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Studies on the effect of endoxan, an anti-tumor substance, to promote the growth of *Nosema cuniculi* *in vivo* and *in vitro*

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Nosema cuniculi was first found in rabbits by Wright and Craighead (1922) and was designated *Encephalitozoon cuniculi* by Levadit *et al.* (1923). The same parasite was found in rabbits and/or mice by a number of authors while they were studying human virus diseases. Nelson (1962) found by the examination of fresh materials that the organisms released a wavy filament and suggested that the parasite might be a member of the Order *Microsporidia*. Lainson *et al.* (1964) was able to release the polar filament from the spore, and also demonstrated it by electron microscope observation and confirmed that the parasite was *Nosema*. Weiser (1964) also expressed the same opinion based on his light microscope observations.

Whether or not *N. cuniculi*, a common parasite of rodents, would be found parasitic in man is a matter of medical importance. Manhuélian & Viala (1924) and Garnham & Roe (1954) inoculated human material into mice and found *Nosema*-like bodies in liver and spleen of the animals. But, there was no proof that these organisms originated from the human material inoculated, because mice are very often infected spontaneously with *Nosema*. Three reports have so far been published in which *Nosema*-like bodies were found directly in human materials. Torres (1927) found *Nosema*-like bodies in brain, muscle and subcutaneous fat tissues of a new-born infant. Coulon (1927) also found *Nosema*-like bodies in the spinal fluid of a 17-year old boy who died of acute meningitis symptoms. However, organisms reported from these two cases are not always morphologically identical with *Nosema*. Matsubayashi *et al.* (1959) found *Nosema*-like bodies in spinal fluid and urin of a 9-year old boy who was suffering from encephalitis symptoms. This is the most probable case of *Nosema* infection in man hitherto reported.

The original intention of this study is to make clear how often man may be infected with *Nosema* and the present work deals with establishment of a method of isolation of the parasite from human being.

Materials and Methods

The strain of *Nosema cuniculi* used in the study.

Mice are believed to be often infected with *Nosema cuniculi*. The only method to detect the infection without sacrificing the animals is to examine the peritoneal macrophage cells. *At random* examinations of the peritoneal fluid, however, rarely demonstrate the parasite, probably due to the extreme paucity in number, even if the animals are infected. Koike *et al.*

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(1960) tried serial blind passages of emulsion of brain, liver and spleen of those apparently negative mice. Among 20 series, 10 became positive for the parasite within four passages. The same procedures were taken in the present study. The passage was made every two week interval and a strain was obtained after 6 passages. This strain is maintained in mice by subinoculation being made every two week interval.

Method of identification of the parasite.

Mouse peritoneal fluid was drawn with a capillary and smears made of it were dried, fixed with methanol and stained with Giemsa. The smears were also wet-fixed by Schaudinn's solution and stained with trichrome stain after Wheatley (1951). The latter method gave a fine view of the parasite which was stained purple red against light blue host cell protoplasm. Parasites gowing in tissue culture were stained by the same methods.

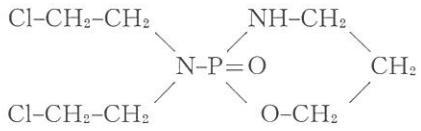
Results

1) Attempt to promote the growth of *Nosema cuniculi* in the pertoneal cavity of mice.

Nosema cuniculi, when inoculated into peritoneal cavity of mice, grows in the macrophage cells. One cell may contain one to fifty or more parasites. The highest population of the parasite is usually obtained about two weeks after the inoculation (Fig. 1). Even at this time, however, the number of the infected cells is very small and it takes much time for the microscopic examinations to find out the parasite. The possibility of missing the parasite is great even if mice being examined are infected. Good growth of the parasite in the peritoneal cavity of mice is highly desirable for the detection of the infection. Matsubayashi *et al.* (1957) tried to promote the growth of the parasite by a number of procedures such as intraperitoneal injections of gastric mucin, hyaluronidase, glycogen, cortisone, antihistamine or by X-ray irradiation, splenectomy etc. All of these procedures had no effect.

In the present study, cortisone was also tried. A daily dose of 5 mg of 17-oxy-11-dehydrocorticosteron was injected intraperitoneally for 2–3 days after the inoculation of the parasite. Mice often died of the toxic effect of the drug and even in cases surviving the effect, the population of the parasite in the peritoneal cavity did not increase.

Endoxan, an anti-tumor substance having the following chemical composition was tried.



This substance is believed to inhibit the protain metabolism. The experiment was carried out with 4 groups of mice. Each group was consisted of 4 endoxan-mice and 4 control mice. They were inoculated with the same amount of the same inoculum which was the mixed emulsion of brain, liver and spleen of an infected mouse. Those organs, added with 10 ml amount of normal saline, were emulsified in a homogenizer and 1.0 ml of the emulsion was inoculated into each mouse. A daily dose of 1.0 mg of endoxan was injected intraperitoneally, starting at the time of inoculation. The population of *Nosema* was estimated by the number of infected cells among 500 macrophages in the peritoneal fluid. The examinations were carried out at 3-day intervals during a period of one month by the end of which the parasite disappeared from peritoneal fluid in most of the animals. The results are shown in Tables 1–4.

In the first group (Table 1) given endoxan for 14 days all mice died within 18 days, probably due to some toxic effect of the drug. The number of infected cells was definitely larger than in control mice at the 9 and 12 day periods after inoculation. In the second group (Table 2) endoxan was given for 6 days. All mice died by the 18th day. The number of infected cells was much larger than in control mice at 9, 12 and 15 day periods after inoculation. In the third group (Table 3) endoxan was given for 4 days. Only one out of 4 mice of endoxan group died by the 12th day. The number of infected macrophage cells was much larger than in the control group, too. In the fourth group (Table 4), endoxan was given for

Table 1 Effect of endoxan on the growth of *Nosema cuniculi* in the peritoneal cavity of mice (Dose : 1 mg/day for 14 days)

	Mouse	Number of infected macrophages among 500 cells									
		Days after inoculation									
		3	6	9	12	15	18	21	24	27	30
Endoxan group	1	0	0	5	1	3	D				
	2	0	1	12	20	D					
	3	0	D								
	4	1	17	17	8	D					
Control	5	0	0	1	1	2	1	1	0	0	0
	6	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	1	0	0	0	0	0	0
	8	0	4	2	1	0	0	0	0	0	0

C : Died.

Table 2 Effect of endoxan on the growth of *Nosema cuniculi* in the peritoneal cavity of mice (Dose : 1 mg/ml for 6 days)

	Mouse	Number of infected macrophages among 500 cells									
		Days after inhculation									
		3	6	9	12	15	18	21	24	27	30
Endoxan group	1	0	0	10	3	5	D				
	2	1	1	5	1	9	D				
	3	0	1	12	4	0	D				
	4	0	0	3	3	4	D				
Control	5	0	0	1	0	1	0	0	0	0	0
	6	0	0	1	0	0	0	0	0	0	0
	7	0	1	3	2	0	0	0	0	0	0
	8	1	0	4	3	0	0	0	0	0	0

D : Died.

Table 3 Effect of endoxan on the growth of *Nosema cuniculi* in the peritoneal cavity of mice (Dose : 1 mg/ml for 4 days)

	Mouse	Number of infected macrophages among 500 cells									
		Days after inoculation									
		3	6	9	12	15	18	21	24	27	30
Endoxan group	1	0	14	9	2	5	2	2	0	0	0
	2	0	19	10	9	11	0	2	0	0	0
	3	1	5	0	D						
	4	1	28	5	1	6	0	4	1	0	0
Control	5	0	6	5	8	2	1	4	1	0	0
	6	0	0	1	1	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0
	8	0	0	2	4	3	6	3	1	1	0

D : Died.

Table 4 Effect of endoxan on the growth of *Nosema cuniculi* in the peritoneal cavity of mice (Dose : 1 mg/ml for 3 days before the inoculation

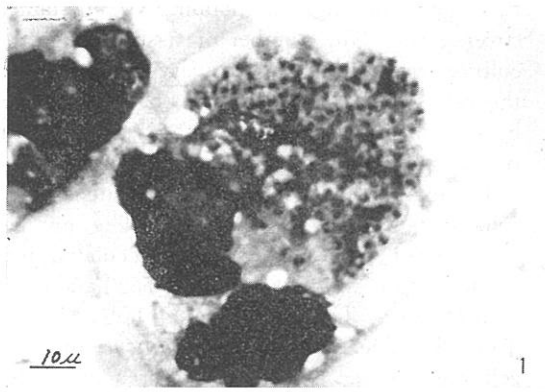
	Mouse	Number of infected macrochages among 500 cells									
		Days after inoculation									
		3	6	9	12	15	18	21	24	27	30
Endoxan group	1	0	0	1	1	8	10	8	2	0	1
	2	0	0	D							
	3	0	0	2	3	5	13	13	5	4	2
	4	0	0	6	4	4	6	3	1	0	0
Control	5	0	0	3	4	4	7	6	2	0	0
	6	0	0	3	6	8	11	1	0	0	0
	7	0	0	0	0	0	0	0	0	0	0
	8	0	0	2	4	3	6	3	1	1	0

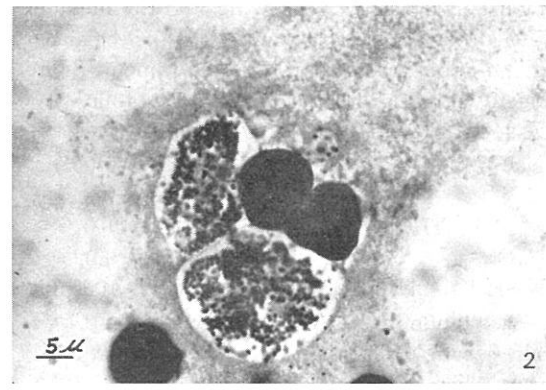
D : Died.

3 days before the inoculation. One mouse died by the 9th day. The number of infected macrophage cells was not always larger than in the control group.

All of these results clearly demonstrated that daily injections of endoxan after the inoculation of *N. cuniculi* definitely promoted the growth of the parasite and it was expected that this substance may be also effective for the provocation of latent infection which is very common among laboratory mice.

As stated above *N. cuniculi* when inoculated intraperitoneally into mice, grows in macrophage cells to attain the highest population usually





Explanation of Figures

Fig. 1 *Nosema cuniculi* in a macrophage cell in the peritoneal fluid of mouse. Light microscope picture. About 4,000×.

Fig. 2 *Nosema cuniculi* growing in HeLa cell. Small dots are the nuclei of spore. A little larger organism having a nucleus surrounded by cytoplasm is a trophozoite. About 1,600×.

two weeks after inoculation. They, however, decrease in number gradually and disappear sooner or later. As shown in Tables 1–4, *N. cuniculi* in control animals disappeared from peritoneal cavity mostly within 30 days.

The experiments were carried out with those mice which had been infected for more than 30 days. They were checked for the absence of *N. cuniculi* in the peritoneal fluid, and injected with a daily dose of 1 mg of endoxan for 6–10 days. The peritoneal fluid was examined for the appearance of *N. cuniculi* every 5 days. The results are shown in the Table 5. The parasites appeared in 5 out of 35 mice examined. They appeared during the period of injections or within 10 days after the last injection. As it rarely occurs that *N. cuniculi* reappear in the peritoneal fluid of mice after they have once disappeared, the results shown in those 5 positive cases may indicate the effect of endoxan injection.

In another experiment, 20 mice which had no previous treatment were injected with endoxan in the daily dose of 1.0 mg for 8 days. The peritoneal fluid of these mice was examined every 5 days after the injection was started. Only 2 of these mice became positive of *N. cuniculi* between 15–25 days after the first injection. Remaining 18 mice were autopsied 3 months later and brains were examined in fresh preparation. *N. cuniculi* was found in 3 of them.

Table 5 Provocation experiments of latent *Nosema cuniculi* infection by the intraperitoneal injections of endoxan (1 mg/day)

No. of mice examined	Day after inoculation	Days of endoxan injection	No. of mice turned positive
7	30	6	0
7	50	6	1
7	60	6	0
7	40	10	3
7	60	10	1

These results indicated that the provocation by endoxan injections seemed to be effective in some cases, but this procedure can not be available for the selection of uninfected mice for experimental purposes.

2) Attempts to grow the parasite in tissue culture.

HeLa cell (uterus cancer cell) and L cell

(mouse fibroblast cell) growing in YLH medium (Yeast extract, lact albumin, Hanks balanced salt solution) was used in this experiment. The constituents of the medium were as follows.

Hanks' balanced salt solution

NaCl	8.0 gm	KH_2PO_4	0.06 mg
KCl	0.4	$NaHCO_3$	0.35
$MgSO_4 \cdot 7H_2O$	0.2	Glucose	1.0
$CaCl_2$	0.14	Phenol red	0.02
$Na_2HPO_4 \cdot 2H_2O$	0.06	Aqua dest	1000 ml

Lact albumin (0.5%), yeast extract (0.1%), calf serum (10%) and Kanamycin (400 γ/ml) were added to Hanks' medium.

HeLa or L cells were suspended in this medium in concentration of $7\text{–}10 \times 10^4$ cells/ml and the suspension was distributed into small square culture tube in amount of 1.0 ml each. A cover glass of 12×32 mm size was put into each culture tube. After these tubes were incubated at 37°C for 48 hours to grow the cells on the cover glass surface, *N. cuniculi* were inocnlated into them.

N. cuniculi were obtained from the peritoneal cavity of mice which had been inoculated 10–14 days before. Sterile saline, 2.0 ml in amount, was injected intraperitoneally into these mice and drawn with the same syringe. The saline and peritoneal fluid mixture containing *Nosema*-infected peritoneal cells was centrifuged at 2000 rpm for 5 minutes, the supernatant was discarded and YLH medium was added to the sediment. The number of the total peritoneal cells in 1.0 ml of this suspension was estimated by hemocytometer. On the other hand, the percentage of the infected cells in the peritoneal cells was estimated beforehand by the examination of stained smear of the mouse peritoneal fluid. From these two figures, the number of the infected cells in 1.0 ml of the suspension was culculated and the suspension was inoculated into tissue culture. The culture medium was exchanged for fresh medium 48 hours after the inoculation and every 3 days thereafter.

Every 5 days after the inoculation, the cover glass in the culture tube was taken out and was stained by Jenner-Giemsa (Ogawa, 1968). The growth of the parasite was determined by the microscopical examination of the stained cover glass. The number of tissue cells in the culture was estimated at every 5 days, after the cells were detached from the cover glass by trypsin treatment, using 0.2% trypsin solution in phosphate buffer.

The experiments to grow the parasite in HeLa cells were repeated five times. The number of infected cells which were inoculated into tissue culture was 60, 200, 640, 5520 and 5520, respectively. The results are shown in the Table 6. The growth of *N. cuniculi* in HeLa cell was not recognized in cultures inoculated with a small number of infected cells (60–640), but in cultures inoculated with a large number of infected cells (5520), the aggregations of the parasites in HeLa cells were found very often (Fig. 2). The number of HeLa cells multiplied in culture was compared between inoculated and control tubes. No signifiant differences were recognized between them.

Experiments with L cell culture have so far been carried out 3 times (Table 7). The inoculum contained 2,580 or 4,200 infected cells and the growth of the parasite was recognized in the cultures inoculated with the larger number of the parasite. The number of L cells multiplied in culture was compared between inoculated and control tubes. No significant differences were recognized between them. In both HeLa and L cell cultures, the parasite appeared from 5 to 15 days after the inoculation.

Table 6 Growth of *Noscma cuniculi* in Hela cell culture

	Number of infected cells on a cover glass					Av. No. of Hela cells in culture (1000/ml)	
No. of infected cells inoculated	60	200	640	5520	5520	Control	Inoculated
Days after inculation							
5	0	0	0	3	1	1,228	1,285
10	0	0	0	8	2	2,128	1,500
15	0	0	0	3	1	2,234	1,603
20	0	0	0	0	0	2,798	2,884

Table 7 Growth of *Nosema cuniculi* in L cell culture

	Number of infected cells on a coverglass			Av. No. of L cells in culture (1000/ml)	
No. of infected cells inoculated	2580	2580	4200	Control	Inoculated
Days after inoculation					
5	0	0	1	460	390
10	0	0	5	607	300
15	0	0	0	1,690	1,440
20	0	0	0	1,890	1,513

Table 8 Comparison of the growth of *Nosema cuniculi* in HeLa and L cell culture

Days after inoculation	Number of infected cells on a cover glass			
	Hela cell		L cell	
5	2	0	0	0
10	7	1	1	0
15	2	1	0	0
20	0	0	0	0

Table 9 Effect of endoxan on the growth of *Nocema cuniculi* in Hela cell calture

	Number of iniected cells inoculated	Dose of endoxon (mg/ml)	Number of infected cells on a cover glass			
			Days after inoculation			
			5	10	15	20
Endoxan group	1200	1	4	1	0	0
	1200	1	16	3	0	3
	1200	1	6	1	0	0
	600	2	0	1	0	0
	2880	2	0	0	0	0
Control	1200	0	12	6	1	0
	1200	0	21	4	1	0
	1200	0	7	0	4	0
	600	0	0	1	0	0
	2880	0	1	3	0	0

For the isolation of *N. cuniculi* in tissue culture, it is necessary to elucidate which of HeLa or L cells would be more competent to the growth of the parasite. The cultures of these two kinds of cells were inoculated with the same inoculum containing 2580 infected cells. The experiments were repeated two times (Table 8). HeLa cell cultures gave rise to better growth than the L cell cultures.

3) Effect of endoxan on the growth of *Nosema cuniculi* in vitro.

Endoxan was added to tissue culture in amount of 1–2 mg/ml to test its effect on the growth of *N. cuniculi* in vitro. It was added at the same time as *N. cuniculi* was inoculated into the culture. After 48 hours, the culture medium was exchanged for fresh medium which did not contain endoxan. Subsequent medium exchanges were carried out every 3 days. Thus, the parasite underwent the action of endoxan during the first 48 hours of incubation. The results obtained by the HeLa cell culture is indicated in the Table 9. No significant difference was seen in the growth of *N. cuniculi* between endoxan and control group. The results obtained by L cell culture is indicated in the Table 10. In this case, endoxan group showed somewhat a better growth than the

Table 10 Effect of endoxan on the growth of *Nosema cuniculi* in L cell culture

	Number of infected cells inoculated	Dose of endoxan (mg/ml)	Number of infected cells on a cover glass			
			Days after inoculation			
			5	10	15	20
Endoxan group	180	2	0	0	0	0
	180	2	0	0	0	0
	200	1	1	0	0	2
	200	1	9	1	3	1
	4200	2	0	16	8	3
Control	180	0	0	0	0	0
	180	0	0	0	0	0
	200	0	0	1	5	1
	200	0	2	0	2	0
	4200	0	0	5	1	0

control group when a large dose of *N. cuniculi* was given.

In these two series of experiment, exposure of the parasite to the action of endoxan was only 48 hours. In the third series of experiment with the HeLa cells culture, endoxan was added to the culture media in 0.5 mg/ml concentration throughout the experiment. In control experiment the parasites were exposed to the endoxan only for 48 hours. Results are shown in the Table 11. No significant difference could be seen in experimental and control group.

Influence of temperature on the effect of endoxan on the growth of *N. cuniculi* in vitro was tested. Endoxan was added to the HeLa cell culture at the same time with the inoculation of the parasite and the culture was incubated in 30°C incubator. Exposure to the endoxan was for 48 hours. The control culture was kept in 37°C incubator. The growth of the parasite was better in 30°C culture than in 37°C culture as shown in the Table 12. Another experiment was carried out to check whether the lower temperature alone may have the effect of promoting the growth of the parasite. Procedures were exactly the same as the previous experiments except the endoxan was not added. In these experiments, no significant difference was seen between the cultures in 30°C and 37°C (Table 13).

Table 11 Effect of endoxan on the growth of *Nosema cuniculi* in Hela cell culture

	Number of infected cells inoculated	Number of infected cells on a cover glass			
		Days after inoculation			
		5	10	15	20
Endoxan group	480	0	20	0	0
	480	3	2	0	0
	480	9	1	0	0
Control	480	0	0	0	0
	480	3	10	0	0
	480	1	10	0	0

Dose: 0.5 mg/ml throughout the period of experiment

Table 12 Effect of endoxan and temperature on the growth of *Nosema cuniculi* in Hela cell culture

Temperature	Number of infected cells inoculated	Dose of endoxan (mg/ml	Number of infected cells on a cover glass			
			Days after inoculation			
			5	10	15	20
30°C	240	0.5	7	3	0	0
	240	0.5	18	0	1	0
	240	0.5	12	2	0	0
	240	0.5	13	13	5	0
	600	0.5	1	8	1	21
	600	0.5	6	4	0	0
	600	0.5	8	18	5	0
37°C	240	0.5	4	9	0	0
	240	0.5	0	4	0	3
	240	0.5	14	3	0	0
	240	0.5	7	11	0	0
	600	0.5	5	0	6	0
	600	0.5	2	2	0	0
	600	0.5	5	1	0	0

Table 13 Effect of temperature on the growth of *Nosema cuniculi* in Hela cell culture

Temperature	Number of infected cells inoculated	Number of infected cells on a cover glass			
		Days after inoculation			
		5	10	15	20
30°C	160	2	2	0	0
	160	1	1	0	0
	160	0	1	0	0
37°C	160	0	0	0	0
	160	1	0	0	0
	160	0	0	0	0

Discussion

In the experimental studies on *N. cuniculi*, it is often desirable to obtain mice free from *Nosema* infection. The only method to detect the infection without sacrificing the animals is to examine the peritoneal fluid. This method, however, does not reveal the chronic infection

in which the parasites are located in the central nervous system and rarely appears in the peritoneal fluid. Unfortunately, most of the spontaneous infection in mice are in this stage. Endoxan, an anti-tumor substance, was found to be effective to promote the growth of *N. cuniculi* in the peritoneal fluid. It was also effective for the provocation of latent infection in mice: by the repeated injections of endoxan into apparently negative mice, the parasite often appeared in the peritoneal fluid. This provocation method, however, was not always successful to detect the latent infection, because some of the mice which were negative after the provocation procedure were found infected in the brain by autopsy. Thus, the selection of negative mice without sacrificing animals is impossible in the present.

If *N. cuniculi* can grow in tissue culture, the infection of the parasite in animals and man may be detected by inoculation of host materials into the culture. In the present experiments, the growth of the parasite in HeLa and L cells cultures was recognized. Chikatsune (1960) was also successful to grow the parasite in HeLa cell culture, but failed to grow it in L cell culture. It was demonstrated, in the present experiment, that the success or failure of the growth mainly depended upon the number of the parasite inoculated: the larger the number, the more the chances of the success can be expected.

Effect of endoxan to promote the growth of *N. cuniculi* in tissue culture was tested. Contrary to the results *in vivo*, endoxan showed little effect on the growth of parasite. According to Shoji and Kimura (1964), endoxan has a very high anti-tumor effect in vivo, while it has little effect to inhibit the HeLa cell growth in vitro. Arnold *et al.* (1958) also obtained similar experimental results. According to the transport or active form theory (Druchery and Raabe, 1952; Druchery *et al.*, 1956), it was maintained that endoxan became effective only after the transport form was transformed to the active form in vivo.

The effect of endoxan to promote the growth of *N. cuniculi* in vivo may not be a direct effect on the parasite, but it may excert some influence on the host cell which in turn may promote the growth of *N. cuniculi*. *In vitro* test, endoxan exerts little influence on the tissue culture cell.

Summary

1. Endoxan is effective to promote the growth of *Nosema cuniculi* in the peritoneal cavity of mice. This effect may not be due to the direct action on the parasite, but may be the result of some changes in the host cell provoked by the compound.

2. Endoxan is effective for the provocation of the latent infection of *N. cuniculi* in mice.

3. *N. cuniculi* can grow in HeLa or L cell culture only when a large number of the parasites are inoculated. HeLa cell culture seems to give rise a better growth of *N. cuniculi* than L cell culture.

4. Endoxan has little effect on the growth of *N. cuniculi* in tissue culture. This may be due to the fact that the compound has little effect on the cells growing in vitro.

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Nosema cuniculi の *in vivo* および *in vitro* における増殖に及ぼす抗腫瘍剤 Endoxan の影響

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Nosema cuniculi を実験的にマウスに感染させた場合，*Nosema* はその腹水細胞中で増殖を行う．しかし感染細胞数は少く，ときには全く腹腔中に見出せないこともある．*Nosema* をマウスに感染させると同時に抗腫瘍剤 Endoxan を腹腔中へ4日間以上投与し続けると腹腔中の感染細胞数は増大した．Endoxan 投与によって *Nosema* が増殖するのは Endoxan が直接虫体に作用したためではなく，宿主細胞に何らかの変化があつたためであろう．また一度腹腔中から虫体が消失したマウスに Endoxan を投与した場合，その腹腔中に再び虫体を見出し得た例もあつた．すなわち Endoxan による誘発の可能性があると考えられる．

組織培養細胞の HeLa 細胞とL細胞とに *Nosema* の接種量が多い場合には細胞内に多数の虫体で作られた胞が認められた．しかし *Nosema* 感染細胞数は非常に少い．培養開始して49時間後に *Nosema* 感染腹水細胞を浮遊させ，Endoxan を加えた培養液(YLH)と培地を交換することにより *Nosema* 接種と Endoxan 投与を行つた．投与後48時間で Endoxan の含まれない培養液と再び交換した．この場合，Endoxan の量，*Nosema* の接種等にかかわらず *Nosema* 感染細胞数は *Nosema* だけを接種した対照群と比較して変らなかつた．

次に接種および Endoxan 投与後の培地交換の時にも Endoxan を加えた培養液を用いて Endoxan を連続投与した．この場合にも，対照群と比較して感染細胞数に変化が認められなかつた．これらの実験では培養温度は全て37°C であつたが，*Nosema* を接種後および Endoxan を投与後は30°C で培養した実験を行つた．*Nosema* 感染腹水細胞を浮遊させ，Endoxan を加えた培養液と培地とを交換して接種および投与をし，48時間後にこの液と Endoxan の含まれない液とを交換する．*Nosema* の接種と Endoxan の投与後30°C で培養した場合，感染細胞数は同様の処理をして37°C で培養した場合と比較してわずかながら増加していた．培養温度と *Nosema* 増殖との関係を調べるため，Endoxan を投与せず *Nosema* だけを接種して30°C と37°C とで培養した場合の感染細胞数の比較を行つた．この場合，両者の感染細胞数にはつきりした差が認められなかつた．