IMMUNOTHERAPY OF POST-SURGICAL ALVEOLAR HYDATID DISEASE (AHD)

by

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ABSTRACT

Immunotherapy of post-surgical Alveolar Hydatid Disease (AHD) was attempted using an attenuated strain of *Mycobacterium bovis* (BCG) in this study. Gerbils were subcutaneously infected with *Echinococcus multilocularis*. Ninety days later the established subcutaneous cyst mass was surgically removed in three groups of gerbils that had been administered with either BCG (a macrophage activator), Carrageenan (CAR) (an anti-macrophage agent) or saline 10-12 days before the operation. In the fourth group of gerbils the established cyst was left intact. At necropsy 30 and 60 days post-surgery, body regions with disseminated cyst foci were recorded and metastatic cyst weights taken in all the experimental groups. Cyst tissue samples were taken for histology and blood smears were examined for differential white blood cell counts.

Metastasis occurred mostly to the draining lymph nodes suggesting that germinal tissue was disseminated mostly via the lymphatic vessels.

All four treatments groups did not show any significant difference in their levels of metastasis. Histological appearance of the qualitative leucocyte cellular response towards the parasite was similar in all the groups.

It is suggested that BCG does not have an enhancing effect on macrophages already suppressed by the chronic parasitic infection. In fact macrophages may be further suppressed, thus not controlling post-surgical growth of metastatic foci.

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DEDICATION

Ne jonyuolna,

Jaduong' Aluoch S. Ogundo kod Mama Adero C. Aluoch.

Tich gi matek ema omiyo achopo kama antieni.

Nyasaye ogwethgi.

INTRODUCTION

Alveolar Hydatid Disease (AHD) is a fatal disease of humans caused by the larval stage of a cestode, *Echinococcus multilocularis*. The rate of development of this solid, tumour-like, vascularized cyst mass is determined by the species or strain of host involved. Thus, the larval stage grows without inhibition in susceptible hosts (e.g. humans) debilitating and eventually killing them (Ali-Khan *et al.*, 1983; Eckert *et al.*, 1983; Kizaki *et al.*, 1991). Humans get accidental infections through ingestion of oncospheres and usually serve as "dead end hosts" in the life cycle. Despite the increasing recognition of this parasite as a disease-causing agent and its impact on human health, only limited progress has been made in research to develop control measures for *E. multilocularis* (Gemmell *et al.*, 1987). Eckert (1992) indicated that there existed a potential infection risk for humans in wider areas of Central Europe than was previously known, as a result of the prevalence and geographical distribution of *E. multilocularis* in foxes in those areas.

Echinococcus species require two mammalian hosts to complete their life cycles. The definitive host, usually a canid, is infected by ingestion of protoscoleces produced by asexual multiplication of the larval stage. Sexually mature adult worms, living in the small intestine of a canid, release oncospheres containing fully-developed embryos which are voided in the faeces. Ingestion of oncospheres by intermediate hosts, omnivores or herbivores, leads to the development of the larval stage in various organs, mainly the liver, lungs and brain. The cycle is completed when the canid feeds on the infected organ.

Hydatidosis.

Hydatidosis refers to infection with the larval stage of an *Echinococcus* cestode. The larval stage of *Echinococcus* cestodes, with its low host specificity, has a high reproductive ability and is considered among the most significant parasitic zoonotic diseases. In *E. granulosus*, the larval stage is subspherical in shape and has a fluid-filled unilocular cyst that proliferates endogenously to form daughter cysts within the original cyst. Proliferation of *E. multilocularis* larval stage occurs both endogenously and exogenously (new cysts created outside the original cyst) resulting in numerous small vesicles embedded in a dense stroma of connective tissue. These vesicles appear grossly as a bunch of grapes hence the name multivesicular or alveolar hydatidosis. The undifferentiated germinal cells of alveolar hydatid cysts may detach and disseminate through lymph or blood to give rise to distant metastatic foci, a feature unique to *E. multilocularis*.

Hydatidosis is a serious disease to the infected individual and costly to those countries in which the parasite is prevalent. Although there are no reliable data on the worldwide human prevalence of the disease, it is generally accepted that *E. granulosus* is cosmopolitan whereas *E. multilocularis* is restricted to the northern hemisphere (Gemmell, *et al.*, 1987). McManus and Smyth (1986) reported that human cases of AHD frequently occur in parts of the USSR, Japan, Alaska and Central Europe, and that there has been an extension to countries previously free of it such as China, India, Iran and USA thus increasing the threat of spread beyond its current southern limit.

Natural secondary hydatidosis is believed to be the result of the leakage of protoscoleces from ruptured parent cysts and the transformation of these larvae into cysts identical to the parent cyst (Kassis and Tanner, 1976). Metastatic foci of *E. multilocularis* do not always grow to attain large sizes in hosts; instead, they usually remain small. Their failure to grow in size is thought to result from suppression by an established larger parent cyst (Rau & Tanner, 1973). This characteristic of AHD is shared with malignant tumours whereby the presence of a primary tumour inhibits growth of its distant metastatic foci. Animals already bearing an established primary tumour are resistant to homologous challenge (Gershon *et al.*, 1968; Marx, 1974). Removal of such established parent cysts favours the growth of the distant metastatic foci as a result of removal of concomitant immunity. Rau and Tanner (1976) showed that there was a flare-up of intrathoracic lesions of AHD in cotton rats after surgical removal of the established subcutaneous cyst mass. More recently, Liance *et al.* (1992) reported a rapid growth of metastatic foci in lungs following removal of infected livers with subsequent liver transplants in human patients. Gershon *et al.* (1968) noticed that excision of a primary lymphoma from hamsters led to the appearance of metastatic foci and that second grafts of such tumours were accepted by the hamsters. Flare-up of distant metastatic foci is a common complication after surgical resection of the initial parent cyst mass in treatment of hydatidosis in man.

Growth of distant metastatic foci can also be enhanced by immunosuppression (Baron and Tanner, 1976; Bresson-Hadni *et al.*, 1992; Liance *et al.*, 1992). Carrageenan (CAR), a polysaccharide of high molecular weight with a long chain

sulphated salt, obtained from the marine plant *Chondrus crispus*, has many biological properties including immune suppression. CAR inhibits the complement system, induces lysis of macrophages and suppresses delayed hypersensitivity reactions (Catanzaro *et al.*, 1971; Ishizaka *et al.*, 1977; Keller, 1980; Schwartz and Catanzaro, 1981). CAR abrogated the protection induced in cotton rats by phytohaemagglutinin (PHA) such that metastatic cyst mass weights were identical to those in infected controls (Reuben and Tanner, 1983).

Echinococcus species do establish and survive in their habitats for considerable periods of time despite the strong immunological reaction of the host (Reuben *et al.*, 1979). This can be explained in terms of immune evasion by the parasite. The larval stage of *Echinococcus spp.* has been reported to have some immunosuppressive properties. Annen *et al.* (1981) showed that *E.granulosus* cyst fluid contains a cytotoxic ingredient that is heat stable, of low molecular weight and could easily penetrate through the cyst wall and interfere locally with immunocompetent cells thus facilitating the long time survival of the parasite. Heath (1986) reported that there appeared to be a toxic product from juvenile cysts of *E. granulosus* that was able to destroy host lymphoid cells in close contact with the cyst. Recently, it has been shown that hydatid and coenurus cysts (cysts of *Taenia multiceps*) produce a secretory or excretory substance that alters the protective functions of accessory cells to become suppressive *in vitro* (Dixon *et al.*, 1982; Rakha *et al.*, 1991a, b & c; 1992). Janssen *et al.* (1992) demonstrated the presence of a secretory toxic substance from hydatid cysts that reduces the viability of macrophages *in vitro*. Anticomplementary components in

hydatid cyst fluid have also been demonstrated (Ferreira and Nieto, 1992).

Treatment

Treatment of hydatidosis has limited success. Chemotherapy with several anthelmintics has been attempted. Ochieng-Mitula (1991) working with gerbils found that Ivermectin was only partially effective in the treatment of AHD, while Wen *et al.* (1992) reported that the widely used benzimidazoles are only parasitostatic and have adverse side-effects on human patients. Different methods of immunotherapy have also been tried. Magambo (1992) reported some success with immunotherapy using activated leucocytes; only early treatment with the activated cells eliminated the hydatid cyst. Reduced success of immunotherapy may be attributed to the ability of taeniid infections to induce immunodepression in their hosts (Williams, 1979). The hosts do have an immunological response towards hydatidosis. Complement proteins are protoscolicidal *in vitro*. Heat-inactivated serum from an infected animal is also cytotoxic to protoscoleces of *E. multilocularis in vitro* and replacement of hydatid cyst fluid of *E. granulosus* with serum from an infected animal can kill the cyst (Kassis and Tanner, 1976). Ali-Khan and Siboo (1981) also reported the presence of specific immmunoglobulins against the cyst membranes that could assist in complement activation and antibody-dependent cell-mediated cytotoxicity. However, the immune response elicited is unable to check the growth and dissemination of the parasite probably due to the immune evasive techniques employed by the parasite.

Surgery is another form of treatment of AHD. Surgical removal of the larval cyst mass is difficult in *E. multilocularis* as a result of exogenous budding of the cyst

and is complicated by periodic relapses of the disease after such a treatment. Bresson-Hadni *et al.* (1992) reported recurrence of AHD in an individual after replacement of a severely infected liver with a new liver transplant, as a result of cerebral metastasis. There was also recurrence on the grafted liver. Liance *et al.* (1992) have shown that patients who had transplants, had a relatively rapid growth of smaller parasite foci in the lungs and peritoneal cavity. Subcutaneous cysts of *E. multilocularis* exhibit a surprisingly high incidence of metastasis. Distinct metastatic foci are frequently found in the thoracic cavity and lungs of infected animals (Rau and Tanner, 1973; 1976; Eckert *et al.*, 1983). Effective surgical removal therefore depends on the success of controlling the recurrences of the distant metastatic foci of the cyst mass. Immunotherapy seems to be a better approach to treatment of AHD as it may control metastases.

Attempts have been made to prevent both the establishment and development of *E. multilocularis* cysts in their hosts. Suppression of growth of *E. multilocularis* cysts has been attempted using an attenuated strain of *Mycobacterium bovis*, BCG (*Bacillus Calmette-Guerin*) or phytohaemagglutinin (PHA). Treatment with BCG, before intraperitoneal infection, completely prevented infection (Rau & Tanner, 1975; Reuben & Tanner, 1979; Reuben *et al.*, 1978, 1979). Treatment after infection reduced dissemination to other sites (Rau and Tanner, 1975). BCG provides protection against several different parasites: *Trypanosoma cruzi* (Ortiz-Ortiz *et al.*, 1975); *Babesia* and *Plasmodium* (Clark and Allison, 1976); and *Leishmania* (Frommel and Lagrange, 1989). Such treatment works by activating macrophages which then become

destructive to various organisms (Keller, 1980; Mantovani *et al.*, 1992). The protection is immunologically non-specific to both neoplastic diseases (Pearson *et al.*,1973; Baldwin and Pimm, 1973; Snodgrass and Hanna, 1973; Wolfe *et al.*, 1976) and infectious diseases (Larson *et al.*, 1972; Nakamura and Cross, 1972; Clark and Allison, 1976; Ortiz-Ortiz *et al.*, 1975; Frommel and Lagrange, 1989). BCG protective effects are said to be independent of a wide range of doses of viable organisms and routes of administration; doses as low as 80 viable organisms were able to protect mice against murine leukaemia (Pearson *et al.*, 1973).

There is no reported information on immunological control of post-surgical alveolar hydatidosis. Armed with the information that the presence of an established cyst of *E. multilocularis* inhibits growth and dissemination of secondary cysts or re-infection and that BCG prevents growth and metastasis of *E. multilocularis*, it can be visualised that the immune response might be manipulated to influence the interaction between the parasite and the host. This might be done such that upon surgical removal of the primary destructive cyst mass, the metastatic foci may be kept small thus not causing any overt pathology while still boosting the immune response to prevent re-infection or parasite growth. The main goal of this study, therefore, was to examine how to control growth of secondary hydatidosis following surgical treatment of AHD. The following three objectives were set based on the above.

Objectives:

(i) To determine the effects of BCG (a known macrophage activator) and the effects of CAR (a known anti-macrophage agent) in modulation of the growth of

metastatic foci after surgical removal of a primary cyst of *E. multilocularis;*

(ii) In conjunction with (i) above, to attempt to reduce or control rapid growth of post-surgical metastatic foci of hydatid cysts; and

(iii) to understand better the immunomodulatory effects of BCG in a host chronically infected with hydatid cysts.

MATERIALS AND METHODS

Experimental animals.

Male gerbils, *Meriones unguiculatus*, five weeks old, were purchased from High Oak Ranch Ltd., Goodwood, Ontario, Canada. The gerbils were fed laboratory Rodent Chow® (Purina, Canada Inc.) *ad libitum* and had free access to water throughout the entire period of the experiment. The gerbils were kept in groups of four per cage of 22 x 42 x 14 cm and were infected with hydatid cyst material one week after acclimation to our laboratory conditions.

Experimental parasite

Echinococcus multilocularis hydatid cysts were obtained from a naturally infected wild rodent by Dr. R.H. Rausch, University of Washington, Seattle. The parasite was then maintained at UNB by serial passage in laboratory gerbils by homogenizing hydatid cyst mass in 100% heat inactivated fetal calf serum and injecting 0.5ml of homogenized material intraperitoneally into naive gerbils as previously described (Lubinsky, 1960; Rau and Tanner, 1973; Kroeze and Tanner, 1986).

Infection of experimental animals.

Sixty, six-week-old gerbils were anaesthetized with Somnotol® (Sodium pentobarbital, 65mg/ml from M.T.C. Pharmaceuticals, Canada Packers Inc.) diluted to a concentration of 21mg/ml with 10% ethanol and administered intraperitoneally at a dose rate of 50 mg/kg body weight. The gerbils were placed on a well-padded dissection plate. Left inguinal area was then shaved clean and disinfected with 70%

ethanol. A 10mm incision was made on the skin. The skin was then undermined by blunt dissection. A parasite cyst mass weighing 0.15-0.20 g and consisting of acephalic vesicles (small vesicles which have no protoscoleces) was inserted under the skin through the incision. The skin incision was subsequently closed using plain or chromic catgut sutures (Ethicon sutures Limited, Peterborough, Ontario) applying an interrupted suture pattern. The surgical area was then disinfected with lugol's iodine.

The gerbils were kept in warm, small plastic cages until they recovered from the general anaesthesia. Thereafter, they were put back into their cages which had clean sawdust bedding.

Treatment groups

Eighty days post-infection (p.i), the gerbils that had developed a palpable subcutaneous cyst mass were randomly divided into four groups as follows:-

(i) BCG group

At 80 days p.i., 14 gerbils were given an attenuated strain of *Mycobacterium bovis*, BCG. The lyophilised BCG vaccine (Connaught Laboratory Limited, Willowdale, Ontario) was reconstituted with the diluent (sterile buffered saline solution) according to the manufacturer's instructions to form a concentration of 1mg/ml in which 1ml contained at least 8.0×10^6 colony forming units (CFU) of organisms at bottling. The organisms were diluted with sterile distilled water (Baldwin and Pimm, 1973) to a concentration of 8.0×10^3 CFU/ml. Each gerbil was given 1.6×10^3 CFU of organisms intraperitoneally.

The gerbils were anaesthetized as stated above at 90 - 92 days p.i. and the

developed subcutaneous cyst mass was removed aseptically by first shaving the surgical area, disinfecting with 70% ethanol, then making a 30 mm incision above the cyst mass. The skin was carefully detached from the cyst mass by blunt dissection using a pair of scissors. The detached cyst mass was held by forceps, and cyst attachment to the abdominal wall was severed making sure that no cut was made on the abdominal wall and that no spillage of cyst contents onto the abdominal wall occurred as described by Rau and Tanner (1976). Lugol's iodine was applied on the surgical area using a cotton swab. The skin incision was then apposed and finally sutured with plain catgut. The excised cyst mass freed, as much as possible, of any adherent host tissue, was then weighed. The gerbils, after recovering from anaesthesia, were taken back to their cages which had clean sawdust bedding.

Necropsy was conducted on 6 of the 14 gerbils at 120±1 days p.i. The gerbils were anaesthetized using ether, followed by cervical dislocation. Blood was collected from the tail just before cervical dislocation to make smears for differential white blood cell (WBC) counts. The euthanised gerbils were then placed on a dissection tray and pinned down on four limbs. The skin was carefully removed starting from the ventral mid line. Metastatic sites under the skin were recorded as was their relationship to the draining lymph nodes. The abdominal and the thoracic cavities were then thoroughly checked for any evidence of metastatic foci. The extent of parasite dissemination to different areas was recorded. All the disseminated parasite foci were removed and weighed. Samples of the parasite foci were taken for histological examination.

Necropsy was conducted on the remaining 8 of the gerbils at 150±1 days p.i. Other procedures were as for the first 6 gerbils stated above.

(ii) Carrageenan group

At 80 days p.i. 10 gerbils were injected with carrageenan, CAR (Gelcarin GP 812, FMC Corporation, Philadelphia; donated by Dr. C.E. Tanner, of the Institute of Parasitology, McGill). The drug was dissolved in physiological saline by heating in a water bath at 60^0 C. for about 20 minutes. Each gerbil was given 0.6 ml intraperitoneally at a concentration of 5mg/ml (Ishizaka et al., 1977; Schwartz and Cantazaro, 1981). Surgical removal of established subcutaneous cysts at 90-92 days p.i. and subsequent necropsy at 120±1 and 150±1 days p.i. were done as in the BCG group.

(iii) Saline group

Twelve gerbils in this group were injected with 0.6 ml of physiological saline solution (0.9%) intraperitoneally 80 days p.i. At 90-92 days p.i., the established subcutaneous cyst mass was removed surgically. Necropsy was done on 5 and 7 gerbils at 120±1 and 150±1 days p.i. respectively as in the two groups above.

(iv) Intact cysts group

Unlike the three groups above, 10 gerbils in this group had their established subcutaneous cysts intact at necropsy 120±1 and 150±1 days p.i. respectively. The subcutaneous cysts were removed at necropsy and weighed. The subcutaneous area, abdominal and thoracic cavities were examined for disseminated parasite foci. If the foci were found, they were removed, weighed and samples taken for histological

examination as in the other groups.

Blood smears

Thin blood smears were made from gerbils of each experimental group.These were air dried and fixed in methanol. The slides were then stained with giemsa and examined under a compound microscope (Olympus) for differential white blood cells (WBC) count. Three to four hundred WBC per experimental group were counted as in Ali-Khan (1974), Panosian & Wyler (1983) and Kroeze & Tanner (1986). Different WBC were noted and the count expressed as a percentage of the total WBC counted.

Phagocytosis.

Phagocytotic activity was assessed by the uptake of latex beads by circulating monocytes and peritoneal macrophages as described by Panosian & Wyler (1983) and Mosser & Handman (1992). Six gerbils with subcutaneous infections were divided into three groups of two gerbils each. The animals in each group had received either 1.6 x 10^3 CFU of BCG, 0.6 ml (5mg/ml) of CAR or 0.6 ml of physiological saline. Blood from gerbils in these groups was obtained by cardiac puncture at necropsy 35 days post-treatment. Monocytes were isolated using a modification of the methods of Kumugai *et al.* (1975), Abo *et al.* (1976) and Lissoni *et al.* (1993). Blood samples from individual gerbils were pooled for each group. Two millilitres of Ficoll-paque® (Pharmacia) were put into a test tube and an equal volume of heparinized blood was then layered onto the Ficoll-paque® according to the manufacture's instructions. The mixture was centrifuged at 1700 rpm for 20 minutes. The top plasma layer was pipetted off and the lymphocyte-monocyte layer removed. To separate monocytes from

lymphocytes, the layer was put on a cover slip (22x50 mm) in a four-well multidish cell culture plate (ICN, Biomedicals, Cleveland, Ohio) and Dulbeccos Minimum Essential Medium supplemented with 10% Newborn Calf Serum (DMEM + 10% NCS) added. The mixture was incubated at 37^0 C. for one hour to allow monocytes to adhere to the cover slip. The non-adherent lymphocytes were pipetted off and fresh DMEM +10% NCS added to the adhered monocytes.

Carboxylated latex beads (0.93µm in size, Seradyn Inc., Indianapolis, Indiana) were added to physiological saline (1:30) and 0.5ml of this was added to the culture medium over the monocytes giving a ratio of monocytes to beads of approximately 1:40. The monocytes were then reincubated for another one hour at 37^0 C, and subsequently observed using an inverted microscope (Olympus, CK2) for any evidence of phagocytosis of the beads. The cover slips with monocytes were then removed, washed in running tap water to remove excess beads, air dried and fixed in methanol before staining with giemsa. The stained cover slips were mounted on slides and examined under a compound microscope (Olympus) for evidence of phagocytosis of beads.

Peritoneal macrophages were collected according to the methods described by Mosser & Edelson (1984) and Mosser & Handman (1992). Ten gerbils were divided into two groups of five gerbils each. One group was infected intraperitoneally with 0.2 ml of homogenized hydatid cysts; the other group was left uninfected. At 28 days p.i. two gerbils from each group were treated with 1.6×10^3 CFU of BCG. Another two gerbils per group were treated with 0.6 ml (5mg/ml) of CAR. The remaining two, one

from each group, were given 0.6 ml of physiological saline intraperitoneally. Forty eight hours post-treatment, the gerbils were anaesthetized and warm physiological saline injected into their peritoneal cavities. The peritoneum was massaged to dislodge the peritoneal cells. The cells were aspirated with a 1ml syringe and needle (gauge 23) and placed onto cover slips in a four-well multidish plate. DMEM + 10% NCS was added onto the cells, the plates covered and the mixture incubated for 1 hour at 37^{0} C. Monocyte adherence, addition of beads and other subsequent procedures were done as for the circulatory monocytes.

Histology.

Tissue samples from each treatment group taken for histology were fixed in a 10% buffered formalin solution for at least two weeks, then left in running tap water overnight. The samples were dehydrated in 50%, 70%, 95%,100% and 100% ethanol; cleared twice (45 minutes each) in a clearing agent (Histoclear™,National Diagnostics, Manville, New Jersey), before impregnation twice (45 minutes each) with paraffin wax. Tissues were then embedded in wax and sections cut at 10 µm with a Rotary Microtome (Reichert, Histo STAT). The sections were mounted on a glass slide that had a thin film of Glycerol-Albumen (Mayer's, BDH Inc.) and left overnight. The sections were deparaffinized using routine procedures before staining with haematoxylin and eosin (Humason, 1979). They were then examined under a compound microscope for a qualitative immunological cellular response.

RESULTS

Macroscopical observations

Necropsy results are summarized in Table 1. In all the treatment groups, a total of 34 gerbils (74%) used in the experiment showed metastases with cyst in different regions of the body.

The percentage of gerbils showing metastasis was high (93%) in the BCG group (administered with 1.6 x 10^3 CFU of attenuated strain of *Mycobacterium bovis* 10-12 days before surgical removal of an established subcutaneous cysts) and lower (50%) in the Saline group (physiological saline given 10-12 days before surgical removal of an established subcutaneous cyst).

Figure 1 shows the averages of the total metastatic cyst weights per gerbil for the different treatment groups. CAR group (given carrageenan 10-12 days before surgical removal of an established subcutaneous cysts) had a higher average total metastatic cyst weight (1.1 grams, range 0.0-4.72) than the other groups, while the Intact Cysts group (no surgical removal of an established subcutaneous cyst mass) had the lowest average total metastatic cysts weight (0.8 grams, range 0.0-2.64). BCG and CAR groups had generally more metastasis than the Saline and/or the Intact Cysts groups. However, the level of difference ($p=0.37$) in the total cyst weights was low among the groups using the Kruskal-Wallis test.

Tables 2a & b show the average total weight of cysts per animal and number of metastatic foci per infected gerbil at necropsy 120±1 and 150±1 days p.i. in the treatment groups. Although there was an increase in the average cyst weight at 150±1

TABLE 1. Number of gerbils infected and number and Percentage of gerbils with metastasis

Treatment group	Number infected	Number with metastasis	Percentage with metastasis
BCG	14	13	93
SALINE	12	6	50
CAR	10	8	80
INTACT CYST	10	7	70
TOTAL	**46**	**34**	**74**

Figure 1.

The means of total metastatic cyst weight in grams per gerbil for each treatment group. The standard error of mean (±SE) for each treatment group is given. There was no significant difference among the treatment groups using the Kruskal-Wallis test at the $p \leq 0.05$ level.

Fig. 1 Means of total metastatic cyst weight per gerbil for each treatment group

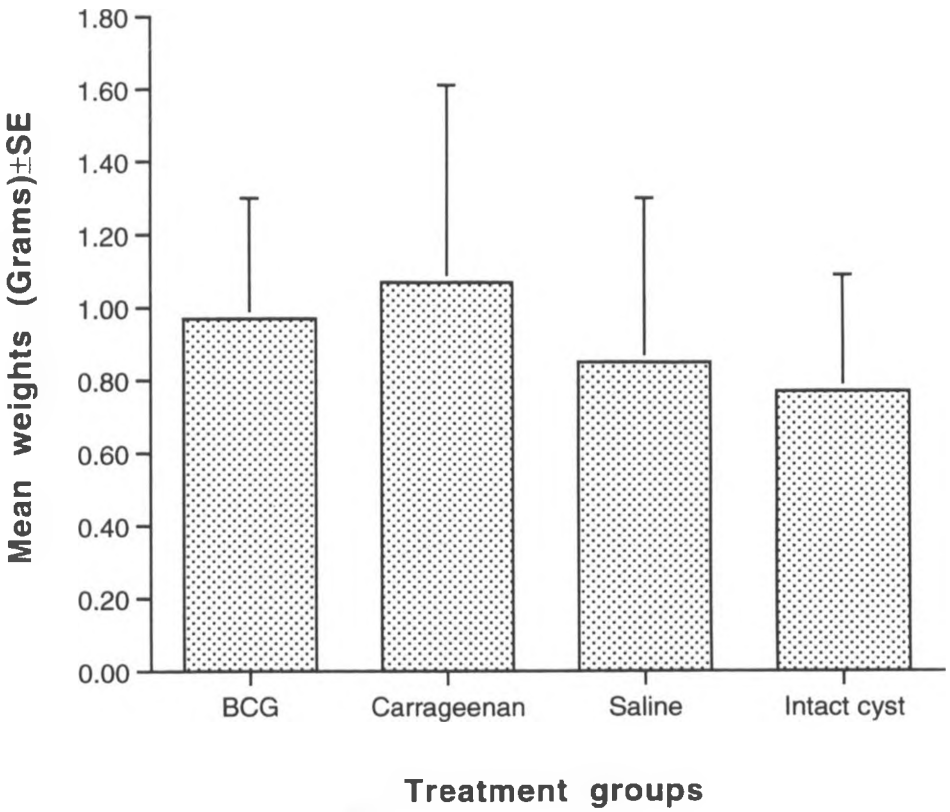


TABLE 2a. Average total cyst weight (in grams) per animal and number of metastatic foci per infected gerbil at necropsy 120 days p.i.

Treatment group	Number of foci per infected gerbil	Average Weight ±SE at 120 days p.i.
BCG	3.5	0.93 (±0.4)
SALINE	2.0	0.54 (±0.5)
CAR	3.4	1.09 (±0.7)
INTACT CYSTS	3.4	0.56 (±0.5)

TABLE 2b. Average total cyst weight (in grams) per animal and number of metastatic foci per infected gerbil at 150 days p.i.

Treatment group	Number of foci per infected gerbil	Average Weight ±SE at 150 days p.i.
BCG	4.1	0.99 (±0.5)
SALINE	1.9	1.06 (±0.7)
CAR	2.8	1.11 (±0.9)
INTACT CYSTS	3.7	0.92 (±0.4)

days p.i., it was not significantly different ($p>0.05$) from those at 120±1 days p.i.

Between the necropsy times, there was an increase in number of metastatic foci in the BCG, Saline and Intact Cysts groups, but a decrease in the CAR group. However, the differences between the groups and the differences in number of cysts at 120±1 and 150±1 days p.i. for each group were not significant ($p>0.05$). The BCG group had many more metastatic cyst foci than other groups (54, range 0-10 per gerbil) but the foci were smaller in size, as indicated by the number of foci in relation to the average total weight of metastatic cysts in the groups.

Although the Saline group had the least number of metastatic foci (23, range 0-8 per gerbil), the cysts were generally larger weighing on average 0.44 grams/focus. The Intact Cyst group had the lowest average cyst weight per focus (0.22 grams/focus) (Table 3). Low level of difference ($p=0.22$) was observed between the groups using the Kruskal-Wallis test.

In all the experimental groups, careful examination of the subcutaneous area, as well as the abdominal and thoracic cavities at necropsy revealed that parasite dissemination occurred mostly to particular regions of the body. Most and heaviest metastatic foci were found on the left side of the gerbils. This was the same side in which the animals were surgically infected. The axial region and the thoracic cavity had most of the foci. One gerbil in the Saline group had a cyst mass weighing 2.18 grams within the thoracic cavity. The left axial region had the highest incidence of metastasis and heaviest cyst masses (Fig. 2). Metastatic cyst foci were also found on the fascia around the kidneys, especially the left kidney. Two of the gerbils, one in the

TABLE 3. Total number of metastatic foci, average number of foci and average focal cyst weight in different treatment groups.

Treatment group	Total number of foci	Average number of foci(±SE)	Average focal cysts weight* (±SE)
BCG	54	3.86 (±0.8)	0.25(±0.03)
CAR	31	3.10 (±0.9)	0.34(±0.07)
SALINE	23	1.92 (±0.8)	0.44(±0.12)
INTACT CYSTS	36	3.60 (±1.2)	0.22(±0.04)

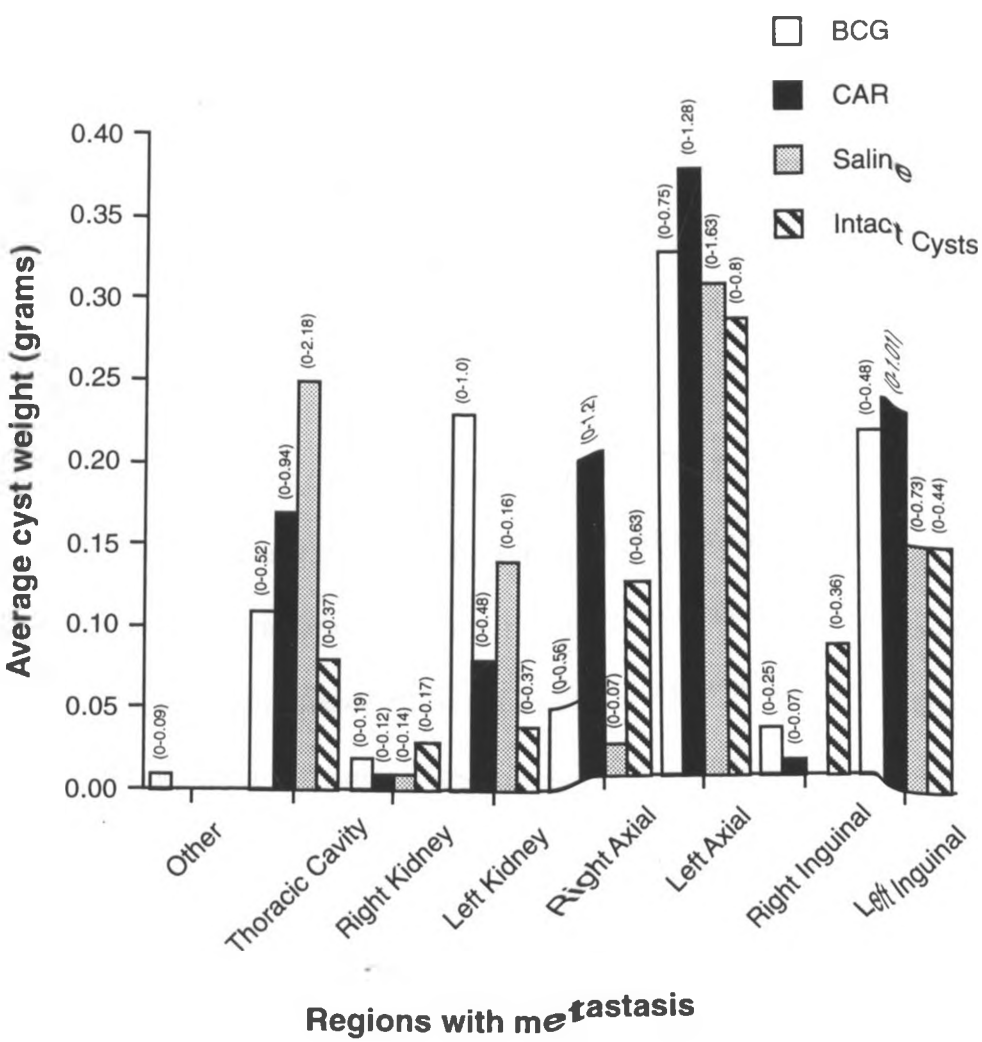
* Average focal cysts weight refers to the total weight of metastatic cysts in a group divided by the total number of metastatic foci in that group (grams/focus).

Figure 2.

The average cyst weights (in grams) in the body regions with metastatic cysts in the different treatment groups. The range of cyst weights in each region for different treatment groups are given in parenthesis.

Other: refers to the rectal lymph node which had a metastatic cyst focus weighing 0.09 grams in one gerbil in the BCG group.

Fig. 2 Average cyst weights in the body regions with metastatic cysts



BCG and the other in CAR groups, had extensive and massive metastasis to several regions of the body. The draining lymph nodes of the affected regions almost always acted as the site for growth of the metastatic foci.

Blood smears.

Table 4 shows the differential white blood cell counts expressed as a percentage of the total white blood cells counted in the groups. Between three to four hundred white blood cells were counted per treatment group. There was a marked increase in the number of monocytes in all the groups. An increase in number of neutrophils and a decrease in number of lymphocytes were seen only in the blood smear from the Intact cysts group. All other cell types remained within the normal range.

Histology.

At least three slides from different sections of cysts taken from representatives of each different treatment group were examined. At low magnification (100x), there was an intense leucocyte infiltration into the tissue surrounding the hydatid cysts in all the experimental groups. Sterile cysts and fertile cysts, with brood capsules with a distinct laminated layer and sometimes protoscoleces (appendix I) were also evident in all the groups.

Under oil immersion (1000x), polymorphonuclear and mononuclear cells were observed in the pericyst tissue. Polymorphs consisting mainly of neutrophils and some eosinophils were seen in close proximity to the laminated layer and sometimes within the cyst lumen, especially so when the cyst appeared to be disintegrating.

TABLE 4. Differential white blood cell count expressed as a percentage of the total white blood cells counted.

Cell type	BCG	INTACT CYST	CAR	SALINE	NORMAL RANGE*
Neutrophils	18	42	16	9	5-34
Lymphocytes	66	44	69	77	60-90
Eosinophils	1	1	2	0	0-4
Monocytes	14	13	14	13	0-3
Basophils	1	0	0	0	0-1

* Source: Harkness and Wagner (1983)

Lymphocytes and macrophages were diffuse and widespread in most of the connective tissue around the cysts. There was no difference in qualitative leucocyte cellular response among the experimental groups. In all groups, occasional disintegrating cysts could be seen. In these a layer consisting of histiocytes, neutrophils, eosinophils and occasionally giant cells surrounded a central necrotic area with some cell debris.

Phagocytosis.

No evidence of phagocytosis by macrophages collected from gerbils in each experimental group was seen using an inverted microscope or after staining the cover slips, mounting on a glass slide and examining under a compound microscope.

DISCUSSION

Alveolar hydatid disease (AHD) caused by the larval stage of *Echinococcus multilocularis* is considered to be one of the most significant zoonotic diseases. The disease resembles a malignant tumour by gross appearance, by the ability to disseminate to other areas and by induction of concomitant immunity (Rau and Tanner, 1973). In man treatment of the disease is mainly through surgical removal of the parasite mass. This is, however, not usually successful because of increased post-surgical growth at distant metastatic foci as well as growth of secondary cysts.

AHD has the potential to metastasize to distal areas. Post-operative recurrence in cystic hydatid disease (CHD) caused by *E. granulosus* may result from accidental damage of the cysts thus leading to spillage of protoscoleces and/or germinal cells into the surgical area (Rau and Tanner, 1973). In AHD, post-operative recurrences also occur as a result of germinal cells having given rise post-operatively to distant metastatic foci (Ali-Khan *et al.*, 1983). Eckert *et al.* (1983) working with gerbils reported that metastatic lesions developed in the regional lymph nodes draining the infected subcutis area and the lungs. This finding is similar to that observed in this study in which the left side of the gerbils, which carried the original infection, had more metastatic foci and the cyst foci were almost always associated with the draining lymph nodes. The axial lymph node and the thoracic cavity had most of the metastatic foci.

Rau and Tanner (1973, 1976) reported that surgical removal of primary cysts in AHD resulted in weakening of concomitant immunity with subsequent rapid growth of

metastatic foci. Riley *et al.* (1986) summarized the phenomenon that the immune mechanism, although ineffective in removing or damaging the established parasite, was highly efficient in preventing secondary infections with the homologous parasites. Protection against growth of metastatic foci of AHD persisted for at least two weeks after removal of the primary cyst (Rau and Tanner, 1976). Control or prevention of relapse of the disease after surgery can increase the success rate of surgical treatment hence the reason for this study. Control of secondary hydatidosis employing cyst-cyst concomitant immunity coupled with non-specific stimulation of the immune response was, therefore, undertaken. In this study, although there was no significant difference at $p \leq 0.05$ level among the treatment groups, the trend in the data suggested that removal of primary cysts resulted in growth of the distant metastatic foci as Intact Cysts group had low average total metastatic cyst weight and also low metastatic cyst weight per focus compared with the other groups.

Several methods have been used so far in an attempt to prevent the dissemination of AHD. Immunoprophylaxis has been the most widely tested method in experimental animals. Non-specific stimulation of the immune response with BCG prior to infection has been shown to protect cotton rats against AHD (Rau and Tanner, 1975; Reuben *et al.*, 1978; Reuben and Tanner, 1979). BCG was also shown to suppress metastasis of hydatid infections but not growth of the cyst mass when administered two weeks after infection (Rau and Tanner, 1975). Development of secondary *E. granulosus* in gerbils was also inhibited by BCG (Thompson, 1976). BCG has been used against several other parasitic infections.

Ortiz-Ortiz *et al.* (1975) were able to vaccinate mice against *Trypanosoma cruzi* infection with BCG, while Clark and Allison (1976) protected mice against *Babesia* and *Plasmodium* with BCG. Treatment of BALB/c mice with BCG reduced the severity of both *Leishmania tropica* and *L. donovani* lesions, as well as *L. major* infections (Frommel and Lagrange, 1989). Some neoplastic diseases have also been controlled or treated with BCG (Pearson *et al.*, 1973; Baldwin and Pimm, 1973; Snodgrass and Hanna, 1973; Wolfe *et al.*, 1976). BCG is, however, not absolutely protective as it failed to protect calves against *Babesia divergens* infection (Brocklesby and Purnell, 1977). Cheng *et al.* (1993) reported that there was variation in protection of humans against tuberculosis in some regions of the world after vaccination with BCG. These observations and those of this study in which BCG appeared not to have controlled metastasis indicate that effective use of BCG in immunotherapy may depend on several factors.

BCG protection is thought to be via activation of monocytes/macrophages. Activated macrophages have an enhanced capacity for the destruction of various organisms such as bacteria, viruses, fungi, protozoa, some helminths and even some neoplasms (Keller, 1980; Mantovani *et al.*, 1992). Russo-Marie (1992) observed that such activated macrophages have a long life and high metabolic activity that enables them to achieve their defensive role. The macrophages release nitrogen intermediates which have potent cytostatic and antiproliferative effects on tumours, bacteria, parasites and lymphocytes (Isobe and Nakashima, 1992; Cunha *et al.*, 1993). In this study, BCG did not prevent development of the metastatic foci as cyst number and

cyst weight were not significantly different (at the $p \leq 0.05$ level) from those of the control groups. It is possible that macrophages in a host with chronic infection with *E. multilocularis* are suppressed such that stimulation with BCG further increases macrophage suppression. It may also be possible that macrophages suppressed by the parasite are unable to ingest BCG and, therefore, are not affected by the treatment. Rau and Tanner (1975) showed that post-infection administration of BCG did not inhibit growth of hydatid cysts. BCG may have immunosuppressive effects. Thompson and Penhale (1978) showed that mice that had been exposed to BCG were more susceptible to the larvae of *Mesocestoides corti*. Use of mycobacteria antigens in Freund's complete adjuvant in rats rendered them more susceptible to rodent cysticercosis infection than the control animals (Williams, 1979).

Many cestode larval stages are known to alter the antigen presenting cells (macrophages) in such a way that they inhibit the proliferation of lymphocytes and thus suppress the host immune response. Rakha *et al.* (1991a) showed that post-infection peritoneal macrophages suppressed *E. multilocularis* culture supernatant induced lymphocyte transformation. *Taenia multiceps* coenurus are said to have the ability to reduce effective interaction between lymphocytes and accessory cells. This is reportedly achieved through a protein in coenurus fluid, factor F24, that alters macrophages to be suppressive for parasite-activated lymphocyte transformation (Rakha *et al.*, 1991b & c). Normal sheep monocytes were also modified *in vitro* by a coenurus antigen from *T. multiceps* (Rakha *et al.*, 1992). It can therefore be said that use of BCG after the macrophages have been altered by the parasite may facilitate

parasite growth and dissemination by activating suppressor cells rather than T-helper cells. AHD being a chronic disease, may turn on genes responsible for immunosuppression as has been reported in other chronic parasitic diseases such as filariosis, trypanosomosis and malaria (Mitchison and Oliveira, 1986). The ability of macrophages to suppress the immune response has been demonstrated previously in a large number of systems. Distinct phenotypes of macrophages may respond differently to activating stimuli such that macrophage suppressor activity is manifested in some cells (Keller *et al.*, 1993; Paulnock, 1992; Isobe and Nakashima, 1992).

Other factors that may affect the action of BCG in the prevention of metastasis are the routes of administration and doses given. Pearson *et al.* (1973) showed that the protective effects of BCG to a murine lymphoid leukaemia were independent of a wide range of doses and routes of administration; a dose of as few as 80 viable organisms was protective against the leukaemia. Reuben *et al.* (1978) showed that optimal protection against *E. multilocularis* could be achieved by injection of 10^3 CFU of BCG in cotton rats. This dose was protective and precluded the formation of granulomatous lesions inherent in higher doses of BCG and led to an increase of leucocytes of the monocyte/macrophage series. Doses below 10^3 were either not protective or were partially protective. The dose used in this study of $1.6x10^3$ was comparable to the dose known to afford protection when given before infection.

BCG used in immunotherapy may be administered either intravenously, intraperitonealy, subcutaneously, or intradermally. Intravenous (i.v.) injection of BCG was reported to be effective in controlling the pulmonary metastases of rat epithelioma

(Baldwin and Pimm, 1973). Injection of BCG (i.v.) was effective in reducing parasiteamia of *L. donovani*, while the intraperitoneal (i.p.) route was not effective (Frommel and Lagrange, 1989). Prior i.v. injection of BCG protected rabbits against *Herpesvirus hominis* type 2 infection (Larson *et al.*, 1972). Administration of BCG (i.p.) in this study may have resulted in activation of peritoneal macrophages without any systemic activation. Rau and Tanner (1975) controlled metastasis by i.p. administration of BCG before infection of host with hydatid cysts. It is known that during inflammatory stimulation or injection of particles into the peritoneal cavity, there is a gradual increase in the number of macrophages in the cavity as a result of concomitant increase of circulating peripheral monocytes and increased production from the bone marrow (Sluiter *et al.*, 1980; Russo-Marie, 1992). The peripheral monocytes are, however, not activated until they join resident macrophages at the site with offending object(s) (Russo-Marie, 1992) and, therefore, could not impart protection against metastasis that takes place either through the circulatory or lymphatic system (Rau and Tanner, 1973; Ali-Khan *et al.*, 1983; Eckert *et al.*, 1983). Functional properties of resting and activated macrophages differ depending on the stimulatory agent (Keller *et al.*, 1993). Baldwin and Pimm (1973) partially protected rats against pulmonary metastases of rat epithelioma tumour by injecting tumour cells in admixture with BCG. Injection of BCG (i.v.) also controlled metastasis following surgical removal of the subcutaneous tumour. Snodgrass and Hanna (1973) and Wolfe *et al.* (1976) showed that intralesional injection of BCG caused a notable regression of hepatocarcinoma and rat sarcoma tumours respectively. Since most cases in which

BCG was effective in control of AHD, other parasitic diseases and tumours, it was either administered into the area where the parasite was to be inoculated or had been inoculated, one may conclude that route of the administration may contribute to the success of BCG immunotherapy and immunoprophylaxis. This is in contrast to the observation by Pearson *et al.* (1973) that BCG effects are independent of the route of administration.

To ascertain whether BCG inhibited or enhanced macrophage activity, CAR was used in a parallel experiment so that the result of the BCG group could be compared with results from experiments using a known anti-macrophage agent. CAR is said to suppress delayed hypersensitivity reaction, inhibit complement system and induce lysis of macrophages (Catanzaro *et al.*, 1971; Ishizaka *et al.*, 1977; Keller, 1980; Schwartz and Catanzaro, 1981). CAR is therefore immunosuppressive and theoretically could enhance the dissemination of AHD since macrophages are the potential effector cells in protection against the disease. Reuben and Tanner (1983) demonstrated that use of CAR enhanced metastasis of *E. multilocularis* in cotton rats. Immunosuppression has been shown to favour growth and dissemination of AHD (Baron and Tanner, 1976; Bresson-Hadni *et al.*, 1992; Liance *et al.*, 1992; Playford *et al.*, 1992, 1993). Macrophages are modified by *E. multilocularis* such that they produce suppressive factors inhibiting lymphocyte proliferation and thus protect the parasite against host reaction (Rakha *et al.*, 1991a). CAR is toxic to macrophages, possibly killing both parasite-altered and normal macrophages, thus favouring the growth of the parasite. It is possible that altered macrophages might not phagocytose

CAR. In this case, normal macrophages may be lysed by CAR while altered macrophages may survive thus favouring parasite growth. These may explain why there was no significant difference among the groups studied. Although there was no significant difference among the treatment groups at the $p \leq 0.05$ level, the trend in the data showed that both CAR and BCG groups had a higher proportion of gerbils with metastasis and relatively higher average total metastatic cyst weights. The extent of metastasis in the BCG group was almost indistinguishable from that of the CAR group. The trend in the data support findings of Williams (1979) and Thompson and Penhale (1978) who showed that use of BCG increased susceptibility to infections.

Although statistical analysis indicated low level difference among the treatment groups, the trends in the data are nevertheless biologically interesting. The rate of growth of the metastatic foci was higher in the Saline group than in any other group as indicated by the total weight of metastatic cysts per focus (Table 3). This may result from suppression of the immune protection by removal of the established subcutaneous cysts which had imparted concomitant immunity to the host (Rau and Tanner, 1976). Growth rate in the Intact Cysts group was the lowest (0.22 grams/focus) as compared to that of the Saline group (0.44 grams/focus) an indication that preexisting established subcutaneous cysts may have inhibited growth of the distant metastatic foci. This agrees with the observation of Rau and Tanner (1973). The BCG group had extensive dissemination of cysts to many areas compared with the other groups but the cysts did not grow as large, since the total cyst weight per focus was lower (0.25 grams/focus). BCG may have initially facilitated parasite metastasis

hence the extensive dissemination as shown by the large number of metastatic foci. BCG could have stimulated macrophages already altered by *E. multilocularis* to become suppressive (Rakha, *et al.*, 1991a &b, 1992). Cyst-cyst interaction may have limited growth of disseminated cysts as indicated by low total metastatic cyst weight per focus (0.25 gram/focus) and lack of difference in average metastatic cyst weight at 120 and 150 days p.i. These findings suggest that metastasis and cyst growth may be controlled by separate mechanisms. Concomitant immunity may be able to control growth of metastatic foci but not metastasis.

Although the CAR group had a high total metastatic cyst weight per focus (0.34 grams/focus), the group had no significant increase in average metastatic cyst weight between day 120 and day 150 p.i. CAR may have induced some immune suppressive effects by causing cytolysis of macrophages (Reuben and Tanner, 1983) and/or inhibiting the complement system which protected the host against the parasite (Catanzaro *et al.*, 1971), but when the cyst attained a certain size, further growth was limited possibly due to cyst-cyst interactions hence no difference in metastatic cyst weight at 120 and 150 days p.i.

In this study, blood smears showed that there was an increase in the number of monocytes in circulation in all the experimental groups. This finding is in contrast to those of Ali-Khan (1974) who did not find such an increase in C57L/J mice but reported neutrophilia and lymphocytopenia. Working with gerbils, Kroeze and Tanner, (1986) showed that there was progressive lymphocytopenia, neutrophilia and monocytosis. Their findings were similar to those of this study except that neutrophilia

and lymphocytopenia were observed only in the Intact Cyst group. One might therefore say that removal of intact cysts alters the cellular response towards the cyst mass and thus favours dissemination of the parasite. Neutrophils have been suggested to act as effector cells in the control of cyst proliferation in a subcutaneous cyst mass (Ali-Khan and Siboo, 1980a) thus a decrease in their number would result in unchecked dissemination rate as seen in BCG, CAR and Saline groups. Lymphocytopenia can be explained in terms of the ability of *E. multilocularis* to secrete a substance that alters macrophages to produce cytokines that inhibit proliferation of lymphocytes (Rakha *et al.*, 1991a). Cestodes are said to secrete a substance that acts on macrophages whereby their positive accessory role in lymphocyte blastogenesis is replaced by a negative depressive function (Cox *et al.*, 1989; Dixon *et al.*, 1982; Rakha *et al.*, 1991b & c, 1992). The disorganization of the pericortical areas (PCA) of draining lymph nodes in chronic AHD (Ali-Khan, 1978; Ali-Khan and Siboo, 1980b) and in other chronic diseases (Ottesen *et al.*, 1977) results in depletion of T cells in the PCA and may also contribute to lymphocytopenia observed in this study. This fact, however, does not explain why there was only lymphocytopenia in the Intact Cyst group as observed in this study unless presence of an established large cyst had a contributory factor.

Histogenesis of a larval cyst mass in AHD is associated with an intense inflammatory response (Ali-Khan *et al.*, 1983). Further increase in size by the cyst mass is accompanied by a succession of inflammatory cells which eventually determine the fate of the parasite cyst. Infiltration of the larval cyst mass by

neutrophils, eosinophils and macrophages forms the initial leucocytic cellular response followed by infiltration of lymphocytes, plasmacytes and later by giant cells during the formation of granulomas (Ali-Khan, 1978; Ali-Khan and Siboo, 1980a; Heath, 1986; Playford *et al.*, 1992). Such a response does result in the disintegration and eventual disappearance of cysts. In cases where there is progressive growth and increase in size of the cyst mass, there are no giant cells or granuloma formation. Playford *et al.* (1992) showed that in severe combined immunodeficient (SCID) mice, there was neither granulomatous inflammation nor giant cells but macrophages, neutrophils and eosinophils surrounded the larval cyst without affecting the growth of the cysts. In this study, there was leucocyte infiltration in the host layers surrounding the cyst mass. The leucocytes mostly included neutrophils, some eosinophils, macrophages and lymphocytes. These cells were seen in the fibrous layer surrounding the laminated layer. Neutrophils were also seen in close proximity to the laminated layer and sometimes within the disintegrating cysts. These observations agree with those previously reported. Granulomatous reactions were sometimes seen and were indicated by the presence of giant cells surrounding a necrotic area. Giant cells and granulomas are abundant in regressive and degenerative cysts as seen in resistant C.B-17 mice (Playford *et al.*, 1992). There was no apparent difference in the histological appearance of the cellular response to parasite, among the different experimental groups.

An attempt to compare the ability of peritoneal macrophages and circulating monocytes to phagocytize latex beads did not yield results in any of the treatment groups. Failure of the macrophages to show phagocytotic activity might have resulted

from a flaw in the procedure.

In conclusion, this study has verified findings by previous workers that metastasis in AHD occurs mostly via the lymphatic vessels as most of the metastatic foci occurred in the draining lymph nodes anterior to the infection site. Although there were no differences that could be detected statistically at the $p \leq 0.05$ level among the experimental groups in terms of control of metastasis, the trends in the data of this study have highlighted some important factors that need to be considered in BCG immunotherapy.

(a) Route of administration: BCG may effectively control growth of metastatic foci of *E. multilocularis* if administered intravenously. However, one must be careful in selecting a dose that will achieve its purpose without forming granulomas within the lungs of the host. Further research needs to be conducted on the route of administration.

(b) Immunosuppression: Care needs to taken when using BCG in immunotherapy. BCG may be suppressive under certain circumstances and thus favour parasite growth. Research needs to be undertaken to find out which type of macrophages are activated to become suppressive rather than protective to the host.

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APPENDIX I

Light micrographs showing some *E. multilocularis* cyst structures and host cellular response towards the parasite.

(1)

Light micrograph showing fertile cyst with protoscoleces (P) of *E. multilocularis* from the Intact Cyst group.

(2)

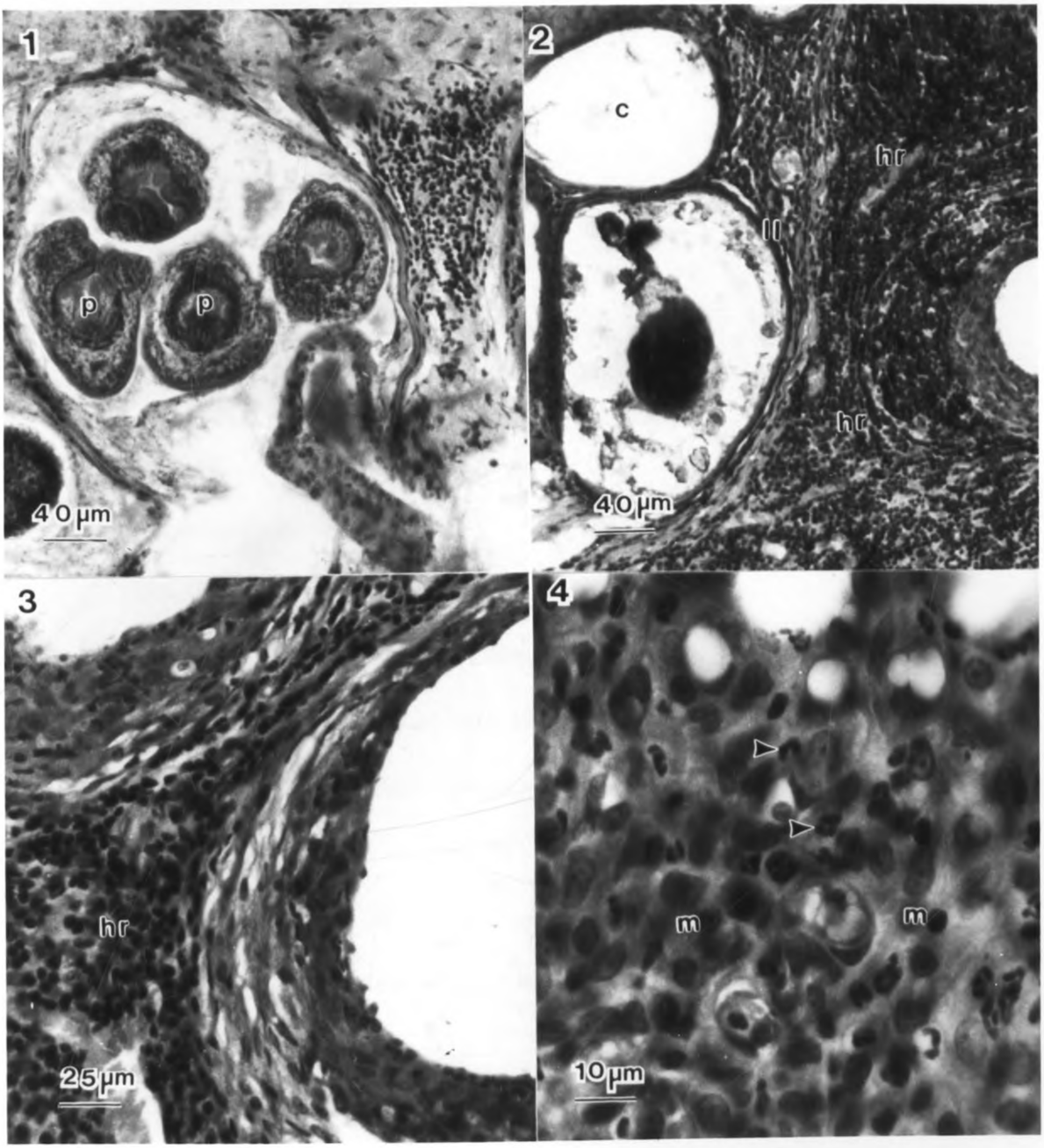
Light micrograph of an intense host leucocyte cellular response (hr) from CAR treated gerbil. Distinct laminated layer (ll) and acephalic cyst (c) are evident.

(3)

Light micrograph of host leucocyte cellular response (hr) surrounding the parasite tissue from CAR treated gerbil.

(4)

Light micrograph showing polymorphonuclear cells (arrows) and mononuclear cells (m) that have infiltrated the host tissue surrounding the parasite.


1
p
p
40 µm
2
c
ll
hr
hr
40 µm
3
hr
25 µm
4
m
m
10 µm

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