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Evaluation of Two Insect Chemosterilants (Hampa and Thiotepa) for Their Effect on *Eimeria vermiformis* Infection in Mice

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ABSTRACT. The mice used in this study were a total of 42; they were divided into seven experimental groups. Each mouse was inoculated with 20,000 sporulated oocysts of *Eimeria vermiformis*. The results showed that hempa and thiotepa exert an effect on the mice bearing *E. vermiformis* when administrated orally at the rate of 0.33 mg per mouse. Doses were calculated from the LD_{50} data for each of the drugs in mice. The doses of hempa apparently caused increased oocyst production. Peak oocyst discharge and patent period were found to be delayed in the treated groups. The doses of thiotepa was kept the same in all animals, but the time of administration was synchronized with the developmental stages of the parasite. It was found that by gradually increasing the duration of the drug administration, oocyst discharge was increased. Both drugs may affect the immune system of the mice and the recognition of antigen of the parasite. It could be useful to use these immunosuppressive agents to prolong the retention of transplant tissues in the body.

Because the development of chemosterilants is still in progress, no definitive statements can be made about all types of compounds with biological sterilization activity. Since over 1,000 such compounds have been described in the literature (Borkovec 1966, Parish and Arthur 1965), the review of literature will be limited to those articles that deal with chemosterilants that were used in the current study; these were Hempa and Thiotepa.

Hempa (hexamethylphosphoric triamide) may be taken as an example of the analogues of alkylating agents proposed for use as chemosterilants. Metabolic effects of hempa have not been extensively investigated and very little is known

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about its biochemistry. Hempa has been reported to be effective in the chelating of metal; its action as a sterilant might be related to this property in that binding of essential metal cofactors might block any of several enzyme systems. It has been reported that the acute oral LD_{50} in rats was greater than 2,500 mg/kg (Kimbrough and Gaines 1966, Jasper *et al.* 1965). Rats receiving a dose in the LD_{50} range showed voluntary urination, mild muscle fasciculation, bloody urine and convulsions (Kimbrough and Gaines 1966).

Thiotepa (tris-l-aziridiny 1) = phosphine is the most reactive of the commonly used chemosterilants (Borkovec 1969). Thiotepa was found to be rapidly converted to tepa in rats, rabbits and dogs (Craig 1959, Mellet and Woods 1960). The acute lethal dose (LD_{50}) for adult rats injected intravenously was 9.48 ± 0.69 mg/kg (Boone *et al.* 1962). The mode of biological action of thiotepa was described by Kaji (1962) who stated that 'thiotepa acted like X-rays or cobalt 60 treatment in causing a reduction of the level of total and fraction III protein in the liver of the rat.' This study was designed to determine the effects of the insect chemosterilants (Hempa and Thiotepa) on the immune system of the mice infected with *E. vermiformis*.

Material and Methods

Selection and Management of Experimental Animals

Since the investigation was designed to study the effect of several chemosterilants on coccidia and its host, and since it has been shown by Renoux and Renoux (1974) that drugs containing sulfur substances may interact with the immune system of the host, mice (*Mus musculus* is the specific host (Ernst *et al.* 1971) for *Eimeria vermiformis*) were obtained from Simonsen Laboratories in Gilroy, California. They were held in cages each measuring 37 cm long, 23 cm wide and 22.5 cm high with mesh floor that permit the feces to go through to a tray containing 2.5% potassium dichromate in water to inhibit build-up of bacteria and for moisturizing feces as well as oocysts.

A total of 42 white mice, each ranging from 4 to 6 weeks in age and weighing 28 to 34 gm, were divided into seven groups, each consisting of six mice. Mice were fed Purina [R]laboratory chow; none were fasted prior to treatment. Water was supplied by bottles with fitted metal tubes.

Inoculation of Animals

Preparation of inoculum

Oocyst inculum was prepared from fecal material collected from infected mice. The fecal material was strained through two sieves, one of 60 meshes per square inch followed by one of 120 meshes per square inch. After straining, the suspen-

sion, including the water used in washing the sieves, was allowed to undergo sedimentation. The water was then siphoned off. The sediment was then rewashed, resedimented and decanted to remove as much organic debris as possible. The final sedimentary product was then suspended in 2.5% potassium dichromate solution to retard bacterial action, and placed in a large stainless steel pan for sporulation. The inoculum was stirred by means of an Eberhard electric motor fitted with a two-bladded stirred. The inoculum was stirred slowly at room temperature in the open vessel until sporulation was complete. It was then stored in a refrigerator until used. Before inoculation, the potassium dichromate was washed from the suspension by decantation. To insure accuracy, bottles containing the inocula were shaken vigorously prior to sampling; samples were then drawn from the suspension and oocyst counts made with the aid of a hemocytometer. The drawn sample was diluted with enough water to produce the dose determined for each experimental animal.

Each mouse was given 20,000 sporulated oocysts of *Eimeria vermiformis* with the aid of a syringe fitted with a piece of plastic tubing of 1.19 mm in diameter *via* a 18 gauge needle.

Treatment of animals

Drugs were obtained from Dr. A.B. Borkovec of the U.S.D.A., Beltsville, Maryland. Fresh preparations of Hempa and Thiotepa were prepared daily from refrigerated stock for each experiment. Hempa was administered to mice at the rate of 0.33 mg/mouse daily for 5 days prior to oocyst inoculation. This dose was selected as a maximum possible dose with less harm to the mice, since it equaled the LD_{50} value for mice (Kapecky and Smejkal 1966).

Fecal sample

Five gm fecal samples of each group were collected each morning, beginning 7-9 days after inoculation and continued until the end of the experiment. The samples were collected in small bottles marked with the number of each group of oocyst count.

The feces were then examined macroscopically for the presence of blood, mucus and tissues. The concentration of oocysts in the fecal samples was determined with the aid of a McMaster chamber. Five gm of feces were mixed with 45 ml of water; 1 ml of this dilution was then mixed with 2 ml of Sheather's solution (Levine 1973). A drop of this mixture was then placed in the McMaster chamber with a syringe, allowed to stand for approximately 15 min, then examined microscopically with 10 × objective and 10 × ocular lenses. The number of oocysts was counted within 1 sq × cm grid etched on the undersurface of the chamber upper glass plate and the total number of oocysts multiplied by 200 to give the number of oocysts present in 1 gm of feces (number of oocysts within 1 sq × cm × 200).

Results

Effects of chemosterilants on both E. vermiformis and mice

Experiment I

This experiment was designed to determine the effect of Hempa (if any) on the development of *E. vermiformis* and its host.

Mice in this experiment were randomized and divided into two groups of six mice each. The mice ranged from 4 weeks to 6 weeks at the time of inoculation with a mean age of 5 weeks. Each of the mice in groups 1 and 2 was inoculated with approximately 20,000 sporulated oocysts of *Eimeria vermiformis*. Group 1 received no treatment and remained as an inoculated untreated control, while each member of group 2 received 0.33 mg/mouse/day of hempa for 5 days prior to oocyst inoculation. The effect of the drug was judged by oocyst production in the treated group compared with the untreated group. Oocyst counts indicated that there was a slight increase in oocyst discharge in the treated mice over the course of the patent period, 444,300 of collected oocysts per 1.2 gm faces for control and 587,480 of collected oocyst per 1.4 gm feces for treated mice. peak oocyst discharge was delayed with occurrence of peak numbers of oocysts at 11 days in treated mice (Fig. 1) while it occurred at 10 days after inoculation in normal mice. In addition, the duration of oocyst discharge, which lasted about 144 hr in control animals, was much longer in mice treated with hempa, being about 168 hr. With these results, no other attempt was made to increase the dose of hempa, since a higher dose would have been harmful to the mice.

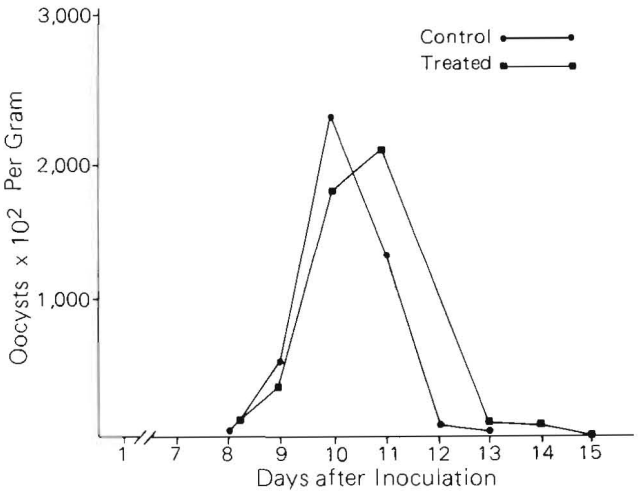

Fig. 1. Mean daily oocyst output of *E. vermiformis* per 1.2-1.4 gm feces from control and treated mice with hempa during patent period starting on the eighth day after inoculation.

Experiment II

This experiment was designed to determine the action of thiotepa (if any) on *E. vermiformis* infections in mice. This was accomplished by timing the administration of the chemosterilant at certain intervals that match roughly with different developmental stages of coccidia (*i.e.*, sporozoites, first-generation schizonts, second-generation schizonts, gamonts, ... etc.).

The mice used in this experiment were similar to those used in Experiment I. Five groups of mice, each composed of six animals, were randomly selected for this experiment. Each mouse in all five groups was given approximately 20,000 sporulated oocysts of *Eimeria vermiformis* in single doses. The treatment was timed to roughly match with the endogenous phases in an attempt to determine the action of thiotepa on the different stages of the life cycle of the parasite.

Group 1 received no treatment and remained as an inoculated untreated control. Group 2 received treatment with thiotepa at the rate of 0.33 mg/mouse for 2 days, 1 day prior to inoculation. To limit drug effect on the first-generation schizont, which normally appears at 2 days after inoculation, timing the drugs for 2 days may have had an effect on that stage. Group 3 was treated the same as group 2, but for 4 days, starting 1 day prior to infection. The timing of the drug could affect

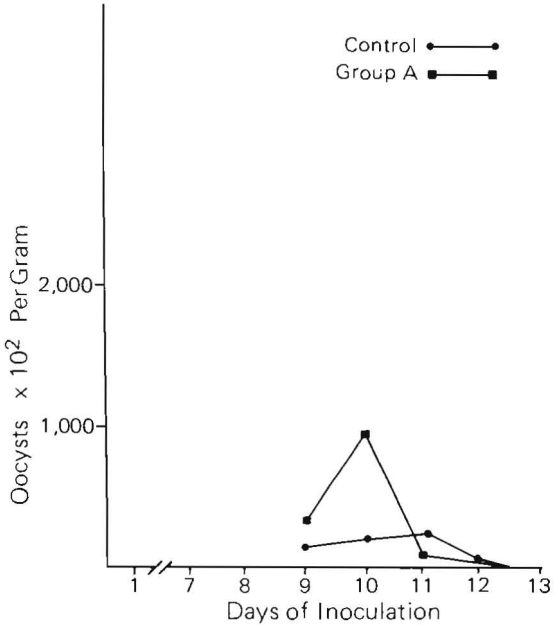

Fig. 2. Mean daily oocyst output of *E. vermiformis* per 1.0 gm feces from control and treated mice (with thiotepa at rate of 0.33 mg/kg for 2 days) during patent period starting on the ninth day after inoculation.

the first-and second-generation schizonts of the parasite. Groups 4 and 5 received treatment with thiotepa at the same dose-level as in the previous groups, but for 6 days and 13 days, respectively, starting 1 day before oocyst inoculation. The drug may have affected the gametogony at these periods in addition to first-and second-generation schizonts.

Gross symptoms of coccidiosis, such as blood and mucus in the feces, appeared more frequently in the treated groups than in the untreated group. Mice in group 2 treated with thiotepa for 2 days showed greater oocyst production when compared with the untreated mice in group 1 (Fig. 2). Groups 3, 4 and 5, treated with thiotepa for a longer period of time, also showed greater oocyst production (Fig. 3, 4 and 5). It is of interest to note that the increase in oocyst production was influenced by increasing the length of time of administration of the drug. Contrary to what may be expected, the longer the duration of treatment (more drug/animal) the higher the number of discharged oocysts. It is possible, therefore, that thiotepa may, in an unknown manner, depress the immune response of mice and such mice may show increased numbers of *E. vermiformis* oocysts in the feces. In addition, it was found that the prepatent period was shorter in treated mice than in untreated animals (Fig. 4). However, no differences were observed in the duration of peak oocyst production among the different groups except in group 5 and the untreated

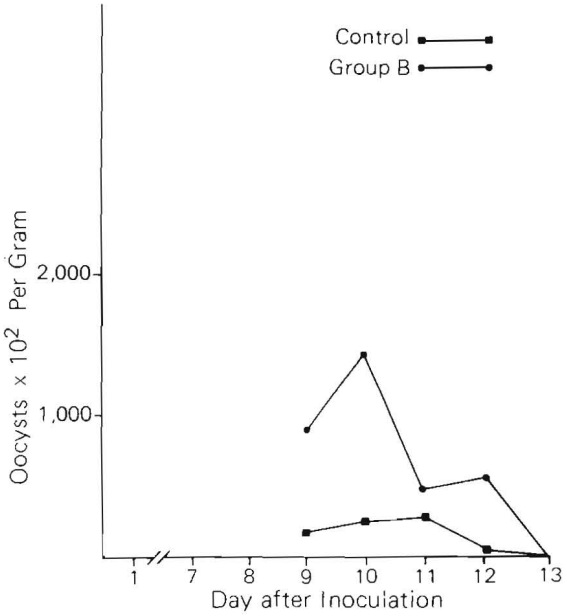

Fig. 3. Mean daily oocyst output of *E. vermiformis* per 1.0 gm feces from control and treated mice (with thiotepa at rate of 0.33 mg/kg for 4 days) during patent period starting on the ninth day after inoculation.

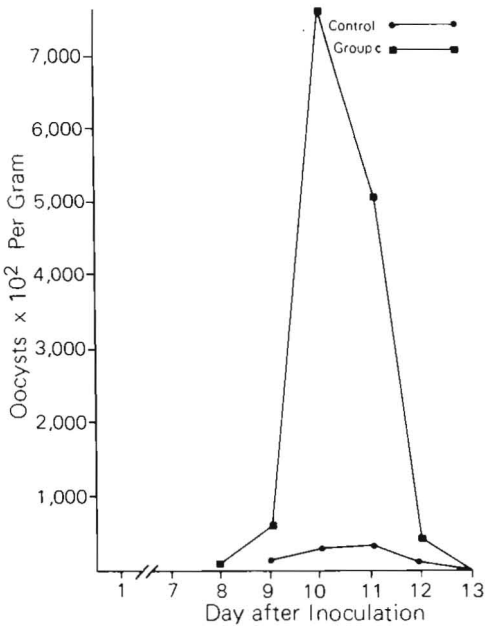

Fig. 4. Mean daily oocyst output of *E. vermiformis* per 1.0 gm feces from control and treated mice (with thiotepa at rate of 0.33 mg/kg for 6 days) during patent period starting on the eight day after inoculation.

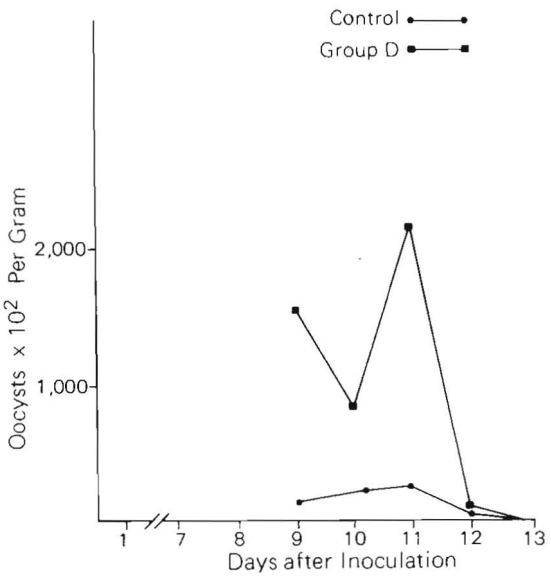

Fig. 5. Mean daily oocyst output of *E. vermiformis* per 1.0 gm feces from control and treated mice (with thiotepa at rate of 0.33 mg/kg for 13 days) during patent period starting on the ninth day after inoculation.

Table 1. Duration number of oocysts and day of peak from mice treated with thiotepa at different days following inoculation with *E. vermiformis*.

Treatment Group	Total Number of Collected Oocysts Per 1.0 gm Feces	Oocyst Discharge	
		Duration (days)	Day of Peak
2 (0.33 mg/kg) 2 days	131,000	5	10
3 (0.33 mg/kg) 4 days	329,960	5	10
4 (0.33 mg/kg) 6 days	1,366,500	6	10
5 (0.33 mg/kg) 13 days	457,000	5	11
Untreated (Control)	60,100	5	11

group (Table 1), where the duration of peak oocyst discharge was longer. The patent period is extended in group 4 (Fig. 4) and the duration of peak oocyst discharge was shorter than in untreated control mice (Table 1).

Discussion

In the present study, mice infected with *Eimeria vermiformis* and a given dose of thiotepa at the rate of 0.33 mg/mouse 9.06 ± mg/kg LD_{50}; (Boone *et al.* 1962) showed a greater increase of oocyst production than did untreated control mice. This increase in number of oocysts discharged in the treated groups might be due to the effect of the drugs on the host, which enhanced the infection. It is probable that administrating the drug for a long period of time enhanced the toxicity of the drugs for mice.

The short prepatent period in treated mice (*i.e.*, 8 days after inoculation with oocysts as compared to 9 days in control) could support the contention that in treated animals or animals that undergo immunosuppression, the prepatent period becomes shorter and the patent period becomes longer than that of normal control animals.

Similar findings were reported in chickens infected with *E. tenella* treated with betamethasone (Long and Rose 1970). Rommel (1970) reported similar results in pigs infected with *E. scabra* and treated with paramethosone and dexamethasone. Long and Rose (1970) found that the life cycle of *E. mivati* in chickens was prolonged when they were treated with corticosteroid.

Thiotepa has been shown to have various effects on different animals. Tarig *et al.* (1977) reported that thiotepa was found to inhibit both RNA and protein synthesis in the testes of albino rats. Also, there is a report by Hanson (1978) of a caffeine enhancement of chromosomal abberations induced by thiotepa in bone marrow. Thiotepa shows its effects both at cellular and tissue levels.

In the experiments using hempa, there were no apparent differences in the number of oocysts produced in the treated mice as compared to the untreated groups. However, peak oocyst discharge occurred at 11 days after inoculation in treated mice compared with 10 days in untreated groups. The patent period in the treated groups was 8 days compared with 6 days in untreated groups. This was probably due to the continuation of schizogony in intestinal tissues. Long and Rose (1970) showed the length of the patent period of *E. mivati* in chickens treated with betamethasone was extended from 12 to 45 days. This was shown histologically to be the result of the continuation of schizogony. Shott *et al.* (1971) found clinical signs of systemic toxicity preceeding death in rats treated with hempa, included alterations of nervous, gastrointestinal and respiratory system.

A report by Kimbrough and Gaines (1966) described the main clinical symptoms observed in acutely poisoned rats which were involuntary urination, mild muscle fasciculation, convulsion and bloody urine. They concluded that hempa possibly affects the reticulo-endothelial system and thus lowers the resistance to infection, as many poisoned animals showed very small atrophic spleens. The lymph nodes of these animals, on the other hand, were enlarged.

Treatment with hempa and thiotepa had a profound depressing effect on immunity to coccidia; similar findings have been reported on the effect of treatment with the corticosteroid betamethasone on primary infection Long and Rose (1970).

The drugs were used may have same effect as antilymphocytic serum that used as an immunosuppressive agents, it can affect the recognition of antigen, immunological memory expression of delayed hypersensitivity, the production of circulating antibody, and can prolong the retention of skin grafts (Sell 1969). In view of this, its effect on immunity to coccidiosis has been tested but on a limited scale Euseby *et al.* (1969).

Conclusion

Results indicated that the administration of hempa at the dose-level of 0.33 mg/kg equal to the LD_{50} dose/mouse for 5 days prior to oocyst inoculation, caused increased oocyst production. The treated group produced a greater number of oocysts than the control, and the patent period was both longer and started later compared with controls. Peak oocyst discharge was found to be delayed for up to 8 days in the treated group.

The dose-level was kept the same in all groups treated with thiotepa, but the time of the administration was synchronized with the time of the developmental stages of *Eimeria vermiformis* in order to match with the presence of sporozoites as well as first-generation schizonts, second generation schizonts and gamont development. The author found that by increasing the duration of thiotepa administration, oocyst production was increased in the treated mice as compared with controls. Clinical signs of coccidiosis appeared in control mice but were more severe in treated mice, which showed mucous and strands of tissue in the feces.

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دراسات تقيمية لنوعين من المركبات الكيميائية التعقيمية (هيمبا وثيوتيبا) على تأثيرهما على الفئران المصابة بالحيوان الطفيلي الأولي *آيميريا فيرميفورميس*

مكي عبدالله العمودي*

قسم علم الاحياء - جامعة ولاية يوتا - لوقان - يوتا - الولايات المتحدة

عدد الحيوانات المستعملة في هذه الدراسة ٤٢ فأرا مقسمة إلى سبع مجاميع، أعطي كل فرد منها جرعة من المرض تقدر بـ ٢٠,٠٠٠ كيس من البيض الناضج من الطفيلي إيميريا فيرميفورميس *Eimeria vermiformis* وقد دلت النتائج أن كلا المركبين الكيميائيين هيمبا *Hempa* وثيوتيبا *Thiotepa* قد أظهرا تأثيراً على زيادة إنتاج البيض في الفئران المصابة بالطفيلي عندما عوملت بجرعات من الدواء بمقدار ٠,٣٣ مجم للفرد الواحد حسبت من الجرعة القاتلة LD_{50} كما أعطت الجرعات الدوائية للمركب هيمبا *Hempa* زيادة واضحة في إنتاج البيض. وقد لوحظ أن القيمة الذروية لإنتاج البيض ومدة الإصابة أبطأ في المجاميع المعاملة.

أما بالنسبة للجرعات الدوائية للمركب ثيوتيبا *Thiotepa* فقد روعي في استعماله أن يكون مستوى الجرعة

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ثابت (٠,٣٣ مجم) بالنسبة لكل المجاميع وأن يكون إعطاؤه متزامنا مع مراحل تطور الطفيلي داخل خلايا العائل. وقد وجـد أنه بالزيادة التدريجية في مدة إعطاء الدواء الكيميائي اعطى زيادة مطردة في إنتاج البيض ومن الاستنتاج أن كلا المركبين الكيميائيين ربما قد أثرا على مناعة الجسم في العائل وعلى معرفة الانتيجين *Antigen* في الطفيلي. وربما يكون ذلك مفيدا في تقليل رفض الجسم للأجسام الغريبة وتطويل بقائها ولا سيما في زراعة الأنسجة.