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Amyloidosis in Hamsters with Leishmaniasis.*

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During the course of an investigation of chemotherapeutic agents in experimental leishmaniasis, the development of anasarca in infected animals was first noted by R. O. Greep in the early stages of the experimental program in the Squibb Institute in New Brunswick, New Jersey. Later, routine histopathological examination as well as chemical investigation of the blood revealed the widespread presence of amyloid deposits and a reversal of the albumin-globulin ratio of the plasma proteins. In human kala azar, the presence of generalized edema is not infrequent and the alteration of the serum albumin-globulin ratio is characteristic; however, neither in clinical nor in experimental leishmaniasis have we been able to find a report of amyloidosis.[1] Nevertheless, it seems likely that the hamsters described in the note just published by Goodwin[2] suffered from amyloidosis. In his animals, as in ours, infection by *Leishmania* might be followed by edema, nephritis, proteinuria, and depression of the level of plasma proteins. The present investigation was undertaken to correlate the development of amyloidosis and the changes in the distribution of proteins in the plasma during the progress of leishmaniasis in hamsters. From the experimental results it was hoped that answers to two questions would emerge: (1) What is the temporal relationship between the deposition of amyloid and the alteration of the plasma protein concentration, and (2) Alterations in which organ, the liver or the kidney, are chiefly responsible for the reduction of the plasma albumin?

Materials and Methods. A single large group of hamsters (*Cricetulus auratus*), six weeks old, served as the experimental animals. They were maintained on pellets of Rockland rat diet ("D" free) supplemented with lettuce, carrots, and whole wheat bread. The animals were divided into two groups at random and at the beginning of the experiment the animals of one group were inoculated intraperitoneally with 20 mg of spleen in a saline emulsion which was obtained from 2 hamsters heavily infected with *L. donovani* (Khartoum strain.) The other group served as the uninfected control. At approximately two-week intervals, 5 representatives each from the control and the infected groups were placed in individual metabolism cages and the urine was collected for 24 hours.

Immediately after the termination of the urine collection, the animals were anesthetized with intraperitoneal evipal sodium (140 mg/kg body weight) and heparinized blood samples were obtained by heart puncture. The animals were then sacrificed; the tissues removed for histological examination were fixed either in Zenker's solution without acetic acid or in Vandegrift's dehydrating fixative.

Owing to the small size of the plasma sample for the determination of non-protein nitrogen, it was necessary to determine this nitrogen fraction by a Nessler procedure which was adopted for all nitrogen determinations. The solutions of protein or non-protein nitrogen were first digested in Pyrex Nessler tubes in the presence of 1 ml of 19 N sulfuric acid; oxidation was completed by a drop or two of 30% hydrogen peroxide. The digested material was diluted with water to 35 ml and 15 ml of Nessler's reagent made according to the directions of Folin[3] were added. The final

* This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

[1] Stitt's *Diagnosis, Prevention, and Treatment of Tropical Diseases* edited by R. P. Strong, Philadelphia, 1943.

[2] Goodwin, L. G., *Nature*, 1945, **156**, 476.

[3] Folin, O., *Laboratory Manual of Biological Chemistry*, New York, 1934.

TABLE I.
Plasma Proteins, Amyloidosis and Edema in Hamsters with Experimental Leishmaniasis.

Days after Infection		Albumin (g%)	S.D.	Globulin (g%)	S.D.	A/G Ratio	S.D.	Edema	Amount of amyloid in: Kidneys (glomeruli)	Adrenal cortex	Spleen	Liver	No. of animals
18	Normal	2.76	0.16	2.06	0.17	1.35	0.18	0	0	0	0	0	3
	Infected	2.12	0.62	3.06	0.61	0.75	0.38	0	0	0	0	0	5
32	Normal	3.09	0.35	2.19	0.43	1.45	0.31	0	M.*1/5	M.1/5	M.1/5	M.2/5	5
	Infected	3.07	0.45	2.76	0.23	1.11	0.20	0	M.1/5	M.2/5	M.1/5	M.1/5	5
46	Normal	2.34	0.33	2.56	0.21	0.92	0.16	0	0	0	0	0	5
	Infected	1.09	0.66	2.72	0.40	0.40	0.22	+2/5	+++5/5	++5/5	+4/5	+4/5	5
60	Normal	3.17	0.17	1.98	0.19	1.60	0.17	0	M.1/5	0	M.1/5	M.1/5	5
	Infected	0.48	0.21	2.22	0.58	0.22	0.07	+++3/3	+++3/3	+++3/3	+3/3	+3/3	3
77	Normal	2.46	0.26	2.26	0.24	1.11	0.06	0	0	0	0	0	3
	Infected	0.61	0.17	2.34	0.29	0.26	0.05	+++4/4	+++4/4	+++4/4	+4/4	+4/4	4

* M = Minimal.

estimates were made in a Beckman photoelectric spectrophotometer at a wave length of 400 mμ. The useful range of amounts of nitrogen per sample was about 0.02 to 0.15 mg. The blank solution was similarly made from reagents alone; with known amounts of nitrogen added as a solution of pure $(NH_4)_2SO_4$, there was a straight-line relationship between optical density and nitrogen-concentration. The method of Howe[4] was employed to separate plasma globulin and thus to determine the concentration of albumin and globulin in plasma. The non-protein nitrogen of urine and plasma was estimated in filtrates containing, after suitable dilution with water, 5% trichloroacetic acid. The histological examinations were made independently by one of us (WJP) and it was not until the experiment was completed that the results of the pathological examination and the chemical changes in the blood were compared. Paraffin sections of the tissues were made and stained with hematoxylin and eosin, Giemsa's stain, or Congo Red which was sometimes combined with Wilder's reticulum stain.

Results. The principal gross pathological findings in infected hamsters were splenomegaly, enlarged lymph nodes, pale kidneys and anasarca. The gross renal change and the anasarca were not evident until 46-60 days after inoculation. There were no significant gross changes in the tissues of the normal controls. The microscopic alterations owing to leishmaniasis conformed to the description of other authors with the exception of the presence of amyloidosis which was found in all hamsters which had been inoculated with *L. donovani* at least 46 days previously. Small amyloid deposits were found in 3 of the 21 control animals; the quantities present were minimal.

The incidence of the amyloidosis is summarized with the other data in Table I. Amyloid was deposited chiefly in the renal glomeruli, the adrenal cortex, the spleen and the liver. Its appearance and distribution were characteristic of "secondary" amyloidosis of man and animals. No intracellular amyloid was identified. The amyloid of the

[4] Howe, P. E., *J. Biol. Chem.*, 1921, **49**, 93.

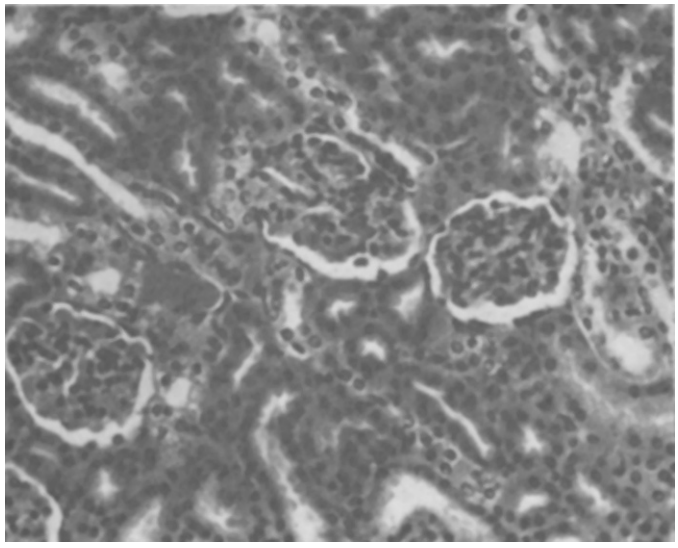

FIG. 1.
Kidney of normal hamster. Hematoxylin and eosin. × 275.

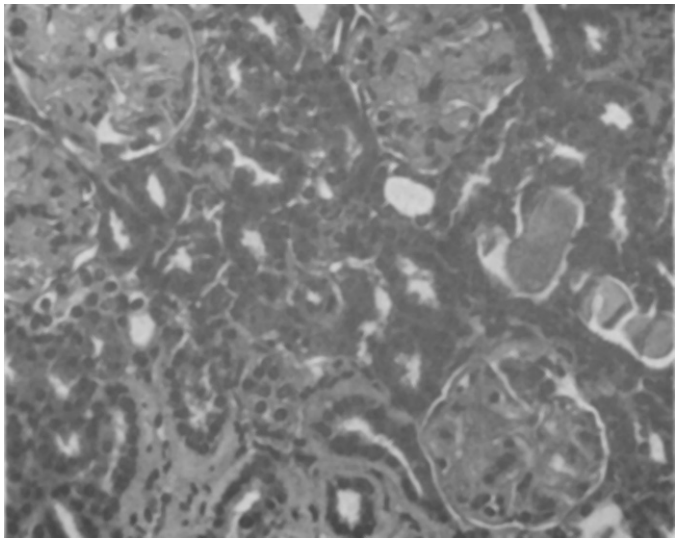

FIG. 2.
Kidney of hamster with amyloidosis 77 days after inoculation of *L. donovani*. Note extensive replacement of glomeruli by amyloid deposited between endothelium and epithelium. The tubular casts are also typical. Amyloid deposited about the tubules and extending into the interstitial tissue is an infrequent finding illustrated in this section. Hematoxylin and eosin. × 275.

kidneys was deposited in the glomeruli and appeared to replace three-fourths or more of this structure in the late stages of the disease. (Leishmania were rarely found in the kidney.) The glomerular amyloid accumulated between the capillary endothelium and the capillary basement membrane; it was never found external to the latter. Trivial deposits of amyloid were observed between the epithelium and the basement membrane of the tubular epithe-

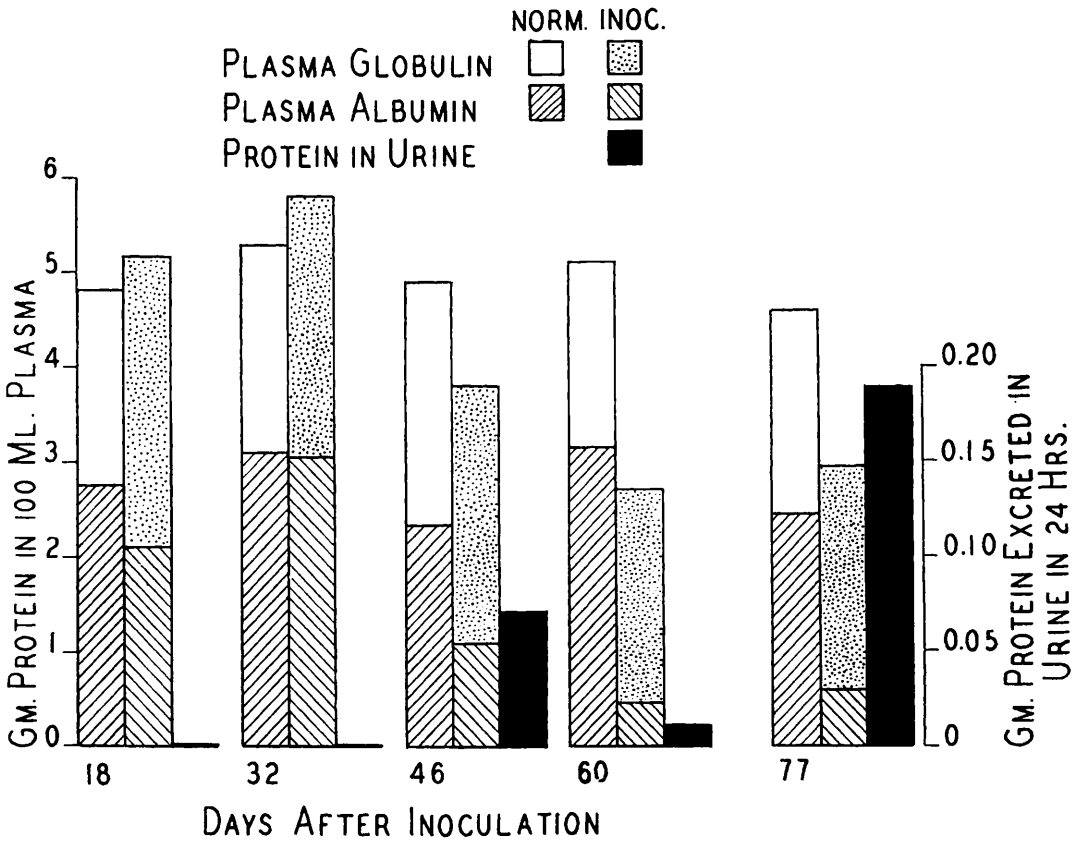

FIG. 3.

The concentration of plasma proteins in normal and infected hamsters. Also shown is the total urinary protein excretion in infected hamsters.

lium. Hyaline droplets or casts could be found in the tubules of the kidneys with severe amyloidosis. (Fig. 1 and 2.) In the cortex of the adrenals there was likewise bilateral deposition of amyloid which in some cases replaced more than half of the normal tissue. The early deposits in the adrenal cortex were clearly between the capillary endothelium and the basement membrane. The amyloid of the spleen was deposited about the widely separated malpighian corpuscles and, to a less extent, in the ring of pulp surrounding the spherical masses of phagocytic cells containing parasites. In the liver, amyloid was first found beneath the endothelium of the hepatic and portal veins from which it occasionally spread into the parenchyma. Deposits sometimes occurred around parasite-filled phagocytic cells. The amounts of amyloid in spleen and liver were always small.

Table 1 also presents the quantitative data on the plasma albumin and globulin and the A/G ratio at intervals after the inoculation of the animals. The standard deviations are also tabulated to indicate the range of the observations and their distribution about the mean. It is to be noted that 46 days after the infection there is a significant decrease in the plasma albumin concentration of the infected animals as compared with the controls. The globulin concentration is not demonstrably different in the two groups and as a result the A/G ratio is significantly lower in the infected animals. It is also to be noted that at this time the deposition of significant amounts of amyloid makes its first appearance and that it is found to be most abundant in the glomeruli of the kidney. Between the 46th day and the 60th day the decrease in plasma albumin concentration in the infected animals

continues precipitously. In the next interval of 17 days there is no further change in the plasma albumin concentration of the infected animals. During the entire time when the striking fall in plasma albumin concentration is occurring, there is no significant deviation of the plasma globulin level in the infected animals as compared with the normal group. Amyloidosis is maximal at the 60th day and the distribution of amyloid is at all times more widespread in the kidney (glomeruli) than in the liver. The presence of edema is correlated with the hypoalbuminemia as one would expect.

The plasma non-protein nitrogen concentration in the infected animals was not significantly different from the control in all the experiments except at 60 days. In this experiment the non-protein nitrogen concentration of the infected group was clearly above the level in the control animals.

Fig. 3 presents the results of the plasma protein studies graphically, and, in addition, summarizes the quantitative estimation of proteinuria. It can be seen that at no time did the control groups show any protein in the urine. In the infected animals, proteinuria made its first appearance after 46 days, when the first observation of hypoalbuminemia was made. Proteinuria was always observed subsequently. The available evidence suggests that the urinary protein was albumin; none could be precipitated in the presence of 1.5 M sodium sulfate.

Discussion. In another series of hamsters attempts to inoculate one strain of *L. infantum* by intraperitoneally-injected splenic emulsion did not produce infection or recognizable edema within the following 6-8 weeks. For this reason, normal spleen was not administered intraperitoneally to the control animals.

In all the experiments, the percentage of globulin in the plasma of the infected animals was higher than that of the normals; however, no statistical significance can be attached to these differences. (Because of the high percentage of globulin in the plasma of the infected hamsters of the first experiment, the A/G ratio was reduced.) There can be no doubt that in hamsters infected with *Leishmania donovani* 46 days or longer, there was a significant fall in the plasma albumin and in the A/G ratio in comparison with corresponding control animals. The highest probability that such a change could occur by chance was less than 0.01 (comparison of albumin-concentrations of experiment 3.) In all other comparisons of plasma albumin percentage in plasma or of the A/G ratio, the probability of chance occurrence was even lower—especially in the last 2 experiments. Albuminuria in the infected hamsters was first detected 46 days after infection when a significant lowering of plasma albumin was also first observed. The small amount of albumin excreted in the urine 60 days after infection is to be explained by the small quantity of urine excreted owing to excessively hot weather. All the protein in the urine may have been albumin since no globulin could be precipitated by warm sodium sulfate solution according to Howe's plasma method; however, no dogmatic statement in this regard can be made since the experimental conditions are seriously altered when urine instead of plasma is the protein solvent and the concentration of protein present is different from that of plasma.

The animals infected 46 days or longer, in contrast with the controls, had hypoalbuminemia, proteinuria, edema (characterized by anasarca in the last 2 experiments) and deposits of amyloid in the kidneys, adrenal cortex, spleen and liver. These changes occurred 46 days after infection. It is concluded that the primary change, amyloid deposition in the renal glomeruli followed later by a similar but slight change in the tubules, led to excessive loss of albumin in the urine with an associated reduction of plasma albumin concentration and lowering of the colloid osmotic pressure of plasma so that excessive amounts of fluid escaped into the tissues. It is not believed that failure of the synthesis of plasma albumin was responsible to any important extent for the lowering of the percentage of this protein in the plasma.

Summary. Forty-six days after successful inoculation of *L. donovani* into hamsters, there appeared edema associated with amyloidosis. There was marked anasarca at 60

and 77 days after infection with extensive deposition of amyloid in the glomeruli and the adrenal cortex. The hypoalbuminemia, which coincided with the edema and a proteinuria, is believed to have been caused by the impairment of glomerular function.

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Occurrence of Two Immunological Groups Within the Genus Listeria. Studies Based upon Precipitation Reactions.

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Thus far, attempts to establish the serological reactions and antigenic structure of microorganisms of the genus Listeria have been based upon agglutination and agglutinin absorption studies. Paterson[1] concluded that the antigenic structure permitted division of the genus into 4 types. He stated, "the bacteriological types do not bear any relation to the zoological species of the host or to the geographical distribution of the places of isolation." Schultz, Terry, Brice and Gebhardt[2] on the other hand found that among 11 different strains of *Listeria monocytogenes* only 2 serological groups occurred. The strains were derived from various animal sources, including man. On the basis of the agglutination reaction Julianelle and Pons[3] divided 8 strains into 2 types which they designated Type I and Type II. Type I represented strains isolated from rodent animals and Type II from ruminants. Strains isolated from the disease in man fell into one or the other of the types. Julianelle[4] later observed that filtrates from broth cultures gave a precipitate only with their type specific immune sera. The precipitate was suggestive of that seen in the precipitation of specific polysaccharides in the presence of immune serum. The purpose of this paper is to describe the precipitation reactions of 16 strains of *Listeria monocytogenes*.

Material and Methods. The precipitinogen was a polysaccharide extracted from the bacterial cell according to the method of Fuller.[5] Chemically, the carbohydrate fractions were characterized by strongly positive Molisch reactions, and Fehling's solution was not reduced. The biuret and ninhydrin reactions were negative. For the preparation of polysaccharide and the immunization of rabbits bacteria in the smooth phase were selected. All cultures were grown in tryptic digest broth containing 0.1% dextrose and incubated 18 hours at room temperature (25-30° C).

Vaccines were prepared from broth cultures, which were centrifuged and the bacterial sediment resuspended in normal salt solution containing 0.2% formalin. The vaccine was injected intravenously into adult rabbits until the precipitin titer of the serum was sufficient to give a marked, compact-disc precipitate in the presence of the specific carbohydrate within a period of 15 minutes at room temperature. The animals were then bled to death from the heart. Two rabbits were immunized against each strain and the sera from the final bleedings pooled. Control sera were taken from all rabbits before the administration of vaccine and tested for the presence of precipitins to the carbohydrate of that strain of organism to be used for the production of immune serum. In no instance were normal precipitins demonstrated.

Precipitation as determined by the ring test using equal amounts of carbohydrate and immune serum was indicative; however, it

[1] Paterson, J. S., *J. Path. and Bact.*, 1940, **51**, 424.

[2] Schultz, E. W., Terry, M. C., Brice, A. T., Jr., and Gebhardt, L. P., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 605.

[3] Julianelle, L. A., and Pons, C. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 364.

[4] Julianelle, L. A., *J. Bact.*, 1941, **42**, 367.

[5] Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 130.