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Relationships between Anthelmintic Effects of Drugs against *Echinococcus multilocularis in Vitro* and *in Vivo*

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Introduction

The most important problem in the chemotherapy of animals suffering from hydatidosis is the discovery of a drug quite effective against larval *Echinococcus*. However, there is no available drug exhibiting apparently anthelmintic action against larval echinococcosis up to the present. Accordingly, many investigators tried to test the anthelmintic action of many drugs against *Echinococcus granulosus*[2,10,11,14] and *E. multilocularis*[5,8,9,12,13] *in vitro*. Recently, some attempts[1,3,4,6,7] at empirical screening of several drugs in respect of their inhibitory actions on the growth of larval *Echinococcus* were made, using laboratory animals suffering from secondary echinococcosis. However they have been limited to either *in vitro* or *in vivo* experiments, and the relationships between anthelmintic actions of drugs *in vitro* and those *in vivo* have not been discussed sufficiently.

For some years, using mice infected experimentally, the author tested the anthelmintic actions of many drugs showing significant scolicidal effects *in vitro*. In the present experiment, the anthelmintic activities of the four salicylanilide derivatives, one carbanilide derivative and one bisphenol derivative against *E. multilocularis in vitro* and in mice were tested.

Materials and Methods

This experiment includes two series as in the following:

1. In vitro screening test

Protoscoleces were collected aseptically from the liver of cotton rats experimentally infected with *Echinococcus multilocuralis*, and were washed several times with Hanks' solution.

Medium 199 (morgan et al. 1950) was used as a basal culture medium. To the medium 199 inactivated calf serum at a rate of 20%, 100 units of penicillin G and 100 γ of streptomycin sulfate per ml were added prior to the incubation of protoscoleces.

The drugs were dissolved or suspended at the concentration of 2 mg per ml in 70% propylene glycol solution. Those solution or suspensions were added to the culture medium in the concentrations of 10, 40 and 100 γ per ml. Three concentrations of each compound were tested three times. The morphological observation of the protoscoleces was carried out for ten days, using standard and phase contrast microscopy and supravital staining

Table 1. Effect of drugs upon the survival rates of protoscoleces *in vitro*

Drug	Dose (γ/ml)									
	10					100				
	Day of incubation					Day of incubation				
	2	4	6	8	10	2	4	6	8	10
Sodium antimony gluconate	87.0	51.0	33.4	24.1	21.2	62.1	35.5	2.6	2.5	0
Thymol	90.6	80.5	42.8	28.2	23.6	21.0	2.7	0	0	0
Chlorothymol	99.7	94.8	37.1	30.2	14.9	39.9	0	0	0	0
Thymol iodide	99.0	49.6	30.4	14.5	8.2	96.8	15.2	5.3	0	0
Dithiazanine iodide	92.9	70.0	49.7	24.1	0.8	64.5	0	0	0	0
Gentian violet	57.0	50.4	0.2	0	0	0	0	0	0	0
Dichlorophen	15.9	0	0	0	0	0	0	0	0	0
Bithionol	0.6	0	0	0	0	0	0	0	0	0
2, 2′-Thiobis (4-chloro-6-nitrophenol)	15.8	9.1	0	0	0	0	0	0	0	0
3, 5-Dibromosalicylanilide	7.4	0	0	0	0	0	0	0	0	0
3, 5, 4′-Tribromosalicylanilide	0.1	0	0	0	0	0	0	0	0	0
3, 5-Dibromo-3′-trifluoromethyl-salicylanilide	3.2	0	0	0	0	0.9	0	0	0	0
3, 5, 4′-Trichlorosalicylanilide	0	0	0	0	0	0	0	0	0	0
3-Trifluoromethyl-4, 4′-dichloro-carbanilide	0.9	0	0	0	0	0	0	0	0	0

Percentage of survivors to average of those in controls

with 0.2% neutral red and 0.02% Janus green. The scolicidal effect of the drugs was indicated by the rates of survivors in each medium to the average of those in controls. Of many drugs screened *in vitro*, the data of 14 drugs used in the therapeutic experiments using mice, were shown in Table 1. To observe the effect of propylene glycol, some of the drugs were suspended in the same concentrations in the medium containing 0.3% sodium carboxymethyl cellulose, and the scolicidal effect of the drug in the medium without propylene glycol was compared with that in the previous medium.

2. Therapeutic experiment in mice

To compare with the *in vitro* screening test, the results of therapeutic treatment with 8 drugs, which were tested *in vitro* in the first experiment, were shown in Table 2.

In the second experiment, the following drugs were tested: 3,5-dibromosalicylanilide, 3,5,4′-tribromosalicylanilide, 3,5-dibromo-3′-trifluoromethylsalicylanilide, 3,5,4′-trichlorosalicylanilide and 3-trifluoromethyl-4,4′-dichlorocarbanilide. The mice used were infected by oral administration of about 300 oncospheres of *E. multilocularis* one month before the treatment. Each drug was prepared as the solution at the concentration shown in Table 3 in 0.1 ml of 70% propylene glycol. Besides the above drugs, Mintes-B (a suspension of 15% 3,5-dibromosalicylanilide and 85% 3,5,4′-tribromosalicylanilide in saline solution with 1% sodium carboxymethyl cellulose, Kaken Chemical Co., Ltd) and a suspension of 2,2′-thiobis(4-chloro-6-nitrophenol) in saline solution containing sodium carboxymethyl cellulose at a rate of 1% were diluted at the desired concentration with the same solution. Each dosage shown in Table 3 was given twice a week for 15 weeks. Mice were dissected a week after the last administration. The foci in the liver were examined macroscopically, with the detection of small foci under a zoom-lens binocular microscope, and the liver was weighed. The sections were made for microscopical investigation. The results shown in Table 3 were evaluated statistically.

Table 2. Inhibitive efficacy of drugs against echinococcal tissue of CF-1 mice*[1] in the first experiment

Drug	Daily dose in mg/kg	Administration		Courses		No. of Mice	Macroscopical findings*[2]					Histological findings*[3]			
		Route	Duration	No.	Interval		++++	+++	++	+	−	++++	+++	+	−
Control						8	5		1		2	2	2	2	
Sodium antimony gluconate	200	im	5 times on alternate days	5	1 week	15	3	2			10	1	1	3	
Bithionol	6.4	im	daily for 5 days	5	1 week	20	14		2		4	11	1	5	4
Thymol palmitate	20	im	5 times on alternate days	5	1 week	32	12	4	4		12	11		11	12
Dithiazanine iodide	2	im	daily for 5 days	5	1 week	8	4		4			8			
Control						4	4					4			
Dithiazanine iodide	2	im	daily for 5 days	4	1 week	30	10	1	4	2	14	9		8	14
Control						10		2	4	4		2	1	7	
Gentian violet	2	im	twice a week	2	0	9	3	6				3	2	4	
Chlorothymol	40	im	3 times a week	4	0	8	1	1	4	2		3	3	2	
Thymol iodide	40	im	3 times a week	4	0	7	2	3			2		1	6	
Thymol iodide	40	im	4 times a week	2	0	7				2	5			7	
Dichlorophen	40	im	4 times a week	2	0	8				5	3	1		7	
Dichlorophen	80	po	4 times a week	2	0	5			1	2	2			5	

*[1] Mice were infected by oral inoculation of about 300 eggs of *Echninococcus multilocularis*.

*[2] Plus signs indicating the macroscopical findings

++++ A great number of large and small cysts are distributed over the liver.
+++ Several large foci consisting of large and small cysts are scattered in the liver.
++ A few small foci consisting of several small vesicles are scattered in the liver.
+ A few free vesicles are scattered in the liver.
− There is no cyst. In some cases, scars are found on the surface of the liver.

*[3] Plus signs indicating the histological findings

+++ Active formation of brood capsule and protoscolex.
++ Initial formation of brood capsule.
+ Cysts without brood capsule and protoscolex formation.

Table 3. Inhibitive efficacy of drugs against echinococcal tissue of CF-1 mice in the second experiment

Drug	Daily dose in mg/kg	Route	LD_{50} mg/kg	Survivors	Liver weight	Percentage of liver weight to control	Macroscopical findings*1					Histological findings*1			
							++++	+++	++	+	−	+++	++	+	−
Control				10/10	7.62 ± 2.29		8	2				10			
Mintes-B*2	15	im	75	8/10	6.97 ± 2.07	91.45 ± 22.75	5	1		1	1	5	2	1	
2,2′-Thiobis (4-chloro-6-nitrophenol)	10	im	209	10/10	8.53 ± 3.43	111.94 ± 47.43	7		1		2	7	1	1	1
Control				10/10	20.19 ± 6.92		8	2				10			
3,5-Dibromosalicylanilide	1	im	108.5	10/10	4.81 ± 4.49	23.85 ± 19.49	3		3	3	1	4	2	3	1
3,5,4′-Tribromosalicylanilide	5	im		7/10	1.88 ± 0.50	9.34 ± 2.49			4	2	1		3	3	1
3.5.4′-Trichlorsalicylanilide	8	im	26.4	8/10	2.05 ± 0.47	10.15 ± 2.38		2	1	2	3	2		3	3
Control				10/10	6.84 ± 1.17		8	2				9	1		
3,5-Dibromosalicylanilide	30	po		9/10	5.32 ± 2.38	77.76 ± 4.31	5	2	1	1		4	3	1	1
3,5,4′-Tribromosalicylanilide	30	po	250	7/10	2.60 ± 0.61	38.06 ± 9.69	1	2	2		2	3	1	1	2
3,5-Dibromo-3′-trifluoromethyl-salicylanilide	30	po	2,000	9/10	3.74 ± 1.01	54.66 ± 14.83	6	1	1	1		5	2	2	
3,5,4′-Trichlorosalicylanilide	15	po		8/10	1.99 ± 0.45	29.17 ± 7.06	3	1	1	3		2	1	3	2
3-Trifluoromethyl-4, 4′-dichloro-carbanilide	15	po	2,250	9/10	1.82 ± 0.22	26.64 ± 7.29	2			4	3	2		4	3
2,2′-Thiobis (4-chloro-6-nitrophenol)	30	po	310	10/10	3.54 ± 1.38	51.77 ± 20.15	5	3	2			7		3	
Mintes-B*2	30	po	250	7/10	2.14 ± 0.73	31.34 ± 11.48	1	2	1	1	2	1	2	2	2

*1 The plus signs represent the same findings as those in Table 2.

*2 Mintes-B is a suspension of 15% 3,5-dibromosalicylanilide and 85% 3,5,4′-tribromosalicylanilide in saline solution with 1% sodium carboxymethyl cellulose.

Results

The survival times of protoscoleces in the media containing drugs at the rates of 10 and 100 γ per ml are shown in Table 1. The most rapid decrease of survival protoscoleces was observed in the medium containing the derivatives of salicylanilide, followed by derivatives of bisphenol and carbanilide. A decrease in the rate of survivors in the media with salicylanilde derivatives became progressively intense in the following order: 3,5-dibromosalicylanilide, 3,5-dibromo-3′-trifluoromethylsalicylanilide, 3,5,4′-tribromosalicylanilide and 3,5,4′-trichlorosalicylanilide.

The scolicidal effect of salicylanilide and bisphenol derivatives suspended in the medium containing 0.3% sodium carboxymethyl cellulose was lower than that of drugs in the medium with propylene glycol.

The results obtained in therapeutic experiment of mice carried out with 8 drugs in the first experiment are shown in Table 2. Of the drugs tested, only dichlorophen and thymol iodide in propylene glycol solution revealed a slight inhibitive effect against the growth of echinococcal tissue in the macroscopical and histological findings.

The liver weight, its ratio to the average of controls, and macroscopical and histological findings of the liver of mice in the second experiment are shown in Table 3. The inhibitive effect of the injection of salicylanilide derivatives in propylene glycol solution was significantly higher than that of the oral administration. In cases in which the drugs were injected intramusclarly, 3,5,4′-tribromosalicylanilide and 3,5,4′-trichlorosalicylanilide having three halogens were more effective than 3, 5-dibromosalicylanilide possessing two bromines. In the histological examination, apparently degenerative changes such as collapsed and ruptured cysts and the organization of degenerated cysts were recognized in the hydatid tissue of the mice injected the suspensions of salicylanilide derivatives having three halogens in propylene glycol. Mintes-B and the suspension of 2,2′-thiobis-(4-chloro-6-nitrophenol) in 1% sodium carboxymethyl cellulose solution were ineffective in the cases in which the drugs were injected intramusclarly. The oral administration of these suspensions was slightly effective. The effect was significant at 1% level. In case of oral administration, 3-trifluoromethyl-4,4′-dichlorocarbanilide was most effective, followed by 3,5,4′-trichlorosalicylanilide. 3,5,4′-tribromosalicylanilide and Mintes-B showed inhibitive efficacy at the 1% level of significance. Concerning the mortality of the treated mice, there was a great tendency to increase simultaneously with the rising of the inhibitive effect of drugs.

Discussion

Lubinsky[6] and Lubinsky et al.[7] reported that some antineoplastic agents inhibited the growth of secondary *Echinococcus multilocularis* in mice. Krotov et al.[4] and Heath et al.[3] reported lethal effect of mebendazole against *E. multilocularis* and *E. granulosus*, respectively. Campbell and Blair[1] reported the effect of thiabendazole and cambendazole against *E. multilocularis*. However, all of these drugs were tested in extremely high or toxic doses.

Schwabe et al.[14], Meymerian et al.[10], Frayha et al.[2] and Sakamoto and Gemmell[12] explored the *in vitro* screening test, using protoscoleces of *E. granulosus*. On the other

hand, Lukashenko et al.[8,9] and Kovalenko[5] tested the inhibitory effects of drugs on the respiratory rate of protoscoleces of *E. multilocularis*.

Sakamoto[11] and Sakamoto et al.[13], and Sakamoto and Gemmell[12] reported that some of halogenized derivatives of salicylanilide and bisphenol showed high scolicidal activity on the larval *E. multilocularis* and *E. granulosus in vitro*, respectively. They observed that the intensity of scolicidal action of those derivatives increased together with the increase of the number of halogen atoms, and that the presence of propylene glycol in the medium shows a tendency to increase the intensity of scolicidal action of drugs insoluble in water. The results obtained in the present *in vitro* screening test were also the same as mentioned above. The relationships between the chemical structure and the cestocidal action of the drugs observed in the above *in vitro* test, were recognized in the therapeutic experiment also. Besides, there was a great tendency, about the toxicity upon the treated mice, to increase with the rising of the anthelmintic effect of drug. As far as the present results are concerned, some relationships lying between the anthelmintic efficacy of drugs *in vitro* and *in vivo* are recognized.

Summary

The protoscoleces of *Echinococcus multilocularis* were incubated in the medium to which drugs were added at the rates of 1, 40 and 100 γ per ml. The survival rates of the treated, and the control protoscoleces were compared. Generally, halogenized salicylanilide- and bisphenol-derivatives showed high scolicidal effect. The intensity of the scolicidal action of salicylanilide derivatives increased with the addition of halogen atoms. The mice inoculated with about 300 oncospheres of *E. multilocularis*, were treated with drugs showing scolicidal activity *in vitro*. The results obtained in the therapeutic experiment were compared with that of the *in vitro* screening test. The inhibitive effect of the suspension of salicylanilide derivatives in propylene glycol by injection was higher than that by oral administration. The relationships between the chemical structure and anthelmintic action of those derivatives *in vitro* as mentioned above were recognized in the therapeutic experiment. Accordingly, it was conjectured that there is something in common between the anthelmintic activity of drugs *in vitro* and that *in vivo*.

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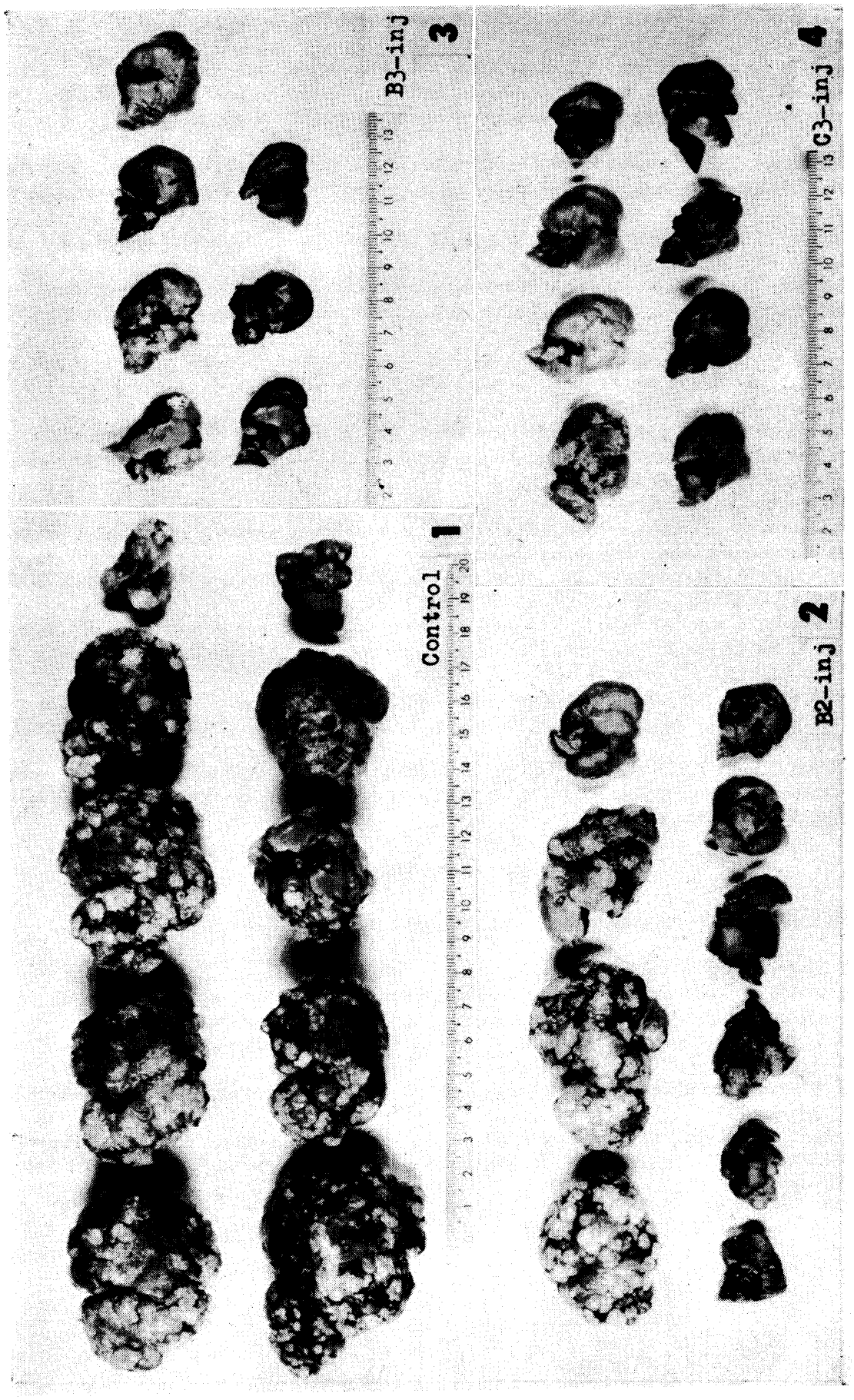
Explanation of plates

Figures 5 ~ 8 are photomicrographs of specimens stained with hematoxylin-eosin.

Fig. 1 Livers of control mice
Fig. 2 Livers of mice treated with intramusclar injection of 3, 5-dibromoscalicylanilide
Fig. 3 Livers of mice treated with intramusclar injection of 3, 5, 4′-tribromoscalicylanilide
Fig. 4 Livers of mice treated with intramusclar injection of 3, 5, 4′-trichlorosalicylanilide
Fig. 5 Hydatid tissue in liver of control mice
Fig. 6 Ruptured hydatid cyst in liver of mice treated with intramuscular injection of 3, 5-dibromosalicylanilide
Fig. 7 and 8 Degenerated hydatid cyst surrounded by granulation tissue in liver of mice treated with intramuscular injection of 3,5,4′-tribromosalicylanilide and 3,5,4′-trichlorosalicylanilide, respectively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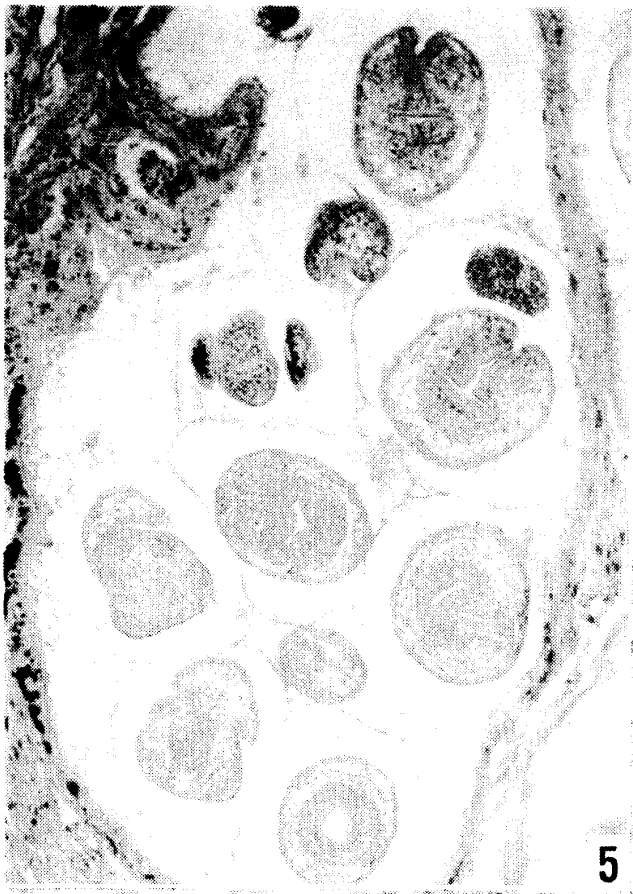
B2-inj 2
B3-inj 3
C3-inj 4



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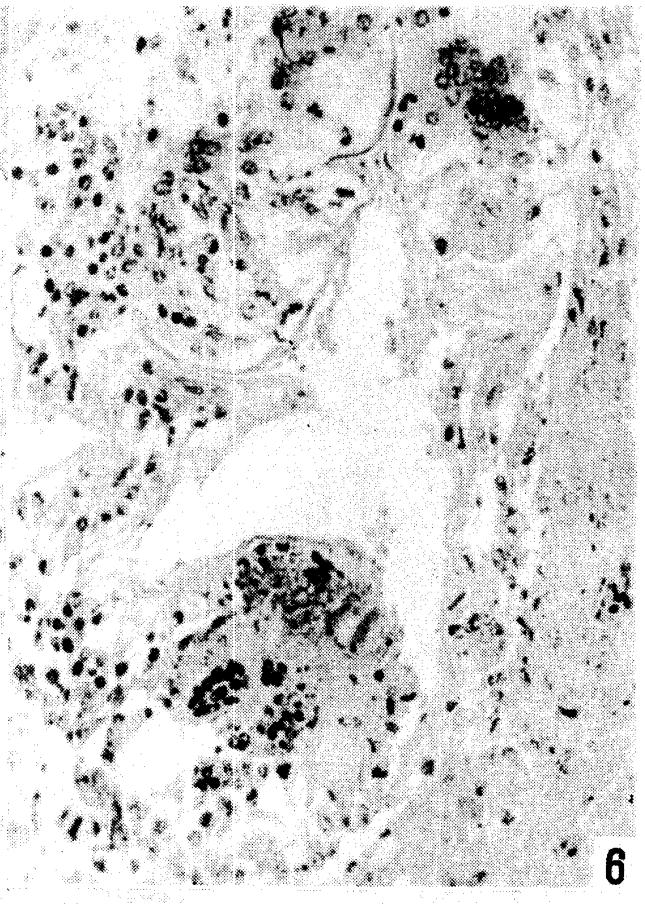

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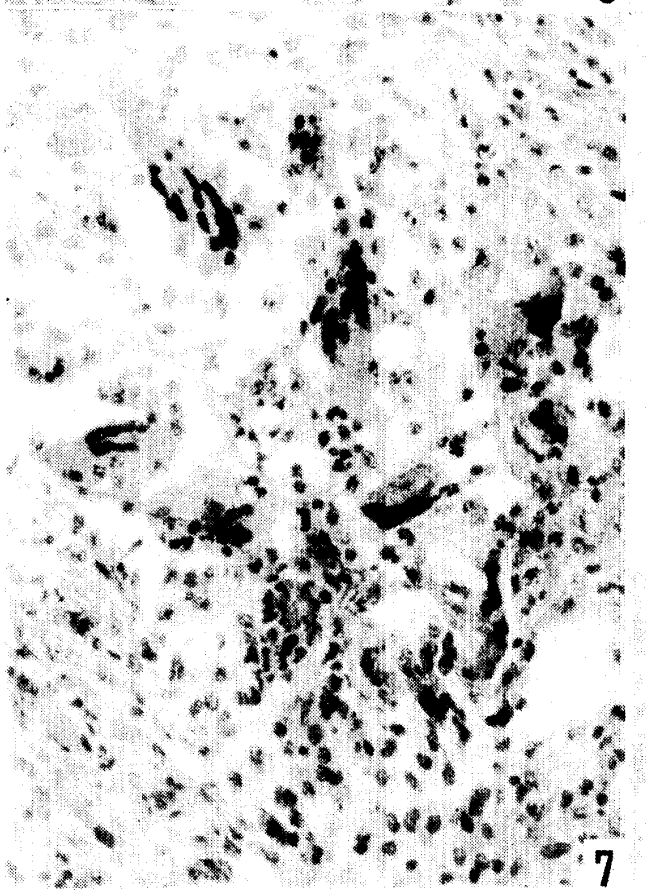

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