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UNITED STATES ARMY
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Fort Detrick, Maryland

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Degradation of fat by pathogenic leptospirae.

by F. Kamanes and L. Lovrekovich.

Personal communication, 15 November 1958.

Blood serum is the most important component of media employed in the culture of leptospirae (Fletcher, 1928; Tarassov, 1928; Korthof, 1932; Schaeffner, 1940; Terskikh, 1945; Stuart, 1946); 8-10% rabbit serum has been found optimal for this purpose.

It has not been established, however, whether the decisive effect is furnished by albumin, amino acids, vitamins or lipoid constituents of blood serum in the culture of leptospirae, which do not utilize carbohydrates and for this reason propagate at a relatively low rate.

Helprin and Hiatt (1957) showed that *L. icterchaemorrhagiae* is capable of utilizing various fatty acids. In this case the blood serum (especially serum albumin) present in the nutrient acts both as an energy source and as a detoxifying agent. In addition, as reflected in the studies of Woratz (1955 and 1957), the reproduction of *L. canicola* is promoted not only by fatty acids, but also by alcoholic extracts of rabbit serum, bovine heart muscle and egg yolk. Small amounts of lecithin (Mifuchi and Kawata, 1953; Fulton and Spooner, 1956) and Liebig's meat extract (Gram, 1953) also further the metabolism and development of leptospirae.

It could be expected, therefore, that leptospirae of various serotypes are able to cleave fats present in connective tissue.

Test method

Initially we used the method employing saturated paper strips to demonstrate degradation of beef tallow by leptospirae (Lovrekovich, 1957); later we developed a more sensitive technique.

The procedure in brief: We place glass plates measuring 12-14x70-80 mm and 1-2 mm thick in Wassermann tubes, add enough melted fat (usually beef tallow, lard or technical tristearin) to cover the glass plates almost completely. The Wassermann tubes filled with fat are then sterilized for one hour in the autoclave at 1 atmosphere excess pressure; the fat is cooled to 50°C and the glass plates are transferred to sterile,

pre-warmed Wassermann tubes. In this manner the surplus fat collects on the bottom of the tube, leaving a thin fat layer on the glass plates.

The glass plates, covered by a thin fatty layer, are then placed under sterile conditions in 4-6-day Korthof cultures of different types of leptospirae which show vigorous reproduction visible to the unaided eye. Demonstration of fat decomposition requires well-developed cultures, since energetic cleavage of fats releases toxic substances that soon impede further development of leptospirae.

Degradation of fats was determined during storage of tubes for 7 days at 28-32°C by nearly simultaneous presence of the following manifestations:

1. The thin layer of beef tallow adhering to the glass plates loses its adhesion (in contrast to controls), becomes wrinkled and separates from the plate after several days (cf. the second tube on the enclosed illustration). The free membrane either sinks to the bottom of the tube or rises to the upper layer of the nutrient (cf. the third tube on the enclosed illustration).

2. The partially loosened fatty layer reveals either numerous small or a few large "oil droplets" 1-3 mm in diameter (probably drops of fatty acids with a high content of C atoms). These drops either separate by themselves from the wrinkled membrane or do so upon shaking of the tube; they collect on the nutrient's surface.

3. Meanwhile the medium, which has a soapy odor, acquires water-soluble, heat-resistant decomposition products (fatty acids) which are demonstrated by the circumstance that they dissolve various types of erythrocytes in high dilutions.

4. The pH of the cultures shifts by 0.3-0.6 in the acid direction.

5. Leptospirae degenerate (becoming stiff and motionless) in proportion to the vigor with which they decompose fat, and perish not rarely within a few days. In cultures without signs of fat cleavage, on the other hand, they occasionally propagate more vigorously than in fat-free media.

Titration of erythrocyte-lysing fatty decomposition products present in the medium was accomplished by mixing lots of 0.5 ml of dilutions, made with normal saline in geometric sequence, with 0.5 ml of a 2% suspension of erythrocytes (sheep, cattle, horse, swine, guinea pig, etc.) which had been washed three times. Hemolysis was analyzed after storage of tubes for 2 hours in a water bath of 38°C; complete lysis was indicated by ++, 50% lysis by +, and trace effects by -.

Results

In the course of our study we examined predominantly the fat decomposition of Hungarian, virulent (freshly grown) strains from our collection. Data on growth, cultural peculiarities and beef tallow degradation of these strains have been compiled in Table 1.

According to the results, only virulent leptospirae belonging to serotypes *icterohaemorrhagiae*, *canicola* and *pomona* are able to cleave beef tallow intensely, i.e., only those strains that cause jaundice and connective tissue or pulmonary hemorrhages in domestic and experimental animals (Syrian hamsters and guinea pigs).

Fat degradation by leptospirae of types *grippotyphosa* and *hebdomadis* takes place on a smaller scale; it should be noted, however, that we did not employ fully virulent strains of these types in our tests.

We were unable to demonstrate cleavage of beef tallow by strains *hyos*, *sejroe* and *saxkoebing*, although we found occasionally that they experienced better initial growth in these fatty media than in control cultures. It is interesting to note that these strains failed to produce clinical symptoms in domestic animals.

We also established that strains which had been grown in pure culture some time ago and had become avirulent without change in antigenic structure, had lost some or all of their original ability to cleave fat and to produce hemotoxin (Kemenes, 1958).

Lard was decomposed by our strains somewhat more intensely than beef tallow, although the membrane adhering to the glass plate was separated at the most in the form of tiny scales. "Oil droplets" were given off on a larger scale, however, yielding a titer of hemolytic decomposition products which was usually higher by one dilution.

Examination of rabbit fat revealed no apparent change in the fatty layer; separation of "oil droplets" was usually absent. On the other hand, strong hemolytic titers (1:64-256) indicated the presence of extensive cleavage.

In the case of technical tristearin melting at 67°C (a product consisting of more than 90% tristearate of glycerol ester), decomposition was indicated only by separation of the hard, fatty layer in a form resembling smooth paper, without production of hemolyzing side products. Surprisingly enough, this medium promoted the initial growth of strongly fat-degrading leptospirae in all cases vis-a-vis control nutrients without this lipid.

Fat decomposition apparently involves an enzymatic effect (lipase). Cultures maintained at 4°C do not attack lipid, although they may be stored for days at this temperature without losing their power of cleavage. Cultures withdrawn from low temperatures behave like those exposed to fatty plates on the same day, by decomposing fats most intensely between 28 and 32°C , to a lesser extent at higher or lower temperatures.

When cultures are placed in a water bath of $56-60^{\circ}\text{C}$ for 10 minutes, lipid decomposition is reduced but little, although leptospirae and the hemotoxin produced by them are inactivated thereby. Inactivation of the fat-cleaving ability normally sets in after 10 minutes in a water bath of 80°C .

It is interesting to note that the original ability to degrade fats is demonstrated as before in non-cellular filtrates of *L. canicola* and *L. pomona*, whereas this power is reduced in filtrates of *L. icterohaemorrhagiae*.

The appropriate hemolytic decomposition products are also produced by the effect of lipase. In contrast to hemotoxin, they are resistant to heat and capable of lysing erythrocytes of ruminants (sheep, cattle and goats) and other species, especially those of guinea pigs, in high titers.

Discussion

As reflected by the study, there are considerable differences among virulent leptospirae of various type with respect to fat decomposition. Our results agree with reports of utilization of different fatty acids and lipoids, particularly on the part of *L. icterohaemorrhagiae* (Mifuchi and Kawata, 1953; Fulton and Spooner, 1956; Helprin and Hiatt, 1957) and *L. canicola* (Woratz, 1955 and 1957). In similar experiments with *L. hyos* and *L. grippotyphosa*, Gothe (1957) was unable to get results of equal weight.

As shown unequivocally by our comparative studies of pancreatic and bacterial lipase (Bertok and Kemenes, 1958), fat decomposition by leptospirae involves the action of lipase. Our experiments show that fats attacked by leptospirae, as in the case of pancreatic and bacterial lipases, are left on the glass plates in the form of a mealy, white deposit after drying, while beef tallow separates easily in the form of a creased membrane. Meanwhile the fatty layer secretes "oil droplets" (probably drops of fatty acid with a high content of C atoms) which collect on the surface of the nutrient and exude a soapy odor; at the same time the degraded fats give off thermostable decomposition products (fatty acids) that act toxically on red blood cells and on leptospirae.

The pathological significance of leptospiral lipoid decomposition lies in the fact that those serotypes that attack fats vigorously can cause jaundice and hemorrhages in the connective tissue, the lungs, muscles, etc.

The circumstance that strains subjected to prolonged laboratory culture become avirulent may be due to the loss of their original ability to produce hemotoxin (Kemenes, 1958) and partial or complete cessation of lipoid degradation.

Summary

A new method (utilizing glass plates covered with a thin layer of fat) was employed in the study of lipoid degradation (especially that of beef tallow) by pathogenic leptospirae from our institutional collection.

The tested fats were attacked most vigorously by virulent leptospirae belonging to serotypes *L. icterohaemorrhagiae*, *L. canicola* and *L. pomona*, to a lesser extent by types *grippotyphosa* and *hebdomadis*. No demonstrable lipoid cleavage was evidenced by virulent strains *hyos*, *sejroe* and *saxkoebing*, or by avirulent strains subjected to protracted laboratory culture. Lipase contained in the leptospirae causes the fats to give off "oil droplets" (probably drops of fatty acid with a high content of C atoms), while releasing toxic, hemolytic decomposition products into the soapy-smelling nutrient. The pathological importance of fat cleavage by leptospirae ought to involve the fact that serotypes which attack lipoid most intensely are usually capable of causing jaundice and hemorrhages in various tissues.

Literature

Bertok and Kemenes: *Acta Vet. Hung.*, to be published. Fletcher: *Tr. Roy. Soc. Trop. Med. Hyg.* 21:265 (1928). Fulton and Spooner: *Exp. parasit.* 5:154 (1956). Gothe: *Inaugural Diss.* Berlin, 1957. Gram: *Zeit. f. Hyg.* 137: 553 (1953). Helprin and Hiatt: *J. of Inf. Dis.* 100:138 (1957). Lovrekovich: *Acta Microbiol. Hung.* 4:363 (1957). Kemenes: *Acta Vet. Hung.* 8:143 (1958). Korthof: *Zent. Bakt. Orig.* 125:429 (1932). Mifuchi and Kawata: *Igaku to Seibutsugaku* 28:128 (1953); *ref. Chem. Abstracts* 48:282 (1954). Schueffner: *Zent. Bakt. Orig.* 145:341 (1940). Stuart: *J. of Path. Bact.* 58: 343 (1946). Tarassov: *Kurs Inf. Bolesnyej*, Vol. 1:346 (1928). Terskich: *Leptospirozi lugyej i saivotnich*, Moscow, 1945. Worats: *Zent. Bakt. Orig.* 162:106:1955 and 169 (1957), p. 269.

Table 1.

Origin, cultural properties and data on beef tallow decomposition by leptospirae of different serotypes.

Leptospiral strains						Data on lipid degradation after incubation at 30°C for 1 week		
Serotypes	Nomenclature	Year of culture	Virulence	Hemotoxin production	Separation of fat layer	Excretion of oil drops	Titer of fatty acid hemolysis (sheep erythrocytes)	pH of cult. Viability of leptospirae in cultures
ictero-haemorrhagiae	Rostock	1956	+	-	+	+	1:64	6.5
	Kondratyev	1948	(+)	-	+	(+)	1:64	6.6
	542(Fuchs)	1946	-	-	-	-	<1:4	6.9
canicola	Budapest I	1957	+	(+)	+	+	1:128	6.4
	Budapest 3	1957	+	(+)	+	+	1:128	6.6
	M 53	1953	(+)	+	+	+	1:64	6.5
	Klimcsok	1953	+	-	+	(+)	1:64	6.5
	Standard	1944	-	-	-	-	1:16	6.8
pomona	Bacsbokod	1956	+	+	+	+	1:128	6.5
	Tarjan	1957	+	+	+	+	1:128	6.6
	Perjespuszta	1956	+-	(+)	+	(+)	1:64	6.6
	Nagymagocs	1955	-	+	+	+	1:64	6.8
	Mezzano I	1941	-	+-	+-	+-	1:32	6.7
	Monyakov	1937	-	-	-	-	<1:4	7.0
	DV-B ₁	1938	-	-	-	-	<1:4	7.1
gripotyphosa	148(Fuchs)	1946	-	-	-	-	<1:4	7.0
	Dj 73	1954	(+)	(+)	(+)	+-	1:32	6.8
	OKI	1951	+-	-	+-	-	1:32	6.6
	Sztrelok	1946	-	-	-	-	<1:4	7.2
	Bovis	1948	-	-	-	-	<1:4	7.2
hebdomadis	Gayot	1954	-	-	-	-	<1:4	7.1
	Akiyami B	1947	-	-	-	-	1:32	6.8
sejro, saakoebing	Hebdomadis	1948	-	-	-	-	1:16	6.9
	Martonvasar	1958	+	-	-	-	<1:4	7.0
	Ecség	1957	+	-	-	-	<1:4	7.2
	Nero	1951	-	-	-	-	<1:4	7.0
	Topino	1950	-	-	-	-	<1:4	7.1
hyos (mitis)	Mus 24	1949	-	-	-	-	<1:4	7.1
	Kecskenet 4	1957	+	-	-	-	<1:4	7.1
	Puesposkl.	1956	+	-	-	-	<1:4	7.1
	Ecség	1958	+	-	-	-	<1:4	7.2
	Eszenyos 298	1955	+-	-	-	-	<1:4	7.1
	Mitis(Johnson)	1939	-	-	-	-	<1:4	7.2
Control 1: uninoculated Korthof medium with fatty plate	DV-A	1938	-	-	-	-	<1:4	7.2
Control 2: Korthof medium without fatty plate							<1:4	7.2
								7.2

Legend: $\nearrow$ = vigorous (virulent)
 $(\nearrow)$ = moderate (moderately virulent)
 $\nearrow -$ = weak (nearly avirulent)
 $-$ = negative (avirulent)

Table 2.

Erythrocytic titration of hemolytic decomposition products in a culture of *L. canicola* on the 7th day of beef tallow cleavage.

Type of erythrocyte	Dilution of canicola culture 1:							
	4	8	16	32	64	128	256	512
Sheep, cattle & goat	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow -$	-	-
Horse, swine & dog	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	-	-	-
Guinea pig	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	$\nearrow\nearrow$	-

Illustrations

Fig. 1. Culture of *L. hyos* with unchanged fat plate. The strain does not decompose fat.

Fig. 2. Culture of *L. icterohaemorrhagiae* with fat plate on the 4th day of exposure. The wrinkled fat layer has separated in part from the fat platelet.

Fig. 3. Culture of *L. canicola* with fat plate on the 6th day of exposure. The fatty layer has separated and has risen to the upper stratum of the culture.