ATTEMPT FOR HEPATIC LESION REPRODUCTION IN DOGS WITH BLOOD FROM HEPATITIS PATIENTS AND GLUCOCORTICOIDS

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The morbidity rate of virus hepatitis shows a tendency for raising in most parts of the world (23), Bulgaria comprised (8). The struggle against this illness is rendered difficult, in addition, by the circumstance that a number of problems, related to its etiology, pathogenesis and patho-anatomy are still awaiting definitive solution (13, 19, 21). The attempts for isolating the etiological agent, though quite numerous and persevering, have failed to provide for equivalent and universally accepted results (1, 23, 24, 28).

The medical science still lacks a secure experimental model of this infectious disease despite the big number of studies on the reproduction of human hepatitis, carried out over a great variety of experimental animals (9, 10, 7, 18, 27). More successful might possibly prove the experiments with animals as dogs and mice, which, by rule are affected by similar hepatic infections, although caused by different vira — the viral agents of the canine and mouse hepatitis (2, 10, 11, 30).

Background of the experimental work

In most cases, the failure to find an experimental model of human virus hepatitis is, very likely, due to the natural protection or resistance of the animals tested to the pathogenic effect of the infective agent. Theoretically, the failure of the experiment might be also explained by the properties of the infectious factor, which might prove insufficient (sub-threshold) for developing a perceptible pathogenic effect: small dose, low virulence or short-duration effect.

In view of the considerations pointed out, we chose the dog as the object of our experimental setting, endeavouring to place the two basic experimental factors (infectious factor and animal macroorganism) in such interrelationships that might provide for the optimal conditions for the development of the infectious process alluded to. First and foremost, we aimed to increase the susceptibility of the experimental animal through preliminary application of glucocorticoid preparation, to the tested infection by way of inhibiting the immunogenesis and reticulo-endothelial macrophages and thereby, to favourably influence the generalization of the infective agent (5, 6, 22). In compliance with the study of Vella and assoc. (30), a group of mice, cortisone treated and infected with the mouse hepatitis virus, proved to be much more susceptible to the infection as compared to another group, treated with triolein or bacterial toxin which, as well known, bring about activation of the reticulo-endothelial system.* However, bearing

* Tessman (29) reports untoward effect of cortisone in tetrachlor-carbon induced, experimental, toxic hepatic lesions.

in mind the favourable therapeutical effect of glucocorticoids in already developed inflammatory process of the liver, stressed by the majority of authors, we applied the preparation in question merely in the period of initial viral aggression, i. e. during the presumed incubation period.

Young animals (dogs) were used on account of their stronger susceptibility to virus infections (7, 9). Deliberately, no racial selection was made in completing the experimental contingent, as a single, pure breed might easily prove refractive of the infection tested. Male dogs were selected in view of avoiding the influence exerted on the biochemical indices by eventual pregnancy. In the group of experimental animals a single female dog was included: its eventual conception might easily serve as a factor, provoking the experimental infection, whilst its offspring — more suitable object of the experimental model.

In order to secure higher concentration and virulence of the infectious material, the whole blood used for the purpose was obtained from patients at the end of the first week of illness. The experimental animals were injected with full citrate blood, never serum or plasma, as it was not precluded that the virus employed be adsorbed on the erythrocytes, thrombocytes and blood fibrin; the latter phenomenon has been demonstrated on grippe virus (25, 26). The pathogenic blow by the infective agent was intensified and prolonged by means of its daily application over a 10-day period.

In the course of the experiment, we started injecting also hepatic homogenate from dog treated with the basic infectious material. Thus, we aimed the testing of eventually intensified activity of the hepatitis virus with this passage.

Experimental Material

The experiment was carried out on a series of 25 dogs, with 15 of the total number serving for the experiment proper, and the remainder (10) — for determination of the normal biochemical indices. In the beginning of the experiment, all the dogs ranged in age from 3 to 6 months except for three newborns. The entire experimental group was isolated and subjected to preliminary observation for a duration of 3 months with a view to await the development of eventual canine hepatitis, acquired by natural routes. During the same period, the dogs underwent deparasitization from worms (helminths), fleas and ticks. Over the full observation period, they were fed on free, general diet. Not a single animals showed (in the beginning or in the course of investigations) macroscopic data for dystrophic changes, which would be related to the nutritive regimen.

Whole citrate blood was used as infectious material, obtained from 13 patients with typical for virus hepatitis clinical and laboratory data, hospitalized and treated at the Clinic of infectious diseases of the Higher Medical Institute in Varna. Hepatic homogenate was also employed as potentially (latent) infectious material, obtained from one of the dogs treated with the blood referred to earlier and sacrificed on the 7th month after the beginning of the experiment.

Experimental Background and Observations

Nine dogs (№№ 1 through 9) were injected intramuscularly with 2—4 ml blood according to body weight for 10 consecutive days. Intramuscular 6-Methyl-prednisolone (Urbason-Hoechst) was also given to 6 of them in a dose 0.7 mg/g body weight, daily. The first injection with this glucocorticoid preparation was made the day the blood inoculations were begun and its daily application continued until the 15th day since initiating the experiment. In the following 10-day period it was applied every other day, and thereafter, till the end of the third month — twice weekly. Three of the animals (№№ 2, 7 and 8) did not receive urbason — controls.

The clinical observation was initiated with the very first infectious-material inoculation, recording chiefly the general condition and behaviour of the animals, the colour of the sclera and visible mucosa. The dynamic follow-up of the laboratory data commenced on the 25th day of the experiment. Approximately every 20 days blood was obtained from the animals for the purpose of performing the following biochemical and serological tests: total serum bilirubin, transaminase activity (SGPT), MacLagan, Weltmann, Burstein-Samay, total protein, proteinogram, antistreptolysin titer and complement fixation reaction. Serum of patients affected with virus hepatitis, in the initial period of the disease, and allantoic fluid from embryo, inoculated with blood from hepatitis patients, were used as antigen in CFR*. After the third month and at the same time intervals, aspiration hepatic puncture was made with a Menghini type needle aiming the removal of material for histologic examination. The topographic orientation during punctures was effected in compliance with the instructions proposed by Telcharov and Kyutukciev (12, 20).

In most of the animals the observations just referred to lasted 9 months, and thereafter they were killed with electric current.

Dog № 3 was sacrificed on the 7th months and the homogenate prepared from its liver was injected to three additional animals (№№ 10, 11 and 12), aged from 3 to 6 months. The homogenate was applied intramuscularly in three different sites, at a dose 3—4 ml each.

Dogs №№ 1, 2, 4, 5, 6, 7 and 8 were sacrificed early in the tenth month. After subjecting their livers to macroscopic inspection, material was obtained for histological examination from several different sites of the liver, lungs, myocardium, kidneys, adrenals and spleen.

Experimental animal № 9 gave birth to 3 puppies on the 9th month of the experiment (№№ 13, 14 and 15).**

The three puppies were subjected to twofold injection in the first days of life, introducing 4 ml whole citrate blood from hepatitis patients to each of them and 4 ml hepatic homogenate from dog № 3. The newborns and animals №№ 10, 11 and 12 as well were observed clinically for a duration of 8 months after the inoculation, when they were sacrificed and subjected to histologic investigation. The latter was unfortunately, discredited owing

* CFR — complement fixation reaction.

** Considering the duration of gravidance in bitches (from 59 to 70 days), she was pregnant during the 7th and 8th month of the experiment.

to technical mistakes. During the same period of time, the clinical observation on the mother of the three newborns proceeded. She was killed on the 15th month and histological preparations were obtained from the liver and other internal organs.

Results

Clinical: During the full period of observation, all experimental animals (№№ 1 through 15) retained their buoyancy and apetite and did not display perceptible deviations from the normal inasmuch their general condition and attitude were concerned. No yellow tinge of the sclera or visible mucosa was noted.

Biochemical: In determination of the already listed biochemical indices in 10 healthy and intact dogs (№ 16 to 25), the following normal values were obtained, fluctuating within the following limits:

Table 1

Total bil. in mg %	Welt. test-tubes	MacLagan in U	B.-Sam. in U	SGPT in U	Total protein in gr %	Proteinogram				
						alb. %	globulins — %			
							α_1	α_2	β	γ
Up to 1.0	6—7 1/2	up to 40	up to 25	up to 46	4.9—8.2	42—63	3—11	3—11	7—22	10—30

In tracing up the analogical laboratory findings in the biochemically investigated 9 experimental dogs (1—9), data worth of special note were obtained merely with respect to the Weltmann test.

As obvious from table 2, on the fourth month of the experiment a tendency appeared for lengthening of the coagulation band, so that the mean arithmetical value of the test in all these animals reached up to 7.77 test tubes (initial value — 7.27). The greatest prolongation was recorded during the fifth months — mean 8.16 test tubes, and thereupon a reverse tendency was manifested — towards normalization. At the end of the observation, the mean value of the Weltmann test was 7.77 test tubes. No differences were established as regards the same test, between the animals treated with blood from hepatitis patients + urbason and those treated merely with blood (controls).

The rest of the biochemical tests in all the investigations of this series, comprising 9 dogs, did not display a perceptible correlation with the Weltmann's reaction shiftings, displaying variable results within the limits of normal.

In the course of 6 months, six-fold determinations were also performed of the antistreptolysin titer in the same experimental animals. During the 3rd and 4th month, mean values were established in all animals, respectively 283 ± 184 U ($m_1 = 82$) and 249 ± 168 U ($m_2 = 84$).* The tendency for the

* According to data reported by Köhler (cited by 16), the normal antistreptolysin titer in dogs amounts to 130 U. Hence, comparatively high values were recorded as early as the first investigation. However, it is not certain whether this is not provoked by the experiment proper, as the first examination was carried out on the third month since its initiation.

Table 2

Investigations / Weltmann in test tubes	Initially	4th month	5th month	Final
6	1	0	0	0
7	4	1	0	0
$7^1/_2$	1	2	1	4
8	3	6	6	5
9	0	0	2	0
Statistical indices	$M_1=7.27$ $\sigma=\pm0.91$ $m_1=0.32$	$M_2=7.77$ $\sigma=\pm0.13$ $m_2=0.01$ $p<0.01$	$M_3=8.16$ $\sigma=0.80$ $m_3=0.20$ $p<0.001$	$M_4=7.77$ $\sigma=\pm0.26$ $m_4=0.07$ $p<0.001$

liter increase reached its maximum during the 7th month, when at intervals of 12 days, the mean value obtained was 666 ± 167 U ($m_3=55$; $p < 0.001$) and 783 ± 132 U ($m_4 = 44$; $p < 0.002$). Since the 8th month thereafter, the titer levels disclosed a rapid fall, with the mean value obtained being 254 ± 232 U ($m_5 = 100$). After nine months, in all the animals (except for that with titer 166) a titer lower than 125 U was recorded. The fluctuations in the antistreptolysin titer in the experimental and control animals were analogical.

Pathoanatomical: Morhological data are available for the same 9 dogs, which prior to dissection and in the course of the observation, were subjected to 8 aspirating puncture biopsies of the liver. The material obtained, after fixation in 10% neutral formalin, underwent staining with hemalum-eosin and after Gomori for the reticular fibers. The dissection material of the liver and other internal organs was subjected to staining with hemalum-eosin, Sudan III and Sudan-black, Best — for glycogen, Brasche for DRNA and Gomori — for reticular fibers.

Cases without glucocorticoid:

Three dogs were studied. The preliminary control puctures and the first puncture during the 6th month since the beginning of the experiment did not reveal deviations in the liver structure (Fig. 1). The second puncture, performed at the same time, exhibited, in some of the preparations, slight clearing of the hepatic cells. This clearing was better pronounced in the punctures made early at the 7th month, when at places nuclear extrusion was also marked (Fig. 2). Heaviest changes were established with the secondary punctures — made during the same month — severe vacuolation with drop-like dystrophy and separate lymphocytic infiltrates. The following month, the infiltrates were intensified and in some cases found in small groups in the puncture material (Fig. 3). Thereupon, the preparations did not exhibit essential alterations of the hepatic structure and merely after the sacrification of the dogs, mesenchymal proliferation was present; the

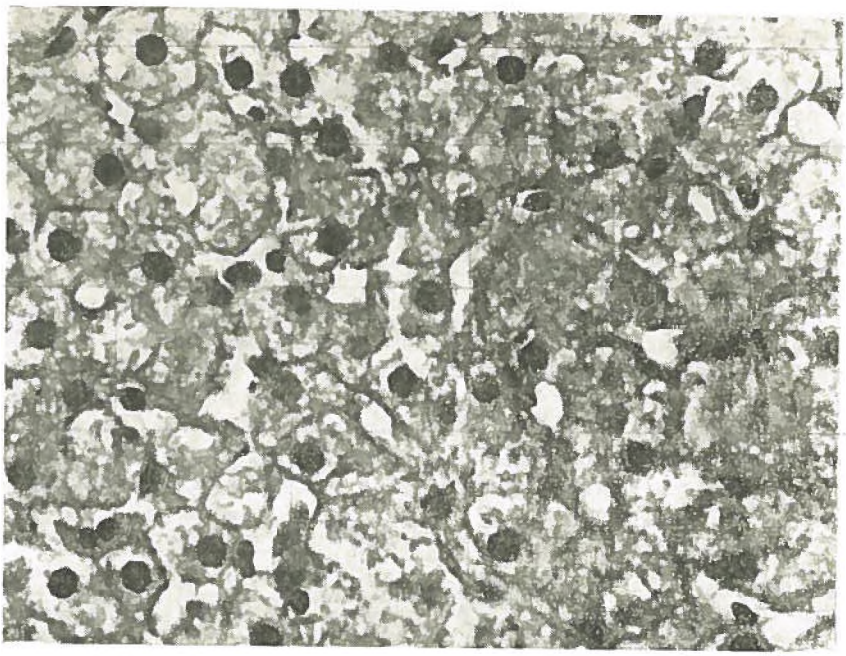

Fig. 1. Dog № 2, biopsy carried out on 3 Febr., 1966; preserved histological structure of the hepatic tissue (hemalum-eosin).

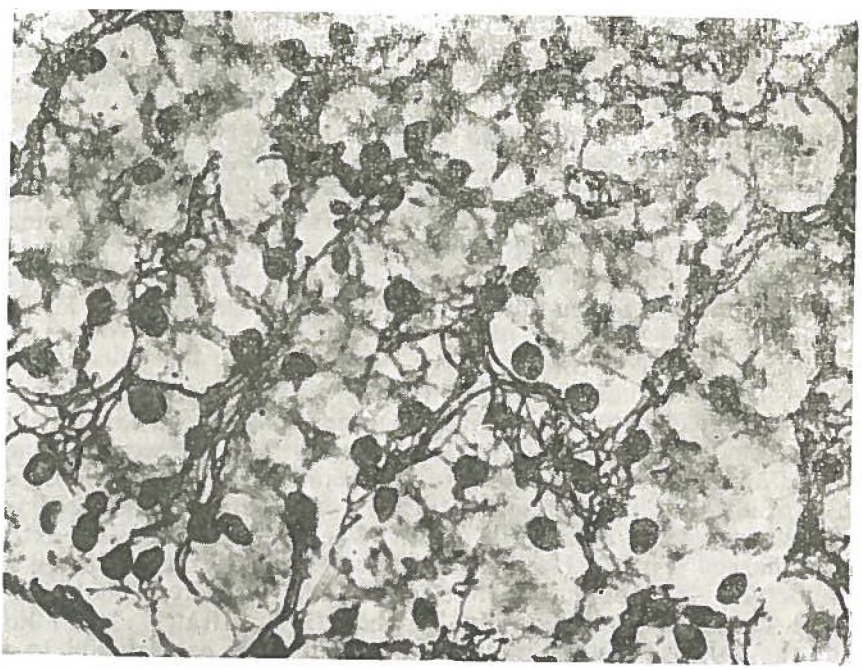

Fig. 2. Dog № 2, biopsy 1/3/1966; delicate reticular fibers, at some places enlarged and interlaced (Gomori).

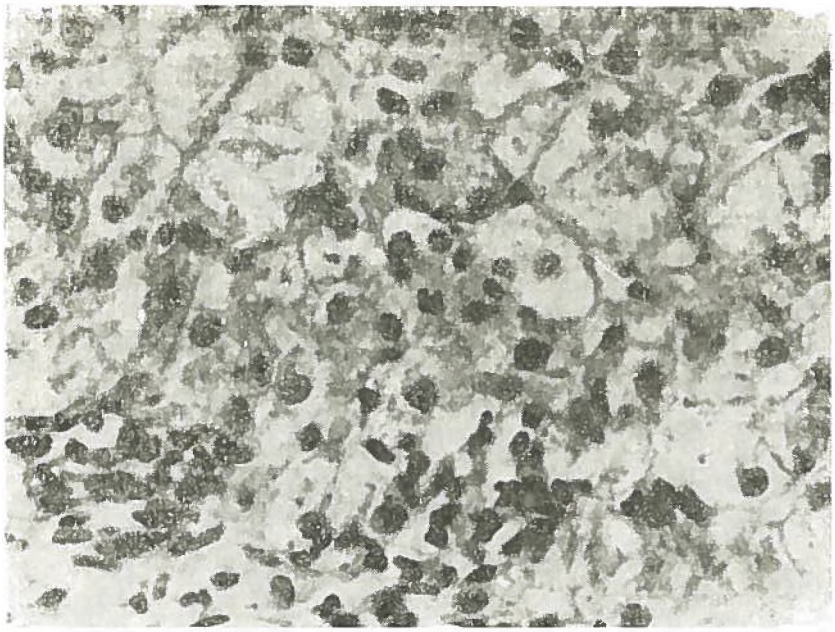

Fig. 3. Dog. № 7 — biopsy 27/5/1966; swelled and inflated cells as well as single and in small groups mesenchymal cells are evident.

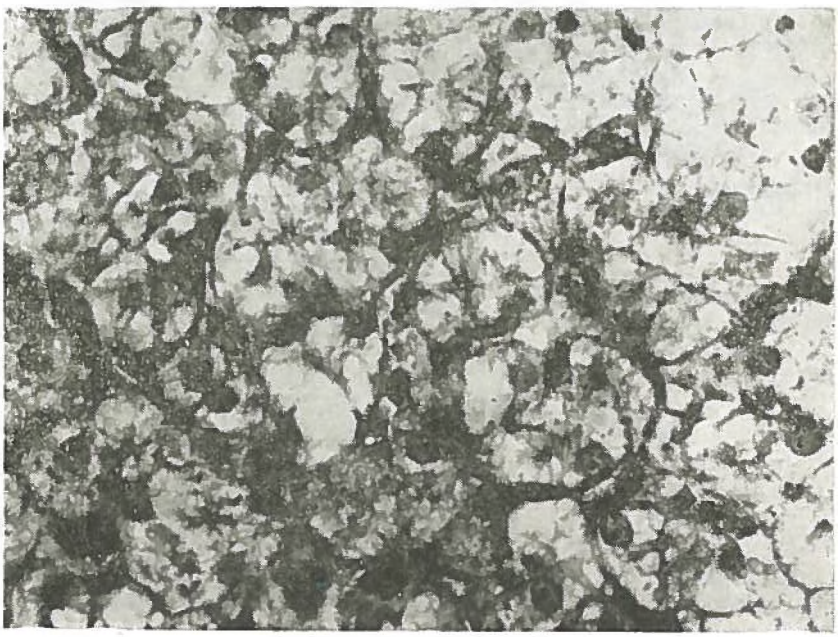

Fig. 4. Dog № 1 — puncture biopsy 3/2/1966; inflation of single hepatic cells, lateral displacement of nuclei (hemalum-eosin).

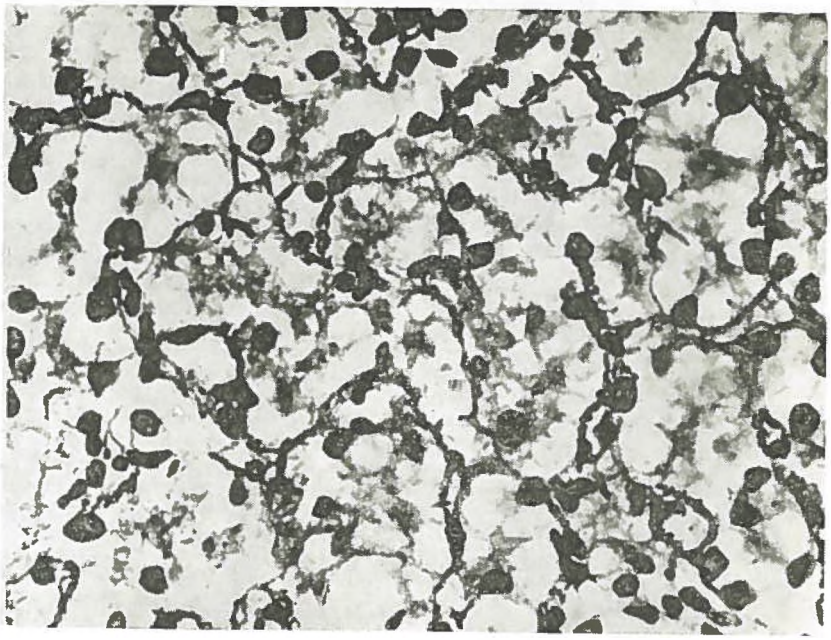

Fig. 5. Dog № 1, biopsy 1/3 1966; well pronounced reticulum, at places individual fibers display slight thickening (stained after Gomori).

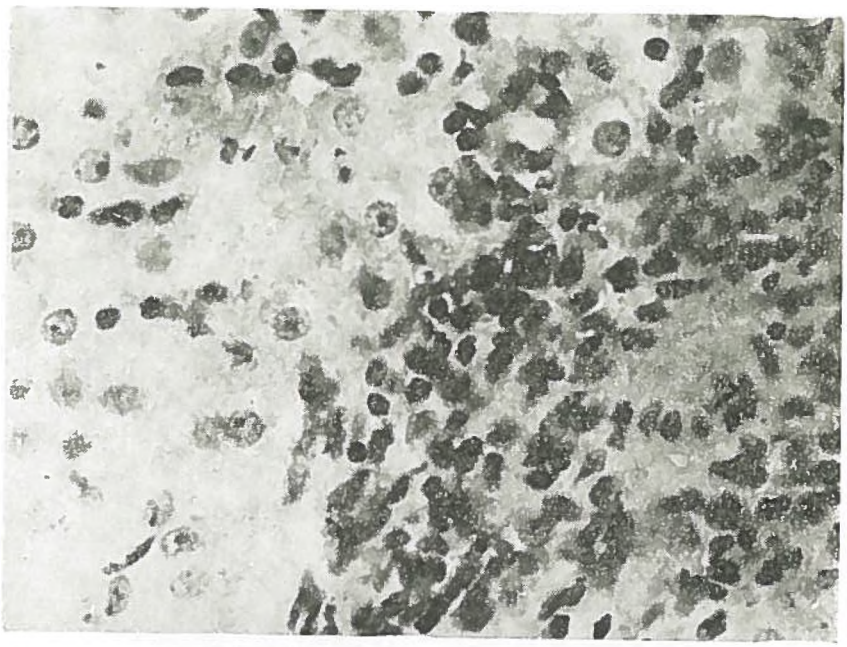

Fig. 6. Dog № 6, sacrificed on 6 June, 1966. Conserved histological structure of the hepatic lobes; scattered focal lymphocytic and histiocytic amassements.

staining for reticular fibers revealed reticular induration. Fibrous alteration was absent.

Cases with methyl-prednisolone:

Six dogs were studied. Early in the sixth month, single cleared hepatic cells were established (Fig. 4). In the punctures during the second half of the 6th month, a greater number of cleared cells and presence of single, round

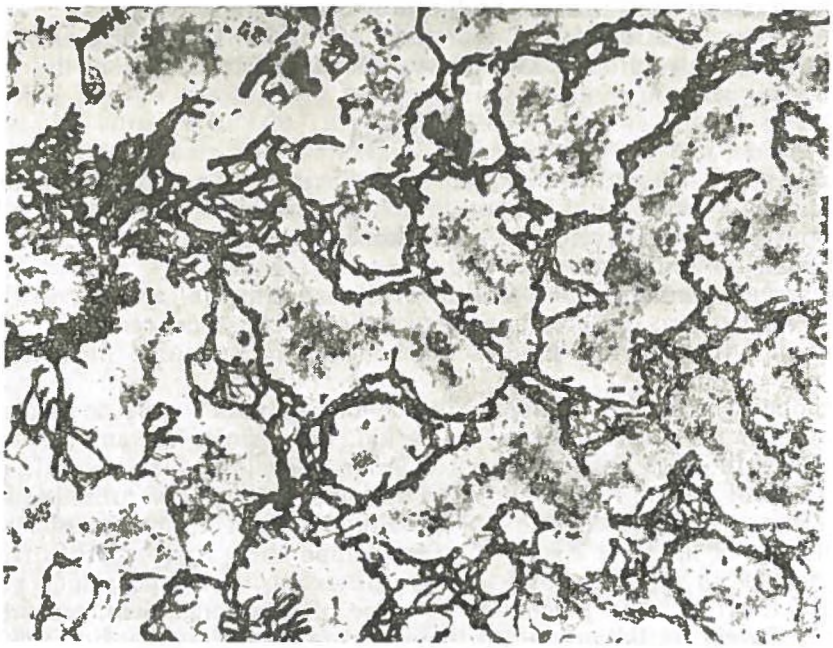

Fig. 7. Dog № 5, sacrificed on 7 June, 1966; great thickening and enlargement of the reticulum, mostly around the central veins (Gomori).

lymphocytic cells were disclosed. A stronger glycogen charge was found in the hepatic cells; the protoplasm appeared to be reticular. The punctures early in the 7th months showed small bubbles in the protoplasm and laterally wise — nuclear extrusion in single hepatic cells. In some places the nuclei were missing. Single lymphocytic infiltrates were detected as well as slight sinusoidal extension. The reticular fibers were well pronounced some of them being slightly thickened (Fig. 5). The secondary punctures of the 7th month displayed pronounced parenchymatic dystrophy and vacuolation of the hepatic cells and scattered lymphocytic elements. The changes found in the punctures of the following month were still heavier — numerous round cells were observed, situated mainly around the periportal spaces. The changes within the hepatic cells were rather stable in the punctures of the 9th month. Upon killing the dogs at the beginning of the 10th month merely roughened reticular fibers were found in the liver and in single cases, multiple monocytoid and lymphoid cells within the interstitium (Figs. 6 and 7).

Therefore, it is impressing that in the cases, treated with cortisone and blood from hepatitis patients, the changes occur earlier and appear to be severer, they persist for a longer time and subsequently, bring about a heavier reticular induration. In none of the cases, however, the picture of pronounced, complete necrosis was established, characteristic of the toxic hepatic parenchyma dystrophy. Neither were pronounced cirrhotic changes found. Presumably, the changes appear to be heaviest during the 7th and 8th month, with subsequent stabilization of the process and recovery.

The macroscopic and histologic study of the remaining organs after the killing of the dogs did not show deviations worth to note from the normal histological structure.

Discussion

The dog was chosen as object for reproducing of an experimental model of the inoculation form of human virus hepatitis owing equally to its biological closeness to man and to its affectability by canine hepatitis, being as contagious for humans.

We assume that the susceptibility of the experimental animals to the infection studied was elevated by way of their bio-resistance reduction through treatment with 6-methyl-prednisolone during the supposed incubation period.

Although perceptible clinical evidence for hepatitis was by no means established over the entire observation period, the histological and some of the laboratory findings prove a hepatic lesion, developing as a result of the experimental setting resorted to. The systematic check-ups provided for the most clearcut detection and follow-up of the lesion. Yet, however substantial the changes of the liver tissue, they were apparently incapable of involving its function, in all likelihood, due to the extensive compensatory possibilities of the liver. The judgement of some of functional derangements was possible merely on the ground of the abnormal findings regarding the Weltmann reaction and height of the antistreptolysin titer. The latter inference should be made with great caution and reserve, for both tests alike might hardly be assumed as characteristic merely for the liver's function. The fluctuations' dynamics in the height of the antistreptolysin titers in the dogs studied, in general outline, corresponds to that observed in humans affected with virus hepatitis — a rise at the onset of the illness and a fall during the reconvalescent period (16). Anyway, contemporaneously, a number of important hepatitis tests diagnosticalwise as MacLagan, transaminase activity and serum bilirubin were within the normal limits. Neither the CFR did provide indicative data, which, in the opinion of some authors (3), might be accepted as helpful in the diagnosis of human virus hepatitis. The effect of the glucocorticoid, similarly employed in the experiment described, was manifested merely in the histologic specimens — the morphological damage was more pronounced in the dogs treated with 6-methyl-prednisolone.

The earliest abnormal findings — prolongation of the Weltmann coagulation band — were established during the 4th month since the initiation of the experiment. Hence, the eventually induced infectious process at the level of the liver displayed an incubation period amounting to several months, i. e. the same as in the serum hepatitis in humans.

Inference

1) A hepatic lesion was induced by means of daily intramuscular injection of whole citrate blood from patients with virus hepatitis into young dogs over a period of 10 days, running a symptomless clinical course and established mainly on the basis of the histological findings and partially — biochemically.

2) The Weltmann test (up to 9 test tubes) was assumed as the earliest sign of the incipient process resulting in certain hepatic disorders, recorded for the first time during the 4th month and reaching its maximum value during the 5th month from the beginning of the experiment. Chronologically, the antistreptolysin titer determination is the second test to be rendered positive, exhibiting maximal values during the 8th month.

3) The morphological changes within the hepatic tissue — parenchymatic dystrophy and vacuolation of the hepatic cells mesenchymal lymphocytic infiltrates — occurred latest and showed highest intensity during the 8th and 9th months. The histological changes in the liver were more pronounced in the experimental animals, treated with 6-methyl-prednisolone (Urbason).

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ПОПЫТКА ВОСПРОИЗВЕДЕНИЯ ПОРАЖЕНИЯ ПЕЧЕНИ У СОБАК, ПРИ ПОМОЩИ КРОВИ ОТ БОЛЬНЫХ ГЕПАТИТОМ И ГЛЮКОКОРТИКОИДАМИ

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РЕЗЮМЕ

Эксперименты были проведены на молодых собаках, третированных кровью от больных гепатитом и 6-метилпреднизолоном (урбазон). Путем ежедневного внутримышечного инъектирования собак, в течение 10 дней, было вызвано известное поражение печени, которое протекло клинически бессимптомно и было установлено преимущественно гистологически и до некоторой степени — биохимически. Самым ранним признаком начинающегося процесса, приведшего к известным смущениям в печени, оказалось удлинение коагуляционной пробы Вельтмана (до 9 пробирок), начавшееся на четвертом месяце и достигшее своего максимума, в течение 5-ого месяца от начала опыта. Хронологически второй позитивировалась проба для определения антистрептолизинового титра — показавшая максимальные значения в течение 8-ого месяца. Морфологические изменения в ткани печени — паренхиматозная дистрофия и вакуолизация клеток печени, мезенхимные лимфоцитные инфильтраты появились позже всего и были самыми интенсивными, в течение 8-ого и 9-ого месяцев. Гистологические изменения в печени были более подчеркнутыми у третированных кровью от больных гепатитом и 6-метилпреднизолоном опытных животных, чем у третированных только кровью.