Short papers

Validity of a modified shuttle test in adult cystic fibrosis

Judy Bradley, Jennifer Howard, Eric Wallace, Stuart Elborn

Abstract

***Background*—The purpose of this study was to provide some evidence of the validity of a modified shuttle test (MST) by comparing performance on the MST with peak oxygen consumption ($\dot{V}O_2$peak) measured during a treadmill test in a group of adult patients with cystic fibrosis.**

***Method*—Twenty patients with stable cystic fibrosis performed a ramped maximal treadmill test (STEEP protocol) and the MST using a randomised balanced design.**

***Results*—The relationship between the distance achieved on the MST and $\dot{V}O_2$peak was strong (r = 0.95, p<0.01) with 90% of the variance in $\dot{V}O_2$peak explained by the variance in MST distance. The relationship was represented by the regression equation (with 95% confidence intervals) $\dot{V}O_2$peak = 6.83 (2.85 to 10.80) + 0.028 (0.019 to 0.024) × MST distance.**

***Conclusion*—This study provides evidence of the construct validity of the MST as an objective measure of exercise capacity in adults with cystic fibrosis.**

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Keywords: modified shuttle test; cystic fibrosis

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Exercise testing is useful in determining prognosis, exercise prescription, and in the evaluation of new and existing treatments in patients with cystic fibrosis. The most precise method of assessing exercise capacity is by formal laboratory tests with online analysis of expired air. However, formal tests are not widely available to clinicians working in cystic fibrosis centres, and there is debate as to the most appropriate protocol for testing. Furthermore, many patients with cystic fibrosis find these tests excessively stressful and are reluctant to perform such tests on a routine basis. Some centres have attempted to use informal tests to assess and monitor exercise capacity in patients with cystic fibrosis, but some studies have highlighted the controversy surrounding the reliability, validity, and sensitivity of many of these tests.[1 2]

The shuttle walking test is an incremental externally paced informal exercise test which overcomes many of the problems associated with existing informal exercise tests. The original authors of the shuttle walking test have shown this test to be a reliable (after just one practice test), valid, and sensitive measure of exercise capacity in patients with chronic obstructive pulmonary disease.[3–5] We have carried out preliminary work with the shuttle walking test in adult patients with cystic fibrosis, and have shown that the walking speeds in the original test (up to a maximum of 2.37 m/s) do not elicit a maximal response in adult patients with cystic fibrosis and minimal disability as well as in patients with more severe disability. On the basis of these preliminary findings the original test was modified by the addition of three levels and, further, by permitting the patients to run. The additional stages to the original 12 stage test were: level 13, 5.63 mph, 15 shuttles; level 14, 6.00 mph, 16 shuttles; and level 15, 6.38 mph, 17 shuttles. It was hypothesised that this modified shuttle test (MST) could be used to measure peak exercise capacity objectively in adult patients with cystic fibrosis. The aim of this study was therefore to compare patients' performance on the MST with peak oxygen consumption ($\dot{V}O_2$peak) measured directly during a treadmill test.

Methods

Twenty patients (14 men) of mean (SD) age 25 (7) years, weight 58 (8) kg, height 1.68 (0.08) metres volunteered for the study. All patients had been familiarised with the MST and the treadmill test prior to entry into the study. Patients undertook the treadmill test on one visit to the hospital and the MST on a separate visit. The order of the tests was randomised in a counterbalanced design. The mean (SD) duration between visits was 7 (4) days. The tests were performed at approximately the same time each day. Baseline spirometric measurements (Vitalograph Alpha), resting oxygen saturations and resting heart rate (Ohmeda Sa_{O_2} monitor with ear probe), and rating of perceived breathlessness (Borg scale[6]) were recorded before the exercise test on each study day. The study was approved by the hospital ethical committee and informed consent was obtained from all patients.

TREADMILL TEST

The treadmill test was a symptom limited maximal exercise test performed according to

the standardised treadmill exponential exercise protocol (STEEP).[7] During the treadmill test measures of $\dot{V}O_2$ (ml/min), $\dot{V}CO_2$ (ml/min), and minute ventilation (VE, 1/min) were recorded at 15 s intervals (PK Morgan). The equation forced expiratory volume in one second (FEV_1) × 35 was used to predict maximum voluntary ventilation (MVV).[8] The heart rate was measured at one minute intervals using a 12 lead electrocardiogram (Model Marguette Case 15) and SaO_2 was continuously monitored using an Ohmeda SaO_2 monitor. At the end of the test the peak heart rate, SaO_2, and peak rate of perceived breathlessness were recorded. Reasons for stopping or failing to maintain the correct pace were also recorded.

SHUTTLE TEST

Using the 15 level MST, patients were required to walk/run at increasing speeds back and forth on a 10 metre course.[3] They were accompanied by an operator during the first minute of the test to help them pace themselves with the audiosignal. At the end of each level the patients were also told to go a little faster and were reminded that they were permitted to run at any time during the test. Patients continued with the test until they were unable to do so or failed to maintain the set pace.[3] Heart rate was measured at 15 s intervals using a short range telemetry device (Polar Sports Tester) and SaO_2 was continuously monitored using an Ohmeda SaO_2 monitor. At the end of the test the peak heart rate, SaO_2, and peak rating of perceived breathlessness were recorded. Reasons for stopping or failing to maintain the correct pace were also recorded.

Results

Lung function (FEV_1) ranged from 17% to 96% predicted normal, indicating that the patients exhibited a wide variety of disease impairment. Table 1 shows that there were no significant differences between study days in baseline test parameters (FEV_1, resting heart rate, resting rating of perceived breathlessness, resting SaO_2). Furthermore, there were no significant differences between tests in comparable physiological responses to exercise.

Table 1 Mean (SD) baseline characteristics and physiological responses to exercise testing of patients

Parameter	*Shuttle test*	*Treadmill test*	*p value*
Baseline characteristics			
Age (years)	25 (7)	—	—
Weight (kg)	58 (8)	—	—
Height (m)	1.67 (0.008)	—	—
FEV_1 (% pred)	49 (23)	48 (25)	0.31
SaO_2	94 (3)	94 (3)	0.39
Resting heart rate (beats/min)	95 (12)	97 (17)	0.56
Resting heart rate (% pred max)	49 (7)	50 (8)	0.46
Resting rating perceived breathlessness	0 (1)	0 (1)	0.89
Physiological responses			
$\dot{V}O_2$peak (ml/kg/min)	32.85 (10.36)	—	—
$\dot{V}O_2$peak (% pred)	73.45 (26.27)	—	—
VEmax (l/min)	57.76 (20.01)	—	—
VE (% pred)	101 (40)	—	—
MST distance (m)	—	929 (335)	—
Max heart rate (beats/min)	171 (23)	169 (24)	0.90
Max heart rate (% pred)	87 (10)	86 (11)	0.95
Peak rate perceived breathlessness	6 (1)	6 (1)	0.90
End SaO_2	89 (7)	88 (7)	0.10

FEV_1 = forced expiratory volume in one second; SaO_2 = oxygen saturation; $\dot{V}O_2$peak = peak oxygen consumption; VE = minute ventilation; MST = modified shuttle test.

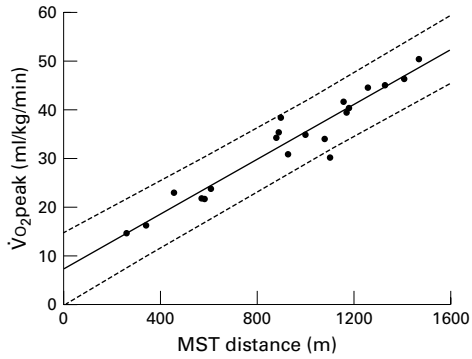

Figure 1 Regression line (with 95% confidence intervals) for the relationship between performance on the modified shuttle test (MST) and peak oxygen consumption ($\dot{V}O_2$ peak) measured during treadmill testing.

A significant and moderately strong relationship was found between the distance achieved on the MST and lung function (MST vs FEV_1% predicted: $r = 0.70$, $p = 0.001$) and between $\dot{V}O_2$peak and lung function ($\dot{V}O_2$peak vs FEV_1% predicted: $r = 0.78$, $p<0.001$). The relationship between the distance achieved on the MST and directly measured $\dot{V}O_2$peak was strong ($r = 0.95$, $p<0.00$) with 90% of the variance in $\dot{V}O_2$peak explained by the variance in the MST distance. Using methods adopted from the authors of the original test,[4] regression analysis was used to further describe the nature of the relationship between $\dot{V}O_2$peak and MST performance. The relationship was represented by the regression equation and 95% confidence intervals:

$\dot{V}O_2$peak = 6.83 (2.85 to 10.80) + 0.028 (0.019 to 0.024) × MST distance (fig 1).

Discussion

The purpose of this study was to determine the validity of the MST as a measure of exercise capacity in adults with cystic fibrosis. The results show that there was a strong relationship between $\dot{V}O_2$peak and MST performance in patients with cystic fibrosis and varying degrees of lung function impairment. Ninety percent of the variation of directly measured $\dot{V}O_2$peak is explained by the variation in MST performance. This compares very favourably with the original test in which the shuttle walking test performance explained 77.4% of the variance in directly measured $\dot{V}O_2$. Many cystic fibrosis clinicians have no access to formal exercise testing equipment and the regression analysis used in this study provides additional information to such individuals on the nature of the relationship between $\dot{V}O_2$peak and MST performance. There was no significant difference between peak heart rate and peak rating of perceived breathlessness recorded during both exercise tests, which indicates the effectiveness of the MST to evoke a symptom limited exercise response in both mildly and more severely compromised adults with cystic fibrosis.

Most of the patients in the present study encroached on their pulmonary reserve during exercise testing (mean peak VE >70% MVV). None of the patients reached their maximum predicted heart rate, as determined by the age related equation (220–age), and "shortness of

breath" and "fatigue" were the most common reasons reported for stopping the exercise test. These findings support the assertion that ventilatory factors rather than cardiovascular factors limit exercise tolerance in cystic fibrosis.[9]

The relationship between lung function and $\dot{V}O_2$peak was moderate. This finding supports previous work which indicated that impaired pulmonary function limits exercise capacity.[10] Lung function is not a good predictor of exercise capacity because of wide intersubject variability of exercise capacity in patients with comparable lung function. In the present study oxygen desaturation (more than 5% fall in SaO_2)[11] occurred in all patients with FEV_1 less than 35% predicted, and in two of the patients with FEV_1 of 43% and 50%. Exercises tests to establish if exercise induced desaturation occurs are therefore a necessary prerequisite to exercise prescription in cystic fibrosis. Oxygen saturation should also be intermittently monitored during exercise programmes and, if necessary, supplemental oxygen should be used to avoid oxygen desaturation.

This study has shown that there is a strong relationship between MST performance and $\dot{V}O_2$peak in adults with cystic fibrosis and thus provides evidence of the validity of this test as a measure of peak exercise capacity in adult cystic fibrosis. Further work is required to establish the intertest reliability, test-retest reliability, and the sensitivity to change of the MST in adult patients with cystic fibrosis.

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Collagen degrading activity associated with *Mycobacterium* species

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Abstract

***Background*—The mechanism of *Mycobacterium tuberculosis* penetration into tissues is poorly understood but it is reasonable to assume that there is a contribution from proteases capable of disrupting the extracellular matrix of the pulmonary epithelium and the blood vessels. A study was undertaken to identify and characterise collagen degrading activity of *M tuberculosis*.**

***Methods*—Culture filtrate protein extract (CFPE) was obtained from reference mycobacterial strains and mycobacteria isolated from patients with tuberculosis. The collagen degrading activity of CFPE was determined according to the method of Johnson-Wint using ^{3}H-type I collagen. The enzyme was identified by the Birkedal-Hansen and Taylor method and its molecular mass determined by SDS-PAGE and Sephacryl S-300 gel filtration chromatography using an electroelution purified enzyme.**

***Results*—CFPE from *Mycobacterium tuberculosis* strain H37Rv showed collagenolytic activity that was four times higher than that of the avirulent strain H37Ra. The 75 kDa enzyme responsible was divalent cation dependent. Other mycobacterial species and those isolated from patients with tuberculosis also had collagen degrading activity.**

***Conclusions*—*Mycobacterium* species possess a metalloprotease with collagen degrading activity. The highest enzymatic activity was found in the virulent reference strain H37Rv.**

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Keywords: *Mycobacterium tuberculosis*; collagenase; metalloprotease

An increasing number of microorganisms, many of which are putative human pathogens, produce enzymes which degrade collagen.[1] The mechanism of penetration of *Mycobacterium tuberculosis* into the tissues and bloodstream is poorly understood but, as with other lung diseases,[2 3] gastrointestinal infections,[4] and necrotic conditions,[5] it is reasonable to assume that there is a contribution from proteases capable of disrupting the

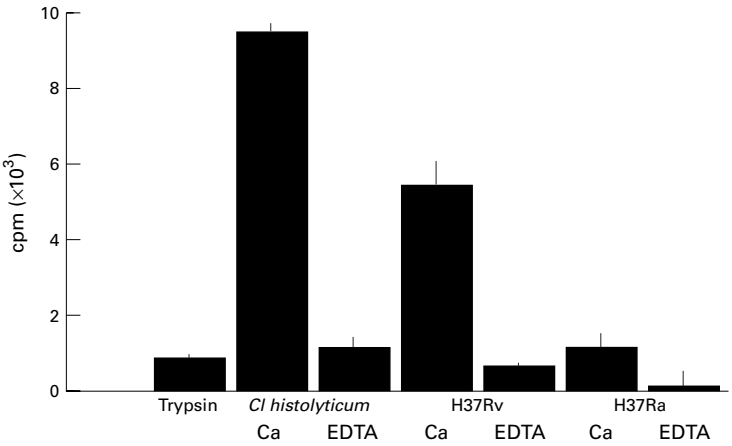

Figure 1 Collagenolytic activity of M tuberculosis H37Rv and H37Ra culture filtrate protein extract in the presence of calcium or EDTA as calcium chelant. Results are expressed as mean (SE). Background counts per minute (cpm) have already been subtracted. Maximum activity, calculated assuming that all the radioactivity (12 500 cpm/well) was liberated, corresponded to Cl histolyticum (75.5).

extracellular matrix of the pulmonary epithelium and the blood vessels. The aim of this study was to identify and characterise collagenolytic activity in *Mycobacterium* reference strains and in those isolated from patients with tuberculosis.

Methods

MYCOBACTERIAL CULTURES

First early morning expectoration samples from two patients with pulmonary tuberculosis and two with miliary tuberculosis, pleural fluid from two patients with pulmonary tuberculosis and one with miliary tuberculosis, and the first early morning urine sample from one patient with renal tuberculosis were collected, seeded, grown, and identified as *M tuberculosis* by the National Tuberculosis Reference Laboratory. The following ATCC reference strains were donated by the referred laboratory and CENID Microbiología, México: *M tuberculosis* H37Rv and H37Ra (virulent and attenuated mycobacteria isolated from a human lung, used for susceptibility testing[6 7]), *M kansasii*, *M fortuitum*, *M microti*, *M terrae*, *M avium*, and *M gordonae*. These strains were seeded in protein-free Proskauer-Beck-Youmans (PBY) medium and maintained at 37°C for 7–9 weeks before bacteria were harvested.[8] Some cultures were maintained for as long as 12 weeks before harvesting.

CULTURE FILTRATE PROTEIN EXTRACT (CFPE)

The culture medium was separated from the bacterial mass by filtration through 0.45 µm and 0.22 µm membrane filters (Millipore Corp). Proteins were precipitated with solid ammonium sulphate crystals (80% final saturation) and centrifuged for 30 minutes at 16 000*g*. The precipitate, suspended in 5 ml phosphate buffered saline (PBS) containing 0.2 mg/ml phenylmethylsulphonyl fluoride (Sigma Chemicals), was dialysed extensively against PBS and 0.5 ml aliquots (6 mg/ml) were stored in liquid nitrogen until used. Some filtrates were precipitated at 40% and 60% $(NH_4)_2SO_4$ final saturation.

COLLAGEN

Collagen was obtained from the skin of two month old female Wistar rats according to the method described by Epstein.[9] The specific activity of radiolabelled collagen was 1×10^6 cpm/mg of protein.

COLLAGENOLYTIC ASSAY

The enzymatic activity was determined according to the method of Johnson-Wint[10] using 120 µg CFPE and 12 500 cpm radioactive collagen/well. Some experiments were performed with 20 mM/well EDTA tetrasodium salt (Sigma Chemicals). Controls included buffer, 0.001% trypsin (type IX from porcine pancreas), and *Clostridium histolyticum* collagenase (high purity, type III fraction A).

COLLAGENASE IDENTIFICATION

The enzyme was identified using a modification of the method described by Taylor and Birkedal-Hansen[11] that included elimination of SDS from the gel with 3% Triton X-100 and overnight incubation at 37°C. The region with collagenolytic activity in the CFPE containing gel (β-mercaptoethanol reduced and boiled sample/lane in 10% slab gels[12]) was located, purified by electroelution, dialysed, lyophilised, and kept at –20°C until used. The non-denatured state of the substrate was corroborated by the inclusion of trypsin, an enzyme incapable of breaking down collagen but highly active upon gelatin.

MOLECULAR MASS DETERMINATION

The molecular mass of the enzyme (75 000 by SDS-PAGE) was determined by Sephacryl S-300 gel filtration chromatography. 500 µg of enzyme partially purified by electroelution were suspended in 0.05 M Tris HCl buffer (pH 7.5) containing 4 mM $CaCl_2$, poured onto a 1.2×100 cm glass column previously equilibrated with the same buffer, and eluted with the Tris HCl buffer at a flow rate of 12 ml/hour. Standards included horse heart myoglobin (17 kDa), bovine serum albumin (66 kDa), purified human IgG (150 kDa), and bovine liver catalase (240 kDa). The activity of each fraction was tested as described above.

Results

The collagenolytic activity of CFPE from H37Rv was four times higher than that from H37Ra (43.4% and 9.1%, respectively). Addition of EDTA to the H37Rv CFPE blocked the enzymatic activity by 88%, thus establishing that the enzyme in the virulent strain was dependent on divalent cations (fig 1). Similar results were obtained with replicate cultures. The enzymatic activity was secondary to a 75 kDa protein which was clearly established by the degradation of collagen containing gels and molecular mass determination by Sephacryl S-300 gel filtration chromatography.

The collagenolytic activity was not changed by the use of ammonium sulphate saturation to precipitate the CFPE, the amount of H37Rv CFPE precipitated with a final ammonium sulphate saturation of 40%, 60% and 80% being

Table 1 Mean (SE) collagenolytic activity of culture filtrates obtained from various mycobacterial species

	With Ca		*With EDTA*	
	Counts per minute (cpm)	*Enzyme activity*	*Counts per minute (cpm)*	*Enzyme activity*
Trypsin	1088 (203)	8.7	528 (18)	4.2
Cl histolyticum	10982 (904)	87.8	2888 (878)	23.1
M microti	3234 (113)	25.8	1094 (140)	8.7
M kansasii	1038 (348)	8.3	888 (282)	7.1
M avium	2621 (229)	20.9	1277 (305)	10.2
M terrae	768 (63)	6.1	433 (69)	3.4
M gordonae	542 (74)	4.3	485 (45)	3.8
M fortuitum	272 (33)	2.1	174 (46)	1.3

Background cpm have already been subtracted. The percentage of activity was calculated assuming that all the radioactivity (12 500 cpm/well) was liberated by the enzyme.

Table 2 Mean (SE) collagenolytic activity of culture filtrates obtained from mycobacteria isolated from patients with tuberculosis

		With Ca		*With EDTA*	
*Diagnosis**	*Source†*	*Counts per minute (cpm)*	*Enzyme activity*	*Counts per minute (cpm)*	*Enzyme activity*
Trypsin		792 (130)	6.3	404 (103)	3.2
Cl histolyticum		9763 (559)	78.1	2367 (362)	18.9
Pulmonary TB	Sputum	1160 (284)	9.2	510 (462)	4.0
Pulmonary TB	Sputum	4198 (204)	33.5	684 (642)	5.4
Pulmonary TB	Pleural fluid	1783 (462)	14.2	348 (90)	2.7
Pulmonary TB	Pleural fluid	1376 (248)	11.0	1142 (108)	9.1
Miliary TB	Sputum	2276 (98)	18.2	874 (138)	6.9
Miliary TB	Sputum	4986 (230)	39.8	566 (32)	4.5
Miliary TB	Pleural fluid	2864 (127)	22.9	814 (175)	6.5
Renal TB	Urine	2316 (224)	18.5	1238 (148)	9.9

*All patients were PPD positive.
†Source refers to the secretion from which the mycobacteria were isolated.
Background cpm have already been subtracted. The percentage of activity was calculated assuming that all the radioactivity (12 500 cpm/well) was liberated by the enzyme.

4380 (304) cpm, 4224 (215) cpm, and 4146 (182) cpm, respectively. To determine the influence of culture time on enzymatic activity, five H37Rv cultures were seeded and harvested after 32, 38, 46, 70, and 103 days of culture and had CFPE activity of 20%, 29%, 34%, 33%, and 15%, respectively, indicating that the collagenolytic activity decays with time.

The collagenolytic activity of CFPE obtained from mycobacteria other than *M tuberculosis* was also tested. *M microti* showed the highest level of activity followed by *M avium*, *M kansasii*, *M terrae*, *M gordonae*, and *M fortuitum* which had the least activity (table 1). Enzymatic variability was also observed in culture filtrates obtained from mycobacteria isolated from patients with tuberculosis (table 2).

Discussion

The presence of collagenolytic activity in the CFPE of *M tuberculosis* H37Rv strain was secondary to a 75 kDa enzyme whose activity was dependent on divalent cations and was blocked by EDTA, suggesting a mechanism of non-competitive inhibition. These characteristics are similar to those reported for *Clostridium histolyticum* collagenase[13] and the majority of collagenases.[14] Even though we recognise the possible influence of collagenase degradation before harvesting, the differences between the CFPE samples of H37Rv virulent and H37Ra avirulent mycobacterial strains were striking and were independent of batch to batch variations, source of seed, and time of culture.

None of the mycobacteria we tested had activity greater than *M tuberculosis* H37Rv. As in the case of catalase activity, once considered a virulence factor,[15] collagen degrading activity is important but not vital in the pathogenesis of the disease since there was no uniform activity in *M avium* and *M kansasii*, both of which induce tuberculosis. *M fortuitum* and the non-disease inducers *M terrae* and *M gordonae* had very poor enzyme activity. Although tissue destruction is secondary to an enhanced cellular immune response, mycobacterial metalloproteinases might be important for bacterial penetration[1 16] as has been shown with other infectious agents.[4 17] Despite the above considerations, it was interesting to observe that *M tuberculosis* isolated from tuberculosis patients with different clinical syndromes (renal, miliary, pulmonary) had greater activity than the avirulent reference strain H37Ra.

Our results confirm the presence of collagen degrading enzymatic activity in the culture filtrate protein extract of all the mycobacteria tested. Extracellular proteases may also play a role in the pathogenesis of mycobacterial infection. However, the recent resolution of the genome for *M tuberculosis* H37Rv (website: www.sanger.ac.uk) predicts at least 38 genes coding for proteins of virulence and more than 250 macromolecular and/or micromolecular degrading proteins. One of these, the product of gene Rv0198c, a zinc metalloprotease with a molecular weight of 73.8 kDa, is similar to the enzyme we describe in this work.

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