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The effect of immunosuppressive therapy on the course of development of *Dictyocaulus viviparus* in guinea-pigs

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Summary.

Three chemical immunosuppressive agents, *viz.* dexamethasone, methotrexate and cyclophosphamide, were administered to guinea-pigs two days prior to their infection with *Dictyocaulus viviparus* infective larvae and onward. The cell mediated immunity of these guinea-pigs was subdued under the influence of these immunosuppressive agents as evidenced by macrophage migration inhibition test but this could not prevent or postpone the rejection of majority of the worm population of guinea-pigs on day 15 post-infection. Methotrexate exerted, besides its cell mediated immunosuppressive action on the host, some inhibitory influence on the general biotic potentialities of the developing worms so that, on day eight post-infection, a reduced number of stunted worms was recovered.

Résumé.

Effet d'un traitement immunosuppressif sur l'évolution d'une infestation de *Dictyocaulus viviparus* chez le Cobaye.

Trois immunosuppresseurs chimiques, dexaméthazone, méthotrexate et cyclophosphamide, ont été administrés aux cobayes à partir de deux jours avant l'infestation avec des larves infestantes de *Dictyocaulus viviparus*. En appliquant le test d'inhibition de la migration des macrophages, il a été démontré que sous l'influence des immunosuppresseurs l'immunité cellulaire était réduite. Ce traitement n'a pas empêché ou retardé l'expulsion de la majorité des vers des cobayes 15 jours après administration des larves. En plus de son action immunosuppressive sur l'hôte, le méthotrexate avait une influence inhibitrice sur la biologie des vers, à telle enseigne que 8 jours après l'infestation on ne trouve qu'un petit nombre de vers freinés dans leur développement.

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An alternative laboratory host in which the pulmonary nematode of cattle, *Dictyocaulus viviparus,* could achieve egg-laying patent infection is not available or perhaps does not exist. The need and importance of such an experimental host, nevertheless, can not be overemphasised. Few workers (Soliman, 1953; Wade, Fox and Swanson, 1960 and Düwel and Schleich, 1971) had exposed a number of small laboratory animals to the infective larvae of *D. viviparus* and unequivocally found guinea-pigs the most satisfactory host for this infection. In this host, however, a strong immunological reaction develops so that the major part of the worm burden is expelled after about two weeks of development (Poynter *et al.,* 1960; Wilson, 1966 and Wieczorowski, 1971). Guinea-pigs are known to be the susceptible laboratory host for another metastrongylid nematode, *Metastrongylus apri,* in which patent infection has been achieved (Dunn and White, 1954 and Düwel and Schleich, 1971).

The present paper reports on the course of development of *D. viviparus* among guinea-pigs which were administered various chemical immunosuppressive agents.

Materials and methods

Animals :

48 random bred albino guinea-pigs of both the sexes, 10 week old and weighed 350 to 450 gm, were divided in four groups, each of 12 animals. These groups were maintained in separate cages and were fed vitamin C enriched commercial pellets, carrots and water *ad lib.*

Infective larvae :

Freshly harvested infective larvae of *D. viviparus* were obtained through the courtesy of Allen and Hanbury, Herts, England. These were stored at 4° to 6 °C and used within 15 days of their harvest for experimental infection of guinea-pigs. The authors had experienced that storage of the larvae for this duration did not influence their infectivity for guinea-pigs. Each guinea-pig received 1,000 such larvae orally. For this purpose the larvae were counted by random count method and inoculated directly into the oesophagus of the guinea-pigs by a *per os* inoculation needle.

Immunosuppressive agents :

The guinea-pigs of the groups I to III were administered dexamethasone (0.2 per cent suspension), methotrexate (Ledertrexate sodium, brand of Lederle Laboratories Division, N.J.) and cyclophosphamide (Endoxan, brand of Janssen Pharmaceutica, Beerse, Belgium), respectively.

The guinea-pigs of group IV served as untreated controls. The general schedule of the experimental plan including the dose rate of immunosuppressive agents and their mode of administration are shown in Table I.

Table I. *Experimental design.*

Groups	No. of guinea-pigs	Immunosuppressive therapy		Infection day	Day of sacrifice and no. of animals		
		Schedule for each animal	Time of initiatiion	Day 0	Day 8	Day 15	Day 19
I (Dexamethasone)	12	0.5 mg. s.c., thrice a week	2 DPreI	1000 larvae orally	3	6	3
II (Methotrexate) ..	12	0.5 mg, i.m., thrice a week	"	"	"	"	"
III (Cyclophosphamide)	12	10.0 mg, s.c., once a week	"	"	"	"	"
IV (Untreated control)	12	—	—	"	"	"	"

Days preinfection abbreviated as DPreI.

Recovery of worms :

From each group, three guinea-pigs were sacrificed on day eight post-infection, six were sacrificed on day 15 post-infection and the remaining three were sacrificed on day 19 post-infection. The choice of the days of sacrifice was based on previous preliminary experiment where it was found that the infected guinea-pigs harboured maximum number of worms between seven and nine days post-infection, that the great majority of the worm burden of the host was eliminated on day 15 post-infection and that on day 19 post-infection it was extremely rare to find the nematodes in the lungs of guinea-pigs. For sacrifice, each guinea-pig was put under deep ether anesthesia before the pleural cavity was opened. From the guinea-pigs sacrificed on day 15 post-infection, however, the peritoneal macrophages were harvested first for carrying out macrophage migration inhibition test as described below before the pleural cavity was opened.

The lungs along with the trachea of each guinea-pig were excised and removed. The left lung and the accessory lobe were neatly separated from this, chopped into fine pieces and put on Baermann's apparatus. It was allowed to stand for about 20 hours before the worms accumulating in the stem of the funnel were recovered. The chopped pieces of lungs were further macerated and washed in physiological saline through a coarse sieve (mesh, 1 mm square) and the washing was examined to ensure that the worms which had failed to escape out of the lung pieces during Baermann's procedure were not lost. The worms collected by these two procedures were pooled and counted.

Length of worms :

The lengths of worms recovered on day eight post-infection of the guinea-pigs of each group were measured. For this purpose randomly selected 20 male and 20 female

specimens were included. The median axes of these specimens were drawn with the help of a camera lucida and the lengths of these axes determined with the aid of the scale of stage micrometer slide.

Macrophage migration inhibition test (MIT) :

MIT was performed with the peritoneal macrophage harvest of the guinea-pigs of various groups which were sacrificed on day 15 post-infection.

Each animal was injected 20 ml sterile liquid paraffin intraperitoneally three to four days before sacrifice for harvesting the peritoneal macrophages. For sacrifice the guinea-pig was put to sleep under deep ether anesthesia. The peritoneal cavity was irrigated with 60 ml of cold Eagle's MEM containing Hank's salts (pH 7.2), the abdominal wall was kneaded gently and then all the fluid content of peritoneal cavity was withdrawn under strict sterile precautions. The available fluid containing peritoneal exudate was centrifuged in cold at 450 $\times$ *g* for 10 minutes. The supernatant was discarded and the sediment was washed twice in Eagle's MEM containing Hank's salts (pH 7.2) by centrifugation as before. The washed sediment was mixed in 20 volumes of cold TC 199 containing 15 per cent heat inactivated guinea-pig serum. Viability of the macrophages was assessed by dye exclusion test using 0.1 % eosin and the concentration of macrophages in the suspension was adjusted in such a way that each ml contained 4-5 $\times$ 10^7 macrophages.

Six microhaematocrit tubes were filled with the homogenous suspension of macrophages from a guinea-pig, sealed at one end with plasticine and centrifuged for five minutes at 1000 r. p. m. Each tube was then cut at a point where the packed macrophages had a depth of about three mm above the plasticine. The cut pieces were placed singly in sterilized migration chambers; the plasticine ends of these "explants" were held in position by a small amount of vasiline so that the cut ends lay in the centre of the chamber. Three of these chambers containing "explants" were filled with TC 199 containing 15 per cent inactivated guinea-pig serum while the remaining three chambers were filled in the same way excepting that in this case each ml of TC 199 and guinea-pig serum mixture contained 1.25 mg of lyophilised hydrosoluble extract of adult *D. viviparus* antigen. The chambers were covered with cover glass and sealed in position by a rim of vaseline without entrapping any air bubble. The chambers were incubated for 24 hours at 37 °C.

The margins of the migration areas were drawn on a mm graph paper through camera lucida image. Planimetry of the migration area was done by precisely counting the number of mm squares covered inside the drawn image.

$$\text{Migration index} = \frac{\text{Mean migration of test group (with antigen)}}{\text{Mean migration of control group (without antigen)}} \times 100$$

Observations

The frequencies of worm recovery from the left lungs and accessory lobes of various groups of guinea-pigs are summarized in Table II. The maximum number of worms were harvested on day eight post-infection of the guinea-pigs of all the groups;

Table II. Frequency of worm recovery from guinea-pigs of various groups after varying periods of infection with *Dictyocaulus viviparus*.

Groups	Mean number of worms harvested from the left lung and the accessory lobe of each guinea-pig on		
	Day 8	Day 15	Day 19
I (Dexamethasone)	278.6	2.3	0
II (Methotrexate)	34.3	0.7	0
III (Cyclophosphamide)	103.3	2.0	1.0
IV (Untreated control)	149.0	2.7	0

The results are based on examination of three, six and three guinea-pigs on day 8, day 15 and day 19, respectively.

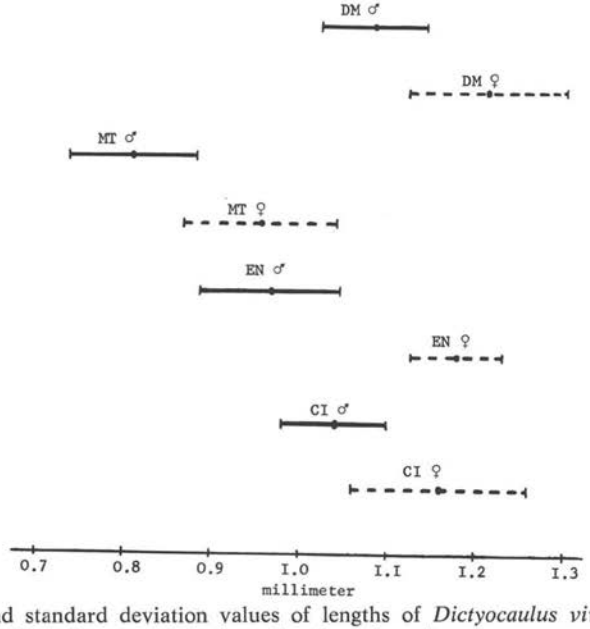

Fig. 1. Mean and standard deviation values of lengths of *Dictyocaulus viviparus* recovered eight days post-infection of guinea-pigs of various groups.
DM : Dexamethasone ; MT : Methotrexate ; EN : Cyclophosphamide ; CI : Untreated control.

there being relative difference in various groups when compared with untreated infected group. The trend of elimination of worm burden of the guinea-pigs of these groups was clearly in evidence on day 15 post-infection. On day 19 post-infection, this process of elimination was most pronounced in so far as none of the three groups of guinea pigs harboured any worm; only the guinea-pigs of cyclophosphamide group yielded one worm each.

The mean lengths of worms of both the sexes harvested on day eight post-infection from various groups of guinea-pigs are illustrated in figure 1. It is observed that the mean lengths of the recovered worms were to a small measure directly proportionate to the frequency of their occurrence.

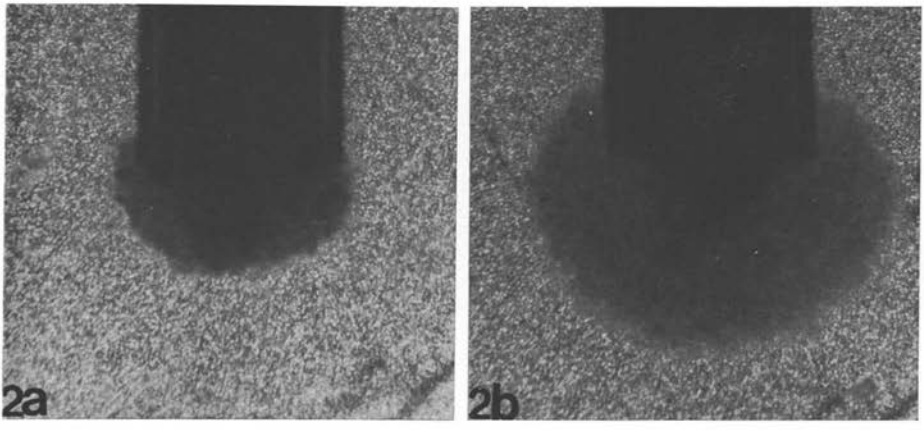

Fig. 2. Macrophage migration inhibition test. 2 *a* illustrates the migration pattern of macrophages of a guinea-pig receiving dexamethasone therapy and infected with *D. viviparus*. The medium of incubation contained antigen. 2 *b* shows macrophage migration pattern of the same animal in the absence of antigen in the medium.

MIT (*fig. 2* a *and 2* b) was performed on the guinea-pigs of each group only on day 15 post-infection to assess the effect of various immunosuppressive therapy on the cell mediated immune response of these animals. The mean values of migration indices of these groups are presented in Table III.

Table III. *In vitro* migration inhibition of peritoneal macrophages of guinea-pigs infected for 15 days with *Dictyocaulus viviparus*.

Groups	Mean value of migration index (%)
I (Dexamethasone)	66.8
II (Méthotrexate)	83.4
III (Cyclophosphamide)	80.3
IV (Untreated control)	36.1

Discussion

The guinea-pigs of the untreated infected group yielded an average of 149.0 worms each on day eight post-infection. The comparable worm recoveries from the remaining three groups were as following; — group I (dexamethasone) yielded 278.6 worms each, group II (methotrexate) yielded 34.4 worms each and group III (cyclophosphamide) yielded 103.3 worms each. Evidently, methotrexate, besides its immunosuppressive action on the host, had some inhibitory influence on the general biotic potentialities of the nematode so that the normal evolution of invasion of guinea-pigs of this group by *D. viviparus* was altered and reduced number of worms was recovered.

Further, the worms recovered from the methotrexate group on day eight post-infection were relatively stunted when their mean lenght was compared with those of other treated or untreated groups. These findings essentially confirm the results of Wilson (1964, 1971) who found that methotrexate, when administered intraperitoneally on alternate days at a rate of 5 mg per guinea-pig two days before infection or on the day of infection, resulted in fewer worm recovery and stunting of these worms when compared with untreated control.

On day 15 post-infection, a great majority of worm burden of the guinea-pigs of all the groups was rejected. None of the three chemical immunosuppressive agents used in this experiment could prevent or postpone this process of rejection of worm burden. While the untreated infected group of guinea-pigs yielded 2.7 worms each on day 15 post-infection, after comparable periods of infection the animals of dexamethasone, methotrexate and cyclophosphamide yielded 2.3, 0.7 and 2.0 worms each, respectively. Accordingly, although on day eight post-infection the guinea-pigs of dexamethasone group yielded maximum number of worms when compared with other groups, on day 15 post-infection the situation is reversed in so far as this group did not yield more number of worms than those of untreated infected group.

The *in vitro* MIT is known to be a direct correlate of cell mediated immunity of the host. When the value of macrophage migration index is higher than 60 per cent, it is suggestive of vaining of cell mediated immune mechanism of the host. In the present experiment all the chemical immunosuppressive agents employed at the stated dose were found to subdue the cell mediated immunity of the guinea-pigs on day 15 post-infection when compared with untreated infected group. This subdued immunity, however, could not exert any influence on the process of rejection of worm burden of the host.

The opinions of various workers concerning the precise schedule of cyclophosphamide administration are somewhat divided. According to Patkowski (1967) and Bratkowska-Seniow (1972), as cited by Martynowicz (1975), cyclophosphamide is most effective as an immunosuppressive drug when it is administered before or simultaneously with antigen. On the other hand, Turk (1964), Dukow and Dietrich (1970) and Many and Schwartz (1970) had used the drug after sensitization of the host. In

the present experiment the weekly administration of cyclophosphamide was commenced two days before infection of the host and the MIT suggested that the schedule followed was effective in suppressing the cell mediated immune response of the host.

Rejection of *D. viviparus* by the infected guinea-pig, atleast in secondary or superimposed infection, is mediated by immunological mechanism as this host acquires an immunity during the primary infection which prevents the establishment of superimposed infections (Poynter *et al.*, 1960). If this is the sole mechanism or other non-immunological mechanisms are operating this phenomenon of rejection in primary infection is not clear. The evidence available from the present experiment shows that the rejection in primary infection is not mediated by specific cell mediated immune response of the guinea-pigs. If this was not the case, suppression of cell mediated immunity of guinea-pigs by chemical immunosuppressive agents could have postponed or prevented the rejection of worms in primary infection. However, since the inhibitory influence of the immunosuppressive agents on the humoral response of the guinea-pigs was not assessed, future studies in this direction could resolve the question of mechanism mediating the rejection of primary infection of *D. viviparus* in guinea-pigs. In any case, the non-immunological mechanisms operating this phenomenon, if any, may not be altered with the use of immunosuppressive agents.

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