THE EFFECT OF IMMUNOSUPPRESSANTS ON EXPERIMENTAL INFECTION WITH FASCIOLA HEPATICA

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Abstract. Results are presented on the effect of immunosuppressive substances such as chlorambucil, cyclophosphamide, azathioprine, amethopterine and a cortizone derivate of betamethasone, on the development of *Fasciola hepatica* in the rat. The suppression of the immune response of the host to immunosuppressants was reflected in an earlier start of migration of the flukes to the common bile duct, and in an earlier onset of egg production as compared with that in the controls. Of the substances employed, cyclophosphamide and betamethasone were the most effective ones within the period from week 2—6 p.i., which is the time during which the migration of the flukes in the liver parenchyma is highest. Pathological changes in the liver of the animals were less marked than those of the infected controls. Evidence was obtained on an increased pathogenicity of infective larval flukes causing a higher mortality of the hosts in comparison with that of the control animals. On the other hand, the administration of immunosuppressants did neither influence the total number of developed flukes nor the appearance of eosinophilia in the peripheral blood of the treated animals.

Immunosuppressants are the most important component of clinical transplantation treatment; they are used also in a number of studies on protein synthesis and antibody production. In addition, they are employed in the study on various immunological processes thus increasing their range of clinical applicability. In clinical parasitology, immunosuppressants are employed mainly in studies on the immune response of the host in that they either expel parasitic worms from the organism or devitalize them in the individual organs. Moreover, these substances have been used successfully for blocking the natural resistance of nonspecific hosts enabling thus the cultivation of various helminths in hosts in which they normally do not survive. The effect of immunosuppressants on the immune response of the host has been described by Wilson (1971) in experiments with the nematode *Dictyocaulus viviparus;* by Ritterson (1968), Kozarová (1969), Čorba and Špaldonová (1974) with *Trichinella spiralis;* by Wiest (1972) with *Trichostrongylus axei* and *T. colubriformis;* by Urquhart et al. (1965) with *Nippostrongylus brasiliensis.* For cestodes, Hopkins et al. (1972) described the effect of these substances in an experiment with *Hymenolepis diminuta;* Domingo et al. (1969) with the trematode *Schistosoma mansoni.*

In an earlier experiment (Čorba and Špaldonová 1974) concerned with studies on the immune response to experimental trichinellosis evidence was obtained on the fact that immunosuppressants administered during the intestinal phase of infection suppressed the resistance of the host and, hence, were responsible for a significant

increase in the number of worms in the muscles. The present study deals with the effect of immunosuppressants on the development of *Fasciola hepatica* in experimentally infected rats, and with the response of the infected host and its organs.

MATERIALS AND METHODS

Male rats of the Wistar strain, weight 70—170 g, were infected by way of a stomach tube with 30 adolescariae of *Fasciola hepatica* suspended in a 0.1 % solution of the detergent Lissapol. Adolescariae were obtained from our laboratory stock of Galba truncatula reared under standard conditions. Each group consisted of 20 animals. The immunosuppressive substances administered to both experimental and control animals were these:

1. Imidazole derivate 6-mercaptopurine — azathioprine (Imuran) produced by Burroughs Wellcome Co, Great Britain
2. p-/N,N-di/β-chlorethyl) aminophenyl butyric acid — chlorambucil (Leukeran) produced by Burroughs Wellcome Co, Great Britain
3. N,N/β-cyclophosphamide (Endoxana) produced by Ward Blenkinsop Co, Great Britain
4. 4-amino-N-methyl of ptero-glutamic acid — amethopterine (Methotrexate) produced by Lederle Labs Division, Great Britain
5. Cortisone derivate — fluor — methylprednisolon — betamethasone (Betsolan) produced by Glaxo Labs Ltd., Great Britain
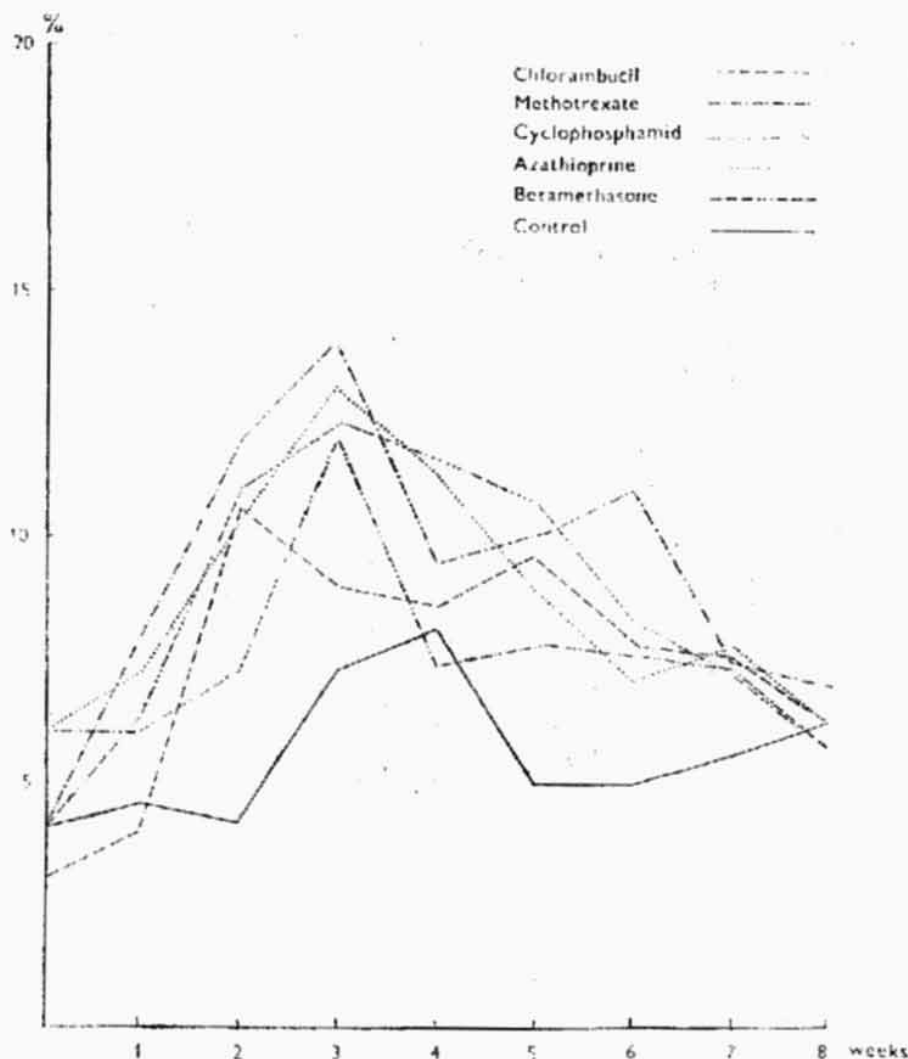

Fig. 1. Percentage of eosinophilic leucocytes in the white blood picture of rats treated with immunosuppressants 3 times a week in intervals of -2 weeks before to + 2 weeks after infection with 30 adolescariae of *F. hepatica* per rat.

The substances were administered either orally or parenterally in doses and intervals shown in tables 1 and 2. From week 4 p.i. onwards, two animals of each experimental and control group were killed in weekly intervals and examined for the location, size and degree of sexual maturity of the flukes, and for the extent and quality of the pathological process in the liver. Simultaneously, two animals were killed of the uninfected control group which had received immunosuppressants in the same intervals as the experimental animals. From the first day of infection onwards, blood was taken from the retroorbital plexus of the animals in weekly intervals using the method suggested by Nöller (in: Cors et al. 1958) for the purpose of examining the differential white blood picture. From week 5 p. i. onwards we examined the faeces of the infected animals for the presence of eggs of *F. hepatica* by means of the flotation-sedimentation method suggested by Breza and Čorba (in press). In addition daily records were made of the general state of health and the rate of mortality for all groups. After week 8 of infection, i.e. at the termination of our experiment, the remaining animals were killed in order to evaluate all criteria under consideration.

RESULTS

Experiment no. I. Evidence was obtained by comparison with the untreated control group (Table 1) that an administration of immunosuppressants commenced two weeks prior to infection and continued for two weeks after infection with *F. hepatica* did not influence significantly the number of flukes recovered from the common bile duct of
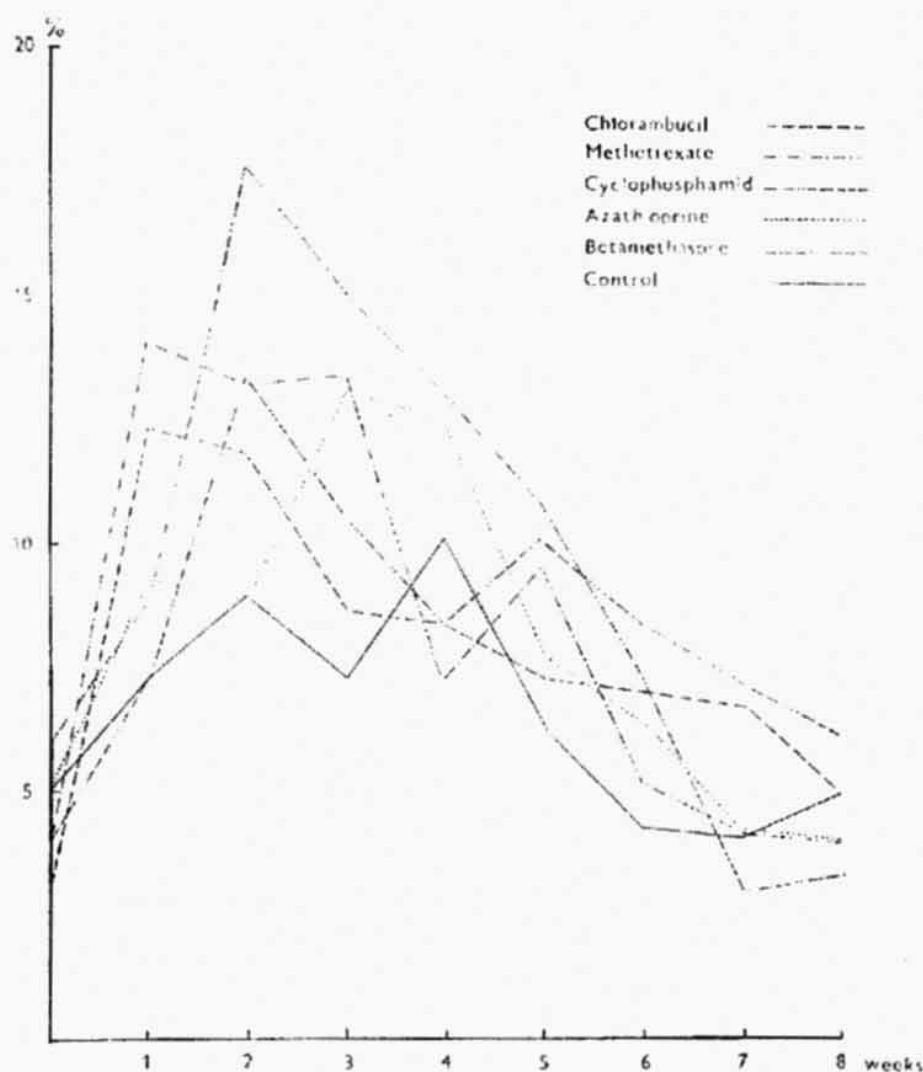

Fig. 2. Percentage of eosinophilic leucocytes in the white blood picture in rats treated with immunosuppressants three times a week in intervals from week 2—week 6 p.i. with 30 adolescariae of *F. hepatica* per rat.

the experimental animals. Neither did it influence the speed of migration of the flukes through the liver parenchyma, their size and degree of sexual maturity, and the expulsion of *F. hepatica* eggs with the faeces. On the other hand, significant differences were observed in the rate of mortality: three animals died in the group treated with amethopterine, up to 5 animals in the group treated with cyclophosphamide, while no case of mortality was recorded in infected and uninfected animals of the control groups.

Experiment no. II. The results shown in Table 2 indicate that also in this experiment differences in the number of flukes recovered after the termination of the experiment were insignificant. Significant, however, were differences in the location and size of the flukes, in pathological changes of the liver and in the number of eggs passed with the faeces. In animals of the groups treated with either cyclophosphamide (dose 1 mg.ip.) or betamethasone (dose 0.5 mg.ip.) a speed-up in the migration of the flukes through the liver parenchyma was observed between week 2 and week 6 p.i., which occurred considerably earlier than under normal condition; on day 35 p.i., the majority of flukes had reached the common bile duct, several of the parasites had attained sexual maturity and harboured formed eggs of *F. hepatica*. Also an earlier onset of egg production in these groups was confirmed by corpological examination: eggs of *F. hepatica* were present in the faeces of the treated animals on day 42 p.i. already. In the control animals, the first flukes appeared in the common bile duct on day 50 p.i., the first eggs appeared on day 56 p.i. The size of the flukes was similar in both experimental and control animals. The extent of pathological changes in the liver of animals treated with either cyclophosphamide or betamethasone was considerably smaller in comparison with that of the controls. After the termination of the experiment, i.e., in week 8 p.i., the appearance of the liver parenchyma of these animals was normal in spite of the fact that mature flukes were present in the common bile duct. Mortality was recorded in all expe-

Table 1. Influence of immunosuppressants administered three times a week from two weeks prior to infection to two weeks postinfection with 30 adolescariae of *F. hepatica* on the rat

Group	No. of animals in experiment/dead	Dose in mg	Individual no. of flukes	Average
Chlorambucil	20/1	0.2	3, 3, 4, 5, 2, 2, 2, 3, 5, 2	3.4
Amethopterine	20/3	0.125	2, 6, 2, 1, 4, 2, 4, 5, 5, 4	3.6
Cyclophosphamide	20/5	1.0	3, 5, 6, 1, 4, 3, 5, 5, 2, 3	3.6
Azathioprine	20/0	2.0	4, 1, 6, 4, 2, 5, 3, 6, 6, 4	4.0
Betamethasone	20/0	0.5	4, 2, 6, 3, 1, 5, 5, 4, 6, 2	3.8
Infected controls	20/0	—	5, 4, 3, 4, 2, 4, 5, 4, 4, 4	3.9

Table 2. Influence of immunosuppressants administered three times a week within week 2—week 6 of infection with adolescariae of *F. hepatica* on the rat

Group	No. of animals in the experiment/dead	Dose in mg	Individual no. of flukes	Average
Chlorambucil	20/3	0.2	5, 4, 3, 1, 6, 3, 2, 5, 3, 3	3.5
Amethopterine	20/5	0.125	5, 5, 3, 2, 6, 1, 3, 5, 5, 6	4.1
Cyclophosphamide	20/4	1.0	4, 3, 5, 2, 6, 3, 1, 6, 5, 4	4.3
Azathioprine	20/2	2.0	5, 1, 1, 6, 3, 2, 6, 7, 6, 5	4.2
Betamethasone	20/1	0.5	5, 4, 3, 5, 2, 1, 6, 3, 3, 5	3.7
Infected control	20/0	—	4, 6, 6, 4, 5, 5, 6, 3, 2	4.2

rimental groups (Table 2); it was highest in the group treated with cyclophosphamide. No death occurred in the uninfected control groups.

Examination of the differential blood picture disclosed a marked development of eosinophilia in both group I and II from week 2 p.i. onwards; it attained its maximum (up to 18 %) in week 3 p.i. and persisted until week 6 p.i. Although a gradual decrease in the number of eosinophils followed after this period, eosinophilia was still higher than in the controls at the end of the experiment. Administration of immunosuppressants to uninfected animals did not affect their white blood picture (see Fig. 1 and Fig. 2).

DISCUSSION

The immunosuppressants used in our experiments have a different mechanism of effect and belong to these groups: a) alkylating agents — chlorambucil (Leukeran), and cyclophosphamide (Endoxana). These have a marked cytotoxic effect on proliferating and, hence, also immunocompetent cells. Cyclophosphamide is ineffective in the form administered. It is activated by the splitting off of its phosphamide component by phosphoramidase present the microsomes of the liver cells, the bone marrow and the kidneys. b) Antimetabolites — imidazole derivate 6-mercaptopurine — azathioprine (Imuran), and the antagonist of ptero-glutamic acid (Methotrexate): these substances inhibit cell mitosis on the basis of nucleic acid synthesis. c) The corticosteriod substance betamethasone (Betsolan) has an effect similar to that of the remaining corticosteroids, i.e., in addition to its antiinflammatory effect it inhibits the lymphoreticular tissue and antibody production.

Of importance in the administration of immunosuppressive substances is the correct timing of the antigenic stimulus and that of treatment. With this in mind we arranged our experiments in this way: administration of immunosuppressants was introduced prior to the antigenic stimulus, i.e., infection with adolescariae of *F. hepatica*, and continued during the early phase of the parasite's development (experiment no. I); in experiment no. II, immunosuppressants were administered at the period of maximum migration of the flukes in the liver parenchyma and, in this case, the antigenic stimulus preceded the therapy. Our results suggested that the administration of several immunosuppressive substances in experiment no. II suppressed the developing (adaptive) defense response of the host causing thus a speedier migration and earlier sexual maturation of the flukes. The absence of defense mechanisms was responsible for an increased pathogenicity of the adolescariae administered and, consequently, for an increased mortality of the treated animals (up to 25 %). The sole responsibility for mortality should be ascribed to migrating forms of *F. hepatica*, since neither symptoms of disease (except for several cases of haemorrhagic enteritis after treatment with amethopterine), or death were recorded for both treated, uninfected und untreated, uninfected control animals.

A considerable individual variability in the number of flukes recovered is shown in tables 1 and 2. Since, in comparison with the experimental group, the number of developed flukes from an infective dose of adolescariae was more stable in the control groups it appears that individuals of the same species respond differently to immunosuppressive substances. The size of sexually mature flukes was not influenced significantly by the introduction of these substances. Therefore, we agree with Kendall (1967) in that the size of the flukes located in the bile duct (or gall bladder) depends directly on their number.

Eosinophilia in the peripheral blood is typical of many parasitic diseases including fascioliasis. Although knowledge of the function of eosinophils is incomplete as yet it

has been suggested that they signal an immune response of the allergic type. Sinclair (1970) inferred that an increased tissue eosinophilia as well as circulating eosinophilia are in correlation with retarded growth of the flukes and with the incidence of haemorrhagic lesions. Our results indicate that eosinophilia was not suppressed in the rats by the doses of immunosuppressants administered although in this case the individual variability in the number of eosinophils was again high.

In a recent experiment (Čorba et al. 1971) the authors observed that a delayed type of hypersensitivity developed in rats infected with *F. hepatica*. This could be transferred to isogenic recipients with the lymphatic tissue of the infected donors. In a provisional experiment (Čorba 1974 — unpublished) transfer of a large amount of immune serum to recipient rats influenced significantly an infection with *F. hepatica;* this confirmed the importance of humoral factors in the immune response to *F. hepatica*. In experiments with allergic skin tests, Flagstad et al. (1972) observed that the immune response of the host to infection with *F. hepatica* did combine both early and delayed type of hypersensitivity. In experiments with calves with a functional or morphological defect of the thymus, these authors did not find any marked difference in the number of developed flukes in the gall bladder of the infected animals in comparison with the control animals. They observed, however, that the animals were incapable to react to the presence of infection with *F. hepatica* with typical signs such as eosinophilia of the peripheral blood, allergic skin response, and a marked cellular reaction of the liver parenchyma to the presence of migrating larvae. Sinclair (1968) found in corticosteroid-treated lambs a speed-up in the development of *F. hepatica* and an increased pathogenicity of the parasite to its host. This was reflected in the death of the animals although, under normal conditions, this infective dose of adolescariae employed would have caused neither mortality nor major clinical symptoms. However, after re-infection, the administration of this substance did not prevent the development of eosinophilia and hyperglobulinaemia. Our results are a contribution to the knowledge of the effect of immunosuppressants on the development of the parasite and on the general response of the host. It may possibly attribute to the elucidation of immune response to this most important helminthiasis of economically important animals.

ВОЗДЕЙСТВИЕ ИММУНОПОДАВЛЯЮЩИХ ВЕЩЕСТВ
НА ЭКСПЕРИМЕНТАЛЬНУЮ ИНВАЗИЮ ТРЕМАТОДОЙ *FASCIOLA HEPATICA*

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Резюме. Авторы изучали воздействие иммуноподавляющих веществ — хлороамбуцила, циклофосфамида, азатиоприна, аметоптерина и кортизонового деривата бетаметасола — на развитие *Fasciola hepatica* у пасюков. Подавление иммунологических процессов в организме хозяина путем подачи иммуноподавляющих веществ имело результатом более раннюю миграцию червей в общий желчный проток и более раннее образование яиц по сравнению с контрольными животными. Самое яркое воздействие имела подача циклофосфамида и бетаметасона в период между второй и шестой неделями после инвазии, т. е. в период максимальной миграции червей в паренхиме печени. Патологические изменения в печени животных оказались менее выразительными чем у контрольных животных. Также наблюдали повышение патогенности инвазирующих начал, и тем и повышенную смертность животных по сравнению с контрольными. Применение иммуноподавляющих веществ однако не оказало влияния ни на общее число развитых червей ни на появление эозинофилии в периферической крови подопытных животных.

REFERENCES

BREZA M., ČORBA J., Porovnanie dvoch novších koprologických metód pri fascielóze hovädzieho dobytka.) Helminthologia (in press.

ČORBA J., ARMOUR J., ROBERTS R. J., URQUHART G. M. Transfer of immunity to *Fasciola hepatica* by lymphoid cells. Res. Vet. Sci. 12: 292—295, 1971.

—, ŠPALDONOVÁ R. Štúdium faktorov ovplyvňujúcich intenzitu invázie *Trichinella spiralis* u bielych myší. I. Vplyv imunosupresívnych látok aplikovaných počas črevnej a rannej migračnej fázy parazitácie. Biológia (Bratislava) 29: 167—173, 1974.

DOMINGO E. O., COWAN R.B.T., WARREN K. S. The inhibition of granuloma formation around *Schistosoma mansoni* eggs. I. Immunosuppressive drugs. Amer. J. Trop. Med. Hyg. 16: 284—292, 1967.

FLAGSTAG T., ANDERSEN S., NIELSEN K., The course of experimental *Fasciola hepatica* infections in calves with a deficient cellular immunity. Res. Vet. Sci. 13: 468—475, 1972.

HOPKINS C. A., SUBRAMANIAN G., STALLARD H., The effect of immunosuppressants on the development of *Hymenolepis diminuta* in mice. Parasitology 65: 111—120, 1972.

KENDALL S. B., SINCLAIR I. J., Barrier to reinfection with *Fasciola hepatica* in the rabbit. Res. Vet. Sci. 12: 74—79, 1971.

KOZAR M., Immunosuppressive influence of Imuran on trichinellosis in mice (Preliminary report). Wiad. Parazyt. 15: 628—629, 1969.

NÖLLER — According to CORS P., JAFFE R., NUSSEN A. Blutentnahme aus der retroorbitalen Venenplexus. Pathologie der Laboratoriumstiere, Part I, pp. 188, Springer Verlag, 1958.

RITTERSON A. L., Effects of immunosuppressive drugs (6-mercaptopurine and methotrexate) on resistance of Chinese hamsters to the tissue phase of *Trichinella spiralis*. J. Infect. Dis. 118: 365—369, 1968.

SINCLAIR R. B., The pathogenicity of *Fasciola hepatica* in previously infected, corticosteroid-treated lamb. Res. Vet. Sci. 11: 209—219, 1970.

URQUHART G. M., MULLIGAN W., EADIE R. M., JENNINGS F. W., Immunological studies on *Nippostrongylus brasiliensis* infection in the rat: the role of local anaphylaxis. Exp. Parasit. 17: 210—217, 1965.

WIEST L. M. Jr., Effect of immunosuppressants on the susceptibility of hamsters to *Trichostrongylus axei* and *T. colubriformis*. Proc. Helm. Soc. Washington 39: 46—51, 1972.

WILSON R. J. M., Effect of methotrexate on the parasitic development of the nematode *Dictyocaulus viviparus* (Metastrongylidae) and on the immune response of infected guinea-pigs. Parasitology 63: 145—156, 1971.

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