

Mitochondrial Dysfunction in Chronic Fatigue Syndrome

Brad Chazotte

1. INTRODUCTION

Chronic fatigue syndrome (CFS), also known as chronic fatigue and immune dysfunction syndrome (CFIDS), myalgic encephalomyelitis, and so forth, has an unknown etiology and a poorly understood pathophysiology. Almost nothing is known of the cellular bioenergetics of CFS patients. The syndrome is a subject of increasing interest, though it is not a new phenomenon; medical literature going back over a hundred years has detailed similar illnesses with similar symptoms. Chronic fatigue syndrome was recently reviewed by Komaroff and Buchwald (1998), and a recent study based on a four-year surveillance of four U.S. sites determined an age-, race-, and sex-adjusted prevalence in CFS of 4.0 to 8.7 per 100,000 and an age-adjusted prevalence of 8.8 to 19.5 per 100,000 for white women (Reyes *et al.*, 1997). An even higher prevalence was reported in another U.S. study, with an estimated range of 75 to 267 per 100,000 (Buchwald *et al.*, 1995).

There is a rough consensus that the CFS patient population is predominantly, but not exclusively, female and more likely white than minority. In fact, CFS is diagnosed 3 to 4 times more frequently in women than in men and about 10 times more often in white Americans than in other American population groups.

1.1. Origins of Chronic Fatigue Syndrome

There is no known specific cause for CFS, but many etiological theories are proposed. The etiologies can be broadly divided into three groups: infectious agents, immunological

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causes, and central nervous system causes. Infectious agent theories (e.g., Straus, 1994, and articles therein; Levy, 1994) are based on the view that an infectious agent either triggers a prolonged response or remains as a chronic infection. Most CFS patients report experiencing a “flu-like” illness prior to the onset of this syndrome (Fukuda and Gantz, 1995; Rasmussen *et al.*, 1994). In some instances there have been “outbreaks” of CFS, including the Royal Free Disease and Lake Tahoe outbreaks (e.g., Hyde *et al.*, 1992, and references therein). Many viral agents have been proposed: Epstein-Barr virus, varicella zoster, cytomegalovirus, various enteroviruses, various retroviruses (e.g., one similar to HTLV-II, spuma viruses), and more. There is some question as to whether a pathological agent akin to chronic brucellosis could give rise to CFS, or whether a “leaky gut” might permit intestinal flora to trigger CFS symptoms (Cho and Stollerman, 1992). To date no specific viral agent has been shown to cause CFS, nor in fact has *any* specific pathological agent been shown to cause it. (e.g., Levy, 1994).

Immunological theories that focus on some defect in patient immune response have also been advanced based on various reports of immune cell abnormalities. These theories suggest that cell populations can be affected, such as low numbers of NK cells (Aoki *et al.*, 1993), or increased populations of apoptotic lymphocyte cells (Vodjdani *et al.*, 1997). Alternatively, it has been suggested the cytokine or immunoglobulin response of immune cells could be altered for example by overproduction of interferon- α (IFN- α) in response to stimulus (Lever *et al.*, 1988)

Central nervous system theories include both physiological and psychological aspects. For example, Demitrack and co-workers (Demitrack, 1994; Demitrack and Crofford, 1998) have argued for a disturbance in neuroendocrine function along the hypothalamic-pituitary-adrenal axis following “acute, often infectious, stress in emotionally susceptible individuals.” Rowe *et al.* (1995) have asked whether neurally mediated hypotension could cause CFS. Others have speculated that CFS may be a primarily psychological disorder. Clearly, there is no agreed upon etiology. Despite increasing scientific literature on CFS, (e.g. Bock and Whelan, 1993; Dawson and Sabin, 1993; Hyde *et al.*, 1992; Jenkins and Mobarary, 1991; Komaroff and Buchwald, 1998; Straus, 1994), there is to date little appreciable increase in knowledge about cellular physiology and, in particular, about cellular bioenergetics in CFS patients, as mentioned. A more detailed exploration of cellular pathophysiology in CFS patients will help in understanding the disorder.

1.2. Symptoms and Case Definitions for Chronic Fatigue Syndrome

Chronic fatigue syndrome has proved elusive to define, identify, and treat. A case definition was proposed by Holms and associates (1988) that was subsequently refined by Fukuda *et al.*, (1994). For detailed descriptions and references, the reader is referred to these. Chronic fatigue syndrome is a clinically defined condition characterized by persistent (i.e., greater than six consecutive months), severe, disabling fatigue: postexertional malaise: and a combination of various symptoms such as concentration and short-term memory impairments, sleep disturbances (e.g., unrefreshing sleep), musculoskeletal pain, multijoint pain, sore throat, and new headaches. The diagnosis is still by elimination, when no other illness can be found (see also Komaroff and Buchwald, 1998). Unfortu-

nately, there is no accepted, simple, clear diagnostic criterion for CFS as there is with, for example, the presence of rubeola virus for measles.

1.3. Role for Mitochondrial Dysfunction in Chronic Fatigue Syndrome

Clinical reports on CFS patients suggest a role for mitochondrial dysfunction at the cellular level. The regular and routine catabolic, anabolic, and energy-transducing metabolic functions performed by mitochondria are essential for normal cellular and physiological function, for organismal homeostasis—even for life itself. The classic symptoms of persistent and debilitating fatigue, chronic muscle weakness, and myalgia (Cheney and Lapp, 1992; Fukuda, *et al.*, 1994) are consistent with mitochondrial dysfunction in other diseases of known mitochondrial etiology (Carafoli and Roman, 1980; DiMauro *et al.*, 1985; Frackowiak *et al.*, 1988; Roe and Coates, 1989). Also, cardiac muscle function under an exercise load appears to be affected in CFS patients compared with matched controls (Montague *et al.*, 1989). Most CFS patients report mental concentration impairment and cognitive deficits (Graftnan *et al.*, 1991; Hyde and Jain, 1992; Sandman *et al.*, 1993; Straus, 1988), which are also seen in mitochondrial dysfunctions (Kartounis *et al.*, 1992; Peterson, 1995). Such reports have generated interest in SPECT and magnetic resonance neuroimaging of CFS patient brain metabolism where some defects have been observed (Natelson *et al.*, 1993; Schwartz *et al.*, 1993a, 1993b). Likewise, many CFS patients report gastrointestinal disturbances similar to known mitochondrial dysfunction (e.g. Peterson, 1995). Kuratsume *et al.* (1994) reported an acylcarnitine deficiency in the serum of CFS patients that apparently correlates with performance level. In one study, CFS patients were treated with L-carnitine; improvement was related to symptom severity at the time the treatment began (Plioplys and Plioplys, 1997). Carnitine is involved in long-chain fatty acid transport for mitochondrial β -oxidation, a major energy source in muscle. Carnitine and acylcarnitine levels are both altered in mitochondrial diseases such as medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (Roe and Coates, 1989), for which carnitine is given therapeutically. In CFS patients, immune disturbances have also been clinically reported (Aoki *et al.*, 1993; Ojo-Amaize *et al.*, 1994; Buchwald and Komaroff, 1991; Caligiuri *et al.*, 1987; Cannon *et al.*, 1997; Jones, 1991). Overall, the symptoms suggest problems in *mitochondrial energy metabolism in more than one tissue*. Clinically, mitochondrial defects are most easily recognized in muscle and brain because these tissues have high energy demands (Barbiroli *et al.*, 1993; Behan *et al.*, 1991; DiMauro *et al.*, 1985; Frackowiak *et al.*, 1988; Peterson, 1995), although they manifest in other tissues as well. For example, Coates *et al.* (1985) demonstrated the utility of using both fibroblast cells and leukocytes for metabolic studies from a group of patients with mitochondrial-based MCAD deficiency by comparison with liver cell studies on these patients, cells much more difficult to obtain. As is universally accepted in the mitochondrial literature, Wallace (1992) points out that toxicological studies show that different organ systems rely on mitochondrial energy to different extents and that as mitochondrial ATP production declines, it successively falls below the minimum levels needed for each organ to function normally. Further evidence for mitochondrial deficiency is found in reports of early muscle acidosis (Jamal and Hansen, 1985; Wong *et al.*, 1992) and deficit in oxygen utilization with low anaerobic threshold during exercise. One report suggests defects in muscle metabolism (McCulley

et al., 1996). These authors had some concerns, however, about matching activity levels of CFS patients with those of controls, and they also speculated that muscle deconditioning in CFS patients was an alternative explanation they could not rule out without additional studies. With respect to a possible role for mitochondrial DNA, there is a report of unusual deletions in CFS patient skeletal muscle, albeit anecdotal because only one individual was studied (Zhang *et al.*, 1995). A strong clinical finding implicating mitochondria involvement in CFS is that ATP levels at exhaustion were lower compared with levels in the control group when measured in gastrocnemius muscle *in vivo* by phosphorous NMR (Wong *et al.*, 1992). Significantly, there is a related finding in fibromyalgia patients, similarly measured in trapezius muscle, that ATP and phosphocreatine levels are 17% and 21% lower, respectively, and that these levels correlate with CFS-like symptoms (Bengtsson and Henriksson, 1989). Interestingly, in the fibromyalgia study not all muscle fibers were similarly affected. Thus there is ample *clinical* evidence to suggest a role for mitochondrial dysfunction in many different tissues and cells in CFS, which could give rise to many of the symptoms. Whether this role is due to a specific mitochondrial defect, perhaps in genetically susceptible individuals, or is an effect of some other problem such as altered cytokine levels that in turn affect mitochondrial function, needs investigation. Due to the difficulty in obtaining human specimens in sufficient quantities per specimen for biochemically based studies of mitochondrial function, there are few (and no detailed) studies of mitochondrial function in CFS patients.

1.4. Cytokine Role in Chronic Fatigue Syndrome

Cytokines (polypeptides, proteins, or glycoproteins of >5000MW) are carefully controlled, critical intercellular and possibly intracellular messengers produced by specialized and unspecialized cells of many tissues and organs. Cytokines tend to act locally unless secreted into the circulatory system, where they remain for a rather short time. Cytokines such as interferons (IFN- α , - β , or - γ), interleukins (IL-1 through IL-18), tumor necrosis factor (TNF), and so on exert their effects at extremely low concentrations (picograms/ml) via cell receptors. Some of the triggered reactions are related to immune and inflammatory responses against pathogenic stimuli such as bacteria and viruses. Administration of IFN- γ and TNF- α to smooth muscle cells in culture reportedly inhibits mitochondrial respiration at complexes I and II (Geng *et al.*, 1992). Likewise, isolated hepatocytes have exhibited signs of mitochondrial impairment, such as decreased cellular ATP levels, upon exposure to TNF- α (Adamson and Billings, 1992), and reports suggest that cytokines may affect mitochondrial β -oxidation (Barke *et al.*, 1991). Thus there is plenty of evidence to indicate that mitochondrial function can be affected by cytokines in a number of cell types, and there is a clinical relevance to these effects. Related evidence suggests that immune dysfunction (e.g., Buchwald, 1991; Klimas *et al.*, 1990; Lloyd *et al.*, 1991) and cytokine imbalances (Ho-Yen *et al.*, 1988; Lever *et al.*, 1988; Lloyd *et al.*, 1991; Straus *et al.*, 1989) occur in CFS and other chronic diseases. One interesting side effect reported for (antiviral) IFN- α therapy is that it induces chronic-fatigue-like symptoms, (e.g., MacDonald, 1987). Finally, and most importantly, we have preliminary evidence that at least one cytokine we studied, IFN- α , lowers mitochondrial membrane potential ($\Delta\psi$) in human cells (Chazotte and Pettengill, 1998, 1999; Chazotte *et al.*, 1996).

2. METHODS

2.1. Cell Isolation, Culture, and Labeling

Two cell types, human mononuclear leukocytes and human fibroblasts, were used in the studies described in this chapter. The mononuclear leukocytes were isolated from human donors and the fibroblasts were obtained from commercially available cell lines. Both cell types were labeled in the same manner.

2.1.1. Human Mononuclear Leukocytes

Leukocytes were used in the CFS phase of our studies, due in part to the relative ease of obtaining individual, intact living cells from patients (and controls) compared with obtaining cells from other tissues. Whole blood was obtained from fasting morning samples of excess patient blood (typically 12 mls) drawn for routine testing at a clinic specializing in CFS. Normal control specimens were obtained as fasting morning specimens of healthy individuals donating blood for control studies at University of North Carolina (UNC) Hospitals. Whole blood was maintained at 4°C during overnight shipment, conditions we have routinely found *not* to decrease the $\Delta\psi$ parameters we measure for leukocyte cells and mitochondria. Control specimens were subjected to the same conditions as patient specimens. Leukocytes were isolated at 18–20 °C as specified in protocols from Robbins Scientific (Sunnyvale, CA) for their commercial preparative gradients that permit separation of mononuclear and polymorphonuclear leukocytes (as well as platelets) from whole blood. The separated leukocytes were then resuspended in appropriate culture medium (e.g., Hanks medium) or phosphate buffered saline (PBS). Isolation and resuspension in a defined medium gave us the important ability to carefully control the extracellular environment for cells from both patients and controls. Our primary focus was mononuclear leukocytes; however, we also examined platelets, which could be co-isolated and distinguished in our confocal specimen chambers and then analyzed separately.

2.1.2. Cultured Human Fibroblast Cell Lines

Human BG-9 or CCD-27sk fibroblast cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and penicillin/streptomycin. Cells were grown in incubators under standard cell culture conditions and were subcultured before confluence and plated according to standard protocols. For confocal microscopy experiments, cells were subcultured on glass microscope coverslips

2.1.3. Cell Labeling

Human mononuclear leukocytes were labeled with the fluorescent $\Delta\psi$ -sensitive probe tetramethylrhodamine methylester (TMRM) (Chazotte and Pettengill, 1998, 1999; Chazotte *et al.*, 1996). TMRM is one of the $\Delta\psi$ -imaging probes most frequently used in confocal microscopy due to its highly advantageous properties. Isolated cells suspended

in PBS or cell culture medium on the coverslip were placed in a cell culture dish. Labeling with 600 nM TMRM was carried out in the dark at an appropriate temperature (37 °C or room temperature) for 25 minutes in an appropriate culture medium or PBS⁺ (PBS with 0.45 mM Ca²⁺ and 0.25 mM Mg²⁺, which enhances cell attachment to the glass coverslip). After 25 minutes, the coverslip was removed from the culture dish and mounted in a special microscope chamber. The chamber was subsequently filled with 150 nM TMRM in culture medium or PBS⁺ (Chacon *et al.*, 1994).

2.2. Confocal Microscopy

Using laser-scanning confocal microscopy (Pawley, 1995) to image $\Delta\psi$ is an important and powerful technique for examining the ability of mitochondria to make ATP in individual, intact, living cells (Chacon *et al.*, 1994; Chazotte *et al.*, 1996; Chazotte and Pettengill, 1998, 1999; Lemasters *et al.*, 1993a, 1993b). The advent of confocal microscopy has allowed thin (one-micron) sections of inherently thick biological specimens to be observed without interference by out-of-focus light. A key benefit of our approach, which looks at and measures individual cells, is that it requires very little material, in contrast to biochemical approaches that require large amounts of material for mitochondrial studies. Biochemical studies of human mitochondria in particular are difficult due to problems in obtaining isolated mitochondria in sufficient quantities for more than several enzyme assays, hence the relatively small number of such studies in the literature. Also, our ability to examine individual cells is an asset if CFS has a viral etiology and affects only a percentage of a specific cell population or subpopulation, such as CD4 cells.

A Bio Rad MRC-600 microscope was used for multichannel image acquisition using a 60 \times objective lens with a 1.4 numerical aperture (NA). The scope was set up and calibrated as described in detail elsewhere in this volume (Chacon *et al.*, 1994; Chazotte and Pettengill, 1998, 1999; Chazotte *et al.*, 1996; Lemasters *et al.*, 1993a). Briefly, the enhancement circuit was at setting 4 to accommodate the wide dynamic range of fluorescence from extracellular to mitochondrial TMRM intensities. Also, the black level was set manually such that half the pixel intensities were distributed in a Gaussian manner at or below gray-level zero, and the imaging focal plane was at the coverslip for a zero TMRM concentration. The gain was set based on cell images such that no off-scale intensities, i.e., greater than gray-level 255 were observed. The cell chambers were temperature controlled, typically at 37 °C. Images were rapidly acquired and signal-averaged sufficiently to reduce noise. We archived them to magnetic or optical storage media for subsequent analysis.

2.3. Membrane-Potential Imaging Analysis

Confocal images analysis using fluorescent probes was accomplished by quantifying the digitized image brightness and converting it to physiologically relevant parameters using a technique that employed look-up tables based on an application of the Nernst equation (Chacon *et al.*, 1994; Chazotte and Pettengill, 1998, 1999; Chazotte *et al.*, 1996; Lemasters *et al.*, 1993a). The Nernst equation calculates the diffusion potential in mV for a charged species in two electrolyte solutions separated by a membrane. The digital 8-bit

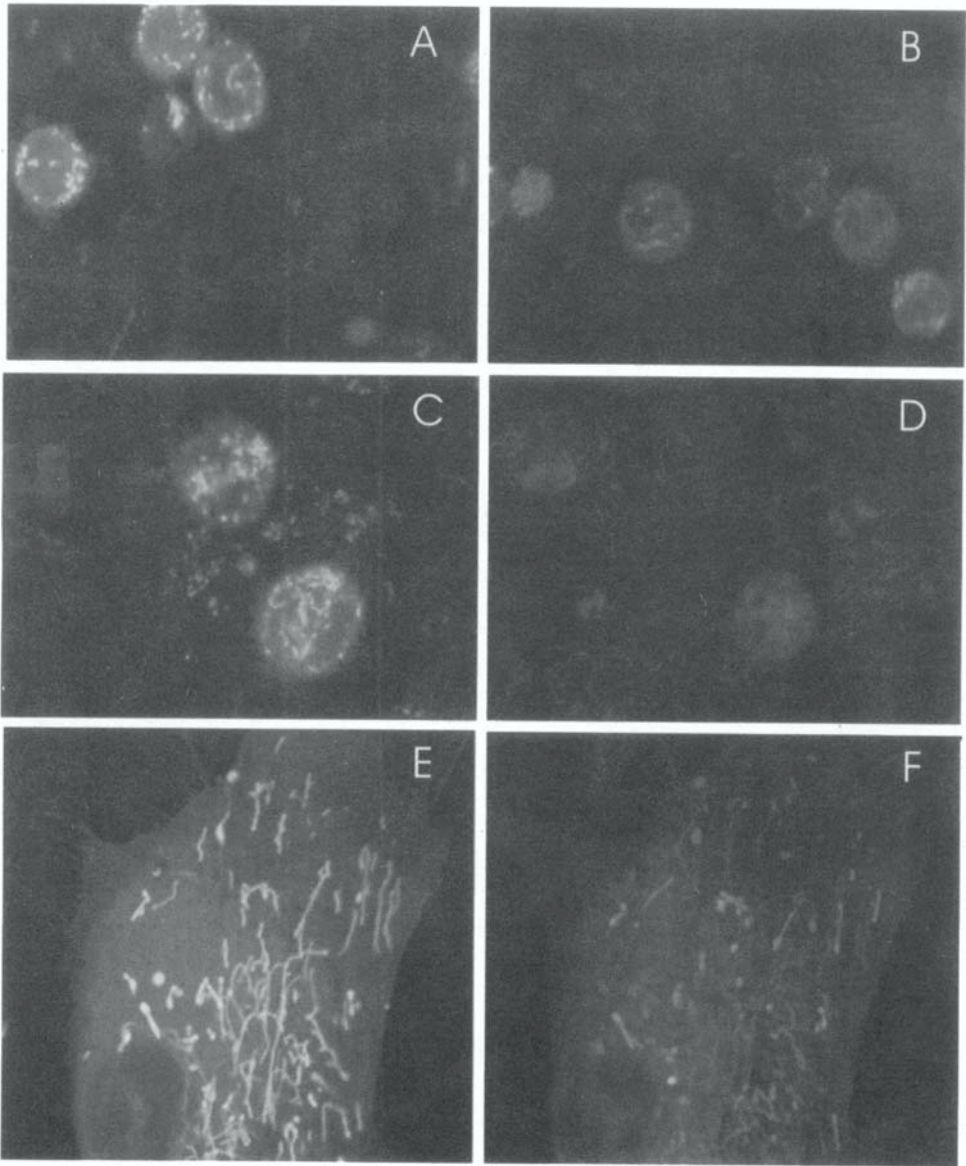


FIGURE 1. Membrane potential images of human cells. Human cells were labeled with TMRM and imaged using laser-scanning confocal microscopy as described in Section 2. (A–D) Mitochondria in leukocytes appear as bright spots. (A, C) Two individuals representative of our control population. (B, D) Two patients in our CFS population. (E, F) Single cultured human fibroblast. Mitochondria appear as bright ribbonlike structures. (E) Before addition of 180 ng/ml of human interferon- α , (F) three minutes after the addition.

confocal or gray-level image (e.g., Fig. 1A, 1C, 1E) shows the most-negative potential regions as the brightest, so the highly negatively charged, fully functional mitochondria in human mononuclear leukocytes appear as bright tubular spots (Fig. 1A, 1C) and ribbons Fig. 1E in fibroblasts. Using a specific look-up table to convert brightness into millivolts of potential, a digital image can be displayed as a pseudocolored image that facilitates visual inspection of the $\Delta\psi$ differences; a color typically represents a 20-mV range. Due to the absence of color plates in this monograph, only gray-level images are shown.

To quantitatively measure membrane potential we adapted long-accepted general approaches from image-intensity analysis. To quantitatively analyze individual cells or any part of the image, a histogram analysis was applied by selecting a specific area and counting the number of pixels (small dots that make up a digital image) at each potential, using in-house custom image-analysis software. The number of pixels at a given potential were counted to determine the area potentials, integrated potential density, and mean (density) potential functions (Fig.2),

The histogram for each cell was further analyzed using custom software to calculate approximately 190 parameters based on area fractions, integrated potential densities, and mean potentials. One set of parameters determined the mean potentials of mitochondria, cytoplasm, and cell. Relative probability density functions were calculated from area fractions to determine what percentage (fraction) of the cell or mitochondria is operating at a given potential or range of potentials (e.g., 30% of the cell at mitochondrial potentials of 120–240 mV). Similarly, the percentages of mitochondrial areas operating at low, moderate, and high potentials were calculated. Likewise, using the integrated potential densities, which are more sensitive membrane-potential-weighted area fractions, relative probability densities were calculated by dividing, say, the total mitochondrial integrated potential density (IPD) by the total cellular IPD, to determine, for example, that 40% of the cell's IPD was at mitochondrial potentials. This is our unique methodology to quantitatively determine an individual cell's energy production capabilities through its membrane potential.

Collectively, these provide powerful analytical tools for quantifying membrane potentials of the cell and its mitochondria. Approximately 190 parameters can be examined for each cell, though experience shows that 56 of them provide the necessary

$$\begin{aligned} \text{Area Potential } (T_1, T_2) &= \sum_{\Delta\psi=T_1}^{T_2} H(\Delta\psi) \\ \text{Integrated Potential Density } (T_1, T_2) \\ \text{(Integrated "Optical" Density)} &= \sum_{\Delta\psi=T_1}^{T_2} H(\Delta\psi) \times \psi \\ \text{Mean Potential } (T_1, T_2) \\ \text{(Average "Density")} &= \frac{\text{IOD}}{\text{Area}} = \frac{\sum_{\Delta\psi=T_1}^{T_2} H(\Delta\psi) \times \Delta\psi}{\sum_{\Delta\psi=T_1}^{T_2} H(\Delta\psi)} \end{aligned}$$

FIGURE 2. Methods for quantitative analysis of digital images. The equations are used to sum the pixel intensities expressed in terms of millivolt potentials as described in the text. **H** is a histogram of the pixel potentials in millivolts, $\Delta\psi$ is the membrane potential in millivolts, and T_1 is the lower limit potential and T_2 the upper limit potential.

information for analysis. The parameters of many individual cells are examined further in standard statistical analyses to calculate means, standard deviations, medians, minimums, and maximums for cell populations for individual cells with respect to a cell population, and/or for populations with populations. The analysis seeks to determine whether mitochondria are able to operate at their normal higher potentials; if not, it would indicate that their *ability* (thermodynamic capacity) to make ATP is impaired.

2.4. Criteria for Patient and Control Populations

Patient selection was carried out at a remote independent clinic specializing in diagnosis and treatment of CFS. Patients filled out extensive medical histories that included medications, date of symptom onset, and questionnaires assessing symptom severity, evaluated by a clinician specializing in CFS. Patients were diagnosed according to Centers for Disease Control (CDC) criteria (Fukuda *et al.*, 1994; Holmes *et al.*, 1988). All patients were classified by age, sex, race, and clinically rated activity level for comparison with controls. As mentioned, a loose consensus exists that the patient population is mainly female and often white. Controls were nonpatient volunteers with no acute or chronic illness taking no known medication, who were donating blood for routine testing at UNC Hospitals.

3. RESULTS

3.1. Confocal Imaging of Membrane Potential in TMRM-Labeled Human Cells

Confocal images of TMRM-labeled human mononuclear leukocytes were acquired from all individuals in the study under the same set of experimental conditions. In accord with our protocol (Sect. 2.1), all images of human mononuclear leukocytes were of cells attached to glass coverslips and placed in special microscope chambers. Cells were labeled as close to the time for actual imaging as was feasible. In experiments studying the effect of cytokines on human fibroblasts, cells were subcultured on the coverslips. Our experience with controls studies showed that over the course of our observations and experiments, isolated cells exhibited no time-dependent decrease in viability and membrane potential. In any event, both CFS and control specimens were subject to the same conditions. Also, control studies found that a confocal zoom factor of 3.0 using a 63×1.4 N.A. lens optimal for human mononuclear leukocytes, because it permitted simultaneous imaging of multiple cells. This zoom factor yielded a sufficient number of pixels per cell and per mitochondrion for numerical analysis and kept the inherent shading problem in the design of the Bio Rad MRC 600 to manageable levels. Human fibroblast cells, due to their larger size, were best followed with a zoom factor of 2.0.

3.2. Patient and Control Populations in this Study

The CFS population of 34 individuals was 70% female and 30% male, ranging in age from 18 to 60, with both a mean and a median age of 40. Similarly, the control population of 15 individuals was 72% female and 28% males, ranging in age from 20 to 50, with both

a mean and a median age of 37. Of the 25 patients with a known date of CFS onset prior to the blood draw, the median length of the illness in years was 5.4, the mean 7.6, the maximum 25, and the minimum 0.6.

3.3. Comparison of Patient and Control Populations

Our data indicate that the mitochondrial membrane potential and the areas at mitochondrial potentials are significantly lower in CFS patients than in normal individuals, which can be shown numerically and visually. The result is most clearly seen by visually comparing membrane potential images of cells from a typical control with those from a typical CFS patient. Mononuclear leukocytes from a control are shown in a laser-scanning confocal gray-level image, wherein the brighter the image the more negative the potential (Fig. 1A); mitochondria are the small bright oval. In contrast, typical CFS cells from our study population (Fig. 1B) have much dimmer cells and mitochondria in the gray-level image. The same comparisons can be seen in another healthy control (Fig. 1C) and another CFS patient (Fig. 1D). This clear and significant difference between CFS patients and normal controls is typically seen when comparing the membrane potential confocal images. The CFS patients cells and mitochondria are at lower potentials, with a smaller total cellular area at potentials defined as mitochondrial.

The $\Delta\psi$ differences for CFS patients vs controls can be shown numerically and graphically using the analyses in Section 2.3. The total cellular and mitochondrial average potentials were lower for approximately 800 mononuclear leukocyte CSF cells compared with approximately 300 control cells (Fig. 3A). The differences were statistically significant at the 99% confidence level using the students *t*-test for a two-tailed hypothesis calculated according to Zar (1985). Similarly, we determined that the relative probability densities of the cytoplasmic and total low- and midmitochondrial IPDs are lower in CFS patients as compared with controls (Fig. 3B). These findings were also calculated to be significant at the 99% confidence level (Zar, 1985). Thus, quantitative differences can be established between CFS patients and healthy individuals.

3.4. Comparison of Male and Female Chronic Fatigue Syndrome Patients

Examining approximately 500 cells from female and 300 cells from male patients in our initial studies, we found values of 78.9 ± 13 mV and 81.2 ± 4 mV for mean (total) cell potential, 60 ± 4.6 mV and 60.5 ± 2.4 mV for the mean (nonmitochondrial) cytoplasmic potential, 110 ± 5.4 and 110 ± 3.5 mean mitochondrial potential, respectively. Likewise, we found cell values of $65 \pm 15\%$ and $60 \pm 5\%$ for the areas at cytoplasmic potentials and correspondingly, $35 \pm 15\%$ and $40 \pm 5\%$ for areas at mitochondrial potentials. We also found that $96 \pm 4\%$ and $99 \pm 0.5\%$ respectively, of the mitochondria area fraction was at low mitochondrial potentials, which does not support an appreciable or statistically significant difference between female and male CFS patients.

3.5. Interferon- α Effects on Human Fibroblasts

Adding human IFN- α to human cells causes relatively rapid and protracted decreases in the average total cellular and mitochondrial potentials and the areas at mitochondrial

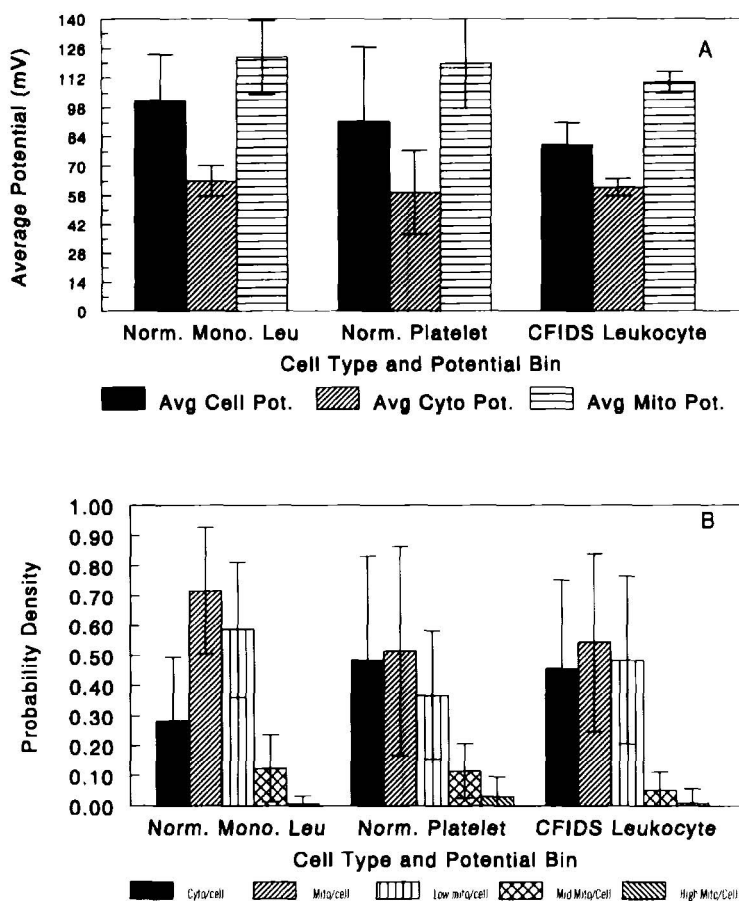


FIGURE 3. Quantitative comparison of mitochondrial membrane potential between a CFS patient population and normal, healthy controls. Data are based on 800 mononuclear leukocytes isolated and prepared from 34 CFS patients and over 300 cells from 15 controls. (A) Average membrane potentials are shown for the total cell, the cytoplasm, and the total mitochondria according to the figure's key. Total cellular and total mitochondrial average potentials were significantly lower in CFS patients compared with controls at the 99% confidence level when analyzed for a two-tailed hypothesis using the student's *t*-test as described in Zar (1985). Average cytoplasmic potential is approximately the same in both groups. (B) Integrated potential density function (IPD) to calculates the relative probability density relative to the total cell IPD. The IPD relative probability densities for total mitochondrial potential and low- and midmitochondrial potentials are lower in CFS patients compared with controls, whereas the cytoplasmic relative probability densities are correspondingly higher. Values are statistically different at the 99% confidence level.

potentials. Adding 100 ng/ml of human IFN- α to a fibroblast cell (Fig. 1E) decreased TMRM intensity in cell's mitochondria (Fig. 1F) within three minutes. This decrease persisted for up to 3 hours (data not shown). The same effect was seen on human mononuclear leukocytes (Fig. 4) when treated with 100 ng/ml IFN- α . The total mitochondrial average potential dropped within 2.5 minutes of adding it and remained depressed over the 60 minutes we observed. Cytoplasmic average potential drops slightly, roughly

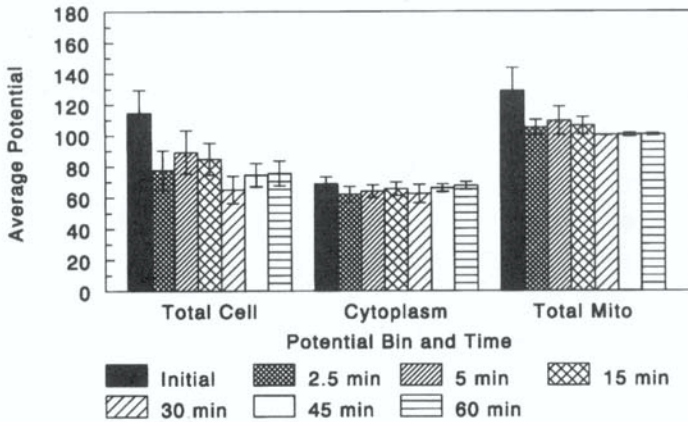


FIGURE 4. Effect of interferon- α on human mononuclear leukocyte membrane potential. Membrane potentials of mononuclear leukocytes were monitored beforehand for 60 min after adding 100 ng/ml interferon- α . Cell populations comprised approximately 50 cells each time. Decreases in total cellular and total mitochondrial average potentials are significantly lower at the 95% confidence level based on a student's *t*-test for a two-tailed hypothesis (Zar 1985).

recovering over the course of the 60 minutes. Total cellular average potential, comprised of the two previous potentials, initially dropped sharply, then oscillates somewhat at the decreased level over the same period. Both human fibroblasts and mononuclear leukocytes showed decreased potentials after IFN- α addition.

4. DISCUSSION

Our confocal microscopy studies of the TMRM distribution in human mononuclear leukocytes from CFS and control populations, which shows lower membrane potentials in the CFS patients, provide the first evidence of a specific cellular site of impairment in the bioenergetics of these patients. Our studies of IFN- α effects on membrane potential in cells provide the first evidence of cytokine-induced effects on the membrane potential and also suggest a possible link between these immune- and cell-signaling agents and alterations in mitochondrial bioenergetic function.

4.1. Membrane-Potential Evidence for a Mitochondrial Dysfunction Role in Chronic Fatigue Syndrome

Our data implicate mitochondrial dysfunction in the CFS population we studied. Our comparison of CFS and control populations reveal a difference in $\Delta\psi$. In CFS patients the total cellular and total mitochondrial average potentials and the IPDs (and area fractions) of the cells at mitochondrial potentials were lower. The 12-mV lower values for the average $\Delta\psi$ in CFS patients compared with controls (122 vs 110mV) should be taken as the minimum difference. This is based on two factors inherent in the quantitative analysis employed (Sect. 2.3). Because the confocal image is defined only by membrane potential, a mitochondrion is operationally defined as having a

potential between 100 and 240 mV. Thus, mitochondria (perhaps dysfunctional) operating below 100mV are treated as cytoplasm. This is why the IPDs and areas fractions are important in the analysis, however. Cells having sub-100mV mitochondria will have smaller mitochondrial IPDs and area fractions, as we observed for CFS patients. In addition, the total cellular average potential, some 21 mV lower in CFS patients (including mitochondrial and cytoplasmic contributions) suggests that the average difference for all mitochondria in the cell may be, effectively, 21 mV. Reports based on the state 4 to state 3 transition in isolated rat liver mitochondria determined a 30-mV average decrease during ATP synthesis (e.g., Petite *et al.*, 1990). Thus, the approximately 12–21 mV lower average potential and approximately 24% lower mitochondrial IPD indicates significant impairment in the ATP-making ability of some or all CSF mitochondria.

Our comparison of male and female CFS patients indicates that the menstrual cycle may not have a significant effect on the parameters we measured. No one knows why more women than men are diagnosed with CFS, although there is speculation that women are more likely to seek medical care (e.g., Hyde *et al.*, 1992; Reyes *et al.*, 1997). Distribution of CFS patients along racial and gender lines has been examined in a number of reports (Buchwald *et al.*, 1995; Gunn *et al.*, 1993; Komaroff, 1997; Reyes *et al.*, 1997), and our patient population was consistent with these reports. Our data indicate that between male and female patients diagnosed with CFS, there is on average no significant mitochondrial difference, and we are unaware of any such distinction in the literature, or of any between male and female physiological need for such a difference consistent with our data.

Our finding of a likely impairment in the ability of mitochondria in CFS patient cells to make ATP may well explain certain widespread CFS symptoms, some of which were mentioned previously (Sect. 1.3.). This is supported by the fact that illnesses of known mitochondrial dysfunction give rise to many of the same symptoms (e.g., Carafoli and Roman, 1980; DiMauro *et al.*, 1985; Frackowiak *et al.*, 1988; Roe and Coates, 1989). Given the disparate but systemic nature of CFS symptoms, our findings are further supported by the fact that mitochondrial dysfunction is expressed differently in different tissues, attributable to the different energy demands of these tissues (Behan *et al.*, 1991; DiMauro *et al.*, 1985; Peterson, 1995; Wallace, 1992). Consequently, muscle and brain tissue are most likely (but not exclusively) to give rise to patient complaints. It is no surprise, therefore, that the major CSF complaints in addition to general fatigue are typically muscle fatigability (often perceived as muscle weakness), cognitive dysfunction, and sleep abnormalities. Of particular interest are reports of the beneficial effects of carnitine, which is involved in mitochondrial β -oxidation of fatty acids—an important source of muscle energy. Ubiquinone, an electron transport chain component in the mitochondrial inner membrane, has also been reported to help some CFS patients, but few studies have truly examined cellular energy production at the cellular and subcellular levels.

Our findings of impaired mitochondrial ATP-making ability in CFS patient cells may explain reports of muscle bioenergetics. Muscle bioenergetics tend to be studied for two reasons: (1) Muscle is the most plentiful tissue, and biopsy samples are somewhat easier to do. Conventional biochemical or microscopic analysis, albeit limited by sample size, can be done (Behan, 1991); (2) Muscle is amenable to the use of P-NMR to study tissue bioenergetics (Jamal and Hansen, 1985; McCulley *et al.*, 1996; Wong *et al.*, 1992).

Nonetheless, conventional studies on CFS patient muscle tissue have yielded conflicting results. Histological studies show sporadic, but not uniform, abnormalities in muscle fibers and their mitochondria, (e.g., Behan, 1991). McCully *et al.* (1996) took the ambiguous position in their NMR studies of arguing for a defect in muscle oxidative metabolism while also maintaining the problems could be a result of muscle deconditioning. NMR studies by Lane and co-workers (1995, 1998a, 1998b), however, showed a significant subset of CFS patients with abnormal lactate responses to exercise, magnetic resonance characteristics indicative of excessive intracellular acidosis and impaired capacity for mitochondrial ATP synthesis, which could not be satisfactorily attributed to inactivity or deconditioning. Our studies using mononuclear leukocytes, which avoid the interpretative problem of the deconditioning phenomenon in muscle, support the reality of a defect in muscle oxidative metabolism. Further, mitochondria in mononuclear leukocytes “look” normal, which would explain many of the histological findings of small ultrastructural abnormalities. Our ability to examine individual cells and their mitochondria using confocal microscopy and our analytical methods (Sect. 2.3) also have an advantage over P-NMR, which inherently must average a great many cells in a given muscle area. One drawback to our technique, however, is that it is not an *in vivo* approach, though the mitochondria might well be considered “*in vivo*” since they are in their normal environment, cytoplasm. Future studies to complement our findings would ideally study the hard-to-isolate-and-maintain individual muscle cells, looking at bioenergetic differences in CFS and healthy controls populations.

It should be clear that our data do not prove that mitochondrial dysfunction causes CFS. Rather, we show that mitochondrial dysfunction is *involved* in CFS. Whether some immune- or cell-signaling abnormality, perhaps due to a genetic predisposition perhaps triggered by a viral or bacterial infection, causes mitochondrial dysfunction remains to be seen. Some of our studies adding cytokines such as IFN- α or IL-2 to human mononuclear leukocytes or fibroblasts, which show for the first time an effect on mitochondrial function, may suggest such a linkage, however.

4.2. Searching the Mitochondrial Pathways for Possible Defects

Our data show mitochondrial dysfunction in CFS patient cells, but further studies are needed to determine if there is a site (or sites) for this dysfunction in the mitochondrial metabolic pathways or if there is a mitochondrial membrane problem. Such studies can be accomplished through the use of various mitochondrial cell-permeable substrates or inhibitors. The $\Delta\psi$ response of control and CFS cells is being examined for differences in their response to: various fatty acid substrates and glucose; uncouplers of oxidative phosphorylation such as carbonylcyanide-*m*-chlorophenyl hydrazone; various inhibitors of specific electron transport enzymes such as antimycin A for the ubiquinol-cytochrome *c* oxidoreductase complex, an inhibitor of ATP synthesis (oligomycin); and so forth. Differences in CSF and control response to one or more of these agents could identify the site or sites of mitochondrial dysfunction.

4.3. Cytokines Affect Mitochondrial Bioenergetics, Cellular Bioenergetics, or Both

The effect of IFN- α on membrane potentials of human cell lines was examined in relation to a possible role in CFS. Our studies revealed that IFN- α has a profound effect

$\Delta\Psi$, causing a marked and prolonged decrease, and hence on mitochondrial and cellular bioenergetics. Our data may also provide a rationale for reports of abnormal IFN- α levels in CFS patients (Levy, 1994; Lloyd, 1991) and the abnormal interferon production of some patients' cells in response to viral infection (Lever, 1988). We reported elsewhere that other cytokines such as IL-2 can also affect mitochondrial and cellular membrane potentials in a similar fashion (Chazotte and Pettengill, 1999). To our knowledge these are the first reports of such cytokine effects on $\Delta\Psi$ and bioenergetics.

Our data examining the IFN- α effect on mitochondrial function is consistent with clinical reports on its effect as an antiviral agent e.g., (MacDonald, 1987): many patients undergoing IFN- α therapy develop chronic-fatigue-like symptoms. Interferons cause cells to enter an antiviral state. Part of this state may be an impaired mitochondrial ability to make ATP, which makes it hard for a virus to utilize the cell's machinery to reproduce and hence, re-infect.

Our data may also be of import to chemotherapy, where it might provide a rationale for the unexplained cardiotoxicity and hepatotoxicity of IL-2 and IFN- α treatment (e.g., Kruit *et al.*, 1994; Nakagawa *et al.*, 1996). Patients undergoing such treatment regimes for renal carcinoma, for example, develop chronic-fatigue-like symptoms as well as organ toxicities. Better understanding of the effects of cytokines on cellular and mitochondrial bioenergetics may lead to more efficacious treatment of cancer with cytokines.

5. CONCLUSIONS

Understanding of mitochondrial dysfunction in CFS will clearly benefit from more study. Our findings warrant detailed studies on larger patient and control populations. The siting of mitochondrial impairment may lead to approaches for alleviating some of the symptoms of CFS. Studies on the effects of cytokines on cellular and mitochondrial bioenergetics are important to see whether cytokines or similar molecules are involved in linking CFS immune disturbances to mitochondrial dysfunction, and to better understand the consequences of cytokine therapies for cancer and viral infection.

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