s-CARBOXYMETHYL-CYSTEINE INHIBITS CARBACHOL-INDUCED CONTRACTION OF EPITHELIUM-DENUDED RAT AND HUMAN AIRWAY PREPARATIONS

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SUMMARY

1. The effects of s-carboxymethyl-l-cysteine (S-CMC), either administered orally to rats or incubated with tissue preparations from rats and humans, on isometric contractions of tracheal smooth muscle were investigated in the present study using an improved in vitro model of tracheal tube or ring preparations. The involvement of the tracheal epithelium in the observed effects was also investigated.

2. The experimental model permitted selective perfusion of the airway tube, luminal-IN or serosal-OUT, and measurement of airway smooth muscle contraction or relaxation in preparations with (+) or without (–) epithelium (Ep), excluding direct effects of airway mucus.

3. We found that oral pretreatment of rats with S-CMC (mixed with water; 200 mg/kg per day for 2 weeks), but not short pre-incubation of preparations in vitro (10–3 mol/L S-CMC for 1 h), diminished the sensitivity of –Ep preparations to carbachol compared with controls (EC50 (–log10 mol/L) values: 5.5 ± 0.1 vs 5.8 ± 0.1, respectively, for IN perfusion (P < 0.005); 5.6 ± 0.1 vs 5.9 ± 0.1, respectively, for OUT perfusion (P < 0.005)), whereas the sensitivity of preparations to aminophylline was not affected. Normal sensitivity to carbachol stimulation was re-established if preparations were pre-incubated with capsaicin.

4. It was also found that longer pre-incubation (4 h) of ring-preparations of human bronchus with S-CMC (10–5 mol/L S-CMC for 1 h), resulted in a diminished response to carbachol stimulation.

5. In conclusion, S-CMC had small inhibitory effects on the sensitivity of rat and human airway smooth muscle to carbachol, particularly in epithelium-denuded preparations. Whether the epithelium was responding to S-CMC by producing some contracting factor(s) requires further investigation.

Key words: epithelium, rat trachea, s-carboxymethylcysteine, tracheal ring, tracheal tube.

INTRODUCTION

The airway epithelium, which separates inhaled gas from the underlying airway tissue, forms an important barrier between living structures and the environment. Preservation of the normal function of the bronchial epithelium is a precondition for maintenance of an effective air–tissue barrier. That barrier may be disturbed in airway inflammation and asthma. One objective of our earlier and the present study has been to test the hypothesis that the bronchial epithelium, either as a diffusion barrier or by actively secreting yet not well defined mediators (prostaglandins, leukotrienes or other mediators), can modulate bronchial smooth muscle sensitivity to various stimulating agents.1–3 Experimentally, we use a system of perfused tracheal tubes because a tube airway preparation allows selective pharmacological stimulation of the epithelial or serosal side of the airway in vitro. In the present study, the model described earlier2,4,5 was improved so that it allowed investigation of contractions of the entire airway tube first and then as a ring preparation.

We centred our study on the mucolytic agent s-carboxymethylcysteine (s-carboxymethyl-l-cysteine; S-CMC; carbocysteine), used across Europe. Improving mucociliary transport is one of the therapeutic approaches in asthma therapy, in which, in addition to anti-inflammatory agents,5,7 mucolytic agents are occasionally used.8,9 Indeed, beneficial effects of S-CMC for chronic obstructive bronchitis have been demonstrated in a large multicentre controlled trial.10 Nevertheless, the mechanism of action of S-CMC remains unclear. It was found that S-CMC could enhance the production of sialomucins at the expense of fucomucins,11,12 which may result in an improvement in mucociliary transport. Several other findings have implicated either reduced or increased sputum viscosity13–16 or even an anti-inflammatory property17–19 that may be related to increased sialoglycoprotein secretion as a mechanism of action of S-CMC.

Nevertheless, asthmatic subjects often have airway hyperresponsiveness that is accompanied by secretion of thick mucus and various degrees of inflammation. Recent evidence indicating that S-CMC may indirectly favour smooth muscle relaxation18,20,21 further justified the aim of the present study, which was to test the effects of S-CMC on airway smooth muscle (ASM) responsiveness. More precisely, we wanted to examine the effect of S-CMC on bronchial epithelium function and/or tracheal smooth muscle in healthy animals and its effect on the isometric contraction of bronchial smooth muscle in cannulated preparations or ring preparations. The second objective of the study was to construct and test a slightly
modified \textit{in vitro} apparatus that can be used with an airway tube or, alternatively, an airway ring preparation.

\textbf{METHODS}

\textbf{Animal preparation}

Experiments were performed on tracheas taken from male Sprague-Dawley rats, weighing 390–420 g. Animal treatment and experimental procedures were in accordance with the recommendations of INSERM and with the local Instructions for Animal Care of Greifswald University. All animals were housed in individual cages and received water and food \textit{ad libitum}. The pretreated group of animals (S-CMCpr) received S-CMC mixed with water (200 mg/kg per day) for 2 weeks. During the 3rd week, rats were killed for the experiments by stunning and quick exsanguination. The tracheas were immersed in modified Krebs’–Henseleit solution (KH; composition (in mmol/L): NaCl 113; KCl 4.8; MgCl$_2$·6H$_2$O 1.3; KH$_2$PO$_4$ 1.2; NaHCO$_3$ 25; CaCl$_2$ 2.5; glucose 5.7) and cleaned from surrounding tissue.

\textbf{Perfusion studies}

Proximal tracheal ends (10 tracheal rings long) were used for the experiments. In half the preparations the epithelium was removed (–Ep) by gently rubbing the luminal side with a cotton-wrapped metal stick; in the other half, the epithelium was left intact (+Ep).

Under magnification (×2–4), two stainless-steel hooks were passed through the tracheal wall around two adjacent cartilaginous rings as close as possible to the tracheal muscle insertion. The tracheal segment was then connected longitudinally to steel tubes built in the ‘carrier block’ of the apparatus (in–out system) and firmly tightened with silk thread. The apparatus used (Fig. 1a–c; EMKA Technologies, Paris, France) was an improved version of the cannulated tracheal system described previously. The improvement consisted of the fact that the lower hook, which served as a ‘fixed point’, was attached via silk thread to the micrometric screw serving to adjust hook tension on the tracheal wall. This allowed precise adjustment of resting tension. The upper hook was connected to a force transducer. The ‘carrier block’ for cannulated trachea could be easily removed and replaced by an identical one which the lower part has been adapted for the mounting of tracheal rings. (c) A photograph of the new tracheal tube preparation. (d) Tracheal tube with the hooks that are placed as close as possible to the end of the tracheal rings and close to the tracheal muscle.

The KH solution (37°C, pH 7.4, gassed with 95% O$_2$–5% CO$_2$) was perfused at a constant flow rate (2 mL/min) through the organ bath (outer perfusion–OUT) and through the lumen of the tracheal segment (inner perfusion–IN) using peristaltic pumps (Watson Marlow 5025, Falmouth, Cornwall, UK).
s-Carboxymethylcysteine and airways

Procedure

Perfusion studies

In this experimental model, studies were conducted using preparations with and/or without epithelium taken from animals pretreated with S-CMC (S-CMCpr) or from control animals preparations.

After a period of stabilization (45—60 min), the tracheal muscle was stretched to its optimal length, corresponding to approximately 1.5 g passive pretension. Preliminary assays were performed to determine the optimal stretch of the muscle, as described previously. The length–tension relationship did not differ significantly between S-CMCpr and control preparations.

In the initial set of experiments, cumulative concentrations of carbachol (10^-7 to 10^-3 mol/L) were perfused IN (–Ep) and then perfused OUT (–Ep or +Ep) in S-CMCpr or controls preparations (n = 8 for each). We concluded from these experiments that responses to IN and OUT stimulation in –Ep preparations were identical (on the basis of EC50 values and maximal responses) and, to further examine the effects of S-CMC in –Ep preparations on IN stimulation with carbachol, we designed another four sets of experiments.

First, to examine the effect of pre-incubation, preparations taken from untreated animals were incubated with 10^-3 mol/L S-CMC (S-CMCinc; –Ep; n = 8) for 60 min and cumulative concentrations of carbachol were perfused luminally (IN).

In the second set of experiments, S-CMCpr (–Ep) preparations were pre-incubated with 10^-6 mol/L indomethacin (INDinc; both sides; n = 8) or 10^-3 mol/L capsaicin (CAPSinc; both sides; n = 6) for 60 min and prior to perfusion of cumulative concentrations of carbachol IN. The effects of S-CMC on the relaxant effects of aminophylline were examined in S-CMCpr (–Ep) preparations that were first precontracted with a medium concentration (10^-4 mol/L) of carbachol OUT and then perfused IN with 10^-4 to 10^-1 mol/L aminophylline (n = 10) and compared with control responses to aminophylline (n = 6). In the third set of experiments, to examine direct relaxant effects of S-CMC, preparations taken from control animals were precontracted OUT with an EC50 concentration of carbachol (10^-3 mol/L) and then perfused IN with cumulative concentrations of S-CMC, ranging from 10^-5 to 10^-1 mol/L (+Ep, n = 7; –Ep, n = 7).

Human airway

Preparations of human bronchi (3–4 mm in diameter, n = 4 in each group) were obtained during thorax surgery (partial lung resection due to lung cancer), stored for approximately 1 h in cold (+4°C) physiological salt solution (PSS; NaCl 0.9%) and then dissected into 2–3 mm ring preparations and incubated with 10^-5 mol/L S-CMC for 4 h. Paired controls were kept in cold PSS during that time. In either one of the paired preparations, the epithelium was removed before the preparations were mounted for in vitro experiments. Owing to scarcity of the specimens, cannulation was not possible and therefore the experiments were performed only on bronchial rings. In these preparations only dose–response curves to carbachol were obtained.

Substances

The following substances were used: carbachol (carbamylcholine chloride, Sigma Chimie, St Quentin Fallavier, France), indomethacin (Sigma Chimie), aminophylline (theophylline–ethylenediamine; Pharmacie Centrale des Hopitaux, Paris, France), capsaicin (Sigma Chimie) and S-CMC (Park-Davis, Orleans, France). The S-CMC was diluted in 10% NaOH, whereas indomethacin was diluted in methanol; final dilutions of both stock solutions were made in KH solution. Final solutions contained less than 0.01% methanol and NaOH; all controls were exposed to the same concentrations of solvent as the treatment groups.

Statistical analyses

Data are expressed as a percentage of the maximal response and in absolute values (g or s) and given as the mean±SEM. Half-maximal concentration (EC50) values were calculated by means of non-linear regression using the Hill–Langmuir equation implemented in GraphPad Prism (GraphPad Software, San Diego, CA, USA) and the results given as the mean of –log10 EC50 values obtained. Statistical analysis was performed using analysis of variance and Student’s t-test for paired or unpaired data adjusted for multiple comparisons (Bonferroni), as appropriate. P < 0.05 was regarded as being statistically significant. The False Discovery Rate (FDR) procedure was also used for multiple comparisons. All statistical analyses were performed using the software package Graph Pad Prism 4 for Windows (Graph Pad Software), except for the FDR, for which a short routine was written in Microsoft Excel (Microsoft Office 2000; Microsoft, Redmond, WA, USA).

RESULTS

Animals pretreated with oral S-CMC did not show any clinical signs of disease or metabolic disturbances. They showed normal cage activity, no disturbances in the sleep–wake pattern, normal spontaneous food and water intake, weight gain ordinary stool consistency and normal eyes and no piloerection.

Perfusion studies

Effects of oral pretreatment and pre-incubation with S-CMC and capsaicin and indomethacin

In preparations taken from S-CMCpr animals, we found diminished sensitivity to carbachol in –Ep, but not +Ep, preparations (Tables 1,2; Fig. 2). Interestingly, the diminished sensitivity following epithelial application (inside, IN) of carbachol in –Ep preparations from S-CMCpr was absent in preparations pre-incubated with 10^-5 mol/L capsaicin (CAPSinc; Table 3; Fig. 3), but was maintained in preparations pre-incubated with 10^-6 mol/L indomethacin.

Table 1 EC50 values for carbachol (IN)

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<thead>
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<th>+Ep</th>
<th>–Ep</th>
<th>P</th>
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<tr>
<td>Control</td>
<td>5.40 ± 0.11</td>
<td>5.85 ± 0.06</td>
<td>&lt; 0.005</td>
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<tr>
<td>S-CMCpr</td>
<td>5.19 ± 0.08</td>
<td>5.52 ± 0.09</td>
<td>&lt; 0.005</td>
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<tr>
<td>P</td>
<td>NS</td>
<td>&lt; 0.005</td>
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Data are the mean±SEM of EC50 values (–log10 mol/L), obtained in preparations with (+Ep) and without (–Ep) epithelium, taken from controls or animals pretreated for 2 weeks with with s-carboxymethyl-l-cysteine (S-CMC; 200 mg/kg per day, p.o.; S-CMCpr), following stimulation from the epithelial side (IN) with cumulative concentrations of carbachol (F = 10.45).

Table 2 EC50 values for carbachol (OUT)

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<th>+Ep</th>
<th>–Ep</th>
<th>P</th>
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<tr>
<td>Control</td>
<td>6.01 ± 0.09</td>
<td>5.92 ± 0.06</td>
<td>NS</td>
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<tr>
<td>S-CMCpr</td>
<td>6.17 ± 0.08</td>
<td>5.61 ± 0.05</td>
<td>&lt; 0.005</td>
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<tr>
<td>P</td>
<td>NS</td>
<td>&lt; 0.005</td>
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</table>

Data are the mean±SEM of EC50 values (–log10 mol/L), obtained in preparations with (+Ep) and without (–Ep) epithelium, taken from controls or animals pretreated for 2 weeks with with s-carboxymethyl-l-cysteine (S-CMC; 200 mg/kg per day, p.o.; S-CMCpr), following stimulation from the serosal side (OUT) with cumulative concentrations of carbachol (F = 11.14).
In control experiments, we observed that pre-incubation of ring preparations with capsaicin did not affect their sensitivity (EC50) or the maximal force developed (T_max) following carbachol stimulation. In contrast with the effects of oral pretreatment, pre-incubation of tissues with S-CMC (S-CMCinc) for 60 min did not affect the sensitivity of –Ep preparations to carbachol IN stimulation (Table 3). However, all +Ep preparations were more sensitive to carbachol OUT stimulation than to carbachol IN stimulation. Removal of the epithelium increased sensitivity to carbachol in all preparations except for control preparations stimulated with carbachol (OUT; Tables 1, 2).

**Table 3** EC50 values for carbachol following pre-incubation with indomethacin or capsaicin

<table>
<thead>
<tr>
<th></th>
<th>–Ep (IN)</th>
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<tr>
<td>Control</td>
<td>5.85 ± 0.06*</td>
</tr>
<tr>
<td>S-CMCinc</td>
<td>5.71 ± 0.07</td>
</tr>
<tr>
<td>S-CMCpr</td>
<td>5.52 ± 0.09</td>
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<tr>
<td>S-CMCpr (INDinc)</td>
<td>5.59 ± 0.09†</td>
</tr>
<tr>
<td>S-CMCpr (CAPSinc)</td>
<td>5.99 ± 0.15‡</td>
</tr>
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</table>

Data are the mean±SEM of EC50 values (−log10; mol/L), obtained in preparations without epithelium (–Ep) following stimulation from the epithelial side (IN) with cumulative concentrations of carbachol. Preparations were taken from controls and pre-incubated in S-carboxymethyl-l-cysteine (S-CMC; 10−3 mol/L; S-CMCinc) or from animals pretreated for 2 weeks with oral S-CMC (200 mg/kg per day, p.o.; S-CMCpr) and pre-incubated with 10−5 mol/L indomethacin (INDinc) or 10−5 mol/L capsaicin (CAPSinc). *P < 0.03 compared with S-CMCpr (F = 5.444; see also Table 2); †P < 0.05 compared with S-CMCinc (FDR procedure); ‡P < 0.05 compared with S-CMCpr (FDR procedure).

In contrast with the effects of oral pretreatment, pre-incubation of tissues with S-CMC (S-CMCinc) for 60 min did not affect the sensitivity of –Ep preparations to carbachol IN stimulation (Table 3). However, all +Ep preparations were more sensitive to carbachol OUT stimulation than to carbachol IN stimulation. Removal of the epithelium increased sensitivity to carbachol in all preparations except for control preparations stimulated with carbachol (OUT; Tables 1, 2).

**Direct effect of S-CMC, T_{max} and aminophylline**

In +Ep or –Ep preparations precontracted with carbachol (10⁻⁶ mol/L) OUT, S-CMC alone in the concentration range 10⁻⁷ to 10⁻³ mol/L IN had neither contracting nor relaxant effects. In addition, the T_{max} developed following stimulation with carbachol did not depend on the side of stimulation (OUT or IN) and was not affected by removal of the epithelium, pretreatment with S-CMC or pre-incubation (INDinc; Table 3).
affected by pretreatment with S-CMC (–log 10 EC50: 3.2 incubated (–H17033) smooth muscle contraction.1–3,24,29 In most of these studies, the clearly demonstrate that tracheal epithelium can modulate tracheal preparations incubated with S-CMC were less sensitive to carbachol). similar small effects in human bronchus denuded of epithelium (i.e. and require an explanation. In addition, we were able to demonstrate with controls (–Ep). These changes, although small, were significant without epithelium less sensitive to carbachol stimulation compared with S-CMC, whereas pre-incubation with S-CMC seemed to diminish sensitivity to carbachol.

**Human bronchial rings**

The carbachol concentration–response curves for human bronchial rings pre-incubated for 4 h with S-CMC, without epithelium, were less sensitive to carbachol stimulation (Fig. 4, Table 4), thus indicating that removal of the epithelium increased sensitivity to carbachol stimulation only in preparations not pre-incubated with S-CMC, whereas pre-incubation with S-CMC rendered preparations more sensitive to carbachol stimulation.25 This may be relevant for a better understanding of different airway pathologies. It has been shown that the bronchial epithelium is damaged in patients with severe asthma, indicating that bronchial epithelial damage and airway hyperresponsiveness could be linked.26 Several studies have demonstrated that the bronchial epithelium constitutes a powerful diffusion barrier2,27 and can attenuate the effects of pharmacological agents applied luminally. The findings of the present study, although demonstrating small effects, support this hypothesis. It appears that the epithelium in rats mediates less inhibition compared with guinea-pigs. In guinea-pigs, we have found bigger differences following serosal compared with epithelial perfusion (D Pavlovic et al., unpubl. obs., 1999). However, it has been quite difficult to demonstrate airway hyperresponsiveness in vivo in airways already hyperresponsive in vitro. The significance of small in vitro changes that we observed may (but do not have to) correspond to more important in vivo changes, which we would like to verify in further experiments.

In the present study, we found that removal of the epithelium rendered preparations more sensitive to carbachol stimulation and, alternatively, that pretreatment with S-CMC rendered preparations without epithelium less sensitive to carbachol stimulation compared with controls (–Ep). These changes, although small, were significant and require an explanation. In addition, we were able to demonstrate similar small effects in human bronchus denuded of epithelium (i.e. preparations incubated with S-CMC were less sensitive to carbachol).

The results of various experimental studies, including our own, clearly demonstrate that tracheal epithelium can modulate tracheal smooth muscle contraction.1–3,24,29 In most of these studies, the inhibitory effects of the bronchial epithelium were found to be small (except in dogs). However, it has been notoriously difficult to identify a single underlying pathophysiological mechanism. In addition, it seems that not only the contracting, but also the relaxing effects of some pharmacological agents are dependent on the presence of an intact epithelium.25 This may be relevant for a better understanding of different airway pathologies. It has been shown that the bronchial epithelium is damaged in patients with severe asthma, indicating that bronchial epithelial damage and airway hyperresponsiveness could be linked.26 Several studies have demonstrated that the bronchial epithelium constitutes a powerful diffusion barrier2,27 and can attenuate the effects of pharmacological agents applied luminally. The findings of the present study, although demonstrating small effects, support this hypothesis. It appears that the epithelium in rats mediates less inhibition compared with guinea-pigs. In guinea-pigs, we have found bigger differences following serosal compared with epithelial perfusion (D Pavlovic et al., unpubl. obs., 1999). However, it has been quite difficult to demonstrate airway hyperresponsiveness in vivo in airways already hyperresponsive in vitro. The significance of small in vitro changes that we observed may (but do not have to) correspond to more important in vivo changes, which we would like to verify in further experiments.

Incubation with S-CMC appears to influence human ring preparations but not rat preparations. However, in the present study, rat preparations were incubated for 60 min whereas human ring
preparations were incubated for 4 h and this may explain the differences observed.

In a recent animal study, it was shown that in SO₂-exposed rats carbocysteine diminished fucose, sialic acid and protein content, as well as the number of inflammatory cells, and reduced free radicals and elastase activity in bronchoalveolar lavage fluid. In addition, an increase in cAMP in tracheal tissue was observed and patch-clamp techniques revealed increased activity and density of cAMP-dependent Cl⁻ channel. These findings could, in principle, explain our observations. However, numerous other mechanisms may also be involved.

There is evidence that blocking enkephalinase (which degrades kinins) by phosphoramidon increases bronchoconstriction similar to removal of the epithelium. Indeed, mechanical removal of the epithelium could promote the release of different mediators from mast cells, which could be responsible, at least in part, for the bronchial hyperresponsiveness observed experimentally. It has also been shown that S-CMC increases the production of sialomucins and suggested that sialomucins could have an antikinin action. The anti-inflammatory activity of S-CMC was confirmed in an animal study where it reduced neutrophil infiltration provoked by intratracheal injection of interleukin (IL)-1. The same study demonstrated that S-CMC diminished smoke-induced bronchial hyperresponsiveness in guinea-pigs. Very recently, it was demonstrated that treatment with S-CMC effectively reduces airway hyperreactivity and airway inflammation at different phases of the response to secondary allergen challenge in mice, implicating the possible importance of the timing of S-CMC administration.

Indeed, capsaicin-sensitive nerve terminals secrete various, although only partially identified, mediators. It is conceivable that some as yet unidentified mediator/s originating from capsaicin-sensitive nerve terminals could have inhibitory effects on tracheal smooth muscle contraction. In addition, mechanical removal of the epithelium could have contributed to the release of such mediator/s. Nerve terminal destruction by capsaicin and the disappearance of the putative inhibitory agent could, in turn, have increased the sensitivity of tracheal smooth muscle to carbachol compared to the present experiments with S-CMC preparations not pre-incubated with capsaicin. It is tempting to hypothesize that S-CMS binds nitric oxide (NO) released from sensory nerves in epithelium-denuded tissues and thereby suppresses the responsiveness to carbachol. As a result of treatment with capsaicin, the NO stores may be depleted and S-CMS will not inhibit carbachol-induced contractions any further.

Other hypotheses, such as an anti-oxidant action for S-CMC, similar to one recently described for carbocysteine lysine salt monohydrate (SCMC-Lys), should be examined. Last, but not least, one simple feed-back mechanism could be proposed that could explain our finding of diminished sensitivity in –Ep preparations with S-CMC or carbachol (in the present experiments) with S-CMC preparations not pre-incubated with capsaicin. It is tempting to hypothesize that S-CMS binds nitric oxide (NO) released from sensory nerves in epithelium-denuded tissues and thereby suppresses the responsiveness to carbachol. As a result of treatment with capsaicin, the NO stores may be depleted and S-CMS will not inhibit carbachol-induced contractions any further. A multicenter, double-blind, placebo-controlled trial.

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REFERENCES


