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Lesion Formation in the Liver of Mice Caused by metabolic By-Products of Hymenolepsis Microstoma

Gary Simpson
Western Kentucky University

LESION FORMATION IN THE LIVER OF MICE CAUSED BY METABOLIC BY-PRODUCTS OF *HYMENOLEPIS MICROSTOMA*

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Gary F. Simpson

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LESION FORMATION IN THE LIVER OF MICE CAUSED BY METABOLIC BY-PRODUCTS OF HYMENOLEPIS MICROSTOMA

Recommended April 18, 1974
(Date)

Jerry N. Gleason
Director of Thesis

D. Hugh Puckett

L. M. Bailey

Approved April 29, 1974
(Date)

Elmer Gray
Dean of the Graduate College

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Gary F. Simpson May 1974 34 pages
Directed by: L.N. Gleason, D.W. Bailey, and D.H. Puckett
Department of Biology Western Kentucky University

The presence of lesions in the liver of mice is associated with the infection of these mice with the common bile duct-dwelling cestode, *Hymenolepis microstoma*. Lesions develop even though there is no direct contact between the cestodes and the liver. It was found in this investigation that administration to mice of Tyrode's solution, in which cestodes had been incubated for 24 hr, resulted in liver lesions comparable to those observed in normal infections. The administration of the causative substance by either *per os* intubation or intraperitoneal injection produced similar lesions. Further studies revealed that the active lesion-causing substance was isolated in a lipid extract of the metabolic by-products. It was also determined that the active material had a molecular weight of less than 10,000.

INTRODUCTION

Dobrovolskaia-Zavadskaia and Kobosieff (1927) first reported the presence of lesions in the liver of mice infected with the bile duct-dwelling cestode, Hymenolepis microstoma. Chandler (1943), Dvorak, et al. (1961), Litchford (1963), Bogitsh (1966), and Gleason (1971) have subsequently reported various aspects of the formation of the lesions and described histopathology associated with the infection of mice by this cestode. It has been postulated by the above authors that lesion formation may be caused by such factors as parasite-induced host nutritional deficiencies, biliary obstruction, or toxic-metabolic by-products. Gleason (1971) reported fully developed focal lesions as early as 6 days after infection, indicating that actual formation of the lesion begins prior to this time. The occurrence of lesions at such an early stage in the infection would seem to exclude blockage of the bile duct and parasite-induced host nutritional deficiencies as possible mechanisms for the formation of the lesions.

This study was initiated to investigate the possibility that metabolic by-products were the causative agent(s) of lesion formation in the liver. Preliminary studies indicated that this was the etiology of the lesion formation and fur-

ther experiments were then conducted to study the effects upon different strains of mice and upon different routes of experimental administration. Also, preliminary characterization of the causative substance was done by preparation of lipid and protein extracts and by determination of the approximate molecular weight using ultrafiltration techniques.

MATERIALS AND METHODS

The *Hymenolepis microstoma* used for this study were obtained from a stock maintained in the Department of Parasitology and Laboratory Practices at the University of North Carolina. The cestodes were routinely maintained in mice and the intermediate host, the confused flour beetle, *Tribolium confusum*. This was done by removing gravid proglottids from mature worms found attached in the common bile duct of the mouse host and placing the proglottids on strips of filter paper which were placed in a petri dish with beetles. The beetles had been starved for 5-8 days prior to being given the proglottids. After the beetles had eaten the proglottids, they were transferred to baby food jars half-filled with flour and containing a few raisins for moisture. At room temperature cysticercoids become infective in 8-10 days. Cysticercoids 2-4 weeks old were used to infect stock mice. Beetles were dissected and the cysticercoids collected in saline (0.9% NaCl). Ten cysticercoids were administered to each mouse *per os* using a 1-cc tuberculin syringe attached to a blunted, slightly curved 16-gauge needle. Following intubation the needle was flushed with saline and the washings checked under a dissecting microscope to insure that all cysticercoids had been delivered. Stock mice were

housed until needed for continuing the life cycle or experimental use.

The experimental study involved several facets. However, certain techniques common to all facets were employed in the investigation. In order to obtain cestodes for the studies, white laboratory mice were infected *per os* with 15 cysticercoids each. (A particular inbred strain of mouse was not used for this part of the procedure because of the excessive cost.) After the worms had been in the mice for the time dictated by the experimental procedures to be described later, the mice were killed by cervical dislocation, the *H. microstoma* removed from the common bile duct and placed in an antibiotic solution containing 1 cc Combiotic/ 99 cc saline. Combiotic (Chas. Pfizer & Co., New York) is an aqueous solution containing 200,000 units procaine penicillin G and 0.25 g of dihydrostreptomycin per 1 cc. The worms were left in this solution for approximately 30 min in order to insure bacteria-free cestodes. Using aseptic techniques, the worms were then removed from the antibiotic solution and placed in sterile petri dishes which contained Tyrode's solution (Rohrbacher, 1957), in the approximate ratio of 3 worms/ml. The worms were then incubated in this medium at 37°C for a period of 24 hr. The cestodes remained viable following incubation. The Tyrode's solution containing the incubated cestodes was then filtered through a 450 mu Millipore filter. Filtrate samples of 0.5 ml were used to inoculate pour plates of nutrient agar and blood

agar to check for bacterial contamination of the filtrate. The filtrate was then pipetted into sterile vials to be used in the experiments outlined below.

The procedure for determining wet and dry weights of the cestodes per ml of Tyrode's solution involved using the worm mass left on the Millipore filter. All the worm mass was placed on filter paper to remove as much moisture as possible using this technique. The worms were then placed in a pre-dried and pre-weighed 2-in diameter, shallow, aluminum pan, and weighed on an analytical balance. The wet weight was determined by subtracting the weight of the pan from the weight of the pan and worms. The pan containing the wet worm mass was placed for 24 hr in a drying oven maintained at 85°C. Subsequent to drying, the pan and worms were placed in a desiccator containing calcium chloride crystals for 2 hr to allow for cooling. The pan and worms were removed and immediately weighed on the analytical balance. The dry weight was determined by subtracting the pan weight from the pan and dried-worm weight. The average worm mass per ml of Tyrode's solution was calculated.

To determine if the active substance was contained in a protein fraction of the metabolic by-products produced during the incubation of the worms, proteins were extracted from the incubation medium using methods described by R Rendina (1971). A 50-ml aliquot of incubation medium was placed in one and one-eighth in diameter dialysis tubing and dialysed against approximately 350 ml of saturated am-

monium sulfate solution for 24 hr. The contents in the dialysis tubing were divided in half and centrifuged in 50-ml plastic centrifuge tubes for 1 hr at 39,100 x gravity using a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge with an SS-34 rotor head. At this point the procedure was discontinued since no protein precipitate was observed. Subsequently, fresh medium was analyzed for proteins using the Waddell method cited in Murphy and Kies (1960). This technique is a protein specific absorbance test to determine the mg of protein per ml. This analysis was done at 25°C using a Bausch and Lomb Precision Spectrophotometer.

Extraction of the lipid fraction of the incubation medium was accomplished using the methods described by Rendina (1971). A 50-ml aliquot of incubation medium was mixed with an equal volume of 2:1 methanol-chloroform solution. The mixture was centrifuged for 1 hr at 39,100 x gravity as described in the protein extraction procedure. The resulting supernatant was collected and an equal volume of 2N KCl was added to produce a biphasic system. The biphasic state was further delineated by centrifugation for 30 min at 39,100 x gravity; the upper phase was removed and discarded. The lower phase was washed with water in a 100-ml separatory funnel, again producing a biphasic system. The lower phase was collected, placed in a crucible, and the methanol-chloroform mixture evaporated in a 65°C oven. The lipid fraction remained in the crucible in approximately 1 ml of solution.

This material was reconstituted to a volume of 50 ml with Tyrode's solution, the total volume including washings of the crucible. The Tyrode's solution containing the lipid fraction was transferred to vials to be used as prescribed in the experimental procedures for the test of the lipid fraction of the worm metabolic by-products.

Ultrafiltration was used to determine the molecular weight range of the active ingredient(s) contained in the metabolic by-products. A 50-ml aliquot of incubated medium was placed in an Amicon Model 52 ultrafiltration cell and forced through 100,000, 30,000, and 10,000 standard molecular weight filters using pressurized nitrogen. The cell was packed in ice during the filtration procedure. The medium was successively passed through filter sizes of 100,000, 30,000, and 10,000; material left on each filter was resuspended in 50-ml aliquots of fresh Tyrode's solution. The filtrate passing through all filter sizes was also collected. This resulted in 4 molecular weight ranges: (1) 100,000 and above; (2) between 30,000 and 100,000; (3) between 10,000 and 30,000; and (4) less than 10,000. The media containing the different molecular weight ranges were placed in vials and used as described in the experimental procedures.

Mice used for the studies as outlined in the experimental procedures were 13-18 g male mice of 2 types: (1) an inbred strain - ICR/DUB - purchased from Flow Research Laboratories, Dublin, Virginia; and (2) an outbred strain maintained in the Biology Department, Western Kentucky

University.

Material to be tested for lesion formation capacity and control materials were administered by either intraperitoneal (i.p.) injection or per os (p.o.) intubation as prescribed by the experimental procedures. The i.p. injections were done with a 1-cc tuberculin syringe and a 27-gauge needle. The needle was inserted under the skin of the abdomen and then through the peritoneum anterior to the point of penetration of the skin. The solution was then injected into the peritoneal cavity. For per os administration, a 1-cc tuberculin syringe and a slightly curved, blunt 18-gauge needle were used. The intubation needle was inserted into the oral cavity and down the esophagus, and the material was intubated directly into the stomach. Between each intubation of the mice the needle and syringe were flushed with saline and air dried.

At the end of each experimental injection or intubation schedule as outlined in the experimental procedures, the mice were killed by cervical dislocation. The mice were eviscerated and the liver removed. The liver was quick-frozen on the microtome-cryostat (International-Harris Cryostat, International Equipment Company) and sectioned at a thickness of 10 microns. Sections were collected and prepared for study as described by Gleason (1971). The sections were collected on clean microscope slides which had been slightly warmed. These were fixed in 10% formalin in 95% ethyl alcohol, washed with running tap water, stained with Harris'

hematoxylin and eosin, dehydrated, cleared in xylene, and mounted in Permount using #1 cover slips.

Microscopic examination of the prepared slides was followed by taking pictures of representative lesions. Pictures were taken on Kodak Panatomic-X fine grain black and white film, using a Wild microscope equipped with an automatic camera.


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EXPERIMENTAL PROCEDURES

Experiment I - Intraperitoneal injection of metabolic by-products from 10-day-old worms:

Both ICR/DUB and outbred strains of mice were used in this study. For each strain, 10 experimental and 5 control mice were used. Each experimental mouse was injected i.p. at 12-hr intervals for 5 days with 1 ml of Tyrode's solution, in which 10-day-old worms had been incubated for 24 hr, thus resulting in a total dosage of 10 ml per mouse. Each control mouse was injected using the same schedule, but using incubated Tyrode's solution in which no worms had been maintained. Twelve to eighteen hr after the 5-day injection schedule, the mice were killed and liver sections were collected and prepared for microscopic examination.

Experiment II - *Per os* administration of metabolic by-products from 10-day-old worms:

This test was initiated to determine if mode of administration affected the ability of the metabolic by-products to cause liver lesions. Both ICR/DUB and outbred strains of mice were employed in this experiment. Ten experimental and five control mice of each strain were used. Each exper-

imental mouse was intubated p.o. at 12-hr intervals with 1 ml of Tyrode's solution in which 10-day-old cestodes had been maintained for 24 hr. The total amount of solution administered to each mouse over the 5-day period was 10 ml. The control mice were intubated p.o. at 12-hr intervals for 5 days with 1 ml of incubated Tyrode's solution in which no cestodes had been maintained. The mice were killed 12-18 hr after the final dose had been administered, and liver sections were collected and prepared for microscopic examination.

Experiment III - Intraperitoneal injection of metabolic by-products from 30-day-old worms:

This study was performed to determine if the age of the worms affected the ability of the metabolic by-products to cause the liver lesions. Both ICR/DUB and outbred strains of mice were used. For each strain, 10 experimental and 5 control mice were used. Each experimental mouse was injected i.p. at 12-hr intervals for 5 days with 1 ml of Tyrode's solution in which 30-day-old cestodes had been maintained for 24 hr. The techniques for the treatment of control mice were identical to those employed in Exp. I. The total amount of solution given to each mouse was 10 ml. Twelve to eighteen hr after termination of the injection schedule, all mice were killed; liver sections were collected and prepared for microscopic examination.

Experiment IV - Per os administration of lipid extract of metabolic by-products from 10-day-old worms:

This experiment was performed to determine if the lesion-causing substance was contained in the lipid extract of the worm metabolic by-products. ICR/DUB strain mice were employed for this test. Each of 5 experimental mice were intubated per os at 12-hr intervals for 5 days with 1 ml of lipid extract, for a total dosage of 10 ml. Two control groups of 5 mice each were used in this experiment. Each mouse of the first control group was given 1 ml of Tyrode's solution, in which worms had been incubated, at 12-hr intervals for 5 days to insure that the lesion-causing material was present. The mice of the second control group were given Tyrode's solution, in which no worms had been incubated, on which the lipid extraction procedure had been performed. This material was administered using the same procedure and schedule as the first control group. The second control group was used to insure that lesion formation was a result of substance contained in the lipid extract and not an artifact produced by the extraction procedure. The mice of the different groups were killed 12-18 hr after the last intubation, and liver sections were collected and prepared for histologic studies.

Experiment V - Per os administration of various molecular weight ranges of metabolic by-products

from 10-day-old worms:

The purpose of this experiment was to determine the approximate molecular weight of the lesion-causing substance. The strain of mouse used in this experiment was the ICR/DUB type. Five experimental mice were used for each of the molecular weight ranges tested. These ranges were: (1) less than 10,000; (2) greater than 10,000, but less than 30,000; (3) greater than 30,000, but less than 100,000; and (4) greater than 100,000. Each experimental mouse was given, per os, 1 ml of the designated molecular weight range fraction at 12-hr intervals for 5 days. The procedure for treatment of the control group used in this experiment was similar to that described for the first control group in Exp. IV. Following final intubation, the mice were killed, and liver sections were collected and prepared for microscopic examination.

Experiment VI - Liver lesions caused by worm infection:

The experiment was conducted to determine if, in the ICR/DUB mice, the lesions formed in a worm infection were similar to those induced passively by injection or intubation of worm metabolic by-products. Ten experimental ICR/DUB mice were intubated, per os, with 6 cysticercoids each, according to the procedures described for routine maintenance of the cestodes. Five days after infection 5 of the mice were killed; liver sections were collected and prepared

for microscopic examination to study the appearance of early lesions. On Day 11 of the infection, the remaining 5 experimental mice were killed. Liver sections were collected and prepared for microscopic examination to study fully developed lesions.

RESULTS

The aseptic techniques employed in this study to insure sterility of the incubation media were effective and contamination of the media did not occur. Results of inoculating aliquots of the media on nutrient agar and blood agar pour plates to test for bacterial growth were negative with the exception of one plate upon which only one contaminant colony was observed.

The average wet weight of the worms was 2.99 mg, resulting in an average of 11.55 mg of wet wt per ml of incubation medium. The average dry weight of the worms was 0.74 mg, resulting in an average of 2.86 mg of dry wt per ml of incubation medium.

Analysis by the Waddell method, cited in Murphy and Kies (1960), of the Tyrode's solution in which cestodes had been maintained revealed very small amounts of protein. The average protein content was only 0.846 mg/ml. This small amount resulted in no perceivable precipitate using the salting out method and thus a protein extract was not prepared.

Microscopic examination of the prepared slides obtained in Exp. I, which involved i.p. injection of metabolic by-products from 10-day-old worms, revealed lesion formation

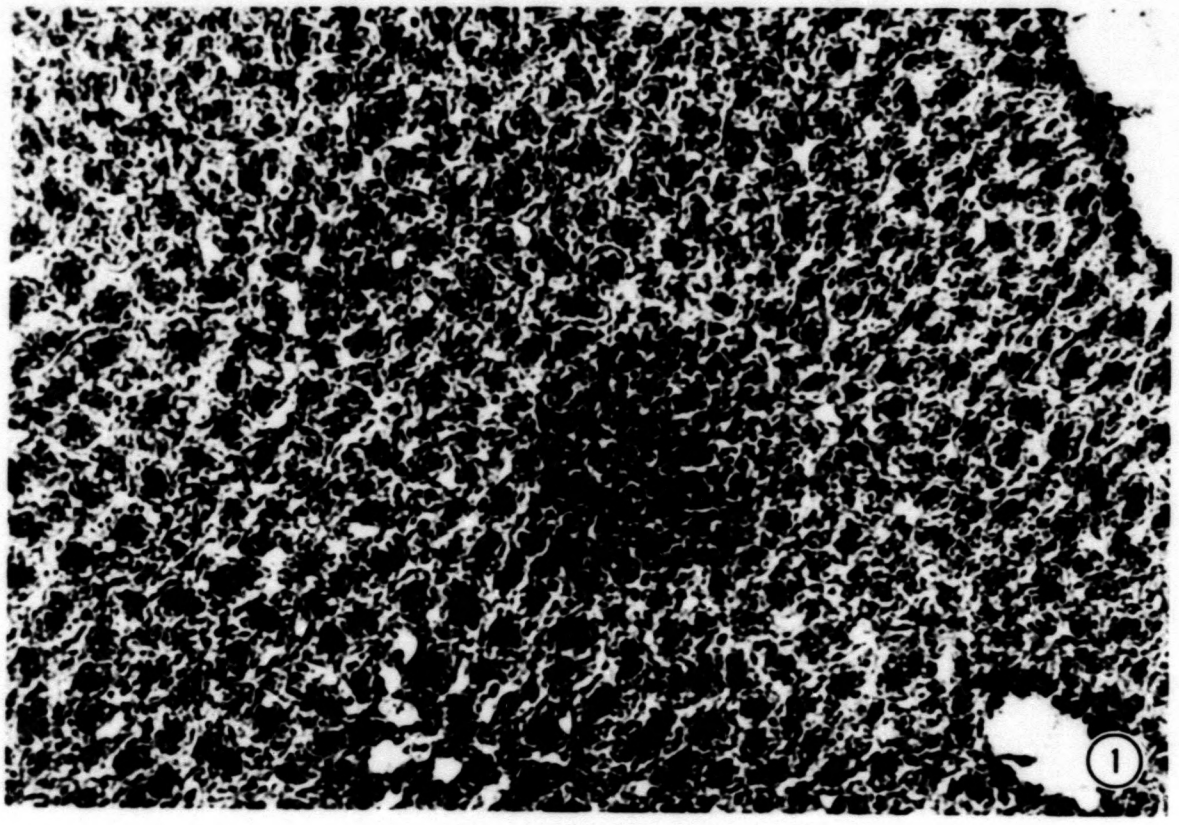
in the liver parenchyma of the experimental animals. Both strains of mice tested showed lesion formation; however, the response in the outbred strain was somewhat greater than that observed in the ICR/DUB strain. In the outbred strain, 9 of 10 mice had liver lesions, while 6 of 10 ICR/DUB mice had lesions. None of the control mice for either experimental group showed evidence of lesion formation.

Lesion formation was quite similar to that described by Gleason (1971) for a worm infection. Early lesion formation was characterized by an infiltration and accumulation of leucocytes into an area of the liver where deterioration and degeneration of hepatic cells had previously begun (Fig. 1). The predominant leucocyte type appeared to be polymorphonuclear leucocytes (PMN's).

Further lesion development involved even more degradation and degeneration of hepatic tissue eventually leading to well-developed focal lesions (Figs. 2-3). These well-developed lesions contained infiltrations and accumulations of leucocytes- predominantly PMN's, accumulations of cellular debris, and areas of extreme internal hemorrhaging. There were also instances where no hemorrhaging was evident (Fig. 4). Many of the well-developed lesions exhibited evidence of the deposition of connective tissue around the necrotic tissue of the lesion. Often this connective tissue deposition was quite pronounced (Fig. 5). Large accumulations of leucocytes- predominantly PMN's- were observed thoughout the connective tissue areas (Fig. 6).

Figure 1. Early lesion in the liver of a mouse resulting from *per os* intubation of metabolic by-products obtained from 10-day-old *Hymenolepis microstoma*. Note the accumulation of leucocytes (predominantly polymorphonuclear leucocytes) at the site of degenerating hepatic cells. x 300.

Figure 2. Advanced lesion in the liver of a mouse resulting from *per os* intubation of metabolic by-products obtained from 10-day-old *Hymenolepis microstoma*. As the necrosis of hepatic cells spreads, there is an increase in cellular debris within the lesion, sometimes hemorrhaging into the lesion (as illustrated in this photomicrograph), and an increase in the number of leucocytes, both in the lesion and in the surrounding tissue. x 300.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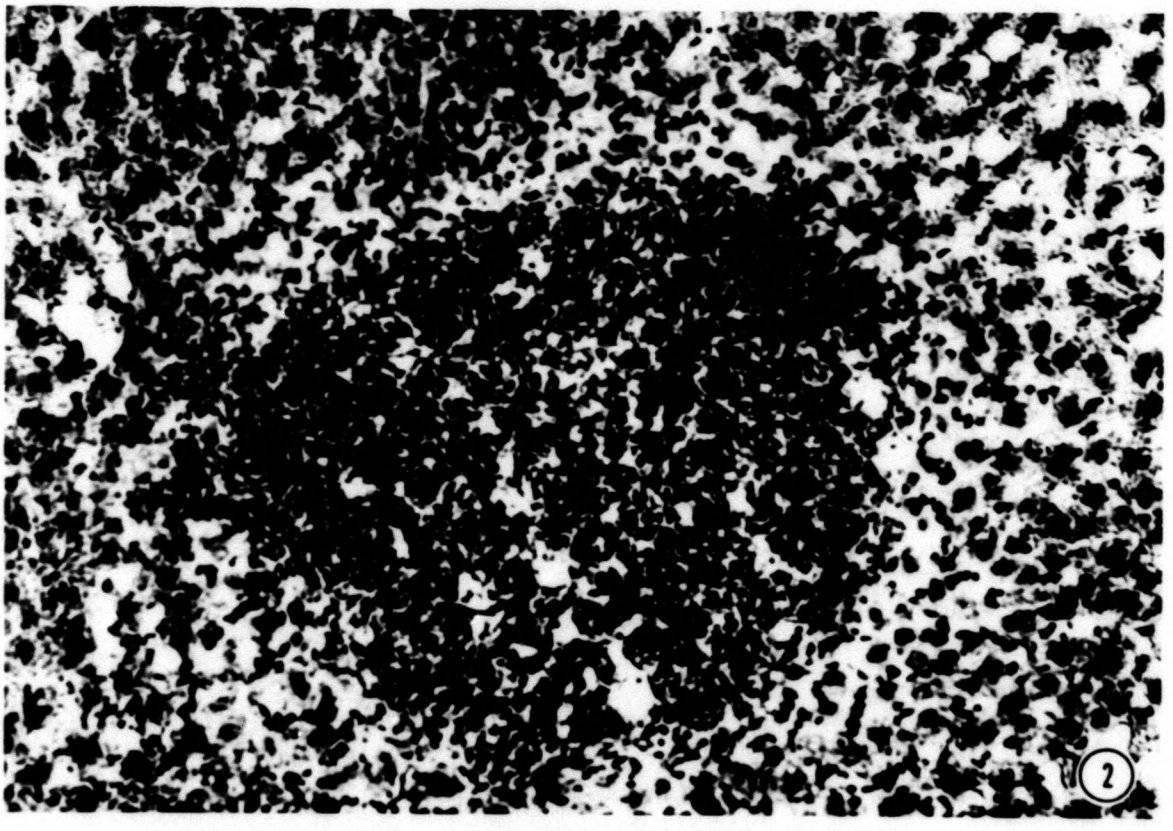
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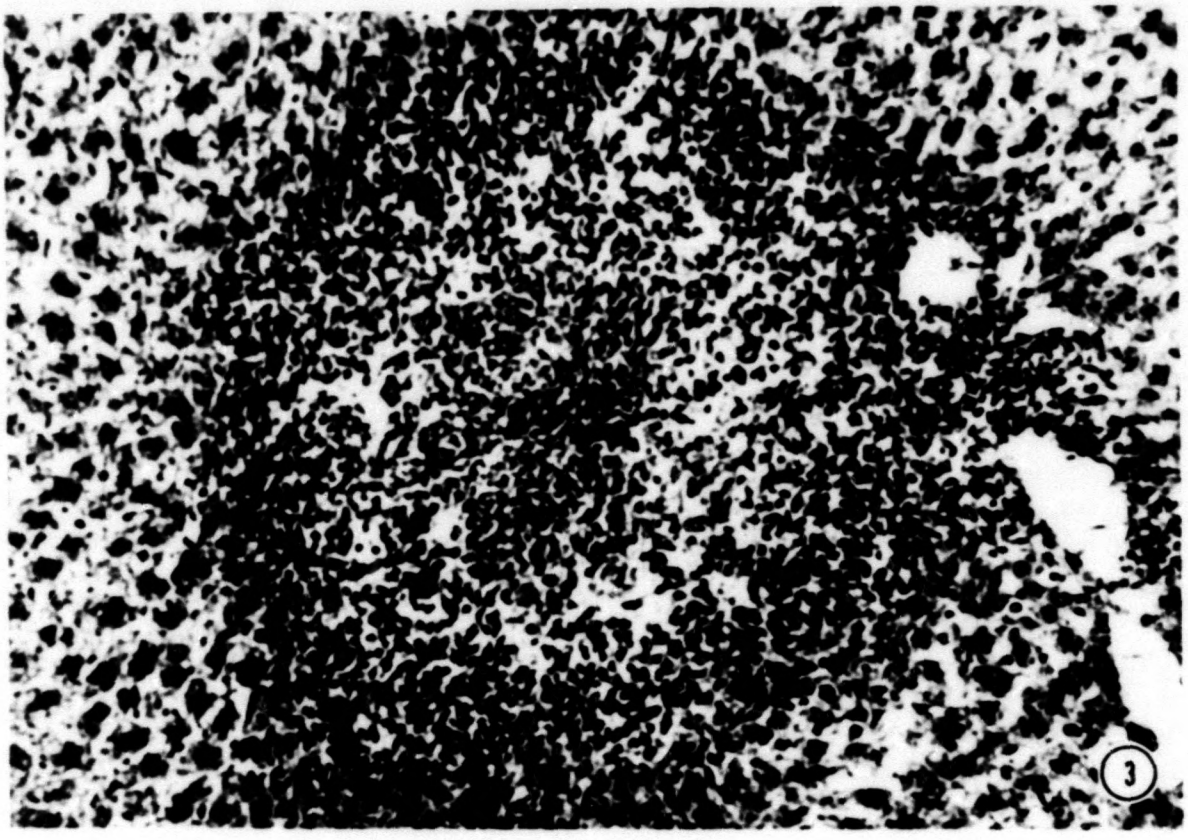
Figure 3. Advanced lesion in the liver of a mouse resulting from intraperitoneal injection of metabolic by-products obtained from 10-day-old Hymenolepis microstoma. Note the similarity between this lesion and the advanced lesion induced by per os intubation as shown in Fig. 2. x 300.

Figure 4. Advanced lesion in the liver of a mouse resulting from intraperitoneal injection of metabolic by-products obtained from 10-day-old Hymenolepis microstoma. The lesion has developed without apparent hemorrhaging. x 300.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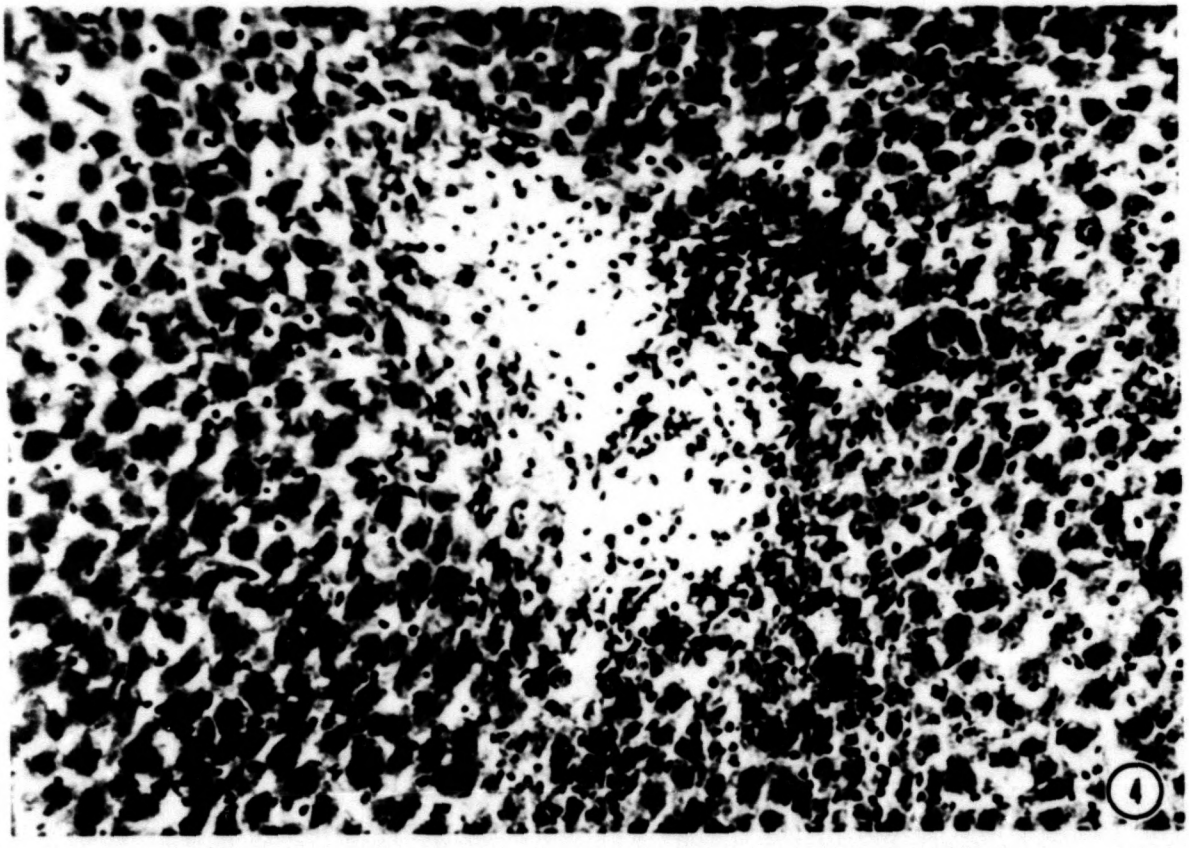
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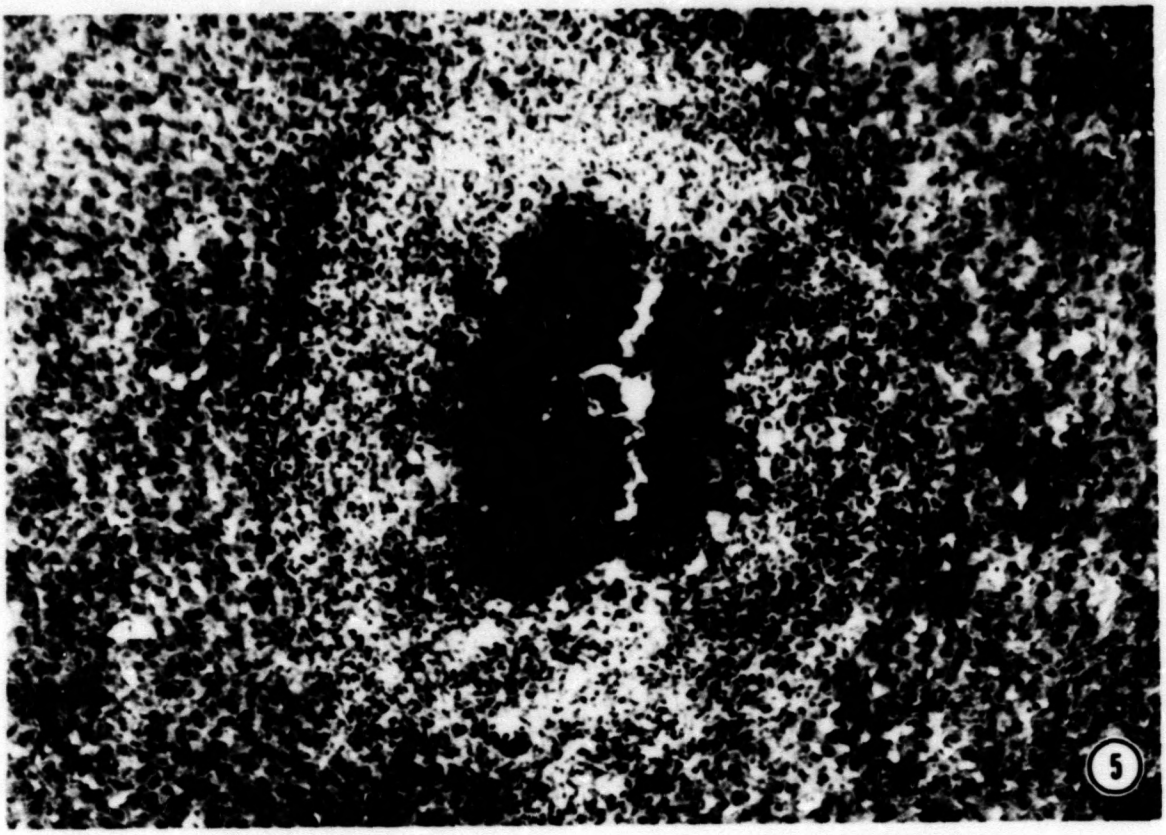
Figure 5. Advanced lesion in the liver of a mouse resulting from *per os* intubation of metabolic by-products obtained from 10-day-old *Hymenolepis microstoma*. Note the deposition of connective tissue around the lesion. x 150.

Figure 6. Connective tissue surrounding a lesion in the liver of a mouse resulting from *per os* intubation of metabolic by-products obtained from 10-day-old *Hymenolepis microstoma*. Normal liver tissue can be observed in the lower left corner of the figure. x 500.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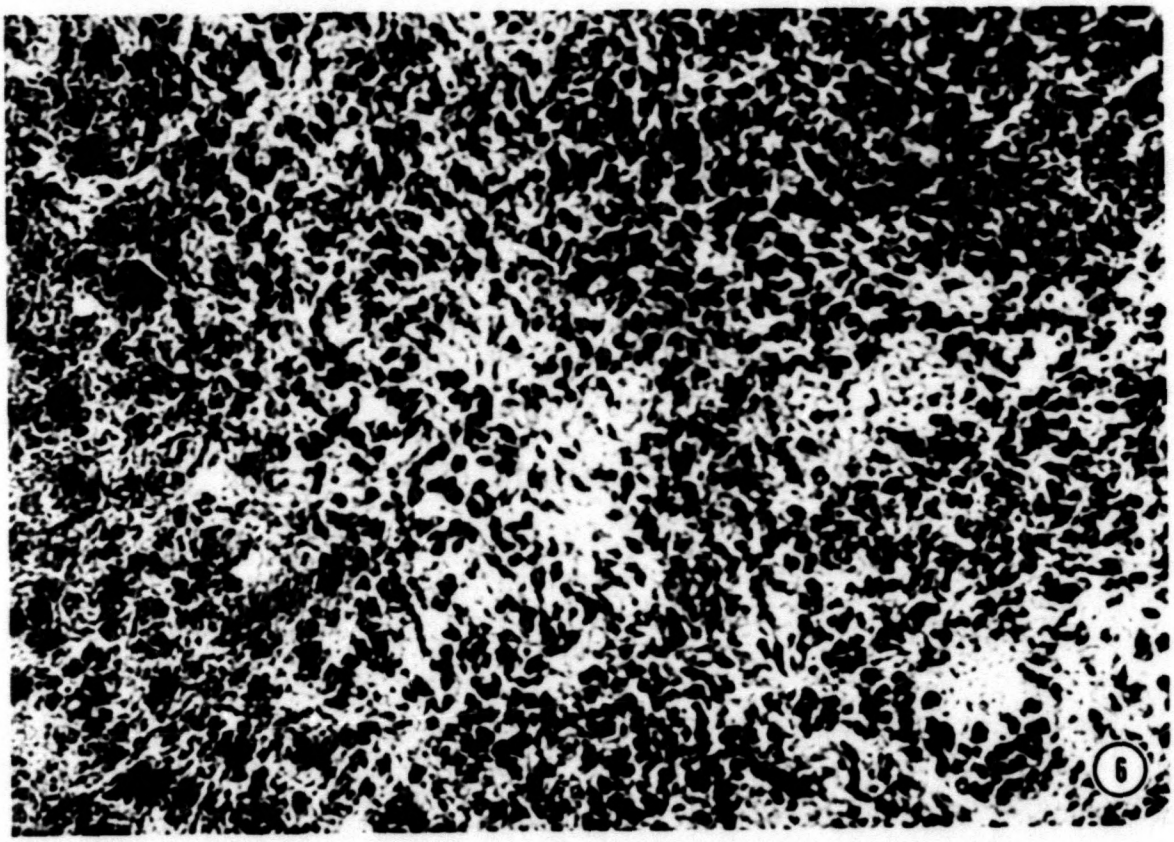
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What appeared to be coalescing of lesions was also evident in the experimental mice. This coalescing appeared to occur in an area where smaller focal lesions were in close proximity (Fig. 7) and expanded in size until they appeared to coalesce to form a much larger lesion (Fig. 8). The experimental mice of Exp. I displayed the various forms and stages of lesion formation just described.

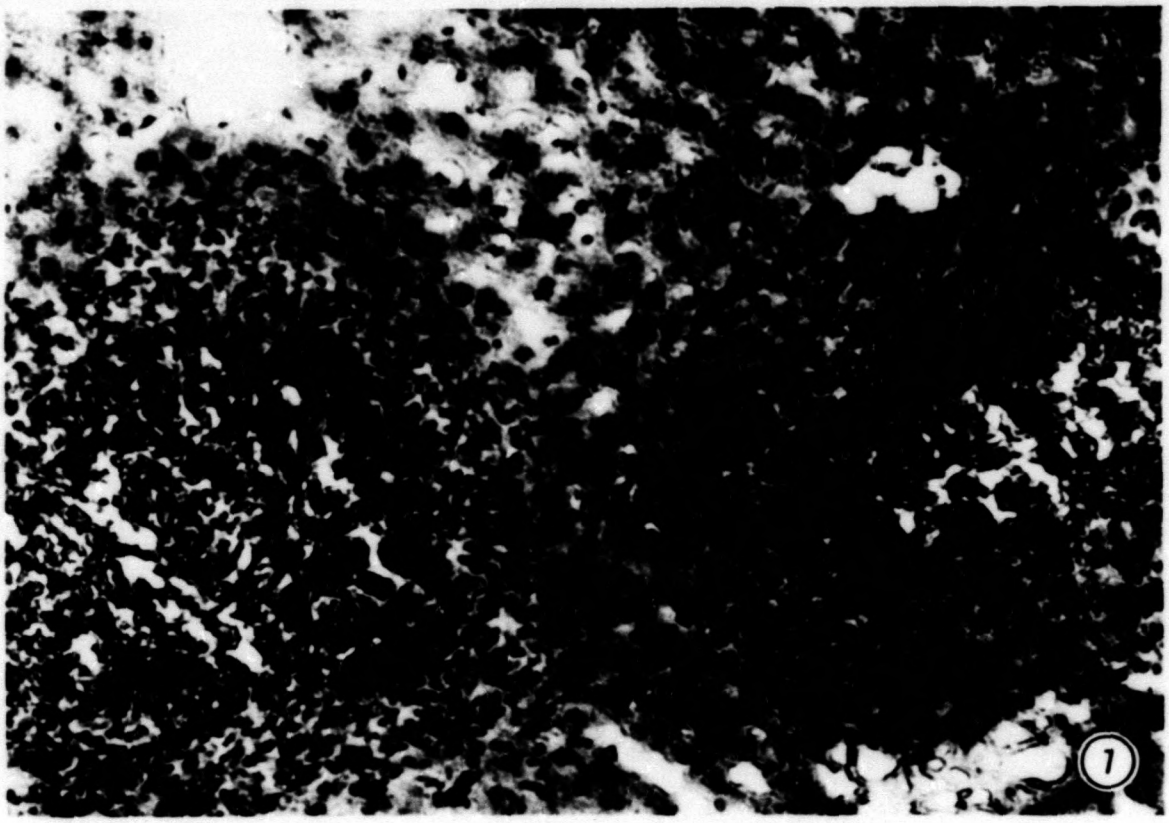
The liver tissue of control mice showed no signs of heavy leucocyte infiltration and accumulation as would be the case in early lesion formation. Also, there was no evidence of deterioration and degeneration of hepatic cells (Fig. 9).

Liver lesion formation in Exp. II, involving per os administration of metabolic by-products from 10-day-old worms, was similar to that described in Exp. I. Again, both strains of mice, outbred and ICR/DUB, showed evidence of liver lesions. In the outbred strain, 8 of 10 mice had lesions, while 7 of 10 ICR/DUB mice had lesions formed. No control mice showed evidence of lesions.

Examination of slides prepared from mice used in Exp. III, in which metabolites from 30-day-old worms were injected intraperitoneally, revealed results which were markedly different from those found in Exp. I and II. Distinct, well-developed lesions were found in neither outbred nor ICR/DUB mice. Evidence of possible lesion development was observed in only 3 outbred and 2 ICR/DUB mice. In these mice, there were a few instances of what might have been

Figure 7. Advanced lesions in the liver of a mouse resulting from *per os* intubation with metabolic by-products obtained from 10-day-old *Hymenolepis microstoma*. Two lesions have formed in close proximity to one another. x 300.

Figure 8. Advanced lesion in the liver of a mouse resulting from *per os* intubation with metabolic by-products obtained from 10-day-old *Hymenolepis microstoma*. The 3 dark areas (within the lesion) of leucocyte accumulation are apparently the sites of individual lesion formation which later coalesced as the lesions increased in size. x 150.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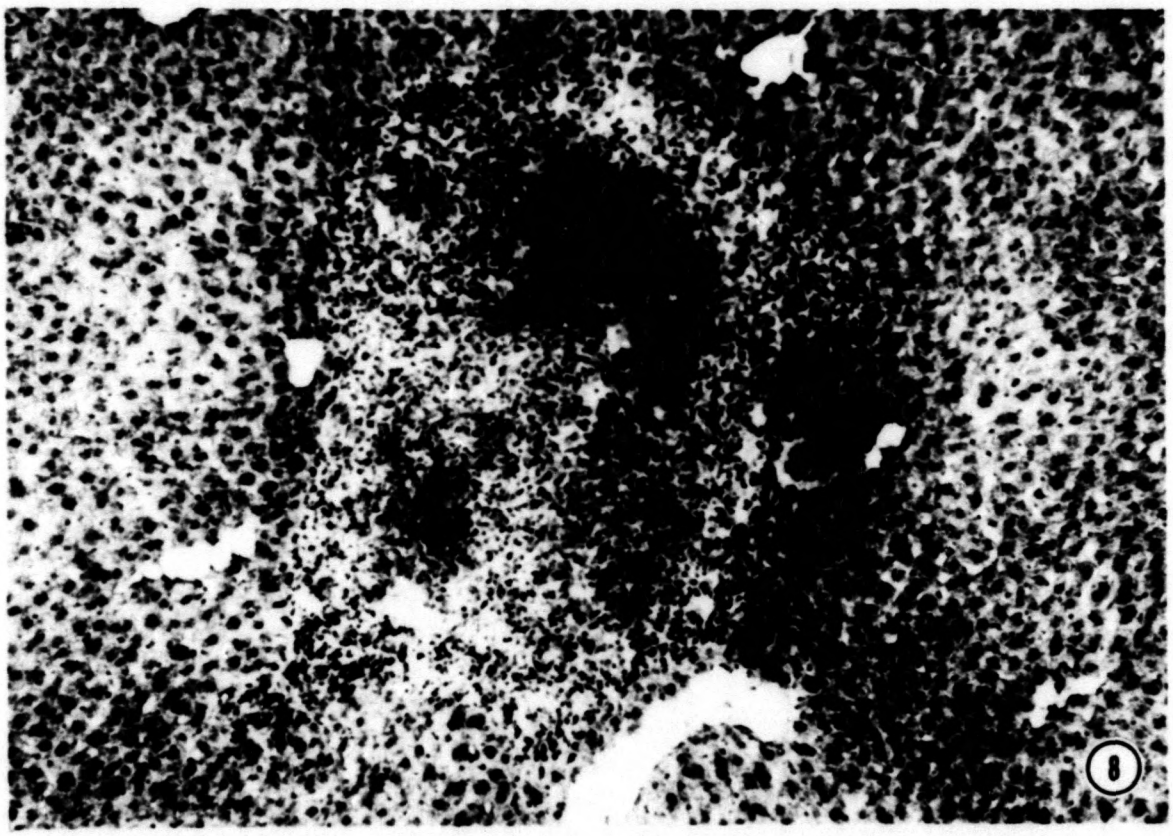
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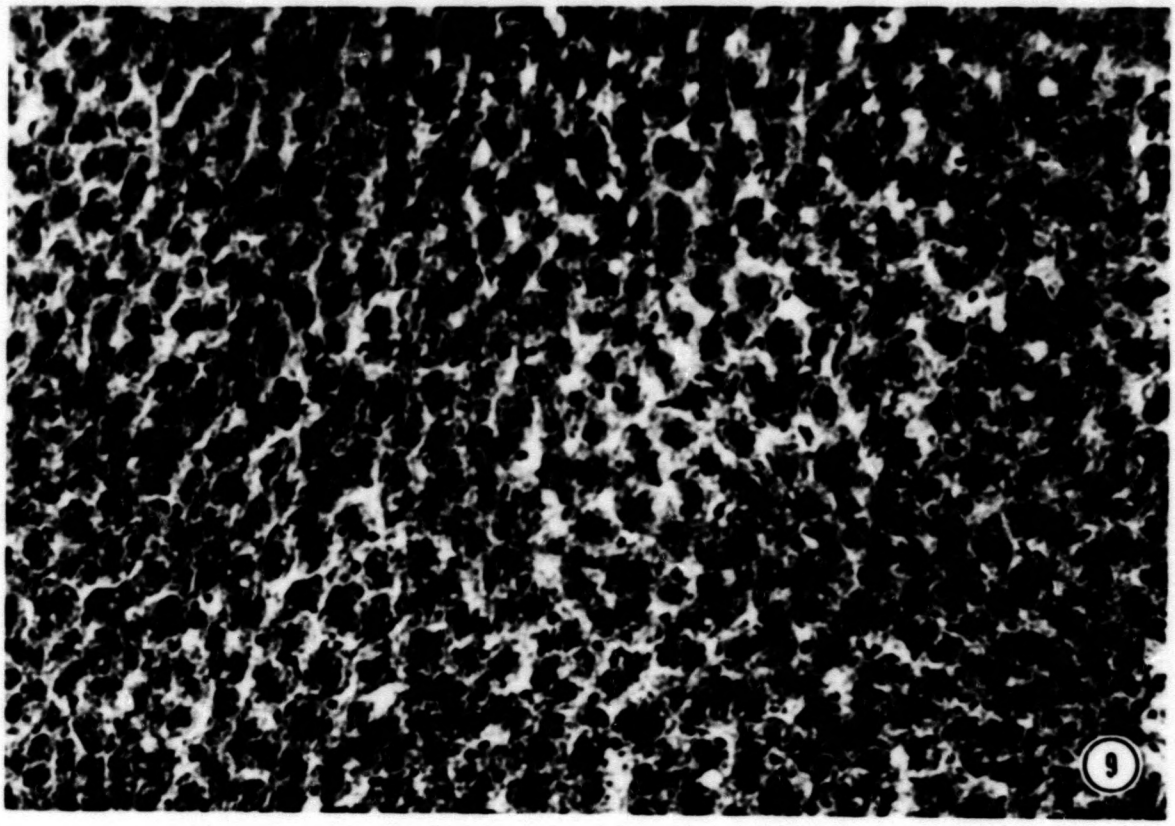
Figure 9. Normal appearing liver tissue of a control mouse injected intraperitoneally with Tyrode's solution. There is no evidence of any stage of lesion formation. x 300.

Figure 10. Advanced lesion in the liver of a mouse resulting from *per os* intubation of a lipid extract of the metabolic by-products obtained from 10-day-old *Hymenolepis microstoma*. Lesion formation is similar to that previously described. x 300.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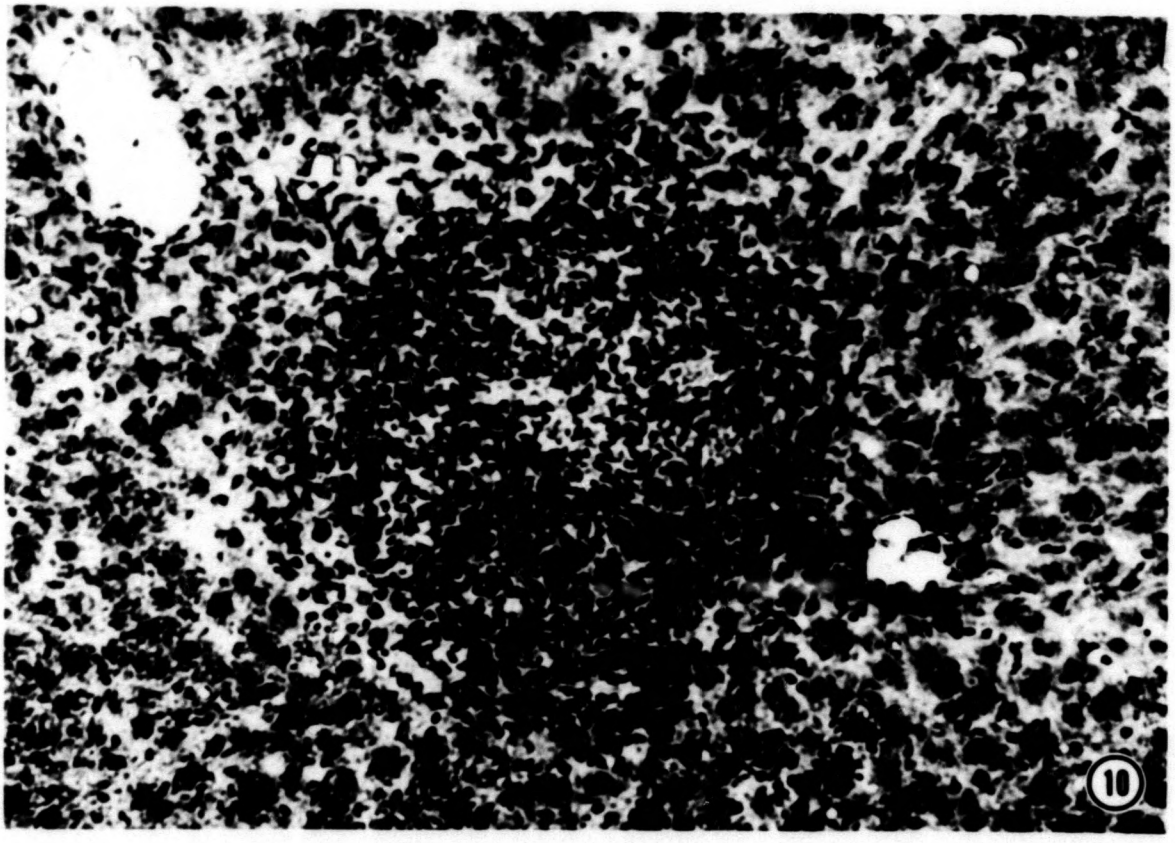
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construed as very early lesion formation or perhaps only extensive leucocyte infiltration. Control mice had no evidence of lesion formation.

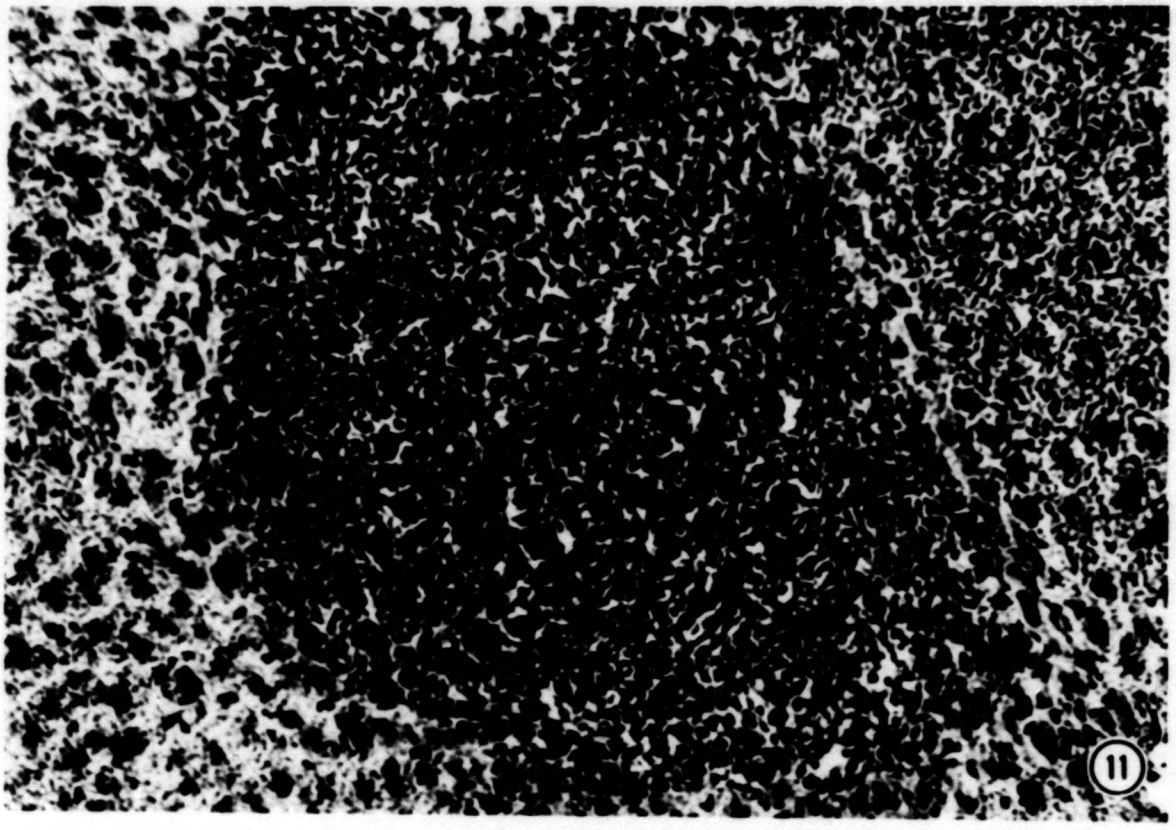
In Exp. IV, per os administration of a lipid extract of metabolic by-products from 10-day-old worms resulted in lesion formation in the liver of 4 of 5 of the experimental mice. Observed lesions were similar to those described previously (Fig. 10). No lesion formation was evident in either of the control groups employed in this experiment.

Of the 4 molecular weight ranges tested in Exp. V, only 1 range resulted in formation of lesions when administered per os to the experimental mice (Fig. 11). This was the less than 10,000 molecular weight filtrate. No formation of lesions resulted in the other 3 ranges: (1) between 10,000 and 30,000; between 30,000 and 100,000; and (3) greater than 100,000. Control mice showed no evidence of lesions.

The lesions formed as a result of 6-worm infections in the experimental mice of Exp. VI were markedly similar to those which were passively induced with metabolic by-products in Exp. I, II, IV, and V. Comparison of Fig. 12 with Fig. 2,3,4,7,10, and 11 readily demonstrated this fact.

Figure 11. Advanced lesion in the liver of a mouse resulting from *per os* intubation of the less than 10,000 molecular weight filtrate of metabolic by-products obtained from 10-day-old *Hymenolepis microstoma*. The lesion has all the characteristics of lesions formed as a result of administration of the metabolic by-products not subjected to ultrafiltration. x 300.

Figure 12. Advanced lesion in the liver of a mouse infected with 6 *Hymenolepis microstoma*. The lesion is comparable to lesions induced by administration of worm metabolic by-products. x 300.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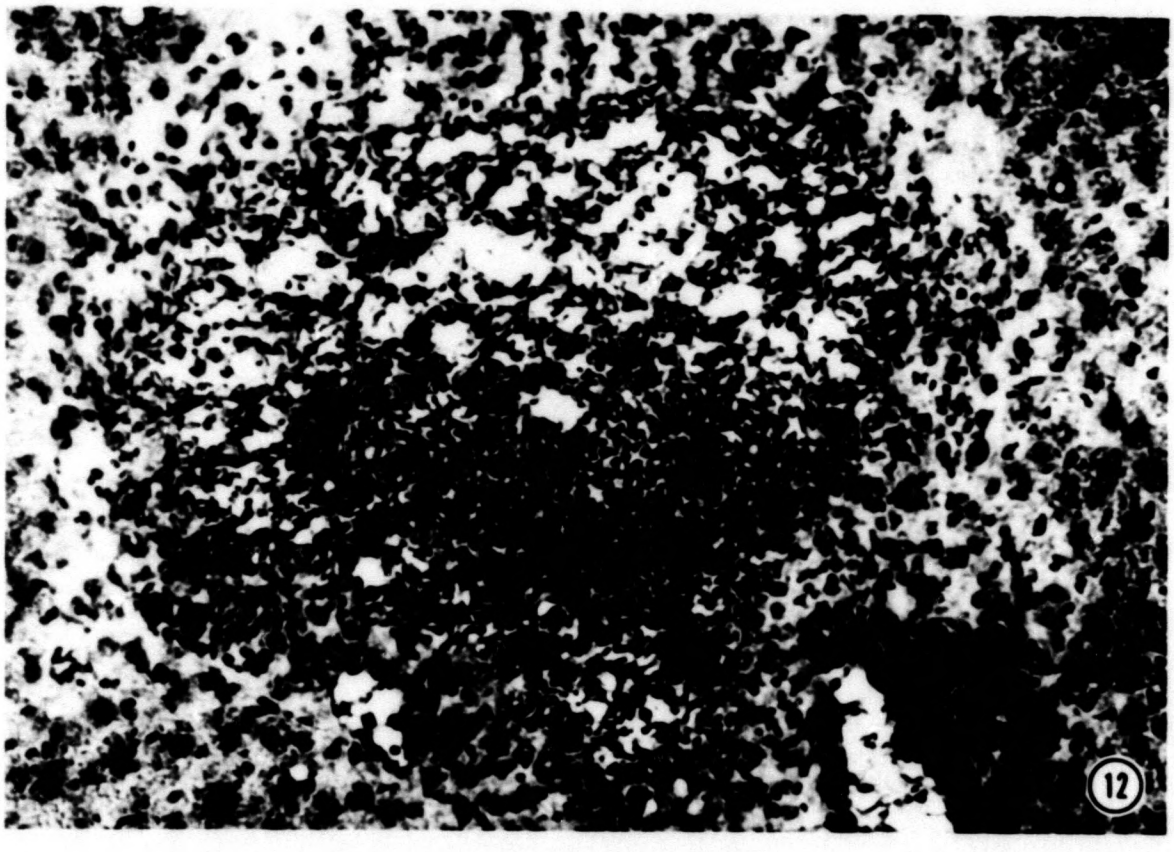
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DISCUSSION

The results of this study indicated that the causative agent of liver lesion formation in mice infected with *Hymenolepis microstoma* is one or more metabolic by-products. The study also revealed that the causative agent is equally effective by either intraperitoneal injection or *per os* intubation as the mode of administration. It was also determined that the causative agent is contained within a lipid extract and has a molecular weight of less than 10,000.

Experiment I and II (i.p. and p.o. administration, respectively, of metabolic by-products from 10-day-old worms) demonstrated that the mode of administration did not affect lesion formation. It is significant that the causative agent, when administered p.o., was not deactivated or destroyed by the digestive juices and acidic conditions of the stomach. Consequently, the causative agent appears to be relatively stable and thus would be easier to work with than some other compound with less stability.

The 2 different modes of administration also lend themselves to different routes of absorption and transportation to the liver of the lesion-causing agent. The intraperitoneally injected by-products could have been carried by the lymph to the circulatory system and then carried to the

liver, or the by-products could have been absorbed into the blood vessels of the peritoneal cavity. The *per os* administered by-products were more than likely absorbed by the intestinal villi and then carried by the hepatic portal system to the liver. Irregardless of the means of transport to the liver, the results of both modes of administration were the same: liver lesion formation similar to that described for mice actually infected with *H. microstoma*.

It is significant that the different stages of induced lesion formation using metabolic by-products were very similar to those observed in mice infected with *H. microstoma*, thus indicating a common origin.

Experiment III, using metabolic by-products from 30-day-old worms, was performed in order to determine if the age of the worms affected lesion formation, since Gleason (1971) reported a decrease in lesion formation after about Day 20 of the infection. Results of this experiment indicated that the age of the worm did affect the lesion formation since the metabolic by-products from 30-day-old worms did not cause well-developed, observable lesions within the parameters of the experimental technique employed. There may, however, be a possible explanation for this. Throughout all the experiments, the worm mass per ml of Tyrode's solution used was held as constant as possible. Consequently, the majority of the worm mass used in Experiment III consisted of mature and gravid proglottids whereas in the experiments using 10-day-old worms, the worm mass consisted predominantly of im-

mature proglottids. Due to these circumstances, it is feasible that in Experiment III the majority of the metabolic by-products were of the type excreted from mature and gravid proglottids whereas the metabolic by-products used in the experiments with 10-day-old worms were from immature proglottids. This could possibly be tested by using metabolic by-products obtained from 30-day-old worms from which the mature and gravid proglottids had been previously removed or by using metabolic by-products from an increased worm mass of 30-day-old worms per ml of Tyrode's solution used, thereby compensating for the decrease in immature proglottids in Experiment III. By use of either of these procedures, one might reasonably expect the same results, in respect to lesion formation, that was obtained using metabolic by-products from 10-day-old worms. It also seems probable that the number of immature proglottids would remain relatively constant during worm development. Consequently, one would not expect the amount of lesion-causing metabolic by-products given off by the immature proglottids to decrease as the age of the worms increased in an infection of mice with H. microstoma. Therefore, a possible explanation for decrease in the rate of lesion formation in infected mice after Day 20 of an infection with H. microstoma may be due to a partial humoral immunity to the metabolic by-products carried by the circulatory system rather than due to the age of the worms.

In experiment IV, the lesions formed by use of a lipid extract were quite similar to those observed in all other

experiments. The route of administration, *per os*, demonstrated once again that the causative agent of lesion formation contained in the lipid extract was not degraded or destroyed in the stomach.

Experiment V, in which 4 molecular weight ranges of the metabolic by-products were tested, yielded results which showed the causative agent to be relatively small. The lesion-causing agent was found in the less than 10,000 molecular weight range and yielded lesions comparable to those observed in the other experiments.

Experiment VI was performed to determine if induced lesions (using worm metabolic by-products) were comparable to lesions formed in an infection with *H. microstoma* and to provide supporting evidence that worm metabolic by-products were instrumental in lesion formation in the liver. The experimental mice, which were given 6-worm infections, were killed 5 and 11 days after initial infection. This was done in order to provide a time range for lesion formation in worm-infected mice similar to that for the lesion-induced mice. Lesions formed in this experiment were comparable in structure to those described for all other experiments. The number of lesions formed, however, appeared to be higher in the mice actually infected with *H. microstoma*. A possible reason for this could be that the amount of metabolic by-products available to cause lesion formation might have been much higher in the mice infected with *H. microstoma* than in the experimental mice which were administered worm metabolic

by-products.

The variation in the number of experimental mice which had liver lesions, in the different groups, could have been the result of individual susceptibility and/or genetic make-up. This was suggested by the differences in the number of lesions formed among the various experimental mice. It appeared that the ICR/DUB strain mice were somewhat more resistant to metabolic by-products lesion formation than were the outbred strain mice. Variation in responses among groups of the same strain could have been due to environmental changes such as stress, temperature, humidity, and other factors which were not controlled with the limited facilities available for animal care.

A correlation between the effects of metabolic by-products of H. microstoma on liver lesion formation and the clinical symptoms of a human infection of H. nana, a serious public health problem when found in small children, is possibly suggested by the results obtained in the present study. Larsh (1964) reported the clinical symptoms of a human infection of H. nana as being abdominal pain, diarrhea, lack of appetite, insomnia, irritability, dizziness, and rarely epileptiform convulsions. Larsh also reported that these symptoms are generally accepted, though not proven, to be due to the absorption of metabolites produced by the cestodes located in the intestinal tract. The present study seems therefore to indicate that the clinical symptoms accompanying a human infection of H. nana could be due to the absorption

into the blood stream of worm metabolic by-products, causing a generalized toxemia.

Additional evidence pointing to the possibility of the effects of worm metabolic by-products in a human infection of H. nana as well as the possibility of actual human infections of H. microstoma, although none have ever been reported, is the close relationship of these two cestodes. In 1961, Dvorak, et al. reported a striking similarity between the eggs of H. nana and H. microstoma, which apparently can only be distinguished by scrutinous microscopic examination by highly trained technicians. The difference is primarily two-fold: (1) a very minute size difference in the eggs themselves, and (2) a difference in the number of polar filaments found within the embryophore of the embryonated egg. Thus, it does not seem unreasonable to assume, since the anatomy and embryogenesis of the two worms are so closely associated, that the aspect of metabolite formation would also possibly be quite similar. Since a fecal smear in which eggs would be found is the positive diagnostic test for either cestode, a technician could easily mistake H. microstoma eggs for H. nana eggs or vice versa.

The conventional treatment for H. nana, an intestional cestode, would not be entirely effective for an infection with H. microstoma, in which the majority of the worm is located in the common bile duct with only part of the gravid proglottid section of the strobila located in the upper intestional tract. Consequently, the study of the worm meta-

bolic by-products of these two cestodes may lead to important discoveries in the field of public health in respect to helminth parasite diseases.

The present study also revealed that the causative agent is a relatively stable compound of low molecular weight and thus would allow for a relatively easy research topic with which to work. This makes possible many research approaches with respect to liver lesion formation in mice using the metabolic by-products of H. microstoma. For example, the causative agent due to its stability could be easily concentrated and analyzed biochemically in several different ways such as determination of exact molecular weight and organic composition. As suggested earlier, metabolic by-products of sexually mature worms, from which only immature proglottids were taken, could be used to determine if the causative agent is actually produced only by immature proglottids. Another area of study could be the administration of worm metabolic by-products to different strains of mice or perhaps to different age and weight mice to determine the factors which play a role in host susceptibility to lesion formation.

SUMMARY

This research showed that metabolic by-products from 10-day-old Hymenolepis microstoma worms, when given to 2 different strains of mice by 2 different routes of administration, resulted in formation of lesions in the parenchyma of the liver of the experimental mice. Similar lesions were formed in an outbred laboratory strain and an inbred ICR/DUB strain of mouse, when given metabolic by-products by either intraperitoneal injection or per os intubation.

Worm metabolic by-products from 30-day-old worms did not appear to cause lesions in the liver of the experimental mice. This could have been due to the experimental procedure used; therefore, further studies need to be conducted concerning the age and type of proglottids of worms from which the metabolic by-products are taken.

Further studies showed that the lesion-causing substance could be isolated in a lipid extract of the metabolic by-products. Molecular weight studies showed that the causative agent appeared to have a molecular weight of less than 10,000.

Lesions formed as a result of administration of metabolic by-products of H. microstoma were comparable to lesions formed as a result of an infection with H. microstoma. This indicates a common causative agent.

Finally, this study gave some support to the assumption that metabolic by-products of cestodes, such as Hymenolepis nana, when found in humans, may actually be the causative agent(s) of clinical symptoms which accompany the infection. This knowledge could become significant in the field of public health.

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