duces a sensitive method for detecting small quantities of viral antigens and for studying differences in antigens related to a single virus.

Summary. A rise in antibodies to types 1-4 of adenoviruses during illness can be detected in human serum by employing hemagglutination technics. Although the original method described does not measure an increase in antibodies directed against a single type of virus, type-specific antibodies can be titrated if serum is mixed with a pool of heterologous viruses before tanned rbc treated with homologous virus are added. This modification measured type-specific antibodies solely in 4 of the 8 sera tested. The antigen adsorbed to tannic acid-treated rbc is probably not the infectious viral particle and is separable from it by high speed centrifugation.

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Variation and Hemolysin Production in Relation to Virulence of *Leptospira pomona.** (24090)

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Properties of leptospirae which contribute to their virulence have not been well established. Fukushima and Hosoya(1) and Higuchi(2) demonstrated substances toxic for guinea pigs in cultures of *Leptospira icterohemorrhagiae* maintained anaerobically or *in vacuo*. Stavitsky(3) was unable to verify these findings. Fibrinolysin, leukocidin, and coagulase could not be demonstrated in filtrates of leptospiral cultures(4). Volland and Brede reported(17) that hyaluronidase was produced by leptospirae *in vitro*. A soluble hemolysin has been demonstrated in cultures(5,6) and it has been suggested that the hemolytic activity of leptospirae is an attribute of a toxin(5). Pathogenic leptospirae decrease in virulence upon continual transfer in culture media(8). Presumably reduction in virulence of a given culture is due to *in vitro* development of avirulent variants. The present investigations concern(1) changes in virulence of *Leptospira pomona* for hamsters following passage in media, and (2) *in vitro* hemolysin production as correlated to virulence.

Materials and methods. Virulence determinations. Two strains of *L. pomona* were employed. Strain W had been isolated from bovine urine(9) and maintained by serial passage in weanling guinea pigs. Although this strain was not lethal for hamsters, it was pathogenic for cattle, swine, sheep, goats, and dogs. Strain O had been maintained in continuous hamster and guinea pig passage since its isolation from porcine urine. Strain O killed hamsters and was pathogenic for sheep, swine, and dogs. The first medium passage of Strain W was obtained as blood culture from a sheep which had been inoculated with infected guinea pig blood. Medium passages of Strain O were initiated by culturing blood from an infected guinea pig. Maximum growth of initial hemocultures was obtained after approximately 30 days. Chang's fluid medium(10),

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containing final concentration of 10% sterile rabbit serum and 0.01% hemoglobin (Difco), was used for cultivation. The medium was dispensed in approximately 10 ml amounts in screw cap culture tubes. Inocula consisted of 0.1 to 0.5 ml of culture from previous medium passage. Cultures were incubated at 29°C. Medium passages and virulence titrations in animals were made when cultures were 5 to 10 days old. At this time growth was optimal and the number of leptospirae was ascertained in a Petroff-Hauser bacterial counting chamber using darkfield illumination at 420x. Inocula for animals were prepared using a diluent which consisted of basic ingredients of Chang's fluid medium(10). Tenfold serial dilutions were made and each animal was inoculated intraperitoneally with 1.0 cc of the respective dilution. Both 3 to 5-week hamsters and 4 to 6-week old guinea pigs were from disease free colonies and raised by the authors. Five hamsters were employed/dilution except in the first medium passage titration of Strain W, in which 7 hamsters were used. In guinea pig titrations 5 animals were employed/dilution and were conducted for medium passages 1, 4, and 8 of Strain W and passages 4 and 11 of Strain O. Hamsters were observed for signs of illness. Blood was obtained from moribund hamsters by cardiac puncture using sterile 2 cc syringe wetted with heparin. The plasma was separated and examined by darkfield microscopy for presence of leptospirae. Concentration of organisms in the plasma was determined using counting chamber method. Animals which survived were sacrificed 20 to 30 days after inoculation and their sera examined for leptospiral antibodies using a modified agglutination-lysis test(11). Virulence of each culture was expressed as number of leptospirae which would kill 50% of inoculated hamsters (LD_{50}). LD_{50} values were calculated by the method of Reed and Muench(12). *Hemolysin determinations.* Cultures of *L. pomona* of various degrees of virulence were inoculated into isotonic buffered medium described by Alexander, *et al.*(5) supplemented with 0.01% hemoglobin (Difco). The cultures were incubated at 29°C for 10 to 14 days, when approximately 10^8 leptospirae/cc were present. Growth phase of cultures was that at which optimum hemolysin production has been reported to occur(5). Following incubation, cultures were stored in refrigerator (4°C). Sheep, cow, hamster, rabbit, and guinea pig erythrocytes were obtained and preserved in sterile Alsever's solution(13). Erythrocytes were washed 3 times in isotonic buffer(5) and resuspended to make a 5% cell suspension. A portion of rbc suspension was used to make standard solutions of 10 to 100% hemolysis. Procedure for titration of hemolysin has been reported by Russell(6). Following incubation, the tubes were gently agitated and centrifuged at 1500 rpm for 10 minutes. A 1 ml amount of supernatant fluid from each tube was diluted with 2 ml of isotonic buffered base to make a total volume of 3 ml. Optical density of hemolysis standards and diluted supernatant fluid was determined in Bausch and Lomb Spectronic 20 colorimeter at 520 mμ. Percent hemolysis was ascertained from standard curve of % hemolysis *vs.* optical density. Hemolytic activity of cultures was expressed as that dilution of culture which produced 50% hemolysis of a 5% washed rbc suspension.

Results. Virulence determinations. Virulence of Strain W for hamsters was determined for medium passages 1, 4, 8, 10, 12, 16, 20, and 24. The number of leptospirae required to infect 50% of hamsters inoculated with the first medium passage of nonlethal W strain was calculated to be 3.5. While death of hamsters did not occur at any dilution of the first medium passage, there were deaths in all subsequent titrations of this strain (medium passages 4-24). Thus, an increase rather than a decrease in virulence occurred during passage. Surviving animals did not have demonstrable serum antibodies. An examination of plasma from moribund animals revealed 10^7 to 10^8 leptospirae/cc. Hamsters also died when inoculated with blood from moribund animals or with cultures of this blood. Cell free culture filtrates of Strain W did not produce death of hamsters following intraperitoneal inoculation.

Virulence of Strain W decreased from pas-

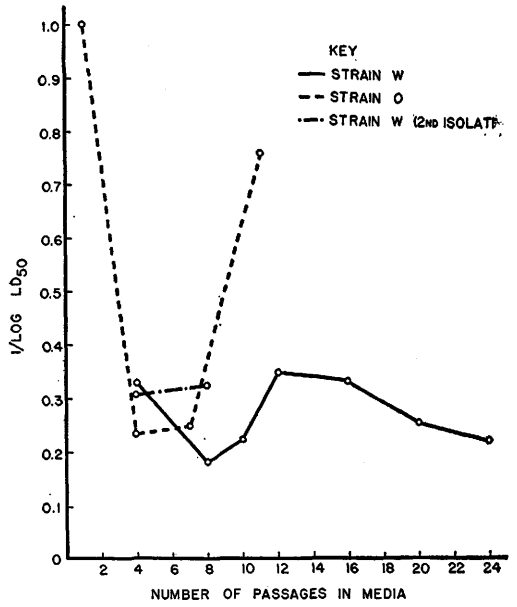

FIG. 1. Effect of passage in media on virulence of *L. pomona* for hamsters.

sage 4 to 8. An increase in virulence was noted for passages 10 and 12 after which virulence decreased again. Lethality for hamsters was again demonstrated for Strain W after 4 medium passages of a culture obtained from another experimentally infected sheep. Virulence determinations with passages 1, 4, 7, and 11 of Strain O indicated an initial diminution of virulence followed by an increase. The LD_{50} of the first passage was approximately 1. The results are summarized in Fig. 1.

Comparison of survival time of infected hamsters and size of inoculum was made. A linear relationship occurred with both strains. The *in vivo* generation time, calculated according to the method of Youmans and Youmans(14), was 7.5 hrs. Cultures of low virulence showed a similar generation time but average survival time was delayed as compared to highly virulent cultures. These findings are in agreement with previous reports employing *L. icterohemorrhagiae* in guinea pigs(15), where the generation time was 8.3 hours.

Hamsters and guinea pigs were equally susceptible to infection with either strain of *L. pomona*.

Hemolysin determinations. A direct relationship between *in vitro* hemolytic activity of *L. pomona* and virulence was not observed. Table I shows that the greatest hemolysin production was associated with a culture of Strain W which had a relatively low degree of virulence ($LD_{50} = 5x10^5$). Cultures which were not lethal for hamsters did not consistently produce lesser amounts of hemolysin. Recent observations (unpublished) have indicated that the hamster lethal variant of Strain W produced a significant *in vivo* hemolytic effect. Marked *in vivo* hemolysis was not observed for Strain O. A consistent difference in hemolysin production *in vitro* for Strains W and O was not evident. Sheep and cow erythrocytes were more susceptible to *L. pomona* hemolysin than were those of rabbits and hamsters. Hemolytic activity was not demonstrated with guinea pig rbc. Relative hemolytic activity of the cultures was not affected by the different rbc types employed.

Discussion. The change from non-lethality to lethality of Strain W for hamsters following cultivation in media is paradoxical but not an isolated incident in evolvement of greater virulence for the genus. Van Riel has been cited(5,7) to have observed an increase in pathogenicity for guinea pigs of *L. icterohemorrhagiae* following 8 months of *in vitro* cultivation. Residence of Strain W in sheep may have enhanced the opportunity for mutation to occur.

The decrease in LD_{50} values (virulence in-

TABLE I. *In Vitro* Hemolytic Activity of *L. pomona* for Sheep Erythrocytes in Relation to Virulence.

Strain	History	LD_{50}	Units of hemolysin
W	Semi-annual transfers in semi-solid medium, 3 yr	Not lethal*	17
"	3 transfers in media following isolation from guinea pig	*Idem*	48
"	4 transfers in media following isolation from sheep	1.5×10^8	80
"	10 transfers *Idem*	1.0×10^8	30
"	25 transfers "	5.0×10^5	230
O	4 transfers in media following isolation from guinea pig	1.5×10^4	57
"	8 transfers *Idem*	3.3×10^8	31

* Culture not lethal for hamsters.

crease) observed with both W and O strains following 4 to 8 passages in media may be explained on the basis of a reverse mutation. The initial decrease in virulence (medium passages 1-4,8) would be due to development of avirulent variants. Subsequently (medium passages 4-11 of Strain O and 8-12 of Strain W) a mutation resulted in production of virulent organisms capable of outgrowing the parent cells *in vitro*. A mutation back to the slower growing virulent cells would account for virulence decrease observed with passages 12 to 24 of Strain W. Data pertaining to variation among leptospirae are meager due to lack of suitable and precise technics. Unfortunately, Cox's solid leptospiral medium(16) did not support growth of either Strain W or O.

Correlation of *in vitro* hemolytic activity and degree of virulence was lacking. The leptospiral hemolysin may contribute some aggressive action *in vivo*, or there may be several hemolytic factors involved with separate systems operating *in vivo* and *in vitro*. It is doubted that the pathogenicity of *L. pomona* is exclusively due to hemolysin, although hemolytic anemia and its cellular ramifications may be the prominent syndrome in severe, acute leptospirosis. The *in vivo* release of hemoglobin is probably beneficial in some degree to leptospirae since hemoglobin or hemoglobin-like compounds are definitely growth stimulative *in vitro*. Probably an endotoxin, entirely independent of the hemolysin, enables the microorganisms to cause damage to tissues and persist in the host. Imamura, *et al.*(18) have recently reported that a toxic product and a hemolysin produced by *L. icterohemorrhagiae* could be distinguished on the basis of thermal stability.

Summary. 1) An investigation was made of changes in virulence of *L. pomona* for hamsters following serial transfers in a fluid medium. *L. pomona* strain W, which did not initially kill hamsters, later produced a lethal effect. Both strain W and O of *L. pomona* decreased in virulence (increase in LD_{50} values) during first 4 medium passages. After 4 to 8 passages in media an increase in virulence was observed. Titrations of subsequent medium passages (12 to 24) of strain W indicated a gradual loss of virulence. 2) *In vitro* hemolysin production could not be correlated with virulence. An explanation for virulence alterations is proposed and the possible significance of hemolytic activity of leptospirae is discussed.

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