Acta vet. scand. 1963, 4, 384—394.

From the State Bacteriological Laboratory and the Department of Virus Research, Caroline Institute, School of Medicine, Stockholm.

STUDIES ON THE VIRUS OF HEPATITIS CONTAGIOSA CANIS (HCC)

V. A STUDY WITH THE AID OF FLUORESCENT ANTIBODIES ON SOME FEATURES OF HCC VIRUS INFECTION IN THE DOG AND GUINEA PIG

By

C. R. Salenstedt

Coffin et al. (2) studied the spread of HCC virus in dogs after inoculation in the anterior chamber of the eye. Virus multiplied and was rapidly disseminated. Already three days after the inoculation it was detected in the liver. Three dogs out of nine died. *Baker et al.* (1) has reported that in dogs the natural route of infection is by the mouth. The present author has used this mode of experimental infection (8). The incubation time depends on the virus amount absorbed, but was considerably delayed in comparison with that observed by *Coffin et al.* (2). If the virus was isolated from the liver at all this could not be done before the fifth post-inoculation day. It points — at least in the first phase of the disease — to another way of virus spread than was the case in dogs given the virus intraocularly. The mortality rate has also been extremely low, less than 2 %. This was in agreement with the results reported by *Rubarth* (5, 6).

The purpose of this paper was, with the aid of fluorescent antibody technique, to confirm some of the results reported in an earlier publication (8), namely to find out, mainly during the incubation phase, the distribution of viral antigen in various tissues in dogs and guinea pigs.

MATERIAL AND METHODS

Virus. The virus pool 4559 (8) originating from an HCC virus strain isolated in this laboratory, was used. The titre was $10^{7.6}$ $TCID_{50}$ per ml.

Tissue Culture. Tissue culture tubes were prepared from dog kidneys. The details have been reported previously (7, 8).

Inoculation of Dogs and Guinea Pigs. The dogs were given virus per os as reported earlier (8). Otherwise, the mode of infection is given in the particular experiment. The guinea pigs were inoculated intraperitoneally with 1 ml. each.

Collection of Test Material. Some of the dogs were anaesthetized and exsanguinated. Organ materials were excised and handled as described in a previous paper (8).

From some of the dogs cell material was taken from the tonsillar surface. It was done in the following way. The dogs were given, depending on the body weight, 1—2 ml. intravenously of a tranquilizer, Plegicil. With the edge of a scalpel some tissue from the surface of one of the tonsils was scraped off. The cells thus obtained were distributed on a slide and the smear was dried and then fixed in acetone.

From some of the killed guinea pigs fluid in the peritoneal cavity was sucked out with a pipette and then poured out on a slide. Only a few drops were used for each slide and the smear was dried in the air before fixation in acetone.

Virus Isolations and Infectivity Titrations. This was done in the same way as reported earlier (4, 8). The titres are calculated per ml. of the 10 % organ extract.

Fluorescent Antibody Technique. A modification of the fluorescent antibody technique described by *Coons et al.* (3) was used.

From the dogs and guinea pigs different organs were excised. A piece 2—3 mm. square was cut out and then frozen in a CO_2 jet. Tissue sections with a thickness of 4 μ were obtained by cutting in a microtome placed in a cryostat. The sections were fixed in acetone for 15 minutes.

Fluorescent antiserum was prepared in the following way. Dogs were infected by giving HCC virus per os. Bleeding was performed 3—4 weeks after infection. The antibody titre in serum was determined by HI test and was found to be between 1/160 to 1/640. Fractionation of the serum was made twice with half-

saturated ammonium sulphate in order to remove most of the albumin. To the globulin solution was added 0.05 mg. fluorescein-isothiocyanate powder per mg. of protein. The dyestuff not bound to the globulin was removed by filtration through a Sephadex column. In order to reduce the nonspecific staining the conjugate was absorbed twice with dog liver powder or guinea pig liver powder. The amount used was 100 mg./ml. of serum. The fluorescent serum was kept frozen when not in use. When comparing the HI titres of the original serums and of the fluorescent ones it was found that the serum titre during the preparation had diminished one to three 2-fold steps.

Staining. For the staining of the antigen the direct method was used. Thus on all fixed slides a few drops of fluorescent antibody solution was poured. After a staining period of 30 minutes the slides were washed in buffered saline and then mounted in buffered glycerol.

Controls. The specificity of the fluorescence was controlled in the following way.

A) Sections and smears from healthy non-infected animals were stained as above.

B) Normal dog serum (without HCC antibodies) was conjugated and used as staining fluid on sections from HCC infected dogs and guinea pigs.

C) Sections from infected dogs were first treated with non-conjugated positive serum and then with conjugated positive serum. A pretreatment period of 30 minutes with the unlabelled serum never gave complete inhibition. However, the intensity was strongly reduced. When the time was extended to 4 hours or more complete inhibition was obtained.

Microscope and Photography. A Zeiss fluorescence microscope with a mercury vapor bulb was mostly used. At the photographic work Kodak Tri-X film was employed. The exposure time varied from 30 seconds to 2 minutes.

Identification of Fluorescent Cells. The identity of the fluorescent cells was established in the following way. After the fluorescence photo had been taken the cover slips were washed away. The sections were fixed once more, this time for 5 minutes in methanol and then stained with hematoxylin and eosin. The slides were mounted with canada balsam. By comparing the

photograph with its fluorescent spots with the picture seen in the ordinary microscope of the restained slide, it was possible to identify most of the interesting cells.

EXPERIMENTAL

A group of 8 dogs were given HCC virus per os. The first dog was killed 72 hours after infection, the last one on the 7th day after exposure to virus. The tonsils were excised and handled in the following way. Five consecutive pieces, 2—3 mm. square, were cut from one tonsil and then treated as said above. From the first piece ten sections were cut. Each of them was separated from the adjacent ones by at least 20 μ, *i.e.* corresponding to 5 sections. From the four remaining pieces only two sections were cut. From the rest of the tonsil an extract was made after disintegration in a mortar and titrated. All sections were stained with fluorescent HCC antibody solution. At the inspection of the slides the number of fluorescent cells in 6 microscopic fields of view was calculated. With the guidance of different structures in the tissue the position of the topographically corresponding fields which should be examined could usually be determined from section to section. This was always the case within the same piece. The magnification was 100 times. A total of 864 fields were counted. They contained 7696 fluorescent cells. The total number for each tonsil is recorded in Table I. In the same table the virus titres of the tonsil specimens as titrated with tissue culture tube technique are also given.

Table I

The total number of fluorescent cells and the virus titres in tonsils from dogs killed at different times after infection with HCC virus.

Tonsil number	Post infection day	Total number fluorescent cells	Virus titre/ml.
1	3	98	N.D.
2	4	1970	2.1
3	4	2065	N.D.
4	5	1235	3.3
5	5	1589	3.7
6	6	640	2.1
7	6	62	1.1
8	7	37	$\leq$ 0.5

N.D. = Not Done.

It is apparent that the ratio between the "fluorescence titre" and the virus titre is not constant. Thus, the virus titre for tonsil number 2 seems to be low in comparison with those for the other tonsils. It is also conceivable that the virus titres of tonsils 4, 5, 6 and 7 are too high in comparison with that of number 2. This may be explained by the assumption that extracellular virus accumulates in the tissue during the next few days which is not possible to reveale with the fluorescence microscope, thus giving another ratio between "fluorescence" titre and virus titre.

The difference between the sections with regard to the number of fluorescent cells was statistically analysed, on the one hand, between the sections from the same piece, on the other, between the first two sections in all five excised pieces in each tonsil. It was found, that there was no statistically significant differences between sections from the same piece. When the sections come from all five pieces within the same tonsil the trend is different. Thus, in five cases out of eight there are highly probable difference. For four of the cases the level of the significance is high ($P < 0.01$), in one case somewhat lower ($P < 0.05$).

By the method of fluorescent antibody technique, an investigation of the distribution of virus and its cellular localization in the body was made. The emphasis has been put on the examination of organs, which in this case are of special interest during the incubation period, namely the tonsils and the small intestine with their draining lymph nodes. Infected dogs were killed at different times after exposure to HCC virus. The first dogs were killed 2 days and the last 7 days after infection.

On the second day specific fluorescence was noted only in the tonsils. The presence of HCC virus antigen in these organs was followed up to 6—7 days after infection. Maximum "fluorescence titre" was recorded on the 4th or 5th post-infection day. During the earliest phase of the virus growth in the tonsil the antigen was detected only in cells in the capsule of the tonsil. Later on the virus spread to the interior of the organ. Different pictures were seen from early changes with only a granular fluorescence lining the nuclear membrane to cells with the nucleus almost completely filled with a fluorescent inclusion body — or bodies. Specific fluorescence was noted within reticulo-endothelial cells and lymphoid cells. In the capsular tissue the epithelial cells often showed varying degrees of fluorescence. Within the follicles antigen was seldom observed.

Explanation of Plates

The pictures are fluorescence photographs of frozen tissue sections stained with fluorescein-labelled antibodies against HCC. The fluorescence depends on specific staining of HCC antigen.

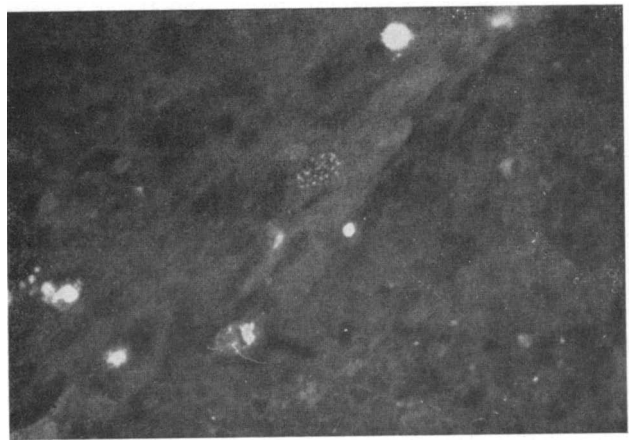

Fig. 1. Tonsil from a dog. Fluorescent cells in and immediately beneath the capsule. In the middle of the photo an epithelial cell with a faint granular fluorescence (early change). Upper right an immature lymphoid cell with its entire nucleus fluorescent (late phase). × 80.

Fig. 2. Superior deep cervical lymph node from a dog. Fluorescent cells in the intermediary sinus. To the right some sinus-endothelial cells, to the left two cells of lymphoid type. × 80.

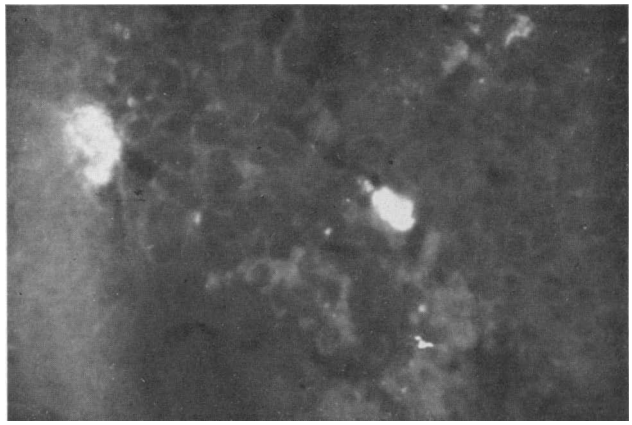

Fig. 3. Mesenteric lymph node from a dog. A sinus-endothelial cell lying in the margin of a lymphoid follicle and a fluorescent cell of lymphoid type inside the follicle. × 200.

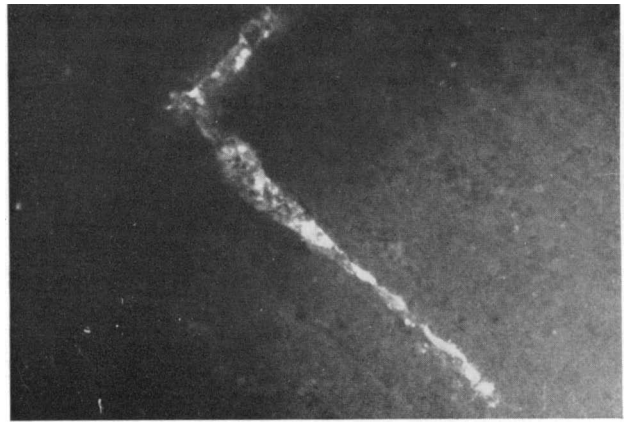

Fig. 4. Small intestinal wall from a dog. Fluorescent epithelial cells within a Lieberkühn's crypt. × 80.

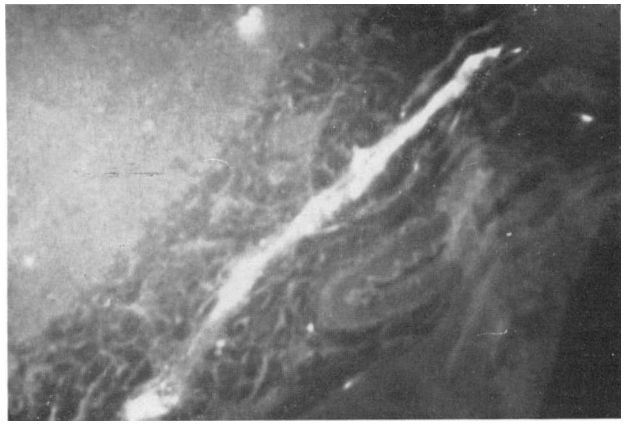

Fig. 5. Lymph vessel in the submucosal tissue of the small intestine from a dog. Fluorescent mononuclear cells in the walls and in the lumen of a lymph vessel. × 80.

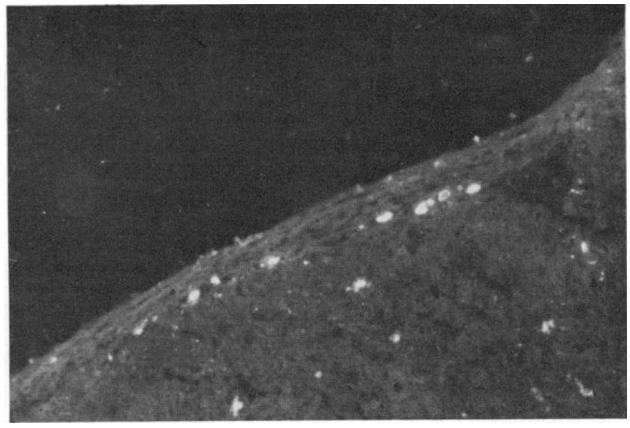

Fig. 6. Liver from a guinea pig. Fluorescent cells in the fibrin coat on the surface of the liver. The cells probably of macrophage type. × 80.

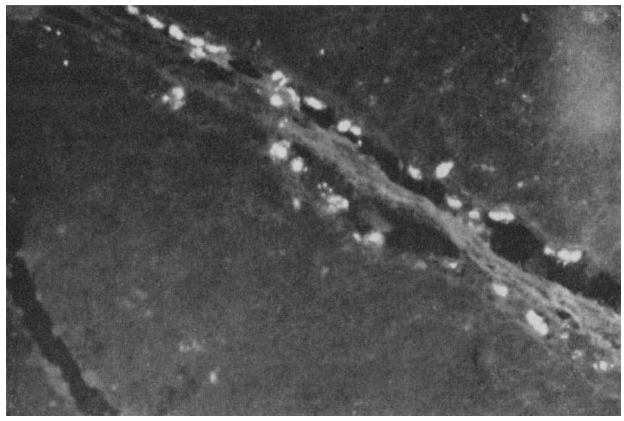

Fig. 7. Liver from a guinea pig. Two lobes joined to each other by a fibrin coat, in which there are a number of fluorescent cells. × 80.

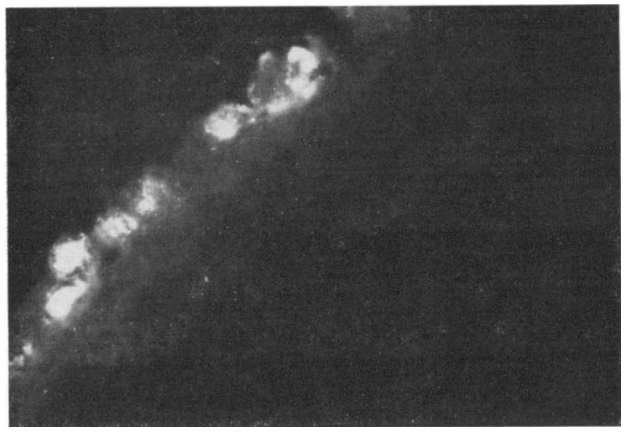

Fig. 8. Spleen from a guinea pig. Fluorescent cells in a fibrin coat on the surface of the spleen. The cells of macrophage type. × 200.

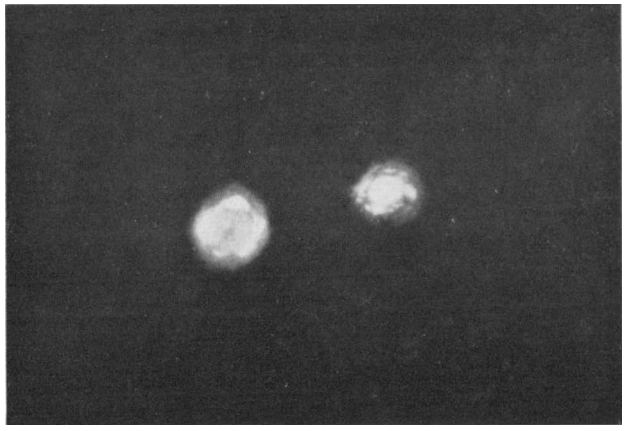

Fig. 9. Tonsil scrape from a dog. Fluorescent epithelial cells. × 320.

On the 3rd post-infection day viral antigen was detected in the superior deep cervical lymph nodes as well. There the fluorescence was seen primarily in sinus-endothelial cells lining the intermediary and subcapsulary sinus but also in some lymphoid cells most often localized in the sinus. As was the case in the tonsil, isolated macrophages in the sinus and in the follicles contained HCC virus antigen. As a whole the type of fluorescence was the same as in the tonsillar sections.

On the following days the spread of the virus in the body was followed. The results obtained were in agreement with those of *Coffin et al.* (2). However, they did not notice the specific changes in the small intestinal wall. With the beginning on the 4th post-infection day fluorescence was found in the mucosal and submucosal tissue. The changes in the mucosa were generally limited to a few intestinal tubules. In advanced cases practically all epithelial cells within these tubules showed fluorescence. In the submucosa viral antigen was detected in endothelial cells lining lymph channels as well as in mononuclear cells in the lumen of these vessels. In the Peyer's patches the fluorescence was observed in sinus-endothelial cells. The picture of the mesenteric lymph nodes was strongly reminiscent of that in the superior deep cervical lymph node but here some fluorescent lymphocytes were also observed.

Attempts to infect guinea pigs by feeding virus have failed. Consequently a direct comparison between the pathogenesis of HCC in the dog and guinea pig cannot be made. However, some features of the infection after an intraperitoneal inoculation in the guinea pigs were studied by the fluorescent antibody method. The animals, killed 3 or 4 days after infection, showed a perihepatitis and perisplenitis. A moderate amount of exsudate was found in the peritoneal cavity. Fluorescent cells were observed in the fibrin coat on the liver and on the spleen as well as in the exudate. The cells were mononuclear macrophages. No fluorescent cells could be seen in the liver or spleen. A series of photographs from different organs of dogs and guinea pigs illustrate what is said above.

The finding that specific fluorescence was observed in the epithelial cells in the capsule of the tonsil during HCC infection in dogs was used as a basis for elaborating a rapid method to diagnose HCC. Four groups of 4 to 7 animals were used in these experiments. In each group 1 to 2 dogs were infected per os with

HCC virus and isolated for some days. After this the dogs were brought back to the litter-mates. During the following days the temperature was measured. When a rise was noticed in an animal a specimen was taken from the tonsillar surface as said in Material and Methods. When the preparation contained fluorescent cells such pictures as shown in Fig. 9 were obtained. The results of one experiment is recorded in Table II.

Table II

Results of specimens from the tonsillar surface of dogs exposed to HCC virus through animals experimentally infected per os. Day after dog number 1 and 2 were infected per os

Dog number	4	5	6	7	8	9	10	11	12	13	14
1	0[1])	+[2])									
2	0	+	+	+	0	0	0	0	0	0	0
3	0	0	0	0	0	+	+	0	0	+	—[3])
4	0	0	0	0	0	—	—	0	0	0	0
5	0	0	0	0	+	+	+	+	+	0	0
6	0	0	0	0	0	0	0	0	+	+	0
7	0	0	0	0	0	0	0	0	0	0	0

[1]) 0 = No Specimen Taken
[2]) + = Positive Specimen
[3]) — = Negative Specimen

In this particular experiment dogs 3, 5 and 6 showed a temperature reaction. The first positive slide was obtained 4 days after the dog came in contact with the infected animals. The last positive reading was made on the 9th day. In two dogs fluorescence in cells from the tonsil capsule could be followed for 5 days. The specimens showed a maximal number of fluorescent cells during the 3rd and 4th days. In order to see whether the dogs with positive slides also contracted hepatitis some of the dogs were killed and examined. Generally fluorescent cells were found in the liver and in tonsil specimens at the same time. The type of fluorescence in the tonsillar cells was the same as earlier. Thus some cells showed only a granular fluorescence along the nuclear membrane, while other cells showed the presence of a big bright inclusion body in the nucleus.

DISCUSSION

To collect information about the virus multiplication in a susceptible host a titration with the use of tissue culture tube technique generally must be done. This is a time-consuming method. If a ratio between the amount of fluorescent cells and the tube virus titre could be established the tube technique could be substituted by a fluorescent cell counting technique. Such an attempt is reported in this paper. However, the ratio was not constant. It must be borne in mind that the fluorescence method gives information about the amount of both viral and soluble antigen, the tube technique about live, complete virus. The phase of viral multiplication within each cell also varies. It is obvious that a cell with a big fluorescent inclusion contains more live virus than a cell with a small fluorescent dot. This difference in amount of live virus can be shown by the tube technique whereas a fluorescent spot is always recorded, independent of its size. It may also happen that virus during the multiplication accumulates in the tissue, which is only detectable by the tube technique.

In addition, the virus distribution within the tonsil was examined. It was found that there was no significant difference between sections from the same small piece of the tonsil. On the other hand, this was often the case when the sections originated from pieces from different places in the tonsil. When the sections are taken from a limited area within the same piece there is a big chance for obtaining material from the same infectious focus. It is by no means certain that this happens when the sections originate from different pieces. Most probably, there are various degrees of viral antigen accumulation within different areas in the same tonsil. Obviously the fluorescence method shows the existence of live virus but gives only a slight idea about the virus titre when judging from a small number of sections.

With the aid of the fluorescent antibody method a study on the spread of HCC virus in the dog was made. Special attention was paid to the tonsils and the intestinal wall, which were considered particularly important from an epizootiological point of view. It was shown that specific fluorescence was present in epithelial cells on the surface of the tonsils. When virus is released from the cells some of it will contaminate the saliva, thus rendering the dogs infectious by the saliva and probably also by the faeces.

In the intestine fluorescence was observed both in the mucosa and in the submucosa. *Rubarth* (5) has reported what he calls "eosinophil degeneration". This was limited to one or a small number of tubules. The present study confirms *Rubarth*'s observation and establishes its specificity as a HCC induced change. It is remarkable how sharply limited the area of virus growth is. In one of the photos it is shown that practically all cells within one tubule show fluorescence while all adjacent are completely negative. The occurrence of antigen in the mucosal and submucosal tissues simultaneously could be shown. The submucosal findings often consisted of fluorescent cells in the lumen or in the walls of lymph vessels. With the results in a preceding paper (8) as a background, it seems probable that the changes in the submucosa are secondary to those in the mucosa. A small amount of virus has been absorbed to some cells in a tubule and after a multiplication virus has been transported by mononuclear cells via the lymph channels to the draining lymph nodes. Part of the virus has surely been shed in the intestinal contents. The photographs show a few fluorescent cells lying in the intestinal lumen.

By the use of the fluorescent antibody technique a possibility of a rapid diagnosis of HCC has been opened. It could in most cases be shown that viral antigen was still present in the capsule of the tonsil when the liver was involved. It is already known that HCC very often gives undefined symptoms. In such cases a tonsillitis may be the only clinical sign of the disease. By the method described the diagnosis can be clarified earlier than would be possible with the aid of virus isolation and "HCC-tonsillitis" can be distinguished in the large group of tonsillitis in dogs. With the knowledge that the amount of antigen in the tonsil decreases rapidly in the advanced phase of HCC it is recommended that dogs admitted to the veterinary clinic with the preliminary diagnosis HCC should as soon as possible be examined in the way described.

REFERENCES

1. *Baker, J. A., M. G. Richards, A. L. Brown* and *C. G. Rickard:* Infectious hepatitis in dogs. Proc. 87th Ann. Meet. Amer. Vet. Med. Ass. 1952, 242.
2. *Coffin, D. L., A. H. Coons* and *V. J. Cabasso:* A histological study of infectious canine hepatitis by means of fluorescent antibody. J. exp. Med. 1953, *98*, 13.

3. *Coons, A. H., H. J. Creach, R. N. Jones* and *E. Berliner:* The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J. Immunol. 1942, *45*, 159.
4. *Kärber, G.:* Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch. exp. Pharmak. 1931, *162*, 480.
5. *Rubarth, S.:* An acute virus disease with liver lesion in dogs (Hepatitis contagiosa canis). A pathologico-anatomical and etiological investigation. Acta path. microbiol. scand. 1947, *24*, Suppl. 69.
6. *Rubarth, S.:* Die Epizootologie und Virologie der Hepatitis contagiosa canis. Arch. exp. Vet.-Med. 1952/53, *6*, 105.
7. *Salenstedt, C. R.:* Studies on the virus of Hepatitis Contagiosa Canis (HCC). I. The isolation of HCC-virus from the tonsils of a dog. Arch. ges. Virusforsch. 1958, *8*, 123.
8. *Salenstedt, C. R.:* Studies on the virus of Hepatitis Contagiosa Canis (HCC). IV. The pathogenesis of Hepatitis Contagiosa Canis in dogs. Acta vet. scand. 1963, *4*, 371—383.

SUMMARY

A statistical analysis of the difference in the number of fluorescent cells between sections from different parts of the tonsil was made. It was found that there was no significant difference between sections from the same small piece, but when the sections originated from different parts of the tonsil significant differences were obtained in some cases. A constant ratio between "fluorescence titre" and virus titre could not be established.

The rôle of the tonsils and small intestinal wall as portal of entry of infection in dogs was studied. It was shown that virus multiplied in epithelial cells of these loci. In addition viral antigen was present in cells of phagocytic nature, in endothelial and in lymphoid cells. The occurrence of HCC antigen in cells on the surface of the liver and spleen as well as in the peritoneal exudate in guinea pigs inoculated with HCC virus was shown.

A rapid method of diagnosing HCC is described. In spontaneously or experimentally infected dogs cell material was scraped from the tonsillar surface. After staining with a fluorescent anti-HCC serum the occurrence of viral antigen could be shown in cells in the collected specimens.

ZUSAMMENFASSUNG

Studien über das Virus des Hepatitis Contagiosa Canis (HCC).

V. Eine Studie einiger Eigenschaften der HCC-Virus Infektion bei Hunden und Meerschweinchen mit Hilfe von fluoreszierenden Antikörpern.

Es wurde eine statistische Analyse unternommen in Bezug auf die Anzahl fluoreszierende Zellen bei Schnitten aus verschiedenen Bezirken der Tonsille.

Soweit es sich um Schnitte aus dem gleichen kleinen Tonsillenbereich handelte, wurden keine signifikanten Unterschiede erhalten, während solche zeitweise gezeigt werden konnten, sobald die Schnitte aus verschiedenen Bezirken der Tonsille genommen waren. Eine konstante Relation zwischen Fluoreszenztiter und Virustiter konnte nicht festgestellt werden.

Es wurde eine Untersuchung in Bezug auf die Rolle der Tonsille sowie der Dünndarmwand als Infektionspforte beim Hunde durchgeführt. Es konnte dabei eine Virusvermehrung in den epithelialen Zellen dieser Organe gezeigt werden. Virusantigen wurde darüberhinaus festgestellt in phagozytierenden, endothelialen und lymphoiden Zellen. HCC-Antigen wurde ebenfalls nachgewiesen in Zellen der Oberflächenschicht von Leber und Milz sowie im Peritonealexsudat bei mit dem Virus geimpften Meerschweinchen.

Schliesslich wurde eine Schnellmethode zur Diagnose von HCC beschrieben. Bei spontan oder experimentell infizierten Hunden wurde Zellmaterial von der Tonsillenoberfläche geschabt. Nach Färbung mit fluoreszierendem Anti-HCC-Serum konnte das Vorkommen von Virus-Antigen in Zellen des entnommenen Materiales nachgewiesen werden.

SAMMANFATTNING

Studier över Hepatitis Contagiosa Canis virus (HCC).

V. En studie med hjälp av fluorescerande antikroppar över några drag av HCC virus infektion hos hund och marsvin.

En statistisk analys av skillnaden i antalet fluorescerande celler mellan snitt från olika delar i tonsillen utfördes. Då snitten härrörde från samma lilla tonsillbit, erhölls inga signifikanta skillnader under det att då snitten kom från olika delar i tonsillen kunde sådana skillnader understundom påvisas. Ett konstant förhållande mellan fluorescenstiter och virustiter kunde ej etableras.

En undersökning över tonsillens och tunntarmsväggens roll som infektionsport hos hunden gjordes. Det kunde visas att virus förökades i epiteliala celler i dessa organ. Virusantigen kunde dessutom påvisas i celler av fagocyterande karaktär, i endotelceller och i lymfoida celler. Ävenså förekom HCC antigen i celler på ytan av lever och mjälte samt i peritonealexsudat hos marsvin, som ympats med virus.

Slutligen beskrevs en snabbmetod för diagnostik av HCC. Hos hundar, spontant eller experimentellt smittade, skrapades cellmaterial av från ytan på tonsillerna. Efter färgning med fluorescerande anti-HCC serum påvisades förekomsten av virusantigen i celler i de tagna materialen.

(Received April 4. 1963).