

Full Length Research Paper

Self-test monitoring of the Th1/Th2 balance in health and disease with special emphasis on chronic fatigue syndrome/myalgic encephalomyelitis

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A simple “self-test” principle is described which allows patients to evaluate their Th1/Th2 balance repeatedly over short periods of time to follow-up the effects of taking pre- and probiotics, nutraceuticals, drugs or any other therapeutic strategy to balance Th1/Th2 status. By analysing a large number of first morning urine samples obtained from individuals with medical conditions associated with an overactive Th2 arm (ulcerative colitis, autism, blastocystis, mercury poisoning and viral infection), a reaction principle was discovered that uses a redox-active colorimetric substrate changing color upon reaction with metabolites present at high concentration in the urine samples of Th2-shifted individuals. The development of color is time-dependent and quantitative. Moreover, 75% of urine samples obtained from chronic fatigue/myalgic encephalomyelitis patients produced a time-dependent and quantitative change of color compared to only 4% of the controls (perfectly healthy population), providing evidence that chronic fatigue syndrome/myalgic encephalomyelitis is a condition associated with an overactive Th2 arm.

Key words: Th1/Th2 balance, urine samples, self test, chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME).

INTRODUCTION

T helper cells (Th cells) are a sub-group of lymphocytes which play an important role in establishing and maximising the capabilities of the immune system (Mosmann et al., 1986). Th cells are specifically involved in activating and directing other immune cells and may differentiate into two major sub-types of cells known as Th1 and Th2 cells (Prete, 1998; Simpson et al., 2002). Whereas Th1 cells are critically involved in the generation of effective cellular immunity, Th2 cells are instrumental in the generation of humoral and mucosal immunity and allergy (Bonecchi et al., 1998; Arestides et al., 2002).

Diseases and particularly immune-mediated disorders

involve dysregulation of the Th1/Th2 balance and can often be classified as Th1- or Th2- mediated (Th1 or Th2 dominant) (Mosman and Coffman, 1989; Nicholson and Kuchroo, 1996; Nishimura, 1999).

However, simple self-tests allowing physicians and patients to follow-up Th1/Th2 balance during therapy are lacking and therefore it still remains very difficult for an individual to evaluate whether the medical treatment he or she is undergoing to restore Th1/Th2 balance is effective. In addition, the effectiveness of over-the-counter products which claim to balance Th1/Th2 status (such as anti-oxidants, pro-biotics and other) should be able to be evaluated on an individual basis. Many patients try to improve their health by “trial and error”, exploring probiotics, nutraceuticals and drugs, without realizing the potential risk of further deterioration to their health by randomly taking products that may worsen their

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Th1/Th2 balance. Several chronic inflammatory diseases have been described as Th1-mediated diseases, including multiple sclerosis (Tremlett et al., 2002), inflammatory bowel disease (Pallene and Monteleone, 1998), Crohn's disease (Brand, 2009), diabetes (Aso et al., 2006; Kyoko et al., 2008), Lupus (Theofilopoulos et al., 2001), Königs disease (Flynn et al., 2004) and rheumatoid arthritis (Harting et al., 2003). Diseases reported as being Th2-mediated include allergic rhinitis (Shahabi et al., 2006), asthma (Tremlett et al., 2002; Hoshimo et al., 2003), autism (Gupta et al., 1998), dermatitis (Makatani et al., 2003), ulcerative colitis (Heller et al., 2005) and blastocystis (Zierdt, 1991). In general, immune-mediated disorders are difficult to treat. Some therapies specifically aim to restore the Th1/Th2 balance by down-regulating Th1 activity and up-regulating Th2 activity, or vice versa (Adorini et al., 1996). Obviously, this requires an accurate diagnosis of the disease, as inappropriate treatment may result in a greater Th1/Th2 imbalance.

However, a specific diagnosis is often difficult to obtain. Indeed, many diseases and conditions share common symptoms, such as fatigue. Therefore, there is a need for broad spectrum assays and kits which make it possible to detect in a simple way and at an early stage whether a patient suffers from a Th1- or a Th2- mediated disease. Here, we describe the development of such an easy-to-perform self-test principle that uses a redox-active colorimetric substrate producing a clearly visible change of color upon reaction with metabolites present at high concentration in the urine of Th2-shifted individuals.

MATERIALS AND METHODS

The Th1/Th2-balance assay is based on the direct interaction of urine and a colorimetric probe under conditions that allow the colorimetric probe to react with metabolites present in the urine sample and that are present in large quantity only in Th2-mediated conditions. These metabolites are substances for which the redox potential is powerful-enough to reduce a colorimetric probe, such as a water soluble tetrazolium salt contained in an appropriate buffer.

Test materials and tools required

1. A recipient (that is, transparent eppendorf vial) with a volume mark containing a redox-active colorimetric probe (that is, tetrazolium salt 100 μ M) in an appropriate buffer (100 mM Tris, pH > 9.00).
2. A plastic container to collect a first morning urine sample.
3. A disposable plastic liquid-dispensing device.
4. A 96-well microplate spectrophotometer (optional).
5. 96-well microtiter plate.

The assay procedure either by visual observation or spectrophotometric reading

Visual observation

First morning urine is collected in the plastic container. Next a urine sample is taken with the plastic dispensing device and added to the

recipient containing the colorimetric probe. The volume of the urine added should not exceed the volume as indicated by the marker. Next, color change is observed over a period of 3 min. This color production can be easily observed with the naked eye (Figure 1).

Spectrophotometric reading

To make a quantitative comparison as a function of time, and for different samples obtained from controls and patients suffering from well-known Th1- or Th2-mediated diseases, we also performed tests on a limited scale and reading of color change [expressed as optical density (O.D.)] using a spectrophotometer [BioRad: O.D._{max} reading (maximum absorption wavelength of the used tetrazolium salt)]. Briefly, 100 μ l solution of colorimetric reagent is contacted with 200 μ l first morning urine in wells of a 96-well Nunc microtiter plate. First the 100 μ l colorimetric solution is added and next 200 μ l of the urine sample. Optical density change at O.D._{max} is read at ambient temperature immediately after adding the urine and after 1, 2, 3 and 15 min, respectively (Table 1).

RESULTS

The assay procedure for visual inspection and observed results are illustrated in Figure 1. We also wanted to investigate whether CFS/ME is indeed a Th2-mediated illness, urine samples obtained from a large population of 472 patients were tested on a "visual" basis for color change after three minutes. The results are shown in Figures 2 and 3.

From the kinetic readings (O.D. reading with spectrophotometer), it is clear that after one minute, a significant color change is obtained with the Th2-mediated conditions (Far Th2), but no color change is seen with Th0- or Th1- mediated conditions. After two minutes, a substantial color change is observed with the Th2-mediated conditions (Moderate Th2), whereas still no significant color change is observed with Th0 or Th1 conditions. After three minutes, a slight color change is observed with healthy controls, and still no color change is observed with the Th1-mediated conditions, and a dramatic color change can be seen with the Th2-mediated conditions. After fifteen minutes a significant color change is observed with healthy controls, and still no significant color change is observed with the Th1-mediated conditions. With the Th2-mediated conditions after 15 min, the optical density of the reaction vial content exceeds the maximum which can be measured by the spectrophotometer. From these results it can be concluded in general that:

1. No color change at all after 3 min is indicative of a Th1-shifted condition. Color change should be followed for another 12 min. If after 15 min no color change is observed, this corresponds to a "far" shifted Th1 condition.
2. No color change or faint color change observed within 3mins corresponds to a "fully" balanced (neutral) Th1/Th2 start to develop
3. Immediate color change, or color change within

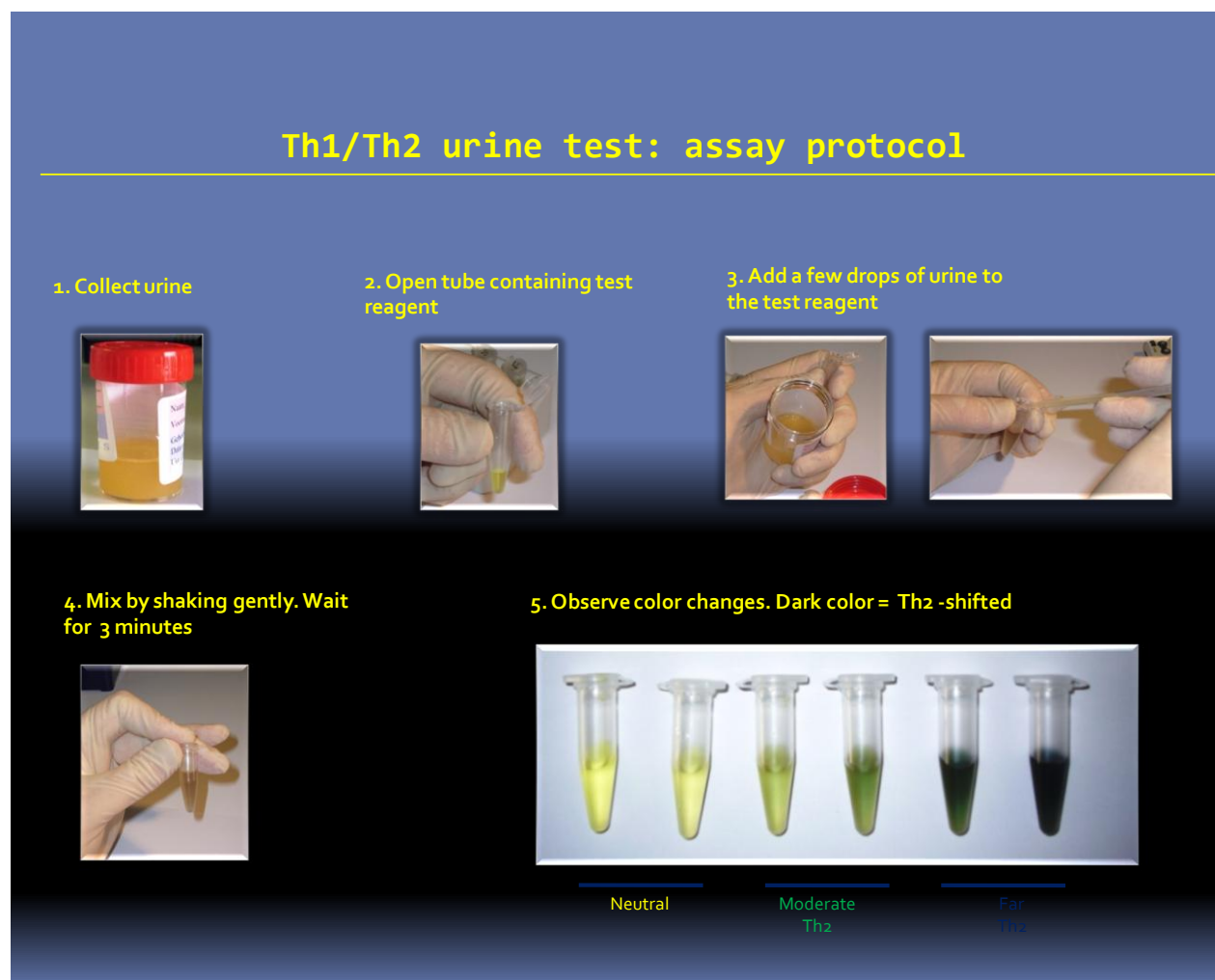


Figure 1. Overall protocol of the Th1/Th2 urine test and visual inspection of color change after 3 min.

3 min is indicative of a Th2-shifted condition. Significant darkening within the first minute is indicative of a “far” shifted Th2 condition.

DISCUSSION

Although the test described is a “broad” spectrum diagnostic test, it provides a very fast and reproducible tool for detecting, diagnosing and/or monitoring Th1 and Th2 mediated conditions. Moreover, the assay provides the basis of diagnostic kits suitable for detecting, diagnosing and/or monitoring conditions or diseases related to inflammatory, infectious and/or chronic immune disease. The color change of the redox indicator dye (due to metabolites characteristic of Th2-mediated condition) occurs rapidly, that is, within half a minute after contacting the urine sample with the colorimetric reagent. Similar metabolites are also found in urine samples

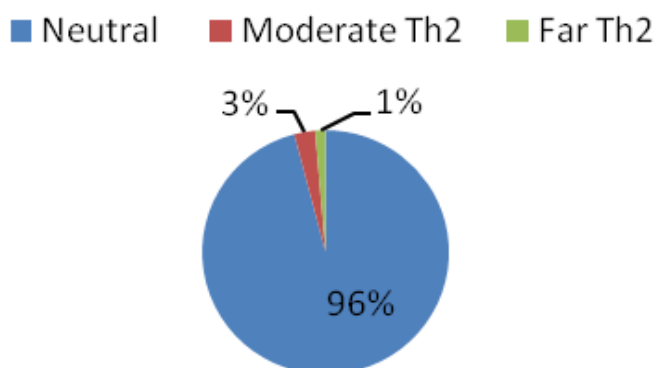
obtained from healthy control samples, although less rapidly reacting: usually a color change is observed after three minutes or longer, although the difference between urine of persons with a Th2-mediated condition and healthy individuals remains visible even after 15 min. Surprisingly, it was found that with urine obtained from individuals with a Th1-mediated condition, this color change does not occur, or occurs far less rapidly, compared to healthy control samples. Therefore, Th1 and Th2 mediated conditions and diseases can be differentiated by an analysis of the same metabolites: wherein Th2-mediated conditions are characterised by an increased concentration of metabolites and Th1-mediated conditions are characterised by a decreased concentration. A Th2-mediated condition may refer to a Th2-mediated disease or infection (that is, ulcerative colitis) or an altered immunological state such as pregnancy (Formby, 1995).

The link between Th2 and altered redox status of the

Table 1. Test results of urine samples from healthy persons and patients diagnosed with various Th1- or Th2-mediated disease conditions.

Condition	Urine/O.D. _{max} (mean) after			
	1 min	2 min	3 min	15 min
Healthy controls (n=73)	0.035	0.060	0.110	1.200
Th2				
CFS/ME (n=50)	0.315	0.650	1.200	>3.000
Ulcerative Colitis (n=10)	0.325	0.920	1.560	>3.000
Food Intolerance (n=5)	0.650	0.955	1.400	>3.000
Blastocystis (n=2)	0.600	1.250	1.800	>3.000
Autism (n=25)	0.400	0.750	1.150	>3.000
Pregnancy (6-9 months)(n=2)	0.195	0.320	0.850	>3.000
Mean O.D. Th2 (n=94)	0.414	0.808	1.326	>3.000
Th1				
Königs disease (n=1)	0.030	0.035	0.035	0.035
Rheumatoïd Arthritis (n=12)	0.025	0.035	0.035	0.125
Bechterew (n=2)	0.030	0.030	0.045	0.040
Lupus (n=5)	0.035	0.035	0.040	0.040
Mean O.D. Th1 (n=20)	0.030	0.033	0.038	0.060

Controls (n=73)

**Figure 2.** Results obtained from urine samples of presumed perfectly healthy controls (n=73).

urine is not surprising in that a cellular Th2 status is obtained by constant depletion of reducing substances, in particular reduced glutathione, other thiol compounds and the like, that may end up in the urine of Th2-shifted individuals (Monick et al., 2003). This test principle can also be used to follow-up any strategy used to manipulate or to balance the Th1/Th2 equilibrium (Ray and Cohn, 2000; Uciechowski et al., 2008; Cheng et al., 2005; Formby 1995; Wilczynski et al., 2000; Kyoko et al., 2008). For instance, it may be important for people out of

balance to know what their actual status is before trying different remedies on a trial and error basis (Charaat, 2007; Kroemer et al., 1996). A typical example is a person believing they should drink green tea to improve a Th2-mediated disease. Green tea however, is a Th2-shifter and therefore will make Th2-mediated condition even worse (Walsh, 2009). It was also found that the majority (75%) of CFS/ME sufferers (Karnofsky score < 60) are moderate or far Th2-shifted. These results correspond with data on cytokine profiles (IL4, IL8, and IL13) published by others (Fletcher et al., 2009; Lombardi et al., 2011) and provide further evidence that CFS/ME is a condition with an overactive Th2 arm. Methods and kits according to the present article can be used to develop *in vitro* diagnostic methods and diagnostic kits that can be used for detecting, diagnosing and/or monitoring a Th1- or Th2- mediated condition.

In particular, it can be used for the detection, diagnosis and/or monitoring of inflammatory, infectious and/or chronic immune diseases, such as infectious diseases (including viral, bacterial, prion-related infectious disease), cancers, mental disorders, autism, immunologic disorders (including auto-immune disease, rheumatoid arthritis and the like), nervous system disorders, disorders of the gut, defective wound healing processes, poisoning (with heavy metals, pesticides and the like), unexplained syndromes, age-related processes, biological clock related processes and diseases with unknown etiology such as fibromyalgia, CFS/ME and the like. The described diagnostic principle can be used to detect the presence of a “disease state” (which may be,

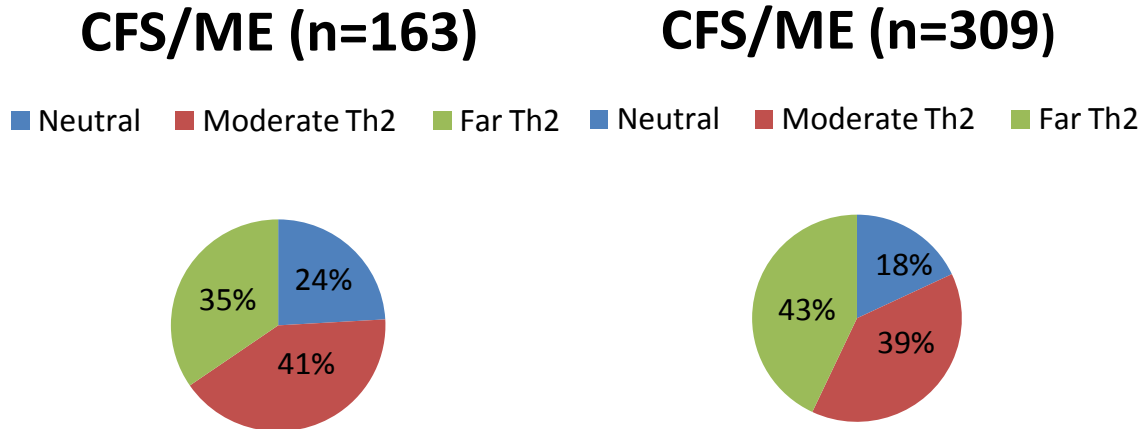


Figure 3. Results obtained from urine samples collected from CFS/ME patients in Europe (n=163) and patients in Australia (n=309).

but is not limited to, any of the disease states listed in this article) or to monitor the severity of a previously identified disease state. This urine test may be useful as a first step in alerting patients to seek medical advice and/or in assisting medical practitioners in recognising a disease state where a disease may be “silent” and difficult to diagnose.

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