defrosted daily immediately prior to NK cell assay (12). In addition, each NK cell assay included cells obtained from a normal volunteer selected randomly from a pool of volunteers repeatedly tested for NK activity in this laboratory. The coefficients of variation for interassay variability using frozen cells ranged from 28 to 30% ( $n = 100$ ) and for fresh cells from 20 to 24% ( $n = 34$ ). On the basis of over 1000 NK cell assays performed with PBMC of 254 normal donors in the laboratory, median NK activity is 142 LU/ $10^7$  cells, with values of 66 and 341 LU/ $10^7$  cells defining the lower and upper limits of the mid-80% normal range (10th percentile to 90th percentile).

## Flow Cytometry

Two-color flow cytometry was performed to determine the proportion and phenotype of circulating NK cells as well as T and B lymphocytes. Peripheral blood MNC were adjusted to the concentration of  $2.5 \times 10^6/\text{ml}$  in phosphate-buffered saline–0.1% (v/v) sodium azide (PBS–azide). Next, 0.2-ml aliquots of this cell suspension were incubated for 15 min at 4°C with 5- $\mu\text{l}$  aliquots of fluorescein- or phycoerythrin-labeled monoclonal antibodies, which were pretitered using normal mononuclear cells to determine the optimal working dilutions. The cells were then washed three times in PBS–azide and fixed in 2% (w/v) paraformaldehyde solution in PBS. Two-color analysis was performed on a FACScan flow cytometer (Becton–Dickinson, San Jose, CA). The monoclonal antibodies (mAbs) employed included anti-CD3, -CD4, -CD8, -CD56, -CD16, and -CD20 (all purchased from Becton–Dickinson). As controls, mouse isotypes IgG<sub>1</sub> and IgG<sub>2</sub> were used in all experiments. To determine the purity of lymphocytes in the “lymphogate,” anti-CD45 (fluorescein isothiocyanate) and anti-CD14 (phycoerythrin) mAbs were used. The lymphogate typically contained >98% lymphocytes. Normal laboratory values for each of the lymphocyte subsets were established by performing flow cytometry on peripheral blood MNC obtained from over 200 healthy volunteers tested over a period of the past 2 years.

## Statistical Methods

Statistical comparisons of cases (affected individuals), unaffected family members (UFMs), and normal controls (NC) were carried out using two methods: (a) *parametric*, in which mixed-effects analysis of variance models were fit to the data, using the method of restricted maximum likelihood, and the models considered the parameters of groups (fixed), draw dates (random), and subjects (random); and (b) *nonparametric*, in which a permutation test, based on the Mack–Skillings statistic, was applied. The *P* values reported are based on a Monte Carlo permutation of the assignment of subjects to groups, since the usual  $\chi^2$  distribution is invalidated by the fact that subjects generally contributed samples on more than one date.

For the parametric tests, NK activity was expressed in terms of log lytic units to normalize the distribution of data. For the same reason, absolute cell counts were transformed to square roots prior to parametric analysis.

The nonparametric test was devised as a check on the parametric test, because of concerns that the parametric analysis might be overly sensitive to the behavior of several very low lytic units found in the data.

Unlike the parametric test, the nonparametric test does not depend on the scale used to express assay results, because it is a function only of data ranks.

## RESULTS

As noted in Table 1, all five siblings and three immediate family members designated as cases met the criteria for CFS according to the initial (1) and the more recently revised (2) case definitions, as recommended by medical panels convened by the Centers for Disease Control and Prevention. Figure 1 summarizes the relationships and provides a clinical classification of the family members with respect to CFS. It is notable that in addition to documented CFS, this family has two members with a history of cancer in childhood: No. 22 with neuroblastoma and No. 42 with thyroid carcinoma. Also, the mother of cases 1 to 5 (No. 12) had cancer (meningioma). While the 5 siblings with CFS developed the disease as adults, all of the three cases of CFS in the next generation occurred in teenage girls (No. 22 at age 11, No. 30 at age 16, and No. 31 at age 18).

### NK Activity

In the initial evaluation of CFS cases 1 to 5 in February 1993 for NK activity, it was observed that all five had levels of NK activity below the mid-80% normal range established for our laboratory (66–341 LU). Therefore, these family members plus three others subsequently found to have symptoms of CFS were studied longitudinally (Fig. 2). At each time point, unaffected family members and/or normal controls were also included in the assay. The NC tested simultaneously with the cases consistently had NK activity within or near the normal laboratory range, and they did not fall below that range. The longitudinal evaluation of NK activity in eight CFS cases indicated that its levels varied between low and borderline normal over time (Fig. 2), but with the exception of one case with consistently normal NK activity (No. 16), they tended to remain at the lower or below lower normal level established in the laboratory. Among cases, the level of NK activity either remained depressed over the 2-year period or fluctuated between the low and the normal range (Fig. 2).

To compare NK activity in the three groups (CFS cases, UFM, and NC), the median LU values for each of the 28 subjects in the study were computed. Table 2 presents median LU values and ranges for each of the three groups and shows that the cases have a lower median level of NK activity than the other two groups. Between-group comparisons of NK activity were tested, using two different statistical methods, as described under Materials and Methods. The two meth-

**TABLE 1**  
Clinical Features<sup>a</sup> of Chronic Fatigue Syndrome in Family Members

	Case 1 CFS 17	Case 2 CFS 14	Case 3 CFS 13	Case 4 CFS 16	Case 5 CFS 15	Case 6 CFS 22	Case 7 CFS 30	Case 8 CFS 31
Age at onset	36	30	<38	36	35	11	16	18
Year of onset	1982	1990	@1981 <sup>b</sup>	1990	1984	1989	1989	1990
Major criteria								
Length of disability (years)	14	6	15	6	12	7	7	6
Fatigue resulting in <50% of premorbid activity	+	+	+	+	+	+	+	+
"Alternative diagnoses"	Seronegative lupus	-	-	-	-	Neuroblastoma (age 11)	-	-
Minor criteria								
Fever	+	+	+	+	+	+	+	-
Sore throat	+	+	+	+	+	+	+	+
Painful lymph nodes	+	+	+	+	+	+	+	+
Muscle weakness	-	+	+	+	+	-	-	-
Myalgia	+	+	+	+	+	+	+	+
Prolonged generalized, fatigue after exercise	+	+	+	+	+	+	+	+
New generalized headache	+	+	+	+	+	+	+	+
Migratory arthralgia	+	-	+	+	+	-	+	+
Cognitive dysfunction	+	+	+	+	+	+	+	+
Sleep disturbance	+	+	+	+	+	+	+	+
Acute onset (<3 days)	+	±	-	+	+	-	+	+

<sup>a</sup> See Holmes *et al.* (1) for details of clinical features.

<sup>b</sup> @1981 means approximately in 1981, not an acute onset of CFS.

ods gave consistent results, in both cases yielding marginally significant *P* values for the three-group comparison: *P* = 0.028 for the nonparametric comparison and *P* = 0.049 for the parametric test.

Since the overall difference was significant, the three-group comparisons were followed by multiple two-group tests. Results of the pairwise tests are shown in Table 3 for the parametric analysis only, as the nonparametric results were similar. Thus, it appears that the cases had significantly reduced median NK activity compared to the NC, while the UFM were intermediate between the two, but the difference between cases and UFM was not statistically significant. The small number of individuals tested did not allow for formal control of comparisons for group differences in age and sex, but such differences do not appear to have contributed to the case-NC difference in NK activity.

The data for NK activity were also plotted in Fig. 3 to show the individual median LU values for each of 28 individuals tested. Figure 3 also indicates the mid-80% normal range for the laboratory and the median NK activity level computed from the data obtained from 46 normal laboratory controls tested in 254 assays. It can be seen that of 8 CFS cases, 5 (62%) had values that fell below the normal range, while among 12 UFM, 4 (30%) had lower-than-normal median NK activity. In contrast, only 2/8 (25%) controls tested in the same assays with the family members had borderline low NK activity. These results indicate that relative to NC sampled at the same time as CFS patients or to a large pool of normal laboratory controls, the cases as well as the UFM group had a considerably increased frequency of individuals with depressed NK activity. Among 46 normal laboratory controls repeatedly tested in 254 assays, 4/46 (8.4%) had chronically low NK activity

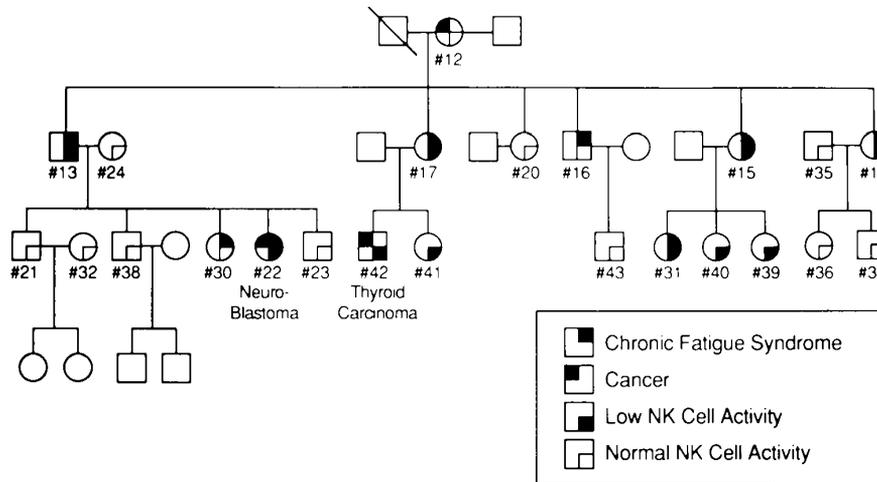


FIG. 1. Pedigree of nuclear family for familial chronic fatigue syndrome.

(data not shown). These data indicate that among cases, an increased proportion of individuals had low NK activity, relative to normal controls. The same trend appears to be true for UFM, but the difference was not statistically significant.

#### Absolute Numbers of NK Cells

Since changes in NK activity may be due to changes in the absolute number of circulating NK cells, we simultaneously studied CD3-CD56<sup>+</sup> and CD3-CD16<sup>+</sup> subsets of circulating lymphocytes in the family members. As shown in Fig. 4 for CD3-CD56<sup>+</sup> NK cells, the median absolute numbers were within the normal laboratory range in all three groups of individuals studied. Similar results were obtained for the absolute numbers of CD3-CD16<sup>+</sup> subset of NK cells (data not shown). In summary, no significant differences were noted between the three groups in the absolute number of circulating NK cells (Table 4).

In addition to NK cells, the following lymphocyte populations were examined for group differences in absolute cell counts (cells/mm<sup>3</sup>): CD4<sup>+</sup>, CD8<sup>+</sup>, CD3<sup>+</sup>, CD19<sup>+</sup>, and CD23<sup>+</sup>. No significant differences were seen. There was, however, a statistically significant between-group difference in the helper/suppressor ratio (CD4<sup>+</sup>/CD8<sup>+</sup>) which was lower in the CFS and the UFM than in the NC group. The overall difference in the CD4<sup>+</sup>/CD8<sup>+</sup> ratio reached a *P* value of 0.029, using the nonparametric and a *P* value of 0.019, using the parametric test. The difference appeared to reflect a higher absolute number of CD8<sup>+</sup> cells in the CFS and UFM groups relative to NC. However, differences in absolute numbers of neither CD4<sup>+</sup> nor CD8<sup>+</sup> lymphocytes individually approached statistical significance.

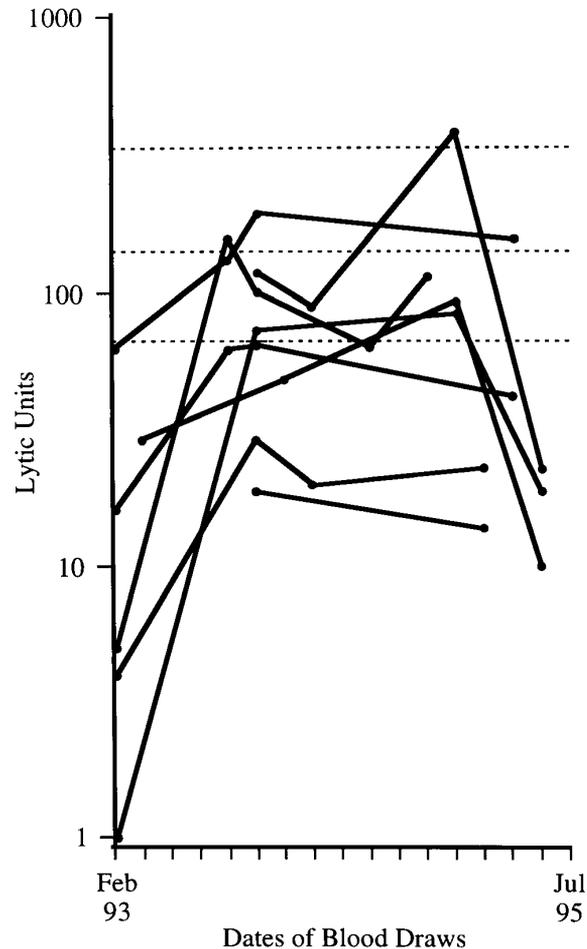


FIG. 2. NK activity profiles for CFS cases. NK activity was serially measured over the 2-year period. The median and the upper and lower boundaries of the mid-80% normal range for NK activity for the laboratory are shown by the dotted lines.

**TABLE 2**

Median NK Activity of the Family Members and Normal Controls

Group	N	Median	Geometric mean	Range
Cases	8	49	52	16–45
UFMs	12	89	80	7–697
NC	8	128	133	62–351

Note. N, number; UFMs, unaffected family members; NC, normal controls. NK activity is expressed in LU, as defined under Materials and Methods.

**DISCUSSION**

This family appears to have CFS largely determined by genetic predisposition: all five index cases developed the clinical features of this disorder in their fourth decade of life while living in very different environments. The role of genetics in the etiology of CFS was first suggested by the strong concordance with allergy (14–16), although CFS has not yet been shown to be due to IgE-mediated abnormalities of the immune system. Also, history of allergy in members of this family did not correlate with the observed immunologic dysfunction. In this family, a hereditary disorder is further supported by the apparent onset of symptoms in three offspring of the affected siblings (No. 22, No. 30, and No. 31) at an early age (i.e., 11, 16, and 18 years) and is suggestive of a hereditary disorder. An earlier age of disease onset in subsequent generations has been expected in hereditary disorders such as familial breast cancer, melanoma, and Huntington's disease (17–19).

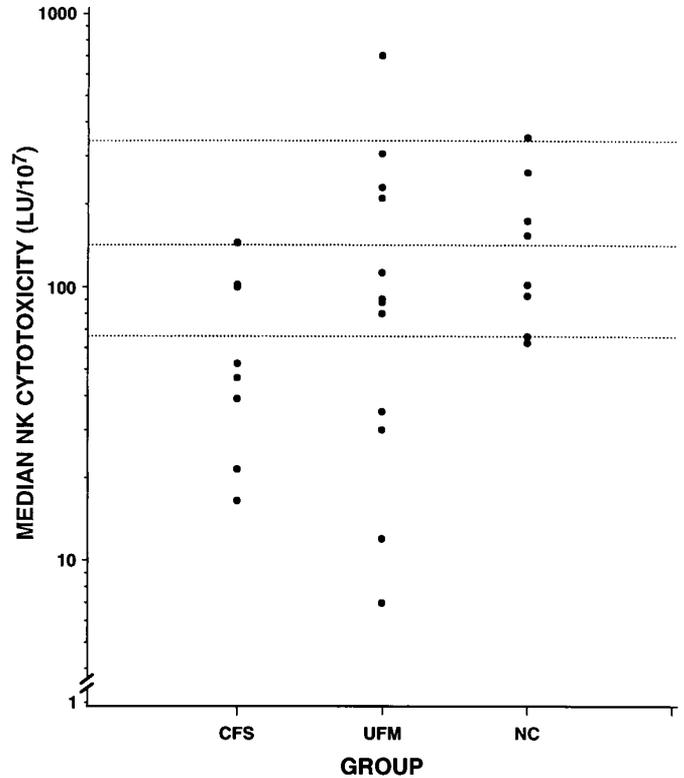
While CFS is a heterogeneous disorder with a variety of triggering environmental agents as well as clinical and laboratory features, low NK activity appears to be a consistent feature in a substantial proportion of cases (8–11, 20, 21). In this family, the decreased NK activity correlated with the diagnosis of CFS, but no direct association with clinical symptoms could be documented. For example, one affected sibling (No. 16) consistently has normal NK cell activity, and four un-

**TABLE 3**

Results of Two-Group Tests for Differences in the Median Level of NK Activity between Cases, Unaffected Family Members (UFM), and Normal Controls (NC)

Groups tested	Parametric <i>P</i> value <sup>a</sup> (two-sided)
Cases vs UFM	0.38
Cases vs NC	0.006
UFM vs NC	0.29

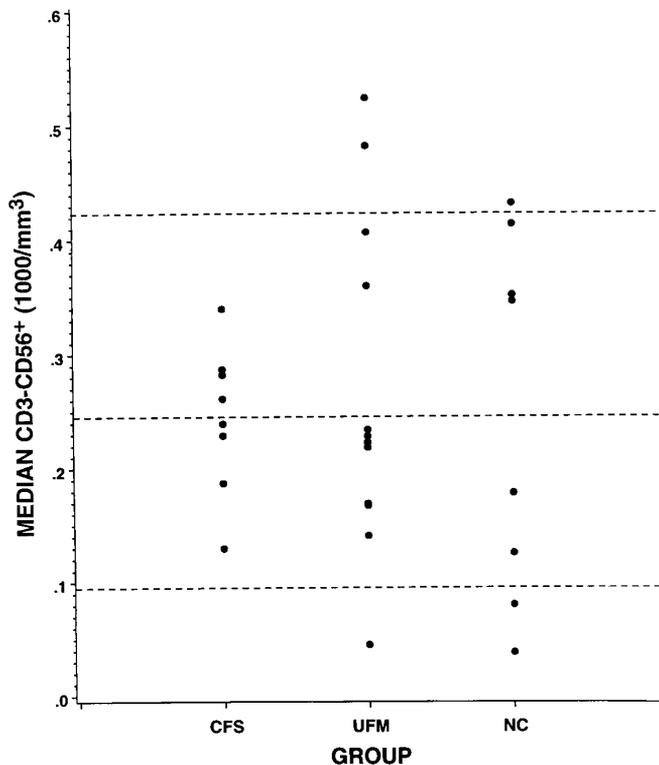
<sup>a</sup> Nonparametric *P* values were similar, with the *P* value for cases vs NC calculated at *P* = 0.014.



**FIG. 3.** Comparison of three groups for NK activity. Since each individual was tested on more than one occasion, median NK activity was calculated for each and is presented. The median and mid-80% normal range are shown as in Fig. 2. CFS, chronic fatigue syndrome; UFM, unaffected family members; NC, normal controls tested concurrently with family members.

affected siblings, including one successfully treated for childhood cancer (No. 42), have low NK cell activity. It is interesting to note that two UFMs (No. 42 and No. 41) are children of CFS case 1 and the other two UFMs are offspring of CFS case 5. The third child of case 5 has CFS with low NK activity. She developed CFS at age 18. These observations suggest that the family members with chronically low NK activity may be genetically predisposed to the development of CFS.

Individuals with persistently low NK activity might be also prone to the development of malignancy. In families with cancer, particularly with familial melanoma or breast cancer, low NK activity has been observed in family members with no evidence of cancer (22–24), suggesting that the low NK activity may be evidence of susceptibility to a future malignancy. The relationship between high familial incidence of cancer and low NK activity was observed in males as well as females and in nonsmokers as well as smokers (24). These studies suggest that genetically determined defects in NK activity may contribute to the initiation of human cancer (25). The fact that in this family two cousins (No. 22 and No. 42; children of cases 3 and 1,



**FIG. 4.** Median values for absolute numbers of CD3-CD56<sup>+</sup> NK cells in the three groups of subjects. The median and mid-80% normal range are shown as in Fig. 2.

respectively) developed cancer and had persistently low NK activity also suggests an association between cancer development and low levels of NK activity, although we have no evidence that low NK activity preceded the onset of cancer in these family members. It will be important to follow the other members of this family, particularly those with no disease symptoms but low NK activity, for evidence of CFS or cancer.

In general, a level of NK activity is a stable individual trait, which fluctuates within a low, middle, or high normal range characteristic for each individual, remaining the same unless disease intervenes (12).

Chronically low levels of NK activity have been described in a subset of normal young individuals, representing 14–15% of a student population (26). Levy and colleagues reported that these individuals have more frequent and more severe upper respiratory infections than their peers and that they have difficulties in handling stressful situations (26, 27). The study of Levy *et al.* linked persistently low NK activity to the inability to cope with stress (26). Numerous other publications confirm the relationship between stress and low NK activity (28–30); therefore it is possible to speculate that low NK activity seen in affected members of this family may be related to stress. On the other hand, in unaffected family members, concerns about health of their relatives, especially CSF and cancer, could lead to stress, low NK activity, and disease.

Among the eight affected family members, the levels of NK activity were persistently low in five, while in the three others, NK activity appeared to reach a normal level at least twice during the 2-year study. As shown in Fig. 2, NK activity in the CFS cases was far from stable over time. It is possible that our results, showing the shifts from low to normal and then back to low levels of NK activity that seem to characterize CFS members of this family, may be strongly influenced by the between-test variability. Furthermore, a retrospective analysis of clinical data showed no correlations between the downward shifts in NK activity and any constellation of clinical or psychological findings in CFS patients. However, the fact that NC bloods shipped together with CFS samples had NK within a normal range (data not shown) suggests that the observed fluctuations in NK activity of CFS patients are not entirely due to interassay variability. In our experience, healthy individuals tested repeatedly over a period of 2 years were found to have a within-individual standard deviation in the base-10 log of NK activity of about 0.2, indicating that a considerable degree of variability exists even among normal donors. Future prospective studies of this and perhaps other families accompanied by more frequent longitudinal monitoring of NK activity might provide a more meaningful clinical-laboratory correlation.

**TABLE 4**

Distribution of the Absolute Numbers of CD3-CD56<sup>+</sup> and CD3-CD16<sup>+</sup> Subsets of NK Cells for Cases, Unaffected Family Members, and Controls<sup>a</sup>

Group	N	CD3-CD56 <sup>+</sup>		CD3-CD16 <sup>+</sup>	
		Median	Range	Median	Range
Cases	8	0.25	0.13–0.34	0.23	0.15–0.35
UFM	12	0.23	0.05–0.52	0.23	0.05–0.64
NC	8	0.26	0.04–0.43	0.20	0.08–0.52

<sup>a</sup> The data are presented as numbers  $\times 1000/\text{mm}^3$  of peripheral blood.

The mechanism responsible for the observed low levels of NK activity in this family remains unknown. It is not attributable to a decrease in the absolute number of NK cells which remained within the normal range throughout this study. Circulating NK cells were defined as CD3-CD56<sup>+</sup> or CD3-CD16<sup>+</sup> subpopulations and we did not measure the number of activated NK cells. It is possible that discrete shifts in NK cell subpopulations, resulting in the presence of an excess of less active NK cells in the blood, as suggested by others (32), are responsible for low NK activity. Alternatively, decreased levels of modulatory cytokines that maintain normal levels of NK activity or the appearance of inhibitory factors might be responsible. To what extent the responsible factors are related to clinical manifestations of CFS remains to be determined, and further studies are necessary to elucidate the intriguing role, if any, NK cells play in this disorder.

Other laboratory investigations, including studies of genetic markers and cytokines, are currently being initiated in the affected individuals and other family members. The availability and cooperation of this family with an unusual pattern of CFS and cancer may provide important information on a number of questions, including the possibility of developing assays identifying susceptibility to CFS and the role of depressed NK cell activity in susceptibility to human cancer.

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