Production of acute bronchiolitis in guinea-pigs by human respiratory syncytial virus

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ABSTRACT: Respiratory syncytial virus (RSV) is the most important cause of acute bronchiolitis in young children, and is implicated in the pathogenesis of paediatric asthma. The present studies were designed to develop a model of acute RSV bronchiolitis in young guinea-pigs, that could be used to study the mechanisms of the acute bronchiolitis and its sequelae.

Anesthetized, one month old guinea-pigs received either 4x10⁵ plaque forming units of Long strain human RSV or uninfected cell culture medium intranasally. Bronchiolar inflammation was assessed 6 days (n=10 RSV-inoculated; n=10 controls) and 14 days (n=10 RSV-inoculated; n=9 controls) postinoculation using a semi-quantitative histological scoring system. Viral replication within the lung was evaluated by culture, and the intrapulmonary distribution of viral antigens was evaluated by immunohistochemistry.

The RSV-inoculated group showed histological evidence of acute bronchiolitis 6 days after inoculation, which subsided by Day 14. Replicating virus was cultured from the lungs of 9 out of 10 RSV-inoculated animals on Day 6, and 2 out of 10 animals on Day 14, with no growth from control animals. Viral antigens were identified primarily within airway epithelial cells on Day 6, and within alveolar macrophages on Day 14.

Intranasal inoculation of human RSV into guinea-pigs provides a model of acute RSV bronchiolitis that may facilitate the study of both the pathogenesis of acute infection and the possible role of RSV in the subsequent development of nonspecific bronchial hyperresponsiveness in children.

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Respiratory syncytial virus (RSV) is estimated to cause 85% of the cases of acute bronchiolitis that affect infants and young children [1]. In addition, several epidemiological studies [2-4] have suggested that acute RSV bronchiolitis predisposes children to develop subsequent episodes of wheezing and asthma. The present studies describe an animal model of acute RSV bronchiolitis that may be useful in investigating the pathogenesis of acute bronchiolitis and its sequelae.

Although bronchiolar inflammation and airway hyperresponsiveness have been described following viral inoculation in natural hosts to such lower respiratory tract pathogens as rat parainfluenza Type 1 (Sendai) virus [5, 6], canine parainfluenza Type 2 virus [7], and canine adenovirus Type 2 [8], practical considerations have precluded similar investigations in natural hosts of human RSV (humans, chimpanzees and cows) [9, 10]. Experimental inoculation of human RSV into small animals, such as cotton rats [11] and mice [12], induces mild bronchiolar inflammation without evidence of clinical respiratory tract disease. Furthermore, the mouse requires a substantial inoculum (>10⁶ plaque forming units (pfu)) of RSV to develop bronchiolar inflammation [12]; in comparison, intranasal inoculation of 500 pfu has been reported to produce infection in 100% of human volunteers [13]. Inoculation of sheep with so-called "ovine" RSV produces subclinical infection characterized by mild bronchiolar inflammation and airway hyperresponsiveness [14]. To date, there is no satisfactory small animal model of acute RSV bronchiolitis in a widely available, easily handled species induced by exposure to a modest amount of human RSV.

The guinea-pig has previously been used as an animal model of acute bronchiolitis and airway hyperresponsiveness to parainfluenza Type 3 virus [15, 16], a member of the same family (Paramyxoviridae) as RSV. Because it is also known to mount a virus-specific humoral immune response following intranasal RSV inoculation [17, 18], we decided to determine whether the virus would produce acute bronchiolitis in the guinea-pig. These studies were based on light microscopic examination of lung sections to test for the histopathological features of human acute bronchiolitis, viral culture of lung digests to test for intrapulmonary replication of RSV, and immunohistochemistry to examine the cellular distribution of viral antigens.
Materials

Animals

Outbred female Cam Hartley guinea-pigs (Charles River, Montreal, PQ, Canada) were randomly assigned into control or RSV-inoculated groups. The two groups were housed in separate rooms, but were maintained under identical conditions of plastic cages containing Bed o'Cobs® corn cob bedding (The Andersons, Industrial Products Division, Maumee, OH, USA) and access to Purina guinea-pig chow, alfalfa hay cubes and water, and 12 h alternating light-dark cycles. Precautions to avoid undesired infections of guinea-pigs included using designated rooms equipped with air filters, and investigators wearing surgical gowns, hats, masks and shoe covers, and using autoclaved instruments and containers. In preliminary experiments [19], 22-29 day old, “juvenile” guinea-pigs (body weight 250-350 g) showed bronchiolar inflammation by Day 4 following either intranasal (n=2) or intratracheal (n=2) instillation of RSV, with maximal inflammation observed on Day 6-7 (n=4) and substantial resolution by day 14 (n=2). Based on this experience, RSV-inoculated (n=10) and control (n=10) guinea-pigs were examined 6 days postinoculation (acute phase), and RSV-inoculated (n=10) and control (n=9) animals were examined 14 days postinoculation (convalescent phase).

Virus

Since subgroup A of human RSV is considered to produce more severe acute lung disease than subgroup B virus [20], we propagated the Long strain of subgroup A human RSV (American Type Culture Collection, Rockville, MD, USA) on HEp-2 cell monolayers at multiplicities of infection from 0.01-0.1 [21], at 34°C in an humidified incubator containing 5% CO₂. Cell culture medium consisted of Dulbecco's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, NY, USA) supplemented with 5% foetal bovine serum (FBS) (Gibco), 0.292 mg·ml⁻¹ L-glutamine (Gibco), vitamins (Gibco), 100 µ·ml⁻¹ penicillin G (Sigma Chemicals, St. Louis, MO, USA), 100 µ·ml⁻¹ streptomycin (Sigma) and 10 µ·ml⁻¹ amphotericin B (Gibco).

Working stocks of RSV for inoculation of guinea-pigs were prepared by addition of autoclaved 3 mm diameter glass beads to infected HEp-2 cell monolayers and agitation with a vortex for 10 s. The lysed cell suspension underwent centrifugation at 800xg at 4°C for 4 min, and the resulting supernatant was transferred to a sterile tube for inoculation into guinea-pigs. Supematant from disrupted uninoculated HEp-2 cells was obtained in a similar manner for inoculation into control animals.

Methods

Inoculation procedure

On the day of inoculation (designated as study Day 0), guinea-pigs were anesthetized via inhalation of 3-5% halothane and each guinea-pig received either 100 µl of RSV-containing supernatant (corresponding to 3.9±0.1x10⁶ pfu (mean±sd) on subsequent plaque assay) or 100µl uninfected supernatant, by intranasal instillation. Animals were observed daily for signs of coryza or shortness of breath over the course of the experiments, and were weighed on the appropriate study day.

Lung tissue processing

The guinea-pigs were anesthetized by intraperitoneal injection of pentobarbital and sacrificed by exsanguination. The chest was opened and the heart and lungs were removed en bloc. The right main-stem bronchus was ligated, and the right lung was isolated and weighed in a sterile vessel. The right middle lobe was dissected and processed for viral culture (see below). The remainder of the right lung was frozen in liquid nitrogen, and stored for archival purposes at -70°C. The left lung was inflated via intratracheal instillation of phosphate-buffered 4% paraformaldehyde, and fixed overnight at 4°C. Parasagittal slices of fixed lungs were processed for paraffin embedding on an Histomatic® Automated Tissue Processor (Fisher Scientific). Serial 4 µm sections were stained with haematoxylin and eosin (H&E) and periodic-acid-Schiff (PAS) stains. At least two slides were prepared for each animal.

Light microscopy

The variability of bronchiolar inflammation induced by intranasal inoculation of RSV was compared to that induced by uninfected cell culture supernatant, using a semiquantitative scoring system based on previous reports in animals [22, 23] and humans [24, 25]. Glass slides were coded, such that the origin of a particular section (control or RSV-inoculated animal) was not known to the microscopist. Membranous bronchioles were evaluated for epithelial necrosis, mononuclear cell infiltrates and oedema, because they are features of human acute viral bronchiolitis [26]; polymorphonuclear (PMN) cell infiltration as an index of concomitant acute inflammatory phenomena [27]; hyperplasia of bronchus-associated lymphoid tissue (BALT) as an additional index of the mononuclear cell response [28], and goblet cell metaplasia as an index of respiratory epithelial repair [29]. For a given airway, each histological feature was scored from 0 (normal) to 2 (moderate to severe changes) by comparison to “standard” photomicrographs of guinea-pig airways (fig. 1). The reproducibility of this histological scoring system between and within observers was tested, as described in the statistical analyses section.

Ten bronchioles were examined per slide, and the observed score for each histological feature was expressed as the sum of individual airway scores (maximum score of 2x10=20 per parameter). Criteria for selecting airways for scoring included examination limited to membranous (noncartilaginous, muscular airways), scoring a given airway once per animal, and avoidance of adjacent cuts of an already scored airway.

Viral Culture

The fresh right middle lobe was minced into fine pieces with a sterile razor blade and digested into single cells
by immersion in sterile 0.25% trypsin-phosphate buffered saline at 37°C for 90 min. Following centrifugation at 800g at room temperature for 4 min, the supernatant was removed and replaced with 1 ml sterile cell culture medium. The resulting suspension was added to HEp-2 cell monolayers growing in 25 ml flasks containing 4 ml cell culture medium at 34°C. Flasks were examined daily under an inverted microscope for signs of RSV cytopathic effect (CPE). The specificity of CPE for RSV on HEp-2 cell monolayers was confirmed by polyclonal anti-RSV antibody (Dako B344), using the immunohistochemistry protocol described below. Cells which did not show CPE were passaged at weekly intervals for up to one month. A culture was classified as negative, when no CPE was observed over the one month period.

Viral plaque assays were performed on the working stocks of human RSV at the time of intranasal inoculation of guinea-pigs and on lung digest from four RSV-inoculated animals on Day 6 according to the method of LENETTE and SCHMIDT [30]. Samples were serially diluted in MEM/5% FBS and 0.5 ml of these diluents were added to confluent HEp-2 cells in plastic multi-well culture plates. Ninety minutes were allocated for viral adsorption at 37°C, after which time the diluent was removed and replaced by 2.5 ml of a liquid mixture of 4 parts MEM/5% FBS to 3 parts autoclaved 1% agarose (Gibco) in distilled water (dH₂O). The plates were kept at 34°C for 5–7 days. Monolayers were fixed for 10 min in 10% neutral buffered formalin, followed by removal of the agarose overlay, staining in 0.5% neutral red for 1 min and gently washing in tap water. Plaques (syncytia) were counted under an inverted microscope.

Immunohistochemistry

The protocol was adapted from NEILSON and YUNIS [31], who documented RSV antigens in formalin-fixed, paraffin-embedded sections of human lung obtained at autopsy. Five μm thick paraffin parasagittal sections of guinea-pig lung were incubated with 0.1% protease, type XIV, (Sigma) in 0.5 M Tris-buffered saline (TBS), pH 7.6 at 37°C for 10 min to disrupt protein crosslinks induced by fixation. Following brief rinses in tap water and 95% EtOH, sections were incubated with 0.9% H₂O₂ in methanol for 25 min at room temperature, to eliminate endogenous peroxidase activity. This was followed by a tap water rinse and 5 min wash in TBS at room temperature.

To prevent nonspecific immunoglobulin G (IgG) binding, sections were preincubated in normal swine serum (Dako, Denmark), diluted 1:20 in primary antibody diluting buffer (Biomedia), for 30 min at room temperature. Incubation with primary rabbit anti-RSV antibody (Dako B344), diluted 1:300 in TBS/2% bovine serum albumin fraction V (BSA)/1% human AB serum was performed for 90 min at room temperature. Negative control slides were incubated in parallel with TBS/2% BSA/1% human AB serum in the absence of anti-RSV antibody. Following
this incubation, sections were washed in TBS for 5 min at room temperature.

Sections were next incubated with biotinylated swine anti-rabbit secondary antibody (Dako), diluted 1:300 in TBS/2% BSA/1% human AB serum, for 45 min at room temperature, followed by a 5 min wash in TBS. A 45 min incubation at room temperature in peroxidase-conjugated streptavidin (Dako), diluted 1:600 in TBS/2% BSA/1% human AB serum, was followed by a 10 min wash in TBS.

The colorimetric peroxidase reaction consisted of developing sections in 20 μl working AEC solution (1 drop 3-amino-9-ethylcarbazole (Sigma), 1 drop 3% H2O2 in 3 ml 0.1 M sodium acetate), pH 5.2 for 15 min at room temperature. Following a rinse in dH2O, sections were counterstained with Mayer's haematoxylin for 1 min at room temperature and rinsed with dH2O. Coverslips were mounted using Immu-Mount aqueous mounting medium.

Formalin-fixed, paraffin-embedded lung tissue from two fatal cases of human acute RSV bronchiolitis (courtesy of J. Dinnick, British Columbia Children's Hospital and L. Holloway, New Zealand) were used as positive controls, and a formalin-fixed, paraffin-embedded block from an adult autopsy lung with no histological evidence of respiratory disease (St. Paul's Hospital, Vancouver, BC, Canada) was used as a negative control.

Statistical analyses

All statistical tests were performed using Systat® Version 5.1 software (Systat, Incorporated, Evanston, IL, USA) on a Macintosh IIci computer. The Student's t-test was used to compare mean right lung wet weights and lung to body weight ratios.

Concerning the reproducibility of the histological scoring system, scores were expressed as a 3×3 matrix (fig. 2). The intraobserver variation (RGH vs RGH; n=50 airways) and the interobserver variation (RGH vs SG; n=50 airways) were evaluated by calculating the Pearson coefficient of mean-square contingency, R2=1−TD(N+1) where T=Pearson chi-squared statistic and N=number of observations for each of the six histological features. The interpretation of R2 was facilitated when expressed as a proportion of the maximum possible value (representing a scenario of 100% agreement in scores), R2max=V(q−1)q, where q=the number of rows (or columns) in a square matrix [32]. An R2/R2max ratio of ≥0.75 was considered to represent acceptable reproducibility.

Upon completion of all scoring, the Mann Whitney U-test was used to compare observed scores (ordinal variables) between the RSV-inoculated and control groups for each histological feature [33]. To account for multiple comparisons, a sequential rejective Bonferroni procedure [34] was used to test for statistical significance at sequential p values ≤0.0083 (0.05/6), ≤0.01 (0.05/5), etc., until all six features had been analysed.

Results of viral culture and immunohistochemistry were reported as either "positive" (unequivocal signal observed) or "negative" (no signal observed). Plaque assays of lung digests from four RSV-inoculated animals on Day 6 were expressed as the mean±SD pfu per g lung wet weight.
Results

Table 1 shows that there were no statistically significant differences between the RSV-inoculated and control animals with respect to mean body weight, mean right lung wet weight or lung:body weight ratio. None of the RSV-inoculated animals showed any clinical signs of coryza or shortness of breath, and gross examination of fresh lung specimens revealed no evidence of consolidation.

Light microscopy

Figure 3 shows the results of the histological scoring system for acute bronchiolitis. On Day 6 postinoculation, there was statistically significant bronchiolar epithelial necrosis, mononuclear infiltrates and PMN infiltrates in the RSV-inoculated group (n=10), compared to controls (n=9) (fig. 3a). One control animal was excluded from analysis on Day 6, due to extensive aspiration pneumonia. On Day 14 postinoculation, none of the six histological features was significantly different between the RSV-inoculated (n=10) and control (n=9) groups (fig. 3b).

Viral culture

Replicating RSV was cultured from the digested lung parenchyma in 9 out of 10 RSV-inoculated guinea-pigs on day 6 (fig. 4), and in 2 out of 10 RSV-inoculated guinea-pigs on day 14. No CPE was observed from the digested lung parenchyma of any control animal on either day 6 or 14. Concerning viral plaque assays, the four RSV-inoculated guinea-pigs had a mean±so of 1.7±0.3×10⁵ pfu per g lung wet weight on day 6.

Immunohistochemistry

On Day 6 postinoculation, RSV antigens were detected primarily in cells within the airway epithelium (figs. 5a and b), with occasional alveolar macrophages and cells within BALT (fig. 5c) showing a positive signal.

On day 14 postinoculation, 6 out of 10 RSV-inoculated guinea-pigs showed positive RSV staining within the lung. In contrast to observations on Day 6, most staining was observed within alveolar macrophages (fig. 5d), with substantially fewer cells within the airway epithelium staining positively. Sections from the positive and negative control human lung sections stained as predicted during each run of the immunohistochemistry protocol. No lung sections from uninfected control guinea-pigs

Fig. 4. – HEp-2 cell culture five days after addition of digested lung from an RSV-inoculated guinea-pig (day 6 study). Note the syncytial formation indicative of RSV CPE (inverted microscope, bar represents 30 μm). RSV: respiratory syncytial virus; CPE: cytopathic effects.

Fig. 3. – Histological scores for a) Day 6 study; and b) Day 14 study. Epith necrosis: respiratory epithelial cell necrosis; Mono: mononuclear cell bronchiolar infiltrate; PMN: polymorphonuclear cell bronchiolar infiltrate; Oedema: bronchial wall oedema; BAL: hyperplasia of bronchi-associated lymphoid tissue; Goblet: goblet cell metaplasia. Each point represents the sum of scores for 10 airways in one animal. ○: controls; ●: RSV-inoculated. **: p<0.002; *: p<0.008; ***: p<0.01. RSV: respiratory syncytial virus.

Table 1. – Body weight, lung weight and lung:body weight ratios between RSV-inoculated guinea-pigs and controls

<table>
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<th>Body wt</th>
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Data are presented as mean±SD. NS: p>0.05. RSV: respiratory syncytial virus; ns: nonsignificant.
Fig. 5. — Immunohistochemistry of guinea-pig lung sections with polyclonal anti-RSV antibody. a) Day 6 study, photomicrograph of an inflamed airway showing staining of cells within the epithelium (arrowheads) (haematoxylin counterstain, bar represents 100 μm); b) Day 6 study, photomicrograph of a membranous bronchiole showing cytoplasmic staining of cells within the epithelium (haematoxylin counterstain, bar represents 50 μm); c) Day 6 study, photomicrograph showing staining (arrow) within BALT (haematoxylin counterstain, bar represents 50 μm). d) Day 14 study, photomicrograph showing staining (arrowheads) within alveolar macrophages (haematoxylin counterstain, bar represents 50 μm). For abbreviations see legend to figure 2.

incubated with anti-RSV antibody showed any positive staining; in addition, no staining was observed in lung sections from either RSV-inoculated or control animals that were not incubated with anti-RSV antibody.

Discussion

The studies reported here show that intranasal inoculation of anaesthetized juvenile guinea-pigs with approximately 4 x 10⁸ pfu of the Long strain of subgroup A human RSV results in acute infection of both lungs, with replication of the virus in the peripheral lung, immunohistochemical evidence of viral antigens within several lung cell types, and changes in the histology of the airways that are consistent with both human disease [26], and other animal models of acute viral bronchiolitis [5, 7, 8, 11, 12, 14]. The absence of clinically apparent respiratory tract illness in guinea-pigs following virus inoculation has also been reported in the cotton rat [11], mouse [12] and sheep [14] models of acute RSV bronchiolitis. The relatively low amount of RSV used for the inoculation of guinea-pigs produced evidence of viral bronchiolitis which may be analogous to subclinical human infection, since only a small minority of children with primary RSV infection develop acute bronchiolitis requiring hospitalization [35].

The overlap in lung histopathology between the RSV-inoculated group and the control group on Day 6, implies that the uninfected cell culture supernatant was capable of producing mild bronchiolar inflammation. However, the semi-quantitative histological scoring system established that the RSV-inoculated animals had statistically significantly higher scores for bronchiolar epithelial necrosis, mononuclear infiltrates and PMN infiltrates on Day 6, which became similar to controls on Day 14 as the inflammatory process subsided. These histological findings could be reproducibly estimated in a single-blind fashion, where the observer was unaware of the infected state of the animal, and proved to be useful in distinguishing inflammation attributable to RSV from the non-specific contribution of the uninfected cell culture supernatant.

The viability of RSV in the lungs of infected animals during the acute phase on Day 6 was confirmed by positive cultures in 9 out of 10 animals inoculated with RSV. The inability to isolate virus from one RSV-inoculated animal, despite positive immunohistochemistry for RSV antigens in the contralateral lung, was probably related to tissue sampling or processing. The results of viral plaque assays for intrapulmonary RSV on Day 6 were similar to previously reported values in cotton rats and mice at a similar time postinoculation [11, 12]. In contrast to the cotton rat and mouse models, replicating RSV was isolated in 2 out of 10 guinea-pigs 14 days postinoculation. This apparently longer time for virus isolation in guinea-pigs may reflect technical differences in
culture methods, since the cotton rat and mouse models used frozen lung specimens, and freezing may inactivate RSV [36]. Alternatively, RSV might be cleared at a slower rate from the guinea-pig lung than from either the cotton rat or the mouse lung. This slower rate of clearance might be a better model of human infections in which RSV may be shed for several weeks after resolution of acute bronchiolitis [37, 38]. Another possibility is that RSV might produce persistent lung infection as a sequela of acute bronchiolitis, which could play a role in the pathogenesis of the chronic complications of acute bronchiolitis.

The immunohistochemical findings of intracellular RSV antigens demonstrate that the virus entered lung cells after intranasal inoculation, and indicate that positive cultures were not solely attributable to free virus left over from the inoculation procedure. Further quantitation of immunohistochemistry results was not attempted, because the protocol was designed to have high specificity (i.e. no false positive signals in control specimens), which was achieved with an inevitable loss of sensitivity. The identification of viral antigens within alveolar macrophages and BALT suggests that RSV was processed by immunocompetent cells, and these observations extend previous descriptions of virus-specific immune responses developing in RSV-inoculated guinea-pigs [17, 18]. The presence of RSV antigens within alveolar macrophages on Day 14 raises the possibility of persistent infection of this cell type, since alveolar macrophages are permissive to RSV infection and may retain virus without cell lysis in vitro [39, 40]. Viral infection of alveolar macrophages may potentially alter host pulmonary defences to inhaled allergens and atmospheric pollutants [41].

The one month old, juvenile outbred guinea-pigs used in these studies modelled a genetically heterogeneous, paediatric human population exposed to RSV [42, 43]. Because so-called "pathogen-free" animals [44] were not used, there exists the possibility that experimental RSV inoculation potentiated the effects of underlying lung infections caused by other micro-organisms. Guinea-pigs are not overly susceptible to viral infections, but may develop spontaneous lung infections with bacteria such as Bordetella and Streptococcus species [45]. In our experiments, the guinea-pigs were randomly assigned into RSV-inoculated and control groups, and no control animal showed clinical or histological evidence of bacterial or viral lung infection. In addition, no CPE characteristic of any virus was observed in cultures from control animals and HEP-2 cells are permissive to numerous viruses in addition to RSV [46]. Furthermore, the specificity of the observed CPE in RSV-inoculated animals was confirmed by antibody staining. Finally, the laboratory animal facilities and technical precautions protected the guinea-pigs against undesired infections.

The wide availability and easy handling of the guinea-pig provides a significant practical advantage over the cotton rat and sheep models, and the relatively low amount of virus required to produce significant bronchiolar inflammation provides an advantage over the mouse model. A limitation of the guinea-pig, cotton rat, mouse and sheep models is that none of these species spontaneously develops a condition resembling human asthma; however, the guinea-pig has been extensively used as an animal model of allergen sensitization and its effects on airway physiology [47]. The genetic heterogeneity of outbred guinea-pigs complicates the study of host genetic factors involved in the development of atopic allergy [48] or acute exacerbations of asthma in atopic children [49] following RSV infection. Inbred strains of guinea-pigs [50] might circumvent some of these difficulties, but these animals are less widely available, tend to breed poorly and, in comparison to the mouse, the genetics of their immune response have not been well characterized. Although the more extensive characterization of leucocyte antigens and antibody responses in the mouse make it an attractive model to study host immunological factors involved in the pathogenesis of RSV lung infection, physiological data will be considerably more difficult to evaluate in this species.

In summary, these studies show that intranasal inoculation of human RSV produced histological evidence of acute bronchiolitis in juvenile guinea-pigs. They also show that viral antigens were present within the host cells of the inflamed airways, and that the virus replicated in the peripheral lungs. Furthermore, the virus continued to replicate in the lung parenchyma when the acute bronchiolitis had resolved. These results suggest that the guinea-pig is a promising animal model for investigating both the acute effects and chronic sequelae of human RSV lung infection.

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References


