CHAPTER I

INTRODUCTION AND

REVIEW OF LITERATURE
Hookworm parasites are among the important intestinal nematodes whose prevalence and concomitant detrimental effects on the body functions have made them to be ranked as one of the important helminthic diseases of man in developing and underdeveloped countries. Although the infection does not cause mortality, it saps the vitality of the hosts already suffering from malnutrition. According to the recent reports, about 1000 million people may be infected with hookworm parasites (Hotez et al., 1987; Crompton, 1989).

"Hookworm disease", in man is mainly caused by the three species - Necator americanus, Ancylostoma duodenale and Ancylostoma ceylanicum. Besides the above three human pathogens, the other parasites pathogenic to livestocks are A.caninum (dog), A.tubaeformae (cats), Nippostrongylus brasiliensis (rats), Nematospiroides dubius (mouse), Bunonstomum trigonocephalum (sheep and goat), B.phelabotomum (cow) and Gaigeria pachycetis (sheep).

Hookworm infection is more prevalent in tropical and subtropical regions of Asia, Africa, Europe and America. In Asian continent the most endemic countries are India, Malaysia, China and Japan. In India, the tropical climate, poor sanitation and poorer health education favour the growth, multiplication and dissemination of all parasitic infections including hookworms. The morbidity and mortality caused by the disease were first reported in tea plantations of Assam, where approximately 90% of the population harbour this infection. The inci-
idence of the disease is also very high in plain where paddy cultivation is predominant (Raghavan, 1967), while the incidence is low in Uttar Pradesh and Pondicherry (Yunus, 1977; Singh et al., 1978).

The period from infection to the first appearance of eggs in the faeces of the host is constant for each species of hookworm. The ovum undergoes four mouls and second moult produces an infective stage i.e. the third stage larvae, L₃. This stage after penetrating the skin moves towards lungs and the third moult takes place. L₄ larvae with a provisional buccal capsule, enters the larynx, then moves to pharynx, oesophageous and finally settles down in the small intestine. The migratory phase occupies about 7-15% of the prepatent period. The last moulting occurs in the intestine, the adults mature, mate and reproduce and thus the cycle continues. Among human hookworm parasites, the shortest prepatent period has been observed in case of A. ceylanicum (18-26 days), while the prepatent period for A. duodenale and N. americanus is longer. Maximum egg output was observed between 12 and 18 months of A. duodenale infection in man and the life span of the worms was 6-7 years (Kendrick, 1934), while that of N. americanus was reported to be 5-6 years.

There are no specific signs and symptoms in the vast majority of cases of hookworm infection and their order of appearance are closely related to the chronology of migration and development of the parasites. The symptoms can con-
Third stage infective larvae (L₃)

Blood circulation (Ground itch)

Oral route

Eggs in faeces

SOIL

Adult intestine (Anaemia, abdominal discomfort)

Stomach

Tracheolarynx (Bronchitis, Asthma)

Oesophagus

L₄

Heart

Lungs

Fig. 1: General life cycle of hookworm parasite.
veniently be divided into two; those associated with migration of $L_3$ and presence of adult worms are primary ones, while the secondary symptoms are the consequences of physiological, biochemical and haematological disturbances that follow the persistence of intestinal infection and constitute what is commonly known as 'hookworm disease'. A primary heavy infection will induce ground itch followed by fever, asthmatic wheezing and a high degree of eosinophilia which will be self limiting. In later stages, the ancylostomiasis is connected with iron deficiency anaemia associated with epigastric pain or discomfort which may be relived by food and mistaken for duodenal ulcer. The functional consequences of this disturbed iron-balance and impaired nutritional status include growth retardation, reduced working productivity and a potential adverse effect on learning and cognitive development. Severe intestinal haemorrhage due to the infection might lead to a drop in haemoglobin (Hb) level as low as 2 g/100 g of blood and in erythrocyte count to below 1000/ml. Chronic and acute, both kinds of anaemic conditions are observed in the patients of hookworm infection. Numerous secondary signs and symptoms attributable to anaemia and hypoproteinaemia accompany chronic hookworm infection. The most important of these are associated with progressive cardiac insufficiency and congestive failure. A wide range of reproductive disorders have also been attributed to the effects of hookworm infection.
Chemotherapy based on efficacious anthelmintic drugs like Mebendazole, Thiabendazole, Bephinium, Levamisole, Tetrachloroethylene etc. remains the starting point of a modern control programme against hookworms (Crompton, 1989). However, these drugs are effective against the adult stage of the parasite and repeated doses cause various types of side effects. Hence, there is an urgent need to evolve an alternate strategy which should be effective against the infective larval stage and can check the infection as soon as it occurs. A realistic approach would be to diagnose the disease at an early stage as well as to develop immunoprophylactic measures, which require a good understanding of the molecular immunology of hookworm infections. These studies will certainly provide the basis for rational vaccine development as well as for immunodiagnosis of the disease. Accordingly, a survey of literature was made along these lines.

REVIEW OF LITERATURE

The efforts to understand the molecular and immunological processes underlying hookworm infection have greatly been limited due to the lack of suitable animal models. Most of the in vivo studies for examining the complete life cycle of Ancylostoma hookworms require the use of dogs or cats, of course with the exception of some studies which were done in primates. Most of the studies have been done using these
animal models but the expense and the time required to maintain the parasites in these laboratory hosts has hampered the studies on hookworm parasites. An important development has been the establishment of human hookworms in laboratory rodents so that controlled experimental studies can be made on these parasites. The maintenance of *A. ceylanicum* in golden hamsters (Carroll and Grove, 1984) and of *N. americanus* in neonatal hamsters (Behnke et al., 1986) has opened up new vistas for laboratory investigations. This achievement has facilitated work on antigen characterisation, immunity and vaccine development. The role of surface antigens and excretory-secretory products which include certain metabolic enzymes like proteases, is still not well established. By using modern techniques, these antigens can be well studied and it will help in developing better immunoprophylactic and immunodiagnostic measures. Studies on immunology of hookworm parasites have mainly been directed towards the two major aspects – one is immunodiagnosis dealing mainly with serological or host immune responses and the other aspect is protective immunity.

**Immunodiagnosis**

The development of immunodiagnostic methods for hookworm infection has received a little attention in view of the relative ease of diagnosis by faecal examination, but, in fact, the hookworm eggs may be sometimes misunderstood with the other intestinal helminth parasite. Hookworm parasites have
been shown to mediate a wide variety of serological reactions in vitro, detected by complement fixation (Magaudda Borzi and Pennisi, 1958), gel diffusion (Singh, 1965), latex flocculation (Rombert et al., 1967); haemagglutination (Desowitz, 1967; Jayawardene and Vijayaratnam, 1968; Ball and Bartlett, 1969), immunoprecipitation (Yamanaka, 1960; Ball and Bartlett, 1969; Ogilvie et al., 1978) and immunofluorescence (de Azevedo et al., 1971; Lewis et al., 1978). A study of immunological reactions during experimental infection of *Namericanus* was done long back by Ball and Bartlett (1969). Later on, Poulain et al. (1976) studied the local systemic responses in rats infected with *Nbrasiliensis* by determining the antibody titers of both sera and intestinal mucosa using passive haemagglutination test and found that a considerable portion of activity was associated with IgA fraction. Specific IgA and other isotypes of antibody to *Nbrasiliensis* antigens have also been detected in sera as well as intestinal secretions by several other workers (See et al., 1976; Sinski and Holmes, 1977; Brown et al., 1981a,b). People have tried to quantitate the antibody titers during the course of infection employing various immunological techniques like counter current immunoelectrophoresis (Bhopale et al., 1981; Menon and Bhopale, 1985), enzyme linked immunosorbent assay (Ogilvie et al., 1978) and radioimmunoassay (Sinski and Holmes, 1978). Counter current immunoelectrophoresis has been shown to have a very limited application in serodiagnosis because of multiple helminth infections as well as cross-reactions among various hookworm species. Radioimmunoassay has successfully
been used for quantifying the specific antibody responses in rats infected with *N. brasiliensis* and it was found to be of local IgA type (Sinski and Holmes, 1978). It has also been reported by the same workers that the local specific IgA antibodies were closely associated with immunity against gastrointestinal nematode. The local antibody responses (IgA, IgM and IgG levels) in rats parasitised with *N. brasiliensis* infection were investigated by measuring the levels of coproantibodies (Wedrychowicz et al., 1983). Recently, the usefulness of radioimmunoassay and reverse enzyme immunoassay using excretory-secretory products from hookworm parasites has been demonstrated in serodiagnosis and prognosis of hookworm infections (Ganguly et al., 1988).

The hookworm infections have been shown to evoke immediate type hypersensitivity reactions as demonstrated by intradermal tests conducted with antigenic preparations from various hookworm parasites (de Hurtado and Layrisse, 1968). The sensitivity of skin reaction was found to be 80% or even more and some results indicated that the intensity of skin reaction may be related to the intensity of hookworm infection (Lobel et al., 1968). In support of these findings a positive relationship has been shown between the skin reaction (diameter of the wheel formed) and the level of infection (faecal egg count) by some workers (de Hurtado and Layrisse, 1968; Lobel et al., 1968). A significant correlation between the wheel size and eosinophilia and presence of signs and symptoms of hook-
worm infection has also been shown by Ishizaki et al. (1961). Antigens used for the skin test include protein or polysaccharide fractions prepared respectively by tryptic digestions of larvae or adult hookworms (Noda, 1953; Sawada et al., 1954; Wei and Kuo, 1958; Shih et al., 1959; Prasad and Mathur, 1962) or by aqueous extraction (Yamanaka, 1960; Ishizaki et al., 1961; de Hurtado and Layrisse, 1968; Lobel et al., 1968). Skin test has been found to be positive in man using antigens from canine hookworm suggesting the cross reactivity among these antigens (Sawada et al., 1954, 1961; Yamanaka, 1960). It has been shown that the sera obtained from hookworm or ascariasis patients also cross-react well with cestode antigens (Correa-Oliveira et al., 1988). The demonstration of these cross reactions points out the problems of serodiagnosis. The heat shock proteins have been shown to have better prospects for a species specific diagnostic test (Newport et al., 1988).

**Immunoprophylaxis**

Though substantial evidence is there for the presence of functional protective immunity during hookworm infection, but the molecular basis of the protective immune responses has not been well defined. The strongest evidence for the acquired immune responses to hookworm infection comes from an early demonstration that the infective larvae of *A. caninum* elicit immunity in dog (Herricke, 1928; McCoy, 1931; Foster, 1935). Different mechanisms have been documented for the active evasion of the host's immune responses in many parasitic
infections and it is supposed that hookworms also employ similar strategies for evading or suppressing the host protective responses (Hotez et al., 1987). The protective immunity to animal hookworm parasites has been investigated in some detail (Miller, 1971; Carroll and Grove, 1984) and has been found to be associated with both serological (Ball and Bartlett, 1969; Ogilvie et al., 1978) and cellular responses (Ogilvie and Jones, 1973; Taylor and Turton, 1976; Maxwell et al., 1987). However, a strong correlation between these phenomena and host protective immunity has yet to be conclusively established.

Although, a number of investigations have been conducted on the systemic immune responses of host to hookworm infection (Kassai, 1982) and the possible role of local antibodies in conferring immunity to the parasites (Poulain et al., 1976; Sinski and Holmes, 1977, 1978; Wedrychowicz et al., 1983a,b, 1984a,b), very little information is available about the antigens important in the stimulation of intestinal antibody responses. It has been suggested that IgA antibodies are induced by antigens irrelevant to parasite survival and these antibodies are supposed to play important role in establishing host–parasite relationship rather than evoking protective immunity (Befus and Bienennstock, 1982). The most striking immunological property of helminth infections is a capacity to provoke high levels of IgE and reagin type antibodies in their hosts. Studies concerning the function of IgE in parasitic infections began on the firm basis with the work on intestinal anaphylaxis
in *N. brasiliensis* infected rats (Urquhart et al., 1965; Jarrett and Bazin, 1977). Both parasite specific and non-specifically potentiated IgE responses have been reported during *N. brasiliensis* infection in rats (Ogilvie, 1967; Orr and Blair, 1969; Bout et al., 1977). However, subsequent studies established that non-specific IgE does not protect against allergic reactions as the passive transfer of immunity could be achieved using antisera devoid of anaphylactic activity (Jones et al., 1970; Leid and Williams, 1974; Jarrett et al., 1980).

Various mechanisms have been proposed for the induction of protective immunity in hosts; these are antibody mediated immunity, cell-mediated immunity, and lastly the local inflammatory responses (Jarrett and Urquhart, 1971; Ogilvie and Jones, 1973; Love et al., 1974). Immunity to mouse hookworm *N. dubius* is believed to have two stages: antibodies damage the worms which are then predisposed to antibody independent expulsion from the gastrointestinal tract (Prowse et al., 1978). This self cure phenomenon seems to be achieved by a combination of all the three mechanisms proposed earlier for protective immunity. However, passive transfer of protective immunity to mice with immune sera against *N. dubius* infection, demonstrated that serum antibody mediate protective immunity (Jones and Robin, 1974; Dobson and Owen, 1978). Later on, it was suggested that passive transfer of anti-*N. dubius* serum directly or indirectly influenced the survival, growth and fecundity of *N. dubius* (Dobson, 1982). It has also been found that the
passive transfer of IgG1 - fraction of anti-N. dubius serum protected the mice and resulted in the retarded growth of the parasites. When combined with adoptively transferred immune mesenteric lymph node cells, enhanced worm expulsion from the intestine was also observed (Pritchard et al., 1983). According to the earlier reports, it was suggested that worms previously damaged by the host immune responses were found to be more susceptible to expulsion mechanism as compared to the undamaged worms (Ogilvie and Love, 1974). However, in some cases, the worms taken from the hosts shortly before expulsion and transplanted to the other hosts were no more susceptible to expulsion (Hopkins et al., 1972; Hopkins and Zajac, 1976; Wakelin and Wilson, 1979). It has been suggested that worm expulsion was due to the metabolic crisis through which the worms were going and the findings support the enteroallergic indirect mechanism for worm rejection (Kassai et al., 1987). Recently, some work has been done regarding the expulsion phenomenon of N. brasiliensis and free radicals (oxygen radical) are said to be involved in the expulsion of primary infections (Smith and Bryant, 1986; Smith, 1989).

Hookworm parasites have also been shown to evoke cell mediated immune responses (Ali et al., 1986). Infection with A. duodenale has been found to be associated with marked cell mediated immunity as manifested by increased T-cell counts, increased inhibition of leucocyte migration and increased lymphoblast transformation. An inverse relationship was proven between
the degree of cell mediated immune responses and the parasite load. Furthermore, some other group of workers have also reported the probable role of cell mediated immunity in vaccinated animals. According to these workers a delayed hypersensitivity reaction has been observed in pups vaccinated with irradiated infective larvae of *A. caninum* and this skin reaction has been reported to correlate with the inhibition of the migration of leucocytes in the presence of larvae or adult worm antigen (Gautum et al., 1986).

A successful induction of protective immunity with soluble extracts from adults of *A. caninum* and *A. ceylanicum* has been shown by the reduction in worm burden by 59% and 74% respectively (Thorson, 1956; Carroll and Grove, 1984, 1986). Dogs immunised with soluble extracts prepared from adult worms have been found to be partially resistant to challenge infection with L₃ of *A. ceylanicum* (Carroll and Grove, 1985). Double dose of irradiated L₃ larvae of *A. caninum* gave 90% protection to subsequent challenge (Miller, 1965, 1971). The route of administration is also an important factor. Intraperitoneal or subcutaneous route has been shown to be less effective as compared to oral administration which gave 100% reduction in worm burden (Gupta and Katiyar, 1985). Menon and Bhonale (1985) have vaccinated the golden hamsters with irradiated larvae of *A. ceylanicum* and obtained 95% reduction in worm burden. However, such vaccines prepared from irradiated attenuated larvae cannot be employed in human beings because
of some ethical reasons, such as the danger of vaccinated host becoming infected.

Characterization of antigens

Attempts to analyse the somatic extracts from adult and larval stages of *A. caninum* and their cross reactivity with sera obtained from *N. americanus* infected patients were made long back by Williams (1970), using immunoelectrophoretic technique. The larval antigens could be separated into 18 precipitin bands whereas the adult antigenic preparation showed the presence of 13 bands with homologous immune rabbit serum. Only one cross reactive component was observed between *A. caninum* and *N. americanus* employing patient sera, whereas the extracts from *N. americanus* adult worms have been reported to show four precipitin bands (Williams, 1970). In contrast to these findings, Desowitz (1962), employing Ouchterlony gel diffusion test has shown more relationship between the same stage of different species than between the different stages of the same species. Later on, a comparative analysis of proteins of *A. ceylanicum* and *A. brasiliensis* was done employing disc gel electrophoresis and an over all similarity between the protein pattern of the two parasites was observed (Yoshida et al., 1977). Antigens of 250, 60, 45 and 30 kD have been found to be common between the adult and larval stages of *N. dubius* (Hurley et al., 1980; Mitchell and Munaz, 1983; Mitchell and Cruise, 1984; Monroy et al., 1985; Monroy and Dobson, 1986; Adams et al., 1987). Affinity purified fraction of adult worms
containing 55 and 20 kD antigen has been shown to induce protection against infection with *N. dubius* (Monroy and Dobson, 1987). Characterization of antigens of *N. brasiiliensis* adult worm have also been done using monoclonal antibodies (Bohn and König, 1985). The antigenic pattern of different developmental as well as the stage specific antigens of *N. americanus* have been studied using immune sera from human (natural host) and hamsters (Carr and Pritchard, 1987).

The antigens of nematode parasites have been shown to be excreted or secreted from the internal cellular and glandular structures during *in vitro* maintenance of parasites. These excretory-secretory (E-S) antigens are supposed to be less heterogenous as compared to somatic extracts and have been found to play important role in diagnosis as well as in protective immunity. The role of E-S products from hookworms in inducing immunity was reported by Otto and Kerr (1939) about a half century ago. The excretory-secretory products of rat hookworm parasite *N. muris* have been shown to induce protective antibodies (Thorson, 1951). Later on, the protective role of E-S products from a number of hookworm parasites was studied by many workers and significant degree of protection was observed in parasitised hosts (Clegg and Smith, 1978; Jain et al., 1979; Wedrychowicz et al., 1983). The immunogenicity of E-S products from hookworm parasites was compared with that of somatic antigens and it was suggested by many workers that E-S products were more immunogenic (Katiyar et al., 1972; Govila
et al., 1973; Murray et al., 1979). The E-S antigens of human hookworm *N. americanus* have been analysed employing immunoblotting technique using serum from infected hamsters as well as from the patients suffering from hookworm infection (Carr and Pritchard, 1986, 1987). Ey (1988) has identified and characterized the E-S products from exsheathed *L*₃ of *Heligmosoides polygyrus* (*N. dubius*) and has also studied the role of E-S products in protection. Besides, hookworms the E-S antigens of other nematodes like *T. canis* (Sugane et al., 1985), *B. malayi* (Kaushal et al., 1982, 1984), *W. bancrofti* (Maizels et al., 1986), *S. cervi* (Malhotra et al., 1987) and *Strongylus stercoralis* (Brindley et al., 1988) have been successfully characterised using immunoelectrophoretic and other immunochemical techniques.

The sex specific antigens present in the secretions of *N. dubius* adults have also been studied (Adams et al., 1988). The E-S products of adult *N. dubius* have been shown to reduce the ratio of male worms to female worms. A highly immunogenic molecule of 66 KD has been found to be present in the secretions and also on the surface of males only and has been supposed to induce protective immunity specific to male worms, thus resulting in the less chances for mating and so the reduction in worm burden. The evidence for the cuticular contribution to E-S products comes from an early demonstration that *N. americanus* sheds significant amount of its surface antigen into the medium during *in vitro* incubation (Pritchard et al., 1985). The adsorption of E-S antigens onto the surface has
been shown in case of *T. spiralis* (Silberstein and Despommier, 1985). Rats immunized with E-S products from *Strongylus stercoralis* adults showed reduction in faecal egg count as well as adult worm burden (Mimori et al., 1987), thereby suggesting the protective role of E-S products.

Surface antigens have also been shown to play a key role in the induction and expression of host immune responses (Ogilvie et al., 1980; Maizels et al., 1982, 1983). The cuticle of helminths including hookworms have been shown to be prone to damage by antibody and complement and by many of the antibody dependent cellular cytotoxicity reactions (Kazura and Grove, 1978; Mackenzie et al., 1978; Kazura and Aikawa, 1980; Ogilvie et al., 1980). The stage specific surface antigen of *N. brasiliensis* have been investigated using surface labelling with 125I, solubilisation with detergent followed by immunoprecipitation and SDS-polyacrylamide gel electrophoretic analysis (Maizels and Ogilvie, 1980; Ogilvie and Phillip, 1980). The cross reactions between the surface proteins of various nematodes, which differ completely in their molecular weight characteristics, have also been documented by several investigators. Maizels et al. (1982) suggested that carbohydrate side chains could be responsible for the cross-reactivity. The cuticular antigens from *N. americanus* adults were studied by Pritchard et al. (1986), using sera from infected hamsters as well as human. A 67 kD antigen of *N. americanus* has been reported to be produced in vitro also in measurable amounts (Carr and Pritchard,
suggesting thereby the involvement of cuticular antigen in inducing immune responses. The surface antigens of *N. americanus* have been shown to be highly immunogenic and have been found to be collagenous in nature which is the main structural component of parasite cuticle (Keymer and Bundv, 1989). It has also been suggested recently by Pritchard et al. (1988a) that natural immune response against surface specific epitopes could be boosted with purified surface antigens to induce sufficiently strong and persistent responses. It has also been suggested that small collagenous molecules and associated disulfide bonds hold together the cuticle of *N. americanus* (Pritchard et al., 1988b).
SCOPE AND PLAN OF WORK

Hookworm disease is a major public health problem affecting millions of people and causing profound morbidity and mortality. Chemotherapy has emerged as the only procedure for temporary cure of the disease. However, repeated doses of anthelmintic drugs are toxic and cause various types of side effects. Therefore, the knowledge about molecular immunology of hookworm infection is of urgent need as it may provide certain leads for development of vaccines.

Several hookworm species are known to infect men and livestocks. Due to the strong host specificity, *Necator americanus* and *Ancylostoma duodenale*; the two human hookworms are less amenable for the studies. Another hookworm parasite *Ancylostoma ceylanicum*, (which infects man besides cats and dogs), being maintained in golden hamsters (*Mesocricetus auratus*) has extensively been used for chemotherapeutic and immunological studies. Many efforts have been made for studying immune responses and protective potential of hookworm parasites, but the antigens responsible for evoking these responses are not well defined. The characterisation of antigens will provide the basic information not only for understanding the host-parasite relationship but also for identifying the antigenic targets of protective immunity. Hence, in the present study an endeavour has been made to analyse the somatic and excretory-secretory antigens of *A. ceylanicum*. The present dissertation deals with the following aspects:
I. Antigenic analysis of somatic extracts from adult and infective larval stages of *Ancylostoma ceylanicum*

1. Protein pattern of adult and larval stages of *A. ceylanicum*.

2. Immunisation of rabbits with somatic extracts and quantitation of parasite specific antibodies in immune sera.

3. Antigenic analysis of adult and larval stages of *A. ceylanicum* using immune rabbit sera.

4. Identification of common or cross reactive as well as stage/parasite specific antigens of adult and larval stages of *A. ceylanicum*.

5. Immunisation of hamsters with adult and larval somatic extracts of *A. ceylanicum* and evaluation of immunised hamsters for protective immunity.

II. Antigenic analysis of excretory-secretory products of adult *A. ceylanicum*

1. Short term *in vitro* maintenance of *A. ceylanicum* adult worms and preparation of excretory-secretory products.

2. Immunisation of rabbits with excretory-secretory products and quantitation of parasite specific antibodies in immune sera.

3. Antigenic analysis of excretory-secretory products using immune rabbit sera.
4. Analysis of common or cross reactive antigens between excretory-secretory products and somatic extracts from adult and larval stages.

5. Immunisation of hamsters with excretory-secretory products and evaluation of immunised hamsters for protective immunity.