

Clastogenic effects of different *Ureaplasma urealyticum* serovars on human chromosomes

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Abstract

The possibility that *Ureaplasma urealyticum* might play an important role in human infertility was first raised more than 20 years ago, but this association remains speculative. Considering the hypothesis that the pathogenicity of *Ureaplasma urealyticum* may depend on its serotypes, the clastogenic effects of different strains of *Ureaplasma urealyticum*, at concentrations of 10^3 CCU (color changing units)/ml, 10^4 CCU/ml and 10^5 CCU/ml, were evaluated *in vitro* in short-term cultures of human lymphocytes. Total or partial mitotic inhibition was produced by *Ureaplasma urealyticum* serotypes 2, 3 and 10 independent of the concentration (10^3 CCU/ml, 10^4 CCU/ml or 10^5 CCU/ml) of the microorganisms employed. In contrast, the clastogenic effects observed with serotypes 1, 7 and 12 varied according to the concentration employed in the test. Mitotic alterations were observed in *Ureaplasma urealyticum* serotypes 5, 6, 7, 8, 9, 11 and 12. Chromatid gaps (53.0%) and chromatid breaks (13.9%) were the most frequent types of alterations observed. The results of this *in vitro* assay demonstrated that the clastogenic effects varied with the *Ureaplasma urealyticum* serotypes evaluated.

Key words

- Mycoplasma
- Chromosome alterations
- *Ureaplasma urealyticum*
- Clastogenic effects

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Introduction

Mycoplasmas are the smallest prokaryotes able to self-replicate and their main feature is the absence of a cell wall. These two facts account for some of the distinctive characteristics peculiar to the group. Mycoplasmas are responsible for many diseases affecting animals and plants, some of them fatal. However, the role of these microorganisms in human pathologies is only partially known. The search for evidence indicative

of any correlation between the presence of these microorganisms and human morbidity and mortality has been the subject of many studies.

Among the human mycoplasmas, *Ureaplasma urealyticum* is a common inhabitant of the urogenital tract and has been associated with non-gonococcal urethritis and chronic prostatitis with direct interference with spermatogenesis, among other diseases. The higher incidence of chorioamnionitis, premature birth, fetal loss, premature rupture

of fetal membranes and low-weight newborns observed among women with *Ureaplasma*-positive placentas is evidence of the vertical transmission of the microorganism. The rate of vertical transmission ranges from 18% to 55% among full-term infants and from 29% to 55% among preterm infants. The high colonization rates in pregnancy make it difficult to validate the concept of fetal/neonatal infection based only on the isolation of *Ureaplasma urealyticum*. However, the possible consequences of this colonization cannot and must not be ignored in pregnant women with problems of reproductive wastage (1,2).

For a long time *Mycoplasmas* sp have been indicated as the microorganisms responsible for many changes in eukaryotic cells with varying deleterious effects (3,4). Different mycoplasma species can lead to different changes in a certain cell, while the same mycoplasma species can lead to different changes in cells of the same type belonging to different host species. Chromosomal aberrations, mitotic inhibition and stimulation and various other cytopathic effects have been observed (5-9).

The difficulties in establishing the role of *Ureaplasma urealyticum* in diseases of the reproductive tract and the antigenic variation among strains suggest that only some of the 14 serotypes are associated with disease (10-12).

The objective of the present study was to determine the clastogenic effects of different *Ureaplasma urealyticum* serotypes on human chromosomes *in vitro*. The understanding of these interactions may be relevant for the study of some of the disease-inducing mechanisms of mycoplasmas.

Material and Methods

Lymphocytes were obtained from a single donor and 11 serotypes of *Ureaplasma urealyticum* (1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12) were obtained from the American Type Cul-

ture Collection. Four supernatants obtained by centrifugation of the culture broths of serotypes 2, 3, 7 and 10 at 14,200 g for 40 min were also assayed.

Strains were prepared by culturing in modified U₁₀ medium (13) and incubating under microaerophilic conditions for 18 to 24 h. A₇ solid medium (14) was used to observe the growth and viability of the microorganisms. The inocula were microtitrated in such a way that 225 µl of U₁₀ medium was added to each microtiter plate well. Twenty-five µl of the culture medium for each serotype was added to the first well after homogenization. Successive dilutions were prepared from 25 µl of this first dilution. After incubation, the highest dilution in which the microorganism grew (this growth was indicated by the change in color of the culture medium) was taken as the titer, and the results were compared with those obtained with a control (U₁₀ medium without the inoculum).

Lymphocyte cultures were prepared by the method of Moorhead et al. (15) with some modifications. For each assay, 20 ml of aseptically collected venous blood was allowed to sediment by keeping the syringe in a vertical position for 2 to 3 h. After red cell sedimentation, the supernatant was transferred to a sterile flask and homogenized and 0.5 ml of this material was added to each flask together with 4 ml of 199 medium supplemented with 10% fetal calf serum and Bacto phytohemagglutinin-P (Difco, cod. 3110) at a concentration of 125 µl/100 ml.

Test culture

Culture broth (0.5 ml) from each serotype was added to the lymphocyte cultures at concentrations, expressed as CCU/ml (color changing units/ml), of 10³ CCU/ml, 10⁴ CCU/ml and 10⁵ CCU/ml, and the preparations were then incubated at 36.5°C for 72 h. After this period, 0.1 ml of 4 x 10⁻⁵ M colchicine was added, and the material was incubated

for 1 h. The lymphocyte viability test was carried out using 1% Trypan blue.

Control culture

The procedure for lymphocyte culture was the same as that used for the test culture, except that *Ureaplasma urealyticum* was not added.

Slide preparation

The contents of the flasks were centrifuged at 300g for 5 min, the supernatant was discarded, 4 ml of hypotonic solution (75 mM KCl) was added, and the flasks were incubated for 20 min. After incubation and centrifugation, a few drops of fixative (methanol/acetic acid, 3:1) were added to the sediment. After homogenization, 4 ml of fixative was added and the preparation was centrifuged again at 300 g for 5 min. The supernatant was removed and the same procedure was repeated once more. Two or 3 drops of this material were then transferred to slides and stained with Giemsa in sodium phosphate buffer, pH 6.8. After staining, the slides were submitted to microscopic analysis for the determination of mitotic index and for chromosome analysis.

Chromosome analysis

Metaphases were analyzed and classified according to the presence of structural aberrations. Metaphases presenting chromosomes with structural alterations were drawn and then photographed. The mitotic index was determined by counting the metaphases obtained out of 1,000 stimulated cells. In this study we considered a mitotic index of less than 10 to indicate partial inhibition of mitosis.

The following structural chromosome abnormalities were considered: chromatid gap (chtg), chromatid breaks (chtb), chromosome break (chrb), rearrangement (rea), ring chromosome (r), premature centromeric

disjunction (pcd) and centromeric decondensation (cd) (16).

Data were analyzed statistically by the Pearson chi-square test to determine the effect of inoculum concentration on the mitotic process, and by the significance test for the difference between two population proportions, applied to determine the significance of the results obtained for the test cultures compared with the control cultures. The level of significance was set at $\alpha = 5\%$ (17).

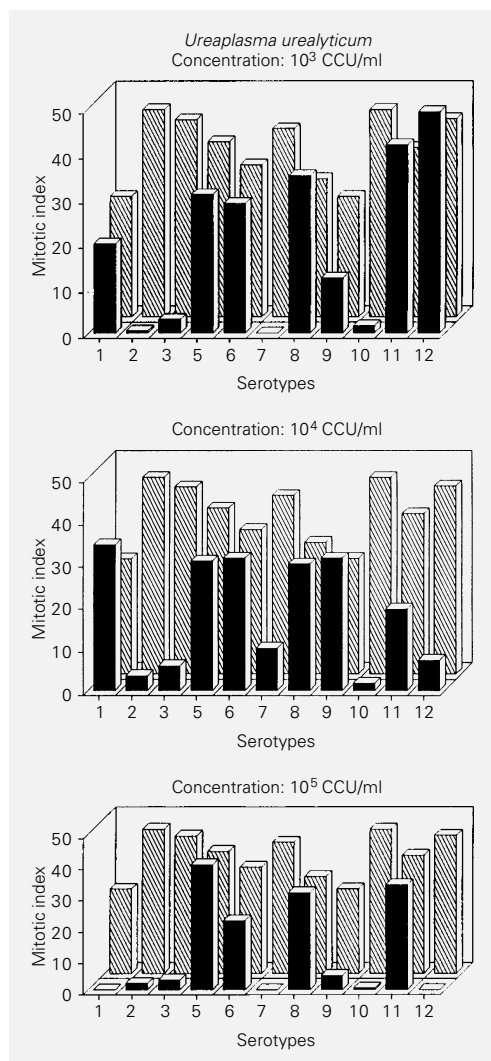
Results

The effects of 11 serotypes of *Ureaplasma urealyticum* on human chromosomes were studied, together with 4 supernatants obtained from cultures of serotypes 2, 3, 7 and 10. All tests were conducted with their respective controls and the slides were analyzed in a blind test.

We studied the effects of the microorganism on mitosis and mitotic index. Among the results observed, the main feature was total and partial mitotic inhibition (mitotic indexes equal to zero and below 10, respectively). Figures 1 and 2 show the values of the mitotic indexes obtained after stimulation with 11 serotypes at concentrations of 10^3 CCU/ml, 10^4 CCU/ml and 10^5 CCU/ml and for the supernatants from serotypes 2, 3, 7 and 10. All control cultures were also submitted to determination of mitotic index and karyotype analysis.

Mitotic indexes equal to zero or below 10 were observed for serotypes 1, 2, 3, 7, 9, 10 and 12. Similar results were obtained for supernatants 2, 3 and 7. Some of these serotypes presented these effects only at certain microorganism concentrations: serotype 1 (10^5 CCU/ml), serotype 7 (10^3 and 10^5 CCU/ml), serotype 9 (10^5 CCU/ml), serotype 10 (10^5 CCU/ml) and serotype 12 (10^4 and 10^5 CCU/ml). Serotypes 2 and 3 caused partial mitotic inhibition regardless of their concentration (Table 1).

Figure 1 - Reduction of human lymphocyte mitotic index by *U. urealyticum* serotypes as a function of concentration. CCU/ml = Color changing unit/ml. ■, Serotype; ▨, control.



The structural chromosome alterations observed were gaps, chromatid breaks, chromosome breaks, acentric fragments, premature centromeric disjunction (partial and total), centromeric decondensation (partial and total), ring chromosomes and chromosome rearrangements. Some chromosome aberrations could not be fully explained, but the statistical significance of their incidence earned them the designation “other alterations”. This designation also includes ring chromosomes and multiradial figures (Figures 3 and 4).

The *Ureaplasma urealyticum* serotypes and concentrations that caused structural chromosome aberrations were serotype 7 at

the concentration of 10⁴ CCU/ml, serotype 8 at concentrations of 10³, 10⁴, 10⁵ and >10⁵ CCU/ml, serotype 9 at concentrations of 10³, 10⁴ and 10⁵ CCU/ml, serotype 11 at concentrations of 10³, 10⁴ and 10⁵ CCU/ml, serotype 12 at the concentration of 10³ CCU/ml, and the supernatants from serotypes 3 and 7. Serotypes 5 and 6 showed chromosome aberrations at concentrations of 10³ and 10⁵ CCU/ml, but these results were not statistically significant when compared to the respective controls. Serotype 8 at concentrations of 10⁴, 10⁵ and >10⁵ CCU/ml, serotype 9 at the concentration of 10³ CCU/ml, serotype 11 at the concentration of 10⁵ CCU/ml, serotype 12 at the concentration of 10³ CCU/ml and the supernatants from serotypes 3 and 7 caused significantly increased alterations compared to their respective controls. Among these, gaps, chromatid breaks, chromosome breaks and partial centromeric decondensation were the most frequently observed.

The highest frequency of gaps and chromatid breaks was observed in the cultures with serotype 8 at concentrations of 10⁴, 10⁵ and >10⁵ CCU/ml and serotypes 9 and 11 at concentrations of 10³ CCU/ml and 10⁵ CCU/ml, respectively.

Chromosome breaks were significantly increased in the serotype 8 culture at concentrations of 10⁵ and >10⁵ CCU/ml. Partial centromeric decondensation was most frequent in serotype 8 at concentrations of 10⁴ and 10⁵ CCU/ml and serotype 9 at the concentration of 10⁵ CCU/ml. The serotype 8 test culture at a concentration of 10⁵ CCU/ml presented metaphases with drastic multiple chromosome alterations that could not be defined and that occurred with statistical significance, thus being classified as “other” alterations.

Sixteen of the 5432 control culture metaphases analyzed presented chromosome alterations (0.3%). The most frequent were gaps (52%), followed by chromosome breaks (24%) and chromatid breaks (16%). These

values did not differ significantly from those obtained for their respective test cultures.

A total of 101 of the 6164 test culture metaphases analyzed presented structural chromosome alterations (1.6%). The most frequent were gaps (53%), followed by chromatid breaks (13.9%), partial centromeric decondensation (9.5%), chromosome breaks (4.4%), total centromeric decondensation (4.4%) and "other alterations" (8.9%).

In serotypes 5, 6, 7, 8, 9, 11 and 12 chromosome alterations only occurred at certain inoculum concentrations.

Discussion

The alterations in chromosome organization observed when the cell is exposed to mutagenic agents represent a typical and easily detectable effect. Short-term cultures of peripheral blood lymphocytes represent one of the most sensitive techniques for the *in vitro* detection of these effects in human genetic material (18). Yet these studies serve only as indicators, because the individual characteristics of the lymphocyte donors can alter the frequency of cells with chromosome alterations (19,20). According to the literature, the average frequency of cells with chromosome alterations ranges from 1% to 20% in normal individuals (21-24). In our control group, the observed frequencies ranged from 0.2% to 2.6%, which indicates that the methodology used was adequate for the experiment.

Various cell systems have been used in attempts to explain some cytopathic effects caused by mycoplasmic infection (25,26). Schneider et al. (27) and Fogh and Fogh (28), in studies of the possible effects of mycoplasmas on cell cultures from amniotic fluid, showed that mycoplasmas are responsible for a significant increase in the number of chromosome alterations of these cells. Our findings demonstrated that, *in vitro*, different *Ureaplasma urealyticum* serotypes may be responsible for structural damage to

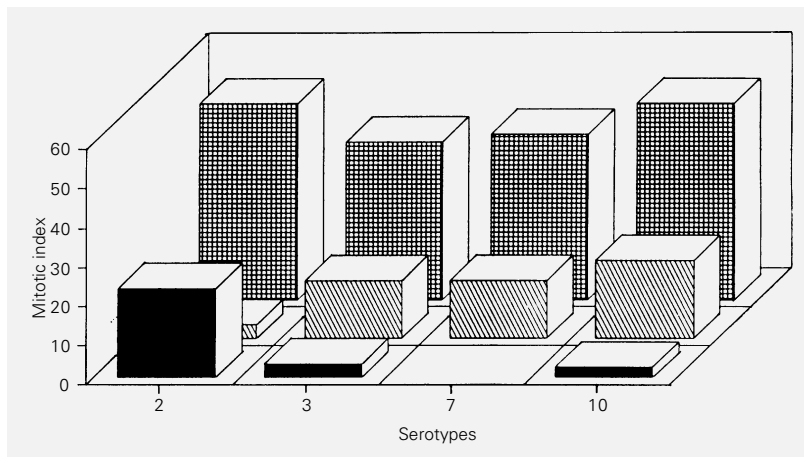


Figure 2 - Effect of supernatants of *Ureaplasma urealyticum* serotypes on the mitotic index of human lymphocytes *in vitro*. S₁ (■) = Supernatants of serotypes 2, 3, 7 and 10 at the concentration of 10⁵ CCU/ml; S₂ (▨) = supernatant duplicate; C_s (▩) = supernatant control.

Table 1 - Effect of serotype and concentration on *U. urealyticum*-induced modifications of human lymphocyte mitosis *in vitro*. Concentration is reported as CCU/ml (color changing units/ml).

<i>U. urealyticum</i> serotypes	Total mitosis inhibition	Partial mitosis inhibition	Metaphases with chromosome alterations	Metaphases without chromosome alterations
1	10 ⁵			10 ³ , 10 ⁴
2		10 ³ , 10 ⁴ , 10 ⁵		
3		10 ³ , 10 ⁴ , 10 ⁵		
5			10 ³ , 10 ⁵	10 ⁴
6			10 ³ , 10 ⁵	10 ⁴
7	10 ³ , 10 ⁵		10 ⁴	
8			10 ³ , 10 ⁴ , 10 ⁵ , >10 ⁵	
9		10 ⁵	10 ³ , 10 ⁴	
10		10 ³ , 10 ⁴ , 10 ⁵		
11			10 ³ , 10 ⁴ , 10 ⁵	
12	10 ⁵	10 ⁴	10 ³	

chromosomes. Thus, amniocentesis of pregnant women highly colonized by this microorganism should detect an abnormal fetal karyotype.

Many studies have been devoted to the controversial role of *Ureaplasma urealyticum* in perinatal pathologies. The high intraspecies antigenic heterogeneity has been responsible for the difficulties in establishing a correlation between the ureaplasmic infection and the appearance of disease. *Ureaplasma urealyticum* comprises 14 serotypes,



Figure 3 - Structural chromosome alterations caused by *U. urealyticum* serotypes on mitosis: ring chromosome (●); chromatid gaps (→); chromatid breaks (▶); partial premature centromeric disjunction (◆); chromosome fragment (→).

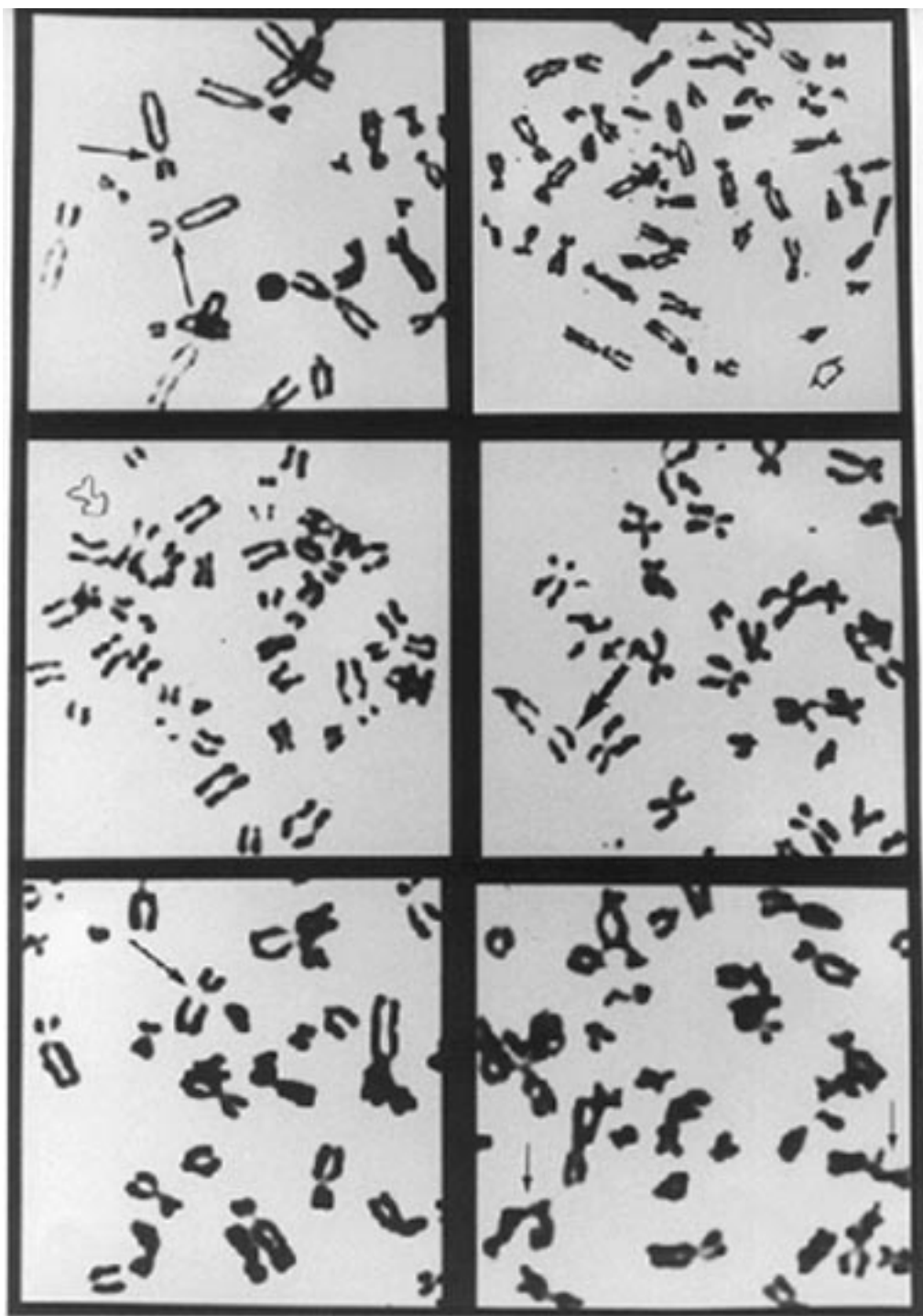


Figure 4 - Structural alterations caused by *U. urealyticum* serotypes: centromeric decondensation (—→); total centromeric premature disjunction (⌘); chromosome fragment (—→); chromatid rearrangements (—→); metaphases with several aberrations (□).

classified into two clusters: A (serotypes 2, 4, 5, 7, 8, 9, 10, 11, 12 and 13) and B (serotypes 1, 3, 6 and 14). Among these, the B cluster serotypes are the most frequently involved in human pathologies, although serotypes 4, 8 and 10 from cluster A also play an important role in some processes (11,29,30).

The aim of the present study was to establish a correlation between the serotypes studied and the structural chromosome alterations observed, and to determine (on the basis of the supernatants) if soluble products produced by the strains may be responsible for these alterations. The results indicated that serotypes 1, 2, 7, 10 and 12 from cluster A and serotype 3 from cluster B were responsible for the partial or total inhibition of mitosis. Metaphases presenting morphological chromosome alterations were the main features among serotypes 8, 9, 11 and 12 from cluster A. Among these, serotype 8 presented a significantly higher frequency of these alterations. In addition, the supernatants of serotypes 3 and 7 showed reduction in the mitotic index and clastogenic effects,

both possibly caused by ammonia release. Consequently, we observed that serotypes from both clusters had clastogenic effects on human chromosomes and on the mitotic process itself. Our results are consistent with the clinical analysis of some investigators who correlated positive cases of *Ureaplasma urealyticum* with the involvement of serotypes 3, 8 and 10 (12,29-32).

The isolation of mycoplasma from human malignant tissues reported by some investigators and the effects on human chromosomes demonstrated here suggest the importance of further studies on the role of these microorganisms in certain carcinogenic processes (33-35).

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