



## Increased neutrophil apoptosis in chronic fatigue syndrome

G Kennedy, V Spence, C Underwood and J J F Belch

*J. Clin. Pathol.* 2004;57;891-893  
doi:10.1136/jcp.2003.015511

---

Updated information and services can be found at:  
<http://jcp.bmjournals.com/cgi/content/full/57/8/891>

---

*These include:*

### References

This article cites 13 articles, 5 of which can be accessed free at:  
<http://jcp.bmjournals.com/cgi/content/full/57/8/891#BIBL>

1 online articles that cite this article can be accessed at:  
<http://jcp.bmjournals.com/cgi/content/full/57/8/891#otherarticles>

### Rapid responses

5 rapid responses have been posted to this article, which you can access for free at:  
<http://jcp.bmjournals.com/cgi/content/full/57/8/891#responses>

You can respond to this article at:  
<http://jcp.bmjournals.com/cgi/eletter-submit/57/8/891>

### Email alerting service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article

---

### Notes

---

To order reprints of this article go to:  
<http://www.bmjournals.com/cgi/reprintform>

To subscribe to *Journal of Clinical Pathology* go to:  
<http://www.bmjournals.com/subscriptions/>

## SHORT REPORT

## Increased neutrophil apoptosis in chronic fatigue syndrome

G Kennedy, V Spence, C Underwood, J J F Belch

*J Clin Pathol* 2004;57:891–893. doi: 10.1136/jcp.2003.015511

**Background/Aims:** Many patients with chronic fatigue syndrome (CFS) have symptoms that are consistent with an underlying viral or toxic illness. Because increased neutrophil apoptosis occurs in patients with infection, this study examined whether this phenomenon also occurs in patients with CFS.

**Methods:** Apoptosis was assessed in patients with CFS in conjunction with concentrations of the anti-inflammatory cytokine, transforming growth factor  $\beta$ 1 (TGF $\beta$ 1).

**Results:** The 47 patients with CFS had higher numbers of apoptotic neutrophils, lower numbers of viable neutrophils, increased annexin V binding, and increased expression of the death receptor, tumour necrosis factor receptor-1, on their neutrophils than did the 34 healthy controls. Patients with CFS also had raised concentrations of active TGF $\beta$ 1 ( $p < 0.005$ ).

**Conclusions:** These findings provide new evidence that patients with CFS have an underlying detectable abnormality in their immune cells.

Chronic fatigue syndrome (CFS) is a debilitating condition that has no known aetiology or pathophysiology. In a search for a diagnostic test, recent gene profiling research has confirmed the heterogeneous nature of CFS,<sup>1</sup> supporting the need for continued clinical screening based on the exclusion of other diseases in association with the presence of specific symptoms. Some of these symptoms are suggestive of an underlying viral or toxic illness associated with persistent infection and immune activation. There have been various reports of immunological disturbances and viral infections in the illness.<sup>2</sup>

“Minor alterations to neutrophil function can have profound immunological consequences, with amplification of the inflammatory response and the production of cytokines”

Neutrophils make up 50–60% of the total circulating white blood cells. They are short lived reactive cells that are fundamental to the functioning of an intact immune system. Minor alterations to neutrophil function can have profound immunological consequences, with amplification of the inflammatory response and the production of cytokines.<sup>3</sup> As part of the resolution of inflammation, accumulated neutrophils are removed by apoptosis, a process where unwanted or damaged cells are eliminated without releasing their toxic contents and enhancing the inflammatory response. It is also a process associated with the release of anti-inflammatory mediators; most specifically, the production of transforming growth factor- $\beta$ 1 (TGF $\beta$ 1),<sup>4</sup> which has been implicated in the pathogenesis of CFS.<sup>2</sup>

Although increased neutrophil apoptosis is present in patients with infection,<sup>5</sup> there have been no reports of

neutrophil function in patients with CFS. The aim of our study was to compare elements of neutrophil apoptosis and TGF $\beta$ 1 in patients with CFS with a control group of age and sex matched healthy subjects.

## METHODS

Forty seven patients selected by clinical examination to fulfil the Centres for Disease Control classification of CFS<sup>7</sup> and 34 sex and aged matched healthy subjects volunteered for the study (table 1). All subjects gave written informed consent and the local medical ethics committee approved the study.

Neutrophils were separated from whole blood after sedimentation with dextran and histopaque density gradient centrifugation. The neutrophils were immediately incubated with annexin V and/or propidium iodide (PI), as described by literature enclosed in the TACS annexin V FITC kit (R&D Systems, Abingdon, Oxfordshire, UK). Exposure of phosphatidylserine on the outer leaflet of the cytoplasmic membrane is characteristic of early apoptosis and is associated with preferential binding of annexin V. Cells with an intact plasma membrane exclude PI and this property is maintained in those cells that are in the early stages of apoptosis.<sup>8</sup> In contrast, necrotic cells have lost their membrane integrity and stain with PI. Neutrophils that were positive for annexin V only were termed early apoptotic, neutrophils that were positive for both annexin V and PI were termed late apoptotic and/or necrotic, and neutrophils that were positive for neither were classified as viable cells. Leucocyte tumour necrosis factor receptor I (TNFRI) surface expression was measured on a second whole blood sample using a fluorescent labelled antibody, as described in the product insert (R&D Systems; FAB225F). The remaining blood sample was used to prepare platelet poor plasma (PPP) and activated TGF $\beta$ 1 were measured in the PPP by enzyme linked immunosorbent assay (R&D Systems). TGF $\beta$ 1 is present in platelet granules and is released after platelet activation; therefore, PPP values are representative of circulating concentrations.<sup>9</sup>

All blood samples were taken at the same time of day and all tests performed by the same person. The data were normally distributed and an unpaired *t* test was used to compare the mean values of all the parameters between the two subject groups.

## RESULTS

Staining for annexin V and PI showed that patients with CFS had significantly more apoptotic ( $p = 0.002$ ) and significantly fewer viable neutrophils ( $p = 0.002$ ) than did healthy controls. There was a trend for patients with CFS to have later apoptotic/necrotic neutrophils ( $p = 0.075$ ).

The patients' neutrophils also showed increased annexin V binding ( $p = 0.001$ ; 37.4% compared with 22.8% in the control group). Mean annexin V expression in the control group (22.8%) was similar to that reported by others

**Abbreviations:** CFS, chronic fatigue syndrome; PI, propidium iodide; PPP, platelet poor plasma; TGF $\beta$ 1, transforming growth factor  $\beta$ 1; TNFRI, tumour necrosis factor receptor I

**Table 1** Comparison of TGF $\beta$ 1, TNFRI, and annexin V expression in patients with CFS and matched controls

	CFS patients	Controls	p Value
Males/Females	18/29	13/21	
Mean age (range) in years	47.5 (19 to 63)	45.9 (19 to 63)	
Duration of illness (range) in years	9.9 (2 to 27)		
TNFRI expression on neutrophils (%)*	12.5 (8.3 to 16.8)	3.9 (1.0 to 6.8)	0.004
TNFRI expression on monocytes (%)*	8.02 (4.5 to 11.5)	3.1 (0.1 to 6.1)	0.068
TNFRI expression on lymphocytes (%)*	2.8 (2.4 to 3.2)	2.2 (1.8 to 2.6)	0.099
TNFRI expression on all WBCs (%)*	7.1 (4.5 to 9.7)	2.6 (1.2 to 4.1)	0.008
Neutrophil annexin V expression (%)*	37.4 (30.5 to 44.3)	22.8 (18.6 to 29.1)	0.001
Per cent of all neutrophils that are			
Necrotic cells*	1.04 (0.8 to 1.3)	1.30 (1.0 to 1.8)	0.310
Late apoptotic/necrotic cells*	3.94 (2.4 to 5.5)	2.1 (1.0 to 3.5)	0.075
Normal viable cells*	63.0 (56.3 to 69.7)	77.0 (70.6 to 81.1)	0.002
Early apoptotic cells*	32.0 (25.7 to 38.2)	19.6 (16.1 to 24.9)	0.002
Active TGF $\beta$ 1 (pg/ml)*	2.24 (2.1 to 2.4)	1.89 (1.7 to 2.0)	0.005

\*Values are mean (95% confidence interval) using the unpaired *t* test.

CFS, chronic fatigue syndrome; TGF $\beta$ 1, transforming growth factor  $\beta$ 1; TNFRI, tumour necrosis factor receptor I; WBCs, white blood cells.

(12.3–35.0%).<sup>6–11</sup> The relatively large range is a result of the methodological differences between various laboratories necessitating good laboratory practice to make certain that all samples are treated identically. The expression of neutrophil TNFRI was also significantly higher in the patient group ( $p = 0.004$ ).

There were significantly greater amounts of activated TGF $\beta$ 1 in the PPP of patients with CFS than in the healthy matched control subjects ( $p = 0.005$ ).

## DISCUSSION

We have shown that there are a greater proportion of apoptotic cells among neutrophils isolated from patients with CFS and that these cells are significantly less viable when compared with those from healthy subjects. The same neutrophils expressed more TNFRI death receptor molecules and had increased binding of annexin V, indicative of phosphatidylserine exposure.

Apoptosis is triggered by signals initiated both by external stimuli and internal sensors. The death receptor mediated pathway, also known as the extrinsic pathway, starts with the binding of TNF family ligands to the death receptor TNFRI. This results in the recruitment of an adaptor protein, TNFR associated death domain, which in turn recruits another adaptor molecule, the Fas associated death domain. The Fas associated death domain then recruits procaspase 8 or procaspase 10 to form a death inducing signal complex. During the formation of the death inducing signal complex, procaspase forms are cleaved and converted, with the release of activated caspase 8 or caspase 10, which directly convert other procaspases to their active forms, thereby initiating apoptosis. However, there is crosstalk between the extrinsic and intrinsic (mitochondrial dependent) pathways, and cleavage of the proapoptotic protein, Bid, by caspase 8 can occur. This cleavage can result in the release of cytochrome C and the triggering of further apoptotic mechanisms.<sup>12–13</sup> In our study, we have shown that the neutrophils of patients with CFS have increased expression of TNFRI and we can only surmise that the accelerated apoptosis of these cells is a consequence of extrinsic factors affecting apoptotic pathways.

“The data presented here are consistent with the fact that many patients with chronic fatigue syndrome have an underlying, detectable abnormality in the behaviour of their immune cells, consistent with an activated inflammatory process”

In addition, we found that the concentrations of activated TGF $\beta$ 1 were significantly raised in the PPP of patients with CFS. An increase in activated TGF $\beta$ 1 in conjunction with neutrophil apoptosis is an important process in the down-regulation of cytokines and eicosanoid production during the chronic inflammatory process.<sup>4</sup> TGF $\beta$ 1 is also crucial in the apoptotic process because it curbs leucocyte adhesion and transmigration,<sup>14</sup> and this impairment of the transmigratory process of neutrophils may independently promote apoptosis. Neutrophils that transmigrate across the endothelium lose TNF receptors and this loss of receptor density is necessary for the survival of the neutrophil.<sup>15</sup> The fact that neutrophils from patients with CFS have increased surface expression of TNFRI is a further indication that such cells are more susceptible to apoptosis.

The neutrophils of patients with CFS have an increased rate of apoptosis and this may impact on the innate immune system of these patients, given that neutrophils are the major effector cells of this system. The control of apoptosis is complex, and the increased rate of apoptosis in patients with CFS may be a consequence of several factors. Accelerated apoptosis is indicative of a persistent or reactivating viral infection or a toxic state, reprogramming of apoptotic pathways by an infectious or toxic agent, or quicker neutrophil turnover, secondary to an abnormal host response to noxious stimuli.

With the advent of gene profiling the search is on for causative agents in CFS.<sup>1</sup> The data presented here are consistent with the fact that many patients with CFS have an underlying, detectable abnormality in the behaviour of their immune cells, consistent with an activated inflammatory process.

## Take home messages

- Patients with chronic fatigue syndrome (CFS) had higher numbers of apoptotic neutrophils, lower numbers of viable neutrophils, increased annexin V binding, and increased expression of the death receptor, tumour necrosis factor receptor I, on their neutrophils than healthy controls
- Patients with CFS also had raised concentrations of active transforming growth factor  $\beta$ 1
- Thus, patients with CFS appear to have an underlying abnormality in their immune cells

**ACKNOWLEDGEMENTS**

The study was funded by the charity ME Research Group for Education and support (MERGE), Perth, UK. Further support was also received from the Sir John Fisher Foundation (educational grant).

.....

**Authors' affiliations**

**G Kennedy, V Spence, C Underwood, J J F Belch**, Vascular Diseases Research Unit, University Department of Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK

Correspondence to: Dr G Kennedy, Vascular Diseases Research Unit, Division of Medicine and Therapeutics, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK; [g.kirk@dundee.ac.uk](mailto:g.kirk@dundee.ac.uk)

Accepted for publication 15 March 2004

**REFERENCES**

- 1 Whistler T, Unger ER, Nisenbaum R, *et al*. Integration of gene expression, clinical, and epidemiologic data to characterize chronic fatigue syndrome. *J Transl Med* [In press.] [2003 Dec 1 Epub ahead of print.]
- 2 Patarca R, Mark T, Fletcher MA, *et al*. Review: immunology of chronic fatigue syndrome. *Journal of Chronic Fatigue* 2000;**6**:69–107.
- 3 Robinson JP, Carter WO, Narayanan P. Functional assays by flow cytometry. Immune cell phenotyping and flow cytometric analysis. In: Rose NR, deMarcaro E, Folds JD, *et al*, eds. *Manual of clinical laboratory immunology*, 5th ed. 1997:245–54.
- 4 Fadok VA, Bratton DL, Konowal A, *et al*. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF- $\beta$ , PGE2 and PAF. *J Clin Invest* 1998;**101**:890–8.
- 5 Nwakoby IE, Reddy K, Patel P, *et al*. Fas-mediated apoptosis of neutrophils in sera of patients with infection. *Infect Immun* 2001;**69**:3343–9.
- 6 Wang SZ, Smith PK, Lovejoy M, *et al*. The apoptosis of neutrophils is accelerated in respiratory syncytial virus (RSV) induced bronchiolitis. *Clin Exp Immunol* 1998;**114**:49–54.
- 7 Fukuda K, Straus SE, Hickie I, *et al*. The chronic fatigue syndrome: a comprehensive approach to its definition and study. *Ann Intern Med* 1994;**121**:953–9.
- 8 Tait JF, Smith C, Wood BL. Measurement of phosphatidylserine exposure in leukocytes and platelets by whole blood flow cytometry with annexin V. *Blood Cells Mol Dis* 1999;**25**:271–8.
- 9 Grainger DJ, Mosedale DE, Metcalf JC. TGF- $\beta$  in blood: a complex problem. *Cytokine Growth Factor Rev* 2000;**11**:133–45.
- 10 Majewska E, Sulowska Z, Baj Z. Spontaneous apoptosis of neutrophils in whole blood and its relation to apoptosis gene proteins. *Scand J Immunol* 2000;**52**:496–501.
- 11 Noble JM, Thomas TH, Ford GA. Effect of age on plasma membrane asymmetry and membrane fluidity in human leukocytes and platelets. *J Gerontol A Biol Sci Med Sci* 1999;**54**:601–6.
- 12 Benedict CA, Norris PS, Ware CF. To kill or be killed: viral evasion of apoptosis. *Nature Immunol* 2002;**3**:1013–18.
- 13 Gupta S. Molecular signalling in death receptor and mitochondrial pathways of apoptosis [review]. *Int J Oncol* 2003;**22**:15–20.
- 14 Smith WB, Noack L, Khew-Goodall Y, *et al*. Transforming growth factor-beta 1 inhibits the production of IL-8 and the transmigration of neutrophils through activated endothelium. *J Immunol* 1996;**157**:360–8.
- 15 Seeley AJE, Swartz De, Giannias B, *et al*. Reduction in neutrophil cell surface expression of tumor necrosis factor receptors but not Fas after transmigration. *Arch Surg* 1998;**133**:1305–10.