The Influence of *Leptospirae* and their Toxins and Antisera on Chick Embryo Fibroblast and He-La Tissues

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A number of investigators have examined the influence of different gram negative and gram positive bacteria upon various tissue cultures, as well as the effects of bacterial toxins and neutralizing antisera. The systems which have been studied by these authors are summarized in Table I. In the experiments described here, we have investigated the influence of various strains of viable *Leptospirae* and their toxins on cultured tissues of chick embryo fibroblast and HeLa cells in the presence and absence of various corresponding anti-Leptospirae-antisera.

Materials and Methods

Leptospirae strains: *L. biflexa Patoc* I, *L. icterohaemorrhagiae Wijnberg*, *L. canicola Hond Utrecht* IV, *L. pomona Pomona*, *L. grippotyphosa Moskov* V, *L. autumnalis Akivami*, *L. australis Ballico*, *L. hebdomadis Hebdomadis*, *L. bataviae S. van Tienen*. All strains were kindly supplied by the Reference Laboratory for Leptospira of the WHO in Amsterdam. They were cultured on Korthoff's medium in 30 °C.

Neutralizing antisera: Rabbits were hyperimmunized with concentrated suspensions of Leptospirae strains to obtain high anti-Leptospirae specifity without antitoxin specifity. The antisera and their titers were: anti *L. icterohaemorrhagiae* 1:15 000, anti *L. grippotyphosa* 1:20 000, anti *L. pomona* 1:8000, anti *L. canicola* 1:28 000, anti *L. autumnalis* 1:12 800, anti *L. australis* 1:25 600, anti *L. hebdomadis* 1:25 600, anti *L. bataviae* 1:12 800.

Leptospirae toxins: Toxins from the Leptospirae strains were isolated by centrifuging 5 to 6 week old Leptospirae cultures at 5000 rpm for 20 minutes, by which time no Leptospirae remained in the toxin containing supernatant (exotoxin).

Preparation of chick embryo tissue culture

Cell cultures were prepared from 9—11 day old chick embryos. After discarding of heads, extremities and bowels the embryos were cut into small fragments. The fragments were washed three times with buffered salt solution and trypsynized at 37 °C, using 0,25 per cent trypsin with continual mixing. Collected cells were centrifugated for 5 min at 1000 rpm. The sedimented cells were suspended in the growth medium, consisting of Hanks solution with 0,5 per cent lactalbumin hydrolysate, and 10 per cent inactivated calf serum.

The suspension of cells ($2 \cdot 10^5$ cells/1 ml) was dispended into tubes with slides and incubated at 37 °C for 1—2 days.

Preparation of He-La cell tissue culture

The matrix of He-La tissue was trypsynized and centrifugated. The sediment was suspended in Hanks liquid ($2 \cdot 10^5$ cells/1 ml) with 0,5 per cent lactalbumin hydrolysate, and 10 per cent calf serum. The cell cultures were then placed in a thermostat at 37 °C for 5 days.

The cells were cultured on cover-slides (for easier staining) introduced in tubes, 3 tubes for every strain and toxin.

Neutralization of Leptospirae and their toxins

To the cultures of Leptospirae on Korthoff's medium (300 million of Leptospirae per 1 ml) homologic or heterologic immune sera (8 ml of culture plus 2 ml of corresponding immune serum) were added and thouroughly mixed. The cultures were then placed in a thermostat at 37 °C for 30 min. In the same way the neutralization of Leptospirae toxins was carried out.

Infection of tissue culture

Monolayers of chick embryo either He-La cells were infected with Leptospirae cultures (300 million of Leptospirae per 1 ml), or with Leptospirae toxins.

Results

The results of a number of the experiments are shown in the ten micrographs and all of the results are summarized in Table 2, 3, 4. It is clear that the saprophytic *Leptospira biflexa Patoc* (Fig. 2) and its toxin do not evoke any cytopathogenic effect on either tissue culture. The control experiments with Korthoff medium and normal sera from man and rabbit were negative (Fig. 1 *).

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* Figs. 1—10 s. Table 564 a and b.

Author	Bacteria	Toxins	Tissue culture				
			c.f.	K.B.	He-La	h.e.	m.k.
1. C. Levaditi (1913)	—	toxin C. dipht.	+	+	0	0	0
2. C. Placido Sousa (1957)	—	toxin C. dipht.	0	0	0	0	+
3. G. Penso (1957)	—	toxin Cl. sept.	0	+	0	0	+
4. G. Vicari (1960)	—	toxin Sh. dysen.	0	+	0	0	+
5. I. Mesrobeanu (1960)	—	toxin Staph.	0	0	0	+	0
6. D. Rogala (1962)	—	toxin C. dipht.	+	0	0	0	0
7. I. Mesrobeanu (1960)	—	toxin Sh. Salm.	0	0	0	+	0
8. M. Korbecki (1964)	—	toxin Staph.	+	0	0	0	0
9. E. I. Shmeleva (1964/65)	*B. pertussis.*	—	+	0	0	0	0
10. V. Strizova (1964)	—	toxin B. pertussis	+	0	+	+	0
11. J. Vosta (1964)	*Leptospirae*	—	0	0	+	0	0

Table 1. Investigations on the influence of different bacteriae and toxins on tissue cultures [1-10]. Chicken fibroblast line — c.f., K.B. line — K.B., He-La line — He-La, Human embryonic line — h.e., Line from monkey kidney — m.k., 0 = not examined.

The other Leptospirae strains: *icterohaemorrhagiae, grippotyphosa, pomona, canicola, australis, hebdomadis, bataviae* and *autumnalis*, all evoke strong cytopathogenic effect in chick embryo fibroblast cultures and where tested, somewhat weaker effects in He-La culture (Fig. 3 – 10). In fibroblast cultures, strong or complete destruction of protoplasm was observed, whereas in He-La culture, which is more resistant to Leptospirae activity, we observed only the protoplasm destruction and formation of symplasts (Fig. 10). When the Leptospirae were neutralized with homologous sera, no cytopathogenic effects were observed (Fig. 4, 6, 9). Leptospirae toxins, on the other hand, evoke cytopathogenic effects even in the presence of the homologous anti-Leptospira antisera (Fig. 5, 10). The cytopathogenic effects of the toxins were found to be dependent on the age of the Leptospira culture; toxins isolated from 22 day culture are less effective than Leptospira culture two or three times older. Because of the hyperimmunization technique employed, the sera show no capacity to neutralize the Leptospira toxins. Since the homologous sera neutralized only the Leptospira infectivity, but not that of the toxin, we might conclude that the pathogenic activity of Leptospirae depends on components in the Leptospirae cells; it is possible that the intact Leptospirae have a different infection mechanism than the toxins, which contain the medium without the Leptospirae (exotoxins).

Conclusions

The saprophytic *L. biflexa Patoc* does not evoke the CPE in fibroblasts and He-La tissue culture. As control the Korthoff-medium as well as the normal human and rabbit serum does not evoke CPE. The pathogenic Leptospirae: *L. icterohaemorrhagiae, L. grippotyphosa, L. pomona, L. canicola, L. autumnalis, L. australis, L. hebdomadis* evoke the CPE in tissue culture. The homologic anti-

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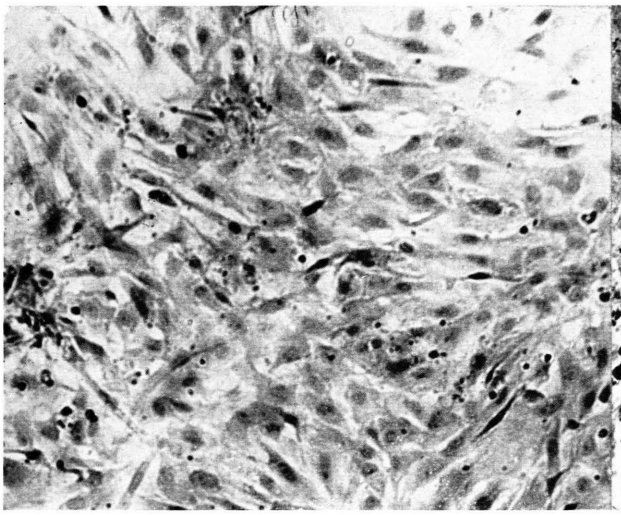

Fig. 1. Control-normal tissue of chicken embryo fibroblast. Eosin and hematoxilin stain. 100 ×.

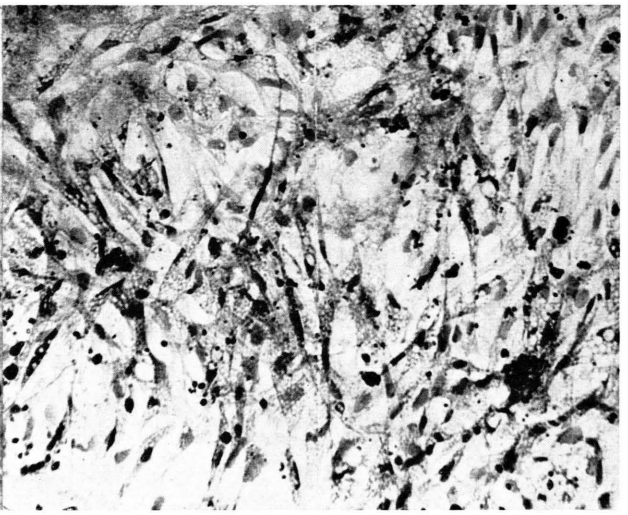

Fig. 2. Tissue of chicken embryo fibroblast infected with *L. biflexa Patoc* (saprophyt), vacualisation is seen. Eosin and hematoxylin stain. 100 ×. (CPE −)

Fig. 3. Tissue of chicken embryo fibroblast infected with *L. icterohaemorrhagiae*, is completely destroyed. Eosin and hematoxylin stain. 100 ×. (CPE +++)

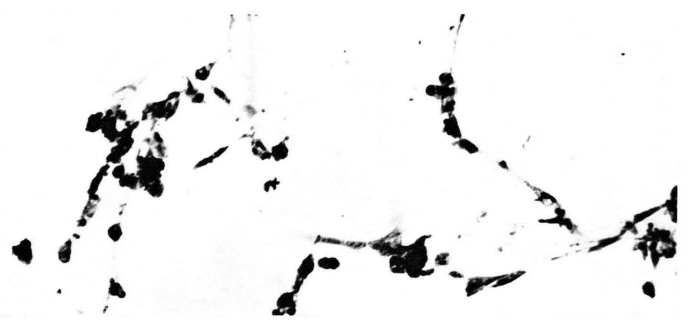

Fig. 5. Total destruction of tissue of chicken embryo fibroblast by *L. icterohaemorrhagiae* toxin. Eosin and hematoxylin stain. 100 ×. (CPE +++)

Fig. 4. Tissue of chicken embryo fibroblast infected with *L. icterohaemorrhagiae* culture neutralized by anti serum *L. icterohaem.* Eosin and hematoxylin stain. 100 ×. (CPE −)

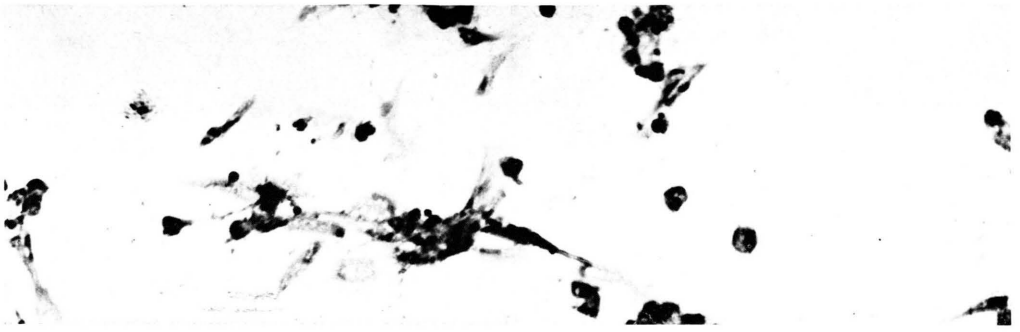

Fig. 6. Tissue of chicken embryo fibroblast with *L. icterohaemorrhagiae* toxin neutralized by anti serum *L. icterohaem.* Lack of neutralization effect. Eosin and hematoxylin stain. 100 ×. (CPE + + +)

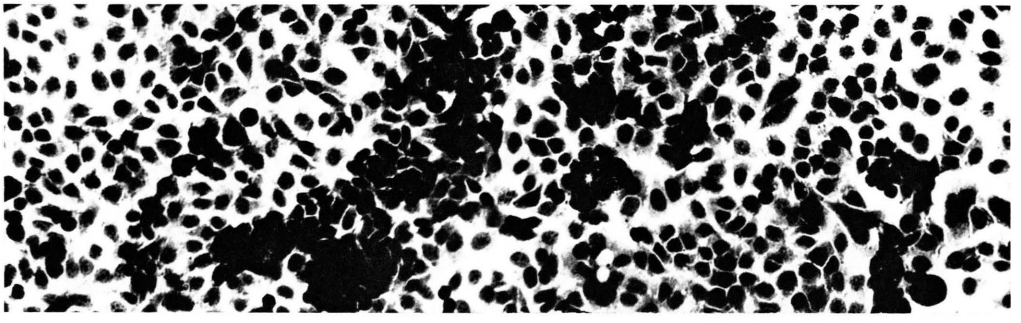

Fig. 7. Tissue culture He-La not infected-control. Eosine and hematoxyline stain. 100 ×.

Fig. 8. Tissue culture He-La infected with *L. icterohaemorrhagiae*, positive effect cytopathogenic, particular interest evokes the destroyed protoplasma cells. Eosin and hematoxylin stain. 100 ×.

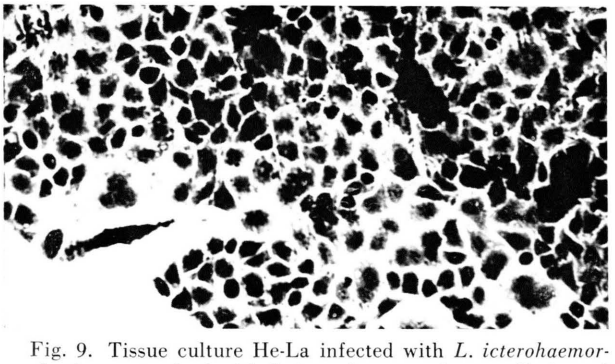

Fig. 9. Tissue culture He-La infected with *L. icterohaemorrhagiae* neutrasized with *L. icterohaem.* antiserum. Eosin and hematoxylin stain. 100 ×.

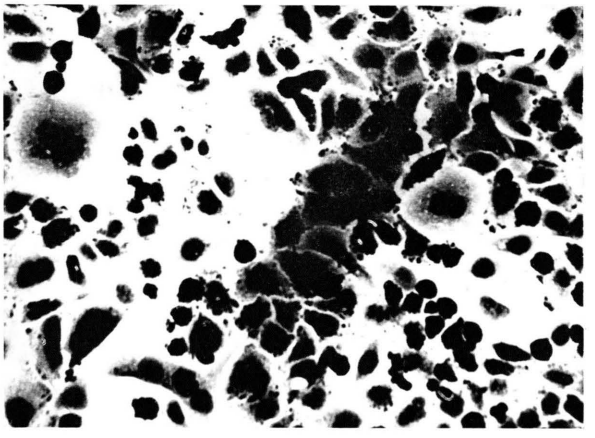

Fig. 10. Tissue culture He-La infected with *L. icterohaemorrhagiae* toxin; great cells are seen (symplast). Eosin and hematoxylin stain. 100 ×.

	Leptospirae	Tissue culture			
		He-La		chick embryo fibroblast	
		cytopathic effect (CPE)			
		24 h	48 h	24 h	48 h
Control	Tissue culture + medium Korthoff	−	−	−	−
Control	Tissue culture + normal rabbit serum	−	−	−	−
Control	Tissue culture + normal human serum	−	−	−	−
Control	*L. biflexa Patoc*	0	0	−	−
Control	*L. biflexa Patoc* toxin	0	0	−	−
	L. icterohaemorrhagiae	+++	+++	+++	+++
	L. icterohaemorrhagiae neutralized with *L. icterohaem.* antiserum	−	−	−	−
	L. icterohaemorrhagiae toxin	+	+	+++	+++
	L. icterohaemorrhagiae toxin neutralized with *L. icterohaem.* antiserum	+	+	+++	+++
	L. canicola	0	0	+++	+++
	L. canicola neutralized with *L. canicola* antiserum	0	0	±	±
	L. canicola toxin	0	0	+++	+++
	L. canicola toxin neutralized with *L. canicola* antiserum	0	0	+++	+++
	L. pomona	0	0	+++	+++
	L. pomona neutralized with *L. pomona* antiserum	0	0	±	±
	L. pomona toxin	0	0	+++	+++
	L. pomona toxin neutralized with *L. pomona* antiserum	0	0	+++	+++
	L. grippotyphosa	+	+	+++	+++
	L. grippotyphosa neutralized with *L. grippotyphosa* antiserum	±	±	±	±
	L. grippotyphosa toxin	+	+	+++	+++
	L. grippotyphosa toxin neutralized with *L. grippotyphosa* antiserum	+	+	++	++

Table 2. The influence of Leptospirae and Leptospira toxins before and after neutralization with homologous sera on tissue cultures −: lack of cytopathogenic, ±: small destroyed tissue foci, ++: partial cytopathogenic effect, +++: complete cytopathogenic effect, 0: not examinated.

Leptospirae sera neutralize the CPE-activity of Leptospirae.

The Leptospirae-toxins evoke also the CPE on fibroblasts and He-La tissue culture. The homologic anti-Lectospirae sera does not neutralize the CPE-activity of toxins.

The heterologic anti-Leptospirae sera does not neutralize the CPE activity of Leptospirae cultures.

These experiments indicate the possibility of application of Leptospirae-CPE activity on tissue culture and the neutralization analysis by homologic and heterologic sera for differentiation of Leptospirae serotypes.

	Leptospirae	Tissue culture chick embryo-fibroblasts cytopathic effect
control	Tissue culture + medium Korthoff	—
control	Tissue culture + normal rabbit serum	—
control	Tissue culture + normal human serum	—
	L. icterohemorrhagiae	+++
	L. icterohemorrhagiae neutralised with *L. canicola* antiserum	+++
	L. icterohemorrhagiae neutralised with *L. icterohaem.* antiserum.	—
	L. canicola	+++
	L. canicola neutralised with *L. icterohaemorrhagiae* antiserum	+++
	L. canicola neutralised with *L. canicola* antiserum	±
	L. pomona	+++
	L. pomona neutralised with *L. grippotyphosa* antiserum	+++
	L. pomona neutralised with *L. pomona* antiserum	±
	L. grippotyphosa	+++
	L. grippotyphosa neutralised with *L. pomona* antiserum	+++
	L. grippotyphosa neutralised with *L. grippotyphosa* antiserum	—

Table 3. The influence of Leptospirae before and after neutralization with heterologous and homologous sera on tissue culture. — – lack of cytopathogenic effect, ± – small destroyed tissue foci, ++ – partial cytopathogenic effect, +++ – complete cytopathogenic effect.

	Leptospirae	Tissue culture chick embryo-fibroblasts cytopathic effect
Control	Tissue culture + medium Korthoff	—
Control	Tissue culture + normal rabbit serum	—
Control	Tissue culture + normal human serum	—
	L. autumnalis	++
	L. autumnalis neutralised with *L. hebdomadis antiserum*	++
	L. autumnalis neutralised with. *L. autumnalis* antiserum	±
	L. hebdomadis	++
	L. hebdomadis neutralised with *L. australis* antiserum	++
	L. hebdomadis neutralised with *L. hebdomadis* antiserum	±
	L. australis	++
	L. australis neutralised with *L. bataviae* antiserum	++
	L. australis neutralised with *L. australis* antiserum	±
	L. bataviae	++
	L. bataviae neutralised *L. hebdomadis* antiserum	++
	L. bataviae neutralised *L. bataviae* antiserum	±

Table 4. The influence of Leptospirae before and after neutralization with heterologous and homologous sera on tissue culture — – lack of cytopathogenic effect, ± – small destroyed tissue foci, ++ – partial cytopathogenic effect, +++ – complete cytopathogenic effect.