Enhanced Retention of Motility and Virulence of Treponema pallidum *(Nichols Strain) in Vitro by the Addition of Gelatin to Anaerobic Medium*

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The effect of gelatin on the survival of *Treponema pallidum* (Nichols strain) in anaerobic maintenance medium was investigated by monitoring of the retention of treponemal motility and virulence. Motility of *T. pallidum* was determined by dark-field microscopy, and virulence of *T. pallidum* was measured by observation of the latent periods of infection after inoculation of rabbits with virulent organisms. Gelatin concentrations of zero, 3%, 5%, 10%, and 15% were tested, and optimal retention of treponemal motility and virulence was observed at 5% and 10% gelatin.

TURNER AND HOLLANDER[1] observed that rabbit testes infected with *Treponema pallidum* became soft and filled with a viscous material as the infection progressed. This material was similar in chemical structure to hyaluronic acid.[1] The helical morphology of *T. pallidum* may be an adaptation for penetration and movement through environments of relatively high viscosity.[2] Spirochetes move better through and are attracted to viscous environments.[3,4] These facts raised the question of whether environmental viscosity is required for survival and growth of *T. pallidum*? We used gelatin in an attempt to determine the effect of increased viscosity on the survival of *T. pallidum* in an anaerobic culture medium.

Materials and Methods

Source of Treponema pallidum

The Nichols strain of *T. pallidum* used in these studies was obtained from Dr. M. Garner of the Institute of Clinical Pathology and Medical Research, Westmead, New South Wales, Australia.

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Propagation of Treponema pallidum

Suspensions of *T. pallidum* were obtained from rabbit testicular syphilomas after inoculation with the Nichols strain of virulent *T. pallidum*.[5] Approximately 5×10^7 *T. pallidum* were inoculated into each testis, and treponemes were harvested 11–13 days later, by which time orchitis had developed. The rabbit was killed by iv injection of 3 ml of a sodium pentobarbitone solution (200 mg/ml), and the testes were removed. With use of an aseptic technique, each testis was coarsely minced in 5–10 ml of gelatin-free maintenance medium with gentle stirring for 2–3 min; the fluid was then removed and examined by dark-field microscopy. Use of a bacterial counting chamber (Weber and Sons Ltd., Lancing, England) showed that an average harvest yielded $\sim 10^7$ highly motile *T pallidum*/ml.

The first elution fluid was discarded because it contained blood and blood clots. The testes were then eluted with successive 10-ml volumes of medium until the required volume of suspension was obtained. The concentration of *T. pallidum* had not fallen significantly after three or four elutions. Tubes containing 10 ml of experimental media were inoculated with 0.5 ml of *T. pallidum* suspension (final concentration, $\sim 5 \times 10^5$ organisms/ml).

Determination of Percentage Motility

Samples were taken every 24 hr, and treponemes were observed by dark-field microscopy. The percentage motility was determined by observation of ≥ 100 treponemes at random and designation of cells as either motile or nonmotile. The time (hr) required for 50% of the treponemes to become immotile in vitro was designated the 50% motility time (MT_{50}). This value

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TABLE 1. Components of *Treponema pallidum* Maintenance Medium (Compositions of Component Solutions are Given in Footnotes).

Components	Amount/ liter
Part A:	
Balanced salt solution*	60.0 ml
Ammonium sulfate (50 g/l)	2.0 ml
Glucose	1.6 g
Fructose	1.6 g
Resazurin solution (0.25 g/l)	4.0 ml
Water	424.0 ml
Glutathione (reduced)	2.4 g
L-Cysteine	1.6 g
Sodium thioglycollate	1.0 g
Part B:	
L-Glutamine (29.2 g/l)	10.0 ml
Amino acid (A), 100×†	10.0 ml
Amino acid (B), 100×‡	10.0 ml
Nonessential amino acids, 100×§	10.0 ml
Vitamins 100×¶	10.0 ml
Solution D**	10.0 ml
Fetal calf serum (heat-inactivated)	250.0 ml
Phosphate solution††	190.0 ml
Buffered bicarbonate solution‡‡	10.0 ml

* Balanced salt solution: NaCl, 40 g/liter; KCl, 4 g/liter; $CaCl_2$, 1.4 g/liter; $MgSO_4 \cdot 7H_2O$, 2 g/liter.
† Amino acid (A) 100 × concentrate, composition per 10 ml: L-histidine·HCl·H_2O, 31 mg; L-isoleucine, 52 mg; L-leucine, 52 mg; L-lysine·HCl, 58 mg; L-methionine, 15 mg; L-phenylalanine, 32 mg; L-threonine, 48 mg; L-tryptophan, 10 mg; L-valine, 46 mg; and L-arginine·HCl, 1.05 mg.
‡ Amino acid (B) 100 × concentrate, composition per 10 ml: L-cysteine, 24 mg; L-tyrosine, 36 mg.
§ Nonessential amino acids 100 × concentrate, composition per 10 ml: L-alanine, 8.9 mg; L-asparagine·H_2O, 15 mg; L-aspartic acid, 13.3 mg; L-glutamic acid, 14.7 mg; L-proline, 11.5 mg; L-serine, 10.5 mg; and glycine, 7.5 mg.
¶ Vitamins 100 × concentrate, composition per 10 ml: choline chloride, 1 mg; folic acid, 1 mg; myo-inositol, 2 mg; nicotinamide, 1 mg; calcium pantothenate, 1 mg; pyridoxal·HCl, 1 mg; riboflavin, 0.1 mg; and thiamine·HCl, 1 mg.
** Solution D, composition per 10 ml: sodium pyruvate, 250 mg; adenine, 5 mg; cocarboxylase, 3 mg; coenzyme A, 50 μg; isobutyric acid, 10 mg; α-lipoic acid, 5 mg; *p*-aminobenzoic acid, 5 mg; biotin, 50 μg; nicotinic acid, 500 μg; pyridoxine·HCl, 2.5 mg; pyridoxamine·HCl, 5 mg; putrescine·2HCl, 10 mg; and cyanocobalamin, 50 μg.
†† Phosphate solution: Na_2HPO_4, 0.6 g/liter; KH_2PO_4, 0.6 g/liter.
‡‡ Buffered bicarbonate solution: $NaHCO_3$, 50 g/liter; K_2HPO_4, 40 g/liter; KH_2PO_4, 8 g/liter.

was determined by plotting of percentage motility against time.

Virulence of T. pallidum

The retention of virulence was detected by inoculation of samples of *T. pallidum* from maintenance medium into the shaved backs of rabbits and observation of the length of the latent period. The length of the latent period is inversely related to the number of virulent *T. pallidum* inoculated into the rabbit[1] and can therefore be used for comparison of the survival times of virulent *T. pallidum* in different media.

Samples of maintenance medium were taken immediately after inoculation with *T. pallidum* (time zero), and at 24, 48, and 72 hr thereafter. The backs of adult male rabbits were shaved, and each one was marked into a grid of 20 squares. Four replicate samples of 0.1 ml of medium were injected intradermally. All experimental and control samples taken at the same time were inoculated into the same rabbit; thus variability caused by slight differences in susceptibility of rabbits to *T. pallidum* was avoided. Rabbits were housed at 16–19 C, shaved regularly, and examined daily for development of lesions as determined by induration at the site of inoculation. Antibiotic-free feed was given ad lib. Occasionally, lesions were scraped, and the exudate was examined for the presence of treponemes.

Preparation of Media

Maintenance medium. The maintenance medium, a modification of a previously described medium,[6] was made in two parts (A and B), which were combined just before inoculation with *T. pallidum*. Its composition is shown in table 1.

The components of part A (except cysteine, glutathione, and sodium thioglycollate) were combined in a conical flask, such that only a small air space remained. The medium was deoxygenated by sterilization in an autoclave at 121 C (15 psi) for 8 min, with slow exhaust. Immediately on removal from the autoclave, the surface of the medium was continually flushed with a stream of oxygen-free nitrogen (obtained by passing nitrogen through a hot copper column) and, at the same time, cooled rapidly in an ice bath.

When the medium was cool, reducing agents (as powders) were added with gentle stirring. The *p*H of the solution was adjusted to 8.6 with 10 M NaOH; the medium was held under a stream of nitrogen until it was added to nitrogen-flushed anaerobic tubes (Bellco Glass Inc., Vineland, N. J.). The tubes were stoppered and autoclaved at 121 C for 15 min in a press clamp. During autoclaving the *p*H fell to ~7.3.

The solutions of part B were either obtained sterile from the Commonwealth Serum Laboratories (CSL, Melbourne, Australia) or were prepared and sterilized separately and combined aseptically. Part B (5 ml) was then added aseptically and under oxygen-free nitrogen to each tube already containing 5 ml of part A. The pH of the complete medium was adjusted to 7.4. The medium was isotonic for rabbit erythrocytes, and its oxidation-reduction potential was always more electronegative than −150 mV (E_{cal}). E_{cal} is the oxidation-reduction potential of the solution using a calomel

reference electrode. The *p*H and E_{cal} were checked daily during the experiment to ensure that they remained constant.

Maintenance medium was used both for harvesting *T. pallidum* and as a control for the experimental media. The latter were prepared in a similar manner and differed only as noted below. After inoculation with *T. pallidum*, the cultures were incubated under nitrogen at 35 C.

Gelatin-containing medium. Gelatin (British Drug Houses, Chemicals Ltd., Poole, England) was used at final concentrations of 3%, 5%, 10%, and 15% (wt/vol). It was added at double strength along with the reducing agents to separate flasks containing part A, allowed to soak for 30 min, and then dissolved by heat (45 C). The *p*H was adjusted to 7.8, and the flasks were autoclaved at 115 C (10 psi) for 10 min. Part B was added as before. The viscosities of the gelatin-containing media (relative to gelatin-free maintenance medium) were determined by use of an Ostwald viscometer (Bartelt Glass Instrument Co., West Heidelberg, Australia).

Results

The Effect of Gelatin on the Viscosity of the Medium

If gelatin-free medium was assigned a value of 1, media containing 5%, 10%, and 15% gelatin had relative viscosities of 4, 9, 18, respectively.

The Effect of Gelatin on Retention of T. pallidum *Motility in Vitro*

Retention of motility by *T. pallidum* was significantly enhanced by gelatin; the optimal concentration was 10% (table 2).

TABLE 2. Effect of Gelatin on the 50% Motility Time (MT_{50}) of *Treponema pallidum* in Vitro.

Experiment No.	MT_{50} (hr) of *T. pallidum* at Indicated Gelatin Concentrations*				
	0	3%	5%	10%	15%
1	42	90	114	114	90
2	42	78	85	93	78
3	48	83	90	122	86
Average	44	84	96	110	85

* The MT_{50} is the time (hr) required for 50% of the *T. pallidum* to become immotile in vitro.

The Effect of Gelatin on Retention of T. pallidum *Virulence in Vitro*

The data presented in table 3 are the means of four independent experiments. There was a significant difference in the latent period between infections induced by treponemes incubated in medium without gelatin and those incubated with gelatin. This difference was statistically significant for lesions induced by treponemes taken after zero, two, and three days of incubation in media containing 5% or 10% gelatin. The results for the samples taken on days 2 and 3 correlated well with the motility data. At time zero, 100% of treponemes in all samples were motile and might have been expected to produce lesions with identical latent periods, although this was not the case. Lesions induced by treponemes incubated with gelatin were also larger than those induced by treponemes incubated without gelatin. The differences in lesion diameter were 3–5 mm. The differences in size between "gelatin" and "nongelatin" lesions were greatest with 5% and 10% gelatin; these results agreed with the results based on motility and latent period of infection (data not shown).

Inoculation of gelatin alone or of killed treponemes with gelatin did not induce any lesions in the rabbits.

Discussion

Addition of gelatin to the maintenance medium significantly prolonged survival of *T. pallidum* as meas-

TABLE 3. Effect of gelatin on the latent period of syphilitic infection in rabbits inoculated with *T. pallidum* obtained from maintenance cultures after incubation for zero time, or for one, two, or three days.

Days of in Vitro Incubation	Latent Period (Days ± SD) of Infection with Indicated Concentration of Gelatin				
	0	3%	5%	10%	15%
0	8.8 ± 2.8	7.8 ± 1.7	7.2 ± 1.0*	7.2 ± 1.5*	7.5 ± 1.5
1	8.5 ± 2.3	8.1 ± 1.5	7.4 ± 1.4	8.0 ± 1.9	8.5 ± 2.4
2	15.9 ± 5.2	13.9 ± 4.0	12.6 ± 3.2*	11.9 ± 2.8*	12.9 ± 4.1*
3	17.1 ± 3.6	14.4 ± 2.6	11.5 ± 3.4*	12.3 ± 4.6*	16.3 ± 1.7
Difference in latent period†	...	−1.5	−2.9	−2.7	−1.3

Note. The latent period is the time between inoculation of the rabbit and the appearance of a syphilitic lesion, as indicated by induration in the skin.

* Significantly different from gelatin-free medium as calculated by Student's *t* test ($P < 0.05$).

† Value given is the average difference between the latent period with gelatin-free medium and the latent period with medium containing gelatin. A minus sign indicates a shorter latent period in gelatin-containing medium.

ured by retention of motility. The optimal concentration of gelatin was 10%. This finding might be explained by the increased viscosity, but it is possible that properties of gelatin other than viscosity are beneficial to *T. pallidum*. Gelatin, which is derived from collagen, is an atypical protein with respect to a number of its constituents.[7]

The observation that treponemes, after incubation in gelatin-containing media, retained virulence to a greater extent than did treponemes incubated in gelatin-free media might be explained in terms of protection against host defenses, e.g., an inhibition of phagocytic cells by a gelatinous, capsule-mimicking, gel-like substance around the treponeme. Gelatin may mimic the natural hyaluronic acid–like material produced by *T. pallidum* in vivo[1] and in tissue culture.[8] Gelatin, alone or in conjunction with heat-killed *T. pallidum*, did not induce any lesion or dermal hypersensitivity in the rabbit.

Turner and Hollander reported that the type of motility exhibited by *T. pallidum* in vitro is dependent on the viscosity of the medium;[1] "corkscrew" motility is observed in mucoid media and "rotatory" motility in serous exudates. These authors observed that the corkscrew type of motility could be converted into the rotatory form when the mucoid material of a syphilitic lesion was thinned by hyaluronidase and that rotatory movement could be converted to the corkscrew type by the addition of methyl cellulose. Fitzgerald and Johnson also observed that the type of motility exhibited by *T. pallidum* was affected by the viscosity of the testicular fluid from the rabbit syphiloma.[9] "Directed" motility was detected under viscous conditions, and this type of motility was also observed in tissue culture medium to which methyl cellulose (0.1–0.4%) but not gelatin had been added.

Since viscosity affects the type of motility exhibited by *T. pallidum*, it may also affect other aspects of survival and growth of these organisms in vitro. One possibility is that increased viscosity slows down the metabolic rate of the treponeme by limiting the rate of diffusion of essential metabolites, including oxygen, and so enhances survival of treponemes by virtue of the slower production of toxic end products of metabolism.

In conclusion, we have observed that *T. pallidum*, when incubated in vitro in gelatin-containing medium, retained virulence for a much longer time than when incubated in an equivalent medium without gelatin. The explanation for this phenomenon is unknown, but enhanced viscosity may be partly responsible for the observed effects.

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