ACTIVATION OF NONSPECIFIC PROTECTION IN EXPERIMENTAL HYDATIDOSIS

BY

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ABSTRACT

Hydatid infections are characterized by the proliferation and activation of peritoneal macrophages which are cytotoxic to protoscolices *in vitro*. Immunoprophylaxis with viable BCG, BCG cell walls or with phytohemagglutinin protects animals against infection with *Echinococcus multilocularis*. Normal cotton rat peritoneal, spleen and peripheral macrophages and lymphocytes are activated *in vitro* to kill protoscolices of *E. granulosus* by phytohemagglutinin, pokeweed, lipopolysaccharide and BCG. Peritoneal and spleen leucocytes of animals treated with BCG, mineral oil or phytohemagglutinin, or infected with *E. multilocularis*, are also cytotoxic to this parasite. Resistance to infection with *E. multilocularis* can be passively transferred to normal recipients with specifically and nonspecifically-activated peritoneal cells. It is shown that replacement of hydatid cyst fluid with BCG-activated peritoneal cells kills the cyst of *E. granulosus*. The results of this investigation are discussed in relationship to the ability of the parasite to survive in its host.

ABREGE

Les infections hydatiques sont caractérisées par la prolifération et l'activation des cellules phagocytaires péritonéales, lesquelles sont cytotoxiques in vitro aux protoscolex des echinocoques. L'immunoprophylaxie par le BCG viable, les parois cellulaires du BCG ou par la phytohémagglutinine, protège les animaux contre l'infection à Echinococcus multilocularis. Les macrophages, ainsi que les lymphocytes originant du péritoine, de la rate et du sang péripherique des rats de "cotton", sont activés in vitro par la phytohémagglutinine, le "pokeweed", le lipopolysaccharide et le BCG et détruisent les protoscolex d'E. granulosus. Les leucocytes du péritoine et de la rate des animaux traités au BCG, à l'huile minérale ou à la phytohémagglutinine, ainsi que les animaux infectés à E. multilocularis, sont aussi cytotoxiques à ce parasire. La résistance à l'infection par E. multilocularis peut être transmise passivement à des réceptifs normaux par des cellules péritonéales spécifiquement ou non-spécifiquement activées. Il est démontré que la substitution du liquide hydatique par des cellules péritonéales activées au BCG détruit les kystes d'E. granulosus. Les résultats de cette recherche concernant l'habilité du parasite à survivre à l'intérieur de son hôte, font l'objet de la discussion.

Suggested Short Title:

ACTIVATION OF CELLS IN ECHINOCOCCOSIS

Author's Claim to Originality

The role of the nonspecifically activated cell in the control of experimental hydatid infections was investigated in detail for the first time in this study. It has been established that relatively low prophylactic doses of BCG are effective in controlling experimental infections of Echinococcus multilocularis, without the concomitant formation of macroscopic granulomatous lesions (Chapter 4). These findings were extended to show that cotton rats pretreated with nonviable BCG cell walls can also be completely protected from a subsequent infection with the parasite (Chapter 5).

Peritoneal cells from infected animals are known to lyse the parasite in vitro. There is, however, no precedence which establishes, as this study has (Chapter 6), that leucocytes could be nonspecifically activated in vitro with mitogens to kill parasites. Although resistance to hydatid infections can be passively transferred with specifically sensitized lymphocytes, it is demonstrated that nonspecifically-activated peritoneal cells can also protect (Chapter 7).

Hydatid cysts acquire large numbers of leucocytes during the course of infection which have migrated into them from the peritoneal cavity. These cells failed to respond to mitogen stimulation, in contrast to the susceptibility of peritoneal cells to activation. The depression in the reactivity to mitogens of spleen and peritoneal cells in hydatid infections are of particular significance as it may explain how this larval cestode establishes and survives in its hosts for such extended periods of time (Chapter 8).

The treatment of established hydatid cysts of E. granulosus by replacing the cyst fluid with BCG-activated cells causes the death of the parasite (Chapter 9). The use of nonspecifically-activated cells in

the treatment of echinococcosis, or for that matter, in any other parasite disease, provides a new perspective in the understanding of the host-parasite interaction.

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THIS THESIS IS DEDICATED TO MY WIFE,

CARMEN, AND TO MY SON, JASON

..... The end is where we start from.

T. S. Eliot

CHAPTER 1

CHAPTER 1

THE BIOLOGY AND LIFE-CYCLE OF ECHINOCOCCUS SPP.

The larval stage of the small tapeworms belonging to the genus Echinococcus (family Taeniidae) is the causative agent of hydatid disease in man and animals. Two species, Echinococcus granulosus Batsch, 1786, and E. multilocularis Leuckart, 1863, are recognized to be of veterinary and public health importance (Rausch, 1968).

The typical life cycle of Echinococcus spp. requires, under natural conditions, two mammalian hosts which are adapted to an obligatory and well-defined predator-prey relationship. The definitive host of these cestodes is a carnivore, while the intermediate host is normally a herbivore, usually an ungulate or a rodent. The adult of E. granulosus matures in dogs and closely allied species, and utilizes sheep primarily as the intermediate hosts; the adult of E. multilocularis matures in foxes and cats and utilizes microtine rodents as intermediate hosts. The larvae of both species will develop in man; however, man is not an important intermediate for E. multilocularis as development in this host is much slower than in the natural intermediate hosts (Rausch and Schiller, 1956; Yamashita et al., 1958).

The intermediate host acquires the infection by ingesting infective eggs that are eliminated in the stool of the definitive host. Following the action of enzymes in the stomach and the small intestine, the hexacanth embryo, the onchosphere, hatches from the embryophore. Bile assists in activating the onchosphere to penetrate the wall of the small intestine (Smyth, 1967) and the hooks of the onchosphere are of importance in the penetration process (Heath, 1971). Upon gaining access to a venule, the onchospheres are passively transported to the

liver, where the majority of them become trapped while others may reach the lungs, kidney, spleen, brain and other organs.

When the onchosphere settles down, the embryo loses its hooks, differentiates and begins to produce an unilocular fluid-filled cyst in the case of E. granulosus, or a multivesiculate metastasizing tumor-like cluster of cysts in the case of E. multilocularis which will contain a multitude of metacestode larvae - the protoscolices.

The protoscolices that are produced within the hydatid cyst have a dual potential: If a cyst ruptures within an intermediate host, or if protoscolices are experimentally inoculated into the body cavity, they are capable of developing into secondary hydatid cysts. If, however, they are ingested by a suitable definitive host, they develop into a hermaphrodite adult. Protoscolices ingested by a definitive host, after peptic treatment in the stomach, evaginate in the upper duodenum in response to a change in pH and to an exposure to bile (Smyth, 1967). Host bile is thought to play a very important role in the specificity of the definitive, and probably also of the intermediate host (Smyth, 1968). Smyth (1962) and Smyth and Haslewood (1963) have shown that specific constituents of the bile must be present before evagination of the protoscolex and the establishment of the parasite in the gut can take place. In a suitable definitive host, the cestodes are found between the villi, and in the crypts within 6 hours after the inoculation of protoscolices (Thompson, 1977). Development of the sexually mature hermaphrodite adult then proceeds; the embryonated eggs which are produced in their uteri are discharged to the exterior in the feces and the cycle perpetuates.

The usual predatory-prey life cycle can be a source of experimental Echinococcus laboratory infections; this is a hazardous

procedure, however, because the onchospheres are also infective to man. Fortunately, it is possible to maintain Echinococcus metacestodes in the laboratory by the vegetative passage of protoscolices into a suitable experimental host, the cotton rat (Sigmodon hispidus) and other rodent hosts. This vegetative serial transfer of protoscolices or acephalic cysts from cotton rat to cotton rat provides a convenient method of maintaining the parasite in the laboratory (Lubinsky, 1960a, b, 1967; Sadun et al., 1957).

The wall of the hydatid cyst consists of a friable membrane which is composed of an outer, non-nucleate, thick laminated membrane of host-parasite origin, and an inner, highly nucleated, thin germinal membrane of parasite origin. The laminated membrane is composed of a network of fine fibers in which scattered dense material is found; the germinal membrane sends finger-like processes into this network whose function is for both nutrition and attachment (Bortoletti and Ferretti, 1973; Vercelli-Retta et al., 1975). Kilejian et al. (1961, 1962) reviewed the chemical composition of the laminated and germinal membranes and have reported that the former membrane consists of a protein-carbohydrate complex containing glycogen in trace amounts; the germinal membrane, however, consists of large amounts of glycogen in addition to numerous amino acids.

The protoscolex is the larval stage within the brood capsules. Each protoscolex has a contractile scolex which can be either evaginated or invaginated, and is equipped with four muscular suckers, in addition to a double crown of rostellar hooks. Chemical analysis of the protoscolex has shown it to contain large deposits of glycogen and only minute amounts of acid and alkaline phosphatases (Kilejian et al., 1961). The tegumental sheath is rich in glycoproteins (Kilejian et al.,

1962); the tegument itself does not show any enzymatic activity but the subtegumental layer has an abundance of enzymes (Reissenweber et al., 1975).

Numerous reports have been published on the chemical composition of the calcareous corpuscles which are found in cestodes and trematodes (Chowdhury et al., 1962; von Brand et al., 1965). The calcareous corpuscles are found to contain inorganic as well as organic material; their function, however, is not clearly known. Some reports have suggested that they could buffer anaerobically-produced acids (von Brand et al., 1960), or may serve as reserve centres for inorganic ions (von Brand et al., 1965). Only recently, a definite critical role for these corpuscles has been identified by Kassis and Tanner (1976b). They found the corpuscles to be able to neutralize the effect of complement produced by the host and thus enable the parasite to evade certain aspects of the immune response in hydatid infections.

CHAPTER 2

CHAPTER 2

THE ACTIVATION OF MACROPHAGES
A LITERATURE REVIEW

INTRODUCTION

During the past few decades immunology has been one of the most rapidly growing branches of biomedical science. Subpopulations of lymphocytes have been identified and their interactions with one another, as well as with macrophages and other cell types, have been analyzed in detail. Various classes and subclasses of antibodies have been distinguished, and already a great deal is known about their structure and how they combine with antigens. Most of the components of the complement system have been isolated and their interactions in the course of activation by the classical and alternative pathways have been defined. All this information has shed considerable light on the specific immune responses of animals to pathogenic organisms and tumors.

The specific responses of immunocompetent cells have been relatively easy to study because they fit into conventional immunological thought; there are other aspects of the immunological response of the host which present considerable conceptual problems, e.g. the nonspecific immunity which results when the infection of an animal with one organism renders it resistant to a simultaneous or a subsequent challenge with an antigenically unrelated pathogen (Elberg et al., 1957) or with syngeneic tumor cells. Similarly, the inoculation of an animal with certain microbial products increases their resistance "nonspecifically" to a wide range of bacterial (Adlam et al., 1972; Collins and Scott, 1974; North, 1973; Senterfitt and Shands, 1970), viral (Remington and Merrigan, 1969) and parasitic infections (Clark et

al., 1976, 1977; Tabarra et al., 1975). Among the agents that are active in this manner are the lipopolysaccharide endotoxins of gram-negative bacteria (Spitznagel and Allison, 1970), zymosan (Edwards et al., 1976), glucan (DiLuzio et al., 1976), double stranded polyribonucleotides (Alexander and Evans, 1971), and synthetic polyanions, such as polyacrylic acid and pyran copolymer (Harmel and Zbar, 1975).

Three distinct, but related, mechanisms responsible for such nonspecific resistance are recognized: one is adjuvant activity, when the agents in question increase the specific immune responses to unrelated antigens (Spitznagel and Allison, 1970; Unanue et al., 1969); a second is the formation of interferon, which is active against viruses as well as other infectious agents and tumors (Younger and Salvin, 1973); and a third mechanism, which is assuming increasing importance, is the activation of macrophages to perform various specific and nonspecific functions.

Macrophage activation is difficult to study in vivo because of the complexicity of the cellular interactions in the animal. It has been more convenient to study cultures of macrophages which respond in ways remarkably similar to those which must occur in vivo. As a result of the studies by many workers, a considerable body of information has been accumulated concerning the mechanisms by which macrophages are activated and the biological consequences of this activation. It is only appropriate that a brief account be given of the natural history of the cells of the mononuclear phagocytic lineage before the consequences of activation are discussed.

A. THE FORMATION OF MONONUCLEAR PHAGOCYTES

Mononuclear phagocytes arise from the same stem cells in the bone marrow that also have the potential to develop into granulocytes. These precursor cells can be cultured in semisolid medium to

produce colonies of either mononuclear phagocytes or granulocytes (Virolainen and Defendi, 1968). In the mouse, colony-forming cells can also be found in the peripheral blood and in the spleen. The proliferation of the precursors can be significantly increased by the colony-stimulating activity (CSA) which is generated during an inflammatory or an immunological response (Chervenick and Lo Buglio, 1972). High levels of CSA stimulate the proliferation of precursor cells to differentiate into granulocytes; low levels of CSA favour differentiation into mononuclear phagocytes. In acute infections, where the stimulation by antigen or the causative agent is intense, large amounts of CSA are produced and many neutrophils are formed by the bone marrow and liberated into the peripheral circulation. Where the stimulus is less intense, lower levels of CSA are produced over a long period and the formation of macrophages is favoured. According to van Furth (1975), the precursor cells differentiate in the bone marrow itself into promonocytes, which already have considerable phagocytic activity. These cells are liberated into the blood, where they differentiate into and circulate as monocytes for an average of 32 hours before emigrating into the tissue (van Furth and Cohn, 1968).

There is also proliferation of macrophage precursors in the lymphoid organs such as the spleen. Upon the intravenous injection of Corynebacterium parvum (CP), or Bacillus Calmette - Guérin (BCG), many of the organisms are taken up by macrophages in the spleen. In the spleen CSA is produced locally and it stimulates the proliferation and differentiation of specific precursor cells into mononuclear phagocytes. In the tissues, these precursor cells are capable of considerable modulation and maturation which takes different forms depending on the particular site in which the cells are found. Evidence indicates that skin macrophages (Volkman and Gowens, 1965), alveolar macrophages (Pinkett et al., 1966), the Küpffer cells of the liver

(Howard, 1970) and the peritoneal macrophages (Balner, 1963; Volkman, 1966) are normally replenished from the marrow-derived blood monocyte pool, rather than by a local proliferation of macrophages. Macrophages in tissues appear to have a very long life-span and ordinarily do not recirculate (Roser, 1970). Under normal conditions, macrophages do not synthesize new DNA nor do they divide, but, under certain special circumstances, they can be stimulated to do so (North, 1969; Virolainen, 1968). The turnover of macrophages in the tissue is quite slow: about 40 days for mouse peritoneal macrophages (van Furth and Cohn, 1968). Mature macrophages are dispersed throughout the body and occur in two large populations: (a) those wandering in the tissues and in the body spaces (e. g. alveolar and peritoneal macrophages, skin macrophages, histiocytes), and (b) those which are fixed to the vascular endothelium to a baffle system in the blood or in the lymphatic system (e. g. the Küpffer cells of the liver and the fixed macrophages of the spleen and the lymph nodes).

B. THE ACTIVATION OF MACROPHAGES

Mononuclear phagocytes respond to a variety of stimuli by an increase in activity; macrophages reacting in this manner have been called activated (Mackaness, 1964). Activated macrophages display a pronounced ruffling of the plasma membrane, an enormously increased capacity for adhering to and spreading on a substratum, an increased capacity for phagocytosis, and increased numbers of phagolysosomes and endocytic vessicles (Fowles et al., 1973; Lejeune and Evans, 1972; Nathan et al., 1971; Simon and Sheagren, 1972). More recently, it has been demonstrated that macrophages activated by various stimuli, in contrast to normal macrophages, secrete large quantities of proteinases including plasminogen activator (Gordon et al., 1974a), a specific esterase (Wiener and Levanon, 1968), and a specific collagenase (Werb and Gordon, 1975a). Activation of macrophages can be induced

in vivo as well as in vitro by a wide variety of stimuli: immunization, chronic intracellular infection, and numerous chemical compounds (Cohn et al., 1966; Lejeune and Evans, 1972; Simon and Sheagren, 1971; Wahl et al., 1974). The literature indicates, however, that the term activation has been used to designate several, perhaps unrelated, kinds of reactions of macrophages (Gordon et al., 1974b; Karnovsky et al., 1975), and it may very well be that not all reactions can be provoked by all stimulants. It is, therefore, imperative that the initial activating agent be identified (and, if possible, also the direct activating agent, e.g. C3b produced by agents acting through the complement system); it should also be stated whether the activating agent has been used in vitro or in vivo (Reikvam et al., 1975), and which biological or biochemical responses have been measured. It must not be assumed that different stimuli will produce the same set of responses, or that the latter are related to one another.

C. THE SECRETIONS OF MACROPHAGES

In recent years, numerous studies have documented the fact that macrophages have important secretory activities. Activated macrophages have been shown to secrete plasminogen activator (Unkeless et al., 1974), collagenase (Werb and Gordon, 1975a), and an esterase-like enzyme (Werb and Gordon, 1975b), in addition to lysozyme (Gordon et al., 1974b); the latter enzyme is also secreted by nonactivated macrophages. Macrophages have also been shown to release B-glucuronidase and other acid hydrolases selectively when they are stimulated by undigestible particles (Davies et al., 1974), lymphokines (Pantalone and Page, 1977), or the products of the activation of complement (Schorlemmer and Allison, 1976). The following sections will deal with each of the above enzymes and their consequences.

Hydrolytic Enzymes

The synthesis and secretion of various hydrolytic enzymes by macrophages participating in acute and chronic inflammatory responses are essential for the effector function of these cells. The characteristics of the substances inducing enzyme secretion, the nature and the identity of the substances released, and the conditions under which these cells secrete have been extensively reviewed (Davies and Allison, 1976; Gordon and Cohn, 1974).

The stimulation of macrophages maintained *in vitro* by numerous substances can lead to an enhancement of the production of various enzymes, such as, lactate dehydrogenase (LDH; a cytoplasmic enzyme participating in energy production and retained within the cell) and hydrolytic enzymes which are released from the viable cell. The hydrolytic enzymes can be divided into at least three classes: acid hydrolases, neutral proteinases, and lysozymes. In general, substances which provoke chronic inflammation induce the release of these enzymes, while inert or readily digestible substances do not (Axline and Cohn, 1970; Davies *et al.*, 1974). Some of the same stimuli that cause the release of acid hydrolases also cause the release of neutral proteinase (Pantalone and Page, 1977), although these can also be released by some stimuli which are considered to be non-inflammatory (Davies *et al.*, 1974; Werb and Gordon, 1975a, b). There is, however, a difference in the timing and in the amount of acid hydrolases and neutral proteinase which are released. Acid hydrolase is released in large amounts shortly after an appropriate stimulus, while the release of neutral proteinase is usually delayed for at least 24 hours but, once initiated, continues over an extended period. Lysozymes are secreted continuously after stimulation and in comparable amounts by cells maintained under widely varying culture conditions (Gordon *et al.*, 1974b; Schnyder and Baggiolini, 1978).

Acid Hydrolases

Macrophages secrete specific substances during pinocytosis and phagocytosis or in response to the binding of immunoglobulin or of complement to the cell surface. Cohn and Benson (1965a) showed that mouse peritoneal macrophages synthesized lysosomal enzymes when cultured in a medium containing serum; lysosomal enzymes are, however, not released from the cell under these conditions (Cohn, 1966). The stimulation of pinocytic activity has been found to be due to the interaction between the macrophage and a macroglobulin (IgM) in the serum used to supplement the culture medium (Cohn and Ehrenreich, 1969). Macrophages stimulated in this manner, however, may not be biologically relevant; they do not secrete enzymes other than lysozymes or kill tumor cells, and they actually kill ingested Listeria monocytogenes less well than unstimulated macrophages.

The correlation of pinocytosis and lysosomal formation do not, however, distinguish between the influences of membrane interiorization and substrate uptake on the accumulation of hydrolase. This question has been approached using phagocytosis as a model in which an increase in acid hydrolase activity and extracellular release of the enzyme occur following the phagocytosis of some types of particulate non-digestible substances. Phagocytosis of inert carbon particles and latex beads do not appear to cause an elevation in enzyme levels in the cell (Axline and Cohn, 1970; Davies et al., 1974), while other non-digestible materials, such as sucrose (Cohn and Ehrenreich, 1969), and digestible particles, like sheep erythrocytes and aggregated globulin (Weissmann et al., 1971), evoked significant increases in the cell levels of lysosomal and other enzymes; there was, however, no extracellular release of these enzymes. The phagocytosis of zymosan by macrophages does cause the release of acid hydrolases, without a change in the viability of the cell (Weissmann et al., 1971). It is of

interest to note that zymosan contains glucans which are capable of inducing inflammation (McCandless, 1965), although the relationship of these substances to the release of the enzymes remains unclear.

Macrophages are regularly present in chronic inflammatory lesions of both immunological and non-immunological origin and probably play a major role in tissue destruction; a number of studies have, therefore, investigated the role of substances known to elicit chronic inflammation in vivo for their effectiveness in causing the release of acid hydrolase from macrophages maintained in vitro. It has been found (Weissmann et al., 1971) that acid hydrolases are rapidly released when macrophages are exposed to many stimuli which induce inflammation; the release of the enzymes is not a general response since biologically unreactive substances do not induce release while potential pathogens are more successful.

The enzymes are usually released from preformed lysosomes although enzyme production de novo may also be stimulated (Davies et al., 1974). The enzymes produced by macrophages include all of the acid hydrolases, such as the cathepsins, glycosides, acid phosphatases, aryl sulfatases, and others. These enzymes may participate in the initial stages and in the continuation of inflammation through their ability to degrade connective tissue substances. These enzymes activate the zymogens of several humoral mediators of inflammation, including those of the complement system, the coagulation and kinin systems; they are also able to modify cell function by the limited hydrolysis of certain plasma membrane constituents (Davies et al., 1974).

D. THE ACTIVATION OF MACROPHAGES BY BACTERIAL CELL WALLS

After a single injection at various sites into experimental animals, a type-specific C-mucopolysaccharide-peptidoglycan complex (PPG) from group-A streptococcal cell walls causes chronic inflammatory lesions in which macrophages are the predominant cells (Ginsburg, 1972a, b; Page et al., 1974a; Schwab et al., 1959). The cell wall material of the streptococci is resistant to the action of lysosomal enzymes (Ayoub and McCarty, 1968) and PPG persists for many months at the site of chronic inflammation causing granulomatous lesions (Page et al., 1974a). The granulomatous lesions are due to the direct effect of the bacterial cell wall material on macrophages, as shown with cultures of these cells exposed to PPG (Davies et al., 1974; Page et al., 1974a). Upon exposure to low concentrations (1-15 μg/ml) of PPG for 72 hours, macrophages undergo rapid and marked morphological changes which persist for many days. In addition, there are significant increases in non-lysosomal enzymes such as LDH. There are less significant increases in the levels of lysosomal enzymes and in B-glucuronidase in the cell; there is, however, no release of these enzymes from macrophages into the culture medium. Higher concentrations of PPG (15-50 μg/ml) induce the release of lysosomal enzymes from viable macrophages in a dose-and time-dependent manner; the release proceeds rapidly and much of the enzyme is redistributed within 4-6 hours of exposure of the cells to the cell wall material (Davies et al., 1974).

E. THE ACTIVATION OF MACROPHAGES BY BACTERIAL ENDOTOXINS

Endotoxins added to cultures of mononuclear phagocytes elicit a variety of responses which are not fully understood. Cells exposed in vitro to concentrations of endotoxin near 1 μg/ml of medium exhibit enhanced pinocytosis and phagocytosis (Bennett and Cohn, 1966);

mouse macrophages treated with endotoxin from Salmonella abortus - equi exhibit increased cell size and granularity and an elevation in acid phosphatase levels in the cell (Cohn et al., 1966). The morphological and biochemical effects of endotoxin on mouse peritoneal macrophages maintained in culture differ from those seen in cultures containing inert particles or streptococcal cell walls (Allison et al., 1973). Endotoxin-stimulated cells exhibit an enhanced spreading on the surface of the culture vessel and an increased granularity very soon after exposure; however, these effects disappear in 24 hours. In cultures exposed for 72 hours to 10 μg of endotoxin per milliliter of medium, the levels of LDH and leucine-2-naphthylamidase increased about twofold over control values. Furthermore, endotoxin-treated cells are far more effective killers of bacteria and various tumor cells than are normal untreated control cells. Experimental animals have been protected, at least in part, against malignant tumor cells by an administration of a nonspecific stimulant like endotoxin (Bober et al., 1976). Macrophages exhibiting the nonspecific killing of tumor cells in vitro can be obtained from animals previously treated with endotoxin (Alexander and Evans, 1971). Also, macrophages can be "armed" or "activated" for the nonspecific killing of tumor cells by exposure in vitro to endotoxin (Evans and Alexander, 1972).

F. THE ACTIVATION OF MACROPHAGES BY DENTAL PLAQUE

Dental plaque is a mixed bacterial population found on the sheltered areas of teeth and there is substantial evidence that plaque is the primary etiological agent in the pathogenesis of chronic peridontitis. Plaque sterilized by irradiation causes changes very similar to those seen when streptococcal cell wall material is added to cultures of macrophages (Page et al., 1974a). Plaque in doses of 5-50 μg/ml causes a rapid and massive redistribution in vitro of acid hydrolases from the cells into the culture medium. Although no net increase in

acid hydrolase levels has been detected in cultures exposed to plaque for 24 hours, there are significant increases in the levels of B-glucuronidase in cultures exposed to concentrations of plaque greater than 10 μg/ml for 72 hours. This observation indicates that depletion of intracellular lysosomal enzymes may act as a stimulus for the replacement of enzymes which have been released into the culture medium. These changes occur in the absence of any detectable cell death. The constituents of plaque which are responsible for this effect are not known, although subsequent studies (Page et al., 1974b) have indicated that sterile homogenates of Actinomyces viscous, which is capable of inducing chronic inflammatory peridontal disease in germ-free rodents, have effects similar to unfractionated plaque.

G. THE ACTIVATION OF MACROPHAGES BY PRODUCTS OF LYMPHOID CELLS (LYMPHOKINES)

It is well known (Mackaness, 1971) that sensitized T lymphocytes reacting with specific antigens liberate products that activate macrophages in vivo to kill endocytosed bacteria more efficiently. Antigens, such as those of schistosome eggs, stimulate T lymphocytes to elicit a chronic inflammatory response (Warren and Boros, 1975). These in vivo effects can be explained by similar in vitro phenomena which occur when products of activated T lymphocytes are cultured with macrophages. Sensitized T lymphocytes, reacting with specific antigen or unsensitized T lymphocytes stimulated by concanavalin A or other lectins, liberate various products which activate macrophages (David and David, 1972). The products include a macrophage chemotactic factor which can recruit monocytes into sites of contact hypersensitivity, infection, or inflammation; the migration inhibition factor which can immobilize macrophages in the lesion; and various other macrophage-activating factors. The most biologically relevant are the many reports of macrophages which have been activated in vitro by

lymphocyte mediators. The stimulation of cultured lymphocytes by mitogens or by previously antigen-sensitized lymphocytes results in the release of lymphokines (Bennett and Bloom, 1968; Pick and Turk, 1972). Some of these substances induce remarkable morphological changes and decreased mobility of the cell (David, 1966), greater adherence to culture vessels (Mooney and Waksman, 1970), increased metabolic activity (Nath et al., 1973; Nathan et al., 1971), and enhanced microbicidal activity (Fowles et al., 1973; Krahenbuhl et al., 1973; Magliulo et al., 1973).

Pantalone and Page (1975) have studied the hydrolytic enzymes in mouse peritoneal macrophages cultured with products of activated lymphocytes. The cells contained more LDH, leucine-2-naphthylamidase and acid hydrolases than the cells of control cultures. While the first two enzymes were retained within the cell cytoplasm, acid hydrolases were secreted in large quantities by otherwise intact and viable cells.

The synthetic and secretory activities induced in macrophages by lymphokines differ markedly in time, magnitude, and pattern from those exhibited by cells exposed to various other substances. For example, large amounts of acid phosphatase activity are released within 15-30 minutes by cells exposed to streptococcal cell walls (Davies et al., 1974), while there is a lag of 36 hours in the release of this enzyme from lymphokine-treated cells (Pantalone and Page, 1975). The release of B-glucuronidase induced by the cell wall material is essentially complete within 4 hours, while the same enzyme is released in an almost linear manner from lymphokine-activated cells over a period of 72 hours. Although definitive experiments along these lines were not made, data relating to the time course, dose-response, and quantity of acid phosphatase release indicate that secretory activity in lymphokine-activated cells may be complete at the termination of synthetic activity.

This does not seem to be the case with cells activated by streptococcal cell walls or by certain bacterial substances (Page et al., 1974b).

H. THE ACTIVATION OF MACROPHAGES BY PRODUCTS OF THE CLEAVAGE OF COMPLEMENT

Mouse peritoneal macrophages in culture can also be activated to secrete hydrolytic enzymes by substances such as zymosan (Edwards et al., 1976), or by carrageenans and dextran sulfate (Burger et al., 1975), all of which induce chronic inflammation in vivo; hydrolase secretion from macrophages occurs in vitro as a consequence of the activation of complement by the alternate pathway (Schorlemmer and Allison, 1976). An early event in such activation is the cleavage of complement component C3 to fragment C3a and C3b. Highly purified C3a, when incubated with mouse peritoneal macrophages in culture, causes the release of both lysosomal hydrolases and LDH, indicating that the cell has been killed (Schorlemmer et al., 1976). In contrast, highly purified guinea pig C3b incubated with mouse and guinea pig peritoneal macrophages in serum-free medium has been found to elicit a dose-and time-dependent release of several hydrolases, but not LDH (Schorlemmer and Allison, 1976). Supernatants from macrophages stimulated with asbestos fibers, but not those from normal macrophages are able to cleave C3 (Schorlemmer and Allison, 1976).

Normal macrophages are known to synthesize C3 (Lai A Fat and van Furth, 1975) and factor B of the alternate pathway (Bentley et al., 1976), both of which are secreted intact. However, when macrophages are activated by C3b they cleave C3a and C3b (or enzymes that cleave C3), thereby generating further C3b and, thus, amplifying complement reactions. This amplification mechanism for complement may very well play an important role in chronic inflammation.

I. THE ROLE OF THE ACTIVATED MACROPHAGE IN PARASITOLOGY

Over the past few years, many examples of nonspecific immunity have accumulated in parasitology, when infection of an animal with one parasite renders it resistant to a simultaneous or subsequent challenge with an antigenically-unrelated organism. Similarly, inoculation of an animal with certain microbial products or a synthetic compound can increase its resistance to a wide variety of protozoa and helminths. Although many of the effector cells and mechanisms involved in this type of resistance are still obscure, the activation of the macrophage and its role in the control or modulation of these infections is increasingly gaining importance. The role of the activated macrophage in host-parasite relationships of a few representative protozoa and helminths is discussed below.

(1) PROTOZOA: Toxoplasma gondii

Toxoplasma gondii is a coccidian protozoan which is capable of infecting a wide range of vertebrate hosts (Frenkel, 1975). Intracellular toxoplasmas reside and multiply in the cytoplasmic vacuoles in macrophages; the vacuoles eventually rupture, releasing the parasites. The mechanism of the entry of toxoplasmas into macrophages has been a subject of discussion for many years. Pulvertaft et al., (1954) considered phagocytosis to be the most important mechanism of entry of toxoplasmas into these cells. Vischer and Suter (1954) also felt that phagocytosis was the mechanism by which toxoplasmas entered peritoneal macrophages, but Schupp et al. (1978) were also of the opinion that active penetration of non-phagocytic cells also occurred. Later, Lycke et al. (1965) suggested that a penetration process was the prime mechanism for entry of toxoplasmas into HeLa cells because penetration of the parasite was enhanced in the presence

of hyaluronidase or lysozyme. Norrby (1971) and Norrby et al. (1968) supported this theory by demonstrating that toxoplasmas contain acid phosphatase-positive granules near the anterior part of the parasite. Jones et al. (1972) however, showed that the plasma membrane of the phagocytic cells remains intact during entry of the parasite; they proposed that the enzymatic factors which were reported by previous workers were released by toxoplasmas to induce phagocytosis, even by cells not usually phagocytic; these enzymes were not used to lyse any portion of the plasma membrane. These authors also demonstrated that nearly all the parasites which entered the non-phagocytic HeLa cells survived and divided, whereas approximately half of the invading parasites were digested upon entry into macrophages. In a subsequent study, Jones and Hirsch (1972) showed that the reason for the survival of the toxoplasmas within the macrophages was due to a failure of the primary lysosomes to fuse with the vacuoles containing the parasites. This lack of fusion with the vacuoles was thought to reflect either a failure to activate the fusion mechanism or a blocking of it by some unusual membrane alteration of the normal fusion-promoting mechanism. It is unclear whether the living parasites play a role in the blocking or activating the fusion mechanism.

Macrophages isolated from mice infected with Toxoplasma gondii are capable of inhibiting or of killing the parasite in vitro (Krahenbuhl and Remington, 1971; Remington et al., 1972), but the mechanisms involved in these processes are not known. There is considerable evidence that thymus-derived lymphocytes stimulate macrophages to inhibit or to kill Toxoplasma (Anderson and Remington, 1974; Anderson et al., 1976; Borges and Johnson, 1975; Hoff and Frenkel, 1974; Lindberg and Frenkel, 1977; Sethi et al., 1975; Shirahata et al., 1976). Immunity to toxoplasmosis in animals can be passively transferred to non-immune animals by spleen or lymph node cells but not by

serum; the protection induced by cell transfer is immunologically specific (Frenkel, 1967) and is consistent with the present understanding of T lymphocytes and their role in cellular immune mechanisms (Mackaness, 1969, 1971).

Conflicting results concerning the relative efficacy of active supernatants versus intact antigen-stimulated lymphocytes in inducing the in vitro activation of macrophages have been found in experimental animal models (Cole, 1975; Fowles et al., 1973; Hoff and Frenkel, 1974; Sethi et al., 1975). Variations in experimental design, host species, infectious agents, antigen or the mitogen employed in these studies may account for the differences obtained. Although it appears that both soluble lymphocyte products and intact antigen or mitogen-stimulated lymphocytes are capable of conferring enhanced microbicidal capacity to normal macrophages, the exact role of cell-free mediators versus direct lymphocyte-macrophage contact in this immunological event is not clear. That lymphocyte products may act after contact of the Toxoplasma with the macrophage membrane is suggested by the work of Shirahata et al. (1976) who demonstrated that supernatants derived from the incubation of sensitized lymphocytes with Toxoplasma antigen stimulated macrophages to inhibit the multiplication of the parasite, even though the organism had entered the macrophage hours earlier. However, Jones et al. (1975) were not able to show a similar effect of lymphocytes or their products on T. gondii once the protozoa were inside the macrophages.

Knowledge of the mechanisms whereby lymphocytes or lymphokines stimulate macrophages to inhibit or to kill Toxoplasma is incomplete. When cultures of lymphocytes from animals or humans infected with T. gondii are incubated in the presence of homologous antigen, the supernatants obtained from the cultures are capable of

stimulating mouse and human macrophages to inhibit the replication of the parasite (Anderson et al., 1976; Borges and Johnson, 1975; Jones et al., 1975; Shirahata et al., 1976). The effects of lymphocytes on macrophages, mediated either by direct contact with lymphocytes or through lymphokines, may elicit a cytotoxic or a cytostatic response from the macrophage which can be specific (Lindberg and Frenkel, 1977) or nonspecific (Araujo and Remington, 1974; Hibbs et al., 1972; Krahenbuhl et al., 1973; Mackaness, 1971; Remington and Merrigan, 1969; Swartzberg et al., 1975). Examples of these are the reports of Lindberg and Frenkel (1977) and Hoff and Frenkel (1974) who described the in vitro arming by immune lymphocytes of elicited normal hamster macrophages to inhibit multiplication of Toxoplasma and the report of Anderson et al. (1976) who showed that supernatants produced by cultures of human lymphocytes stimulated with streptokinase-streptodonase (SK-SD) or with concanavalin A (Con A) can induce human macrophages to kill the parasite. In the latter experiments, control supernatants, to which either SK-SD or Con A had been added, were capable of partially activating macrophage monolayers, when compared to control untreated macrophages. However, this effect on T. gondii was less than that seen in stimulated macrophages exposed to active supernatants prepared by incubating homologous lymphocytes with either SK-SD or Con A.

Recently a new lymphokine, called Toxoplasma Growth Inhibitory Factor (Toxo-GIF), which inhibits the intracellular multiplication of T. gondii in non-immune mouse macrophages has been described (Shirahata et al., 1977). Immune spleen or lymph node cells were shown to produce large amounts of Toxo-GIF when cultured with Con A, phytohemaggutinin (PHA), or Toxoplasma lysate antigen. By the addition of supernatants from immune lymphoid cell cultures stimulated with Con A or PHA, non-immune macrophages showed nearly the same enhanced anti-Toxoplasma activity as demonstrated by monolayers

cultured in supernatants from immune lymphocytes after the inoculation with specific parasite antigen. Treatment of immune lymphocytes with anti-lymphocyte serum (ALS), plus complement, abolishes the ability of lymphocytes to produce Toxo-GIF. However, when immune lymphocytes were stimulated with lipopolysaccharide (LPS), the resultant supernants, as well as those from non-immune lymphocytes stimulated with T-cell or B-cell mitogens, did not show any significant Toxo-GIF acticity; hence, it appears that the production of this factor arises from a population of T cells. This conclusion is in agreement with work previously done by Borges and Johnson (1975) in humans and Sethi et al., (1975) in mice.

The production of Toxo-GIF is inhibited by the treatment of immune lymphocytes with the protein inhibitors puromycin and cycloheximide. Therefore, Toxo-GIF appears to be a substance synthesized de novo by sensitized T lymphocytes in response to specific antigen stimulation (Shirahata et al., 1977).

(2) NEMATODES: Trichinella spiralis

The nematode Trichinella spiralis provides a model in which host responses to helminth infections have been studied by a number of authors. In trichinellosis, there are three phases against which host resistance might develop: First, resistance may be directed against adult forms which develop in the gut after inoculation of the host with infective larvae. Second, resistance may be directed against the newborn larvae, which penetrate the gut mucosa and pass to the skeletal muscles by way of the bloodstream. Finally, it may be directed against larvae in the muscle cells, which mature into infective third-stage larvae 2-3 weeks after inoculation (Villela, 1970). However, the majority of the work done on the host immune response to this parasite has been directed to understanding the mechanism of expulsion of the

adult worm from the intestine of the host (Larsh, 1963, 1967, 1968).

Both humoral and cell-mediated immunity have been shown to play a role in the expulsion of the adult worm from the intestine of animals infected with T. spiralis (Catty, 1976). The cellular events responsible for expulsion are, however, poorly understood, but it has been suggested that the mechanisms include the action of lymphoid cells (Larsh et al., 1964, 1966), antibodies and lymphoid cells (Love et al., 1976; Wakelin and Lloyd, 1976a, b; Wakelin and Wilson, 1977a), lymphoid cells and non-lymphoid bone-marrow-derived cells (Wakelin and Wilson, 1977b).

Although resistance is detected against adult worms (Despommier, 1977; Love et al., 1976), destruction of the newborn larvae may be one of the primary mechanisms of resistance in natural infection with T. spiralis. Faubert (1976) has suggested that the immuneodepression observed in experimental trichinellosis is due to the action of migrating newborn larvae. Depletion of eosinophils, which are thought to be effector cells (Grove et al., 1977), increases the recovery of muscle larvae and Kazura and Grove (1978) have recently described an antibody-dependent eosinophil-mediated destruction mechanism which is specific for the newborn larval stage of this parasite. Furthermore, a collaborative effort by macrophages and eosinophils in the inflammatory response to the parasite has also been reported (Walls et al., 1974).

The role of macrophages in the immunity to T. spiralis has not been investigated extensively. Wing and Remington (1978) have suggested a role for these cells: they showed that, when normal mice and mice chronically-infected with Toxoplasma gondii were challenged with T. spiralis larvae, the peak intestinal worm burden was significantly lower in the animals with Toxoplasma. Moreover, there is a consider-

able increase in the number of peritoneal exudate cells in mice after an oral inoculation with T. spiralis which coincides with the elimination of adult worms from the gut (Wing et al., 1979). Animals infected with this parasite are also susceptible to challenge with Listeria monocytogenes 7 or 21 days after infection with the helminth (Cypess et al., 1974a). Experimental trichinellosis also inhibits a superimposed infection with Trypanosoma equiperdum and T. lewisi in rats (Meerovitch and Ackerman, 1974). Peritoneal macrophages taken from mice infected with T. spiralis inhibit the synthesis of DNA by leukemia cells (Meerovitch and Bomford, 1977), and by EL-4 tumor cells (Wing et al., 1977). Infection with T. spiralis also potentiates the delayed-type hypersensitivity response to BCG in mice; this potentiation is dependent on the stage of the parasite infection (Cypess et al., 1974b; Molinari et al., 1974). There are several possible explanations for the observed enhancement of the cell-mediated responses in T. spiralis infected animals, including a potentiation of the antigenic stimulation induced by the second immunogen, the nonspecific activation of the RES and an enhancement of T cell function.

(3) TREMATODES: Schistosoma mansoni

Effector mechanisms which protect animals in experimental schistosomiasis differ from the two-step hypothesis for immunity to nematodes proposed by Ogilvie and Love (1974). Immunity in schistosomiasis is induced by the adult trematode but the effector mechanisms are only active upon reinfection of the schistosomulum, the immature invading stages (Smithers and Terry, 1969a). A number of hosts are known to acquire resistance to S. mansoni (Smithers and Terry, 1976). Among these, the rat and the mouse have been shown to develop an immunity which destroys invading schistosomula within the first 5 days following re-exposure to cercariae (Perez et al., 1974; Sher et al., 1974). In the rat, this immunity is controlled by a mechanism which

requires the participation of antibodies (Phillips et al., 1975).

Clegg and Smithers (1972) were the first to demonstrate that young schistosomula of S. mansoni are killed in vitro in the presence of complement when cultured with the serum of monkeys which have been exposed to S. mansoni. Dean et al. (1974, 1975) have reported that schistosomula could be killed in culture by immune IgG and normal neutrophils acting in concert; damage to the worms was due to the complement-dependent release of lytic enzymes from these cells. Butterworth and co-workers (1974, 1977) demonstrated antibody-dependent human eosinophil-mediated damage to schistosomula in vitro which was independent of complement; in a murine system Mahmoud et al. (1975) have observed a decrease in resistance to re-infection in immune animals given anti-eosinophil serum. Recently, the role of the macrophage in schistosomiasis has been proposed (Capron et al., 1975, 1977a; Perez and Smithers, 1977) and Capron et al. (1977b) have suggested that macrophages are activated in schistosomiasis by immune complexes containing IgE. In numerous other studies, macrophages have been activated by BCG (Bast et al., 1976; Civil and Mahmoud, 1977, 1978; Civil et al., 1978; Maddison et al., 1978; Mahmoud et al., 1979), by cord factor (Mahmoud et al., 1977), by Toxoplasma gondii (Mahmoud et al., 1976) or by Corynebacterium parvum and T. spiralis (Mahmoud et al., 1979) to kill schistosomula in vitro or to protect animals from infection with S. mansoni. Similarly, preparations of heterologous animal proteins or of killed bacteria have been reported to induce protective effects against S. mansoni (Smith et al., 1975).

The mechanisms whereby BCG or other substances suppress schistosome infections in experimental animals has yet to be elucidated. Perez and Smithers (1977) have, however, demonstrated that peritoneal macrophages stimulated by proteose peptone adhere to schistosomula

and damage their surface when sensitized with inactivated immune rat serum. Recently, a mechanism of adherence and killing of schistosomula in vitro that is independent of complement but which requires specific opsonizing IgG antibodies and eosinophils or macrophages has been suggested (Kassis et al., 1979). More recently, monolayers composed of macrophages that were activated in vivo by BCG or C. parvum when cultured with larvae of T. spiralis or with schistosomula of S. mansoni released soluble factors that could kill schistosomula of S. mansoni in vitro (Mahmoud et al., 1979).

(4) CESTODES

The importance of humoral factors in the immunity to larval cestode infections was first demonstrated by the successful passive protection of normal rats against Taenia taeniaeformis by serum from artificially immunized rats or from animals experimentally infected with this parasite (Miller, 1932a; Miller and Gardiner, 1932a, b, 1934). Subsequent studies have demonstrated the successful passive immunization of rabbits against T. pisiformis (Campbell, 1938a, b; Kerr, 1934) and of mice against Hymenolepis nana (Hearin, 1941). The transfer of immunity to T. taeniaeformis from the mother to her offspring under experimental conditions has also been shown (Miller, 1932b, 1935). Larsh (1942, 1944) extended these findings to show that protection in mice against H. nana could be transferred in utero or via the colostrum.

Much less success has been achieved in protecting animals against either the intestinal or the cystic stage of Echinococcus. Early workers tried to immunize animals against this parasite and some of them met with success. Dévé (1927) was unable to produce an artificial immunity against E. granulosus in rabbits injected with hydatid sand. Attempts to produce immunity in white rats by means of specific sero-

therapy (Dévé, 1933) or by the injection of hydatid membrane material (Dévé, 1934) were also failures. Turner et al., however, (1933, 1935, 1936, 1937) observed a remarkable decrease in the incidence and in the degree of intestinal infection with E. granulosus in dogs that had been immunized with antigens prepared from hydatid cyst material.

Forsek and Rukavina (1959) vaccinated dogs with hydatid fluid, extracts of protoscolices, or extracts of germinal membrane and have also reported smaller worm burdens in the immunized animals. Sweatman et al. (1963) obtained only a partial 9 month resistance in sheep following a high dose of E. granulosus eggs. More recently, mice have been treated against E. granulosus with extracts of protoscolices, hydatid cyst fluid or by infection with live protoscolices (De Rycke and Pennoit-De Cooman, 1973). This study has shown that hydatid fluid conferred little protection, but that the other 2 vaccines gave some degree of immunity. In particular, it was found that a first infection normally produced a negative challenge, while a successful challenge occurred only when the first inoculation did not result in an infection. It has been reported that puppies injected with irradiated hydatid fluid or with protoscolices of E. granulosus harboured fewer adult worms and the development of these cestodes was retarded, irrespective of the mode of immunization (Aminzhanov, 1978).

Unhatched eggs and artificially hatched and activated embryos have proved to be promising protective antigens. A series of extensive studies carried out on T. ovis, T. hydatigena, T. pisiformis and E. granulosus by Gemmell (1964, 1965a, b, 1966, 1967) and by Gemmell and Johnstone (1977) have shown that injections of the eggs of homologous or heterologous species may confer a marked degree of protection against the pre- and post-encystment stages. In some cases, this immunity is absolute, although its longevity has not been determined; in other cases, however, no immunity developed. Thus, activ-

ated embryos of T. hydatigena gave 100% protection against this species at the pre- and post-encystment stages. In contrast, live eggs of T. pisiformis gave no protection whatsoever against challenge with T. hydatigena, T. ovis, or E. granulosus, although substantial protection was obtained against the homologous species. Some degree of protection against the adult stages of E. granulosus in dogs has been induced by onchosphere vaccines from heterologous species; protection was not, however, absolute in all dogs (Gemmell and Soulsby, 1968). In general, lumen-dwelling cestodes do not evoke reactions which protect against re-infection. In the case of H. diminuta, an inhibiting effect has been reported on challenge with a new infection, but crowding and competition for nutrients between worms may be sufficient to account for this apparent acquired resistance (Weinmann, 1966).

The ability of cestodes to survive for extended periods of time within the tissues of immunologically competent hosts is very intriguing. Several hypotheses have been put forth to account for this phenomenon; these hypotheses are based upon either antigenic sharing between host and parasite as a result of natural selection (Damian, 1964; Dineen, 1963a, b), phenotypic adaptation (Smithers and Terry, 1969b) or on blocking antibodies (Rickard, 1974). The presence of host-like proteins in the membranes and in the fluids of the hydatid cyst and the permeability of these membranes to serum proteins have been the subject of a number of reports (Cameron and Staveley, 1957; Coltorti and Varela-Diaz, 1972, 1974, 1975; Kagan and Agosin, 1968; Varela-Diaz and Coltorti, 1972, 1973). Coltorti and Varela-Diaz (1975) have suggested that the permeability of the hydatid cyst to host immunoglobulins might be due to passive transport or to a random process since gerbil IgG was detected in the fluid of only 29.5% of the cysts that had been transplanted from mice into the peritoneum of gerbils. More recently, Kassis and Tanner (1977a) have demonstrated

the presence of host serum proteins, including IgG and IgM, within the cyst membranes and on the surface of protoscolices of E. multilocularis.

Although many workers have studied the effects of antisera on cestodes, the role of complement seems to have been disregarded until recently (Santoro et al., 1979). The involvement of complement in the immunological mechanisms of the host response in echinococcosis has been suggested in the last few years by several workers and there are a few reports concerning the lysis and death of cestodes after incubation in fresh normal or immune sera (Heath, 1973; Heath and Pavloff, 1975; Heyneman and Welsh, 1959; Silverman, 1955). The observation that some reactions of antisera against cestodes can be abolished by heat-inactivation (Smyth, 1969a) or enhanced by the addition of complement (Heath, 1973; Heyneman and Welsh, 1959) have strongly suggested the involvement of complement.

The lysis of protoscolices of E. granulosus and E. multilocularis has been demonstrated following incubation in the normal sera of a number of different host species (Herd, 1976; Kassis and Tanner, 1976a); heat-inactivation of the serum at 56°C renders it non-cytotoxic (Kassis and Tanner, 1976a). Human sera lose their complement-dependent protoscolicidal activity following treatment with EGTA (ethyleneglycoltetraaceticacid) or EDTA (ethylenediaminetetraaceticacid); the addition of calcium to EDTA-treated sera restores this activity (Kassis and Tanner, 1977a). The presence of antibody and the fact that chelating agents can inhibit the lysis of protoscolices of E. multilocularis by normal serum led Kassis and Tanner (1977a) to conclude that the activation of complement was mediated through the classical pathway. In contrast, Herd (1976) and Rickard et al. (1977) have been unable to demonstrate antibody on the surface of E. granulosus protoscolices from sheep and horse hydatid cysts. However, the complement component C3

was demonstrated by immunofluorescence on the surface of protoscolices of this parasite previously incubated in normal human serum (Rickard et al., 1977). The fact that serum from guinea pigs deficient in C4 was as effective as normal serum in its ability to lyse protoscolices has led these latter authors to conclude that E. granulosus protoscolices are lysed by the alternate pathway. This discrepancy on the mechanism of activation of complement in echinococcosis may be the result of biological differences which are known to occur between different strains of parasite (Matossian et al., 1978). These in vitro studies have suggested that both the classical and alternate complement pathways, in the presence or absence of antibodies, may be activated and may have a role in the control of hydatid infections. There is some evidence to suggest that complement does play such a role in experimental hydatid infections: Inoculation of protoscolices of E. multilocularis into rats previously depleted of complement with cobra venom factor, resulted in the development of significantly larger cyst masses and a greater number of infection foci and metastases as compared with untreated infected rats (Kassis and Tanner, 1977b).

In spite of the fact that the larvae within the cysts are coated with parasite-specific antibody (Kassis and Tanner, 1977a; Pozzuoli et al., 1972; Varela-Diaz et al., 1974), the larvae are not lysed by complement action in the cyst. Kassis and Tanner (1976a) have shown that the cyst contains calcareous corpuscles which have anti-complementary activity and hence effectively protect the larvae from the lethal action of the antibodies with which they are coated. It was further sugtested (Kassis and Tanner, 1976b) that fresh patients' serum can be used to kill E. granulosus cysts in difficult locations, thereby eliminating the necessity for major complicated surgery (Saidi, 1976).

At the time of this writing, there is no effective medical treatment for hydatid cysts in any organ system; the treatment of all

hydatid cysts is strictly surgical (Saidi, 1976). E. granulosus cysts are often "benign" and generally operable, whereas those of E. multilocularis are, because of their rapid dissemination by metastases, inoperable and often fatal. The present study has dealt largely with E. multilocularis because of an interest in resolving the immunobiology of the control of this human parasite and partly because of its resemblance to a proliferating neoplastic tumor; these infections have the added attraction that they can be relatively easily maintained in the laboratory by the vegetative passage of protoscolices or small cysts (Lubinsky, 1960a, b). Studies investigating the nature of the control of Echinococcus infections have suggested that parasite-parasite interactions are similar to some tumor-tumor interactions (Laird, 1964). Rau and Tanner (1973) have demonstrated that relatively large subcutaneous cysts of E. multilocularis effectively inhibit the establishment, growth, and metastatic spread of subsequent intraperitoneal inoculations of the same parasite. This control is so effective that a large cyst mass suppresses the development of its own metastatic foci of infection (Rau and Tanner, 1976a); the removal of a subcutaneous cyst mass will permit the growth of intrathoracic metastases which will kill the experimental animal by asphyxiation.

Hydatid infections are characterized by an important proliferation of cells in the peritoneal cavity (Ali-Khan, 1974; Schwabe et al., 1959), in the spleen (Ali-Khan, 1978 a, b, c; Rau and Tanner, unpublished data) and in the peripheral circulation (Rausch, 1954). The principal cell in peritoneal exudates of animals with an intraperitoneal infection of E. multilocularis is the macrophage (Reuben et al., 1978) which is activated by this infection to kill the parasite in vitro (Rau and Tanner, 1976b); inactivated immune serum adds to this activity, but by itself, is not very lethal. Attempts to identify a specific macrophage activation factor in the supernatant of spleen cells incubated in vitro

with hydatid fluid, protoscolices, or small cysts have been unsuccessful to date. The fact, however, of the presence of activated phagocytic cells in this infection has suggested that "nonspecific" activation of macrophages with BCG could enhance the regression of the parasite cyst mass. This is, in fact, the case since BCG treatment of the animal host before inoculating the parasite almost abolishes the growth and metastasis of the cyst (Rau and Tanner, 1975; Thompson, 1976); treatment with BCG after the cyst has established does not, however, suppress growth but does effectively limit the metastatic dissemination of the infection (Rau and Tanner, 1975). Infection by other cestodes can also nonspecifically activate an anti-Echinococcus cytotoxicity by peritoneal cells (Baron and Tanner, 1977).

The potential of in vitro and in vivo activation of cytotoxic macrophages from normal or infected animals with BCG or with lectins could provide an alternative approach to the prophylaxis and therapy of hydatid disease. It was the purpose of this study to investigate the effectiveness of nonspecifically-activated cells for the rational immunobiological control of echinococcosis.

CHAPTER 3

CHAPTER 3

MATERIALS AND METHODS

Parasite and the Animal Host

The strain of Echinococcus multilocularis used in the present study was derived from an Alaskan strain which was originally isolated by Rausch and Schiller (1951). It has since been maintained at the Institute of Parasitology by the vegetative transfer of the metacestode in cotton rats (Sigmodon hispidus), according to the technique described by Lubinsky (1960b). Briefly, this involves the intraperitoneal inoculation of ground cyst material obtained from infected cotton rats, into a new set of animals every 90 days.

The usual rodent-carnivore life cycle can be used as a source of E. multilocularis larvae but it is hazardous because the onchospheres are infectious to man. The cotton rat (Sigmodon hispidus) is a highly susceptible host (Sadun et al., 1957). Vegetative serial transfers in which protoscolices or brood capsules are transferred intraperitoneally from cotton rat to cotton rat provide a convenient method for maintaining the parasite (Lubinsky, 1960b, 1967). The growth of cysts in these animals is fast since over 40 g of cysts can be produced within 90 days after the inoculation of one acephalic cyst (Baron et al., 1974a). These rats are therefore a convenient host in which stock infections can be maintained as a source of the large quantities of infectious material necessary for experimentation with large numbers of animals.

Experimental Infection of the Cotton Rats

To minimize biological variation, all replicate infections were induced by the surgical implantation of an acephalic brood capsule

(approximately 0.25 cm in diameter) obtained from a single cotton rat harbouring a 60 day-old infection. These acephalic cysts were collected from a larger biomass which was teased gently apart in a moderate volume of medium 199 (Difco, Detroit, Michigan) to free daughter cysts; the daughter cysts were counted under a stereo dissecting microscope.

The inoculum was gently placed into the peritoneal cavity of cotton rats lightly anesthesized with Nembutal (Abbott Laboratories, Montreal) through a small incision into the central hypogastric region. The incision into the peritoneum was sutured (Silk 5-0, Ethicon, Peterborough) and the abdominal wall was closed using a surgical wound clip (9 mm, Clay-Adams).

Necropsy Procedure

At the termination of each experiment, all animals were exsanguinated by cardiac puncture under ether anesthesia. At autopsy, each animal was weighed, total and differential leucocyte counts were made of the peritoneal cells, the spleen was weighed, and, whenever present, the total weight of granuloma and/or parasite cyst mass within the peritoneal cavity was obtained.

Harvesting of Peritoneal Exudate Cells

A small incision was made in the abdominal wall of exsanguinated animals, and a 10 µl volume of the exudate was drawn into a Unopette (Becton-Dickenson, Rutherford, N. J.); a total cell count was obtained with the aid of a hemocytometer. An additional 5 µl sample was used to prepare two coverslips for differential cell counts. Briefly, this was done by placing a 5 µl sample of the exudate on a coverglass (22 x 22 mm^2, No. 2 thickness) and a second coverglass was placed over the first. The specimen will spread in a very thin film and, just as the

film stops spreading and before it begins to coagulate, the coverglasses are quickly pulled apart firmly in a plane parallel to their surfaces. This method is especially recommended, since all the leucocytes in the 5 µl sample will be found on the two coverglasses and, thus, errors due to unequal distribution can be excluded by counting all the leucocytes.

The remaining peritoneal cells were collected using a modification of the method of Evans and Alexander (1970). Briefly, 5 ml of tissue culture medium 199 (Difco, Detroit, Michigan) was injected into the peritoneal cavity of the animal; the abdominal cavity was gently massaged and the cells aspirated with a Pasteur pipette. The above procedure was repeated with an additional 5 ml of medium; the 2 aspirations were pooled and a total leucocyte count done.

Staining of Coverglasses for Differential Leucocyte Counts

The coverglasses were allowed to dry in air, fixed in methanol for 2 minutes, and then they were placed in Wright's stain (diluted 1:3 in Giordano's buffer, pH 6.4) for 6 minutes. At the end of this time, the coverglasses were placed in Giemsa stain (diluted 1:30 in Giordano's buffer, ph 6.4). The coverglasses were left in the Giemsa stain for an additional 9 minutes, and then they were rinsed in tap water, air dried, and mounted onto microscopic slides. Differential leucocyte counts were obtained by counting 1000 leucocytes on each stained coverglass.

Cytochemical Demonstration of Acid Phosphatase in Peritoneal Cells

The demonstration of acid phosphatase activity in the peritoneal cells from control and treated cotton rats was done according to the method described by Kaplow and Burstone (1964) using unfixed smears and azo dye coupling techniques, with naphthol AS-MX phosphates (Sigma, MO.) as the substrate. All coverslip smears were

stained and examined within 24 hours after collection.

The substrate used was napthol AS-MX phosphate (Sigma, MO.) with fast red violet LB salt (ESBE Laboratory Supplies, Toronto) as the coupling agent. Incubation was carried out for 2 hours at 37°C in a mixture of freshly prepared filtered solutions containing 5 mg naphthol AS-MX phosphate dissolved in 0.3 ml dimethyl formamide. Sixty ml of 0.1M citrate buffer, pH 5.2 and 30 to 40 mg of fast red violet (LB salt) was then added. Following 2 hours incubation, the coverslips were washed in running tap water for 20 to 30 seconds, air dried and counterstained with Mayer's aqueous hematoxylin for 5 to 8 minutes. Glycerol-gelatine was used as a mounting medium and the preparations were examined with the oil immersion lens. Negative controls consisted of coverslips inactivated by immersion in boiling water for 5 minutes prior to incubation in the staining solution. Coverslips were also incubated in solutions in which the substrate and the diazonium salt were omitted from the incubation mixture.

Preparation and Staining of Histological Sections[1]

The tissue to be examined histologically was fixed for 6-8 hours in Gomori's Fixative. The tissue was dehydrated in a graded series of alcohols, transferred to benzene, infiltrated and embedded in paraffin (melting point 56°C). The embedded tissue in paraffin was sectioned at 7 μm and stained with Mayer's Hematoxylin and counterstained with eosin.

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1. Humason, G. L. 1972. Animal tissue techniques. Third edition. W. H. Freeman and Co., San Francisco.

Statistical Analysis

Student's t-distribution test was used to analyze the data of certain experiments. This test is used to relate data obtained from untreated controls to those obtained from treated groups. The data were considered statistically significant if the t value was above the 5 percent level (Steel and Torrie, 1960).

The analysis of variance (ANOVA) and Duncan's multiple range test (Steel and Torrie, 1960) were used to help analyze some of the data of the experiments with BCG. When a number of mean values were to be compared, the F values were estimated by the analysis of variance and, when this was above the 5 percent level, the mean value of each group was compared with that of its control by Duncan's test. All data in the graphs are represented as the mean $\pm$ 95% confidence intervals.

When the range of the data gave unnecessary weight to extraneous values in an estimate of dispersion, the Q Test was applied to eliminate those values which failed to pass the screening test (Dean and Dixon, 1951). The Q Test, applicable to ten or fewer independent observations, is defined as $(x_2 - x_1) / W$, where $(x_2 - x_1)$ is the distance of a double observation from its nearest neighbour, and W is the range. If the calculated Q value exceeds the tabulated value, then the questionable observation may be rejected with 90% confidence.

CHAPTER 4

CHAPTER 4

IMMUNOPROPHYLAXIS WITH BCG OF EXPERIMENTAL ECHINOCOCCUS MULTILOCULARIS INFECTIONS

INTRODUCTION

Although many of the effector cells and mechanisms involved in the nonspecific immunity to parasite infections are still obscure, the activation of the macrophage and its role in the control and modulation of experimental hydatid infections is well recognized. Pretreatment of cotton rats (Rau and Tanner, 1975) and Mongolian gerbils (Thompson, 1976) with 26×10^6 colony-forming units (CFU) of BCG protected these animals against a subsequent infection of E. multilocularis and E. granulosus, respectively; the marked effect of BCG on developing cysts of both these infections is due to the non-specific activation of macrophages. In spite of the obvious immuno-prophylactic effects of BCG in hydatid disease, many animals develop numerous small granulomatous lesions in the peritoneal cavity as a consequence of the treatment. Consequently, a study was initiated to determine the minimum effective prophylactic dose of BCG for experimental E. multilocularis infections and to relate any protective effects to changes in the leucocyte population induced by this treatment and/or the parasite infection.

MATERIALS AND METHODS

The Parasite

Brood capsules of E. multilocularis used in the present study were obtained from stock infections. To minimize biological variation, all replicate infections were induced by the implantation of

acephalic brood capsules (approximately 0.25 cm in diameter) obtained from a single infected cotton rat.

Animals

Eight-week old laboratory-reared cotton rats of both sexes (85-120 gms) were used throughout this study. To reduce variation further, cotton rats were grouped by body weight to constitute blocks and/or replicates. The animals received a single injection of BCG and were inoculated with the parasite 14 days later. All replicates were inoculated with the parasite at the same time; the experiment lasted for 8 weeks.

Bacillus Calmette-Guérin (BCG)

The strain of BCG used in this study was a lyophilized vaccine, specifically prepared for use in cancer immunotherapy by the Institut Armand-Frappier, Montreal. The vaccine contained, according to the manufacturers, 8.9×10^6 CFU/mg (lot number 1750-4); the range of the viable counts was $8\text{-}12 \times 10^6$ CFU/mg. The lyophilized vaccine was rehydrated with phosphate buffered saline (PBS), pH 7.2 according to the instructions of the manufacturers; suspensions containing 10^1, 10^2, 10^3, 10^5, and 10^7 CFU per ml were prepared in PBS from this stock.

Determination of the Level of Stimulation by BCG Two Weeks After Treatment

In order to determine the level of stimulation induced by BCG at the time of the inoculation of the parasite, 1 ml doses of 10^1, 10^2, 10^3, 10^5, and 10^7 CFU of BCG in PBS were each injected intraperitoneally (i.p.) into 4 cotton rats; 4 control cotton rats were injected i.p. with 1 ml PBS. Fourteen days later, all the cotton rats

were exsanguinated by cardiac puncture under ether anesthesia. At autopsy, each animal was weighed, total and differential leucocyte counts were made of the peritoneal cells, the spleen was weighed, and, whenever present, the total weight of granuloma within the peritoneal cavity was obtained.

Determination of the Minimum Effective Prophylactic Dose of BCG Against Echinococcus multilocularis

Cotton rats were placed in groups of 10 or 12 individuals according to body weight, as described above. The animals in each group were treated i. p. with the appropriate dose of BCG suspended in a 1 ml volume of PBS, pH 7. 2. Two weeks later, half of the animals from each treated and control group were anesthetized with Nembutal (Abbott Laboratories, Montreal) and a brood capsule was surgically implanted into the peritoneal cavity of each animal. All other animals were sham-inoculated, as controls.

Forty-two days post-inoculation of the parasite, all animals were exsanguinated by cardiac puncture under ether anesthesia and processed as described above, and the weights of the parasite cysts were obtained.

Harvesting and Staining of the Peritoneal Exudate Cells

The harvesting of peritoneal cells was done according to the procedure outlined in Chapter 3, Materials and Methods. The coverslips of peritoneal cells were stained with both Wright's and Giemsa stains. Differential leucocyte counts were obtained by counting 1000 leucocytes on each stained coverslip. The data were analyzed by the analysis of variance (ANOVA) and Duncan's test for multiple ranges.

RESULTS

The results of the total and differential leucocyte counts of the peritoneal exudate of animals that had been treated two weeks previously with either 10^1, 10^2, 10^3, 10^5 or 10^7 CFU of BCG or PBS is summarized in Figure 4. 1.

Only those cotton rats which had been treated two weeks previously with an intraperitoneal injection of 10^3 or 10^7 CFU exhibited total leucocyte counts that were significantly higher ($p \leqslant 0.05$) than those of control animals treated with PBS. Total leucocyte counts were not significantly elevated in animals that had been treated with 10^1, 10^2, or 10^5 CFU. The increase in the number of leucocytes was due principally to a significant elevation in the population of monocytes; neither the polymorphonuclear cells, nor the lymphocytes, were significantly increased at any of the doses studied. Macroscopic BCG granulomas were found in those animals that had been treated with 10^5 and 10^7 CFU of the vaccine.

The total and the differential peritoneal cell responses of cotton rats treated with BCG, and subsequently inoculated with one brood capsule of the parasite, is illustrated in Figure 4.2. The results presented here are of two separate experiments: in the first experiment, cotton rats were treated with 10^1, 10^2 and 10^3 CFU of BCG; in the second experiment, doses of 10^3, 10^5 and 10^7 CFU were used (appropriate PBS controls were done in both cases). Since the total leucocyte counts of the peritoneal exudates of animals treated with PBS or 10^3 CFU of BCG in both experiments were not significantly different ($p \leqslant 0.01$), the results of the two experiments were pooled and presented together in Figure 4.2.

Figure 4. 1 Levels of stimulation of peritoneal leucocytes of cotton rats two weeks after inoculation with various doses of BCG or PBS. Vertical bars depict the mean ± 95% confidence intervals.

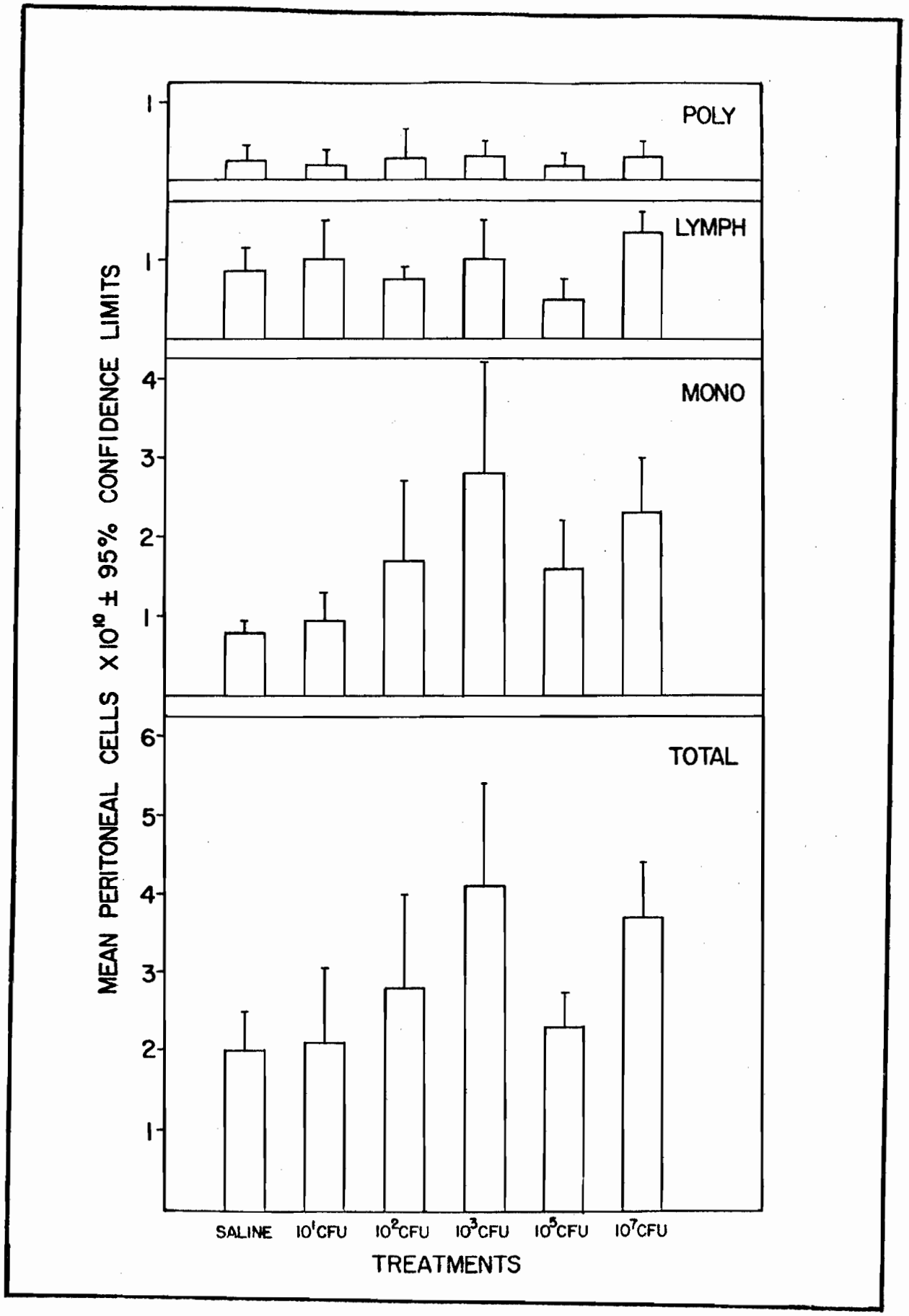
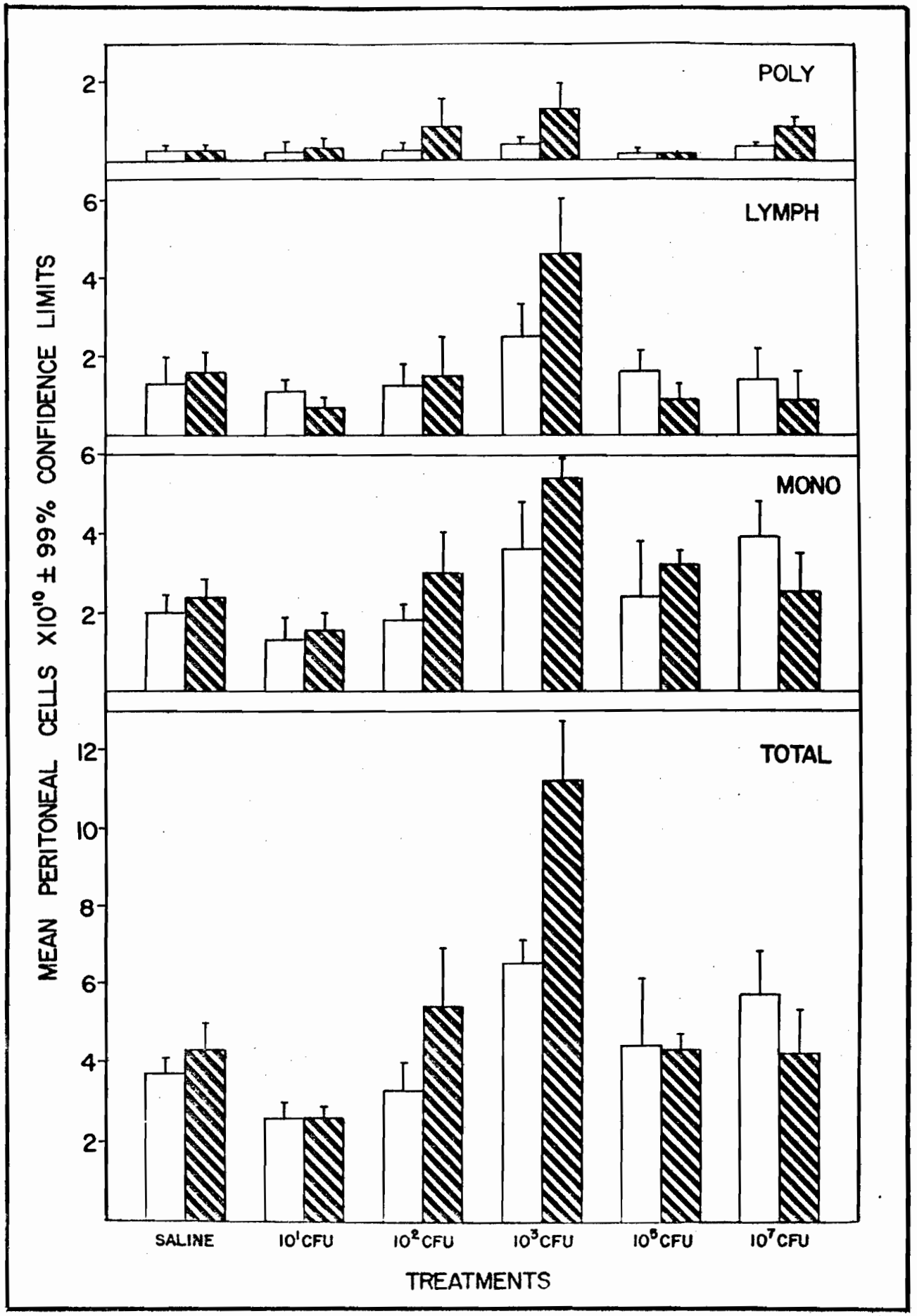

POLY
LYMPH
MONO
TOTAL
MEAN PERITONEAL CELLS X 10^10 ± 95% CONFIDENCE LIMITS
SALINE
10^1CFU
10^2CFU
10^3CFU
10^5CFU
10^7CFU
TREATMENTS

Figure 4.2 Levels of stimulation of peritoneal leucocytes of cotton rats inoculated with BCG and E. multilocularis. Cross-hatched bars represent animals treated with BCG or PBS and subsequently inoculated with the parasite. Open bars represent sham-inoculated controls previously treated with BCG or PBS. Vertical bars depict the mean ±99% confidence intervals. (POLY = polymorphonuclear leucocytes; LYMPH = lymphocytes; MONO = monocytes/macrophages; TOTAL = total leucocytes.)


POLY
LYMPH
MONO
TOTAL
MEAN PERITONEAL CELLS X10¹⁰ ± 99% CONFIDENCE LIMITS
SALINE
10¹CFU
10²CFU
10³CFU
10⁵CFU
10⁷CFU
TREATMENTS

The number of total leucocytes tended to increase with an increase in the dose of BCG, up to 10^3 CFU; at higher doses, a decline in total leucocytes to normal, or nearly normal, values was evident. The concomitant parasite infection enhanced the total peritoneal leucocyte numbers in cotton rats treated with 10^2 and 10^3 CFU. Cotton rats treated with 10^3 CFU and subsequently inoculated with the parasite exhibited the greatest elevation in leucocytes of all the groups; this elevation was reflected in a significant increase in the total numbers of monocytes and lymphocytes. None of the other doses produced significant increases in the total or differential cell counts.

Complete protection of cotton rats from experimental E. multilocularis infections was achieved with a prophylactic dose of BCG as small as 10^3 CFU, without the induction of any apparent granulomatous reaction. Two of the 5 animals treated with 10^2 CFU also did not develop a hydatid infection. Protection against E. multilocularis was also achieved at the higher doses of 10^5 and 10^7 CFU, but this was accompanied by the formation of distinct granulomas. Animals treated with 10^1 CFU were not protected at all against the parasite, since E. multilocularis infections in these cotton rats reached sizes statistically indistinguishable from those in the controls (Table 4. 1).

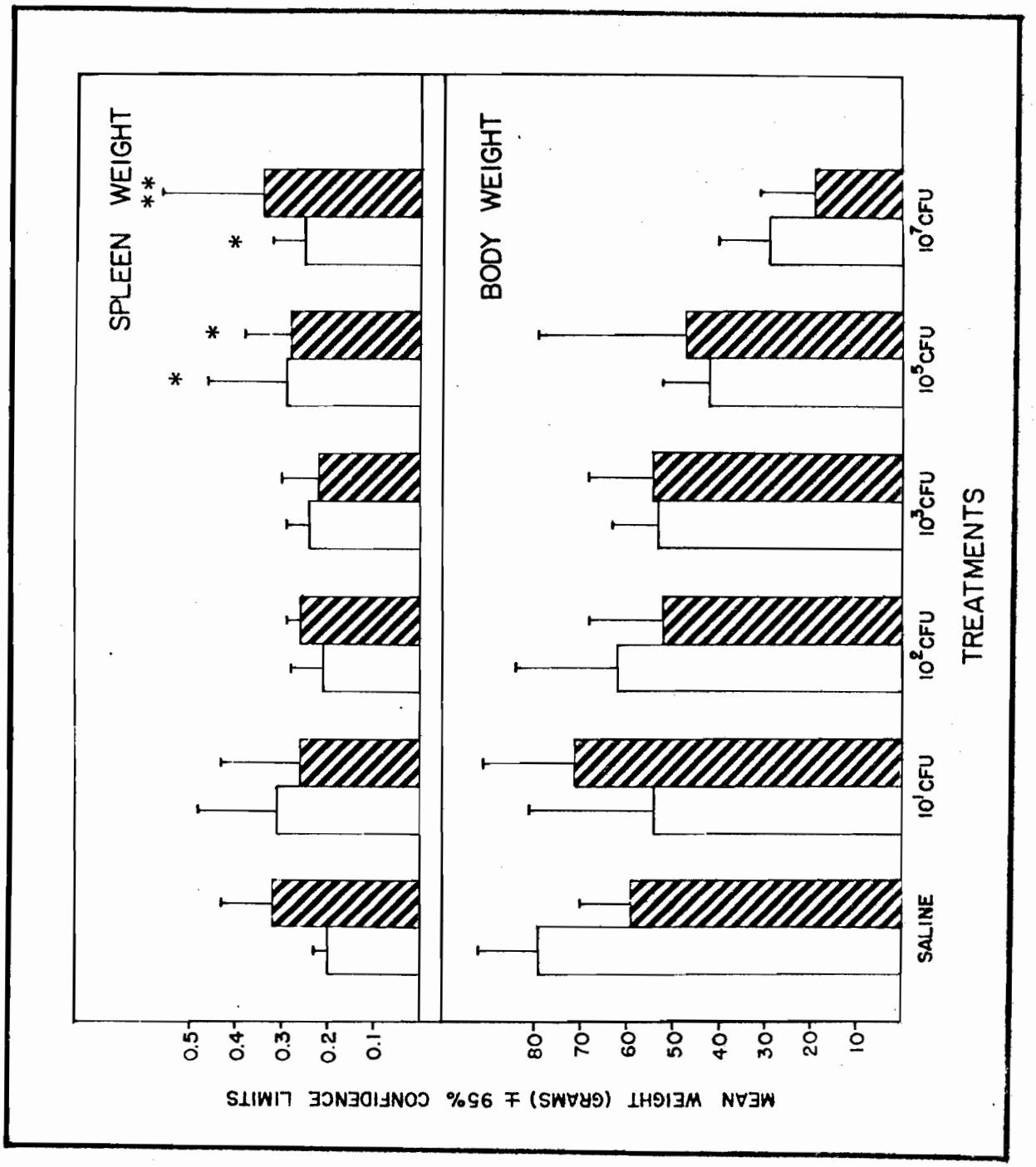
There was no splenomegaly (Figure 4. 3) in any of the treatment groups. There was, however, a tendency for those animals treated with 10^5 and 10^7 CFU of BCG to gain less body weight from the time they were treated to necropsy than those animals treated with lower doses of BCG, or PBS (Figure 4. 3).

TABLE 4.1

The weight of E. multilocularis cysts in control (saline treated) cotton rats and in animals treated intraperitonealy with 10^1 CFU and 10^2 CFU of BCG.

Treatment	Saline	10^1 BCG	10^2 BCG
Mean cyst weight in grams	0.85	0.60	0.27
± Standard error	0.38	0.27	0.26

Figure 4.3 Cross-hatched bars represent the spleen and total body weights at necropsy of cotton rats treated with BCG or PBS and subsequently inoculated with E. multilocularis. Open bars represent sham-inoculated controls previously treated with BCG or PBS. Vertical bars depict the mean weight ±95% confidence intervals. (* represents animals with granulomas of ≤0.1 grams; ** represents animals with granulomas of ≥0.1 grams).


SPLEEN WEIGHT
BODY WEIGHT
MEAN WEIGHT (GRAMS) ± 95% CONFIDENCE LIMITS
0.5
0.4
0.3
0.2
0.1
80
70
60
50
40
30
20
10
SALINE
10¹CFU
10²CFU
10³CFU
10⁵CFU
10⁷CFU
TREATMENTS
*
*
*
**

DISCUSSION

Mycobacterium bovis, strain BCG, has received much attention because of its ability to enhance the defense mechanisms of the host against neoplastic diseases. However, little is known concerning the dose-response relationship in the anti-tumor activity initiated by this organism. In most instances, reducing the dose of BCG eventually eliminates its stimulating effect on the immunological response (Bartlett and Zbar, 1973; Chung et al., 1973), although BCG can be effective at doses as low as 800 colony-forming units (CFU) (Pearson et al., 1973). Rau and Tanner (1975) have found that intraperitoneal (i. p.) pretreatment of cotton rats with 2.64×10^7 CFU of BCG inhibited the growth and metastasis of an inoculum of 200 protoscolices of Echinococcus multilocularis. Similarly, Mongolian gerbils were protected from an intraperitoneal inoculation of protoscolices of the non-metastasizing E. granulosus given seven days after treatment with 2.65×10^7 CFU of BCG (Thompson, 1976). Many of the animals in both these studies developed intraperitoneal granulomatous lesions as a consequence of the BCG treatment. Other workers (Snodgrass and Hanna, 1973) have also reported the formation of chronic granulomatous lesions both at the site of BCG inoculation and within the regional lymph nodes; this response has, however, been considered necessary for the elimination of tumor cells. Nevertheless, it would be desirable to develop a treatment schedule that does not induce the formation of tubercular granulomas in the host, since these bacterial infections might deflect some of the protecting responses of that host away from its primary target.

The purpose of this study was to determine the minimum dose of BCG which can protect cotton rats from experimental infec-

tions of E. multilocularis, without the induction of granulomatous lesions. Animals could not be protected against the parasite by treatment with 10^1 CFU but partial protection was achieved with 10^2 CFU. Complete protection against hydatid infections was achieved with doses of BCG of 10^3 CFU and greater, but protection with 10^5 and 10^7 CFU was accompanied by the formation of BCG granulomas.

Previous studies (Civil et al., 1978; Kassis and Tanner, 1977b; Rau and Tanner, 1973) suggested that the environment into which the parasite is inoculated may determine its success or its failure to establish. The assessment of the cellular response of animals two weeks after treatment with BCG showed that the significant elevation in total leucocytes after treatment with 10^3 CFU was, not unexpectedly, almost exclusively attributable to a significant increase in the numbers of peritoneal monocytes. Furthermore, this dose elicited not only the highest monocyte response, but also completely suppressed the growth of parasite without the induction of granulomas. This observation supports the hypothesis of Baron and Tanner (1977) that monocytes are the primary effector cell in this host-parasite system.

The level of cell proliferation two weeks after treatment with 10^5 and 10^7 CFU of BCG was, apparently, somewhat lower when compared with the response obtained with 10^3 CFU. This result is consistent with the delayed hypersensitivity reaction induced by BCG which develops, peaks and diminishes more rapidly with higher than lower doses (Mackaness et al., 1974; Miller et al., 1973). Thus, the cellular responses of animals treated with 10^3 CFU may have represented a maximum stimulation at 2 weeks.

A dose of 10^3 CFU of BCG was determined to be optimal for stimulating cell proliferation and is also an effective dose for suppressing the growth of the parasite. Doses of 10^1 and 10^2 CFU produced an inadequate level of stimulation of the leucocyte population. At doses of 10^5 and 10^7 CFU, however, the bacilli provoked a host response which ultimately resulted in the formation of granulomas; these granulomas were first observed 2 weeks after treatment and were considerably larger after 8 weeks.

Forty-two days following inoculation of the parasite, leucocyte levels in animals treated with 10^5 and 10^7 CFU of BCG were lower than following treatment with 10^3 CFU. Since tubercular granulomas have been shown to deplete circulating lymphocytes and monocytes (Olson et al., 1971), the lower levels of leucocytes in animals given 10^5 and 10^7 CFU may have been due to a sequesteration of cells for the formation of the granulomas. Furthermore, the reactions to the tubercle bacilli could, conceivably, compete for cells with the hydatid cyst; such reactions surrounding the bacteria would inhibit a maximum anti-parasite effect by the cellular response.

It is well documented (Olson et al., 1971) that an excessive number of BCG can have a disorganizing effect on lymphoid tissues, such as the spleen, so as to interfere with its immunological capabilities. Splenomegaly has been demonstrated in mice treated with BCG and inoculated with E. granulosus (Thompson, 1976). Although we were unable to detect any splenomegaly, there was less gain in body weight in animals treated with 10^5 and 10^7 CFU (Figure 4.3), indicating that high doses of the vaccine may be detrimental to the host.

The results of these experiments indicate that relatively low prophylactic doses of BCG are effective in controlling experimental Echinococcus multilocularis infections without the concomitant formation of macroscopic granulomatous lesions. Nevertheless, the interpretation of these results would be less complicated if the use of viable organisms could be avoided.

CHAPTER 5

CHAPTER 5

THE PROTECTION OF COTTON RATS AGAINST EXPERIMENTAL ECHINOCOCCUS MULTILOCULARIS INFECTIONS WITH BCG CELL WALLS

INTRODUCTION

The results presented in the previous chapter have demonstrated that cotton rats can be protected against a metastasizing growth of Echinococcus multilocularis by pretreatment with as few as 1000 colony-forming units of BCG. In addition, the formation of granulomatous lesions was shown not to be a prerequisite for this effect; nevertheless, it would be desirable to achieve comparable protection without the complication of the growth of viable micro-organisms.

Recently the cell walls of BCG have been shown to be at least as effective in inducing the regression of tumors as the viable organisms (Ribi et al., 1973; Zbar et al., 1973). BCG cell walls retain some properties of the intact organism, including the ability to induce granulomatous lesions (Anacker et al., 1967); however, as they are non-viable, the interpretation of the results of experiments in which they are used is not complicated by the growth of the micro-organisms. Consequently, in this chapter the possibility of a non-living mycobacterial cell wall preparation protecting cotton rats against infections of E. multilocularis is examined; protective effects are related to changes in the leucocyte population induced by the treatment and/or the parasite infection.

MATERIALS AND METHODS

The Parasite and Animals

Maintenance of stock infections of E. multilocularis in cotton rats, the method of inoculation of the animals, and their management has been previously described in Chapter 3.

Preparation of the BCG Cell Walls

Mycobacterium bovis strain BCG (Montreal substrain, Institut Armand-Frappier) was incubated on Sauton's medium for 13 days at 37.5°C. The bacilli, harvested by filtration through a Buchner funnel, were washed on the filter with distilled water and air dried. The biomass (43.7 g, wet weight) was suspended in 172 ml distilled water and the bacilli were disrupted at 35,000 p.s.i. in a Ribi cell fractionator at 4-8°C. The homogenate was centrifuged for 60 minutes at 12,500 r.p.m. (Lourdes "Beta-fuge", model A-2, Rotor 9RA) and the sediment, resuspended in distilled water, was centrifuged under the same conditions. Additional washings with distilled water were carried out until the cell walls were found free of debris by electron microscopic examination (4 additional washings). The final suspension, containing clean cell walls and some undisrupted bacilli, was lyophilized to yield 1.49 g of dry cell walls.

The preparation of the BCG cell wall (BCG-CW) for inoculation into animals was done according to the method of Zbar et al. (1972). The control treatment preparations were made in the same way except, of course, for the omission of the cell walls.

The Effect of the Treatments on the Cellular Response

In order to determine the level of stimulation of the cellular response induced only by the treatment, six cotton rats in each of

four control groups were treated as the test animals (see below), but were not inoculated with the parasite. Fourteen days later, all of these control animals were exsanguinated by cardiac puncture under ether anesthesia. At necropsy, each animal was weighed, a total and a differential leucocyte count was made of the peritoneal exudate as previously described (Reuben et al., 1978); the peritoneal cells were also stained for acid phosphatase activity (see below); the spleen was weighed, and, whenever present, the total weight of granuloma within the peritoneal cavity was obtained.

The Effect of Treatment with BCG Cell Walls on the Establishment of E. multilocularis

Cotton rats of the test groups were placed in groups of 12 individuals according to body weight, as previously described in Chapter 4; each animal in the test groups was treated with a single intraperitoneal injection of 0.1 ml containing 150 µg BCG cell walls (BCG-CW), emulsified in mineral oil in 0.1 ml of 0.2% Tween 80 in saline, or in 0.1 ml of mineral oil in 0.2% Tween 80 in saline, or in 0.1 ml 0.2% Tween 80 in saline, or in 0.1 ml saline. Two weeks later, half of the animals in each treatment group were anesthesized with Nembutal (Abbott Laboratories, Montreal) and a brood capsule was surgically implanted into the peritoneal cavity of each. All other six animals in each group were sham-inoculated controls. Forty-two days after the inoculation of the parasite (eight weeks after treatment), all the test and control animals were exsanguinated by cardiac puncture under ether anesthesia and processed as described above; the weights of the parasite cysts were obtained.

Acid Phosphatase Activity

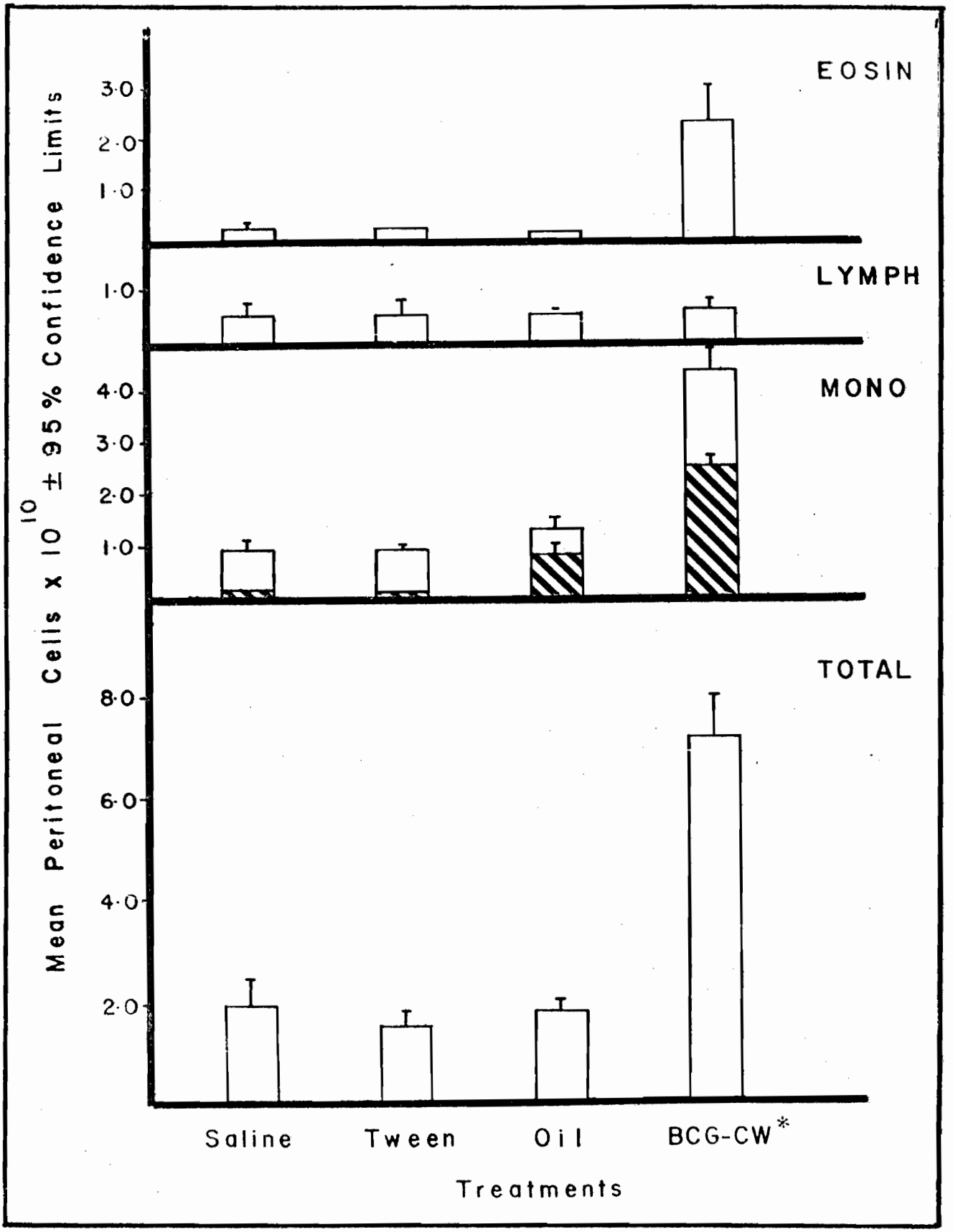
A coverslip smear obtained from the peritoneal exudate of each animal was stained for acid phosphatase activity, since it has been shown that activated macrophages possess more lysosomal enzyme than non-activated cells (Dandenburg, 1968; Dandenburg et al., 1968). The cytochemical demonstration of acid phosphatase in these cells was done using the technique described by Kaplow and Burstone (1964). Five hundred monocytes were counted on each stained smear and the total "active" monocyte population was calculated from the percentage of cells possessing acid phosphatase activity. The data were analyzed by the analysis of variance.

RESULTS

The total and differential leucocyte counts of the peritoneal exudate of animals that had been treated two weeks previously with either BCG cell walls emulsified with mineral oil in Tween-saline (BCG-CW), a mineral oil emulsion in Tween-saline, Tween-saline or saline alone are summarized in Figure 5.1.

The cotton rats which had received an intra-peritoneal injection of 150 µg BCG-CW two weeks previously exhibited total leucocyte counts that were significantly higher ($p \leq 0.05$) than those of animals in the other control groups. The increase in the number of leucocytes in the animals treated with BCG-CW was accounted by significant elevations of both monocytes and eosinophil leucocytes; there was no significant increase in the number of lymphocytes. The total number of monocytes exhibiting acid phosphatase activity was, not unexpectedly, highest in the animals treated with BCG-CW, indicating the activation of the cells in these animals. The acid phosphatase response was lower in animals treated with mineral oil and insignificant

Figure 5.1 Levels of stimulation of peritoneal leucocytes of cotton rats two weeks after treatment with BCG-CW or control preparations. Vertical bars depict the mean $\pm$ 95% confidence intervals. The cross-hatched areas represent the number of monocytes possessing acid-phosphatase activity in relation to the total monocyte population. (EOSIN = Eosinophils; LYMPH = lymphocytes; MONO = monocytes/macrophages; TOTAL = total leucocytes; BCG-CW* = BCG cell walls emulsified in mineral oil and Tween-saline).


EOSIN
LYMPH
MONO
TOTAL
Mean Peritoneal Cells x 10^{10} ± 95% Confidence Limits
3·0
2·0
1·0
1·0
4·0
3·0
2·0
1·0
8·0
6·0
4·0
2·0
Saline
Tween
Oil
BCG-CW*
Treatments

in cotton rats treated with either Tween-saline or saline.

The total and differential peritoneal cell response of the cotton rats treated with either BCG-CW, mineral oil, Tween-saline or saline, and subsequently inoculated with one brood capsule of E. multilocularis, is illustrated in Figure 5.2.

The total number of leucocytes in the sham-inoculated groups was highest in animals treated with saline, and progressively lower in animals treated with BCG-CW, Tween-saline, and mineral oil, respectively. However, patent infections of E. multilocularis significantly elevated the leucocyte populations over those in the corresponding sham-inoculated controls in animals pretreated with Tween-saline or mineral oil. The greater numbers of leucocytes were due to increases in the numbers of both monocytes and eosinophils in the peritoneal cavity of these animals. In animals pretreated with BCG-CW, inoculation of the parasite did not significantly change the total leucocyte counts between the inoculated and the sham-inoculated homologous control group. Inoculation of the parasite also did not significantly enhance the number of cells above that in homologous controls in animals that had been previously treated with saline.

The dose of 150 µg of BCG-CW completely protected cotton rats from active infection with E. multilocularis, without the induction of granulomatous lesions. No protection was afforded by any of the other treatments; indeed, there were more foci of metastatic growth in the control animals which had been pretreated with Tween-mineral oil, or Tween-saline. However, there was no significant difference between the size which the cysts attained in these animals and those in the saline-treated controls. A summary of the size of the infections is presented in Table 5.1.

Figure 5.2 Levels of stimulation of peritoneal leucocytes of cotton rats treated with BCG-CW or with control preparations and then inoculated with E. multilocularis. Open bars represent sham-inoculated controls previously treated with various treatments. Hatched bars represent animals given various treatments and subsequently inoculated with the parasite. Vertical bars depict the mean $\pm$ 95% confidence intervals. (EOSIN - Eosinophils; LYMPH = lymphocytes; MONO = monocytes/macrophages; TOTAL = total leucocytes; BCG-CW* = BCG cell walls emulsified in mineral oil and Tween-saline).

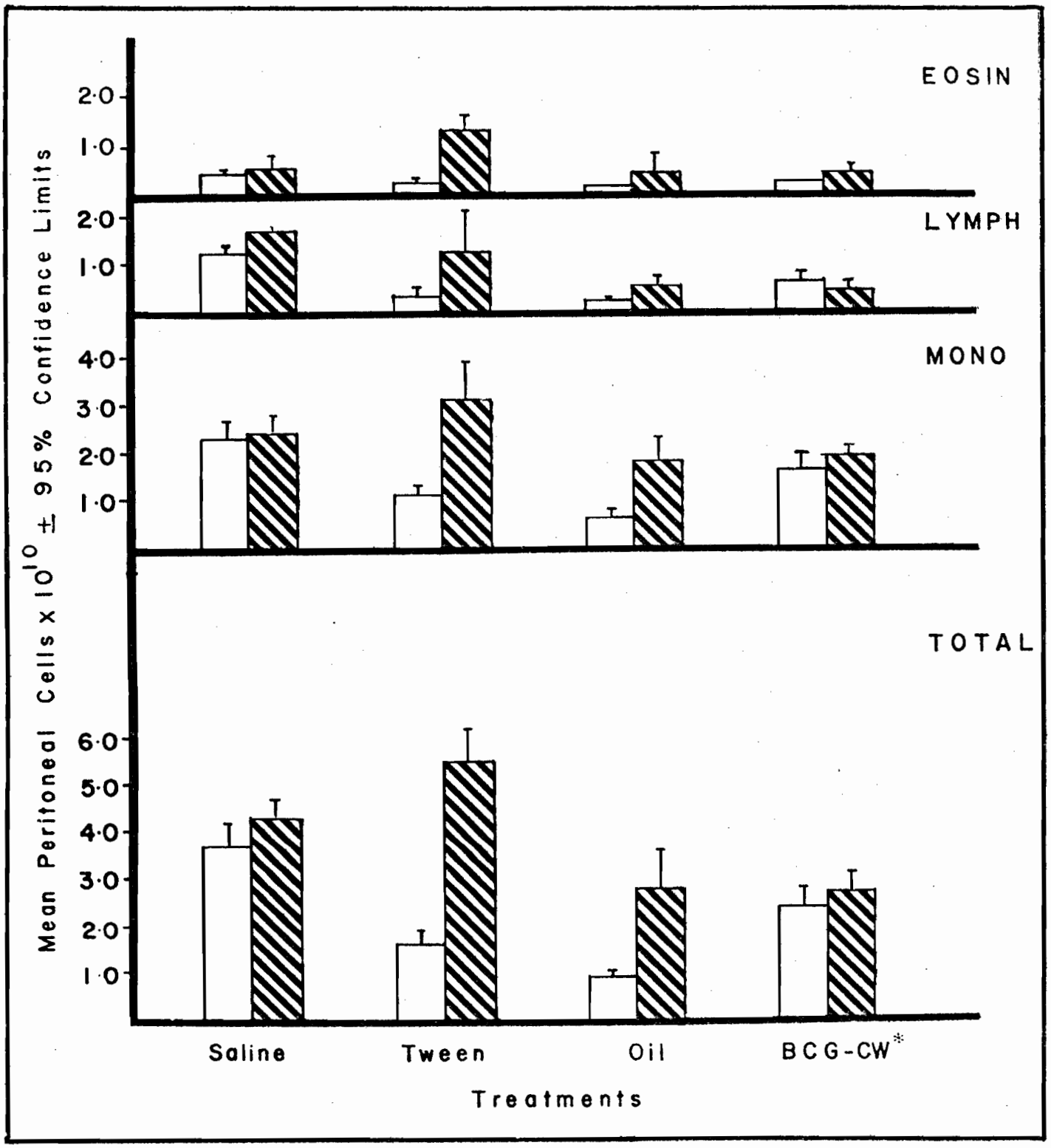

EOSIN
LYMPH
MONO
TOTAL
2·0
1·0
2·0
1·0
4·0
3·0
2·0
1·0
6·0
5·0
4·0
3·0
2·0
1·0
Mean Peritoneal Cells x 10^{10} ± 95% Confidence Limits
Saline
Tween
Oil
BCG-CW*
Treatments

TABLE 5.1 - Mean Cyst Weight and Average Number of Foci in Infected Animals After Various Treatments

	TREATMENTS			
	Saline	Tween	Oil	BCG-Cell Walls
Mean Cyst wt (g) ± 95% Confidence Limit	0.90 ± 0.20	0.50 ± 0.20	0.65 ± 0.20	no growth
Average Number Metastatic Foci	1.20	13.15	14.8	--

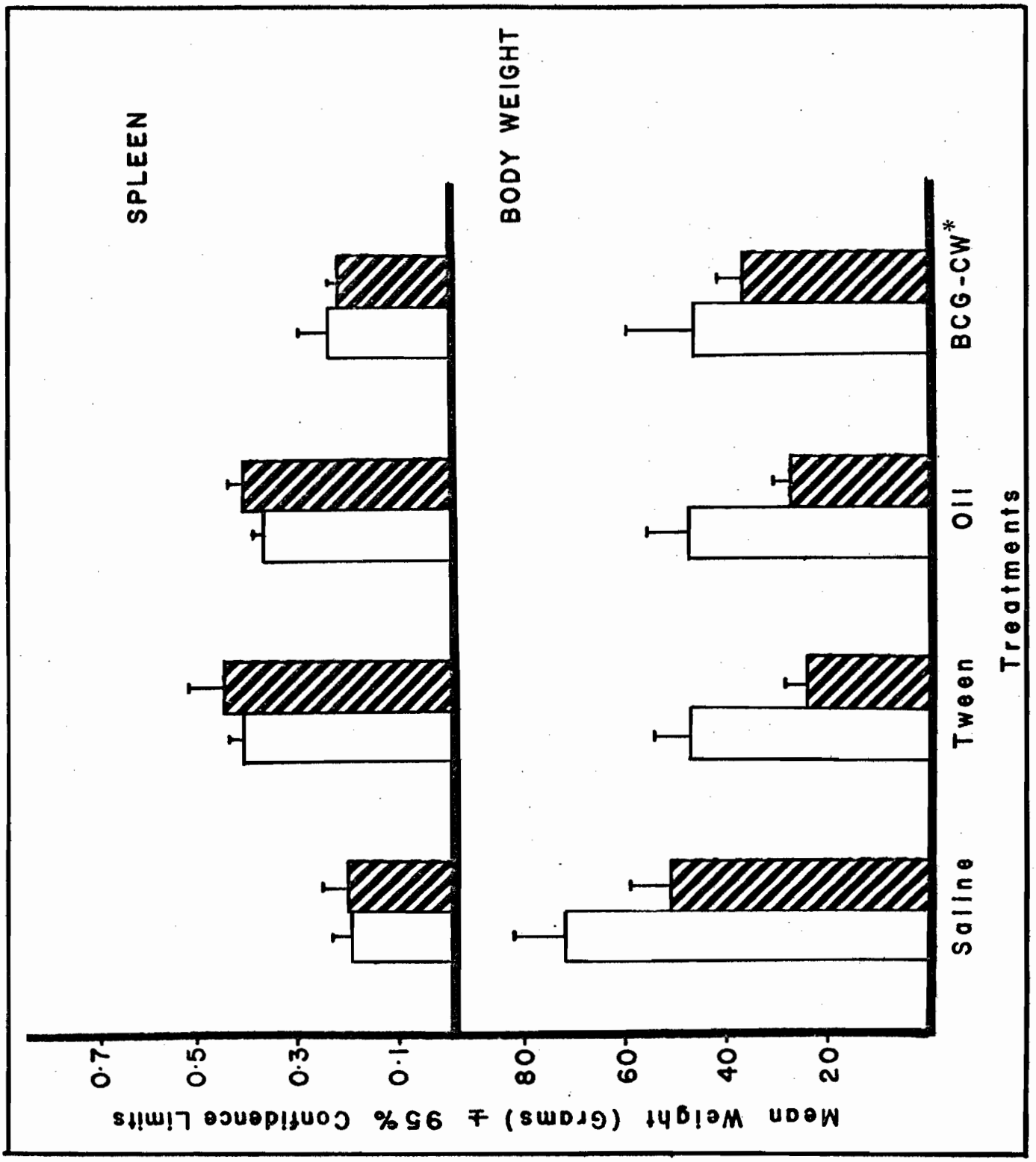
There was splenomegaly (Figure 5. 3) in the control animals treated with Tween-saline or with mineral oil emulsified in Tween-saline. There was no splenomegaly in the cotton rats treated with the cell walls or with saline. All the treatments significantly reduced the body weights of all the animals; inoculation of the parasite further significantly reduced the weight of the cotton rats, except in those that had been treated with BCG-CW.

DISCUSSION

Previous work from our laboratory has demonstrated that viable BCG will protect cotton rats from infections with E. multiloc-ularis (Rau and Tanner, 1975); subsequently it has been shown that granuloma formation is not a prerequisite for this protection (Reuben et al, 1978). In the present study, it is conclusively shown that cotton rats pretreated with non-viable BCG cell walls are completely protected from a subsequent infection with E. multilocularis.

Earlier studies have suggested that the environment into which E. multilocularis is inoculated determines its success or failure to establish (Baron and Tanner, 1976; Civil et al. ,1978; Kassis and Tanner, 1977b; Rau and Tanner, 1973; Reuben et al., 1978). The assessment of the cellular response of animals two weeks after treatment with BCG cell walls (BCG-CW) showed that the significant elevation of the total numbers of leucocytes in the peritoneal cavity was, not unexpectedly, due to significant increases in the numbers of both peritoneal monocytes and granulocytes. This observation is consistent with our previous findings (Reuben et al., 1978) and with a recent report that mice injected intraperitoneally with BCG yield high numbers of peritoneal granulocytes and macrophages (Buhles and Shifrine, 1978).

Figure 5.3 The spleen and total body weights at necropsy of cotton rats given various treatments and subsequently inoculated with E. multilocularis. Open bars represent sham-inoculated controls and the cross-hatched bars represent the inoculated animals. Vertical bars depict the mean $\pm$ 95% confidence intervals.


SPLEEN
BODY WEIGHT
Mean Weight (Grams) ± 95% Confidence Limits
0·7
0·5
0·3
0·1
80
60
40
20
Saline
Tween
Oil
BCG-CW*
Treatments

The present study indicates that there is a correlation between the increased numbers and activation of monocytes, as judged by acid phosphatase activity, with protection (Figure 5. 1). There was also an elevation in the number of peritoneal eosinophils following treatment with BCG-CW (Figure 5. 1). The role of the eosinophil leucocyte in the control of this parasite infection remains to be clarified. These cells are phagocytic, although perhaps less effective than macrophages, neutrophils and periphereal blood eosinophils (Klebanoff et al., 1977). The number of lymphocytes was not elevated in our animals treated with BCG-CW.

Although it has been suggested that mineral oil alone can activate macrophages to the same extent as that induced by mycobacteria (Brunda and Raffel, 1977; Metcalf, 1974), this did not occur in our study. This result may be due to either the kinetics of macrophage activation by mineral oil (Kelly, 1976) or to the fact that mineral oil stimulates a number of different classes of peritoneal macrophages each of which possesses a different phagocytic ability (Rice and Fishman, 1974). In addition, little lysosomal activity has been detected in oil-induced peritoneal exudate macrophages (Cohn and Wiener, 1963). It is also conceivable that 2 weeks after this treatment the cellular responses of cotton rats inoculated with oil droplets may have already returned to normal values, while the responses of the animals treated with the cell wall preparation may represent a peak of stimulation.

Tween-treated animals exhibited no significantly different level of cellular proliferation 2 weeks after treatment. Treatment of cotton rats with Tween, mineral oil-Tween and BCG-CW in a mineral oil-Tween suspension, however, appears to have long-term effect on

the cell numbers in the peritoneal cavity. This effect is evident in the fact that the total and differential leucocyte counts of the treated (but uninoculated) control animals eight weeks after the injection were significantly lower than in the cotton rats given saline. The spleens of these animals were also significantly enlarged in comparison with the saline controls. Splenomegaly is usually associated with a marked hyperactivity of the reticuloendothelial system which is reflected in an increase in the phagocytic index of splenocytes; the acid phosphatase activity of the cells from the animals in this experiment were, however, indistinguishable from saline controls. Splenomegaly can also be related to the extent and duration of the parasite burden (Ali-Khan, 1978a; Ferrante et al., 1978). It is also of interest to note that the infected animals treated with Tween-saline or mineral oil-Tween (without the cell walls) bore a greater number of metastatic foci of E. multilocularis than the untreated infected controls. Detergents depress the growth of some tumors but, at the same time, they paradoxically enhance the extent and intensity of metastatic proliferation (Carter et al., 1971; Cotmore and Carter, 1973; Fisher and Fisher, 1965). A similar mechanism may have enhanced the numbers of metastatic foci of E. multilocularis in the cotton rats treated with Tween-saline or mineral oil-Tween.

Animals pretreated with BCG-CW were completely protected from infection by the parasite. It is therefore not surprising that the number of leucocytes had returned to control levels when the experiment was terminated eight weeks after treatment. The results of these experiments indicate that non-viable cell wall preparations of BCG are effective in protecting cotton rats from infections with Echinococcus multilocularis, without the concomitant formation of macroscopic granulomatous lesions. These results also indicate that

Tween, which is used extensively as an emulsifier in the preparation of the cell-wall vaccines, may enhance metastases of the parasite infection and hence it should be used with caution.

CHAPTER 6

CHAPTER 6

ECHINOCOCCUS GRANULOSUS: NON-SPECIFIC ACTIVATION OF CYTOTOXIC LEUCOCYTES IN VITRO AND IN VIVO

INTRODUCTION

Previous studies have demonstrated that macrophages from the peritoneal cavity of laboratory animals infected with larval cestodes of Echinococcus multilocularis (Rau and Tanner, 1976b) or Taenia crassiceps (Baron and Tanner, 1977) or nonspecifically sensitized with either Bacillus Calmette-Guérin (BCG) (Rau and Tanner, 1975; Reuben et al., 1978) or BCG cell walls (Reuben et al., 1979) are capable of killing protoscolices of E. multilocularis in vivo. It was of interest to determine whether in vitro activation of peritoneal cells would also be possible for in vitro killing of the parasite. Fidler (1974)has demonstrated that syngeneic macrophages sensitized in vitro to cells of the melanoma B16 were capable of inhibiting the ability of the tumor to produce pulmonary metastases. Siegler et al.(1972) have effectively treated patients with advanced malignant melanoma with autologous lymphocytes sensitized in vitro with BCG, phytohemagglutinin, or tissue-cultured melanoma cells. Cheema and Hersh (1972) have successfully treated melanoma patients with in vitro PHA-activated autologous lymphocytes injected directly into cutaneous tumor nodules.

In order to determine whether cells could be activated in vitro against Echinococcus, a study was made of the ability of peritoneal, spleen and peripheral blood leucocytes sensitized in vitro and in vivo with the mitogens phytohemagglutinin (PHA), lipopolysaccharide (LPS), or pokeweed (PWM) and BCG to kill protoscolices of E. granulosus in vitro.

MATERIALS AND METHODS

Animals and Parasite

The animals used in this experiment, the infection and the maintenance of Echinococcus multilocularis in cotton rats have been described previously (Chapter 3).

The protoscolices of E. granulosus used in the in vitro assays of cytotoxicity were withdrawn aseptically from lung cysts of naturally-infected moose; the parasites were washed on a sterile Teflon grid (40 µm sieve opening) with an excess of sterile Eagle's Minimum Essential Medium (MEM; Gibco), without proline. The protoscolices were incubated for 30 min. at 37°C in heat-inactivated immune serum from an infected cotton rat and washed in an excess of MEM, prior to the addition of the activated effector cells.

It has been previously shown that there is no blocking antibody in hydatid infections and that the presence of specific antibody on the protoscolex targets makes them more susceptible to attack by immune macrophages and lymphocytes (Baron and Tanner, 1977). The phenomenon in which target cells, coated with very small amounts of specific antibody are killed by non-immune effector cells is known as antibody-dependent-cellular-cytotoxicity (ADCC) and has been well documented (Fuson and Lamon, 1977; Malewicz et al., 1977; Shore et al., 1977).

Media

The medium used for culturing lymphocytes with the mitogens was RPMI-1640 with bicarbonate buffer and containing 10% heat-inactivated normal cotton rat serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (200 mM); 25 mM HEPES, pH 7.3, was added to enhance the buffering capacity in the medium. Eagle's

Minimum Essential Medium without proline (MEM; Gibco) was used in assays to determine cytotoxicity by measuring the uptake by protoscolices of labelled ^{3}H-proline (see below).

Isolation of Cells

Peripheral blood lymphocytes (PBL) were isolated from the heparinized venous blood of healthy, normal cotton rats by floatation in a 'Lymphoprep' (Nyegard and Co.) gradient according to the method of Böyum (1968). After centrifugation at 400 x g for 20 min. at 20°C, the cells at the interface were collected and washed three times with RPMI-1640 medium containing glutamine. The cells were resuspended to a final concentration of 1.25×10^{6} cells/ml; on the basis of trypan-blue exclusion, the cells were more than 95% viable.

Spleen cells were obtained from minced spleens which had been pressed through a sterile, stainless steel wire mesh (60 gauge, 80 mesh) with a sterile glass pestle; the cells were suspended in 5 ml of ice-cold RPMI-1640 medium. Mononuclear cells were isolated on 'Lymphoprep' gradients as described above; the final concentration of cells was made to 1.25×10^{6}/ml; the viability of these cells was 95% by trypan-blue exclusion.

The isolation of peritoneal cells from cotton rats has been described earlier (Reuben et al., 1978). Adherent and non-adherent cell suspensions were prepared after allowing the peritoneal cells to adhere to glass. The final concentration of each cell-type was adjusted to 1.25×10^{6}/ml.

In vitro Activation of Cells

1.25×10^{6} adherent, non-adherent peritoneal cells and lymphocytes from the peripheral blood and the spleens of normal cot-

ton rats were cultured in RPMI-1640 for 72 hours at 37°C in a humidified atmosphere of 5% CO_2 in air with a 1:80 dilution of PHA-P (Difco, Detroit, Michigan), or with 10^5 colony-forming units of BCG. The doses of mitogens used gave the maximum stimulation of DNA synthesis of each cell-type in preliminary experiments using the microassay of Strong et al. (1973). At 72 hours, the cell cultures were centrifuged, washed 3 times with fresh medium and resuspended in 200 µl of medium containing 15 protoscolices that had been preincubated with heat-inactivated immune serum (see above). The cultures of activated cells and protoscolices and the appropriate controls were incubated further. The protoscolicidal activity of each cell culture was determined visually 24 and 72 hours later, as described by Rau and Tanner (1976b). The cultures were examined through an inverted microscope and the numbers of living and dead protoscolices were determined. Lack of motion, loss of hooks, tegumental 'bubbling' and opacity were some of the criteria used to judge the death of the parasite (Baron and Tanner, 1977). Percent protoscolicidal activity was expressed as by the formula:

$$\frac{\text{number of dead protoscolices}}{\text{total number of protoscolices}} \times 100$$

In vivo Activation of Cells

Fifteen cotton rats were divided into 5 equal groups: group 1 received an intraperitoneal injection of 1 ml of mineral oil, group 2 was treated with 40 µg PHA, group 3 received 10^5 colony-forming units of Bacillus Calmette-Guérin (BCG), and the animals in group 5 were injected with 10 ml of Eagle's MEM. A brood capsule of E. multilocularis was surgically implanted into the peritoneal cavity of the animals in the 4th group.

The animals which had been given mineral oil or medium were killed three days after treatment, whereas, the animals receiv-

ing BCG or the parasite were killed 14 days later. Peritoneal cells and the lymphocytes from the spleen and the blood were collected; cell cultures were prepared in MEM to contain 2.5×10^5 cells in 200 µl of medium and 100 protoscolices of E. granulosus which had been previously incubated for 30 minutes at 37°C with heat-inactivated immune serum. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air; there were six replicated for each treatment and each cell-type. Twenty-four hours later, 2 µCi of ^{3}H-proline (New England Nuclear, Boston, Mass.) were added to each culture and the incubation was continued for another 20 hours; control wells contained medium alone, protoscolices alone or effector cells alone. The cultures were harvested onto fibreglass filter discs using the Multiple Automated Sample Harvester II (MASH-II) described by Thurman et al. (1973). The filter discs were placed into 5 ml glass scintillation vials with 3 ml of scintillation fluid (Formula 950-A, New England Nuclear) and counted in a Nuclear Chicago scintillation counter. The percent survival of protoscolices were calculated as:

$$\frac{\text{uptake of isotope (protoscolices + effector cells) - uptake by cells alone}}{\text{uptake of protoscolices in medium + serum alone}} \times 100$$

If this ratio is designated x, then percent protoscolicidal activity is calculated as 100-x (Brooks et al., 1978).

Statistical Analysis

The arcsine transformation was applied to the percent protoscolicidal activity (calculated above). The representation of the data in all figures is as the mean arcsine percent $\pm$ 95% confidence intervals (Sokal and Rohlf, 1969).

RESULTS

It was found that the macrophages and the lymphocytes from the peritoneum, spleen and the peripheral blood of normal cotton rats could be nonspecifically activated to kill the protoscolices of E. granulosus by treatment in vitro with the mitogens phytohemagglutinin, pokeweed or lipopolysaccharide and with viable BCG. Figure 6.1 shows the nonspecific protoscolicidal activity of mitogen- and BCG-activated adherent and non-adherent peritoneal cells. The activated adherent cells, the majority of which were macrophages by the examination of stained smears, killed significantly more of their protoscolex targets than the non-activated control macrophages. There was no significant difference in the effect of the different treatments on the ability of the nonspecifically-activated adherent cells to kill the protoscolices. The non-adherent peritoneal cells, which consisted principally of lymphocytes, were not activated as effectively as the macrophages to kill the target protoscolices. Nevertheless, all treatments, with the exception of PHA, were able to activate the lymphocytes to kill significantly more protoscolices ($p \leq 0.05$) than the cells incubated only in medium, as controls.

Figure 6.2 represents the protoscolicidal activity of the lymphocytes of the spleen and the peripheral blood (PBL) of normal cotton rats which had been activated nonspecifically in vitro with the several mitogens and with BCG. The cytotoxicity of the activated PBL against the protoscolices was as effective as that exhibited by the activated peritoneal adherent macrophages (Figure 6.1); the cytotoxicity of these cells was significantly higher than non-activated controls ($p \leq 0.05$). The cytotoxicity of the activated PBL did not differ between the treatments. The protoscolicidal activity of the nonspecifically activated spleen cells was less marked than that of the peripheral

Figure 6. 1 The protoscolicidal activity of adherent (6. 1a) and non-adherent (6. 1b) peritoneal cells nonspecifically activated in vitro with phytohemagglutinin (2), lipopolysaccharide (3), pokeweed mitogen (4), and Bacillus Calmette-Guérin (5); untreated controls are designated 1. The vertical bars show the mean protoscolicidal activity, $\pm$ 95% confidence intervals; * represents a significant difference at $p \leq 0.05$.

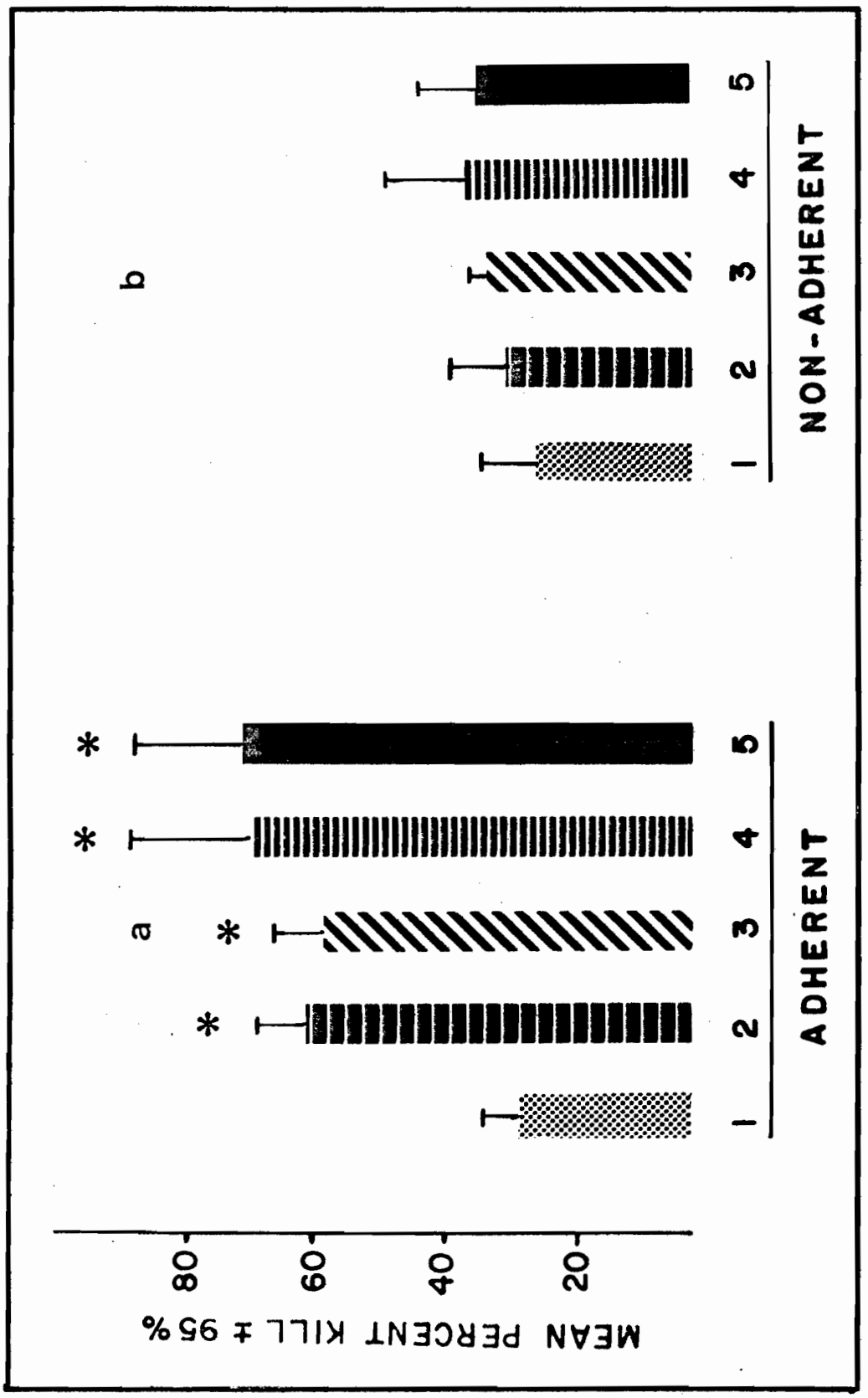
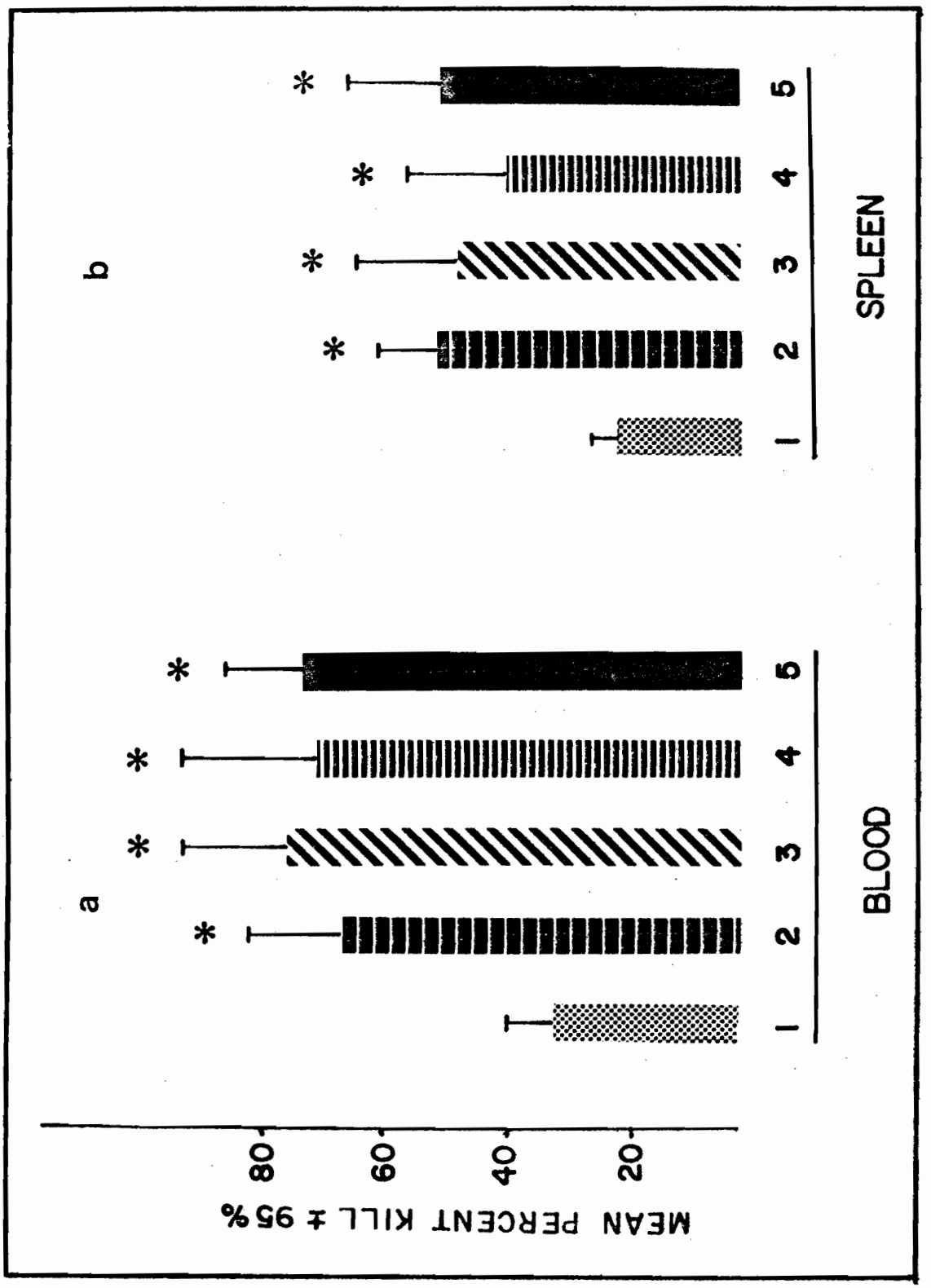

MEAN PERCENT KILL ± 95%
80
60
40
20
ADHERENT
NON-ADHERENT
1
2
3
4
5
a
b
*

Figure 6.2 The protoscolicidal activity of leucocytes from the peripheral blood (BLOOD; 6.2a) and the spleen (SPLEEN; 6.2b) nonspecifically activated in vitro with phytohemagglutinin (2), lipopolysaccharide (3), pokeweed mitogen (4), and Bacillus Calmette-Guérin (5); untreated controls are designated 1. The vertical bars show the mean protoscolicidal activity, $\pm$ 95% confidence intervals; * represents a significant difference at $p \leq 0.05$.


MEAN PERCENT KILL ± 95%
80
60
40
20
BLOOD
SPLEEN
1
2
3
4
5
a
b
*

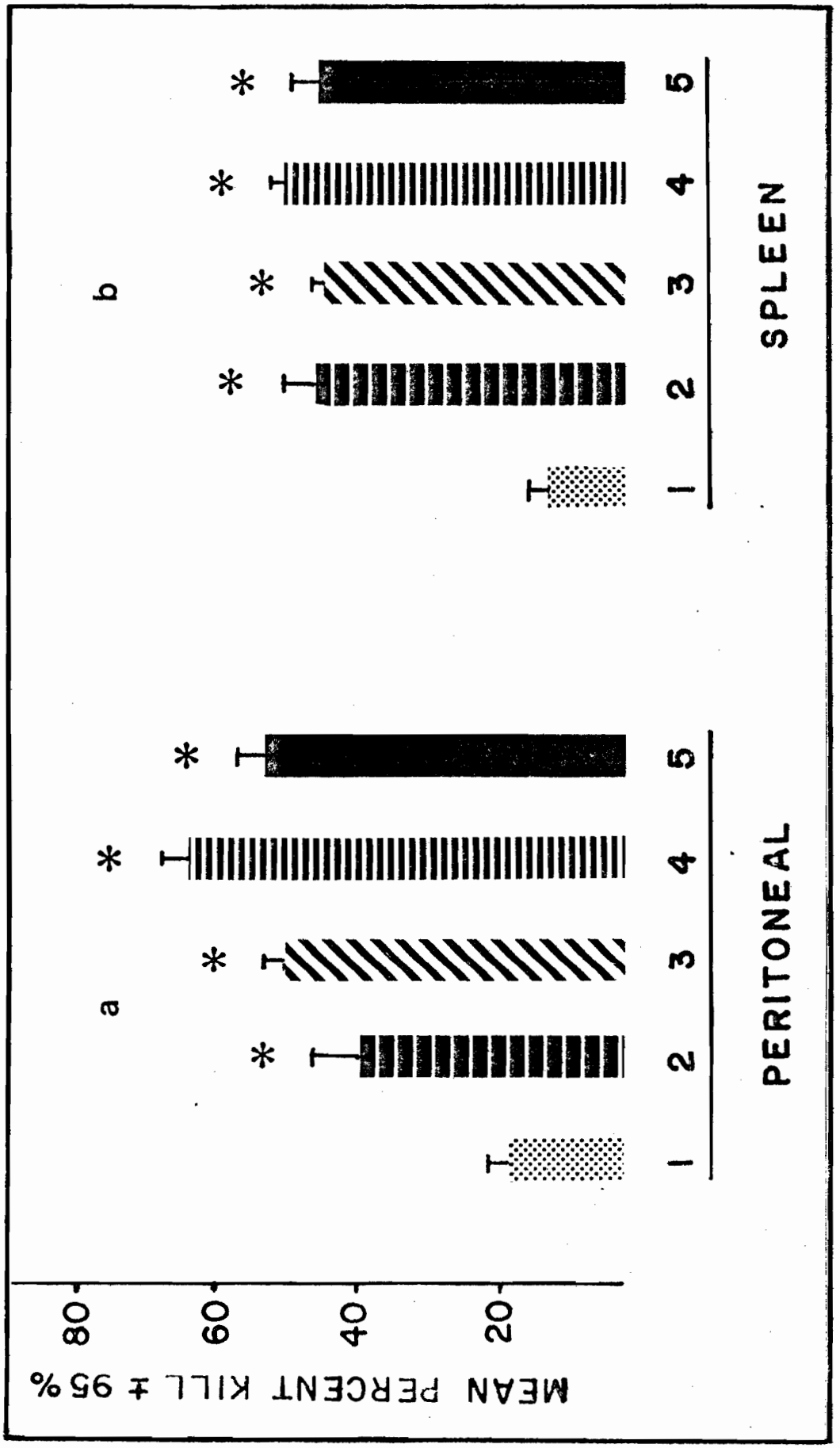
leucocytes but, nonetheless, was significantly higher than in the controls ($p \leq 0.05$). As exhibited by the other cell population, there was no difference between treatments.

The cytotoxicity against protoscolices of E. granulosus of the peritoneal and the spleen cells of cotton rats stimulated in vivo with mineral oil, 10^5 CFU of BCG, PHA and by an infection of E. multilocularis, is shown in Figure 6.3. It is clear that all the treatments activated the peritoneal and the spleen cells to kill significantly higher numbers of protoscolices of E. granulosus than were killed by the cells of the control animals. The maximum cytotoxicity by the peritoneal cells was achieved through activation with BCG; less activation was produced by infection with E. multilocularis and by the PHA and mineral oil treatments. This protoscolicidal activity was significantly higher than that of the cells from control animals, although there was no differences, again, in cytotoxicity between treatments. Similar results were obtained with spleen cells nonspecifically activated in vivo; the protoscolicidal activity of these cells was, however, lower than that of the cells from the peritoneum. Again, there was no significant difference in cytotoxicity that could be ascribed to the treatments.

DISCUSSION

The capacity of murine tumors to immunize experimental animals and, thus, to generate cells capable of inhibiting tumor growth in vivo and in vitro is well documented (Hellström et al., 1968). Mouse lymphocytes can be immunized against syngeneic plasma cell tumors by incubation in vitro with the tumor cells (Röllinghoff and Wagner, 1973; Wagner and Röllinghoff, 1973a, b). Similar studies have shown that the activation of lymphoid cells in vivo and in vitro by phytohemagglutinin (PHA) or Staphylococcus culture filtrates (Holm

Figure 6. 3 The protoscolicidal activity of the peritoneal (6. 3a) and spleen (6. 3b) cells of cotton rats treated with saline (1), mineral oil (2), phytohemagglutinin (3), Bacillus Calmette-Guérin (4) and following an infection of E. multilocularis (5). The vertical bars show the mean protoscolicidal activity, $\pm$ 95% confidence intervals; * represnets a significant difference at $p \leq 0.05$.


MEAN PERCENT KILL ± 95%
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et al. ,1964; Ling et al. , 1965), or lipopolysaccharide (LPS; Alexander and Evans, 1971) is also effective in killing tumor cells in vitro, as well as inhibiting the growth of the neoplasm in vivo. More recent studies have demonstrated that mouse lymphocytes stimulated in vitro by PHA are capable of prolonging the life of mice infected with Rauscher leukemia virus; these nonspecifically stimulated lymphocytes were much more effective killers than normal lymphocytes (Lair and Lozzio, 1974).

It has been previously shown that peritoneal cells from cotton rats infected with Echinococcus multilocularis are protoscolicidal in vitro (Rau and Tanner, 1976b); this activity can also be induced in A/J mice by infection with BCG or with the larvae of the cestode Taenia crassiceps (Baron and Tanner, 1977). In the present study it is demonstrated that it is possible to activate in vitro normal peritoneal, spleen, and peripheral blood mononuclear cells with phytohemagglutinin, lipopolysaccharide or pokeweed mitogen and with viable BCG: these activated cells are cytotoxic in vitro to the protoscolices of Echinococcus granulosus. The adherent peritoneal cells were highly cytotoxic ($p \leq 0.05$) to protoscolices (Figure 6. 1a) whereas the activity of the non-adherent population was not significantly different from the unstimulated controls (Figure 6. 1b). This result is consistent with the previous reports of Baron and Tanner (1977) and Mahmoud et al. (1979), who used peritoneal cells that had been activated in vivo with BCG.

Peritoneal cells from animals stimulated in vivo with 40µg of PHA killed 50. 04% of their target protoscolices of E. granulosus in vitro, as assayed by the uptake of labelled proline by the surviving protoscolices (Figure 6. 3a). Although macrophages are the majority cell-type in peritoneal exudates, the participation of lymphocytes in this lytic process cannot be eliminated since peritoneal exudates are

known to contain cytotoxic T cells (Brunner and Cerottini, 1971) and non-phagocytic cells (Nathan et al., 1977) whose adherence properties are similar to those of macrophages.

In previous experiments we were unable to protect cotton rats against an infection by E. multilocularis with mineral oil (Reuben et al., 1979). The results of that study were probably due to the induction of only a small amount of lysosomal enzyme in the monocytes that were produced; Hibbs (1974) has shown that the production of lysozymes is essential for the cytotoxicity of tumor cells by macrophages. The results presented in Figure 6.3 indicate, however, that the treatment of cotton rats with mineral oil activates cells in the peritoneal cavity and in the spleen which are cytotoxic to the protoscolices of E. granulosus. It has been suggested that such differences might be due to the distinct possibility that macrophage populations with different functions are stimulated by mineral oil (Rice and Fishman, 1974); it is also almost certain that the same cell is not equally cytotoxic in vivo as in vitro (Meltzer et al., 1975).

Peritoneal cells were successfully activated in vivo with BCG and with an infection of E. multilocularis to become cytotoxic in vitro to the protoscolices of E. granulosus. The mechanisms by which BCG activates peritoneal macrophages are not completely understood. BCG may activate macrophages as a distinct result of the inflammation which results from the intracellular infection of the organism and/or mediated through a specific mycobacterial antigen and sensitized lymphocytes (Evans et al., 1973). Macrophage activation during infection with E. multilocularis is very likely, at least in part, due to the inflammatory changes which are induced by the parasite (Ruco and Meltzer, 1977).

The protoscolicidal activity of lymphocytes from the peripheral blood of normal animals stimulated in vitro with PHA,

LPS, PWM and BCG is shown in Figure 6.2a. These results show that nonspecific stimulation of these lymphocytes with mitogens and BCG significantly augment the cytotoxicity of these cells against anti-serum-treated protoscolex targets of E. granulosus. These results are similar to those of Connolly et al. (1975) where it was shown that human peripheral blood lymphocytes, pretreated with PWM, Con A or PHA, augmented the cytotoxicity of anti-serum-treated target cells.

Spleen cells from normal animals stimulated in vitro with PWM, LPS, PHA and BCG (Figure 6.2b) and in vivo with oil, PHA, BCG or an infection of E. multilocularis (Figure 6.3b) also enhanced the cytotoxicity of these cells against anti-serum-treated protoscolices of E. granulosus. These results are similar to those of Forman and Möller (1973) who observed that pretreatment of mouse lymphoid cells with the B cell mitogens, lipopolysaccharide or pneumococcal polysaccharide S III, augmented cytotoxic effector cell activity. These workers reported further that macrophages are not required for the generation of LPS-induced cytotoxicity. The results which are reported here (Figure 6.1b) with non-adherent peritoneal cells show that macrophages are, however, necessary for the expression of cytotoxic activity in vitro against E. granulosus protoscolices by peritoneal cells activated by LPS: the removal of the adherent population significantly reduced the susceptibility of this cell population to activation by LPS. These results are consistent with those reported by Glasser et al., 1976).

It is important to relate these present results to those observed in other studies of cytotoxicity induced by mitogens. The data on the types of cells involved are somewhat conflicting since it has been recently shown that, depending on the source of the lymphoid cells, the stimulating agent, and the target cells, either T cells, B

cells, or macrophages can acquire cytotoxic activity (Muchmore et al., 1975) after treatment with mitogens. Although the effector cell in antibody-dependent cellular cytotoxicity is thought to be a non-T cell (Foreman and Möller, 1973; Harding et al., 1971; Kirchner and Blease, 1973), there are indications that, since activated T cells have Fc receptors (von Boxel and Rosenstreich, 1974; Yoshida and Anderson, 1972), perhaps activated T lymphocytes may act directly as effector cells against anti-serum-treated targets.

The effector capacity of these different cell populations implies that there are several mechanisms which can induce damage to target cells. Hibbs (1974) has reported a novel mechanism of macrophage killing involving the extrusion of lysosomes into target cells with subsequent heterocytolysis. This may be another mechanism by which macrophages in this model exert their cytotoxic potential; indeed, previous studies in this laboratory tend to support this hypothesis (Reuben et al., 1979). However, the mechanism of lymphocyte killing remains to be elucidated. It has been shown that lymphocytes and macrophages from normal animals can be stimulated in vitro with mitogens and BCG or in vivo with oil, PHA, BCG or an infection with E. multilocularis to kill anti-serum-treated protoscolices of E. granulosus. The mechanisms by which the killing is accomplished is unknown, but it is independent of complement.

In light of these results, it would be of interest to investigate what effect, if any, nonspecifically stimulated cells could have on the dynamics of an experimental hydatid infection with Echinococcus multilocularis.

CHAPTER 7

CHAPTER 7

THE PASSIVE TRANSFER OF NONSPECIFICALLY STIMULATED PERITONEAL CELLS IN EXPERIMENTAL ECHINOCOCCOSIS

INTRODUCTION

Depression of the host immunological system by infection with helminths has been well documented and attempts to actively stimulate both nonspecific and specific anti-parasite immune reactions in these hosts might be thwarted by intrinsic defects in the ability of the infected host to respond. In experimental echinococcosis, however, infection with the parasite does not reduce immunological competence since the peritoneal cells of the infected animal can effectively kill protoscolices *in vitro* (Baron and Tanner, 1977; Rau and Tanner, 1976b); the infection, nevertheless, continues. The passive administration of immune serum (Kassis and Tanner, 1976b) or the adoptive transfer of immune spleen cells (Araj *et al.*, 1977) can prevent the establishment of the parasite, arrest proliferation or kill the hydatid cyst.

In the previous chapter it was demonstrated that normal peritoneal, spleen and peripheral blood leucocytes can be stimulated with various lectins and BCG *in vitro* and with PHA and mineral oil *in vivo* to kill protoscolices of *Echinococcus granulosus in vitro*. Consequently, a study was done to investigate the possibility of protecting cotton rats against a challenge infection of *E. multilocularis* by the adoptive transfer of peritoneal cells that had been stimulated *in vivo* nonspecifically with PHA. Animals were treated with PHA or received an adoptive transfer of cells from PHA-treated animals or from animals that had been immunized with homogenate of a cyst of *E. multilocularis*.

MATERIALS AND METHODS

Animals and the Parasite

The animals used in this experiment and the inoculation and maintenance of E. multilocularis infections in cotton rats have been described in Chapter 3.

Treatment of Animals

Sixty, eight-weeks old (80-100 g) cotton rats were placed in 8 separate groups and labelled A to H. On day -3, the three rats in group G were each injected intraperitoneally with 40 µg phytohemagglutinin (PHA - P; Difco, Detroit, Michigan). On the same day the 3 rats in group H were each inoculated intraperitoneally with 1 ml of a homogenate of a 7.4 gram cyst of Echinococcus multilocularis which had been made by aseptically forcing the cyst through a coarse stainless steel mesh into 20 ml of medium 199.

On day 0, each of the 9 rats in group A received an intraperitoneal (i. p.) inoculation of a pool of 16.7×10^6 peritoneal cells harvested from the 3 cotton rats in group G and on the same day, each of the animals in group B received intraperitoneally a pool of 20×10^6 peritoneal cells which had been harvested from the 3 rats in group H. The 9 rats in group C and the 12 animals in group D each received an i. p. injection of 40 µg PHA, while the 9 rats in group E were treated i. p. with 1 ml saline. A brood capsule of E. multilocularis was surgically implanted intraperitoneally into each of the 9 animals in group F.

Three days following the treatments of day 0 , 3 animals of groups C, D, E and F were sacrificed; a total and a differential leucocyte count was done on the peritoneal exudate cells of each animal as previously described in chapter 3. A brood capsule of the parasite,

measuring 1 mm in diameter, was surgically implanted intraperitoneally into each of the treated animals in groups A, B, C and E. The 6 animals which had received a brood capsule of the parasite on day 0 (group F) were each injected intraperitoneally with 20×10^6 peritoneal cells pooled from the animals in group D that had been sacrificed on the same day for the differential cell counts. The cell suspensions were made in medium 199 and had been kept in an ice bath until used. The 9 remaining animals in group D each received an intraperitoneal injection of 5 mg carrageenan (Ca-carrageenan[1], Lot No. 251010, Sea Kem, Marine Colloids Inc., Springfield, N. J.) dissolved in 0.5 ml 0.9% saline on day 3; on day 6, three of these animals were killed and a total and a differential leucocyte count was performed on the peritoneal exudates. The remaining 6 animals in this group were then surgically implanted intraperitoneally with a brood capsule of the parasite. Forty-two days after the inoculation of the parasite, the remaining animals in all groups were killed and the presence or absence of the parasite was ascertained. For easier reference, the protocol of this experiment is presented in Table 7.1.

RESULTS

The differential counts of the cells in the peritoneal cavity of cotton rats 3 days after treatment with PHA, PHA and carrageenan, or adoptively transferred with cells from PHA-treated animals or from cotton rats treated with a homogenate of a multilocular cyst or from cotton rats inoculated intraperitoneally with one brood capsule of the parasite are shown in Table 7.2.

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(1) The Ca-carrageenan was a gift from Dr. W. Yaphe, Department of Microbiology and Immunology, McGill University, Montreal, Canada.

Table 7.1 Protocol for the treatment of animals in groups A-H. All treatments were administered intraperitoneally (ip); inoculation of the parasite was accomplished by the intraperitoneal surgical implantation of a brood capsule of E. multilocularis which measured 1 mm in diameter. The number of animals are in the parenthesis. WBC = differential leucocyte count. PHA = phytohemagglutinin. PEC = peritoneal exudate cells.

Group	Treatment at various days post-inoculation of parasite			
	Day-3	Day 0	Day + 3	Day + 6
A	-	16.7 x 10^6 PEC from G (9)	Parasite (9)	-
B	-	20 x 10^6 PEC from H (6)	Parasite (6)	-
C	-	40 ug PHA (ip) (9)	(a) WBC (3) (b) Parasite (6)	-
D	-	40 ug PHA (ip) (12)	(a) WBC (3) (b) Carrageenan (9)	(a) WBC (3) (b) Parasite (6)
E	-	Saline (ip) (9)	(a) WBC (3) (b) Parasite (6)	-
F	-	Parasite (9)	(a) WBC (3) (b) 20 x 10^6 PEC from D	-
G	40 ug PHA (ip) (3)	-	-	-
H	1 ml cyst homogenate (3)	-	-	-

Table 7.2 The differential leucocyte counts of the peritoneal cells (PC) in animals 3 days after various treatments and the results of the autopsy on these animals 42 days after the inoculation of the parasite.

The asterisk refers to the differential leucocyte count on day 6 of cotton rats that had been treated intraperitoneally with PHA on day 0 and with carrageenan on day 3.

G→A: animals in group A which received peritoneal cells from cotton rats in group G.

H→B: animals in group B received peritoneal cells from cotton rats in group H.

The number of animals are in parentheses.

Mean Cell Counts ($\times 10^6$) $\pm$ S. E.

Group	Total	Monocyte	Lymphocyte	Polymorphs	Results of Autopsy on Day 42
C	132.01 $\pm$ 3.12	69.00 $\pm$ 5.56	44.44 $\pm$ 2.67	18.48 $\pm$ 4.18	4 rats protected 2 rats had opaque cysts (9, 7 mm)
D	160.09 $\pm$ 10.92	81.62 $\pm$ 2.36	41.84 $\pm$ 3.98	36.29 $\pm$ 5.53	All rats with metastatic growth 2.17 $\pm$ 1.33 g
	* 60.80 $\pm$ 3.49	45.05 $\pm$ 4.26	14.31 $\pm$ 1.26	7.38 $\pm$ 1.65	
E	16.13 $\pm$ 1.79	10.40 $\pm$ 1.03	3.62 $\pm$ 0.76	2.12 $\pm$ 0.46	Cyst metastatic 2.27 $\pm$ 1.46 g (6)
F	35.12 $\pm$ 3.42	26.10 $\pm$ 5.60	5.94 $\pm$ 0.81	3.07 $\pm$ 1.45	Complete protection (6)
G→A	158.29 $\pm$ 5.20	82.22 $\pm$ 3.83	43.60 $\pm$ 8.05	32.52 $\pm$ 4.10	All rats protected 2 necrotic cysts (0.38, 0.23 grams)
H→B	92.30 $\pm$ 4.91	76.15 $\pm$ 7.14	14.32 $\pm$ 4.40	1.82 $\pm$ 1.45	All rats protected

Cotton rats that had received an intraperitoneal injection of 40 µg PHA (groups C and D) and killed 3 days later had significantly higher ($p \leq 0.05$) number of cells than the saline controls. The principal cell in these exudates was the macrophage, with less significant elevations in the lymphocyte and polymorphonuclear cell populations. When cotton rats that had been treated with PHA were subsequently treated with carrageenan in vivo (group D), this cellularity was drastically reduced and all cell-types were affected. Homogenates of cyst material of E. multilocularis injected into the peritoneal cavity of cotton rats (group H) also elicited a large cell response in the treated animals. Surgical implantation of a brood capsule (group F) into the peritoneal cavity, on the other hand, caused only a slight increase in the number of cells in the peritoneum 3 days later. These stimulations all yielded significantly higher cell responses than the saline control animals.

The adoptive transfer to normal recipients of peritoneal cells stimulated in vivo by 40 µg PHA or with an active infection of E. multilocularis was successful in protecting these cotton rats against a subsequent challenge infection with this parasite. The passive transfer of peritoneal cells that had been stimulated by PHA 3 days before the challenge infection, protected 7 out of 9 animals; each of the two remaining animals had only one small necrotic cyst which measured less than 10 mm in diameter. No apparent infection was found in rats that received either 20×10^6 PHA-stimulated peritoneal cells 3 days after the inoculation of the parasite or 20×10^6 immune peritoneal cells from animals treated with the homogenate of the cyst 3 days before challenge with the parasite.

The stimulation of the immune system of the host with 40 µg PHA 3 days prior to the inoculation of the parasite also protected these

animals against the establishment of the parasite: 4 out of the 6 animals in this group (C) were completely protected; each of the other two rats had an opaque cyst that was unattached in the peritoneal cavity. These cysts measured 9 and 7 mm in diameter respectively, and contained no protoscolices or brood capsules within them. When the stimulation of cells by PHA was abrogated by treatment with carrageenan (group D) protection was also abolished. All the carrageenan-treated animals had infections that were metastatic; the mean weight of the cyst mass was indistinguishable from that in the saline-treated animals (group E).

DISCUSSION

The establishment of a cyst in primary and secondary echinococcosis depends on the speed with which a protective laminative membrane is layed down around the protoscolex or brood capsule to safeguard the parasite against the mounting host immunological response (Smyth, 1969a). The depletion of complement (Kassis and Tanner, 1977b) and the stimulation of the host's reticuloendothelial system with BCG (Rau and Tanner, 1975; Reuben et al., 1978, 1979) prior to challenge with the parasite are known to influence the establishment and growth of Echinococcus multilocularis in cotton rats. In the former situation (depletion of complement) the infection is enhanced, whereas BCG treatment suppresses the parasite.

In the present study, cotton rats that had been treated prophylactically with an intraperitoneal injection of PHA had significantly elevated cellular responses and were protected against infection with the parasite. When PHA stimulated animals were treated with carrageenan to deplete them of their macrophages (Allison et al., 1966; Bice et al., 1971; Catanzaro et al., 1971), the cotton rats showed a drastic

reduction in the cellular response. The infection in these animals was highly metastatic and indistinguishable from those in the saline controls (group E). This result is consistent with previous studies (Baron and Tanner, 1976; Reuben et al., 1978, 1979) where it was shown that the level of cell proliferation at the site and at the time of the challenge is crucial to the establishment of an infection.

There are many examples in the literature where it is shown that the passive transfer of immune peritoneal, spleen or mesenteric lymph node cells protect recipient animals against challenge infections with helminths (Khoury et al., 1977; Larsh and Weatherly, 1975; Wakelin and Wilson, 1977a). In an attempt to identify the cell responsible for this activity, Jenkins (1977) has shown that the B cell alone is responsible for the passive transfer of immunity in Trichuris muris infections, while immunologic memory to reinfection appears to reside in the T cell.

Efforts to vaccinate dogs against the cestode, Echinococcus granulosus, have concentrated on the adult form of this parasite since eggs voided by these animals complete the life cycle when these eggs are ingested by sheep, cattle and, accidentally, by man (see review Clegg and Smith, 1978). Gemmell (1962) demonstrated that the vaccination of dogs with crude antigens prepared from lyophilized protoscolices or adult worms resulted in a reduction of the number of adult worms developing from a challenge infection with protoscolices. The worms which established in vaccinated dogs had fewer and less developed terminal and sub-terminal segments than those from control dogs; egg production was, hence, effected. The evidence so far, however, indicates that vaccination of dogs against the adult form of E. granulosus does not result in high levels of protection. Attempts to protect mice

(De Rycke and Pennoit-De Cooman, 1973) and sheep (Moya and Blood, 1969) against secondary echinococcosis with hydatid fluid have been unsuccessful and only partial protection has been achieved in mice treated with extracts of protoscolices (De Rycke and Pennoit-De Cooman, 1973).

In a more recent study complete protection has been reported in mice which had received immune spleen cells at the same time as an inoculum of 2000 protoscolices (Araj et al., 1977). In the present study, immune peritoneal cells from 14 day-old infections of E. multilocularis are shown to passively protect cotton rats (group B) against a subsequent infection of E. multilocularis. The principal cell-type transferred in this case was the macrophage.

This author is not aware of any study in parasitology where cells, nonspecifically stimulated by a lectin, have been able to protect normal recipients against a subsequent challenge parasitic infection, whereas there are numerous examples of the effectiveness of these cells in tumor immunotherapy (Chema and Hersh, 1972; Lair and Lozzio, 1974). The larval stages of E. multilocularis resembles a metastasizing tumor because the individual cysts bud and metastasize to new sites in the cotton rats. On the basis of this resemblance the approach to understanding the control of this parasitic infection has paralleled those taken in neoplasms.

The mechanism(s) by which PHA-stimulated lymphocytes and macrophages can protect normal recipients from a challenge infection with E. multilocularis is not known. However, it is reasonable to assume that nonspecifically stimulated cells are capable of releasing soluble factors which may induce inflammatory responses (Crowle, 1975). Similarly, the tissue damage which is characteristic of infec-

tion with E. multilocularis (Schwabe et al., 1959) also elicits a non-specific inflammatory response from the host which, in turn, should not provide a very condusive environment for the parasite to survive. Therefore, it matters not whether the immunologic event involves an immediate or a delayed-type hypersensitivity reaction, as has been proposed by Larsh et al. (1964), since the sequence due to tissue injury could be the same. After all, similar mechanisms have been proposed to explain immunity to certain other infectious agents; for example, the destruction of a diverse group of intracellular agents by activated macrophages (Hibbs et al., 1978).

In conclusion, cotton rats can be protected against an infection with E. multilocularis if they are treated with either immune or PHA-stimulated peritoneal cells before the inoculation of the parasite or with PHA-stimulated peritoneal cells after the inoculation of the parasite. In addition, animals can be protected prophylactically with a single injection of PHA and this protection can be abrogated by reducing the leucocyte cell proliferation by the treatment of these animals with carrageenan prior to challenge with the parasite. The mechanism of control of experimental infections with Echinococcus multilocularis is, therefore, largely a nonspecific immunological event.

CHAPTER 8

CHAPTER 8

THE STIMULATION OF CELLS AT VARIOUS TIMES DURING THE COURSE OF AN INFECTION WITH ECHINOCOCCUS MULTILOCULARIS

INTRODUCTION

Despite much interest in the subject, the chronicity of hydatid infections in apparently immunologically-competent hosts has not been adequately explained. The survival of the cyst in a sensitized host and the mechanisms by which it nullifies the defenses of the host are the central questions in understanding this host-parasite relationship. Schwabe et al. (1959) were the first to show a direct involvement of cell-mediated immunity (CMI) in hydatid infections. Miggiano et al. (1966) and Yusuf et al. (1975) have also suggested a role for CMI in hydatid patients. Baron and Tanner (1976) demonstrated CMI in experimental hydatid infections by showing that thymectomy and the transfer of anti-lymphocyte serum favours the survival and the metastasis of cysts in mice infected with Echinococcus multilocularis. In the present study, the response of cells isolated from infected cotton rats and treated with phytohemagglutinin (PHA) in vitro is analyzed. The mitogen reactivity of splenocytes, peritoneal exudate cells and the cells from the cysts of E. multilocularis is examined at various intervals post-infection.

MATERIALS AND METHODS

Animals and Infection

The animals used in this experiment and the inoculation and maintenance of E. multilocularis infections in cotton rats have been described in Chapter 3.

Harvesting of Cells from the Cyst Mass

The cyst was minced and pressed through a sterile, stainless steel wire mesh (60 gauge, 80 mesh) with a sterile glass pestle; the cell suspension was then suspended in 5 ml of ice-cold RPMI - 1640 medium. An equal volume of Plasmagel (supplied as a sterile solution by Laboratoire Roger Bellon, Neuilly, Hauts-de-Seine) was then added to this cell suspension and, after mixing, the blood and debris were allowed to settle for 30-40 min. at 20°C. The supernatant, containing the leucocytes, was then removed and centrifuged twice at 400 g for 5 min. The supernatant was discarded and the cells were resuspended to a final concentration of 1.25×10^6 cells/ml.

Lymphocyte Transformation

Spleen and peritoneal cells and the cells from the cyst mass were cultured according to the technique described in Chapter 6. Briefly, single cell suspensions from spleens, peritoneal exudates and hydatid cysts were made in medium RPMI 1640 supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 200 m M glutamine, 25 m M HEPES, pH 7.3, and 10% inactivated, pooled normal cotton rat serum. The washed cell suspensions contained at least 95% viable cells, as determined by the exclusion of trypan blue. 2.5×10^5 cells were cultured in 200 µl of culture medium together with 20 µl of 1:80 dilution of PHA-P (Difco, Detroit, Michigan) in the wells of a Microtest II tissue culture plate (No. 3040, Falcon Plastics, Oxnard, Calif.) at 37°C in a humidified atmosphere of 5% CO_2 in air.

The cultures were incubated for 48 hours, after which 2 µCi of ^3H thymidine (sp. act. 6.7 Ci/m mole; New England Nuclear, Boston, Mass.) were added and the incubation was continued for another 16-24

hours. Cultures were harvested by a Multiple Automated Sample Harvester II (MASH II, Microbiological Associates Inc., Los Angeles, Calif.) on glass fiber filters. They were placed into 5 ml glass scientillation vials containing 3 ml of scintillation fluid (Formula 950-A; New England Nuclear, Boston, Mass.) and counted in a Nuclear Chicago scintillation counter. The results are expressed as the mean stimulation index (SI) by calculating the ratio of the mean counts per minute of stimulated cultures to the mean counts per minute of unstimulated cultures $\pm$ 95% confidence intervals. Student's t test was applied to determine significance.

RESULTS

Differential leucocyte counts of the peritoneal exudate of cotton rats (Table 8. 1) 7 and 14 days after inoculation of the parasite show that 80% of the cells of the exudate are mononuclear, consisting of approximately equal populations of macrophages and lymphocytes; the remaining 20% of cells were polymorphonuclear cells. As the infection progresses, the composition of the peritoneal exudates shows a decrease in the number of lymphocytes, with a concomitant increase in the population of macrophages. Along with these changes, there is also an increase in the total number of peritoneal exudate cells, up to the 21st day of the infection; at this time a steady decline occurs in the number of cells,corresponding with the appearance of the parasite cysts as small "milky spots" scattered throughout the peritoneum. As these small cysts grow and metastasize, the total peritoneal cell count drops.

Impression smears show these cysts to consist primarily of macrophages, a few lymphocytes and, to a lesser degree, polymorphonuclear cells.

Table 8.1 The mean differential leucocyte counts, $\pm$ 95% confidence intervals, of the peritoneal exudates of cotton rats various days after inoculation with Echinococcus multilocularis.

Days Post-Infection	Mean Counts ($\times 10^6$) $\pm$ 95% Confidence Intervals		
	Monocytes	Lymphocytes	Polymorphs
7	416.00 $\pm$ 219.17	451.67 $\pm$ 97.15	149.33 $\pm$ 221.11
14	581.67 $\pm$ 83.23	351.67 $\pm$ 217.05	63.00 $\pm$ 147.65
21	624.33 $\pm$ 89.24	344.33 $\pm$ 120.70	57.67 $\pm$ 65.27
28	679.67 $\pm$ 144.21	206.67 $\pm$ 174.00	108.67 $\pm$ 79.36
42	724.00 $\pm$ 279.10	204.67 $\pm$ 219.19	63.00 $\pm$ 71.00

The cultures of splenocytes from infected cotton rats with PHA showed significant ($P \leq 0.05$) blastogenesis at days 14 and 21 of the infection, after which it declined to normal values (Table 8.2). In contrast, a significant increase in the stimulation index (SI) of peritoneal cells was obtained on days 7, 14, 21 and 28. The stimulation peaks on day 14, declines on day 21 and returns to normal values by day 42. The decline in the SI at day 21 coincides with the appearance of small cyst masses scattered throughout the peritoneum.

The cells isolated from these cyst masses were unresponsive to PHA stimulation. The SI of these cells was always less than unity and progressively decreased as the age and size of the cyst increased.

DISCUSSION

Early lymphocyte transformation responses to parasite antigens have been measured in various host-parasite systems such as rats infected with Nippostrongylus brasiliensis (Bloch et al., 1977), in guinea-pigs with Trichostrongylus colubriformis (Dobson and Soulsby, 1974), in mice and rats infected with Trichinella spiralis (Hall et al., 1979; Ottesen et al., 1975), in Dipetalonema viteae-infected hamsters (Weiss, 1978), and in mice and patients (Araj et al., 1977; Miggiano et al., 1966; Yusuf et al., 1975) infected with Echinococcus granulosus. The present study was designed to investigate a correlation between changes in leucocyte cell populations during a primary infection with E. multilocularis and the response to stimulation with PHA of spleen, peritoneal cells and of host leucocytes that migrate into the cysts of this parasite.

The significant rise and fall of the blastogenic responses of

8.2 The mean stimulation indices, $\pm$ 95% confidence intervals, of spleen and peritoneal cells and of cells from the cyst of E. multilocularis exposed to PHA at various intervals after inoculation with E. multilocularis.

Days Post-Inoculation	Mean Stimulation Index ± 95% Confidence Intervals		
	Spleen	Peritoneal Cells	Cyst
0	1.00 ± 0.21	1.00 ± 0.12	-
7	1.08 ± 0.25	2.24 ± 0.60	-
14	2.86 ± 0.66	7.67 ± 2.26	-
21	1.95 ± 0.65	4.39 ± 0.63	0.57 ± 0.08
28	1.43 ± 0.21	3.50 ± 0.54	0.37 ± 0.15
42	0.56 ± 0.05	1.23 ± 0.31	0.26 ± 0.08

spleen cells during a primary infection coincided with the pre-cystic stage and the period of proliferation of the cyst mass (Ali-Khan, 1974, 1978a, b; Baron et al., 1974a). The pre-cystic stage of E. multilocularis is characterized by a slow initial growth of the cyst mass and an intact and functional host cell-mediated immunity (Ali-Khan, 1978b; Baron and Tanner, 1976). The depression in responsiveness of these cells to PHA suggests a suppression or a relative depletion of T cells, or accessory cells, which are necessary for an optimum response to this T-cell mitogen. This depression also corresponds with a disorganization of lymphoid tissues and the depletion of T-cell dependent areas in the spleen which is a consequence of the accelerated proliferation of the cyst (Ali-Khan, 1974, 1978a, b), resulting in a depressed CMI response to homologous antigen (Ali-Khan 1974, 1978a, b). An unresponsiveness to PHA of spleen cells isolated from jirds infected with Brugia pahangi (Portaro et al., 1976) and from mice infected with Trichinella spiralis (Hall et al., 1979) has also been reported. In the former case, depression coincided with the appearance of adult filarial worms in the spleen while in the latter case, depression occurred during migration of the newborn larvae.

The marked inflammatory reaction observed in the peritoneum during the pre-cystic phase of echinococcosis (Rau and Tanner, unpublished data; Schwabe et al., 1959) may be responsible for a more extensive immune response than hitherto appreciated. The cellular response in the peritoneal cavity at days 7 and 14 post-inoculation of the parasite consisted of numerous macrophages and lymphocytes (Table 8.1). As the infection progressed, proliferation was primarily due to monocytes and, with a change in the ratio of macrophages to lymphocytes, there was a concomitant decrease in the PHA stimulation index at days 21 and 28. It is also of interest to note that at day 21 the multi-

locular cyst was only conspicuous as "milky spots" adhering to the omentum. A differential count of impression smears of these cysts revealed its composition as similar to that of their immediate microenvironment, the peritoneal cavity: The majority of cells being macrophages; there were a few lymphocytes and, to a lesser degree, some polymorphonuclear cells. This observation suggests that peritoneal cells migrate into the cyst wall, which acts as a "sink" for these cells.

In previous unpublished work, Tanner, Rau and Lim have found that the spleen cells of cotton rats inoculated with 100 protoscolices of E. multilocularis showed a significantly elevated blastogenic response to lipopolysaccharide (LPS)and concanavalin A (Con A) on the 14th day of the infection; this response was normal on the 30th day and declined to values significantly below normal on the 60th day of the infection. Sixty days after a heavy inoculation with a cyst mash, the spleen cells of the infected animals were also significantly less responsive to the mitogens Con A and LPS than uninfected animals. In contrast to the results presented here, the peritoneal cells of animals inoculated with 100 protoscolices were not significantly different from normal cells in their responsiveness to Con A or LPS; after the large inoculation of a cyst mass, however, peritoneal cells were significantly less susceptible to LPS stimulation.

The decline in the PHA stimulation index of the peritoneal cells at day 21, and thereafter, in the results obtained here and those of Tanner, Rau and Lim for spleen cells correspond to the logarithmic growth phase of the cyst in cotton rats (Baron et al., 1974a); the decline in the index is certainly influenced by the probable intensive antigenic stimulation to which the infected animal is subjected (Ali-Khan, 1978b). Chronic infections such as echinococcosis have been shown to cause macrophages to release mediators capable of depleting and causing the

malfunction of T cells (Bryceson et al., 1972; Nath et al., 1974).

Peritoneal monocytes penetrate the growing cyst clusters at a logarithmic rate and, presumably, form an important part of the structure of the cyst. The role of these cells in the immunobiology of the cyst and in the control of this infection is of considerable interest, especially since it is known that peritoneal leucocytes from infected animals can kill protoscolices of E. multilocularis in vitro (Baron and Tanner, 1977; Rau and Tanner, 1976b). The stimulation index of the cells isolated from the cysts, however, was extremely poor when compared with both normal cells and, more strikingly, with the peritoneal cells of the corresponding infected animal. A similar observation has been made of the cytotoxic capacity of macrophages recovered from a progressing Moloney sarcoma; the inhibition in the cytotoxic activity of intra-tumor macrophages was attributed to a lack of activation of these cells by neighbouring lymphocytes (Russell and McIntosh, 1977). It is, therefore, reasonable to assume that a progressing Echinococcus cyst mass may produce factors which may interfere with the ability of macrophages to be stimulated, or factors which suppress normally activator T cells.

The significance of a depressed ability of leucocytes to be stimulated by PHA is controversial; however, the same phenomenon has been observed in diseases where defects in cellular immunity have been documented (Bryceson, 1974). Thus, depressed responses to PHA in the spleen, the peritoneal cells and in the leucocytes from the cyst itself at the logarithmic growth phase of the parasite suggest that these cells are less competent in phenomena which require lymphocyte proliferation for the generation of an immune response (Kirchner et al., 1975).

There is no evidence at the moment for any specific cause for the unresponsiveness of leucocytes in experimental hydatid infections. A depletion of antigen-sensitive T-cell clones in the spleen, the stimulation of suppressor T cells, and the influence of the increased numbers of (perhaps suppressor) macrophages and their products may all have a role. At any rate, whatever the nature of the suppression in cotton rats infected with E. multilocularis, its existence is of interest since it opens up an avenue for revealing how larval cestodes survive in their hosts.

CHAPTER 9

CHAPTER 9

THE IMMUNOTHERAPY OF HYDATID CYSTS WITH NONSPECIFICALLY ACTIVATED CELLS

INTRODUCTION

The cysts of E. granulosus are often 'benign' and generally operable, whereas those of E. multilocularis are, because of their rapid dissemination by metastases, inoperable and fatal. Although present knowledge is incomplete, the previous chapters (Chapters 4-8) have described certain basic aspects of the host cellular immune response to these metacestodes.

Rau and Tanner (1976b) have demonstrated that peritoneal cells from cotton rats infected with E. multilocularis are protoscolicidal in vitro; this activity can also be induced in A/J mice by infection with BCG or by the larvae of the cestode Taenia crassiceps (Baron and Tanner, 1977). Furthermore, Kassis and Tanner (1977a) have shown the presence of IgG and IgM within the cyst membranes and on the surface of the protoscolices. In the present study, it has been shown that peritoneal, spleen, and peripheral blood leucocytes can be nonspecifically activated with mitogens as well as with BCG and mineral oil to kill antibody-coated protoscolices of E. granulosus in vitro (Chapter 6). Based on this information, it was hypothesized that, although stimulated cells could kill protoscolices in vitro, they are unable to reach their targets within the cysts because of the barrier of the laminated membrane. If activated cells were allowed access to these protoscolex targets, they should kill the parasite in vivo. The result presented in this chapter indicates that the parasite is, indeed, protected from its activated immunological habitat and suggests that immunological treatment of

hydatid disease with activated cells may be effective and biologically sound, especially when the cyst occurs in difficult locations.

MATERIALS AND METHODS

Animals and the Parasite

Cotton rats were inoculated in the peritoneal cavity through a surgical incision with a minced cyst of E. granulosus from an infection in A/J mice. The mice had been inoculated eight months previously with protoscolices obtained from a fertile cyst obtained from an infected moose (Alces americanus). Twenty-four months later, exploratory laparotomy was performed on the inoculated cotton rats and those animals which possessed two unilocular peritoneal hydatid cysts were selected for further study.

Treatment of Cotton Rats with BCG

Three male cotton rats weighing 100-120 grams were inoculated intraperitoneally aseptically with 10^5 CFU of BCG in 1 ml of saline. Fourteen days later, these animals were sacrificed and the peritoneal cells harvested aseptically in RPMI-1640 (Gibco) medium, pooled and washed once in the same ice-cold medium. A differential leucocyte count of this pool of peritoneal exudates revealed that the majority cell-type was the macrophage (80%), while lymphocytes and granulocytes, in approximately equal proportions, made up the remaining population.

Treatment of the Hydatid Cysts of E. granulosus

The cyst fluid was aspirated aseptically from the two cysts and examined for the presence of viable protoscolices and brood capsules. The fluid in one cyst was replaced aseptically with the same volume of medium RPMI-1640 containing 30×10^6 BCG-activated peritoneal

cells; the other cyst received an identical treatment, without the activated cells. The experiment was terminated 90 days later and the viability of the cysts was determined both macroscopically and histologically, as well as by the presence of viable protoscolices in the cysts.

The Staining and the Culturing of Tissues for the Presence of Acid Fast Bacilli

In order to determine whether the injection of peritoneal cells into the cyst from donors activated with BCG had transferred any mycobacteria, peritoneal cells and aseptic homogenates of the spleen, liver, kidneys, lungs and the cyst tissue were cultured for 6 weeks at 37°C on Lowenstein-Jensen slopes in the Laboratory of Clinical Bacteriology, Department of Microbiology and Immunology, McGill University, Montreal.

Histology

The preparation and the staining of histological sections of E. granulosus is described in Chapter 3.

RESULTS

The cultures of peritoneal exudates from BCG-treated cotton rats and the spleens, livers, lungs, kidneys, peritoneal cells and the hydatid cyst mass of animals treated with the activated cells were all negative. Direct smears for each of the above, stained for acid-fast bacilli, were also negative. The hydatid cysts that had had fluid replaced with medium RPMI-1640 alone showed no pathological effects of the treatment and continued to grow, sending outward extensions of growth from the parent cyst.

As a result of replacing the fluid of the hydatid cysts with

BCG-activated peritoneal cells, gross signs of necrosis and arrested growth of the cyst were readily apparent (Figure 9. 1). The protoscolices which characterized the cyst at the time of the treatment with the activated peritoneal cells were no longer apparent. Instead, the lumen was filled with a proteinaceous coagulum with foci of microcalfication. The inner germinal membrane was absent and the area was infiltrated by polymorphonuclear cells. Proximal to this particular infiltration of cells, there was a mixture of polymorphonuclear cells, with macrophages and the occasional lymphocyte. The outer lining of the cyst wall was completely collagenized, with a majority of fibroblastic cells and, to a lesser extent, lymphocytes, polymorphonuclear cells and macrophages - a characteristic reaction of a 'walling-off' phenomenon (Figure 9.2). In contrast, the cyst whose fluid was replaced with the tissue culture medium only showed the typical aspect of a normal cyst: an acellular laminated membrane which contained no nuclei and which showed no sign of a host response (Figure 9. 3).

DISCUSSION

The fact that complement-rich immune serum is able to kill protoscolices *in vitro*, while not exerting an apparent controlling function in the sensitized host in spite of the fact that immunoglobulins diffuse passively into the cyst, suggested to Kassis and Tanner (1976b) that there are anti-complementary substances within the cyst. They proposed that, upon entering the cyst, complement was inactivated by a chelating action of calcareous corpuscles which bind the necessary cofactors for the activation of complement; this mechanism, it was proposed, insured the survival of the established hydatid cyst by protecting the parasite from a complement-dependent immune cytolysis.

In a previous chapter (Chapter 6) it was shown that nonspec-

Figure 9. 1 Effect of the treatment with BCG-activated cells on hydatid cysts: (a) cyst treated with medium 199, (b) cyst treated with cells.

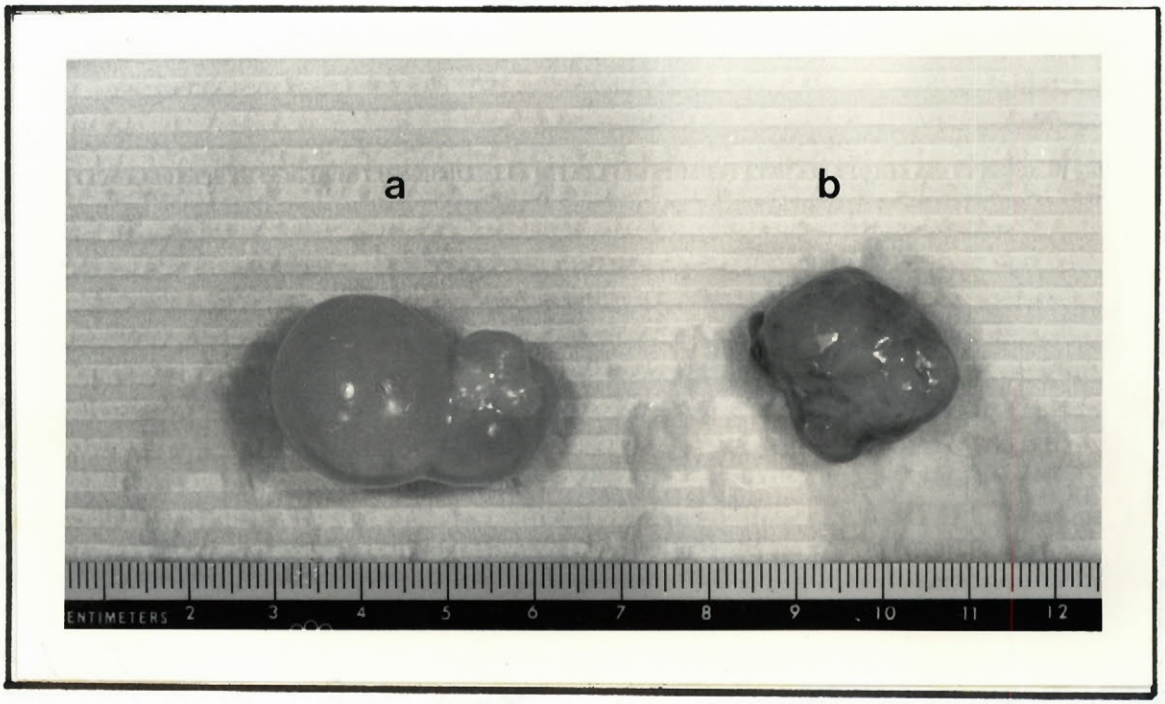

a
b
ENTIMETERS 2 3 4 5 6 7 8 9 10 11 12

Figure 9.2 Histological section of a treated hydatid cyst (a) lumen of the cyst; (b) laminated membrane; (c) infiltration of cells in the pericyst; (d) collagenized tissue (x 160).

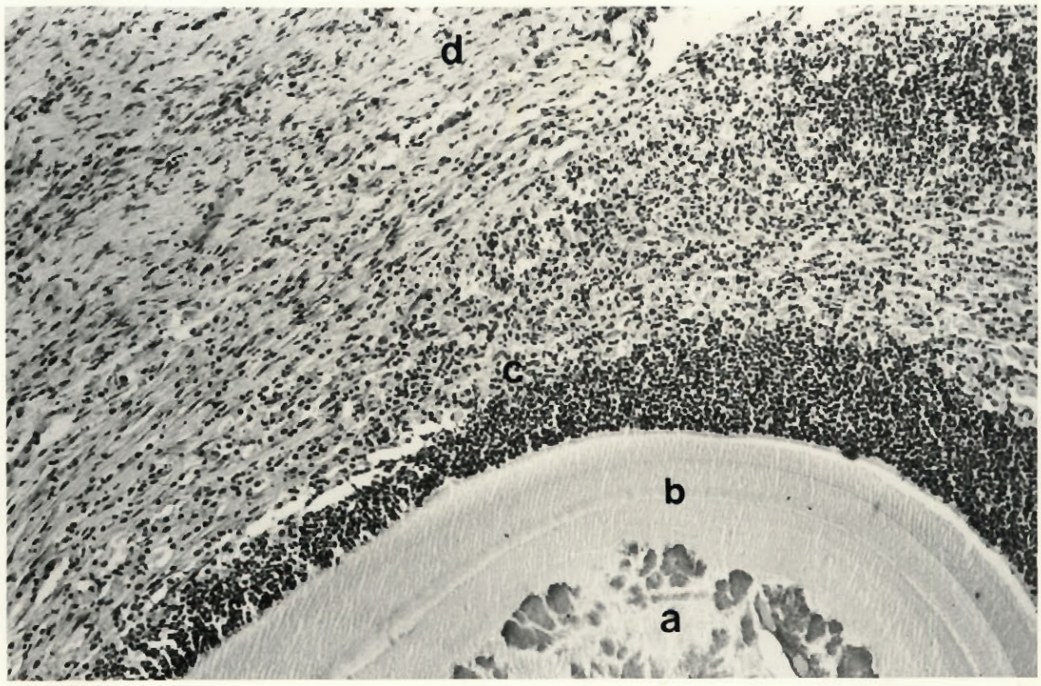
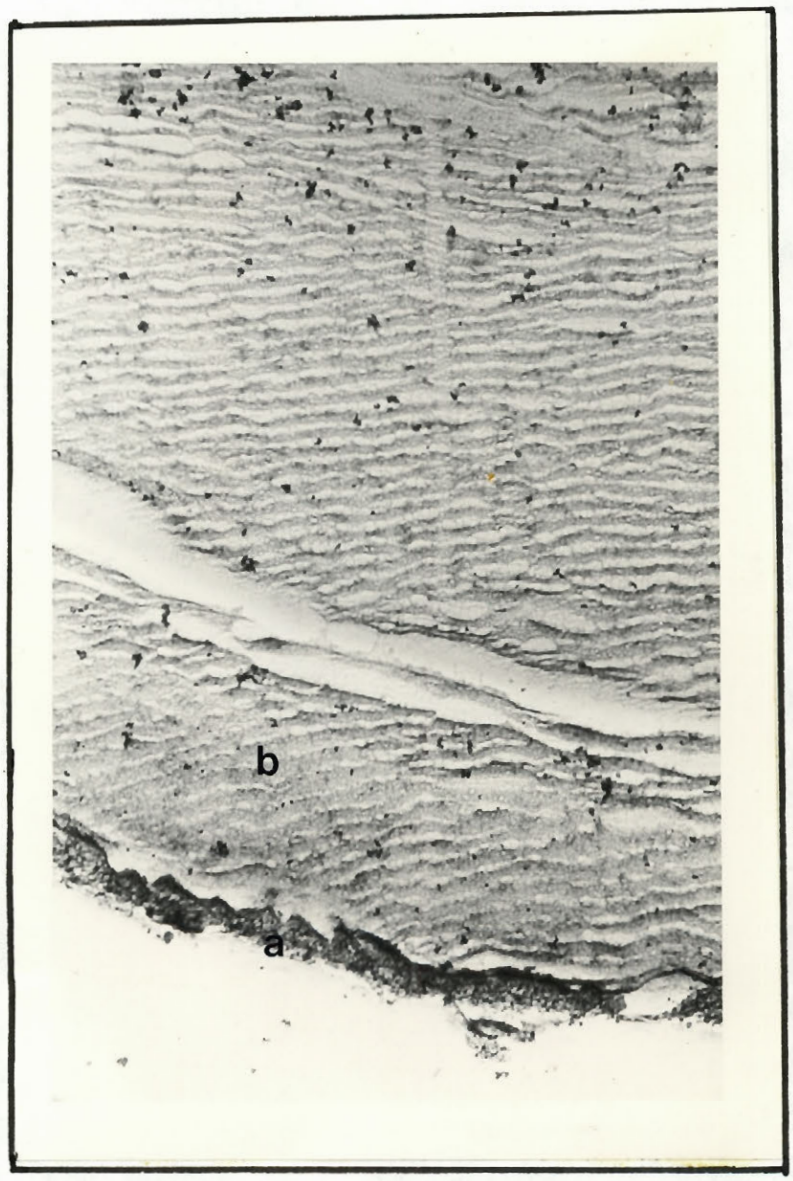

d
c
b
a

Figure 9.3 Histological section of a hydatid cyst treated with medium 1640. (a) germinal membrane; (b) acellular laminated membrane (x 160).


b
a

ifically-activated peritoneal, spleen and peripheral blood leucocytes are capable of killing antibody-coated protoscolices by a mechanism that is independent of complement. This working hypothesis has been confirmed in this chapter, offering a practical approach to the biological and/or medical treatment of E. granulosus cysts. This present finding also substantiates the suggestion by other workers that the only route for effectively attacking an established hydatid cyst is from within the cyst itself (De Rycke and Pennoit-De Cooman, 1973).

Histological sections of the cyst that had been treated with peritoneal cells from cotton rats which had been primed with 10^5 CFU of BCG intraperitoneally showed that the lumen of the cyst contained a proteinaceous coagulum with a few foci of microcalcification. There was also an intensive degeneration of the inner germinal membrane which could have been brought about by the same mechanisms by which activated leucocytes kill protoscolices (Smyth, 1969b). The germinal membrane was replaced by a massive polymorphonuclear infiltration, which suggested tissue damage. Distal to this cellular infiltration, and progressing outward from the lumen of the cyst, there was marked infiltration made up primarily of polymorphonuclear cells and macrophages close to the lumen and distally, in the region of collagenized tissue, the cell population was primarily lymphocytic and polymorphonuclear. The deposition of collagen around the periphery of the treated cyst is characteristic of a primitive 'walling-off' phenomenon (Hibbs et al., 1978). The succession of cell types which surrounded the treated cyst suggests that different cellular mechanisms were active in the reaction: principally an inflammatory reaction in the region proximal to the lumen and a reaction with relatively strong immunological overtones in the regions more distant. It is, of course, impossible to affirm whether this stratification of cell types was static, or whether the dist-

ribution of the different cells changed in the ninety days that elapsed from the initial treatment of the cyst with activated cells and autopsy.

This new approach to the treatment of hydatid cysts of Echinococcus granulosus may provide a practical immunotherapeutic method for the control of this metacestode by cells nonspecifically-activated with BCG.

CHAPTER 10

CHAPTER 10

GENERAL DISCUSSION

Hydatid disease continues to be a public health problem since there is no effective medical treatment for this cestode infection. Attempts at treatment with chemotherapeutic agents have been frustrating: For any drug to be effective, it must be sufficiently stable to remain in the circulation at high concentrations for a considerable period of time and to be able to penetrate the laminated membrane of the cyst while not being toxic. To date no such drug has been found. Anthelmintics, such as mebendazole, are currently being evaluated and it may be possible to utilize them where surgery is contra-indicated, but so far the results of chemotherapy have not been properly assessed (Aarons, 1979).

Surgery is the only approved treatment for hydatid cysts (Saidi, 1976). This procedure is, however, often unsuccessful because the spillage of the contents of the ruptured cysts can result in anaphylactic shock and secondary hydatidosis (De Rycke and Pennoit-De Cooman, 1973). Although hydatid cysts of E. granulosus are generally operable, there are occasions when a cyst is embedded in a vital organ, such as the heart or the brain, thus contra-indicating surgery. As an alternative to conventional surgery, Kassis and Tanner (1976b) have proposed aspirating the cyst fluid and replacing it with fresh complement-rich serum from the patient. This procedure has been shown to result in a regression of E. granulosus cysts in experimental echinococcosis in cotton rats. In experimental infections with E. multilocularis, however, complement-treated cysts are enhanced and continue to grow by surface budding.

The present study has been concerned with E. multilocularis largely because of an interest in resolving the immunobiology of the con-

trol of this human parasite, but partly because of the resemblance of the cystic stage of this cestode to a proliferating neoplastic tumor. The dissemination of infections with the larvae of E. multilocularis can proceed even in the absence of protoscolices (Lubinsky 1968) by a process of peripheral herniation and detachment of small vesicles (Virchow, 1856). The presence of free, apparently spontaneously-disseminated , protoscolices in the abdominal cavity of infected cotton rats (Lubinsky 1960b) suggested that the parasite-parasite interactions in echinococcosis are similar to some tumor-tumor interactions (Laird, 1964). Rau and Tanner (1973) have demonstrated that relatively large subcutaneous cysts of E. multilocularis effectively inhibit the establishment, growth, and metastatic spread of subsequent intraperitoneal inoculations of the same parasite. This control is so effective that a large cyst mass can suppress the development of its own metastatic foci of infection (Rau and Tanner, 1976a); the removal of a subcutaneous cyst mass will permit the growth of intrathoracic metastases.

Whereas in E. granulosus infections the host tissues are content to form a fibrous layer around the unilocular cyst, there is no such limiting laminative membrane around the cyst of E. multilocularis. Moreover, E. multilocularis elicits an intense and persistant local inflammatory reaction in the surrounding host tissues (Ali-Khan 1974; 1978a, b, c; Schwabe et al., 1959). This cellular reaction is probably in response to the direct contact between the host and the parasite because of the lack of a hyaline-like membrane. The principal cell in the peritoneal exudates of animals with an intraperitoneal infection of E. multilocularis is the macrophage (Reuben et al., 1978) which is activated by the infection to kill the parasite in vitro (Rau and Tanner, 1976b). Infection by other cestodes can also nonspecifically activate an

anti-Echinococcus cytotoxicity by peritoneal cells (Baron and Tanner, 1977).

The fact, however, of the presence of activated phagocytic cells in this infection has suggested that "nonspecific" activation of macrophages with BCG could enhance the regression of the parasite cyst mass. This is, in fact, the case since BCG treatment not only activates macrophages to kill the parasite in vitro, but it also protects cotton rats and gerbils against infection by Echinococcus (Rau and Tanner, 1975; Thompson, 1976). Although it is an effective stimulator of macrophages, BCG is a live vaccine and, when administered at high dosages, it can induce the formation of tuberculous granulomas. In most instances, reducing the dose of BCG eventually eliminates its stimulating effects on the immunological response (Bartlett and Zbar, 1973; Chung et al., 1973). Civil et al. (1978) were unable to protect mice against infection with Schistosoma mansoni when the stimulating dose of BCG was reduced from 10^7 to 10^5 colony-forming units (CFU). Similarly, Maddison et al. (1978) have reported a 60% reduction in the worm burden of S. mansoni in mice treated with a dose of 5×10^7 CFU, whereas a half dose (2.5×10^7) was only half as effective in protecting these animals against the parasite.

Granulomatous lesions are a constant feature of treatment with BCG in the majority of the studies which have been reported. Rau and Tanner (1975), Thompson (1976) and Maddison et al. (1978) have all reported massive granulomas in the peritoneal cavity and tissues of animals treated with relatively large doses of BCG. This phenomenon, which is a characteristic of chronic inflammatory response, has been thought to be necessary for the elimination of schistosomula from the lungs of mice (Civil et al., 1978; Fauve and Dodin, 1976). It is argued in this thesis that, rather than being useful for the elimination of

the parasite, tubercular granulomas probably deflect some of the protecting responses of the host away from its primary hydatid cyst target.

In order to test this hypothesis, cotton rats were treated with a single intraperitoneal injection of graded doses (10^1, 10^2, 10^3, 10^5 and 10^7 CFU) of lyophilized BCG two weeks before inoculating the animals with one small acephatic cyst of E. multilocularis intraperitoneally (Chapter 4). The animals were killed six weeks after the parasite was inoculated and the intensity of the infection was determined. The population of leucocytes in the peritoneal cavity, two weeks after treatment with BCG, was characterized by the gradual rise in the numbers of monocytes, in parallel with the increase in the dose of BCG, without a significant change in the numbers of lymphocytes or granulocytes. The fewer monocytes in animals treated with 10^5 or 10^7 CFU of BCG than in those treated with 10^3 CFU (Figure 4. 1) was most certainly due to the trapping of cells in the tubercular granulomas induced by the BCG.

The effects of BCG on the cell proliferation in the peritoneal cavity of the treated and infected animals is also quite marked. Although the population of macrophages rises with increasing doses of BCG, the lymphocyte population is significantly raised only with 10^3 CFU. The significant decline in the numbers of monocytes at the higher doses of BCG (10^5 and 10^7 CFU) is greater in the infected animals than in the uninfected (but treated) controls (Figure 4.2). This decline in the number of monocytes is probably the result of an augmented 'sink' effect because of the migration of the cells from the peritoneal cavity to the tubercular granulomas and to the parasite cyst mass at the higher levels of treatment with the BCG.

This evidence suggests that high doses of BCG (10^5 and 10^7 CFU) may produce pathogenic effects and that treatment with large numbers of tubercle bacilli should be avoided. Complete protection against E. multilocularis was induced by treatment with 10^3 CFU in the gross absence of granuloma formation.

There is currently a great deal of interest in purified subcomponents and nonviable fractions of BCG for immunotherapy. Interest in these arose because of the serious complications and side-effects which accompany immunotherapy with the viable organisms of BCG (Chapter 4). One of the subcellular preparations which are used is the skeleton of BCG cell walls which are prepared by the lysis of the BCG organism. It has been used extensively in the immunotherapy of lung cancer (Yamamura et al., 1976) and in the intralesional immunotherapy in melanomas (Yamamura et al., 1975). There is only one example in parasitology, however, where another subcomponent of BCG, cord factor, has been shown to enhance the resistance of animals against the helminth Schistosoma mansoni (Mahmoud et al., 1977). In the present study, a single injection of 150 µg of BCG cell walls, emulsified in mineral oil and saline protects cotton rats against a subsequent infection with E. multilocularis (Chapter 5).

The protection, as that induced by the whole organism, is correlated with an increase in the numbers of monocytes and, as judged by acid phosphatase activity, an activation of these cells (Figure 5.1) at the time of the inoculation of the parasite. Although it has been suggested that mineral oil alone can activate macrophages to the same extent as that induced by whole mycobacteria (Brunda and Raffel, 1977; Metcalf, 1974), this result was not obtained in this present study. The results presented here agree with those of Cohn and Weiner (1963) who showed that peritoneal macrophages induced by mineral oil possess

little lysosomal activity. Tween-treated animals also exhibit no significantly different enhanced level of cellular proliferation or lysosomal activity at the time of inoculation of the parasite.

Whereas animals pretreated with BCG cell walls were completely protected from infection with E. multilocularis, those animals that were pretreated with Tween-saline or mineral oil-Tween alone were not protected. In fact, the animals treated with Tween or mineral oil bore a greater (but, unfortunately, not significant) number of metastatic foci of the parasite than the untreated controls. It is not surprising that the spleens of animals treated with mineral oil or with Tween were significantly enlarged in comparison with saline controls (Figure 5.3) since splenomegaly is related to the extent of the parasite burden (Ali-Khan, 1978a; Ferrente et al., 1978). The results of these experiments indicate that nonviable cell walls preparations of BCG are effective in protecting cotton rats from infections with E. multilocularis in the absence of granulomas.

The possibility that peritoneal cells from cotton rats treated with BCG, mineral oil, PHA, or an infection of E. multilocularis might have a cytotoxic effect on protoscolices of E. granulosus was predictable (Figure 6.3), bearing in mind previous studies which have demonstrated such an effect in this and in other parasite (Civil et al., 1978; Clark et al., 1976; Maddison et al., 1978; Mahmoud et al., 1979) and tumor cell/host relationships (Cheema and Hersh, 1972; Siegler et al., 1972). As BCG is known to not only nonspecifically increase the immune response of the host to a wide range of antigens (Kuperman et al., 1972; Meyer et al., 1975; Miller et al., 1973; Paranjpe and Boone, 1974; Sparks and Breeding, 1974), but also known to activate T cells and macrophages (Kitamura et al., 1976; Laucius et al., 1974; Ohmichi et al.,

1976; Rau and Tanner, 1975; Thompson, 1976; Yarkoni et al., 1977; Chapters 4 and 5), it is reasonable to assume that the cytotoxic effect of BCG observed in this study is brought about by the augmentation of the immunological mechanisms of the host. There is, however, no precedence in parasitology which establishes the nonspecific stimulation of cells in vitro with mitogens to kill protoscolices of E. granulosus, or any other parasite, for that matter. In Chapter 6 of this study it is successfully demonstrated that normal peritoneal (Figure 6.1), spleen and peripheral blood (Figure 6.2) mononuclear cells, become cytotoxic to antibody-coated protoscolices of E. granulosus when activated in vitro with PHA, LPS or PWM and with viable BCG. The adherent peritoneal cells were highly cytotoxic to these protoscolices (Figure 6.1a), whereas the activity of the non-adherent population was not significantly different from the unstimulated controls (Figure 6.1b).

In relating these results to those of other studies of the cytotoxicity induced by mitogens, the data on the types of cells involved are somewhat conflicting. It has recently been shown that T cells, B cells or macrophages can all acquire cytotoxic activity (Muchmore et al., 1975) after treatment with mitogens. This fact implies that several mechanisms are possible in the destruction of target cells. Hibbs (1974) has proposed that the killing of tumor cells by macrophages is through the release of lysosomal enzymes; the mechanism of lymphocyte killing, however, yet remains to be elucidated. From the present study, the mechanisms by which these activated cells may kill the parasite is unknown, but killing is clearly independent of complement. This fact is most interesting since previous studies by Kassis and Tanner (1976a,b,; 1977a,b) and Rickard et al. (1977) have shown complement to play a major role in the control of both primary and secondary hydatidosis. Protoscolices may, however, vessiculate and escape the lytic action of

complement, thereby successfully establishing a primary infection. The complement-independent cell-mediated cytotoxic mechanism described in Chapter 6 may prove to be active in preventing the establishment of a secondary infection, especially in the plateau phase of this infection when complement levels drop (Kassis, 1976).

Cotton rats can be protected against primary hydatidosis by the passive transfer of immune or PHA-stimulated peritoneal cells (Chapter 7); protection with immune spleen cells has also been reported (Araj et al., 1977). The mechanism of control in this protective response, however, is not known for either the specifically or non-specifically-stimulated cells. It is suggested that protection is mediated largely through nonspecific immunological events, since stimulated cells are capable of releasing soluble factors which may induce inflammatory responses (Allison, 1978; Crowle, 1975; Page et al., 1978). In addition, the tissue damage which is characteristic of infections with E. multilocularis (Schwabe et al., 1959) also elicits a non-specific inflammatory response from the host which, in turn, should not provide a very happy environment for the parasite. In spite of the massive proliferation of leucocytes during primary hydatid disease and the demonstration by several workers of the effective killing in vitro of protoscolices by these cells, the parasite manages to establish and proliferate. These present studies suggest that a closer examination of the functional correlation between these leucocytes and the establishment of a primary infection will prove fruitful.

There are numerous studies in parasitology where the mitogenic responses of leucocytes from various host-parasite systems has been evaluated following stimulation with both specific and nonspecific antigens (Araj et al., 1977; Bloch et al., 1977; Dobson and Soulsby,

1974; Hall et al., 1979; Miggiano et al., 1966; Weiss, 1978; Yusuf et al., 1975). The spleen cells from infected cotton rats cultured in the presence of PHA showed significant blastogenesis when sampled at days 14 and 21 of the infection; the blastogenic response declined to normal values thereafter (Table 8.2). In contrast, a significant increase in the stimulation index of peritoneal cells was obtained when these cells were sampled on days 7, 14, 21 and 28 of the infection. The stimulation reached a peak on day 14, declined on day 21 and returned to normal values by day 42. The decline in the mitogenic response of peritoneal cells to PHA coincided with the appearance of small cyst masses in the peritoneum.

The cellular response in the peritoneal cavity at days 7 and 14 after the inoculation of the parasite consisted of numerous macrophages and lymphocytes (Table 8.1). As the infection progressed, proliferation was primarily due to monocytes and, with a change in the ratio of macrophages to lymphocytes, there was a concomitant decrease in the PHA-stimulation index of cells isolated on days 21 and 28 of the infection. It is also of interest to note that impression smears of the minute cyst masses first seen on day 21 suggest that they are composed of leucocytes that have migrated into them from the peritoneal cavity since the cell composition of the cyst resembled that of the peritoneum. The cells isolated from the cyst masses failed, however, to respond to PHA, in contrast to the susceptibility of peritoneal cells to activation by this mitogen.

The significance of a depressed ability of the leucocytes in the hydatid cyst to be stimulated by PHA is problematical; the same phenomenon has, however, been observed in diseases where defects in cellular immunity have been well documented (Bryceson, 1974). This

depression in hydatid infections is of particular significance as similar depression is observed in the spleen and in the peritoneum in the logarithmic phase of growth of E. multilocularis. It is noteworthy that it is at this stage that the host experiences the challenge which results in splenomegaly (Ali-Khan, 1978b), leaving the host in a compromised position.

There is no evidence at the present time to identify any prime mechanism for this immunosuppression. However, a depletion of antigen-sensitive T cell clones in the spleen, the stimulation of suppressor T cells and/or suppressor macrophages, and their products must be considered seriously. Chronic infections, such as echinococcosis, are well known to induce macrophages to release mediators capable of depleting and causing a malfunction of T cells (Bryceson et al., 1972; Nath et al., 1974). All these aspects of the host-parasite relationship in echinococcosis need considerable more investigation as they might explain how larval cestodes are able to establish and survive in their hosts for such extended periods of time.

Although the inflammatory peritoneal cells, that are effective killers of the parasite in vitro, migrate into the cysts of E. multilocularis, they are, apparently, unable to arrest the growth and metastases of the infection. This phenomenon could be due to the fact that dissemination of E. multilocularis infections can proceed in the absence of protoscolices (Lubinsky, 1968) by a process of peripheral herniation and detachment of small vessicles (Virchow, 1856). Control of the infection with E. granulosus is more manageable. Kassis and Tanner (1976b) have suggested that a hydatid cyst of E. granulosus can be treated and the parasite killed by replacing the fluid within the cysts with complement-rich fresh serum from the infected hosts. Several

theoretical considerations exist, however, which limit the potential usefulness of immunotherapy of hydatid disease with immune serum (Rosenberg and Terry, 1977). One of these limitations might be the transfer of a serum factor which is suppressive to the cellular response of the host (Chapter 8). The transfer of BCG-stimulated cells into the cysts, however, produced a calcification of the contents of the cyst and elicited an intense polymorphonuclear leucocyte response in the inner germinal lining of the cyst. Furthermore, the outer lining of the treated cyst was composed of collagenized tissue, mainly containing fibroblasts, mixed with lymphocytes, polymorphonuclear cells and macrophages - a response typical of a 'walling-off' phenomenon (Chapter 9).

This new approach provides, for the first time, a practical immunotherapeutic method with nonspecifically-activated cells for the treatment and management of Echinococcus granulosus hydatid cysts which are non-resectable.

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