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IMMUNIZATION OF THE RAT AGAINST TAENIA TAENIAEFORMIS

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IMMUNIZATION OF THE RAT AGAINST TAENIA TAENIAEFORMIS

By

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the resistance stimulated by orally administered saline soluble antigens, whether the adjuvant was given together with the antigen or separately in the musculature. Both antigens were shown to confer a systemic resistance when given per os.

Low levels of homocytotropic antibody were detected in rats immunized intramuscularly with either antigen using *B. pertussis* or aluminum hydroxide as adjuvants. None was detectable in those rats immunized orally or intramuscularly using Freund's complete adjuvant with either antigen.

Passive transfer of resistance was not observed in recipients of serum from rats that had been vaccinated orally or intramuscularly with either antigen employing *B. pertussis* as an adjuvant.

Dedicated to my People and the Struggle

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To my friends and folks, including my parents, wife and children; - God bless you all for your moral support, prayers and inexhaustible patience.

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Introduction

Cysticercoses affecting man and domesticated animals are currently increasing on a global scale and their socio-economic impact cannot be taken lightly. In the absence of suitable chemotherapeutic agents, and in the face of difficulties and failures with other control measures, more and more attention is being focussed on the exploration of immunological alternatives to control these infections. Laboratory models of cestodiasis like *T. taeniaeformis* in the rat are a vital part of these experimental immunological investigations. The hope that information gathered from such models would be relevant and contribute to studies on eventual immunization against the important cysticercoses of livestock is now being realized to some degree. The work described herein centers on vaccination of rats against *T. taeniaeformis* using two types of antigens derived from the metacestode. The effects of routes of administration of the antigens and the adjuvants employed are investigated.

The literature review has therefore been organized to provide, in the first part, a background of information on taeniid cestodes of medical and veterinary importance. The second part deals with experimental cysticercoses and immunity. In this section, the general characteristics of acquired resistance

to cestode infections in animals are described, followed by a detailed account of the immune mechanisms in *T. taeniaeformis*. These include protective humoral responses, and other humoral and cellular aspects of host-parasite interactions. In the third part, the intestinal lymphoid tissue and its associated immune responses are examined. The topics dealt with are immunoglobulins produced in the intestinal secretions; the immune response to antigens and infections; the phenomenon of immune exclusion by the gut, and orally-induced systemic tolerance. Lastly, a discussion is presented of the nature of adjuvants and their use in protective immunization against helminth diseases.

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LITERATURE REVIEW

The phylum Platyhelminthes contains many members in the class Cestoda, order Cyclophyllidae, Family Taeniidae which are of both medical and veterinary importance. Adult tapeworms in this family parasitize the intestinal tract of their definitive hosts, whereas the metacestodes, or larval forms, occur in various tissues of the intermediate hosts. Man and domestic animals are commonly involved in these cycles, but several of the taeniids of rodents serve as excellent models for laboratory investigations on host-parasite relationships in cestodiasis. In recent reviews of the literature on natural and experimental taeniiasis, Leid (1973), Musoke (1975), Ansari (1975) and Hustead (1976) have provided comprehensive accounts of the biology, immunology, epidemiology and clinical and economic importance of the major taeniid parasitoses. An exhaustive account of these aspects will not be attempted here, but for the purpose of providing background to the specific areas addressed in this thesis a brief overview is appropriate.

Life cycles and medical and veterinary importance of Taeniid cestodes

Adult taeniid cestodes in the gut of the definitive host shed terminal proglottids, or segments, which contain large numbers of eggs, within each of which is an embryo. Both segments and eggs pass out with the faeces into the environment, and the

intermediate host usually becomes infected by ingesting dispersed eggs. Through the action of gastrointestinal fluids the embryos, or oncospheres, are liberated and actively penetrate the intestinal mucosa. They reach their sites of predilection via the blood or lymph, and by migrating through intervening tissues. At these sites they differentiate into sessile cystic larval forms which must survive in the tissues until consumed by the carnivorous definitive host in order for the life cycle to be completed. The larval forms develop into adult ribbon-like tapeworms in the gut in about 6 weeks.

It is the growth and development of larvae in intermediate host tissues which leads to the public health and veterinary importance of taeniids. For example, *Taenia saginata* occurs as a relatively benign adult tapeworm in the intestine of man, but the cystic larvae (cysticerci) occur in the muscles of cattle, leading to disfiguring lesions throughout the carcasses. *Taenia ovis* lives as an adult in the gut of domestic dogs, but its cysticerci invade the muscles of sheep leading to considerable economic and animal protein loss through carcass condemnation. In some instances the picture is complicated by the accidental development of larvae in the tissues of man, and such is the case for *T. solium* (man - pig cycle) and *Echinococcus granulosus* (dog - sheep cycle). There the infection of human tissues is of no significance in terms of continuation of the life cycle, but the growth of cystic masses in vital organs in humans (e.g. brain, eye, spinal cord) has major clinical consequences.

On a global basis, the prevalence of the cysticercosis-

hydatidosis complex of diseases has shown an upward trend over the past few decades. Both developed and developing countries have experienced this trend, though the behavioral and socio-economic factors which have contributed to the increases are clearly different in these distinct environments. However, certain biological characteristics of the taeniids are important determinants of the success of this group under all circumstances. Taeniid eggs are known to survive for months to years, depending on the environment, and there are no practical chemical disinfectants available which will kill them. Adult worms themselves are long-lived and prolific, and although certain drugs are useful in treatment, none of these is entirely satisfactory. The metacestodes often survive in tissues for years and no satisfactory chemotherapeutic agents are presently at hand for treatment of infected animals or humans. Polyembryonic multiplication of metacestodes occurs in some species and this also enhances the infection potential of taeniids. Finally, although a marked acquired resistance develops to superinfection in hosts infected with metacestodes, there is, as yet, no practical approach to artificial immunization as a preventive measure.

The realization that tapeworm infections are occurring with a disturbing frequency, and that their propagation may even be enhanced by the advent of modern hygienic sewage treatment and distribution measures (Pawlowski and Schultz, 1972), has led to renewed interest in research on these parasites in recent years. Experimentally, some new chemical anthelmintics have been shown to have lethal effects on tapeworm larvae (Salazar et al, 1972;

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Campbell and Blair, 1974; Heath and Chevis, 1974; Thienpont et al, 1974), but a great deal of work needs to be done on toxicology, tissue residues and effective dose levels. Likewise, important advances have been made in our understanding of immunology of cestodes, but much remains to be determined. The exploration of host-parasite relationships in laboratory animals is one of the promising avenues which has been pursued in the work reported in this thesis.

Experimental Cysticercosis and Immunity

General characteristics in animal infections

It has been established conclusively that resistance to cysticercosis may occur in the intermediate host as a result of active infection or artificial immunization, and that passive transfer can be achieved with serum or colostrum. Miller and his coworkers (Miller, 1931, 1932, 1935; Miller and Gardiner, 1932, 1934; Miller and Kerr, 1932; and Kerr, 1935) convincingly demonstrated these characteristics for *T. taeniaeformis* and *T. pisiformis*. Campbell (1936, 1938a and b) confirmed most of the results of Miller's earlier work, and resistance to superinfection against *T. pisiformis* was confirmed by Solomon (1934), Nemeth (1970), and Heath (1973b and c). Acquired resistance is now known to occur to *T. saginata* (Penfold et al, 1936; Urquhart, 1958; Soulsby, 1963; Froyd, 1964), *T. hydatigena* (Sweatman, 1957), *T. ovis* (Gemmell, 1962a) and *T. solium* (Herbert and Oberg, 1974). Passive transfer of resistance with serum and colostrum has been achieved against *T. hydatigena* and *T. ovis*

(Blundell-Hassell et al, 1968; Gemmell et al, 1969; Rickard and Arundel, 1974).

In early work on artificial immunization, Miller and most other workers used crudely ground-up worm homogenates. Subsequent workers have focussed their attention on parenteral inoculation of viable eggs and/or activated embryos, and excretory-secretory products released during the *in vitro* culture of metacestodes. Generally water-in-oil adjuvants have been used as vehicles for antigens in these studies. Gemmell (1964, 1965, 1966), using viable eggs and activated oncospheres, successfully immunized sheep against challenge infection with *T. hydatigena*, *T. ovis* and *E. granulosus*. Following studies on the hatching and activation of cestode ova by Silverman (1954), and Silverman and Maneely (1955), great improvements have been made on the *in vitro* culture of taeniid metacestodes. These improvements (Heath and Smyth, 1970; Heath and Eldson-Dew, 1971; Heath, 1971, 1973a) opened a new avenue to artificial immunization against cysticercosis. Antigens produced *in vitro* by oncospheres of *T. ovis* were first shown to be effective when used in vaccinating lambs against challenge infection by Rickard and Bell (1971a). They also showed that larvae of *T. taeniaeformis* and *T. ovis* contained in membrane diffusion chambers and implanted into peritoneal cavities of rats and lambs, respectively, conferred significant resistance against challenge infection (Rickard and Bell, 1971b). Heath (1976) succeeded in immunizing rabbits against *T. pisiformis* with *in vitro* products. Vaccination of calves with activated oncospheres of *T. saginata* also produced a highly significant

resistance against challenge infection (Wikerhauser et al, 1971; Wikerhauser et al, 1974). Their success was followed by that of Rickard and Adolph (1976) in which *in vitro* products plus Freund's adjuvant were used to immunize calves against *T. saginata*.

Attempts to characterize protective antigens have not been carried out to any great extent to date. Campbell (1936, 1939) found that proteinaceous fractions of ground-up worm material provided protective immunity in vaccination trials against *T. taeniaeformis*, whereas polysaccharide fractions were ineffective. Chromatographic fractionation of crude *in vitro* products of *T. pisiformis* (Rickard and Katiyar, 1976) failed to yield any fraction that produced protective immunity. Using gel-filtration on Sephadex G-200, Kwa and Liew (1977) claim to have purified and characterized protective antigens from crude worm homogenates and excretory-secretory products derived from *T. taeniaeformis* metacestodes maintained *in vitro*. They found that the protective activity in both types of antigens resided in a sample with a molecular weight of approximately 140,000, but the criteria of purity and the assessment of immunity in their experiments are less than satisfactory.

Immune mechanisms in Taenia taeniaeformis infection

Protective humoral response

Advances made recently in research in the immunological response of the rat and mouse to *T. taeniaeformis* serve as important indicators for investigations on other cysticercoses. Leid and Williams (1974a) have confirmed the earlier observations of Miller

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and Gardiner (1932, 1934) and Campbell (1938a and b) by showing that serum taken 14, 21 and 28 days after infection with *T. taeniaeformis* passively protected recipient rats against challenge infection. They fractionated 28 day - immune serum and found that the protective capacity resided in the IgG_{2a} immunoglobulins. Subsequent work (Leid and Williams, 1974b) led to the characterization of antibodies in the IgE immunoglobulin class which were also shown to be stimulated during infection with this parasite. These reaginic antibodies appeared at a time when passive transfer of resistance with serum from infected rats was maximal. It was shown that fractions enriched for reagin conferred a highly significant degree of resistance on recipient rats. However, these reagin-enriched fractions contained other immunoglobulins which by themselves, passively protected recipient rats. The exact role that IgE may play in protective immunity not only against cysticercosis but in other helminthic infections remains a matter of controversy (Murray, 1972). Some recent evidence suggests that in cysticercosis IgE-mediated reactions may accelerate humoral antibody attack on challenge organisms (Musoke et al, 1978).

In mice infected with *T. taeniaeformis*, Musoke and Williams (1975a) showed that fractions of immune serum containing both IgG_1 and IgG_2, but not those containing only IgG_2, had protective activity. Hence it appeared that, unlike in the rat where this activity localized in IgG_{2a}, it probably resided in IgG_1 in the mouse. Intraperitoneally implanted cysticerci in the rat also provoked a high degree of resistance to oral challenge with eggs

(Musoke and Williams, 1976), but the protective activity in immune serum obtained from such rats was found to reside mainly in the IgG_1 and IgM antibody classes. Cysticerci implanted intraperitoneally in rats with concurrent hepatic infections were killed whereas those implanted in uninfected animals survived even when immune serum was given simultaneously.

Further studies by Musoke and Williams (1975b) demonstrated that as infection in the rats progressed, the protective activity in their serum was extended to other immunoglobulin fractions. It was also found that hepatic cysticerci were susceptible to antibody up to five days post infection, but thereafter parasites attained insusceptibility both *in vivo* and *in vitro*. The susceptibility prior to the fifth day was shown to be complement-dependent. Colostral antibodies from infected rats were also shown to play a significant role in conferring passive resistance to young rats (Musoke et al, 1975). Immune colostral IgA conferred resistance when fed or when injected into the intestinal lumen, while immune colostral IgG_1 and IgG_2 were effective when given parenterally. These observations as a whole indicate that the protective mechanism may involve distinct antibody types, depending upon the duration and nature of the exposure of the rat to parasite antigens.

Other aspects of host-parasite interactions

It has become increasingly clear that many components of the immune mechanism may be involved in the response to infections with *T. taeniaeformis* although the role of these reactions in the overall host-parasite relationship remains to be clarified.

Ansari and Williams (1976) found that during the course of a primary infection in the rat, there is a peripheral eosinophilia starting in the second week post infection which peaks before the fifth week and thereafter declines. There was a marked eosinophilic infiltration in the liver tissue during the 2-5 week post infection period with a wide zone of eosinophilic cells surrounding the developing larvae. When challenged orally with eggs, infected rats also showed a brisk secondary eosinophilic response within a week. Rats given intravenous doses of immune serum similarly had a secondary eosinophilia within a few hours after an oral challenge with eggs (Ansari et al, 1976). Mast cell numbers also increase significantly in the tissues of rats with *T. taeniaeformis* and their increased numbers may be important in IgE-mediated reactions to challenge (Cook and Williams, 1978).

Hustead and Williams (1977a) showed that *T. taeniaeformis* larvae are capable of absorbing a variety of proteins, and that the absorbed macromolecules retain their structural and functional integrity following transport. Host serum immunoglobulins were taken up both *in vitro* and *in vivo*, and were shown to retain both antigen binding capacity and biologic functions associated with the Fc regions. By employing radiolabelled macromolecules it was further shown that the rate of transportation of these molecules into the metacestode was increased by the presence of both antibodies and complement (Hustead and Williams, 1977b). *In vitro* the larvae were shown to deplete functional complement levels in the surrounding medium, thus restoring the normal permeability control.

Further investigations by Hammerberg and Williams (1978a) showed clearly that parasite larval products interacted with complement *in vitro* and *in vivo*. Cyst fluids and *in vitro* products from *T. taeniaeformis* larvae consumed complement by both the alternate and classical pathways, cleaved C3 and generated anaphylatoxin *in vitro*, depressed circulating hemolytic complement activity and caused alterations in vascular permeability *in vivo*. The anticomplementary substances were shown to be present in other cestodes too (Hammerberg et al, 1976, 1977; Herd, 1976; Kassis and Tanner, 1976). Physico-chemical characterization of these complement-interacting factors from *T. taeniaeformis* larvae (Hammerberg and Williams, 1978), suggested that they are sulfated polysaccharides or proteoglycans composed of carbohydrate, protein, sulphate and hexosamine, free of sialic and uronic acids and heteregeneous in net negative charge but resistant to proteolysis.

The occurrence of intact and biologically functional host immunoglobulins, including those of the IgG class that have been shown to be protective, in the cyst fluids of *T. taeniaeformis* larvae leaves several questions unanswered. How do these molecules get there and what is their function once in the cystic fluids? Transportation of macromolecules across the cestode membrane has been suggested to be by simple diffusion (Varela-Díaz and Coltorti, 1972) possibly through tegumental pores. Ultrastructural investigations on a variety of taeniid metacestodes have not revealed evidence of pores that might serve this function (Morseth, 1966; Bortoletti and Ferretti, 1971, 1973; Lascano et al, 1975). Ultrastructural and functional analogies have been drawn between the

tegument of larval cestodes and intestinal epithelium of neonatal mammals (Beguin, 1966; Slais, 1966). Pinocytosis has been postulated by Brambell (1966) and Rodewald (1973) to be the process by which proteins are specifically taken up by the neonate intestinal epithelial cells. This process may account for the translocation of macromolecules across the tegument of taeniid metacestodes, and recent evidence suggests that this is the case in *T. taeniaeformis* (Picone, 1977). Whether host macromolecules in the cyst fluids serve in osmoregularity control (Dixon et al, 1973), provide a source of nutrients for the parasite or are there for some mutual benefit to host and parasite is not clear. There is presently no direct evidence to support any proposal.

From the foregoing it is possible to construct a picture of the sequence of events which follow infection in the rat. Some aspects of this picture have been established experimentally while others are susceptible to future investigation. It appears that the parasites rapidly stimulate a complement-dependent humoral antibody protective mechanism which protects the host against further challenge. During and after oncospheral reorganization, the metacestode evolves a mechanism of elaborating and releasing anticomplement factors. *In vivo* their effects are exerted locally around the cyst by a non-immunological triggering of complement consumption through both the classical and alternate pathways. There is no marked inflammatory process around the metacestodes after the first several weeks of infection when eosinophils predominate. It is possible that the host enzymes deactivate the anaphylatoxins locally leading to reduced inflammatory activity.

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There is histochemical evidence of release of proteases by *T. taeniaeformis* in situ (Lewert and Lee, 1957). Other factors released by the parasites may serve to affect, directly or indirectly, the differentiation, infiltration, and function of inflammatory cells such as eosinophils and mast cells. The net result is prolonged survival of the larvae, without local rejection, in hosts which can mount an immediate and highly effective rejection of new challenge organisms.

The character of the antigens which provoke this protective response remains to be seen. The response itself probably occurs at the level of the gut at least in part. IgE-sensitized mast cells living in the gut wall may accelerate the process, or enhance access of complement and protective antibody to the site of invasion.

It seems possible that local gut-related immune reactions may occur which are not detectable in experiments on parenteral transfer of systemic antibody or cells. This aspect of cysticercosis has been little studied, but in recent years the nature of immune events in the intestine has been characterized in many ways which are relevant to this discussion of taeniiasis, and some of these are reviewed below.

Intestinal lymphoid tissue and the immune response

Gut-associated lymphoid tissue

The gastro-intestinal epithelium is exposed to a wide variety of potential antigens. These include dietary and food contaminant antigens, microorganisms and drugs. It is not surprising therefore

that portions of the gut wall are well endowed with lymphoid and other elements capable of immunological response. In most of the mammalian species studied, these elements have a similar pattern of organization in the intestinal wall, the major portion being made up by the so-called "Gut Associated Lymphoid Tissue" (GALT).

From 10 to 15 percent of cells within the intestinal epithelium are thelio-lymphocytes, a term introduced by Fichtelius (1968). Ultrastructurally these cells resemble lymphocytes, but they contain granular inclusions reminiscent of mast cells (Bienenstock, 1974). Their origin is unclear. Bienenstock (1974) suggests that they are derived from mast cells, and Guy-Grand, et al (1974) are of the opinion that they are of T cell origin. Their function is unknown, although their resemblance to lymphocytes and their strategic position in the epithelium suggest that they may have a role to play in the local immune response.

In the lamina propria below the epithelium are numerous cells including lymphocytes, plasma cells, macrophages, eosinophils and mast cells. About 80 percent of the plasma cells contain IgA, 15 percent IgM and 3 percent IgG. IgE, and in some species, IgD-containing plasma cells are present in lesser numbers (Jones, 1972). Within the lamina propria are found organized lymphoid tissues, the "Peyer's patches". They are composed of three structural elements; the domes, the follicles and the thymus-dependent areas (Waksman, 1973). The follicles are found below the domes and the thymus-dependent areas in between the follicles. Towards the lumen the domes are covered by a specialized epithelium which has pinocytotic capabilities (Bockman and Cooper, 1973).

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Currently it is suspected that Peyer's patches function in the immune response after antigenic material enters through the pinocytotic cells overlying the dome. The antigens then interact directly or indirectly with B lymphocytes, inducing them to proliferate and triggering them into terminal differentiation. These cells make their way into the draining lymphatics and hence into the blood stream, from whence they preferentially 'home' to the intestinal lamina propria and mature into antibody-secreting plasma cells (Bienenstock, 1974; Cooper et al, 1974).

Immunoglobulins in intestinal secretions

Some of the immunoglobulins produced by these plasma cells are detectable in secretions, though others cannot be found, perhaps because of their susceptibility to proteolytic degradation. IgG, IgD, and IgE fall into this category. IgM on the other hand, can be present in active form in secretions and may be protected by the addition of secretory polypeptide chains, similar to IgA, which is the most abundant immunoglobulin in the lumen. In the human IgA forms about 13 percent of total serum immunoglobulins (Roitt, 1974). It occurs in a variety of forms - monomeric, dimeric or higher polymers. The polymers including the dimer are held together by a J (joining) chain. The dimer which is the main one found in the intestinal lumen is thought to be secreted by a special mechanism (Heremans, 1974). The monomers are assembled in the lamina propria plasma cells in which the J chain, also produced in these cells, is used to join them into a dimer. The dimer then passes into the epithelial cells by an as yet

undetermined means (Threadgold, 1967; Allen et al, 1973). The dimer with J chain is then combined with yet another molecule, the secretory component, formed in the epithelial cells (Brandtzaeg, 1974; Roger and Lamm, 1974). This secretory IgA (S-IgA) is then released into the intestinal lumen by an unknown mechanism. It has been suggested that the secretory component confers resistance to proteolysis on S-IgA (Tomasi and Grey, 1972). When IgA is deficient, IgM producing cells may increase and secretory IgM in the lumen may compensate for the lack of secretory IgA (Brandtzaeg et al, 1968).

Immunization or infection leads to an increased synthesis of immunoglobulins at the local level. Intestinal stimulation also leads to the development of circulating antibodies (mainly IgG and IgM), and these may then enter the intestinal lumen. Whether their origin is local or circulatory, the question remains as to the protective function of antibodies in the intestinal environment.

Through properties such as neutralization, agglutination, precipitation or opsonization, (Nossal and Ada, 1971) antibodies generally serve to identify or recognize antigens for subsequent destruction by effector systems which may involve phagocytic cells, complement cascade, etc. It has been suggested that IgA may play a rather different protective role in the gut lumen by reacting with bacteria and preventing their adherence onto the intestinal mucosa, and hence interfering with successful colonization (Freter, 1972; Fubara and Freter, 1973; Gibbons, 1974). Walker and Hong (1973) conducted studies with soluble antigens and found

that in orally immunized animals, subsequent administration of homologous antigen resulted in reduced uptake. It was later shown that this reduction was due to enhanced degradation of antigen within the intestinal lumen (Walker et al, 1974). An additional hypothesis, then, is that IgA combines with soluble antigen in the lumen and prevents its uptake, leading to enhanced enzymatic degradation.

Although complement components may occur in the intestinal secretions, complement fixation does not occur in the lumen (Mestecky and Lawton, 1974). IgA antibodies do not ordinarily fix complement, and effector mechanisms involving either the classical or alternate pathways are not considered likely to be important in the intestinal lumen. It has, however, been shown that secretory IgA is bacteriocidal in presence of complement and lysozyme (Adinolfi et al, 1966; Hill and Porter, 1974). Secretory IgA also agglutinates efficiently and may opsonize antigen for phagocytosis (Bienenstock, 1974), but agglutinated or neutralized antigen would be quickly disposed of by peristalsis or proteolysis, so that phagocytosis does not seem to be an essential part in the intestinal immune system.

Intestinal immune responses to antigens and infections

Crabbe, et al (1969) showed that immunization of mice with ferritin orally resulted in IgA producing cells in the intestine and exclusively IgA antibodies in the serum. Parenteral immunization resulted in IgM and IgG antibodies in the serum and IgA producing cells in the intestine. Rothberg, et al (1973) suggested that oral immunization of rabbits with bovine serum ablumin (BSA) was

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followed by local antibody production and a gradual sensitization of systemic lymphoid tissue by the dissemination of sensitized lymphocytes. In later studies (Rothberg et al, 1974) they further suggested that, as well as antigen-reactive cells, systemic lymphoid tissue could be sensitized by immunogenic concentrations of absorbed antigens. There is now plenty of evidence that local immune mechanisms operate in the intestine in response to some viral, bacterial and helminth infections.

Viral

Studies on poliovirus (Ogra et al, 1974) showed that only after oral immunization did significant antibody titers occur in the nasal and intestinal secretions. This antibody was entirely IgA and was considered to protect by preventing the poliovirus from crossing the nasopharyngeal and gastrointestinal mucosae into the circulation. Parenteral immunization, in contrast, produced predominantly IgG with some IgM and IgA, but in the serum.

Recent experiments with an attenuated strain of Transmissible Gastroenteritis Virus have indicated that it is possible to immunize newborn pigs orally with the strain and confer resistance to a subsequent challenge with a virulent strain (Furuuchi et al, 1976).

Bacterial

Extensive work has been carried out on the immune response to cholera in both laboratory animals and humans. Freter (1956) was able to confer protection against fatal enteric cholera in guinea pigs by active oral immunization using heat killed *Vibrio cholerae*. Passive immunization was achieved by oral dosing with antibody from vaccinated donors. In human volunteers (Freter

and Gangaraso, 1963) a heat killed oral *V. cholerae* vaccine induced formation of locally produced coproantibody in the intestinal tract. Oral immunization resulted in production of both local and systemic antibodies in humans given an apathogenic strain of *V. cholerae* (Sanyal and Mukerjee, 1969), and in humans and rabbits given lysates of L-forms of *V. cholerae* (Agarwal and Ganguly, 1972a and b). The predominant locally produced antibodies (coproantibodies) were of the IgA class, although IgM and IgG also occurred (Fubara and Freter, 1972a and b; Shimamura, 1972). Fubara and Freter (1972a, 1973) have demonstrated protective immunity to cholera with secretory IgA, and Pierce and Reynolds (1974) showed that humoral IgG antitoxin conferred protection in passive immunization. To be effective against the cholera infection protective antibodies must be antitoxic as well as antibacterial to prevent the toxin effect and reduce colonization of the gut by the vibrios. Exactly what roles locally and systemically produced antibodies play after oral exposure remains to be clarified (Tomas and Grey, 1972). Recently Agarwal and Sundararaj (1977) have provided evidence that cell-mediated immunity occurs after oral immunization of rabbits with Ribonucleic Acid-Protein fractions of *V. cholerae*.

Escherichia coli antigens as dietary additives for oral immunization of pigs (Porter et al, 1973) have been shown to improve the performance of piglets. Further detailed studies by Porter, et al (1974) showed that the antibody activity in the locally produced secretions in the intestine of piglets was predominantly associated with IgA, although IgM and IgG were also

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detectable. Lysozyme, but not complement, was present in the secretion leading to the suggestion that bacteriostasis was the most likely antibacterial mechanism attributable to the IgA. A single dose of antigen followed by a second one 3 to 4 weeks later did not result in increased antibody secretion, suggesting a lack of memory in the secretory immune system. Repeated antigen administration for up to 3 weeks was necessary to induce maximal numbers of antibody producing cells in the lamina propria.

Helminth Infections

It has been convincingly demonstrated that many parasitic gastrointestinal helminths evoke immune responses which are manifested in the gut of their hosts. These responses have been claimed to result in effects such as reducing the reproductive capacity of worms, and/or stunting and/or expulsion of parasites. Only a brief mention of some examples will be made here.

Among the nematodes, the immune mechanism leading to the expulsion of *Nippostrongylus brasiliensis* has been the most extensively examined. It is now generally accepted that this expulsion involves three components of the immune response. The worms are first damaged by antibodies (Jones and Ogilvie, 1971) and they become susceptible to an expulsive step which is dependent upon both lymphoid and myeloid factors (Keller and Keist, 1972; Dineen et al, 1973; Dineen and Kelly, 1973; Kelly et al, 1973). Rothwell and Merritt (1974) have demonstrated IgA and IgM antibodies against *Trichostrongylus colubriformis* in serum of infected sheep, and thymus-dependent lymphocytes were shown to play a role in the resistance of guinea pigs to this nematode (Dineen and

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Adams, 1971). Both humoral and cell-mediated responses against *T. colubriformis* have since been conclusively demonstrated (Dobson and Soulsby, 1974) and histamine and 5-hydroxytryptamine have been shown to participate in its expulsion in the guinea pig (Rothwell et al, 1974). IgA and IgG antibodies have been demonstrated in the gut extracts of rabbits infected with *Trichinella spiralis*, whereas the serum contained IgM antibodies in addition (Crandall et al, 1967; Crandall and Crandall, 1972). The gut IgG and the serum IgG and IgM antibodies were shown to be specific for *T. spiralis*. In mice infected with *Hematospiroides dubius*, IgM, IgA and antibodies of the IgG class occurred in the serum but only IgG antibodies were detected in the intestine (Crandall et al, 1974).

Among the cestodes, immune mechanisms have been implicated in the resistance of birds to secondary infections with *Raillietina cesticillus* (Gray, 1973) and in the successful immunization of the dog against *E. granulosus* (Turner et al, 1933; Gemmell, 1962b; Herd and Chappel, 1975). Most studies on adult cestodes have been done on *Hymenolepis* species, particularly *H. nana*, *H. diminuta* and *H. microstoma*. In his recent studies Befus (1975) has demonstrated antibody responses to experimental infections with *H. diminuta* and *H. microstoma* in the mouse. By immunofluorescence techniques, he showed that the teguments of these worms are covered with IgA, IgG, IgG_2 and IgM. C3 was also found to be fixed to the tegument of *H. diminuta*. Correlating a number of factors he argues that these immunoglobulins are in fact, specific antibodies. These factors include the time of appearance of the immunoglobulins

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and the intensity of the infection, the rate of their accumulation on the worms and the surface area they cover. Even if the antibodies were specific their role in the immune events harmful to the worms remains to be clarified. The possibility that they represent nutrient material in the process of being taken in through the tegument cannot be discounted.

There is no direct evidence of immune responses to cestode larvae occurring at the gut level. Unlike the lumen dwelling nematodes and cestodes these larvae rapidly penetrate the intestinal epithelium by the combined action of their penetrating glands and hooks (Silverman and Maneely, 1955; Banerjee and Singh, 1969a and b; Heath, 1971). The time taken to complete the penetration of the intestinal wall and to reach the site of predilection will most likely vary with each taeniid larva. For *T. taeniaeformis* penetration of the villi is complete within 15 minutes, and in 3 to 4 hours the parasites reach the liver (Banerjee and Singh, 1969a). If any mechanism is to affect these larvae at the gut level, be it specific or non-specific, immunological or non-immunological, it has to operate in the gut lumen or in the gut wall. Gut level immunity to taeniid larvae was proposed to occur in rabbits immunized against *T. pisiformis* by Leonard and Leonard (1933) but substantive evidence in support of this has not yet been developed. A gut phase of resistance was considered by Banerjee and Singh (1969c) to be present in rats infected with *T. taeniaeformis*. The oncospheres given subsequent to primary infection showed very reduced migration through the intestinal tissues.

Immune exclusion by the gut and orally induced systemic tolerance

Initial presentation of some antigens through the gastrointestinal tract has been observed to alter significantly the response of the organism to a subsequent exposure. Thomas and Parrot (1974) showed that the ability to mount a humoral antibody response to bovine serum albumin (BSA) was significantly lowered in rats which had been fed BSA previously. In these experiments, they were unable to demonstrate anti-BSA producing cells in any lymphoid tissue, including that of the lamina propria, and no antibody activity could be detected in the intestinal juice or feces. Recently, David (1977) showed that rats which had been fed ragweed or horse serum developed specific unresponsiveness to anaphylactic sensitization. They did not form IgE antibodies and maintained a normotensive response to intravenous challenge with antigen.

Prefeeding Ovalbulmin followed by homologous intragastric challenge in mice resulted in a lowered amount of ovalbumin reaching the circulation, and there was no accompanying increased clearance (Swarbrick et al, 1977). It was concluded that antigen feeding induces immune exclusion by the gut. These mice also showed a reduced antibody response when subsequently challenged with ovalbumin parenterally. The phenomena of immune exclusion and orally induced systemic tolerance seem to be simultaneously induced.

Both mechanisms are antigen specific (Thomas and Parrott, 1974; David, 1977). Administration of small but frequent doses of the antigen leads to a more complete state of unresponsiveness

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than feeding of large amounts at longer intervals. David (1977), in his experiments, suggests that age is also an important factor in induction of tolerance. Termination of antigen feeding for 2 months restored the ability to become anaphylactically sensitized.

To explain immune exclusion Walker, et al (1974) have proposed an appealing hypothesis where IgA antibodies combine with antigens preventing their uptake. It has further been suggested that such IgA antibodies blanket normal flora and food antigens protecting them from other immune responses. On the other hand the induction of systemic tolerance has been difficult to explain. Thomas and Parrott (1974) have suggested two explanations: firstly they suggest that antigens may be formed of both immunogens and tolerogens which are separated from each other by the gut, with the immunogens producing a local immune response in the wall of the gut and the tolerogens being absorbed to produce tolerance at more distant sites. Secondly, they feel that both immunogens and tolerogens may be absorbed, but that the liver sequesters the former, allowing the latter to exert their effect.

Pathophysiological disturbances have also been observed by feeding soya protein in calves and piglets (Barrett et al, 1977). Whereas there was no evidence of tolerance, intestinal biopsies showed that there were morphological disturbances following soya protein ingestion. In orally sensitized animals inhibition of peristaltic flow occurred in Thiry-Vella loops into which soya antigen was perfused.

It is clear from the above discussion that the response of an animal locally and systemically to antigens presented via the

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gut depends on many factors which need further investigation. The species of the animal and its age, the nature of the antigen (Bernstein and Ovary, 1968) and the period of exposure to antigen all seem to be important. Further research will undoubtedly reveal some more factors. David (1977) suggested that the potential of feeding antigen as a prophylactic measure in the management of some allergic diseases should not be ignored. The potential for immunization against infectious micro- and macro-organisms via the gastrointestinal route is even greater. It appears that each antigen/animal relationship merits special considerations of factors that will favor a desired response.

Adjuvants and helminth infections

Adjuvants are substances which potentiate the immune response to antigens, and they have been used in much of the experimental work on artificial immunization of animals against helminths with materials extracted from or secreted by parasites. The selection and route of administration of adjuvants for these studies appear to have been arrived at empirically, rather than being based on specific characteristics which are known and desirable. For the most part, Freund's complete adjuvant has been used, often without adequate controls for the effects of water-in-oil emulsions on non-specific host resistance mechanisms. Consequently, it is difficult to interpret the results of such vaccination trials, and in the event that significant protection was produced it is hard to determine what the effect can be attributed to, or what the contribution of the adjuvant was.

There are many substances which are known to have adjuvant activity, including microbial products, nucleic acids, certain minerals, emulsions and even vitamins (Jollés and Paraf, 1973; World Health Organization Technical Report, 1975). Two levels of adjuvant activity may be recognized. At the antigen level the adjuvant may induce conformational changes at specific sites, transform a non or poorly immunogenic molecule into a strongly immunogenic one and lower the catabolic rate of the antigenic molecule. At the host level the activity is directed at both specific immunocompetent cells and non-specific mechanisms, such as those regulating protein synthesis and cell multiplication.

Among the most widely used adjuvants for experimental work are aluminum compounds (including aluminum phosphate, aluminum hydroxide and aluminum oxide), Freund's incomplete and Freund's complete adjuvant which are water-in-oil emulsions, and *Bordetella pertussis*. The aluminum compounds and the Freund's adjuvants form a slow-releasing repository of antigen at the injection site. Antibody-producing plasmacytes form in the draining lymph nodes and around the local granuloma as it develops. In addition to the adjuvants acting as vehicles for antigens, macrophages ingest both antigen and adjuvant material and help disseminate them to lymphatic tissues further away, elevating the overall immune response (Freund, 1953, 1956; White 1967; Allison, 1973; World Health Organization Technical Report, 1975). *B. pertussis* activates macrophages and is a strong lymphocytosis inducer (Dresser et al, 1970; Maillard and Bloom, 1972; Allison, 1973; Finger, 1974; World Health Organization Technical Report, 1975). Light and

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electron microscopic studies have shown that the effectiveness of emulsions as immunological adjuvants depends on the anatomical associations formed by their components (Dvorak and Dvorak, 1974). It is also now evident that the adjuvant effects of microorganisms are due to their subcellular or even molecular components. Wax D (or its derivatives) is thought to be one of the active portions in most mycobacteria (Freund, 1953, 1956; White, 1967). Mota, et al (1974) showed that the adjuvant activity of *B. pertussis* is in a lipopolysaccharide fraction. A histamine-sensitizing factor isolated from *B. pertussis* (Lehrer et al, 1974, 1975, 1976) has also been found to possess adjuvant activity.

In the clinical practice of active immunization against infection adjuvants have found their greatest use in vaccines for diseases caused by microorganisms, especially bacteria and viruses. There are presently no vaccines involving adjuvants for active immunization against helminth infections, although some promising findings have been reported. Silverman, et al (1962), using aluminum hydroxide, were able to enhance the protective ability of *in vitro* antigens from *Dictyocaulus viviparus* and *Trichostrongylus colubriformis* in guinea pigs and from *Strongyloides papillosus* in rabbits. Freund's adjuvant was successfully used in similar vaccinations against *Ascaris lumbricoides* and *A. suum* in the guinea pig (Soulsby, 1957, 1963).

Immunization against *T. ovis* with *in vitro* products and Freund's complete adjuvant has been achieved in lambs (Rickard and Bell, 1971; Rickard et al, 1976) and against *T. saginata* in calves (Rickard and Adolph, 1976). However, no vaccination

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experiments have been done in which adjuvant was not used, and there is no indication of the site at which the protective response operates. Kwa and Liew (1977) employed Freund's adjuvant in immunization against *T. taeniaeformis* in the rat using both somatic and excretory-secretory antigens, but the effect of the adjuvant in their experiments is not clear. Varela-Diaz, et al (1974) and Musoke and Williams (1976) have shown that Freund's adjuvant alone may significantly reduce the survival of implanted taeniid metacestodes in rodents, and similar results have been obtained in schistosomiasis (Capron et al, 1969). Clearly, a more detailed examination is necessary of the role of adjuvants in enhancing the development of protective immune mechanisms against helminths, and especially taeniid cestodes, where artificial immunization has great practical potential.

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ARTICLE

THE IMMUNOLOGICAL RESPONSE OF THE RAT TO INFECTION WITH TAENIA TAENIAEFORMIS VII. IMMUNIZATION BY ORAL AND PARENTERAL ADMINISTRATION OF ANTIGENS.

The immunological response of the rat to infection with *Taenia taeniaeformis* VII. Immunization by oral and parenteral administration of antigens

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Summary

Rats immunized with *in vitro* (IVP) products and saline soluble antigens (SSA) derived from *Taenia taeniaeformis* were found to be significantly protected against challenge infection. Oral and intraperitoneal administration of antigen solutions alone was effective in stimulating resistance. However, adjuvants were required for successful immunization when single doses of antigens were inoculated intramuscularly. *Bordetella pertussis* and aluminum hydroxide were able to improve markedly the protective effects of antigens given parenterally by either route, but Freund's complete adjuvant (FCA) was not effective as an adjuvant in this system. Reaginic antibodies to parasite antigens were detected in the serum of rats vaccinated with IVP or SSA and *B. pertussis* or $Al(OH)_3$, but none were detected in those given FCA. The possible role of reaginic antibodies in immunity to *T. taeniaeformis* is discussed.

Single doses of antigens given orally produced significant protection. Increasing the number of daily doses of antigen administered orally enhanced the degree of protection to a limited but significant extent. However, there did not appear to be any advantage to giving large doses (>1 mg protein) of antigen, or extending the immunizing schedule over several weeks. Reaginic antibodies were not detected in the serum of rats immunized orally, but these animals were resistant to both oral and intravenous challenge infection with parasites. These observations are discussed in relation to the phenomena of immune exclusion of antigen

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Serum from rats immunized by all routes was found to be ineffective in conferring resistance upon recipients when given in quantities up to 1.5 ml per rat. Furthermore, serum from donors vaccinated intramuscularly with saline soluble antigens and *B. pertussis* increased the susceptibility of recipient rats to infection with *T. taeniaeformis*. This is in sharp contrast to our previous experiences in which we have shown that serum from rats with an active infection is highly effective in passive transfer. Possible reasons for these observations are discussed. The requirements for adequately controlled immunization procedures to assess the contributory effects of adjuvant type and the route of antigen inoculation in immunizing against taeniid infections are emphasized in the discussion. The potential for vaccination per os against other helminthiases is also raised.

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INTRODUCTION

It has been known for many years that rats can be successfully immunized against challenge infections of *Taenia taeniaeformis* with inoculations of homogenates or extracts of dead metacestodes (Miller, 1931; Campbell, 1936). Comparable results have been obtained with the rabbit-*T. pisiformis* system (Miller and Kerr, 1932; Kerr, 1935; Heath, 1976), but in domesticated ruminants artificial immunization schemes for cysticercosis have generally been most promising when animals were exposed to living parasites, which were either inoculated at abnormal sites or implanted in diffusion chambers (Gemmell, 1964, 1965a,b, 1966; Gemmell et al, 1968; Wikerhauser et al, 1971, 1974; Rickard and Bell, 1971a). However, recent advances in the *in vitro* cultivation of cestode larvae have lead to the development of an experimental vaccine for sheep which consists of taeniid parasite antigens collected in culture media (Rickard and Adolph, 1976; Rickard et al, 1976), and the potential for application of this procedure in cattle is currently under field test (Rickard, personal communication).

Despite these successes very little is known about the nature of protective immunogens of taeniid helminths or the responses which they provoke in vaccinated animals. The rat-*T. taeniaeformis* model provides an excellent opportunity for study of these characteristics, and Kwa and Liew (1977) have described the partial purification of a potent immunizing factor in metacestodes of this species. We report here our observations on the influence of antigen source, route of administration and adjuvant selection on the efficacy of immunization against *T. taeniaeformis*. Protective antigens

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were detected in parasite extracts and in *in vitro* culture products, and these produced highly significant immunity to challenge, even when they were administered orally. *Bordetella pertussis* was an effective adjuvant in potentiating the immune response, and the results suggest that the mechanism of resistance in vaccinated animals is different from that which develops in rats which become immune following active infection.

MATERIALS AND METHODS

Experimental Animals

Female 21 day old Spartan [Spb(SD)BR] rats were used for the experiments, except where specified. They were purchased from Spartan Research Animals, Haslett, Michigan. The animals were given proprietary brand food and water *ad libitum*.

Preparation of Antigens

i) *In Vitro* Products (IVP)

Rats infected with *T. taeniaeformis* for over 3 months were killed with CO_2 vapour. The metacestodes were carefully dissected from hepatic cysts and washed three times each in triple distilled water and sterile physiological saline. Fifty strobilocerci were placed in culture vials each containing 150 mls of Hank's BME (Grand Island Biological Co., Grand Island, New York) with 1000 µg/ml of streptomycin, 1000 U/ml of penicillin, 500 U/ml of polymixin B and incubated at 37°C. After 24 hrs the culture medium was collected, dialysed for 48 hrs against triple distilled water and then for 12 hrs against phosphate buffered saline (PBS).

The medium was then concentrated by either negative vacuum dialysis, or by dialysis against polyethylene glycol (Carbowax, Union Carbide). When concentration was by the latter method, the medium was further dialysed against PBS for 48 hrs. The protein content per ml was then determined. These *In Vitro* Products (IVP) were then frozen and kept at -20°C until used.

ii) Saline Soluble Antigens (SSA)

Mature strobilocerci obtained as described above, were ground in PBS in a glass mortar. The ground material was left to stir for 12 hrs at 4°C after which it was centrifuged at 50,000g for 2 1/2 hours. The supernatant was carefully withdrawn, its protein content per ml determined and then stored at -20°C until used.

Protein Determination

The protein concentration of the IVP and the SSA was determined by the Folin-Ciocalteau reaction (Williams and Chase, 1968), a modification of the method of Lowry, et al (1951).

Adjuvants

Freund's complete adjuvant (FCA, Difco, Detroit, Michigan) was emulsified with an equal amount of SSA or IVP when used in the intramuscular immunizations.

Where *B. pertussis* (BP) vaccine cells (kindly supplied by the Michigan Department of Public Health, Lansing, Michigan) were used as an adjuvant, a suspension was prepared such that 1 ml contained 1×10^{10} organisms and 1 mg of the appropriate parasite antigen.

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Homologous passive cutaneous anaphylaxis (PCA)

PCA tests were carried out as described by Leid and Williams (1974a). The backs of retired female breeder rats were shaved and 0.1 ml quantities of undiluted or doubling dilutions of appropriate serum were injected intradermally. 72 hours later the rats were challenged intravenously (IV) with the antigen to be tested. Each rat received 1 ml containing 1 mg of the antigen, after the latter had been mixed in a 1:1 ration with Evans blue. Reactions were read 15 to 30 minutes after challenge. Any reaction showing intense blueing of more than 3 mm in diameter was classified as positive. Negative and positive sera were included in each recipient and two rats were used for each sample tested. The negative serum was obtained from normal rats, and the positive one from rats that had been harbouring an active *T. taeniaeformis* infection for at least five weeks.

General Experimental Procedure

Groups of rats were vaccinated in various ways (see results) at 21 days of age. Three weeks later they were challenged with a dose of 250 eggs obtained from *T. taeniaeformis* and counted as described by Leid and Williams (1974a). After an additional three weeks the rats were killed with CO_2 vapour, their livers dissected out of the abdominal cavities and the number of hepatic cysts was counted: Further procedural details are described with each experiment.

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Analytical Assessment of Resistance

The number of cysts per liver was transformed into $\sqrt{y+0.5}$, where y = number of cysts. This transformed data was then analyzed by either the Bonferroni t procedure (Miller, 1966) or the modified Scheffé's method (Gill, 1977). The former was used when the variance of all groups in a single experiment was homogeneous, and the latter when it was heterogeneous. These methods permitted an unlimited number of comparisons or contrasts to be made between two or more groups. For both types of analyses, P values of only 0.05 and 0.01 can be determined from tables currently available. The two values were considered significant, and highly significant respectively. The data given in the tables are the results of a single experiment in each case, but unless otherwise indicated the statistical significance of the differences between groups was the same in replicate experiments.

RESULTS

In the preliminary experiments groups of six rats were immunized intramuscularly (IM), per os (PO) and intraperitoneally (IP). Each rat received not more than one mg of antigenic protein material in a ml of fluid. Intramuscular vaccination was achieved by dividing the dose of antigen into four equal portions, and injecting these into the muscles of each of the four limbs on the first day of each experiment. For intraperitoneal and oral vaccination, the antigen dose was given in a series of equal daily portions for five days.

Oral and intraperitoneal vaccination with IVP conferred

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significant resistance against subsequent challenge (Groups B and C; $P < 0.05$ and $P < 0.01$, respectively - Table 1a). Unexpectedly, immunization with IVP in FCA (group A) did not confer any significant resistance as compared to the untreated control (group D). No effect on resistance was shown with intramuscular FCA alone, or intraperitoneal PBS (groups of E and F).

Highly significant resistance resulted from immunization with SSA in both oral and intraperitoneal routes (groups B and C; $P < 0.01$, Table 1b). Vaccination with SSA in FCA, FCA alone intramuscularly, and PBS intraperitoneally did not result in any resistance (Groups A, E and F - Table 1b). In both experiments (a and b) the rats in the FCA control groups (E) had a higher average number of parasites than did the untreated control groups (D), although this was not statistically significant in either case.

In a subsequent experiment, intramuscular administration of the antigens conferred no significant resistance, although when combined with *B. pertussis* they stimulated a highly protective immunity (groups A, $P < 0.01$ Table 2a,2b). The groups given *B. pertussis* alone intramuscularly and BME orally (E and G) were not significantly different from the untreated control group (D) (Table 2a).

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Table 1. Immunization of rats against *T. taeniaeformis* with IVP and SSA using FCA

a.) IVP

Group and Treatment	Group A IM+FCA	Group B PO	Group C IP	Group D Untreated control	Group E FCA	Group F IP-PBS
Mean No. of parasites and range ( )	33.5 (16-63)	3.7 (0-13)	1.3 (0-4)	32.8 (17-60)	50.5 (16-64)	41.8 (27-75)
Contrasts and P Values	A vs D, NS	B vs D, $P<0.05$	C vs D, $P<0.01$		E vs D, NS	

b.) SSA

Group and Treatment	Group A IM+FCA	Group B PO	Group C IP	Group D Untreated control	Group E FCA	Group F IP-PBS
Mean No. of parasites and range ( )	24.5 (5-53)	3.8 (2-7)	2.0 (1-4)	35.5 (26-50)	49.7 (24-70)	35.8 (21-58)
Contrasts and P Values	A vs D, NS	B vs D, $P<0.01$	C vs D, $P<0.01$		E vs D, NS	F vs D, NS

NS = Not Significant

Table 2. Immunization of rats against *T. taeniaeformis* with IVP and SSA using *B. pertussis* as adjuvant

Table 2. Immunization of rats against *T. taeniaeformis* with IVP and SSA using *B. pertussis* as adjuvant

a.) IVP

Group and Treatment	Group A IM+BP	Group B PO	Group C IP	Group D Untreated Control	Group E BP	Group F IM	Group G PO BME
Mean No. of parasites and range ( )	1.0 (0-2)	11.7 (4-15)	1.7 (0-5)	36.2 (17-51)	37.2 (28-45)	38.7 (24-48)	29.7 (26-71)
Contrasts and P Values	A vs D, P<0.01	B vs D, P<0.05	C vs D, P<0.01				

b.) SSA

Group and Treatment	Group A IM+BP	Group B PO	Group C IP	Group D Untreated Control	Group E PB	Group F IM	Group G PO BME
Mean No. of parasites and range ( )	3.3 (0-9)	14.8 (3-25)	13.7 (2-33)	33.0 (24-41)	ND	21.8 (15-24)	36.5 (20-66)
Contrasts and P Values	A vs D, P<0.01	B vs D, P 0.05	C vs D P<0.01			F vs D, NS	

*ND = Not done NS = Not Significant

B. pertussis was also found to be effective as an adjuvant when given by the intraperitoneal route (Table 3). Whether in combination with *B. pertussis* or not, single doses of both antigens stimulated highly significant resistance (groups A,B,D, and E, $P < 0.01$), as compared to the control rats (group G). However, the levels of protection conferred when the antigens were combined with *B. pertussis* adjuvant and given intraperitoneally (groups B and E) were themselves significantly different ($P < 0.01$) from those obtained with antigen alone (groups A and D). Intramuscular immunization with *B. pertussis* as an adjuvant (groups C and F) also conferred high levels of protection ($P < 0.01$).

Aluminum hydroxide [$Al(OH)_3$] was almost as effective as *B. pertussis* as an adjuvant, when given intramuscularly using both antigens (Table 4). Significant ($P < 0.05$) and highly significant ($P < 0.01$) resistance was obtained with the SSA and the IVP respectively (groups C and F), compared to the untreated controls (group H). As before FCA was ineffective (groups A and D), whereas *B. pertussis* was highly effective in enhancing the protective ability of the antigens (groups B and E; $P < 0.01$).

It was also found that rats which developed resistance to challenge infection (groups B,C,E, and F - Table 4) had detectable levels of circulating homocytotropic antibody beginning at the second week post-vaccination. Antibodies detectable by PCA were present 21 days post-vaccination at which time the rats were challenged. The titres of the antibody were depicted in figure 1.

The development of resistance following oral administration of antigen was further investigated in order to establish the most effective dosing regimen.

Table 3. Immunization of rats against *T. taeniaeformis* with SSA and IVP. Effect of *B. pertussis* when given with antigen by the intramuscular or intraperitoneal route.

Table 3. Immunization of rats against *T. taeniaeformis* with SSA and IVP. Effect of *B. pertussis* when given with antigen by the intramuscular or intraperitoneal route.

Group and Treatment	Group A IP-SSA	Group B IP-SSA+BP	Group C IM-SSA+BP	Group D IP-IVP	Group E IP-IVP+BP	Group F IM-IVP+BP	Group G Untreated Control
Mean No. of parasites and range ( )	8.0 (5-10)	1.3 (0-2)	2.0 (1-3)	7.8 (4-9)	0.0	0.2 (0-1)	27.2 (21-33)
Contrasts and P Values	A vs G, P<0.01	B vs A, P<0.01 B vs C, NS	C vs G P<0.01	D vs G P<0.01	E vs D, P<0.01 E vs F, NS	F vs G P<0.01	

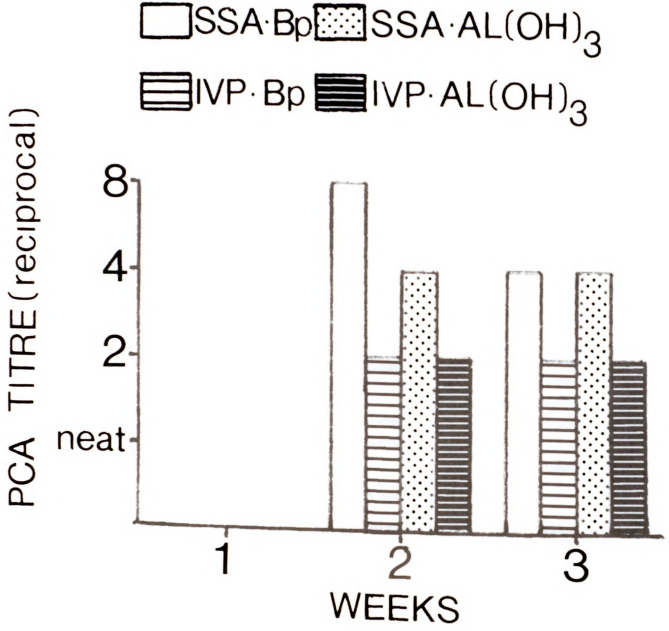
NS = Not Significant

Table 4. Intramuscular immunization of rats against *T. taeniaeformis* with SSA and IVP. Effects of FCA, BP and $Al(OH)_3$ as adjuvants.

Group and Treatment	Group A SSA+FCA	Group B SSA+BP	Group C SSA+$Al(OH)_3$	Group D IVP+FCA	Group E IVP+BP	Group F IVP+$Al(OH)_3$	Group G $Al(OH)_3$ Control	Group H Untreated Control
Mean No. of parasites and range ( )	92.17 56-147	21.5 (2-40)	41.8 (31-56)	77.0 (54-117)	0.2 (0-1)	2.5 (0-9)	98.3 (68-141)	94.2 (73-146)
Contrasts and P Values	A vs H, NS	B vs H, P<0.01 B vs A, P<0.05 B vs C, NS	C vs H, P<0.05 C vs A, P<0.05	D vs H, NS	E vs H, P<0.01 E vs B, P.<0.01 E vs F, NS	F vs H, P<0.01		

NS = Not Significant

Figure 1. Reaginic antibody titres in the serum of rats immunized with SSA and IVP using *B. pertussis* or $Al(OH)_3$ as adjuvants.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When IVP was given in a single dose or divided in equal amounts over 2, 3, 4 or 5 days, the group averages for parasites after challenge were 25.0, 18.2, 25.2, 26.8 and 14.2 respectively. The untreated control group had an average of 47.3 and each immunized group was very highly protected ($P < 0.01$ in all cases). The group of rats that had received their immunizing dose over a five day period was significantly more resistant than those immunized once ($P < 0.05$), but was no more resistant than the group dosed for only two days.

Four groups of rats were immunized with SSA orally with a total of 1, 3, 9 and 15 mg of antigen. Doses were given in equal portions on the 1st, 7th and 14th day. A fifth group was orally immunized with 1 mg of SSA combined with *B. pertussis* as adjuvant. The average numbers of parasites after challenge infection in these groups were 39.3, 54.5, 43.5, 36.8, 53.2. The untreated control rats had an average of 109.2. All vaccinated groups were highly resistant ($P < 0.01$ in all cases). There were no significant differences in protection among the vaccinated groups, including those rats which received *B. pertussis* orally.

When *B. pertussis* was given to rats intramuscularly followed by oral SSA on the following day, and the degree of immunity compared to that in rats given SSA orally alone, the average numbers of parasites in the two groups were 11.7 and 11.8 respectively. Parallel groups were challenged after immunization with intramuscular SSA + BP, intramuscular BP (control) or no treatment (control). These had parasite counts of 1.0, 34.3, and 35.5, respectively. The resistance conferred in the two groups that received SSA

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orally was significant ($P < 0.05$), but they did not differ from each other. Rats in the group immunized with SSA + BP intramuscularly were highly significantly resistant ($P < 0.01$).

In order to establish whether the resistance in immunized rats was manifested in whole or in part at the gut level, four groups of 16 rats each were immunized orally with either SSA or IVP. Half of the rats in each group were then challenged orally (Table 5a), and the other half intravenously (Table 5b). Intravenous challenge was achieved by injecting a suspension of *T. taeniaeformis* larvae through the mesenteric vein after exposure via a midline abdominal incision. The larvae used for challenge were obtained from the livers of donor rats using the procedure described by Musoke and Williams (1975). Table 5a shows that rats which were challenged orally were very significantly protected ($P < 0.01$) by both antigens (groups A and B). SSA was significantly more protective than IVP ($P < 0.01$). In response to intravenous challenge (Table 5b) both antigens again proved to be highly protective ($P < 0.01$), but there was no significant difference between the SSA and IVP groups. These results were confirmed in a duplicate experiment.

We then attempted to protect recipient rats with serum from artifically immunized donors. The results of the first experiment are shown in Table 6. Rats in groups of 8 were immunized intramuscularly with SSA or IVP (Table 6a). After 21 days half the rats in each group were killed with CO_2 vapour and blood was collected from their thoracic cavities by severing the blood vessels to the heart. Serum was obtained from the clotted samples and stored at -20°C. This serum was used for passive immunization of recipients

Table 5. Oral immunization of rats against *T. taeniaeformis* with SSA and IVP.

a.) Oral challenge

Group and Treatment	Group A SSA	Group B IVP	Group C Untreated Control
Mean No. of parasites and range ( )	20.3 (15-27)	36.0 (21-54)	60.1 (37-85)
Contrasts and P Values	A vs C, P<0.01 A vs B, P<0.01	B vs C, P<0.01	

b.) Intravenous challenge

Group and Treatment	Group A SSA	Group B IVP	Group C Untreated Control
Mean No. of parasites and range ( )	23.5 (14-39)	26 (18-43)	55.5 (44-64)
Contrasts and P Values	A vs C, P<0.01 A vs B, NS	B vs C, P<0.01	

NS = Not Significant

within 3 days of collection. The remaining half of the rats in each group were challenged with eggs on the 21st day. Table 6a shows that groups A and B were highly resistant ($P < 0.05$ and < 0.01 respectively). Recipient groups of six rats each were passively immunized on the day of challenge with 1 ml each of the antiserum obtained as described above, but did not show any resistance (Table 6b). In fact, group A which received the antiserum from rats immunized with SSA + BP intramuscularly had a significantly higher number of cysts ($P < 0.05$) when compared to the controls that had received normal rat serum (NRS). Group D received immune rat serum (IRS) obtained from rats which had had an active infection for 21 days, and was almost wholly protected. This experiment was repeated with identical results except in the instance where rats received serum from donors immunized with SSA + BP. Higher burdens of parasites developed in these recipients (135.7) than in rats given NRS(78.2) but this time the difference was not significant.

Table 6. Intramuscular immunization of rats against *T. taeniaeformis* with SSA and IVP, and passive transfer of serum to recipients.

a.) Donors

Group and Treatment	Group A SSA+BP	Group B IVP+BP	Group C Untreated Controls
Mean No. of parasites and range ( )	19.8 (2-35)	0.5 (0-1)	92.8 (82-109)
Contrasts and P Values	A vs C, $P<0.05$	B vs C, $P<0.01$	

b.) Recipients

Group and Treatment	Group A anti-SSA+BP	Group B anti-IVP+BP	Group C NRS Control	Group D IRS Control
Mean No. of parasites and range ( )	142.0 (73-279)	25.7 (5-54)	47.3 (35-74)	0.5 (0-1)
Contrasts and P Values		B vs C, NS	C vs A, $P<0.05$	D vs C, $P<0.01$

NS = Not Significant

Oral immunization of donors with SSA resulted in highly significant protection ($P < 0.01$) when compared to that resulting from IVP (Table 7a). However, antiserum obtained from orally immunized donor rats was ineffective in conferring resistance on recipients (Table 7b). Immune rat serum taken from rats with an active infection conferred absolute resistance on recipients (Group D - Table 7b).

PCA tests were done with serum obtained from donor rats. All those vaccinated intramuscularly using SSA or IVP and *B. pertussis* had detectable homocytotropic antibody, but the titres were low (1:4 at 21 days). Orally immunied donor rats did not have any detectable reaginic antibody at 21 days post-vaccination.

Table 7. Oral immunization of rats against *T. taeniaeformis* with SSA and IVP, and passive transfer of serum to recipients.

a.) Donors

Group and Treatment	Group A SSA	Group B IVP	Group C Untreated Control
Mean No. of parasites and range ( )	20.3 (9-28)	37.0 (17-58)	44.2 (36-60)
Contrasts and P Values	A vs C, P<0.01 A vs B, P<0.01	B vs C, NS	

b.) Recipients

Group and Treatment	Group A anti-SSA	Group B anti-IVP	Group C NRS Control	Group D IRS Control
Mean No. of parasites and range ( )	74.8 (49-116)	110.7 (78-158)	63.0 (49-72)	0
Contrasts and P Values	A vs B, NS		C vs A, NS C vs B, NS	D vs C, P<0.01

NS = Not Significant

DISCUSSION

The results of our experiments confirm and extend the observations on artificial immunization against *T. taeniaeformis* which had been made over 40 years ago by Miller (1931) and Campbell (1936). Both extracted (SSA) and secreted-excreted materials (IVP) evidently contained potent immunogens, although the route of administration had an important influence on the effectiveness of the immunization schedule. Likewise the nature of the adjuvant employed played a large role in determining the outcome of the immunization procedure. These factors have not been examined experimentally in past work on the development of immunization regimens

Miller (1931), Kerr (1935), and Campbell (1936) used crude whole worm homogenates to immunize laboratory animals against taeniid cestodes, and they generally administered them intraperitoneally (IP). In more recent work intramuscular inoculations have been used with Freund's adjuvant as vehicles in vaccinations of ruminants against *T. ovis* and *T. saginata* (Rickard and Bell, 1971b; Rickard and Adolph, 1976; Rickard et al, 1976). Kwa and Liew (1977), immunized rats with antigens of *T. taeniaeformis*, and used the subcutaneous route with Freund's complete adjuvant as a vehicle. Intraperitoneal dosing produced the most consistent protective response in our experiments, and, in the absence of adjuvant, single intramuscular inoculations of antigenic preparations were ineffective. Surprisingly, however, emulsification of parasite antigens in FCA was of no value in enhancing immunogenicity. On the contrary, animals given antigens in FCA consistently developed more parasites per liver than controls.

In contrast, those given *B. pertussis* as adjuvant were markedly more resistant than those given antigens alone, regardless of the route of parenteral administration. *B. pertussis* has been used in many immunologic studies in rodents because of its tendency to cause the development of reaginic antibodies, but its role of enhancing protective responses has not been seriously explored. Circulating reagins were detected in low titers in the serum of rats immunized with SSA and IVP together with *B. pertussis* at the time when they were challenged, but the role which these antibodies play, if any, in the resistance mechanism is not yet clear. It is interesting, however, that aluminum hydroxide, another adjuvant which causes reagin production in rats, was also effective in stimulating protective responses to IVP and SSA. Reagins were present in the serum of these animals at the time of challenge but no reagins were detected in rats immunized with antigens in FCA. Reagins were also undetectable in the serum of rats immunized orally with either antigen, even though these rats developed significant resistance against challenge infection. It is noteworthy that reagins are a prominent feature in the response of the rat to an active infection with *T. taeniaeformis* (Leid and Williams, 1974b), and may be involved in enhancing the onset of immune attack on challenge organisms (Musoke et al, 1978).

Limited attempts have been made at the use of helminth extracts or their *in vitro* products in oral vaccination, possibly due to the belief that most antigens administered via this route are not effective in provoking protective immunity. However, Spindler (1933) showed that immunogens extracted by digesting trichinous meat with

artificial gastric juice were able, to a certain extent, to protect rats, rabbits and guinea pigs against challenge. There is recent evidence of successful immunization against bacterial and viral infection via the oral route (Freter, 1956; Porter et al, 1973; Porter et al, 1974; Furuuchi et al, 1976). Our finding that SSA and IVP, when given orally to rats, stimulated significant resistance against *T. taeniaeformis*, suggests that more exploration is needed into the potential for immunization by the oral route against helminthiasis.

In our system even a single exposure to antigen was shown to be effective, although increasing the number of doses did seem to enhance protection. However, there did not appear to be any advantage to giving large doses of antigen, or administering doses at 7 day intervals before challenge. There was no demonstrable effect of *B. pertussis* when given either orally or parenterally, despite the effectiveness of this adjuvant in enhancing the immunogenicity of antigens given intramuscularly or intraperitoneally. The mechanism of resistance in rats given SSA or IVP orally was not entirely gut-related, but seemed to have a systemic component, since rats challenged intravenously were protected to a significant degree.

The fact that we were unable to improve upon the level of immunity by feeding increased amounts of SSA is consistent with the observations and explanation of Walker and Hong (1973). Their studies showed that oral administration of homologous antigen in previously exposed animals resulted in reduced antigen uptake. They later demonstrated that this was due to degradation of antigen within the intestinal lumen, and speculated that IgA immunoglobulins combined with the

antigens, preventing their uptake and increasing enzymatic degradation (Walker et al, 1974). Swarbrick, et al (1977) have termed this phenomenon, "immune exclusion by the gut". It seems possible that in our experiments reduced uptake of immunogens might have limited the amounts which reached the lymphoid tissues outside the intestinal wall. If stimulation of extra-intestinal lymphocytes were necessary for an anamnestic response to enhance the protective mechanism then retention of antigen in the gut might prevent this from developing. There is some evidence that the major lymphoid tissues of the gut (Peyer's patches) are not able to mount a secondary response (Porter et al, 1974).

Thomas and Parrot (1974), and Swarbrick, et al (1977) have presented evidence that systemic tolerance may be induced by presentation of antigens through the gastrointestinal tract. David (1977) was able to show that oral administration of allergens could inhibit reagin formation in rats. Homocytotropic antibody (reagin) was not detectable in our orally immunized rats even though they became resistant against challenge infection. Further work will be necessary to determine if this was due to gut-induced tolerance effects.

The fact that we were unable to passively transfer resistance of artificially immunized rats using serum contrasts sharply with the situation in rats which become immune after infection. Immune serum from these animals can protect recipients in quantities as low as 0.4 ml, and the antibodies involved have now been well characterized (Leid and Williams, 1974a). It seems that different protective mechanisms may be involved in immunity produced by

artificial vaccination, although the slight but insignificant degree of resistance transferred with serum of IVP-immunized rats suggests that antibodies do make a contribution in that case. It is also possible that qualitative as well as quantitative differences occur in the antibody response to artificial administration of antigens, and that this could account for our observations. The reason why serum from SSA immunized rats increased the susceptibility of recipients is not at all clear at this time, but it does indicate that circulating factors may have an enhancing effect on the infectivity and survival of taeniid parasites as suggested by Varela-Díaz, et al (1972) and Rickard (1974).

Our results clearly demonstrate that protective antigens are contained within the tissues of well developed metacestodes of *T. taeniaeformis*, and are released by them *in vitro*. Similar results have been reported recently by Kwa and Liew (1977). On the other hand, in *T. pisiformis* Heath (1973) has concluded that the capacity to stimulate protective immunity wanes with age of the larvae. He found that whereas implanted post-oncospheral stages stimulated resistance, implanted live mature cysticerci were ineffective. However, his observation that a single subcutaneous inoculation of killed mature larvae stimulated a high degree of resistance is not consistent with this conclusion. In some of their experiments Heath, and Kwa and Liew employed FCA as a vehicle for vaccination and immunizing doses were given subcutaneously or intramuscularly. It is difficult to assess if the adjuvant contributed significantly to the enhancement of immunity, and not at all clear what effect the route of administration

may have had. Controls were not included for the immunizing effects of antigens or adjuvants alone, and the influence of the route of administration was not examined. Our experience would suggest that FCA has very little positive effect on the development of resistance, and that the route of administration can be a crucial factor on the outcome of vaccination. We feel that much more care needs to be given to the selection and evaluation of adjuvants and methods of antigen dosing in future immunization against taeniid infections.

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