IMMUNITY TO TAPEWORMS: VACCINATION AGAINST *HYMENOLEPIS DIMINUTA* AND ROLE OF THE BURSA OF FABRICIUS IN REJECTION OF *RAILLIETINA CESTICILLUS*

THESIS

for the

Degree of Doctor of Philosophy

by

Elsayed Elsiddig Elowni
(B.V.Sc., M.V.Sc.; Khartoum)

Department of Zoology, University of Glasgow

November 1980

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ACKNOWLEDGEMENTS

I am very grateful to Professor C. A. Hopkins for his guidance and encouragement during the course of this study.

I am indebted to Dr. D. Wakelin for the many valuable suggestions and for his scholarly criticisms.

My thanks are due to Sidi, Tim, Redwan, Paul, Peter, Melody, Richard and Hassan for much helpful discussions and advice.

The sincere help of Helen, Iona, Delia, David, Jane, Carrie and Anne is greatly appreciated.

I extend my gratitude to Dr. J. S. Gray, Dublin, for providing the strain of Raillietina cesticillus.

I am very grateful to Mrs. Fiona Harvey for carrying out so efficiently the daunting task of typing the manuscript.

This study was carried out on a scholarship from the British Council.

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SUMMARY

Attempts were made to vaccinate mice against infection with Hymenolepis diminuta, a tapeworm rejected from these hosts by an immunologically-mediated mechanism. Various putative antigenic preparations from the strobilated worm were tested. Marginal protection, as compared with that induced by an oral infection from cysticercoids, was obtained when mice were orally vaccinated with multiple doses of whole worm antigens. Marginal protection was also obtained when tegument antigens were given by this route. Results from two other experiments with tegument antigens, however, did not confirm this finding. No protection was obtained following vaccination with antigens from sonic disruption of somatic cells, exoantigens, saline extracts or egg antigens.

On the basis of evidence from other experimental models, it is proposed that this failure to evoke strong protective immunity by vaccination with killed worm antigens was possibly due to one or more of the following factors:

(i) the tapeworm protective antigens were not present initially in most of the preparations injected or that they were present in too low a quantity to stimulate immunity

(ii) the worm protective antigens were highly labile and they were destroyed during preparation, probably by enzymes released by the disintegrating worms themselves, or inactivated by chemical reactions in the stomach or intestine when these preparations were given orally or intraduodenally

(iii) the physicochemical characteristics of these protective antigens had been altered during preparation

(iv) the presence of a wide array of worm antigens, e.g. in a homogenate, dissipated the host immune response and masked the presence of the protective antigens possibly as a result of antigenic competition

(v) the route of antigen administration was the crucial factor that militated against the induction of functional immunity rather than the antigens themselves

(vi) the duration of antigenic stimulation was not long enough and a longer period, analogous to that required for the development of immunity from an enteric H. diminuta infection, was needed

(vii) the regimes of vaccination described were conducive to the induction of tolerance.

To elucidate the validity of these assumptions, experiments were carried out with live worm antigens. The rationale behind each approach is described separately in the text. Strobilate 8-day-old worms, apparently capable of surviving for appreciable periods of time when implanted subcutaneously or intraperitoneally, did not immunize mice against challenge. Implantation of a strobilate 8-day-old worm surgically into the duodenum conferred only weak protection. The in vivo process of excystation, which is bypassed when immunization is performed by implantation of worms directly into the duodenum, had no influence on the ability of the worm to stimulate immunity. These findings suggest that the failure to induce strong immunity by parenteral implantation of a live 8-day-old was possibly

due to the fact that the 8-day-old worm is, in itself, inefficient in inducing a pronounced protective response against challenge, even when presented enterally. The fact that a weak protective response was induced by the intraduodenal administration of the 8-day-old worm and not by parenteral implantation of these worms suggests that the enteric route is more efficient in the induction of functional immunity against the tapeworm than either the subcutaneous or intraperitoneal routes. In other experiments it was established that the young worm is more efficient in the stimulation of protective immunity against challenge than older worms. It is of interest that the older worms, when implanted surgically into the duodenum, present the host with significantly larger amounts of strobila antigens per unit time than do the younger ones. This observation casts doubt on the significance of the strobila as the major source of H. diminuta protective antigens. The logical explanation for the failure to immunize mice by vaccination with the killed worm antigens is that this is possibly because the antigens used were derived mainly from strobilar tissue obtained from worms even older than the 8-day-old parasites whose poor immunizing potential was demonstrated.

Live excysted worms, which provide only scolex and neck antigens, were capable of inducing a protective response when administered intraperitoneally. Irradiated worms, incapable of growing strobilae, were as immunogenic as worms of the same age which were not irradiated. Immunization of mice with an irradiated vaccine is advantageous in the sense that the immunizing infection can be denoted as self-limiting resulting in the prevention of propagation of the parasite

to the intermediate host, at a time when specific protective immunity is raised in the definitive host.

Two independent investigations were undertaken to locate the origin of H. diminuta protective antigens. The techniques of chemical abbreviation of immunizing infections and irradiation were used for this purpose. The results provided evidence that the induction of functional immunity against H. diminuta in mice is independent of the presence of a strobila: is determined by the duration of an antigenic stimulus deriving from the scolex and/or neck regions. The degree of this immunity is also determined by the number of worms in the immunizing infection. The conclusions drawn from the present investigation as to the origin of H. diminuta protective antigens and the immunogenic potential of irradiated worms are at variance with those reached by other investigators.

In the second part of this thesis the mechanism of the immunologically-based rejection of Raillietina cesticillus from chickens was investigated. Chickens whose ability to produce antibodies was abrogated by bursectomy and irradiation developed protective immunity against the tapeworm as did the controls with specific anti-worm antibodies in their sera. It is suggested that antibody is not the crucial component of the mechanism affecting the growth and development of R. cesticillus in the immune chicken.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Parasitic diseases, undoubtedly, constitute serious problems of great economic importance. Despite the organized efforts to control these diseases or alleviate their severity by chemotherapy, vector control, public health education and many other procedures, they remained an incessant threat to the well-being of man and his domesticated animals. In recent years, however, the progress in the field of parasite immunology and the results obtained from experimental animal models initiated a strong comeback of ideas advocating an immunological approach to parasitic disease control (Terry, 1968) and strengthened the beliefs that vaccination could significantly contribute to the prevention of disease (Silverman, 1970).

A large number of cestodes including species of the genera Echinococcus, Taenia, Diphyllobothrium, Hymenolepis, Dipylidium, Davainea and Raillietina are of medical or veterinary importance and the larvae of some species are often very serious pathogens (reviewed, Smyth & Heath, 1970). Vaccination of animals against infection with larval tapeworms, particularly those of the Taeniidae, has proved partially successful on a number of occasions (reviewed, Gemmell & Soulsby, 1968; Clegg & Smith, 1978). On the other hand, vaccination against the adults received little attention and the results obtained have been much less promising than those obtained for the larval stages (reviewed, Gemmell et al., 1968).

The first part of this thesis describes attempts to vaccinate a mammalian host against infection with an adult tapeworm, Hymenolepis diminuta. Since the original report that the mouse

rejects this tapeworm by an immunologically-based mechanism (Hopkins, Subramanian & Stallard, 1972 a, b), several of the parameters pertaining to this host/parasite association have been investigated in this laboratory and the system was suggested as a suitable model for studies concerned with acquired immunity to intestinal-dwelling helminths. Although the project was primarily intended to test the value of various putative antigenic preparations in raising protective immunity (SECTIONS 1 & 2), the results obtained, particularly when viable worm materials were used, led to several of the experiments (SECTION 3) being designed to locate the origin of the functional (protective) antigens in the strobilate worm.

Protection against most parasites often involves intricate components which may involve immunologically specific as well as non-specific elements (Wakelin, 1978). The second part of this thesis is concerned with the mechanisms of protective immunity against Raillietina cesticillus, an intestinal-dwelling davaineid tapeworm of galliform birds. It is ranked among the potentially pathogenic forms of adult cestodes (Dutt, 1961) and is rejected from chickens by an immunologically-based mechanism (Gray, 1973).

The domestic fowl, Gallus gallus domesticus, is one of the more frequent hosts of adult tapeworms and more than 50 species, belonging to 10 different genera, were known to parasitize this host (Elowni, 1977). Fowls can also be infected with tapeworm larval forms as hydatid cysts, tetrathyridia and spargana, all of which have zoonotic significance. Distinct from other vertebrates, Aves have a hindgut lympho-epithelial organ, the bursa of Fabricius. The original

observation of Glick, Chang & Jaap (1956) on the immunological function of this organ in influencing antibody-forming capacity in chickens later led to the formulation of the concept of dissociation of immunological responsiveness in birds (Warner, Szenberg & Burnet, 1962a; Szenberg & Warner, 1962; Warner & Szenberg, 1964) based on thymic influence on the ontogeny of cellular immunity and the bursa on the development of immunocompetence in cells destined to make immunoglobulins. The domestic fowl, thus, presents an ideal laboratory model for the study of adult and larval cestode immunity where the humoral component can be depleted by selective abrogation of bursal function. On the basis of the aforementioned concept of the dichotomy in the role of the central lymphoid organs, the bursa and thymus in birds, the immunologically-based rejection of R. cesticillus was analysed in bursa-deficient chickens.

The thesis is completed by conclusions and suggestions for further investigation.

CHAPTER ONE

VACCINATION OF MICE AGAINST INFECTION WITH *HYMENOLEPIS DIMINUTA*

INTRODUCTION

INTRODUCTION

(1) Immunogenicity of Adult Tapeworms

Adult tapeworms are obligate parasites which predominantly inhabit the alimentary tract. The evidence obtained from experimental and epidemiological studies indicates that immunologically-mediated protective mechanisms influence the outcome of associations between a variety of hosts and these parasites. In mice previously infected with Hymenolepis microstoma (Tan & Jones, 1968; Howard, 1976) or Hymenolepis citelli (Weinmann, 1966), these effector mechanisms are manifested by inhibition of growth and/or a decrease in the rate of survival of secondary worms. Destrobilation (loss of strobila leaving only the scolex and neck) and subsequent expulsion of worms have been attributed to functional immunity against R. cesticillus in chickens (Gray, 1973) and H. diminuta in mice (Hopkins et al., 1972 a). Inhibition of oogenesis was described as a feature of the immune response against Echinococcus granulosus in dogs (WHO, 1965). The possibility of immunological interference has also been suggested to account for the difficulty in establishing superinfections in dogs harbouring Diphyllobothrium latum (Wardle, Gotschall & Horder, 1937) and in human subjects with Taenia saginata or Taenia solium infections (Brumpt, 1949). The relatively short-lived Moniezia expansa in sheep (Seddon, 1931) may prove to be another example of host immune reaction against an adult tapeworm. Not only do adult tapeworms stimulate immunity against the homologous challenge, but this effect can, at times, extend to heterologous infections. H. microstoma, for example, strongly protects mice against challenge with H. diminuta (Hopkins,

Goodall & Zajac, 1977) and prior infection with Multiceps multiceps renders dogs more resistant to the establishment of E. granulosa (Ramazanov, 1971).

(2) Vaccination Against Tapeworms

(a) Larval Forms

Results from extensive investigations (reviewed by Weinmann, 1970; Gemmell, 1976; Williams, 1979) showed that larval tapeworms are capable of stimulating strong protective immune responses in a wide range of vertebrate hosts and several attempts have been made to protect these hosts by vaccination (reviewed, Gemmell & Soulsby, 1968; Gemmell & Macnamara, 1972; Clegg & Smith, 1978). Homogenates of strobilar or cyst materials of Taenia taeniaeformis have been shown to provide partial to fairly high levels of protection against infection with live eggs of the parasite (Miller, 1930; 1931; 1932; Campbell, 1936; cited by Gemmell et al., 1968). Similar results have been obtained with Hymenolepis nana (Larsh, 1944) following repeated intraperitoneal injections of extracts or freshly macerated homogenates of strobilar tissue. Although injection of dried protoscoleces and germinal membranes of hydatid cysts effectively stimulated protection against the post-encystment phase of E. granulosus in sheep, this procedure failed to induce any resistance against the pre-encystment phase (Turner, Dennis & Berberian, 1937).

Vaccination with live eggs or artificially activated oncospheres of tapeworms has also been shown to evoke significant protective immune responses in the hosts. Gemmell (1969), for example, was able to obtain high levels of resistance in sheep vaccinated with eggs or

oncospheres of Taenia hydatigena and Taenia ovis. The activated oncospheres, however, have the hazardous potential of developing into cysts at the sites of injection and freezing (to -15°C) or treatment with formalin before injection did not prevent some of these embryos from developing into cysts (Gemmell, loc. cit.). On the other hand, treatment of eggs by sonication or freezing (-70°C) before injection had no such undesirable effects but the immunity stimulated was of a lower order than that engendered when live eggs or oncospheres were given (Gemmell, 1964; 1969). When eggs of T. saginata were exposed to radiation and fed to calves, however, the animals showed no evidence of cysticerci at autopsy and their resistance to challenge was substantial (Urquhart, 1961). Since vaccination with killed larval tapeworms induces no significant protection (Weinmann, 1970), it was proposed that secretion and excretion antigens produced by the parasites during their development could be the protective antigens (Smyth, 1969). When activated oncospheres of T. ovis or T. taeniaeformis contained in diffusion chambers were implanted into the peritoneal cavity of lambs and rats, soluble antigenic products released by the larvae during their first week of development were reported to be responsible for raising protective immunity (Rickard & Bell, 1971 a). Since Heath and Smyth (1970) developed the method for in vitro culture of cestode larvae, the technique offered a unique advantage over classical vaccination procedures; animals can be vaccinated with antigens harvested from culture media without their being exposed to the hazards of injecting whole living parasites. Using this technique, Rickard et al., (1971 b), Heath (1973) and Rickard & Adolph (1976) successfully

vaccinated lambs against T. ovis, rabbits against T. pisiformis and calves against T. saginata respectively. Only a few larvae from an initial inoculum were found to survive in culture media, diffusion chambers (Rickard et al., 1971 a, b) or following subcutaneous implantation (Heath, 1973). It was, therefore, argued that the protective immunity obtained by vaccination with tapeworm larval antigens collected from culture media or with antigens from embryos in the diffusion chambers could have resulted either from the administration of somatic antigens released by the disintegrating dead larvae or from exogenous antigens elaborated by the living embryos (Heath, 1976). From an experiment with T. pisiformis, however, Heath (loc. cit.) was able to show that the major protective antigens of developing larvae were exogenous antigens elaborated into the culture medium by intact living organisms. Subsequently, Rickard & Adolph (1977) collected protective antigens from short-term (24 to 48 h) in vitro culture of activated embryos in a tissue culture medium unsupplemented with serum indicating that potent immunogens were released by the early developing larval stages. Secretory and excretory antigens prepared from short-term incubation of tetrathyridia of Mesocestoides corti in a simple medium (Krebs' - Ringer solution) have also been shown to protect mice against challenge (Kowalski & Thorson, 1972).

(b) Adult Forms

Apart from experiments carried out with E. granulosus, vaccination of hosts against infection with adult tapeworms has received little attention (reviewed, Gemmell et al., 1968; Clegg et al., 1978). Presumably this is because the adult tapeworms are rarely serious

pathogens (Rees, 1967) and, until recently, those with no tissue-phase of development have been widely regarded as poorly immunogenic or immunologically inert (Cultberson, 1941; Chandler, 1939; Heyneman, 1962). The few attempts that were carried out often gave inconclusive or conflicting results thus augmenting the contention that intestinal-dwelling cestodes are not immunogenic. As early as 1933, however, Turner and his co-workers protected dogs against E. granulosus by a series of intramuscular injections of crude antigenic preparations from dried hydatid cyst germinal membranes or dried protoscoleces. In a much larger trial, about half the vaccinated dogs resisted challenge compared to 95% infection level in the controls (Turner et al., 1936). The implication of E. granulosus larval antigens in the artificial stimulation of protective immunity against the adult worm was independently confirmed by Matov and Vasilev (1955), Foresk and Rukavina (1959) and De Rosa et al. (1974). From his experiments with E. granulosus, Gemmell (1962) provided evidence that antigens from the adult worm were more effective than larval antigens obtained from protoscoleces. Intravenous or intramuscular injection of artificially activated oncospheres of E. granulosus, T. hydatigena or T. ovis ' appeared' to give a degree of protection against E. granulosus in dogs (Gemmell et al., 1968). The protection so induced, however, was not absolute in all dogs. Results from such experiments probably led to the conclusion that the use of larval antigens in immunizing against adult tapeworms could be an 'anomaly' (Smyth, 1969) since a better protection is more likely to be induced with antigens from the adult worm.

Another approach to vaccination against adult cestodes is to obtain antigens from living worms that are attenuated. Possibly ionizing radiation is one of the most popular techniques of attenuating infective stages of parasites without considerably affecting their immunogenicity. Effective irradiated vaccines were developed against Dictyocaulus viviparus (Jarrett et al., 1959), Haemonchus contortus and Trichostrongylus colubriformis in sheep (Mulligan, 1961) and Ancylostoma caninum in dogs (Miller, 1971). Indeed, a high level of protection was produced by orally administering to dogs larvae of E. granulosus irradiated with X-rays (Movsesijan, 1968). With this species, however, vaccination with irradiated worms was considered potentially dangerous (Herd et al., 1975) since some of the worms exposed to radiation doses as high as 20 Krad were found to be capable of regenerating the potential to produce eggs.

The value of extracts or crude homogenate preparations in vaccination against parasites was doubted by many investigators, particularly those working with nematodes and protozoa (Terry, 1968; Cox, 1978). As Ogilvie (1974) wrote '... the helminth antigens which stimulate the hosts immune defence are substances released by the living parasites and present in relatively small quantities in extracts of the worms.' These antigens were suggested to be enzymes (Soulsby, 1963; Wakelin, 1978). There was considerable progress in the use of antigens harvested from in vitro culture media of nematodes (reviewed, Soulsby, 1963; Thorson, 1970; Silverman, 1970) and trematodes (Clegg et al., 1978). With adult tapeworms, however, development of such a technique lagged considerably and it is only relatively recently

that a successful technique for in vitro culture of a tapeworm, E. granulosus, was established (Smyth, 1967; 1969). The immunizing effect of culture antigens derived from this tapeworm has been investigated by Herd et al. (1975). Secretory antigens collected from a protein-free medium were separated from worm excretory antigens and culture medium components by ultrafilteration and administered with adjuvants to dogs. A significant blocking effect on egg production and suppression of proglottid formation were obtained. In a subsequent study, however, Herd (1977) was unable to attribute specificity to the antigens collected in this way since worms showing arrested development were also recovered from control dogs injected with the adjuvants alone.

So far, the results obtained from vaccination experiments with tapeworms other than E. granulosus have been much less promising. The subcutaneous injection of saline suspensions of dried whole worms conferred no protection on rats against infection with H. diminuta (Chandler, 1940), nor did the intraperitoneal administration of oncospheres, cysticercoids or adult worm homogenate (Swietlikowski et al., 1978). Partial protection against T. taeniaeformis was obtained in kittens given oral doses or subcutaneous injections of an emulsion prepared from the contents of the larva of the parasite (Ohira, 1935). This author was also able to induce partial protection against Diphyllobothrium mansoni in puppies subcutaneously injected with emulsions from the sparganum larvae of the worm. The injection of ground up proglottids was claimed to protect sheep against M. expansa (Seddon, 1931). Although Larsh (1944) demonstrated a weak protective

response by injecting killed or fresh tissue of H. nana, Hearin (1941) was unable to induce any resistance against this tapeworm by intraperitoneal implantation of live homologous strobilar tissue.

(3) H. diminuta and Host Immunity

H. diminuta is a cyclophyllidean cestode that inhabits the small intestine of rats, mice, hamsters and, occasionally, other mammals including man (Smyth, 1976). It has an obligate life cycle, with cysticercoids developing in various larval or adult insects. Unlike H. nana, this tapeworm causes no obvious damage to the gut mucosa (Turton, 1968; Andreassen, Hindsbo & Ruitenberg, 1978a), a phenomenon once considered a prerequisite for the induction of immune responses against intestinal-dwelling cestodes. This was the opinion of Rees (1967) who wrote '... in all instances immunity develops only if the mucosa of the host's intestine is invaded.' The categorical statement of Chandler (1939) that '... premunition in tapeworm infections is due to crowding rather than to immunity in the ordinary sense' was to remain unchallenged till Weinmann (1966) provided evidence that prior infection with a non-invasive tapeworm, H. diminuta, stimulates protective responses in mice. The mechanism by which mice reject this tapeworm was documented as having an immunological basis (Hopkins et al., 1972 a, b). It is now well established that antigenic materials, in immunogenic quantities, can be taken up sufficiently by an intact gut mucosa to elicit immunological responses (reviewed, Hemmings, 1978). This lends support to the view that adult tapeworms confined to the lumen of the gut can elaborate antigens conducive to the stimulation of immunity.

(i) H. diminuta in the Rat

The rat is widely regarded as a good host for H. diminuta which may survive for the life of the host (Read, 1967). Although Harris and Turton (1973) found no loss of worms from a five-worm infection, Andreassen, Hindsbo and Hesselberg (1974) reported the expulsion of worms from rats infected with 100 cysticercoids and demonstrated protective immunity against a challenge secondary infection.

(ii) H. diminuta in the Mouse

The short longevity of this tapeworm in the mouse was reported by Weinmann (1966) '... almost all worms persisted for at least 7 days ... few survived 10 days in multiple infection or 12 days in single worm infection; none survived 15 days or reached maturity'. The loss of H. diminuta from mice was confirmed by Turton (1968, 1971) who also described the phenomenon of destrobilation of the worm. Hopkins et al. (1972 a) found that over 90% of the cysticercoids they administered to mice established, the worms grew for about 10 days, destrobilated and were subsequently expelled. They proposed an immunological explanation which they later supported by experiments in which immunosuppressants were used (Hopkins et al., 1972 b). Further evidence for the involvement of host immunity in the rejection of H. diminuta from mice came from experiments in which Hopkins (1978) demonstrated a strong and long lasting memory to infection.

Prior infection with H. diminuta in mice suppresses the growth and decreases the rate of survival of worms in a secondary infection (Hopkins et al., 1972 a). The degree of this inhibitory response is

dependent on the intensity of both the immunizing and secondary infections (Befus, 1975 a). In infections with other hymenolepid cestodes in the mouse, for example H. citelli (Hopkins & Stallard, 1974) or H. microstoma (Howard, 1976; Hopkins et al., 1977), there are thresholds (as defined by Hopkins, loc. cit) of antigenic stimulation beyond which rejection occurs. In H. diminuta infections, however, there appears to be no low threshold since antigenic stimulation from a single worm results in the worm being rejected and immunological memory engendered (Befus, 1975 a). The reduced variability in immunological responsiveness of mice infected with six worms, however, suggests that this worm burden provides antigenic stimulation to exceed the threshold of all mice (Befus, loc. cit.).

Various strains of mice, including Swiss albino (Weinmann, 1966), C57, C3H, CBA, CD1 (Hopkins, 1980), B_{10}LP (Isaak, Jacobson & Read, 1975), Balb C (Andreassen et al., 1978 a, b) were found to reject H. diminuta. Direct comparisons between strains, however, have rarely been attempted. Hopkins et al., (1972 a) found no difference between the CFLP and the Porton strains in establishment of the tapeworm through destrobilation and commencement of expulsion occurred one day earlier in the former strain. Establishment, growth and rejection in CFLP and NIH mice were reported by Befus (1975 a) to be fundamentally similar but the CFLP mice supported a greater mass of worms. Mice of the CFLP and NIH strains were also used in the present investigation and, although both strains were good responders to the tapeworm, the results obtained from NIH control groups were consistent and predictable when mice were

autopsied at specific times during the course of infection (see General Materials & Methods).

Little difference in the rate of growth and time of rejection was observed in young 6-8 weeks old male and female mice but in older mice larger variations between the sexes were occasionally encountered (Hopkins, 1980).

(iii) Nature of the Rejection Mechanism

Although H. diminuta has been shown to evoke a protective immune response in both mice and rats (see above), the origins and identity of the antigens that stimulate these responses are unknown. Christie (1978), by implanting worms of different ages into the duodenum of mice and recording their rates of injection, suggested the strobila of the worm as a major source of the protective antigens. He concluded, however, that his experiments did not rule out a role for scolex antigens. In a study with nude mice, Andreassen, Hindsbo and Vienberg (cited by Andreassen et al., 1978 a as unpublished results) found that five worms in an infection were rejected at a time when their combined weight was less than that reached by two worms which were not rejected by the mice. For this reason Andreassen et al (1978 a) suggested that 'the functional antigens are related to the scolex and not to the total worm biomass or surface area'.

The precise mechanism of elimination of H. diminuta from mice is also not well-defined. Worm expulsion has been shown to be T-cell dependent (Isaak et al., 1975; Bland, 1976 a, b), partially deficient in young immunologically immature mice (Befus & Featherston, 1974), suppressed by cortisone, methotrexate and anti-lymphocyte serum

(Hopkins et al., 1972 b) and delayed in lactating and pregnant mice (Goodall, 1973; Christie, 1979). Adult thymectomized lethally irradiated mice do not reject H. diminuta following reconstitution with bone-marrow (Bland, 1976 b) suggesting the involvement of a radio-sensitive component in the mechanism of rejection. Andreassen et al. (1978 a), however, claimed that congenitally athymic mice rejected a 5-worm infection albeit later than in their thymus-bearing littermates. They concluded that 'host protection was dependent on the number of worms and that worms can be expelled in the absence of functional T-cells'.

Mice have been shown to produce serum antibodies to H. diminuta (Choromaniski, 1978; Andreassen et al., 1978 a). The tapeworm also stimulates antibody production in rats (Harris & Turton, 1973) and humans (Turton, Williams & Harris, 1975). Whether or not humoral antibody is involved in the rejection of H. diminuta from mice is unknown. Mice whose ability to produce antibody was abrogated by treatment with rabbit anti-mouse IgM were found to expel the tapeworm as rapidly as did the control, non-suppressed mice (Isaak, 1976). Serum from mice immune to H. diminuta did not passively transfer worm expulsion potentiality to normal mice (Isaak, 1976; Hopkins, 1978) or to nude recipients (Andreassen et al., 1978 a). Moreover, incubation in vitro of immune serum with H. diminuta cysticercoids and complement was reported by Isaak (1976) to have no effect on the infectivity of these cysticercoids. He (Isaak) suggested that 'specific humoral antibody is not the crucial thymus-dependent component of the immune system responsible for the expulsion of H. diminuta from mice'.

Evidence for a possible role of antibody in mediation of protective immunity against H. diminuta was presented by Befus (1977). Using an immunofluorescent technique, he was able to demonstrate an increase in the level of intestinal immunoglobulins, particularly at the time of worm rejection. It was suggested that these immunoglobulins were antibodies interacting with tegument antigens and that they were involved in immunological damage to the tegument (Befus & Threadgold, 1975; Threadgold & Befus, 1976). These authors, however, were careful to point out that there was no evidence that these immunoglobulins were specific anti-worm antibodies, as distinct from those produced in response to other intestinal antigens. Also they did not rule out the possibility that these immunoglobulins were non-specifically absorbed on to the polyanionic glycocalyx of the tegument.

Andreassen et al. (1978 a) reported an increase in the number of intestinal mast cells and globule leucocytes in mice to day 10 by which time rejection of the tapeworm was completed. Although nude mice also rejected H. diminuta, such mice had no demonstrable intestinal mast cells or globule leucocytes (Andreassen et al., loc. cit.). Hopkins (1980) found no significant slowing in rejection following the administration of antihistamines and anti-5-hydroxytryptamine and he suggested that rejection of the tapeworm is not dependent on a mast cell mediated inflammatory response.

GENERAL MATERIALS & METHODS

GENERAL MATERIALS & METHODS

The materials and methods described herein are those which apply widely to experiments in CHAPTER ONE. Specific techniques, however, are described separately where appropriate.

1. Animals

(a) Mice: Tapeworm-free CFLP (outbred) and NIH (inbred) strains were purchased from Anglia Laboratory Animals, Huntingdon. Both strains were categorized 3 star [see Register of Accredited Breeders and Recognized Suppliers (Sept. 1978), Medical Research Council, Laboratory Animal Centre] . Infections with the pinworms Aspiculuris tetraptera and Syphacia obvelata were detected in a few occasions and mice were given piperazine treatment (see 'Anthelmintic Treatment' below). On no occasion was infection with a cestode other than the inoculated species encountered when mice were autopsied at the end of experiments. Mice were first treated when 6 to 8 weeks old. In any one experiment animals were of the same strain, age and sex.

(b) Rats: Outbred CFHB (Wistar-derived) rats were bred in this laboratory and were used for maintaining H. diminuta.

2. Maintenance

Mice were obtained from the suppliers and acclimatized for at least 4 days before the beginning of experiments. They were kept in a conventional animal house with the temperature controlled at 20-22 ^{o}C. The light was maintained automatically on 12 h cycle in winter but followed day length in summer. They were caged in lots of four,

five or six in polypropylene 48 x 15 x 13 cm cages (North Kent Plastic Cages Ltd.) and sawdust litter was replaced twice weekly. Pellet food (Rat and Mouse Breeding Diet, Grain Harvesters Ltd.) and tap water were provided ad libitum. In experiments involving laparotomy or parenteral implantation of viable worm tissue, oxytetracycline hydrochloride ('Terramycin', Pfizer Ltd.) was given in the drinking water at a concentration of 165 mg/l for 3 consecutive days. This concentration provides an approximate daily dose of 30 mg/kg body weight. Sham-operated and injection controls were given similar antibiotic treatment.

3. Parasite

The strain of H. diminuta used in the present study was described by Hopkins et al. (1972 a). It was originally acquired from Rice University in Houston, Texas in 1963 and has been maintained in this laboratory since this time by cyclical passage through rats and the flour beetle, Tribolium confusum.

4. Infection

Terminal gravid proglottids from rat worms were homogenized in water using a blender [Measuring & Scientific Equipment Ltd., (MSE), Crawley, Sussex] fitted with a 3-ml micromasticator assembly and operated at top speed (approx. 14,000 r.p.m.) for 45 sec. Eggs in the homogenized material were allowed to settle in a round-bottomed crystalline dish and the supernatant removed by suction. The sediment was resuspended in water and the process repeated. Eggs were then fed on moist filter paper to beetles which had been starved for 5-6 days (T. confusum) or 3 days (Tenebrio

molitor). Occasionally finely-shredded gravid segments were mixed with whole wheat flour and fed to the starved beetles. After about 24 h, beetles were transferred to glass jars containing whole wheat flour (T. confusum) or oat flakes and pieces of apple (T. molitor). They were kept in a relatively humid incubator set at 25°C. Larvae and pupae were sifted monthly from the stock to prevent dilution of the infected colony. Cysticercoids, not more than 3 months old, were recovered by mechanically disrupting infected beetles in modified Hanks' balanced salt solution (HBSS, see Hopkins & Stallard, 1974) using MSE homogenizer operated for 30 sec at approximately 7,000 r.p.m. If few cysticercoids were required, these were recovered by dissecting beetles with needles and fine forceps. The first procedure was advantageous since it allowed for large numbers of cysticercoids to be recovered and the animals infected within a short period. The author found no evidence of a decrease in infectivity of cysticercoids using this technique. Cysticercoids, not more than 30 min after collection, were administered by intragastric intubation of ether-anaesthetized mice and rats. Cysticercoids of the required number were drawn into the first 2-3 cm of a 20-30 cm long Portex polythene tubing (0.80, 1.00, 1.27 mm OD for NIH, CFLP and rats respectively) attached to a hypodermic needle fitted to a syringe containing HBSS. Cysticercoids were flushed into the stomach with 0.05-0.15 ml (mice) or 0.3 ml (rats) of the HBSS. Sham-infected controls were anaesthetized and injected with equal volumes of HBSS as in the experimental groups. Oral infections were performed with cysticercoids exclusively from T. confusum. Cysticercoids grown in T. molitor were used in

experiments in which mice were inoculated parenterally with viable worm tissue. Mice from a cage were infected in succession and groups of mice to be compared were infected alternately. Infections in any particular day were carried out with cysticercoids from the same batch of beetles. Groups of mice were autopsied in the order they were infected. Throughout the thesis, the day of the first infection/injection is Day 0.

5. Anthelmintic Treatment

The anthelmintic oxyclozanide, (trade name 'Zanil', I.C.I.), was reported to be effective against H. diminuta when given orally to mice at a dose of 120 mg/kg body wt (Hopkins, Grant & Stallard, 1973). In experiments where mice were given a primary infection, it was necessary to eliminate primary worms before giving a challenge infection and measuring the effect of the immune response on the challenge. Zanil, which is a 3.4% suspension of oxyclozanide, was diluted in distilled water and doses of 250 mg/kg body wt were calculated from a formula dW = 68; where 'd' is the dilution factor and 'W' is the mean wt (in grams) of mice from a cage. A final volume of 0.5 ml of the diluted anthelmintic was given. At this dose, the drug was found to be reliably effective and well tolerated by mice.

Praziquantel (Bayer AG, Leverkusen) is an effective compound against juvenile and adult cestodes in rodents (Thomas & Gönnert, 1975) and was found to be a 100% effective against H. diminuta of various ages (Appendix) when administered to mice as a single oral dose of 100 mg/kg body wt.

Infections with the pinworms A. tetraptera and S. obvelata in

mice were terminated with piperazine citrate ('Citrazine', Loveridge Ltd.) administered in the drinking water at a concentration of 3 g/l.

6. Parenteral Injections

Subcutaneous (s.c.) injections were given to mice behind the interparietal bone (cortisone acetate injections and live worm implants) or in the flank region (killed antigenic preparations, adjuvants and saline injections). Intraperitoneal (i.p.) injections were given by entering the abdominal cavity close to or at the linea alba (worm implants, saline and anaesthetic injections). With the exception of cortisone, all preparations were administered to mice while under ether anaesthesia.

7. Transplantation of H. diminuta

The efficacy of a regime of vaccination was evaluated by a direct test of the ability of vaccinated mice to mount an enhanced rejection of challenge worms by a selected day after challenge (see below). Challenge was performed by surgical transplantation of strobilate worms from donor mice or rats or by the oral administration of cysticercoids. Eight-day-old donor worms were recovered from mice infected with four cysticercoids. These mice were then treated with 1.25 mg of cortisone acetate ('Cortistab', Boots Ltd.) injected s.c. on days 2, 4 and 6 post-infection (p.i.). Seven-day-old donor worms were also obtained from mice infected with four cysticercoids but the mice received cortisone only twice, on day 3 and day 5 p.i. Seven-day-old rat worms were grown in donors fed 10 cysticercoids, the rats received no cortisone treatment.

The technique of surgical transplantation of H. diminuta into the

duodenum of mice was originally described by Hopkins & Zajac (1976). Because most of the experiments in this thesis involved surgical transplantation of worms, minor differences from their technique are described below. Anaesthesia was induced by ether and maintained by sodium pentobarbitone ('Sagatal', May & Baker Ltd.). CFLP mice were injected with 0.08 ml/10 g body wt of an 10% solution of the barbiturate made up in HBSS containing 1% ethanol. NIH recipients appeared to be slightly less responsive to sodium pentobarbitone than the CFLP and a dose of 0.09 ml/10 g was used satisfactorily. Using this procedure, third stage anaesthesia was maintained for at least 2 h and death from intoxication was minimum.

Worms from donor mice or rats were washed and kept at room temperature (22°C). Only worms of normal gross morphology were selected for transplantation. The skin over the upper abdomen of the anaesthetized recipient was shaved and swabbed with 70% ethanol. A small oblique incision (less than 1 cm) was made through the skin a few mm posterior to the costal region and away from the xiphoid cartilage. To avoid rupture of blood vessels, which ramify in this region, abdominal muscles were not incised but the fibres were separated by first introducing the closed ends of a fine forceps which were then drawn apart in situ. The duodenum was retracted from under the liver with a round blunt-pointed metal hook. Worms for implantation were drawn up, scolex first, into Portex tubing attached to a hypodermic needle fitted to a syringe containing HBSS. The end of the tube was introduced through a small hole on the convex side of the duodenum in the direction away from the stomach. The worm

was slowly delivered with 0.2-0.3 ml of HBSS. The hole in the duodenum was occluded by a transfixion suture and a complete circle hitch using 6/0 Mersilk ('Mersutures', Ethicon Ltd., Edinburgh). The retracted duodenal loop was sprayed with an aersol mixture of neomycin, polymyxin B and bacitracin ('Rikospray', Riker Ltd.) before being replaced. . Two Mersilk interrupted stitches were inserted in the abdominal muscle. The skin was closed with two interrupted mattress sutures and the wound sealed with 'Nobecutane' (Astra Chemicals Ltd.). Worms were normally transplanted not more than 45 min after the donor had been killed. Sham-operated mice underwent similar surgical procedures but no worms were administered. Mice were left on top of an incubator (37°C) to recover from anaesthesia and then transferred to clean cages. With this technique, worm recoveries were as high as 100% and post-operative deaths were as low as 5%.

8. Recovery of Worms

Mice were killed by chloroform inhalation or by cervical dislocation and the abdomen was opened. The small intestine was severed at the ileocaecal junction, freed from the mesenteries and finally cut at the pylorus. Intestinal contents were flushed out with HBSS from a syringe fitted with a wide-bore blunt-ended canula inserted at the anterior end of the intestine. The contents, in a crystalline dish, were shaken gently to free worms from debris. Small worms were searched for under x6 and x12 magnifications of a binocular dissecting microscope using transmitted light. If the recovery was <100%, a method similar to that described by Hopkins et al. (1972 a) was

used for recovery of microscopic worms which remained attached to the intestinal mucosa. The intestine was slit opened longitudinally and divided into four approximately equal segments. Each segment was incubated separately at 37°C for 1-2 h in small glass Petri dishes (51 x 18 mm) containing HBSS. The contents were then scanned under a dissecting microscope (see above) at least twice during the period of incubation. Such procedures usually reveal the presence of worms which are often <0.1 mg dry wt ($\lesssim$1 mm long). At this stage, if the total recovery was still <100%, it was assumed that either

(i) the missing worms had been immunologically rejected; or

(ii) the worms had not established initially, presumably because the transplanted worms or cysticercoids had not all been infective, or because of an experimental error deriving from the techniques of infection and/or recovery of worms. In the latter case, the error was estimated from recovery figures of worms from naive control mice which would not have actively expelled their worms by the day of autopsy (see below).

All worms over 2-3 mm were washed free from debris in HBSS, blotted dry on filter paper and transferred to aluminium foil cups. They were dried at 95-100°C for 24 h and weighed to the nearest 0.1 mg using a Mettler Type H15 balance, Zürich. When mice were challenged with six cysticercoids, all worms from a mouse were counted and weighed en masse to give (worm biomass/mouse). Single worms transplanted surgically were weighed individually.

9. Computation and Interpretation of Results

The problem of how best to measure rejection in an H. diminuta

infection was elaborately discussed by Hopkins (1980). The total number of the worms recovered, number of worms excluding destrobilated worms, mean worm weight and worm biomass are the principal parameters. Each of these parameters has its own limitations and, in the writer's opinion, they provide a reliable measurement of difference only if they are taken collectively. Moreover, ambiguity often arises when decisions are to be made on the logic of statistical inference from biological data of unknown distributions. The criteria set out below relate the markers of worm rejection in a defined period during the course of the challenge infection to those established in investigations performed in this laboratory. The requirements and assumptions pertaining to biometrical analysis of results were those defined by Colquhoun (1971), Phillips (1973) and Castle (1979) and experiments were designed accordingly. One-tailed, nonparametric tests were used. When vaccinated, naive and immune (infected with 6 cysticercoids orally) control CFLP or NIH were challenged by surgical implantation of strobilate worms, they were killed 6 and 5 days after challenge respectively. By this time the naive controls were not expected to reject the challenge infection since a strobilate H. diminuta transplant is not rejected in less than 7 days by a naive mouse compared to a period of about 4 days in the immune recipient (Hopkins et al., 1976). Therefore, the difference between a naive and an immune mouse in rejecting a worm transplanted surgically should be obvious by day 5 (in NIH) or day 6 (in CFLP) after challenge if not earlier. Hence, for a vaccine to be effective, the survival and growth patterns of transplanted worms in the vaccinated mice

should depart from those patterns in the naives and approach those observed in the immune. The significance of this directional departure was determined by the one-tailed statistical tests. The inclusion of immune control groups in nearly all experiments also allows for direct comparisons to be made between the immunity induced with a vaccine and that stimulated by a natural infection. It also ensures that the mice treated are not refractory to worm antigens at the time of the experiment.

When mice were challenged with six cysticercoids, they were autopsied 8 days later. Results from preliminary experiments, and consistently throughout this study, showed that day 8 of challenge revealed the greatest difference between a naive and an immune mouse in rejecting an infection starting from cysticercoids. Although it is difficult to directly compare results from different investigations, data obtained by Hopkins et al. (1972 a) and Befus (1975 a; particularly Fig. 1) showed that loss of worms from previously uninfected mice (Porton, CFLP and NIH strains) do not commence before day 8 of infection. By this time, worm rejection by immune mice should be well underway. The argument presented above concerning the making of decisions from data obtained on a specific day after challenge also applies here. Paradoxically, should the naive controls manifest a sign of rejecting their worms on day 8, e.g. when an adjuvant non-specifically potentiates the loss of challenge worms or impedes their growth [see Fig. 3 (2) & 10 (2)], the assumptions governing the use of a one-tailed test are not justified and hence a two-tailed test of significance is applied.

(i) Growth and Survival of Single-Worm Challenge Transplants

An 8-day-old worm from cortisone-immunosuppressed donor mice when transplanted (day 0) has a mean dry wt of approximately 1 mg (Appendix). Assuming that a tapeworm almost doubles its weight each day (see Hopkins et al., 1976), such worms are expected to reach a weight of about 32 mg (dry wt) by day 5 or 64 mg by day 6 of transplantation. In the growth figures, therefore, a point at '0' mg dry wt indicates that no worm was recovered from a mouse. A worm of <0.1 mg is a destrobilated worm; it is in the process of being rejected (Hopkins et al., 1976) and has therefore been excluded from recovery figures. Worms >1 mg are worms that have grown. The rate of their growth is determined by the statistical test. Growth data from vaccinated, naive and immune control mice were interchangeably compared by the Wilcoxon two-sample test (Colquhoun, 1971). The rates of survival of challenge worms were analysed by the Fisher exact probability test (Siegel, 1956).

(ii) Growth and Survival of Worms from a Challenge of Six Cysticercoids

Worms of <0.1 mg are very small worms (about 1 mm long) consisting of only scoleces and necks. It is established that, on day 8 of a secondary infection, worms of this size are worms that have grown slowly or destrobilated having been affected by the immune response (see Hopkins et al., 1972 a; Befus and Featherston, 1974). Such worms were not weighed and were shown in growth figures as being 0 mg. Assigning each worm of this category to the rank position of 0.1 mg severely distorts the data by accentuating the potential error

of finding worms of this size in the intestine. Worms of $\geq$0.1 mg are segmented worms. They are worms that have grown and the effect of the immune response on their rate of growth was determined by the Wilcoxon test.

Worms from an experimental and a control group were considered as two bivariate samples of discrete variables (wts $<$0.1 mg and $\geq$0.1 mg). The numbers of worms of these two categories in the different groups were tested for statistical distinction by the χ^2 test, Yates' correction (Siegel, 1956). The total recovery (number of worms recovered from a group of mice, regardless of worms weight) was tested for difference from the controls by the same test.

In all tests, the null hypothesis (Ho) was rejected when the results observed in a vaccinated group of mice differed in the predicted direction (see above) and when they were of such magnitude that the probability (p) of their occurrence under Ho was equal to or less than $p = 0.05$. A regime of vaccination was considered effective if it was associated with a significant reduction in the growth and survival of challenge worms or if it was associated with suppression of worm growth alone (see Befus, 1975 a).

SECTION 1

VACCINATION WITH NON-LIVING WORM ANTIGENS

I. WHOLE WORM ANTIGENS [SOMATIC ANTIGENS (SA)]

1.1 SA Given Orally

1.1.1 Materials & Methods

The mice used were 7-week-old male CFLP. Somatic antigens were prepared from immature, strobilate H. diminuta of different ages (12-14 days) recovered from rats each given 10 or 80 cysticercoids. Worms were washed free from debris in successive changes of HBSS, blotted on filter paper and weighed. They were homogenized in phosphate-buffered saline (PBS; pH 7.8) at 4°C for 1½ min using an MSE homogenizer fitted with a 3-ml micromasticator assembly and operated at top speed (approx. 14,000 r.p.m.). Immediately after homogenization, mice which were previously starved for a few hours (Table 1) were anaesthetized and the homogenate was administered by a stomach tube in a volume of 1.75 ml. To minimize the possibility of degradation of the supposedly immunogenic materials in the homogenate by prolonged exposure to the acidic environment of the stomach, mice were treated before the administration of the homogenate with 0.25 ml of 1.4% $NaHCO_3$ orally and Metoclopramide (Beecham Pharmaceuticals, Tadworth) subcutaneously (10 mg/kg body wt).

[Metoclopramide HCl is a specific antiemetic. Its action is twofold (see Wood-Smith et al., 1973): "... it is effective centrally at the chemoreceptor trigger zone, and also has a peripheral action, ... and hastening gastric emptying". Its effect on the rate of gastric emptying was monitored in a pilot experiment using methylene blue as a tracer marker. An homogenate-methylene blue mixture was

Table 1. Immunization procedure of CFLP mice given H. diminuta somatic antigens orally*

	Immunizing dose (mg worm tissue/mouse)									
Days:	0	2	3	4	6	8	9	10	12	15
Vaccinated (6 doses)	390 (2)		200 (7)		270 (4)		320 (7)		280 ($5\frac{1}{2}$)	290 ($6\frac{1}{2}$)
Vaccinated (7 doses)	220 (2)	200 (7)		320 ($5\frac{1}{2}$)	230 ($4\frac{1}{2}$)	320 (7)		350 (6)	290 ($4\frac{1}{2}$)	

* Numbers in parentheses are the hours of starvation.

administered orally to mice previously starved for 1 h. The mixture was transmitted from the stomach down to 89%, 87% and 62% of intestinal length (pylorus to caecal junction) in 15, 17 and 20 min after administration respectively.

To exclude a remote possibility that intact viable scoleces could escape mechanical disruption by the homogenizer and subsequently establish in the intestine (following rapid conveyance via the dilated pyloric sphincter), another pilot experiment was carried out. Nine worms (1-2 cm long) were homogenized for $1\frac{1}{2}$ min and the homogenate was examined under a microscope. No intact scoleces were found in this material.]

1.1.2 Design

Mice received at 3-day intervals 6 doses of the homogenate or 7 doses at 2-day intervals. The dose levels are shown in Table 1. Two naive control groups received 1.75 ml of PBS, 0.25 ml of 1.4% $NaHCO_3$ and Metoclopramide over similar periods of time. A fifth group was infected with six cysticercoids and the infection was eliminated with Zanil on day 16 of infection (immune control group). Each mouse was challenged 21 days after the initial infection/injection by surgically transplanting into the duodenum a single 8-day-old worm recovered from cortisone-immunosuppressed mice. All mice were killed on day 27.

1.1.3 Results

The growth and survival of the challenge worm transplants in vaccinated and control mice are shown in Fig. 1. A reduction in growth of these worms was observed in the group vaccinated with six doses of the somatic antigen preparation as compared with the naive controls. This reduction is just statistically significant ($p = 0.05$).

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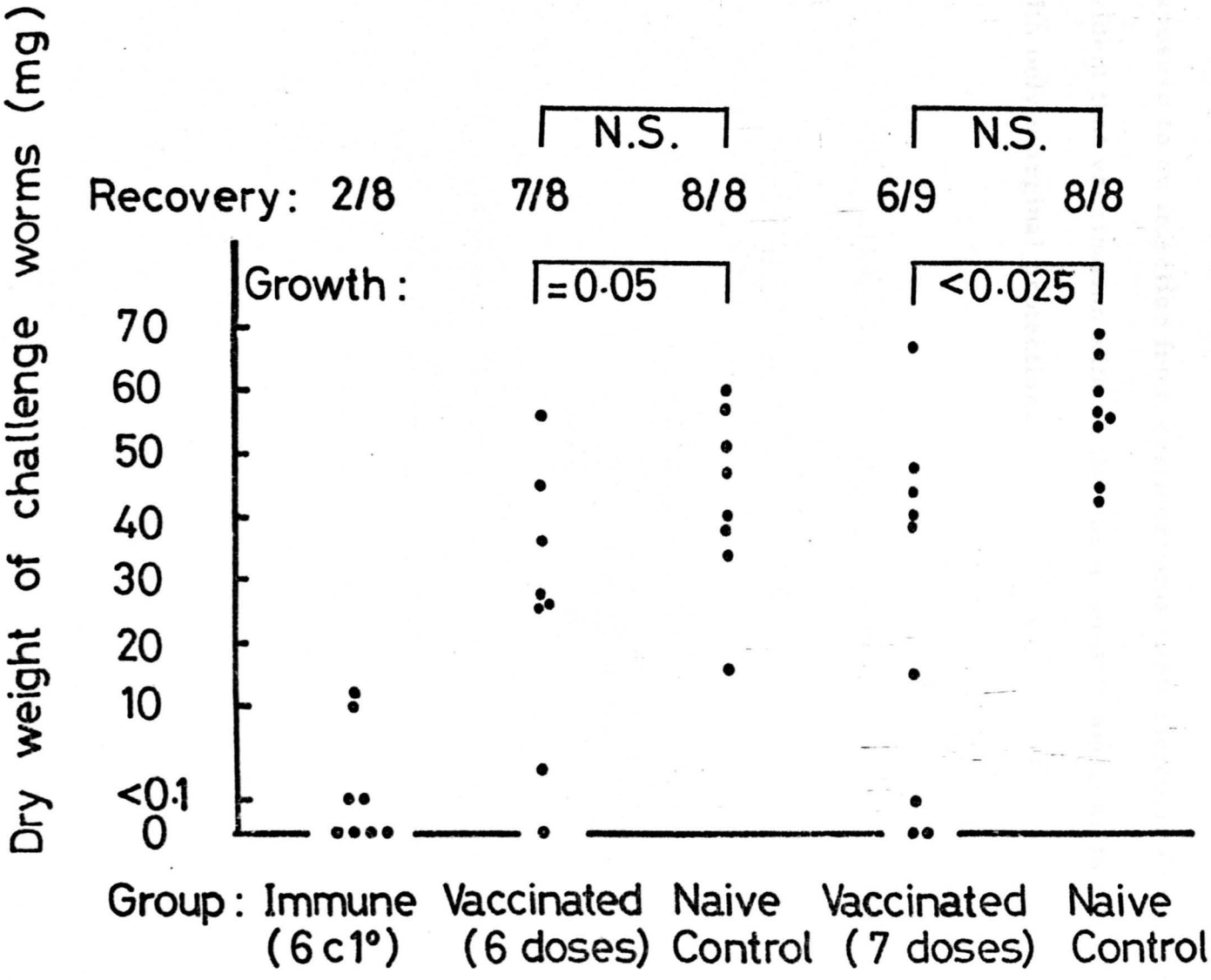
Figure 1.

Growth and survival of 8-day-old H. diminuta single-worm transplants in mice orally vaccinated with tapeworm homogenate and in the immune and naive recipient controls.

N.S. = Not significant


N.S.
N.S.
Recovery: 2/8 7/8 8/8 6/9 8/8
Growth:
=0·05
<0·025
Dry weight of challenge worms (mg)
70
60
50
40
30
20
10
<0·1
0
Group: Immune (6 c1°) Vaccinated (6 doses) Naive Control Vaccinated (7 doses) Naive Control

Mice vaccinated with seven doses harboured significantly lighter worms than the naive controls ($p < 0.025$). In both vaccinated groups, worm survival was not affected significantly. If the reduction in worm growth in the vaccinated mice is compared with that evoked by prior exposure to an infection from cysticercoids (immune controls), it is evident that vaccination with whole worm somatic antigens is associated with only marginal protection.

1.2 S.A. Given s.c.

1.2.1 Materials & Methods

The mice used were 7-week-old male NIH. Eleven 28-day-old H. diminuta were recovered from three rats each given five cysticercoids. Somatic antigens were prepared from the first 8 cm of each worm to reduce the proportion of egg materials in the preparation. Following successive washings in HBSS, worms were blotted on filter paper and weighed. They were finally rinsed in HBSS containing antibiotics ('Crystamycin', Glaxo) at a concentration of 100 units sodium benzyl-penicillin and 100 ug streptomycin sulphate per ml. Worms were then homogenized in cold tris-maleate buffered saline (TMBS, pH 7.2 and osmolarity of 295 mosmol; see Appendix) in an MSE homogenizer operated for $1\frac{1}{2}$ min at top speed. Crystamycin was added to the homogenate (same concentration as above). The homogenized material was centrifuged at 200 g for 30 min at 4°C to sediment particulate worm tissue and eggs. The supernatant formed the antigen which was administered to mice s.c. immediately after preparation.

1.2.2 Design

Subcutaneous injections, containing 2 mg wet weight of worm tissue emulsified in an equal volume (0.11 ml) of Freund's complete adjuvant (FCA, Difco), were given. Each mouse received the dose as two injections on the flanks. Naive control mice were injected with an emulsion of FCA and TMBS (0.22 ml). Mice were challenged on day 21 with an oral infection of six cysticercoids and they were killed on day 29.

1.2.3 Results

The growth and survival of challenge worms are shown in Fig. 2-1 and Fig. 2-2 respectively. Vaccination with whole worm somatic antigens administered s.c. under the conditions specified conferred no protection on mice against challenge.

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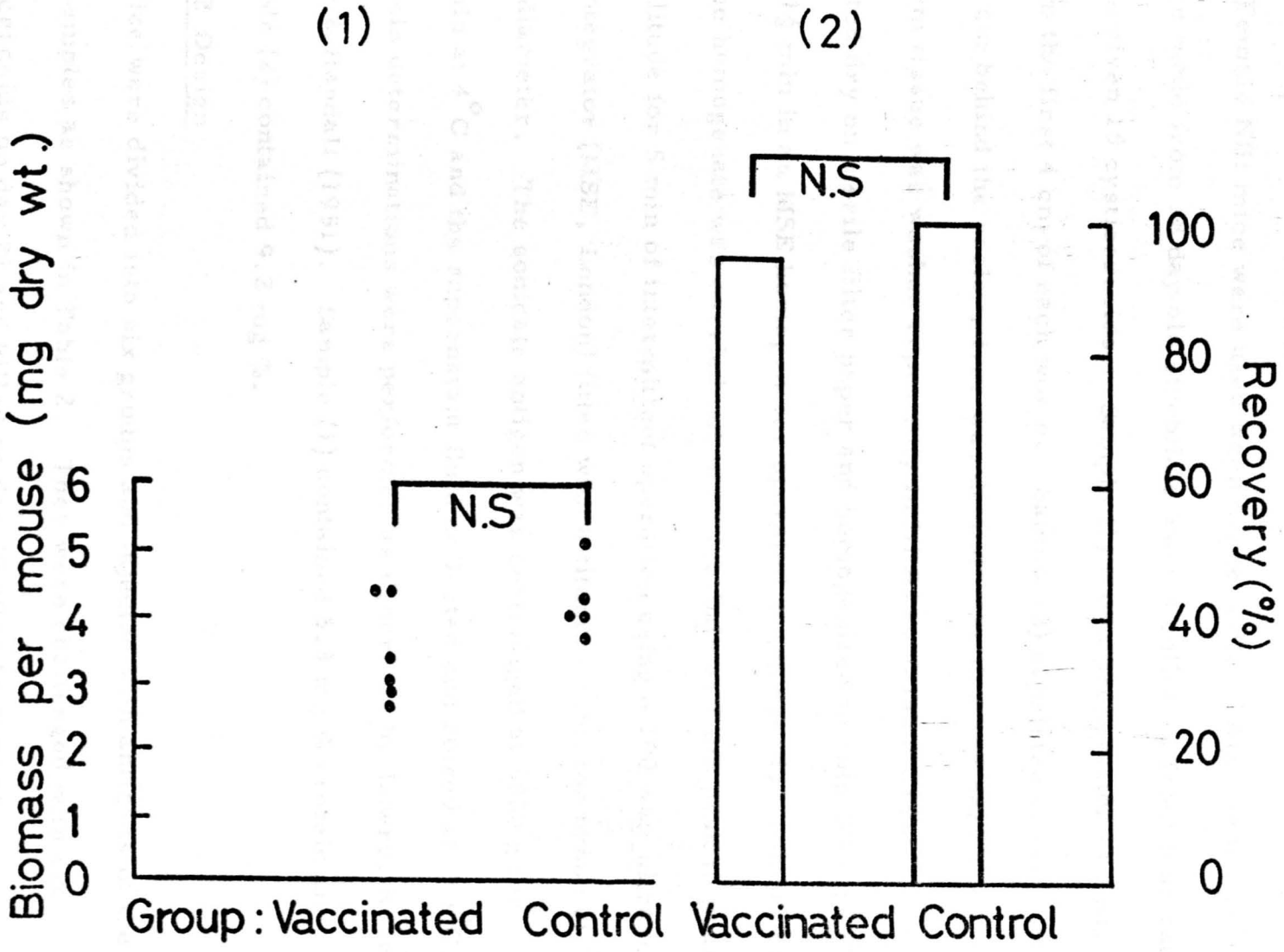
Figure 2.

Growth (1) and survival (2) of worms from a six-cysticercoid challenge infection in mice vaccinated s.c. with H. diminuta homogenate and in the naive controls.

All worms recovered are $\geqslant$ 0.1 mg.

N.S.: Not significant


(1)
(2)
N.S
N.S
Biomass per mouse (mg dry wt.)
0
1
2
3
4
5
6
100
80
60
40
20
0
Recovery (%)
Group : Vaccinated
Control
Vaccinated
Control

2. SONIC DISRUPTION ANTIGENS (SDA)

2.1. SDA Given i.p. or s.c.

2.1.1 Materials & Methods

Female NIH mice were used when 6-weeks-old. Antigen preparations were made from 18-day-old strobilate worms collected from four rats each given 15 cysticercoids. Sample (1) was a preparation of antigens from the first 4 cm of each worm. Sample (2) consisted of segments 3-5 cm behind the scolex, i.e. was devoid of scolex and neck antigens. Worm tissue was washed repeatedly in HBSS containing Crystamycin, blotted dry on sterile filter paper and homogenized in cold PBS (pH 7.2) for $1\frac{1}{2}$ min in an MSE homogenizer operated at top speed. Sonication of the homogenate was carried out in an ice-bath at 8 um peak to peak amplitude for 5 min of intermittent operation using a 100 watt ultrasonic disintegrator (MSE, London) fitted with a titanium vibrator probe of $^3/_8$" end diameter. The sonicate antigen was centrifuged at 5000 g for 15 min at 4°C and the supernatant fluid collected and stored at -15°C. Protein determinations were performed as described by Lowry, Rosebrough, Farr & Randall (1951). Sample (1) contained 5.3 mg % protein and Sample (2) contained 9.2 mg %.

2.1.2 Design

Mice were divided into six groups and injected with antigens of the two samples as shown in Table 2. They were challenged with six cysticercoids on day 21 and killed on day 29 after treatment.

2.1.3 Results

Fig. 3-1 and Fig. 3-2 show the growth and survival of the challenge worms respectively. Vaccination with sonicate antigens from somatic

Table 2. Course of immunization with antigens from sonic disruption of worm tissue.

Group	Antigen sample/ control	dose (protein)	route	adjuvant (0.1 ml)	day
1. Vaccinated	(1)	5.3 ug; 0.1 ml	s.c.	FCA	0
	(1)	5.3 ug; 0.1 ml	s.c.		12
2. Naive control	PBS	0.1 ml	s.c.	FCA	0
	PBS	0.1 ml	s.c.		12
3. Vaccinated	(2)	9.2 ug; 0.1 ml	s.c.	FCA	0
	(2)	9.2 ug; 0.1 ml	s.c.		12
4. Vaccinated	(2)	13.8 ug; 0.15 ml	i.p.		0
	(2)	13.8 ug; 0.15 ml	i.p.		12
5. Naive control	PBS	0.15 ml	i.p.		0
	PBS	0.15 ml	i.p.		12
6. Immune	6 cysti-		orally		0
control	cercoids		s.c.	FCA	0

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Figure 3-1

Growth of worms from a six-cysticercoid challenge infection in mice vaccinated s.c. or i.p. with H. diminuta sonic disruption antigens and in the immune and naive controls.

A = Immune control

B = Vaccinated s.c. with two doses of Sample 1.

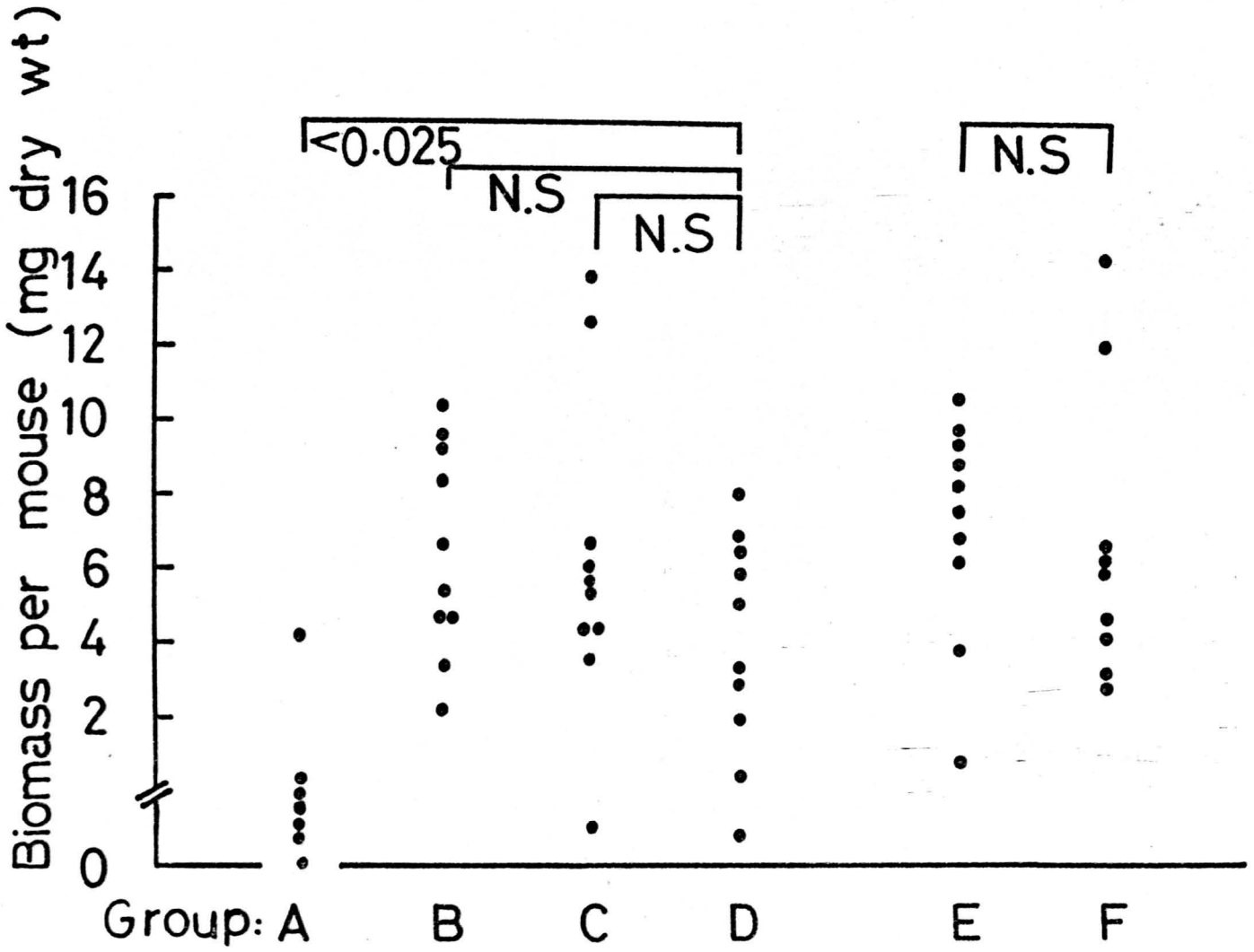
C = Vaccinated s.c. with two doses of Sample 2.

D = Naive controls given PBS s.c.

E = Vaccinated i.p. with two doses of Sample 2.

F = Naive controls given PBS i.p.

N.S. : Not significant


Biomass per mouse (mg dry wt)
16
14
12
10
8
6
4
2
0
<0·025
N.S
N.S
N.S
Group: A
B
C
D
E
F

Facing page 42

Figure 3-2

Survival of worms from a six-cysticercoid challenge infection in mice vaccinated s.c. or i.p. with H. diminuta sonic disruption antigens and in the immune and naive controls.

Shaded portions of bars represent worms $\geq$ 0.1 mg; open portions, worms $<$ 0.1 mg.

(a): Level of significance (total recovery)

(b): Level of significance (worms $\geq$ 0.1 mg)

*: χ^2 test, 2-tailed

A: Immune controls

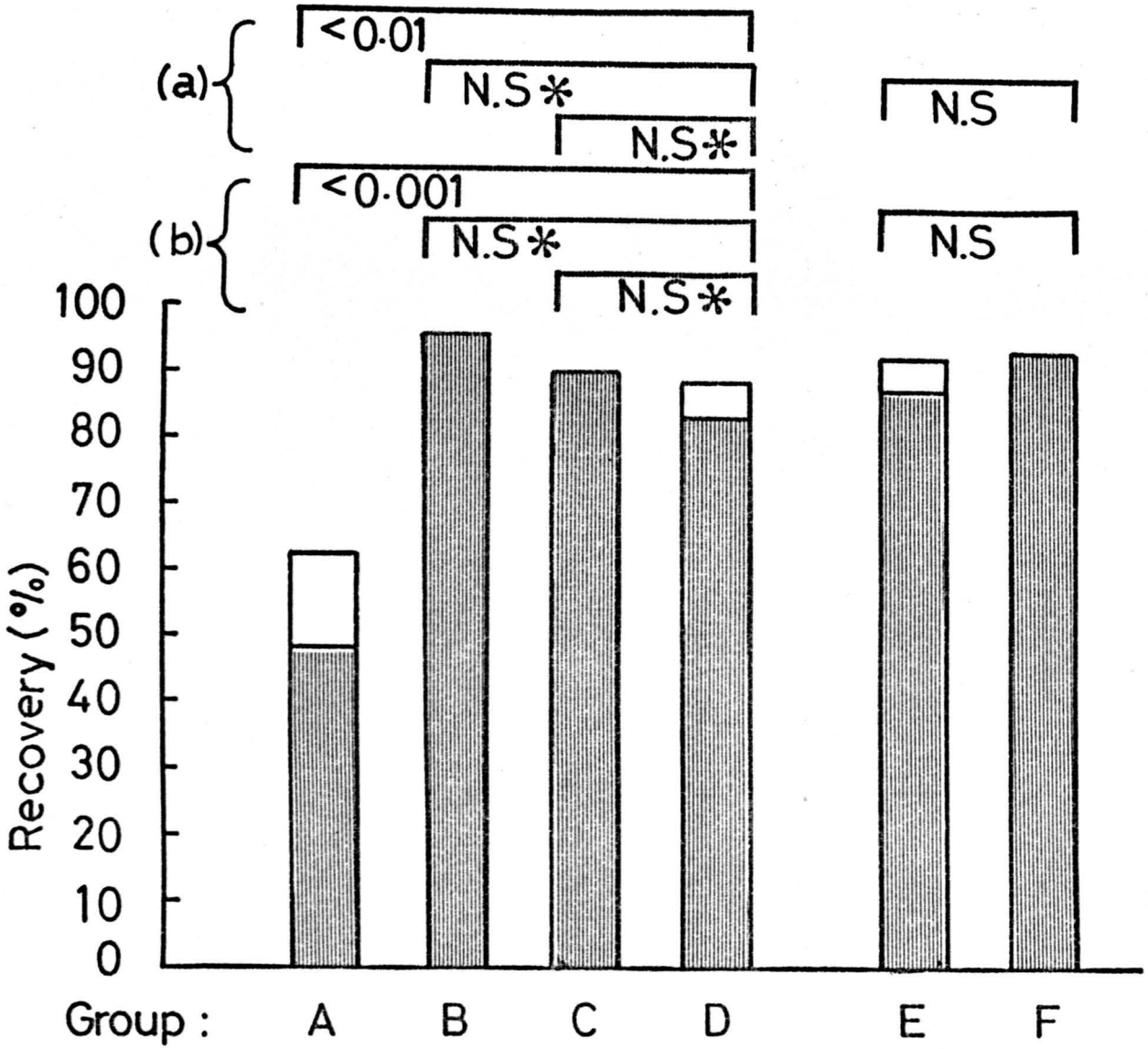
B: Vaccinated s.c. with two doses of Sample 1.

C: Vaccinated s.c. with two doses of Sample 2.

D: Naive controls given PBS s.c.

E: Vaccinated i.p. with two doses of Sample 2.

F: Naive controls given PBS i.p.


(a)
<0.01
N.S*
N.S*
N.S
(b)
<0.001
N.S*
N.S*
N.S
Recovery (%)
100
90
80
70
60
50
40
30
20
10
0
Group :
A
B
C
D
E
F

cells of whole worms or worm strobilae alone conferred no protection on mice against challenge. On the other hand, an infection from cysticercoids was efficient in inducing strong protection.

3. SALINE EXTRACT ANTIGENS (SEA)

3.1 SEA Given s.c.

3.1.1 Materials & Methods

Mice were female CFLP vaccinated when 7-week-old. Strobilate worms (12-day-old) were obtained from rats. After repeated washings in HBSS, worms were taken up in cold PBS (pH 7.2) and disrupted in an MSE homogenizer. The homogenate was left overnight (18 h) at 4°C and centrifuged at the same temperature at 1500 g for 30 min to separate and subsequently remove coarse particulate matter. The supernatant fluid was ultracentrifuged at 180,000g for 1 h at 4°C. Protein in the supernatant fluid (saline extract antigens) was determined by the method of Waddell & Hill (1956).

3.1.2 Design

For injection, the saline extract preparation was made into oil-in-water emulsion with FCA by repeated mixing of antigen and adjuvant. One group of mice received s.c. 2 mg protein in the adjuvanted form. Another group was given 2 mg but without adjuvant. A third group acted as an injection control and was given FCA alone. Experimental and control mice were challenged by surgical transplantation of single 6-day-old worms from rat donors 14 days after treatment. They were killed on day 21.

3.1.3 Results

Fig. 4 shows the growth and survival of the challenge worms in vaccinated and control mice. Saline extract antigens given s.c. with or without adjuvant were ineffective in stimulating protective immunity against challenge.

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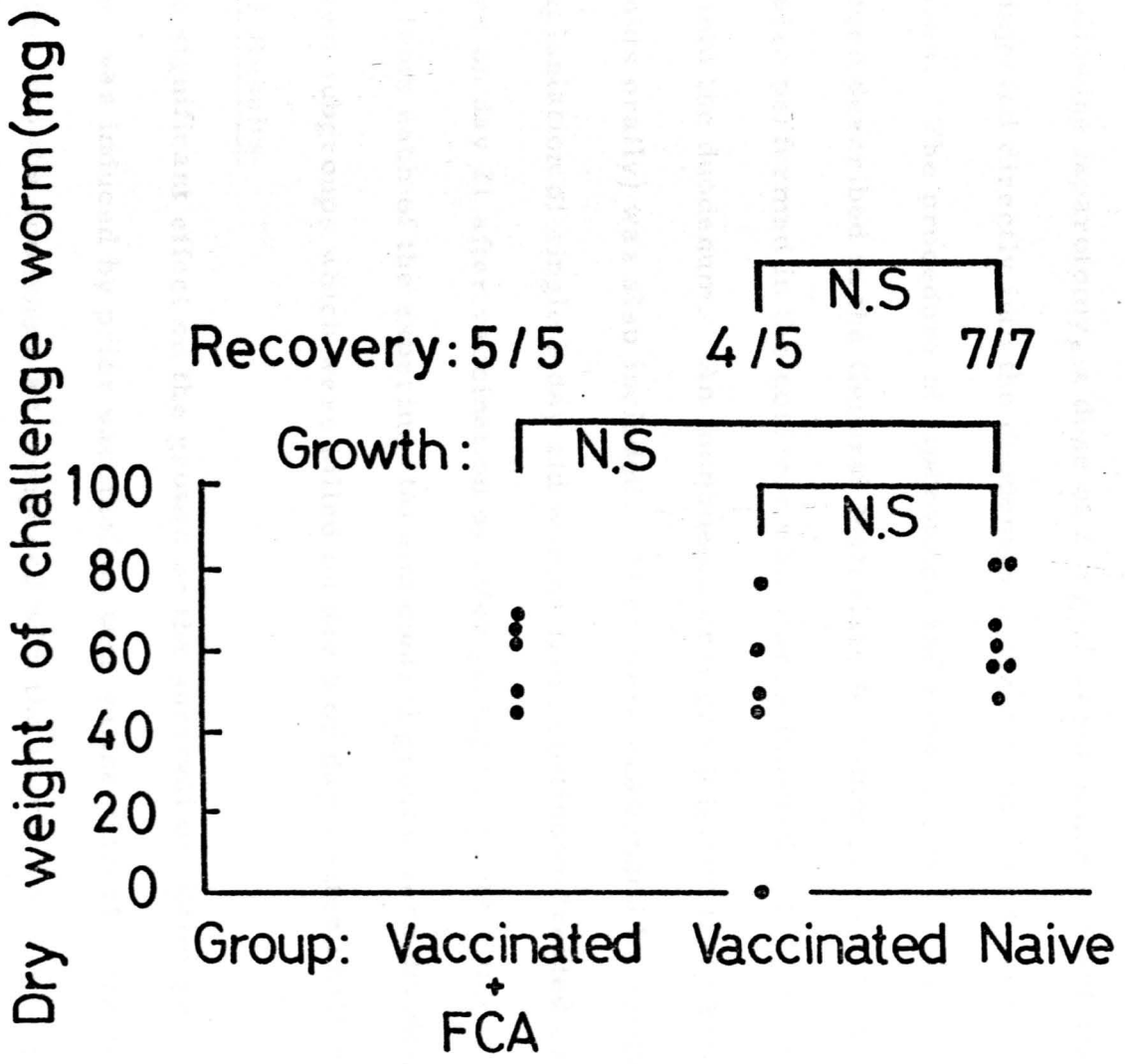
Figure 4

Growth and survival of 6-day-old H. diminuta single-worm challenge transplants in mice vaccinated s.c. with saline extract antigens and in the naive recipient controls.


N.S
Recovery: 5/5
4/5
7/7
Growth:
N.S
N.S
Dry weight of challenge worm (mg)
100
80
60
40
20
0
Group: Vaccinated + FCA
Vaccinated
Naive

3.2 SEA Given Intraduodenally

3.2.1 Materials & Methods

Female CFLP mice were vaccinated when 7-week-old with the antigen preparation used in the previous experiment (3.1.1).

3.2.2 Design

Following laparotomy, a dose of 2 mg of worm saline extract antigens was injected directly into the duodenum in the direction away from the stomach. The procedure of laparotomy and exposure of the duodenum has been described in the General Materials & Methods. Laparotomy was also performed in the controls which were injected with 0.18 ml of PBS into the duodenum. An immune control group (given five cysticercoids orally) was also included. Mice were challenged by surgical transplantation of single 7-day-old worms from cortisone-treated mouse donors on day 21 after vaccination or after giving the control injections. Mice from each of the experimental and control groups were divided into two subgroups which were killed on day 5 or day 7 after challenge.

3.2.3 Results

No significant effect on the growth or the survival of challenge worms was induced by prior vaccination with saline extract antigens injected into the duodenum as compared with the naive controls (Fig. 5). On the other hand, mice which were given five cysticercoids were strongly protected against challenge.

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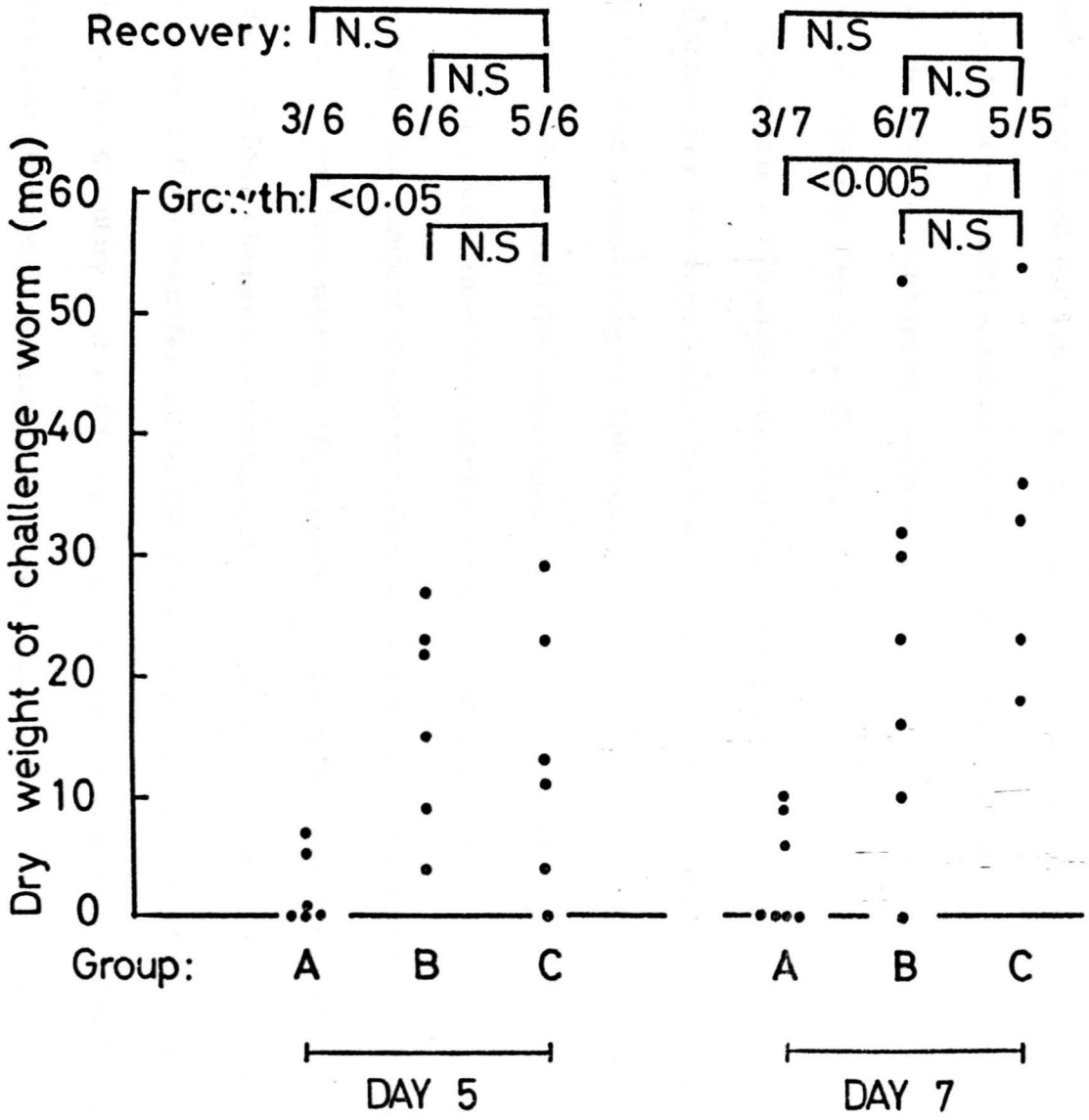
Figure 5.

Growth and survival of 7-day-old H. diminuta single-worm transplants in mice vaccinated intraduodenally with saline extract antigens (B) and in the immune (A) and naive (C) recipient controls.


Recovery: N.S
N.S
3/6 6/6 5/6
N.S
N.S
3/7 6/7 5/5
Growth: <0·05
N.S
<0·005
N.S
Dry weight of challenge worm (mg)
60
50
40
30
20
10
0
Group: A B C
A B C
DAY 5
DAY 7

4. TEGUMENT ANTIGENS (TA)

4.1 Isolation of the Tegument

The technique of isolating the tegument of H. diminuta was originally described by Oaks, Knowles & Cain (1974) using the plant glycoside, saponin and low shear-force agitation. In the present study, a different buffer was used for isolating the tegument but all other procedures were fundamentally similar to those described by the above authors. The membrane disruption solution (MDS) was prepared as follows: saponin (Sigma Chemical Co.) - 1% w/v; $CaCl_2$ - 10 mg/ml; and Crystamycin - 100 units sodium benzylpenicillin and 100 ug streptomycin sulphate per ml were added to tris-maleate buffered saline (TMBS, pH 7.2 and osmolarity of 300 mosmol). The MDS was centrifuged for 1 h at 20,000 g and the supernatant was stored at -15°C.

A pilot experiment was carried out in order to estimate the tegument yield and the amount of worm tissue required for a vaccination experiment. Strobilate worms (10-days-old) were recovered from rats, rinsed in four changes of HBSS and distilled water for a final rinse. They were then transferred to 250 ml conical flasks and MDS was added in a quantity just sufficient to immerse the worms. The flasks were transferred to a Gallenkamp water-bath incubator with a shaker speed set at approximately 110 strokes/min and a temperature of 4-5°C. After 10 min, worms and the bathing MDS were poured into test tubes and vibrated with a Vortex mixer (Scientific Industries Ltd.). Worm carcasses were taken out and the tegument-containing MDS was centrifuged at 200 g for 3 min at 4°C to sediment heavy contaminants

such as proglottids and worm eggs. The supernatant, containing tegument antigens, was transferred to dialysis tubes ('Dialysis Tubing - Visking', Medicell International Ltd.) and dialysed against TMBS overnight. The antigen was concentrated by covering the dialysis tubes with 'Aquacide'. All procedures were performed under refrigeration. Protein was determined by the Lowry et al. (1951) method. The value obtained is equivalent to 5.7 mg tegument protein per gram worm wet weight.

4.2 TA Given Orally

4.2.1 Materials & Methods

Male NIH were used when 6-weeks-old. Three antigen batches were prepared from 23, 23 and 30 g wet wt worm tissue agitated in 15, 33 and 25 ml of MDS respectively. Protein in the three antigen preparations was estimated (Lowry et al., 1951) as 5.22, 12.6 and 15.07 mg/ml equivalent to 3.4, 18.08 and 12.56 mg protein/g wet wt of tapeworm tissue. Antigens in these batches were pooled, dialysed and concentrated as described earlier (4.1). The pooled antigens were administered orally by a stomach tube to mice pretreated with 0.25 ml of 1.4% $NaHCO_3$ given by the same route.

4.2.2 Design

Each mouse received 1.75 mg of antigen protein. Naive controls were given TMBS. An immune control group was also included. Mice were challenged on day 21 with an oral infection of six cysticercoids. They were killed on day 29.

4.2.3 Results

Oral vaccination with 1.75 mg of H. diminuta tegumental protein was associated with a statistically significant reduction in both growth (Fig. 6-1) and survival (Fig. 6-2) of challenge worms as compared with the naive controls. The proportion of worms $\geq$0.1 mg dry wt was also significantly lower in the vaccinated than in the naive controls (Fig. 6-2). Nonetheless, the degree of the protection induced by vaccination was weaker than that stimulated by an infection starting from cysticercoids.

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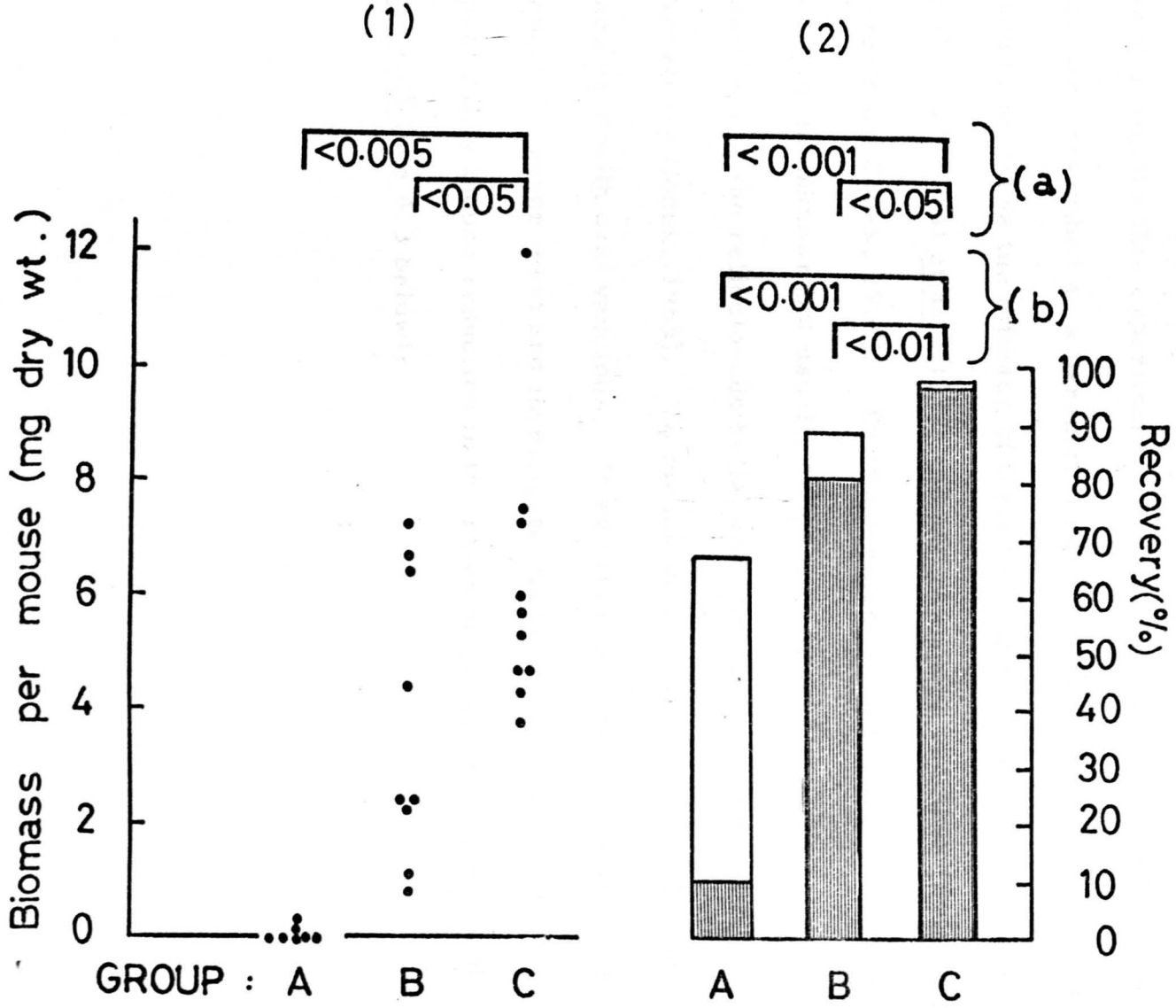
Figure 6.

Growth (1) and survival (2) of worms from a six-cysticercoid challenge infection in mice vaccinated orally with 1.75 mg of H. diminuta tegument (B) and in the immune (A) and naive (C) controls.

Shaded portions of bars represent worms $\geq$ 0.1 mg; open portions, worms $<$ 0.1 mg.

(a): Level of significance (total recovery)

(b): Level of significance (worms $\geq$ 0.1 mg)


(1)
(2)
<0.005
<0.05
<0.001
<0.05
(a)
<0.001
<0.01
(b)
Biomass per mouse (mg dry wt.)
0
2
4
6
8
10
12
GROUP : A B C
Recovery(%)
0
10
20
30
40
50
60
70
80
90
100
A
B
C

Since the naive controls were injected with TMBS (which contained no saponin), it is a possibility that the protection induced in vaccinated mice could have been non-specifically induced by the saponin incorporated in the membrane disruption solution, a point which I overlooked when designing this experiment. This seemed possible as saponin has been described as a parenteral adjuvant (Gall, 1966), and has been used to increase the efficacy of trypanosome vaccines (Johnson, Neal & Gall, 1963; Neal et al., 1977) and anthrax spore vaccines (British Veterinary Codex, 1965). The mechanism by which saponin acts as an adjuvant is unknown but usually appears to be mediated by cellular reactions of the reticuloendothelial and lymphatic systems (British Veterinary Codex, 1965). To my knowledge, this adjuvant has not been used with oral vaccines. It was therefore deemed necessary to repeat the experiment and investigate the potential of saponin to non-specifically induce reduction in the rates of growth and survival of H. diminuta (see 4.3 below).

4.3 TA Given Orally (A Replicate)

4.3.1 Introduction

This experiment is a replicate of experiment 4.2. It was designed to establish whether H. diminuta tegument antigens are the functional antigens. The experiment also investigates the effect of increasing the amount of the injected antigen and the possibility that saponin could act as a non-specific immunopotentiator against a tapeworm residing in the intestine.

4.3.2 Materials & Methods

Male NIH mice were used at the age of 6 weeks. Tegument antigen was prepared from 10-day-old worms (66 g wet wt) obtained from 14 rats each given 10 cysticercoids. Tegument membranes were disrupted in 70 ml of MDS. Protein was estimated (Lowry et al., 1951) as equivalent to 6.03 mg/g wet wt of worm tissue (cf. 4.1; 4.2). The dose of saponin to be received by control mice was calculated from the concentration factor of MDS after dialysis of the antigen preparation (x 11.7). $NaHCO_3$ was also given to mice in this experiment.

4.3.3 Design

The regimen of vaccination is shown in Table 3. Mice were challenged on day 21 with six cysticercoids and they were killed on day 29.

4.3.4 Results

The results are shown in Fig. 7-1 and Fig. 7-2. It is evident that these results are at variance with those reported in the previous experiment (4.2); vaccination with a dose of 1.75 mg of tegument protein did not stimulate protection against challenge. Vaccination

Table 3. Regimen of oral vaccination of mice with tegument antigens

Group	Treatment
1. Vaccinated	1.75 mg (0.3 ml)
2. Saline control	TMBS (0.3 ml)
3. Saponin control	MDS (0.3 ml)
4. Vaccinated	2.0 mg (0.4 ml)
5. Saponin control	MDS (0.4 ml)
6. Immune control	6 cysticercoids

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Figure 7-1

Growth of worms from a six-cysticercoid challenge infection in mice vaccinated orally with 1.75 or 2.0 mg of H. diminuta tegument and in the immune and naive controls.

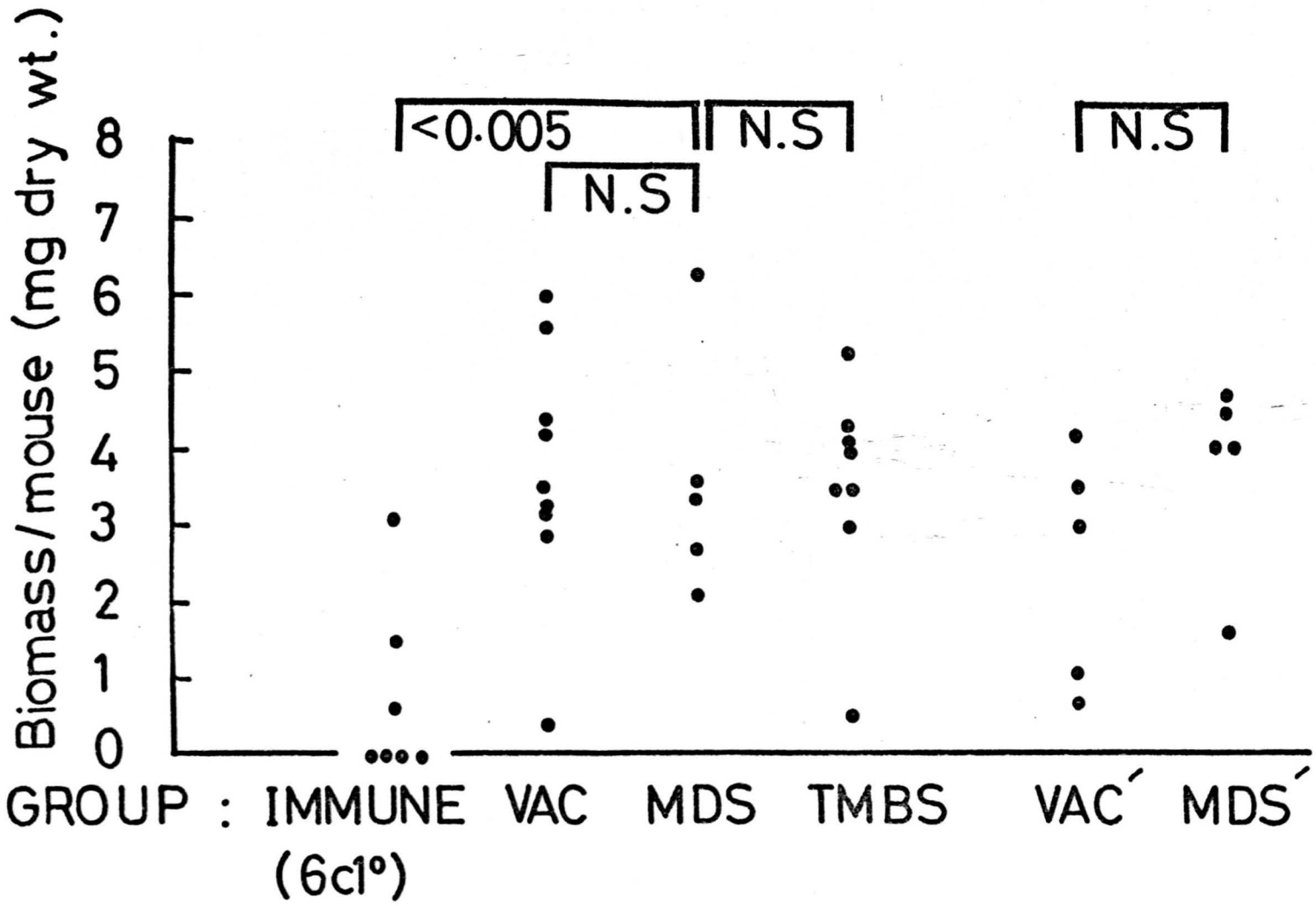
VAC: Vaccinated with 1.75 mg tegument.

MDS: Naive controls given saponin.

TMBS: Naive controls given no saponin.

VAC′: Vaccinated with 2 mg tegument.

MDS′: Naive controls given saponin.


Biomass/mouse (mg dry wt.)
0
1
2
3
4
5
6
7
8
<0·005
N.S
N.S
N.S
GROUP : IMMUNE
(6c1°)
VAC
MDS
TMBS
VAC´
MDS´

Facing page 56

Figure 7-2

Survival of worms from a six-cysticercoid challenge infection in mice vaccinated orally with 1.75 or 2.0 mg of H. diminuta tegument and in the immune and naive controls.

Shaded portions of bars represent worms $\geqslant$ 0.1 mg; open portions, worms $<$ 0.1 mg.

(a): Level of significance (total recovery)

(b): Level of significance (worms $\geqslant$ 0.1 mg)

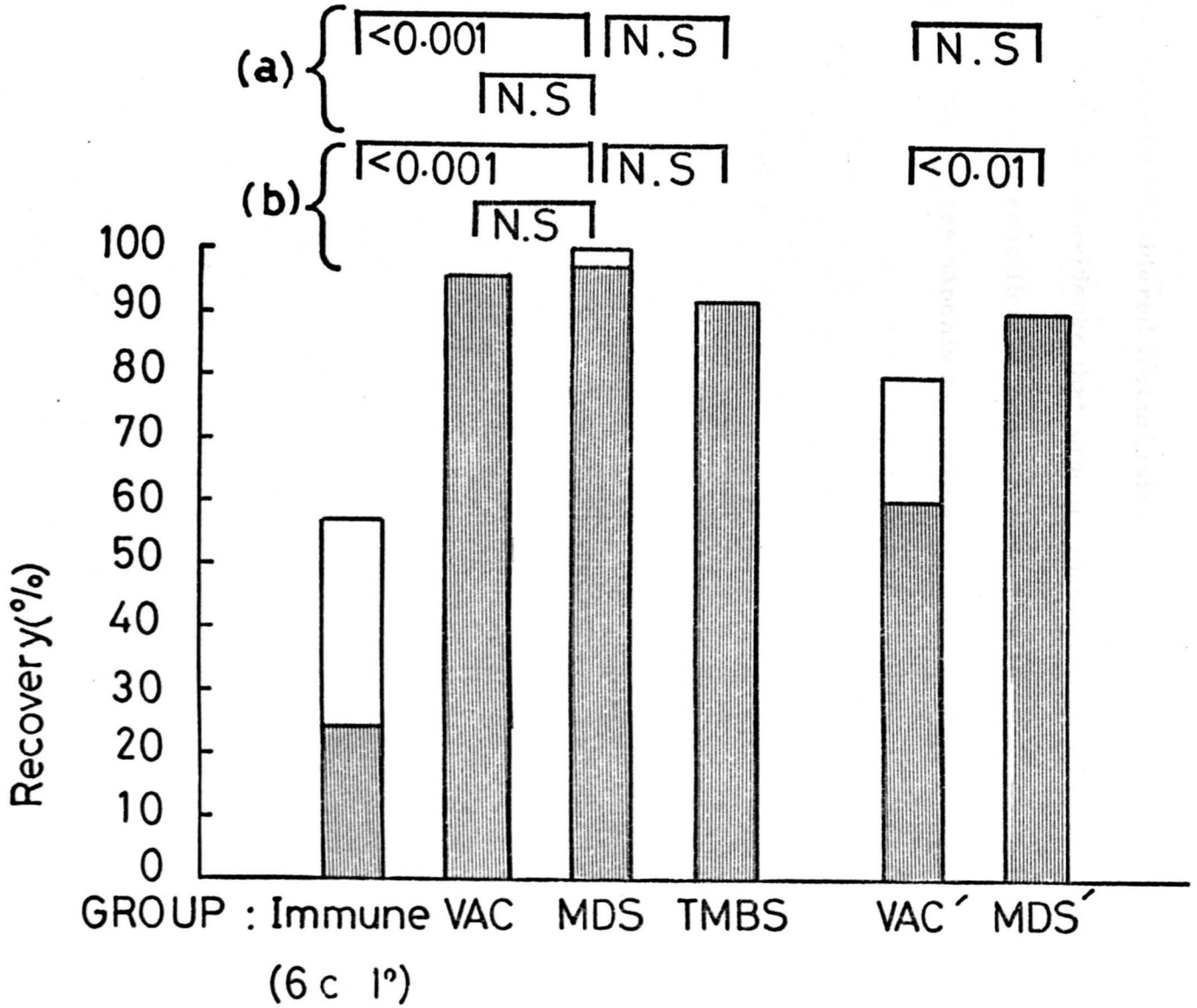
VAC: Vaccinated with 1.75 mg tegument.

MDS: Naive controls given saponin.

TMBS: Naive controls given no saponin.

VAC′: Vaccinated with 2.0 mg tegument.

MDS′: Naive controls given saponin.


(a)
<0·001
N.S
N.S
N.S
(b)
<0·001
N.S
N.S
<0·01
100
90
80
70
60
50
40
30
20
10
0
Recovery(%)
GROUP : Immune VAC MDS TMBS VAC´ MDS´
(6 c l°)

with a larger dose of 2 mg induced no significant reduction in total worm biomass as determined by the Wilcoxon test, but the proportion of worms $\geq$0.1 mg dry wt was significantly lower in the vaccinated mice than in the naive controls which also received saponin (χ^2 test). Since the number of mice vaccinated with 2 mg is relatively small (five), the results are considered inconclusive.

There is no evidence that saponin incorporated with the tegument antigens non-specifically induce a reduction in growth or survival of H. diminuta (see saponin and saline controls in Fig. 7-1 and Fig. 7-2).

4.4 TA Given Orally: Effect of Varying the Regime of Vaccination

4.4.1 Introduction

Because no conclusive evidence was obtained from either of experiment 4.2 or 4.3 as to the potential of tegumental antigens in raising protective immunity against challenge, this experiment was carried out. It is similar to both experiment 4.2 and 4.3, but three variations were introduced: increasing the size of the inoculated dose, administration of a booster dose and starving mice before antigen administration.

4.4.2 Materials & Methods

Six-week-old male NIH mice were used in this experiment. The tegument was isolated from 25-day-old worms (92.6 wet wt) obtained from rats each given six cysticercoids. Antigen and $NaHCO_3$ were administered orally to mice previously starved for $3\frac{1}{2}$ h.

4.4.3 Design

The protocol of vaccination is presented in Table 4. Mice were challenged on day 21 with six cysticercoids and were autopsied on day 29.

4.4.4 Results

Vaccination with 2.25 mg of tegument protein had no protective effects. Mice vaccinated with two doses harboured significantly lighter worms than the saponin-injected naive controls (Wilcoxon test) as shown in Fig. 8-1. The χ^2 test (Fig. 8-2), however, showed that neither the survival rate of the challenge worms nor the proportion of worms ≥ 0.1 mg dry wt was affected significantly. A

Table 4. Schedule of oral vaccination of mice with tegument antigens

Group	1st dose (day 0)	booster (day 10)
1. Vaccinated	2.25 mg (0.35 ml)	
2. Saponin control	MDS (0.35 ml)	
3. Vaccinated	2.25 mg (0.35 ml)	2.0 mg (0.31 ml)
4. Saponin control	MDS (0.35 ml)	MDS (0.31 ml)

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Figure 8.

Growth (1) and survival (2) of worms from a six-cysticercoid challenge infection in mice vaccinated orally with different doses of H. diminuta tegument and in the naive controls.

Stippled portions of bars represent worms ≥ 0.1 mg; open portions, worms < 0.1 mg.

(a): Level of significance (total recovery)

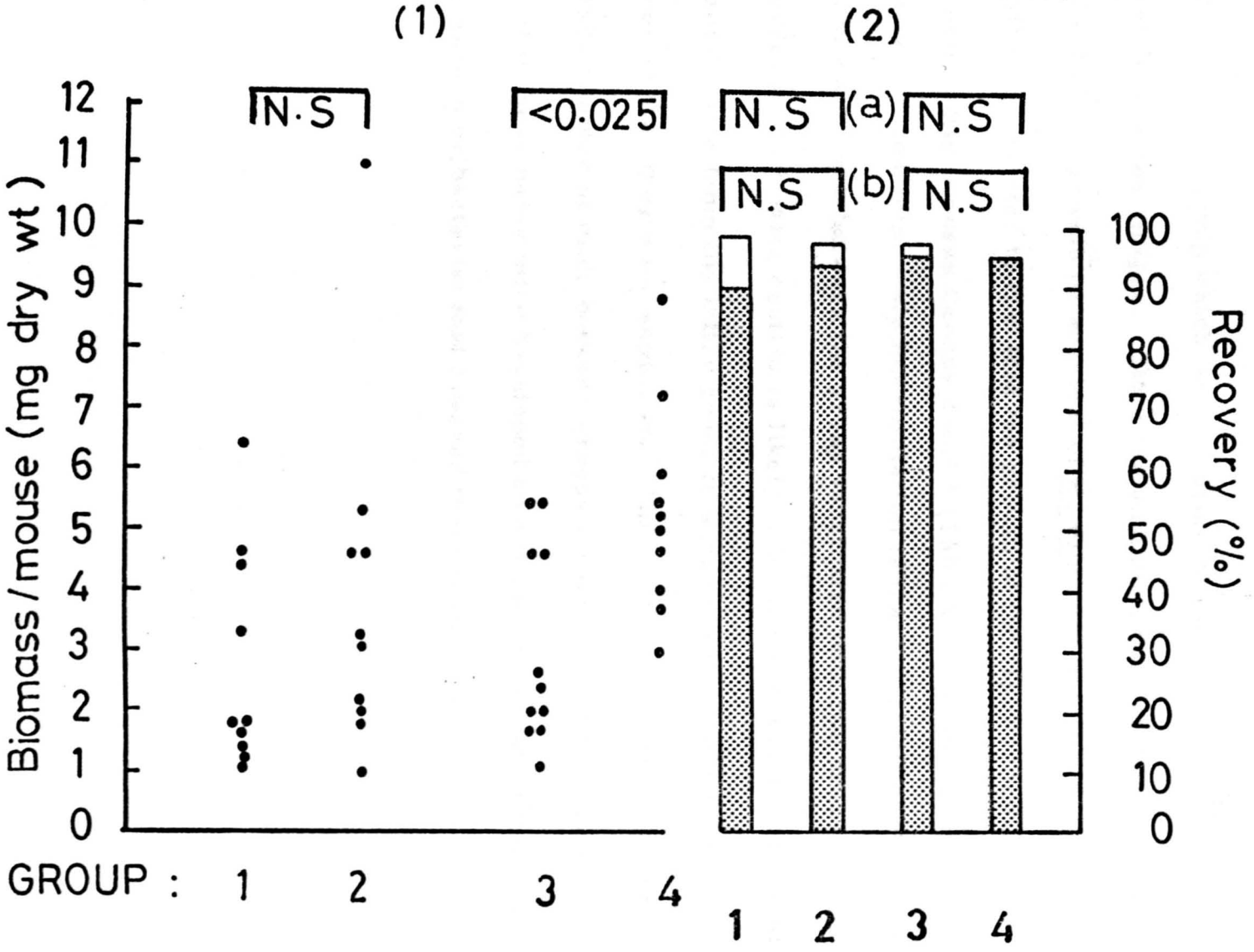
(b): Level of significance (worms ≥ 0.1 mg)

Group 1: Vaccinated with one dose

2: Naive controls given one injection of MDS

3: Vaccinated with two doses

4: Naive controls given two injections of MDS


(1)
(2)
N.S
<0.025
N.S
(a)
N.S
N.S
(b)
N.S
Biomass/mouse (mg dry wt)
Recovery (%)
0
1
2
3
4
5
6
7
8
9
10
11
12
0
10
20
30
40
50
60
70
80
90
100
GROUP :
1
2
3
4
1
2
3
4

crucial point to consider is that the growth figures of challenge worms in mice vaccinated with two doses are not different from those in naive mice receiving MDS on day 0 only (Table 4; Figs. 8-1 and 8-2). They are also indistinguishable from the growth figures of challenge worms in the group which was not protected by the administration of one dose of the antigen. Since the administration of saponin has no effect on the growth or survival of H. diminuta in mice (4.3), it was rather unexpected to find that the growth of the tapeworm differed significantly between Groups 2 and 4 (Table 4, Fig. 8-1), both of which are naive controls. My interpretation is that the significant difference in worm growth between the group vaccinated with two doses and the corresponding naive control is likely to be attributed to a factor that caused mice from the latter group to support a mass of worms greater than they were supposed. This factor could be the difference in the number of mice between groups (fewer naive mice in a cage) or that these naive mice developed an asymptomatic infection, e.g. protozoan or bacterial that lowered their resistance.

5. EXOANTIGENS (EXA)

5.1 EXA Given s.c. or i.p.

5.1.1 Materials & Methods

Mice were female CFLP of 6 weeks of age. For the collection of secretory and excretory antigens (exoantigens, EXA), rats were given 60 or 63 cysticercoids each. Ten days after infection, worms were recovered and washed several times in HBSS to remove adherent debris. They were rewashed through four successive changes, 15 - 25 min each, of HBSS (autoclaved at 34474 Pa) to which Crystamycin was subsequently added (100 units sodium benzylpenicillin and 100 ug streptomycin sulphate per ml). Worms were then incubated in four sterile 250 ml conical flasks each containing 100 ml of tissue culture medium 199 (1X, Gibco) with Hanks' salts, HEPES buffer and L-glutamine. To each 100 ml of medium was added 5 ml of 6.5% glucose (autoclaved); 2 ml of 4.4% $NaHCO_3$ (autoclaved); 1 ml of 2% KCL (autoclaved); 10,000 units of sodium benzylpenicillin and 10 mg of streptomycin sulphate ('Crystamycin', Gibco); 500 ug of 'Fungisone' (Gibco) and 20,000 units of polymyxin B sulphate (Gibco). The final pH was 7.3. Incubation was performed at 37 ^{o}C in a shaking incubator (65 strokes/min). The culture incubate medium was collected after 20 h of incubation by which time all the worms were dead.

Assuming that the worm protective antigens were of a high molecular weight (mol. wt), protein antigen in the culture medium was determined (Lowry et al., 1951) in samples dialysed against PBS (pH 7.3) in an Amicon 8MC Ultrafilteration Unit using a Diaflo filter membrane (UM10) of 10,000 mol. wt exclusion limit. The samples from the

four flasks contained 100, 250, 350 and 400 ug protein (mol. wt > 10,000) per ml. Antigen preparations were pooled before administration to mice.

5.1.2 Design

Antigen injections were given according to the schedule set out in Table 5. Mice were challenged by surgical transplantation of single 8-day-old worms from cortisone-treated mice on day 21. They were killed on day 27.

5.1.3 Results

Fig. 9 shows that vaccination with exoantigens of the tapeworm had no significant effect on either the growth or the survival of the homologous challenge. The mice exposed to cysticercoid infection were strongly protected.

Table 5. Schedule of vaccination with exoantigens

Group	day 0		day 11	
	dose	route	dose	route
1. Vaccinated	275 ug	i.p.	275 ug	i.p.
2. Naive control	PBS	i.p.	PBS	i.p.
3. Vaccinated	550 ug	i.p.		
4. Naive control	PBS	i.p.		
5. Vaccinated	40 ug in FCA	s.c.	400 ug	i.p.
	140 ug	i.p.		
6. Naive control	PBS in FCA	s.c.	PBS	i.p.
	PBS	i.p.		
7. Immune control	6 cysti-cercoids	oral		

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Figure 9.

Growth and survival of 8-day-old H. diminuta single-worm challenge transplants in mice vaccinated with exoantigens and in the immune and naive recipient controls.

A: Immune controls.

B: Vaccinated i.p. with two doses.

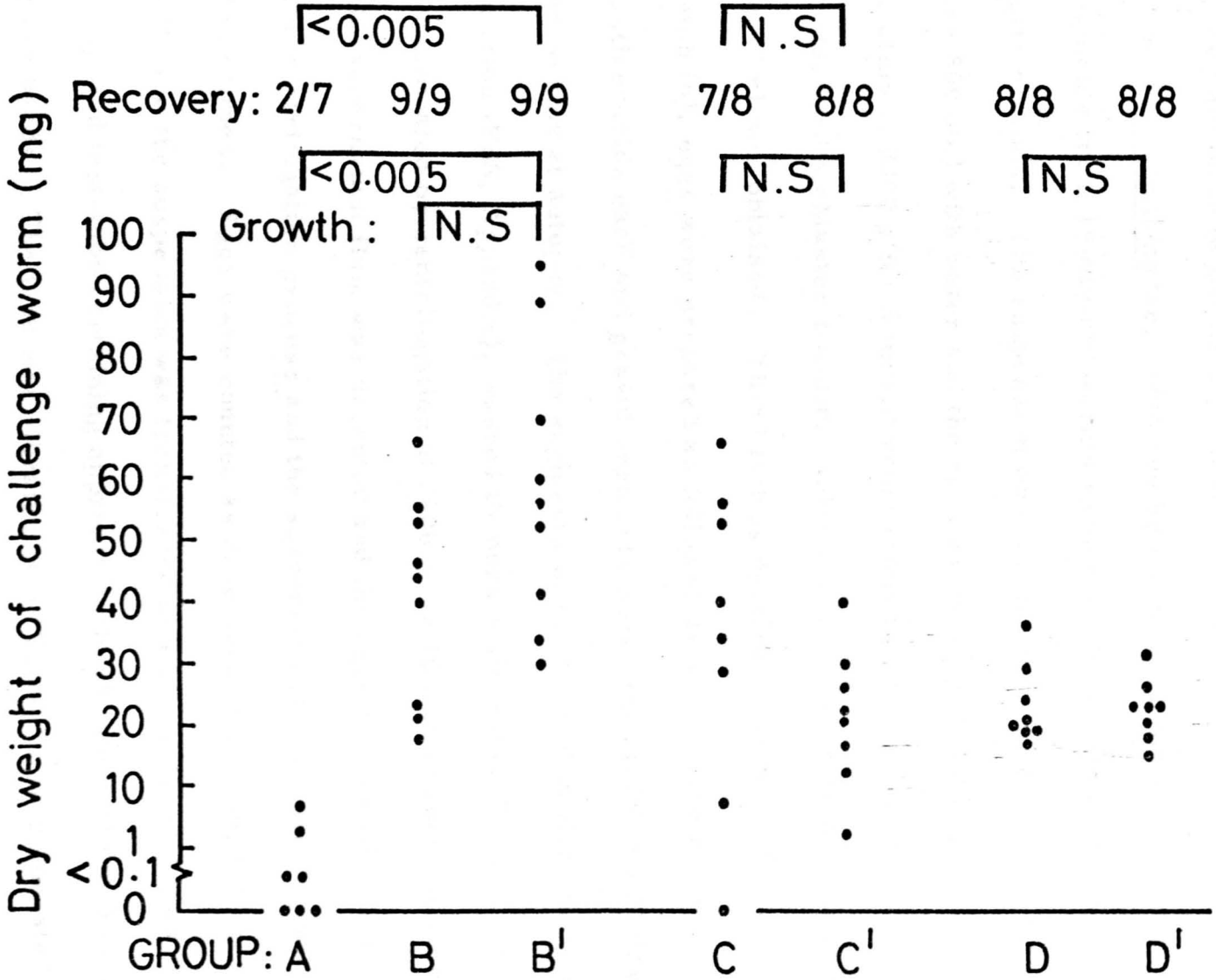
B′: Naive controls for group B.

C: Vaccinated i.p. with one dose.

C′: Naive controls for group C.

D: Vaccinated s.c. and i.p.

D′: Naive controls for Group D.


<0·005
N.S
Recovery: 2/7 9/9 9/9 7/8 8/8 8/8 8/8
<0·005
N.S
N.S
Growth : N.S
Dry weight of challenge worm (mg)
100
90
80
70
60
50
40
30
20
10
1
< 0·1
0
GROUP: A B B' C C' D D'

6. EGG ANTIGENS (EA)

6.1 EA Given s.c. or Orally

6.1.1 Materials & Methods

Female mice of the CFLP strain were used at 6 weeks of age. Eggs of H. diminuta were obtained by chopping in tap water gravid segments from 19-day-old worms recovered from rats each given 10 cysticercoids. The suspension was washed through a sieve (aperture size 800 um) with water and the eggs were concentrated by centrifugation at 2000 g for 5 min at room temperature. They were counted in standard McMaster counting slides and a concentration of 60,666 eggs/ml was obtained. This batch is designated as batch (a). In batch (b), eggs were prepared as follows: two rats were given 10 cysticercoids each and gravid segments were collected from 20-day-old worms at autopsy. The segments were chopped in Earle's salt solution (ESS, Appendix), washed through a sieve and the eggs were concentrated by centrifugation at 2000 g for 10 min at room temperature. The supernatant fluid was decanted and the eggs resuspended in ESS. The centrifugation process and the suspension of eggs was repeated three times. Eggs were counted as described above (16,000 eggs/ml), and the suspension was transferred to a 25-ml 'Exelo' glass stoppered test-tube containing approximately 15 mm depth of 3 mm glass beads. The tube was vibrated on a 'Vortex' mixer operated for 5 min to break eggs' outer shell layers. Crystamycin was added in a concentration of 100 units sodium benzylpenicillin and 100 ug streptomycin sulphate/ml.

6.1.2 Design

Eggs were administered to three groups of mice according to the schedule set out in Table 6. In group (1) the eggs were given with an adjuvant. Whether or not these eggs lose viability after incorporation into the adjuvant was not investigated. Eggs from batch (a), which were given orally to mice, were viable eggs. Those from batch (b) were eggs in which the shells had been disrupted. The inclusion of this experiment under the title 'Vaccination with Non-Living Worm Antigens' was based on the assumption that incorporation of eggs into FCA affects their viability which can also be affected by inoculation of the eggs into the subcutis. Mice were challenged with six cysticercoids on day 21 and they were killed on day 29.

6.1.3 Results

Viable eggs given s.c. without adjuvant or given orally did not protect mice against challenge (Fig. 10-1 and Fig. 10-2). Eggs given s.c. in the adjuvanted form (presumably as non-viable egg forms) also conferred no protection on these animals.

Table 6. Regimen of vaccination of mice with eggs of H. diminuta

Group	antigen batch	dose	route
1. Vaccinated	(a)	6,000 eggs (0.1 ml) in 0.1 ml FCA	s.c.
2. Naive control		ESS (0.1 ml) in 0.1 ml FCA	s.c.
3. Vaccinated	(b)	1,600 eggs (0.1 ml)	s.c.
4. Vaccinated	(a)	60,000 eggs (1 ml)	oral
5. Naive control		water (1 ml)	oral
6. Immune control		6 cysticercoids	oral
		FCA (0.1 ml)	s.c.

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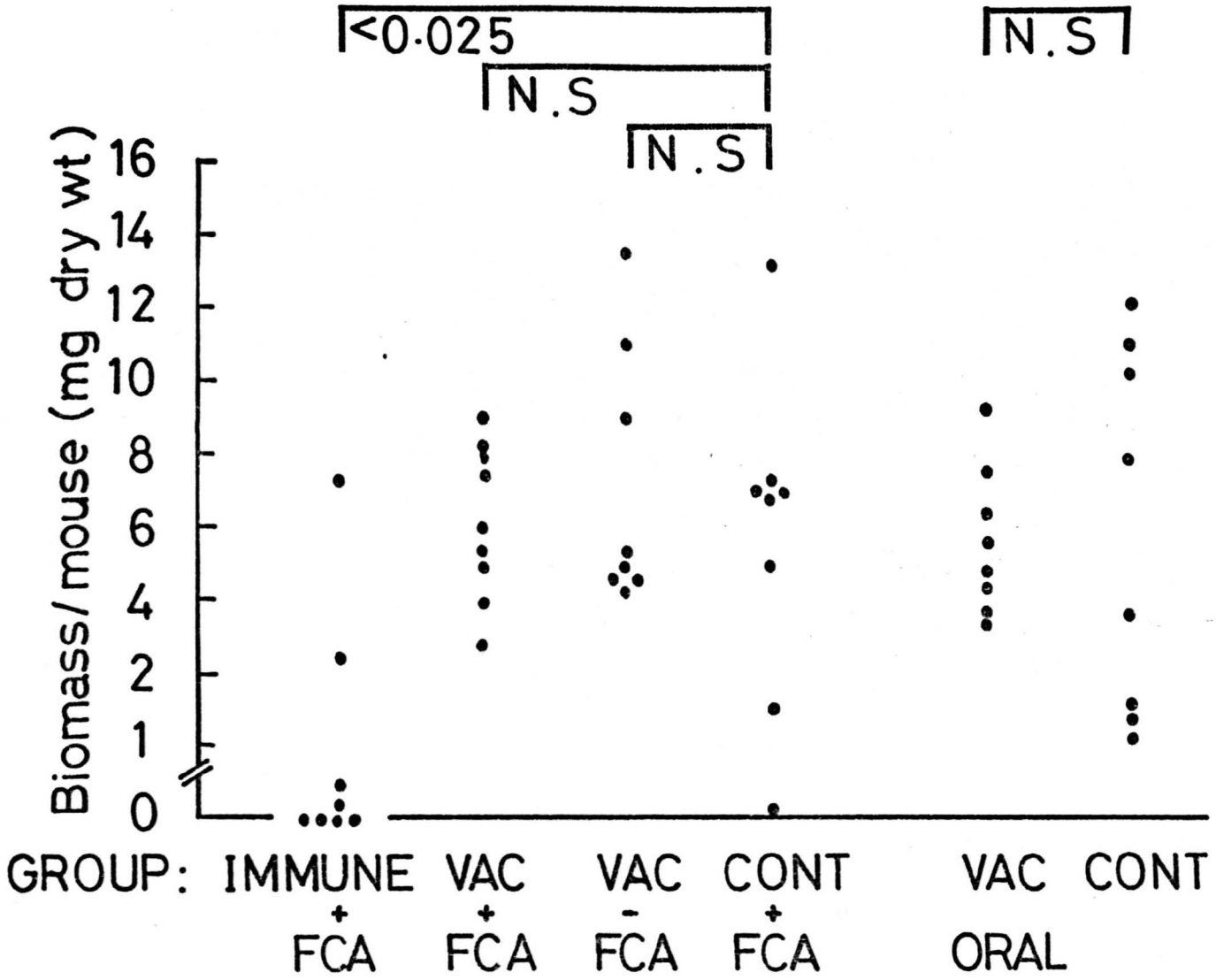
Figure 10-1

Growth of worms from a six-cysticercoid challenge infection in mice vaccinated with H. diminuta egg antigens and in the immune and naive controls.

VAC: Vaccinated

CONT = Controls

FCA = Freund's complete adjuvant.


<0·025
N.S
N.S
N.S
Biomass/mouse (mg dry wt)
16
14
12
10
8
6
4
2
1
0
GROUP: IMMUNE + FCA
VAC + FCA
VAC - FCA
CONT + FCA
VAC ORAL
CONT

Facing page 70

Figure 10-2.

Survival of worms from a six-cysticercoid challenge infection in mice vaccinated with H. diminuta egg antigens and in the immune and naive controls.

Shaded portions of bars represent worms $\geqslant$ 0.1 mg; open portions, worms $<$ 0.1 mg.

(a): Level of significance (total recovery)

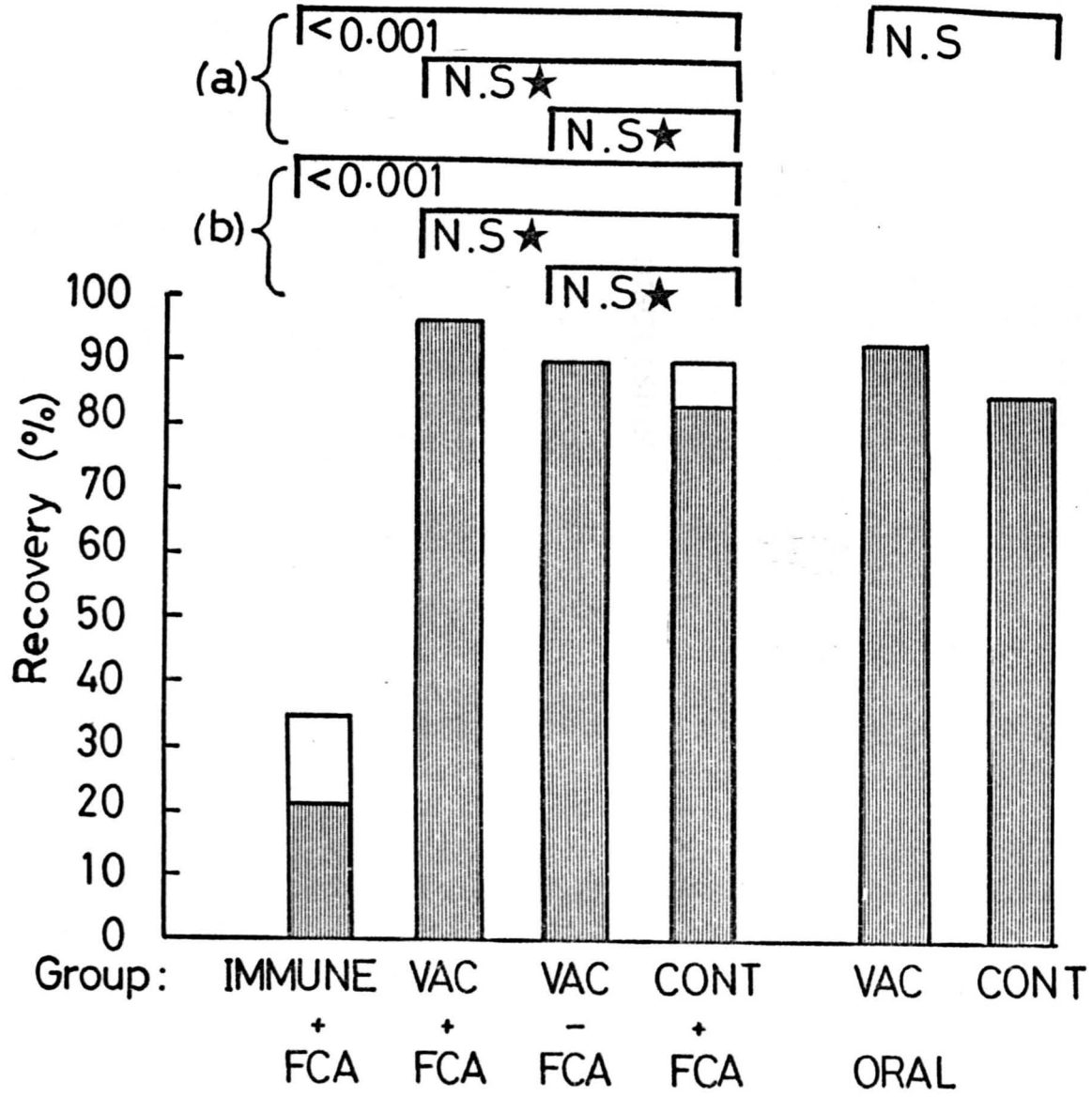
(b): Level of significance (worms $\geqslant$ 0.1 mg)

★: χ^2 test (2-tailed)

VAC: Vaccinated

CONT = Naive controls

FCA = Freund's complete adjuvant.


(a)
<0·001
N.S★
N.S★
(b)
<0·001
N.S★
N.S★
N.S
Recovery (%)
100
90
80
70
60
50
40
30
20
10
0
Group:
IMMUNE + FCA
VAC + FCA
VAC − FCA
CONT + FCA
VAC ORAL
CONT

DISCUSSION

DISCUSSION

The results obtained from experiments in this section have shown that vaccination with non-viable H. diminuta antigens (whole worm somatic antigens given s.c., sonic disruption antigens, saline extracts, exoantigens and egg antigens) induced no protection against challenge. Although a statistically significant reduction in the growth of challenge worms was observed in mice receiving six and seven oral doses of tapeworm somatic antigens (1.1), the protection so evoked was of low order and the regimes of vaccination described were therefore regarded to have little practical significance. I was unable to reproduce satisfactorily the results from experiment 4.2 in which vaccination with a single oral dose of 1.75 mg protein of worm isolated tegumental fraction appeared to induce a weak protection against challenge. No immunity was induced in the replicate of this experiment (4.3) nor was resistance stimulated when higher doses of 2.0 mg and 2.25 mg were given (4.3 and 4.4). In one trial the oral administration of 2.25 mg of protein followed 10 days later by a booster dose of 2.0 mg resulted in a significant decrease in the growth of the challenge worms (4.4). The proportion of worms < 0.1 mg dry wt (i.e. worms definitely affected by the immune response), however, was not increased significantly by administration of the vaccine. The growth of worms in this vaccinated group was no different from that in naive mice of the same age and sex forming another control group (Fig. 8-1 and Fig. 8-2). Ogilvie & Jones (1973) suggested that, since trematode and cestode teguments are consistently being shed and renewed from beneath, the membranes released in this way could be the source of immunogens.

In addition to what may be structural proteins, the tegument of H. diminuta, which is a metabolically active surface, contains intrinsic membrane-bound enzymes (Arme, 1976). Its glycocalyx was considered by Lumsden (1975) as a dynamic structure for which there is evidence of turnover. The demonstration of Befus (1977) that the tegument of H. diminuta binds immunoglobulins and that it is liable to damage that is possibly immunologically-mediated (Befus et al., 1975), furthermore, justifies the contention that vaccination with an isolated tegument fraction of H. diminuta (this section) is likely to induce immunity. Indeed, effective antigens have been extracted from the surface coat of trypanosomes (Cross, 1975) and antigen bound to the membrane of adult Schistosoma mansoni was found to be effective in stimulation of antibodies lethal to schistosomula grown in vitro (Sher et al., 1974). The results I obtained during the present investigation provided no conclusive evidence that tegument antigens are effective in raising protective immunity against H. diminuta in mice.

The administration of whole organisms or homogenates of parasites was recognized in various situations to elicit serological responses in the hosts (Terry, 1968). These responses, however, are not invariably associated with protective immunity and it is only occasionally that the administration of whole parasite antigens stimulated significant protection [for instance, the nematode Trichostrongylus colubriformis in guinea pigs (Rothwell, 1974), the trematode Fasciola hepatica in mice (Lang et al., 1977), the cestode T. saginata in calves (Gallie et al., 1976) and the protozoan Plasmodium knowlesi in monkeys (Butcher et al., 1978)] . Despite the fact that somatic

antigens from some parasites can confer protection on the vaccinated hosts, rarely have the antigens responsible for this protection (protective/functional antigens) been characterized (Despommier & Müller, 1970; Cross, 1975; Herd et al., 1975; Jenkins & Wakelin, 1977).

The use of parasite extracts was suggested by Cox (1978) to have many advantages over the use of whole parasite antigens. Indeed, highly immunogenic substances have been extracted from selected glands of nematodes such as Trichinella spiralis (Despommier et al., 1970) and Trichuris muris (Jenkins et al., 1977). Soluble antigens of Ascaris suum were also found to be protective when administered to guinea pigs (Stromberg & Soulsby, 1977).

Several attempts were described in which animals were vaccinated with antigens from somatic cells of parasites disrupted by sonication. Using this technique, Lang et al. (1977) successfully vaccinated mice against infection with F. hepatica. Antigens from embryos of T. ovis disrupted by sonic oscillation induced some resistance in sheep to the homologous challenge but no effect was observed when embryos of T. saginata were used (Gemmell, 1969).

Another approach to vaccination is the use of larval antigens in the stimulation of protective immunity against the adult forms of parasites. Experiment 6.1, in which mice were vaccinated with H. diminuta egg antigens, conformed to this concept. Although an adult cestode constitutes a histogenic continuity of the larval stage, its antigens, particularly those elaborated by metabolic processes, could exhibit fundamental structural differences from those of the larvae and may therefore induce an utterly different immunological response.

Nonetheless, in a species like H. nana, the adult worm was shown to share antigens with both the egg and the cysticercoid (Heyneman & Welsh, 1959) and in E. granulosus, the injection of artificially activated embryos of the parasite or those from T. hydatigena or T. ovis was reported by Gemmell et al. (1968) to induce protection against the parasite in dogs. This implies that the functional antigens of E. granulosus are present in embryos of the homologous or even heterologous species. Gemmell and his associate (1968), however, were unable to obtain a protective response in dogs against infection with E. granulosus by the inoculation of activated embryos of M. multiceps, T. pisiformis or T. serialis and nor did Swietlikowski et al. (1978) who attempted vaccinating rats against H. diminuta by injecting eggs or cysticercoids of the parasite. Dogs were also protected to some degree against E. granulosus by injections of protoscoleces or germinal membranes from hydatid cysts (Turner et al., 1936; quoted by Gemmell et al., 1968) but antigens from adult worms were reported to induce stronger immunity (Gemmell, 1962).

Parasitic helminths are complex organisms and the immune response they evoke in their hosts often involves intricate processes. Mice exposed to an H. diminuta infection have been shown to reject their worms immunologically (Hopkins et al., 1972 a, b). Despite the extensive studies carried out on the H. diminuta/mouse system, the origin of the worm protective antigens, the nature of these antigens and the precise mechanism(s) of worm rejection are obscure. It is therefore very difficult to relate the failure to vaccinate mice with the non-viable H. diminuta antigens (this section) to a single factor.

The most attractive explanation is that the tapeworm's protective antigens were not present initially in the preparations injected or that they were present in too low a quantity to bind with and subsequently stimulate the antigen-sensitive cells to cause their proliferation and differentiation. It may be that the worm's functional antigens are highly labile and that they were destroyed during preparation, despite the precautions undertaken, probably by enzymes released by the disintegrating worms themselves. It is also possible that worm immunogens administered by the oral route could have been inactivated by chemical reactions in the stomach or intestine. Since a natural infection of H. diminuta consistently conferred on mice protection against challenge (see immune control groups), refractoriness of mice to the killed worm antigens possibly suggests that functional immunity against this species relates to the living worm and its induction requires the continuous release of antigens.

A crude preparation from a large strobilate worm like H. diminuta undoubtedly contains a variety of antigens. Disruption of the normally protective tegument, as occurs in preparing a homogenate, is likely to present to the host antigens not normally met during the course of an infection (for instance, exposure to reproductive organ antigens). The presence of this wide array of 'non-functional' antigens could dissipate the host immune response and mask the presence of the functional antigens possibly as a result of antigenic competition. As an example, when haemoglobin, which does not itself stimulate detectable antibody in the chicken, is given simultaneously with bovine serum albumin (BSA), the response to BSA is lowered (Abramoff, 1955).

Problems stemming from competition of different antigens used concurrently in vaccinating animals are common. For instance, McHardy et al. (1978) found that all the single strain vaccines from Trypanosoma cruzi epimastigotes gave good protection in mice against the homologous as well as the heterologous challenge. The inclusion of more than one strain in the vaccine, however, not only failed to increase protection but, in some occasions, appeared to reduce it. The authors attributed their observations to antigenic competition.

Observations from other experimental models may provide further explanations for the failure to induce protection in mice with H. diminuta non-viable antigens. For example, the induction of an immune response to an antigen is known to be modulated by the physiochemical characteristics of the antigen. Ebersole & Molinari (1978) reported that local immunization with particulate bacterial antigens was capable of stimulating and maintaining a higher rate of antibody secretion than similar sensitization with soluble antigen preparations. Results obtained by both Waldman et al. (1970) and Strannegard et al. (1969) indicated that particulate antigens elicit a substantially better local response while soluble materials were more successful in inducing systemic antibody formation. Observations of this kind were attributed to the ability of intact particles to be retained for longer periods at the site of stimulation (Waldman et al., 1970). On other occasions, the administration of a soluble antigen can completely inhibit the development of the immune response. An example was provided by Baldwin & Robin (1977) who observed that a transplantable rat sarcoma was completely rejected when a simultaneous contralateral inoculum

of tumour cells and BCG was given. This response, however, was abrogated by simultaneous administration of solubilized antigen and BCG. Soluble antigens were suggested to be more tolerogenic than the aggregated forms (Roit, 1977).

As early as 1891, P. Ehrlich reported the appearance of serum antibodies against ricin in mice fed this protein. Since then, several publications demonstrated unequivocally that uptake and transport of immunologically significant amounts of substances (e.g. bovine and rat gamma globulin, ferritin, horseradish peroxidase, starch granules, BSA and botulinis toxin) occur across the intestinal wall of neonatal and adult animals resulting in immunological responses in intestinal and extraintestinal sites (reviewed, Bazin, 1979; Williams, 1979 b). Such responses to the orally administered antigens have been found predominantly of the IgA class of antibody (Crabbe et al., 1969; Bazin et al., 1970; Dolezel & Bienenstock, 1971). In other studies, oral immunization was shown to result in the formation of serum antibodies (Rothberg et al., 1967; 1973; Strannegard et al., 1969). Antigens given orally were also found to stimulate local and systemic cell-mediated immune responses (Frederick & Bohl, 1976; Huntley et al., 1979). An H. diminuta infection is restricted to the intestine of the host where antigenic recognition and stimulation occur and expression of the effector immune mechanism(s) is expected to take place. Therefore the logical approach to testing the immunogenicity of a preparation from the tapeworm is to give this preparation orally, as in the natural immunizing infection, or by a direct injection into the duodenum. A preparation containing the protective antigens administered in this

way would be expected to stimulate at least some degree of protection. If this is a logical speculation, one could surmise that in experiments where no protection was stimulated with non-living worm antigens, the route of antigen administration was probably the crucial factor that militated against induction of protective immunity rather than the antigens themselves. Evidence in favour of this hypothesis came from the observations of Hepler (1977) who demonstrated that of all the routes he used to immunize mice against Nematospiroides dubius '... the oral route was by far superior.' This is by no means meant to imply that N. dubius and H. diminuta are similar in all aspects. The example was quoted to show how crucial the route of antigen administration is in the stimulation of protective immunity against intestinal helminths. Although the hypothesis put forward above may seem somewhat far fetched, it is not necessarily invalidated by the fact that oral immunization failed to stimulate protective immunity against the tapeworm on more than one occasion (see below). Moreover, should it be that the antigenic preparations injected contained sufficient quantities of the tapeworm functional antigens, the administration of these antigens by a route other than the oral route might have resulted in an immune response that was utterly different from that required for protection. For example, many allergens have the potential to induce in atopic individuals reaginic antibody responses (IgE) if they gain access to host tissues via mucous membranes (Terry, 1968). The reaction of cell-bound reagins on re-exposure to the allergens mediates hypersensitive reactions. If the allergens are given by direct inoculation into the tissues, however, they stimulate a different class of antibody

(IgG) which does not sensitize cells to the same extent and fails to induce hypersensitivity. Parenteral injection of antigen was shown to lead to the formation of antibody-forming cells in the lamina propria of the gut (Crabbe et al., 1969; Dolezel et al., 1971) and blast cells from antigen stimulated peripheral lymph nodes were demonstrated to have the potential to migrate to the intestine (Parrott, 1976). Nonetheless, it is widely accepted that parenteral antigen administration does not satisfactorily induce mucosal immunity (Ogra, 1971; Pierce et al., 1975) and that vaccines administered in this way have a poor performance in the control of enteric infections (Porter & Allen, 1978). In cholera infections, for example, there is no definite evidence that parenteral immunization effectively stimulates local immunity in the intestinal tract (Freidman, 1978). Because of the poor performance of parenterally administered immunogens, it was proposed that the best prospect for initiating an adequate defence in the host against enteric infections lay in exploiting the intestinal secretory immune mechanisms by oral vaccination (Porter et al., 1978). This approach, however, has a disadvantage since the secretory immune system apparently lacks memory in contrast to that associated with systemic immunity (Tomasi et al., 1972;). Immunity in the alimentary canal following oral sensitization with Vibrio cholerae was shown to be short-lived in both experimental animals and human subjects (Freter & Gangarosa, 1963; Carpenter & Pierce, 1971) and with vaccines like polio, it was found necessary to administer repeated doses for the maintenance of detectable coproantibody. In this respect, it is significant that intestinal IgA plasma cells in mice have a half-life

of only 4.7 days indicating that they do not play a significant role in long-term memory and suggesting that the persistence of antibody production would depend on the recruitment of further immunocompetent cells (Mattioli & Tomasi, 1973). It has been frequently reported that immunization does not invariably follow antigenic stimulation via the oral route. Antigens as BSA (Thomas & Parrot, 1974) or ovalbumin (Bazin, 1977) can induce in animals a state of antigen-specific unresponsiveness or immunological tolerance. Immunization of animals against tolerogenic antigens may subsequently lead to interference with the absorption of these antigens through the intestine and to an increase in the rate of their breakdown and degradation (Andre, Vaerman & Heremans, 1978; Walker et al., 1975).

It has been clearly established that the amount of antigen presented and the duration of antigenic stimulation are important factors in the induction of the immune response (Tomasi & Bienenstock, 1968; Bienenstock, 1974; Bazin, 1976). A short antigenic exposure cannot easily induce an initial response (Andre et al., 1973; Pierce et al., 1975). It was postulated that the elicitation of an immune reaction or the induction of tolerance is governed by a sensory system at the single cell level where an immunocompetent cell would discriminate between immunogenic and tolerogenic amounts of antigen (Diener, 1974). In this case, a subimmunogenic or supraimmunogenic dose administered over some period may induce tolerance indicating that there is a 'low zone' and a 'high zone' for the induction of tolerance in terms of antigen predosage (see Roit, 1977). Perhaps Hopkins (1980) is correct when he wrote, commenting on the results I obtained from

experiment 1.1, '... there is also the problem that the amount of antigen that is needed to sensitize may in fact be very small ..., so the administration of large (gram) quantities of homogenate, in the hope that at least some of the important protective antigens are included, by inducing tolerance ..., may be self defeating'. In this situation, however, the failure to induce a protective response against the tapeworm is more likely to be attributed to dissipation of the immune response and competition between the protective and 'non-protective' antigens rather than by induction of tolerance to the non-protective antigens. The dose of antigen also seems to be of some relevance to the type of the immune response which is produced: experiments with SRBC in CBA mice have shown that IgM antibodies have a shallow dose-response curve and IgG, a very steep curve (Torrigiani, 1974).

A crucial factor in oral immunization is the refractory period following upon the primary response to an antigen given by this route (Andre et al., 1972). The exact period of this hyporeactivity is unknown or when the animal regains the potential to respond to the antigen after the cessation of the administration of this antigen. In the mouse, induction of an IgA response after mucosal immunization is followed by a refractory period where it is difficult or even impossible to induce a new oral response (Andre et al., loc. cit.). Therefore, supposing that an H. diminuta challenge is given during this refractory period following upon the administration of an oral vaccine, the worm antigens will then no longer be capable of stimulating any response because of an inhibitory effect of antibody manufactured

in the preceding period of local immunization. If antibody is not the crucial component of the mechanism by which H. diminuta is rejected from mice (Isaak, 1976), a challenge worm in an orally vaccinated mouse should continue to survive despite the interaction between its antigens and specific antibody raised by oral immunization and its rejection has to be brought about by a regimen of vaccination that triggers an effector mechanism other than antibody. Studies on the dynamics of H. diminuta infections show that immunizing mice by an oral infection did not prevent the recognition of challenge worm antigens and expression of a vigorous response to these antigens for a period of one to three weeks following termination of the primary infection (this thesis). Mice immunized in this way retain the potential to respond to the challenge for at least six months without waning of memory to the homologous challenge (Hopkins, 1981). Nonetheless, the fact that a live immunizing infection is not associated with unresponsiveness to the challenge antigens does not necessarily mean that a similar situation is created when killed worm antigens are given.

The immune response usually depends on complex interactions between antigen and one or more cell types. An adjuvant can act on any one of these cells to induce immunopotentiation (WHO, 1976). Small amounts of antigen acting over prolonged time e.g. when given in an adjuvanted form, stimulate antibody production against any given determinant whose average affinity increases with time. Cellular immunity is not usually detectable following conventional active immunization with soluble or particulate antigen alone, the immune response induced is only transient and is highly sensitive to antigen

excess (WHO, loc. cit.). FCA (which was predominantly used in this study) holds an established place as a means of inducing in animals high levels of antibody against antigens and it facilitates the development of delayed hypersensitivity (WHO, 1976). The properties of saponin as a parenteral adjuvant has been discussed earlier (4.3.1). It is also an irritant that causes lysis of red blood cells (see Kingsbury, 1975). In pilot experiments, mice vaccinated s.c. with an H. diminuta tegumental fraction, which contained saponin acquired from the membrane disruption solution, died within 24 h. Mice given the same preparation orally showed no adverse effects (4.2; 4.3 & 4.4). Despite an exhaustive dialysis, using ultrafilters of exclusion limits of up to 10,000 mol. wt, the dialysed antigenic materials from the tegument retained residues of saponin which were sufficient to cause death in mice following s.c. injection. After the experiments with tegument antigens (4.2; 4.3 & 4.4) were completed, Knowles & Oak (1979) published a paper in which they described the difficulties they encountered in separating saponin from the tegument of H. diminuta. In this second publication of theirs, they also referred to the ability of saponin to interfere with protein and carbohydrate determinations. Such property of the saponins to interfere with protein determination could, in part, explain the discrepancy between results from experiments 4.2; 4.3 & 4.4 probably due to errors in estimating the protein content of the antigens administered in each case. The possibility that such discrepancies could have been due to qualitative differences between the antigens used in these experiments (e.g. due to differences in the age of the worms used or the number of these), however, cannot be ruled out.

To elucidate further what is presented above as possible determinants of the outcome of the vaccination experiments with the non-viable H. diminuta antigens, it was found necessary to examine these propositions in detail. Experiments in SECTION 2 were therefore designed to reproduce artificially the protective immunity stimulated by a natural infection using live worm antigens. The questions to be answered pertain to the effects of:-

(i) the route of antigen administration

(ii) size of the inoculated dose

(iii) frequency of antigen administration, and

(iv) time lapse between cessation of antigen administration and challenge.

SECTION 2

VACCINATION WITH LIVE WORM ANTIGENS

VACCINATION WITH LIVE WORM ANTIGENS

A live H. diminuta infection acquired via the oral route, undoubtedly, evokes strong protection against challenge (SECTION 1). Therefore, if the hypothesis presented earlier (Discussion, SECTION 1), relating the development of functional immunity only to the live worm, is correct, then it is likely to obtain at least some degree of protection by presenting antigens from living worms parenterally. The hypothesis is based on the assumption that the parenteral administration of antigens from live worms, supposedly the protective antigens, is not inhibitory to the development of immunity at the level of the gut. The validity of these suppositions is investigated by implanting live strobilate worms in the subcutis and peritoneal cavity of mice.

7. STROBILATE WORM ANTIGENS

7.1 Strobilate Worms Implanted s.c.

7.1.1 Materials & Methods

Six-weeks-old female CFLP mice were used in this experiment. Eight-day-old worms were recovered from mice treated with cortisone. They were washed repeatedly in HBSS containing 'Crystamycin' and implanted s.c. using a 16G needle. The duration of antigenic stimulation from the 'viable' worm after implantation under the skin was, subjectively, evaluated by measuring the ability of these worms to establish on subsequent surgical transference to the duodenum of naive mouse recipients. Single worms which had been previously implanted under the skin for 1, 2 or 3 days were implanted into the duodenum of CFLP mice and the recipients were killed 2 days after surgery. The establishment of the transplanted worms is shown in Table 7. Worms implanted under the skin for as long as 3 days were capable of establishing in the intestine of the recipients. This suggests that these worms were not seriously damaged in the abnormal and hostile environment of the subcutis and hence that antigens from these apparently normal worms were presumably released into the tissues of the host for at least 3 days.

7.1.2 Design

One group of mice received s.c. single worm implants on days 0, 7 and 14. Another group was given s.c. injections of HBSS (0.2 ml) over similar periods of time. A third group was used as an immune control. All mice were challenged on day 21 by transplanting into the duodenum single 8-day-old worms from donor mice. They were killed on day 27.

7.1.3 Results

The s.c. implantation of viable strobilate worms had no significant effect on the growth or the survival of challenge worms as compared with the naive controls (Fig. 11). A primary infection in the immune controls, however, strongly protected mice against challenge.

Table 7 Establishment of H. diminuta previously implanted in the subcutis of mice on subsequent surgical transference to the duodenum of naive recipients

Duration of s.c. implantation (h)	No. of recipients	No. of worms establishing
$26\frac{1}{2}$	5	4
46	6	5
$70\frac{1}{2}$	3	3

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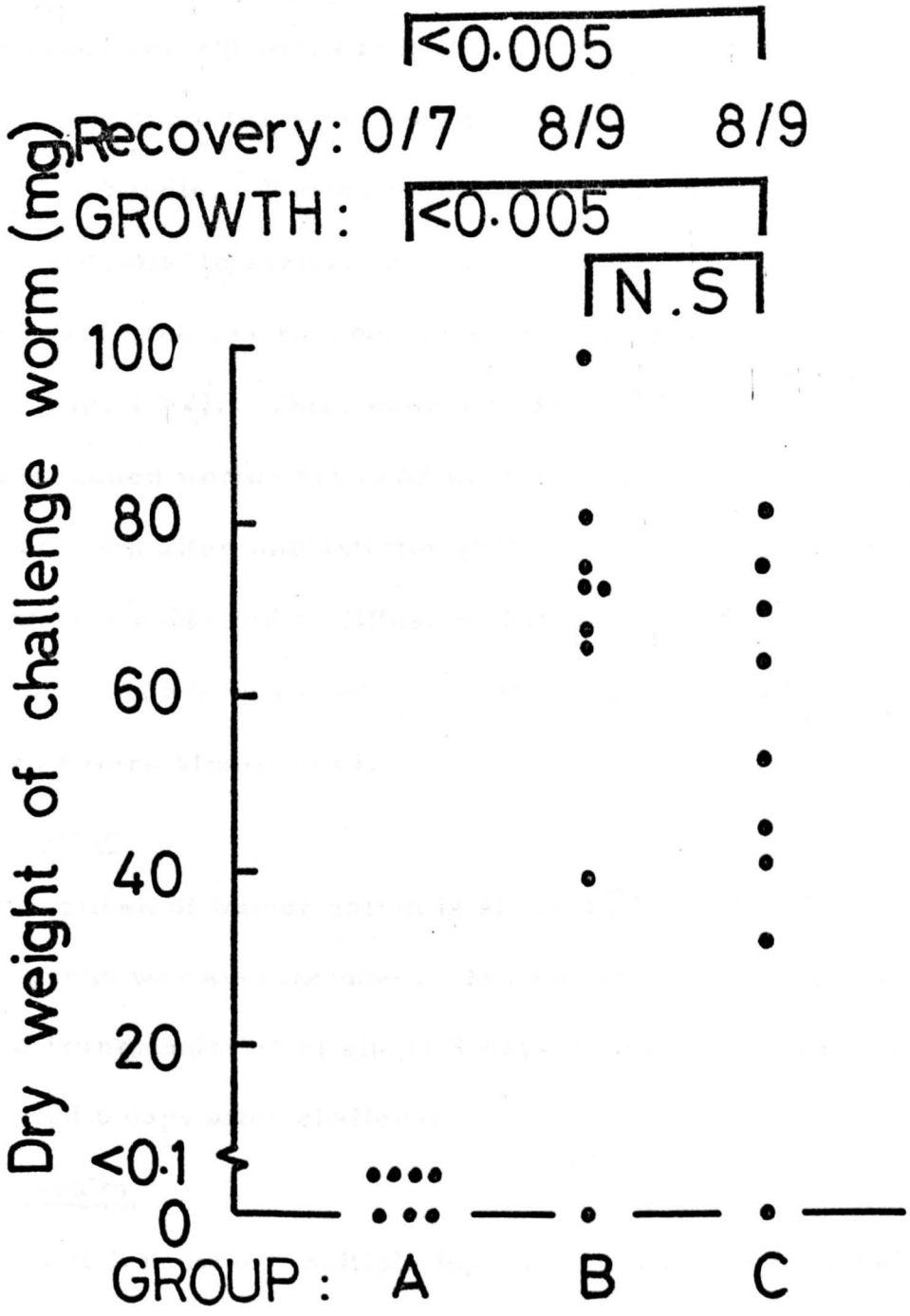
Figure 11.

Growth and survival of 8-day-old H. diminuta single-worm transplants in mice vaccinated s.c. with live strobilate worms (B) and in the immune (A) and naive (C) recipient controls.


<0.005
Recovery: 0/7 8/9 8/9
GROWTH: <0.005
N.S
Dry weight of challenge worm (mg)
100
80
60
40
20
<0.1
0
GROUP: A B C

7.2 Strobilate Worm(s) Implanted i.p.

7.2.1 Materials & Methods

Mice were female CFLP of 7 weeks of age. Worms to be implanted were obtained as 8-days-old from mice treated with cortisone. They were washed several times in HBSS to which 'Crystamycin' was added and introduced into the peritoneal cavity with 0.2-0.3 ml of HBSS using a 16G needle. Worms implanted into the peritoneal cavity have the potential to survive in this environment for at least 24 h, before host tissue reaction becomes pronounced and the worms encapsulated (Fig. 12-2). This, however, does not necessarily imply that all encapsulated worms are dead worms; a worm recovered from one mouse at 144 h after implantation still retained a sluggish activity. Single worms contained in diffusion chambers (0.45 um, Millipore U.K. Ltd.) and administered i.p. survived for up to 72 h. By 96 h the worms were almost dead.

7.2.2 Design

The regimen of immunization is shown in Table 8. An immune control group was also included. Mice were challenged on day 21 by surgical transplantation of single 8-day-old mouse worms. They were killed 6 days after challenge.

7.2.3 Results

Mice which received multiple injections (Groups 4 & 6, Table 8) developed severe peritonitis with resultant adhesions in the viscera, hepatomegaly and ascites. These two groups were therefore excluded. Apart from the granulomata encapsulating the worms, the peritoneal cavities of mice given single injections of one or three worms were

Table 8 Schedule of immunization with strobilate worms implanted i.p.

Group	Injections (days)			
	0	7	11	14
1	1 worm			
2	3 worms			
3	HBSS			
4	2 worms		2 worms	
5	HBSS		HBSS	
6	2 worms	2 worms		1 worm
7	HBSS	HBSS		HBSS

HBSS = Hanks' balanced salt solution

apparently normal. Results from these groups are shown in Fig. 12-1. Implantation of either a single worm or three worms had no significant effect on either the growth or the survival of the challenge transplants as compared with the naive controls. Strong protective immunity was obtained when mice were orally immunized with an infection of six cysticercoids.

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Figure 12-1

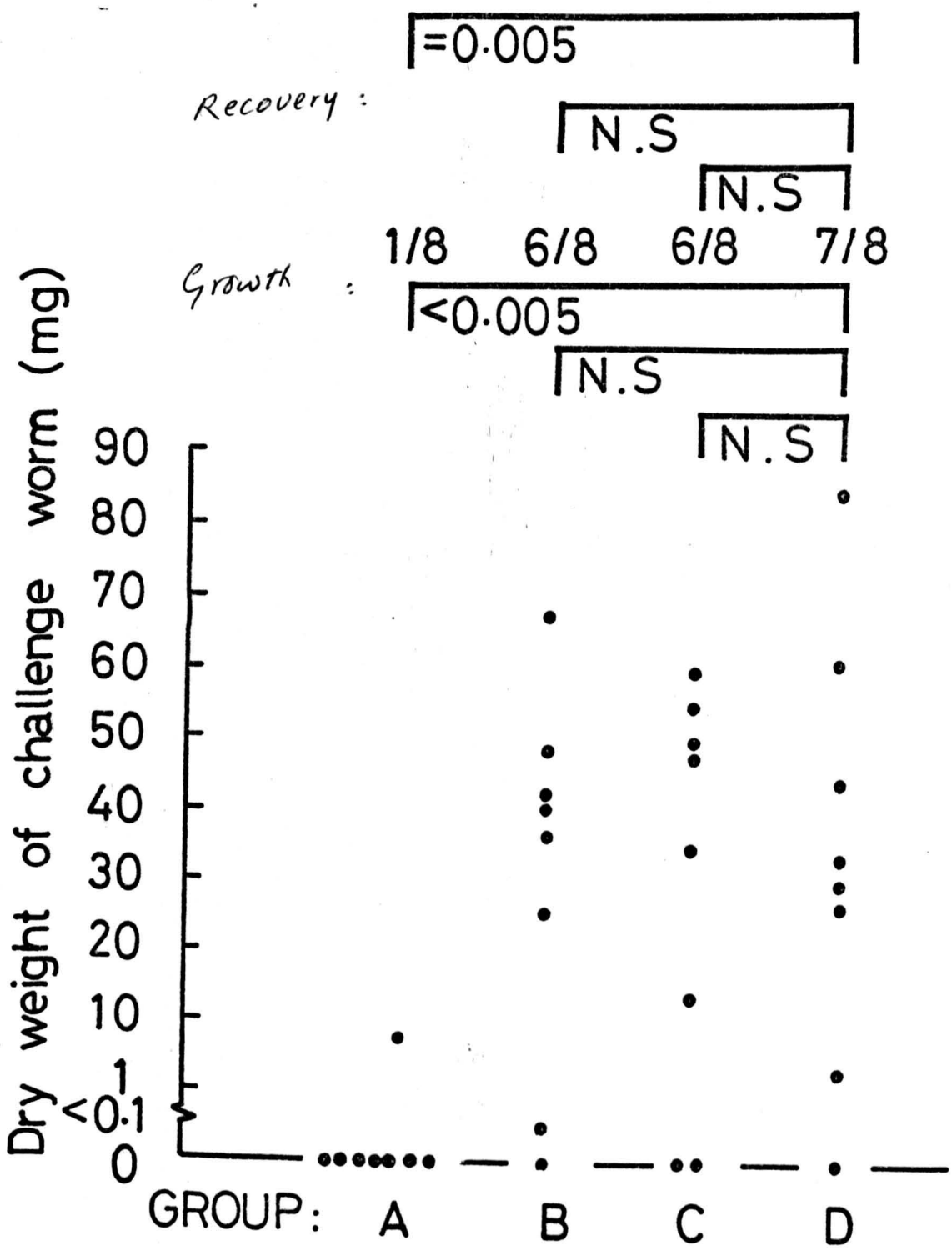
Growth and survival of 8-day-old H. diminuta single-worm transplants in mice vaccinated i.p. with live strobilate worms and in the immune and naive recipient controls.

A: Immune controls

B: Vaccinated with a single worm

C: Vaccinated with three worms

D: Naive controls


=0·005
Recovery :
N.S
N.S
1/8
6/8
6/8
7/8
Growth :
<0·005
N.S
N.S
Dry weight of challenge worm (mg)
90
80
70
60
50
40
30
20
10
1
<0·1
0
GROUP: A
B
C
D

Facing page 94

Figure 12-2.

Viability of H. diminuta previously implanted into the peritoneal cavity of mice as assessed by visual motility after incubation in HBSS at 22°C.

Each point represents a single worm from a mouse.

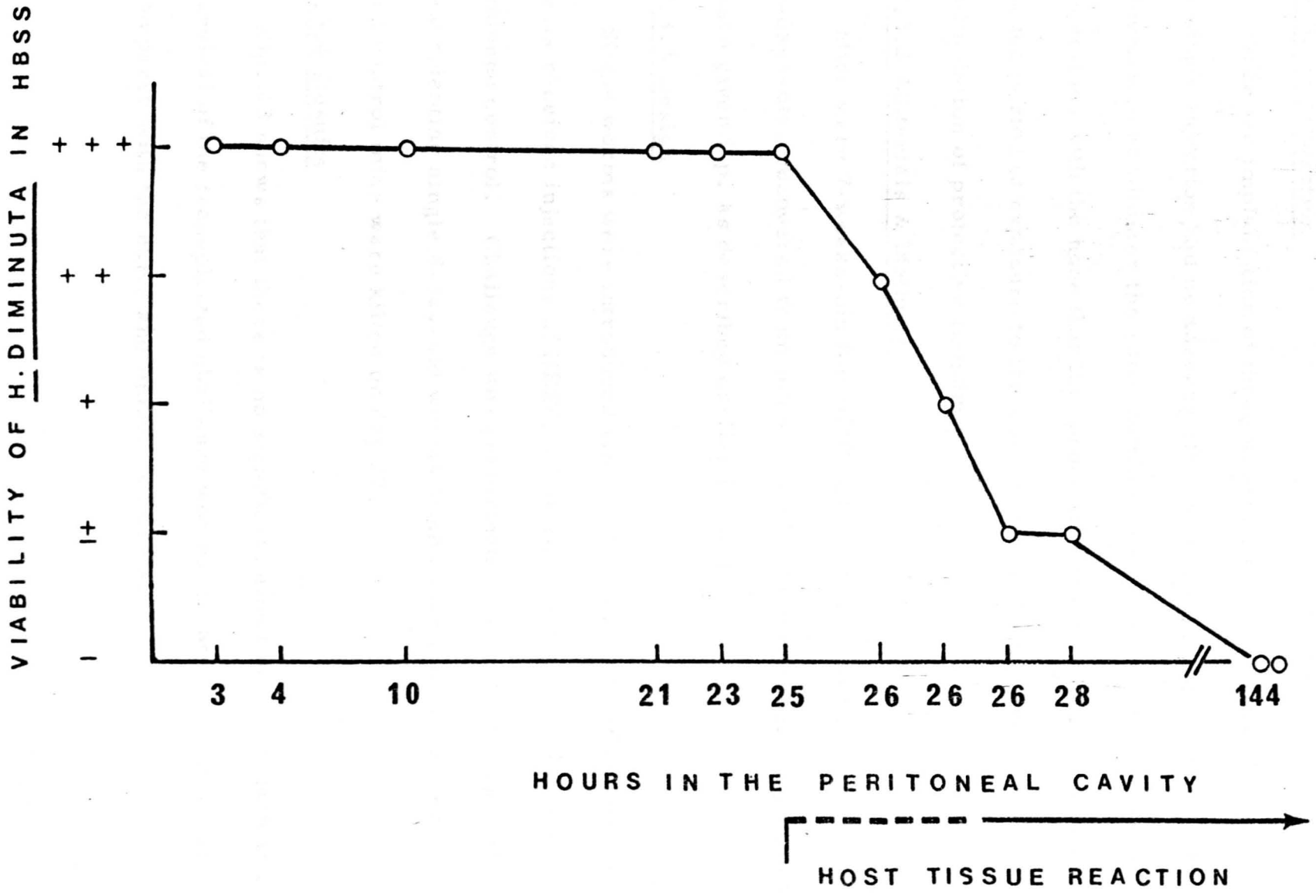
+++ : Very active (as a normal worm)

++ : Active

+ : Activity diminished

± : Very weak activity

- : Completely encapsulated by host tissue.


VIABILITY OF H. DIMINUTA IN HBSS
+ + +
+ +
+
I+
–
3
4
10
21
23
25
26
26
26
28
144
HOURS IN THE PERITONEAL CAVITY
HOST TISSUE REACTION

7.3 A Strobilate Worm Implanted i.p. (3 injections)

7.3.1 Introduction

Since the implantation of three worms into the peritoneal cavity by a single injection had no adverse effects on the mice (7.2.3), it was decided to administer the same number of worms as three separate injections with the hope that this procedure,possibly by prolongation of the period of exposure to the live worm's antigens, would lead to stimulation of protective immunity.

7.3.2 Materials & Methods

Mice were 7-weeks-old female CFLP. Worms to be implanted were 8-days-old, recovered from mice treated with cortisone. Injections were given i.p. as described earlier (7.2.1).

7.3.3 Design

Single worms were introduced i.p. on days 0, 7 and 14. Sham controls received injections of HBSS. A third group was used as an immune control. Challenge was performed on day 21 by surgically transplanting single 8-day-old worms from donor mice. Experimental and control mice were killed on day 27.

7.3.4 Results

Fig. 13 shows that there is no significant effect on the growth and survival of the transplanted challenge worms in vaccinated mice as compared with the naive and immune controls.

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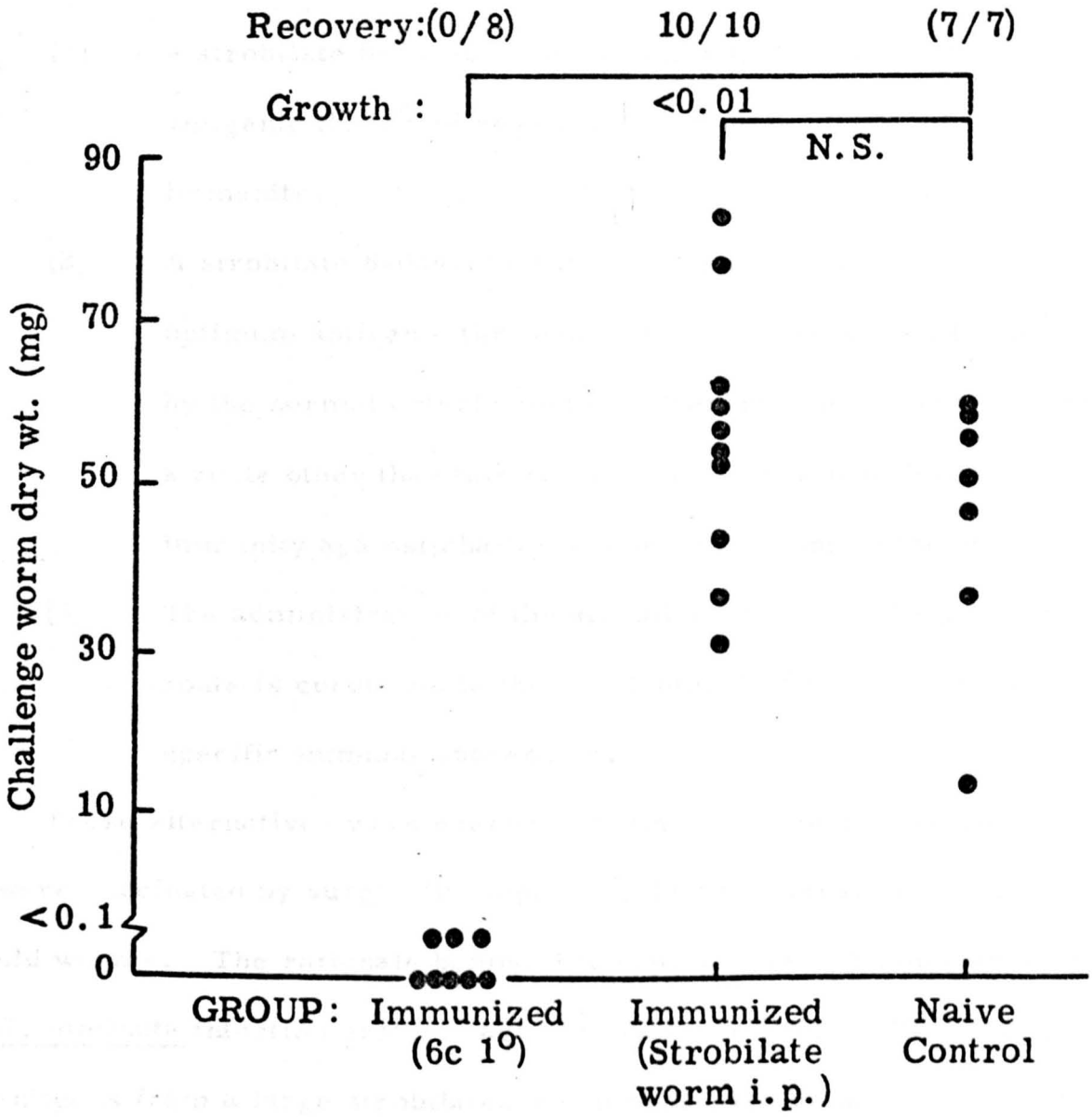
Figure 13.

Growth and survival of 8-day-old H. diminuta single-worm transplants in mice vaccinated i.p. with three single-worm injections and in the immune and naive recipient controls.


Recovery:(0/8)
10/10
(7/7)
Growth :
<0.01
N.S.
Challenge worm dry wt. (mg)
90
70
50
30
10
<0.1
0
GROUP:
Immunized (6c 1°)
Immunized (Strobilate worm i.p.)
Naive Control

7.4 A Strobilate Worm Implanted Intraduodenally

7.4.1 Introduction

Results from the previous experiments showed that strobilate 8-day-old worms, apparently capable of surviving for appreciable periods of time when implanted s.c. or i.p., did not immunize mice against challenge (7.1, 7.2 & 7.3). This suggests either:-

(1) a strobilate 8-day-old worm does not initially provide the antigenic threshold required for the stimulation of protective immunity.

(2) A strobilate 8-day-old worm is capable of providing this optimum antigenic threshold but only if presented to the host by the normal enteric route. Presentation of the worm by a route other than this route does not result in functional immunity against challenge worms residing in the gut.

(3) The administration of the strobilate worm by the parenteral route is conducive to the development of specific or non-specific immunosuppression.

These alternatives were examined in an experiment in which mice were vaccinated by surgically implanting into the intestines single 8-day-old worms. The rationale behind this approach is that since an enteral H. diminuta infection protects against challenge (INTRODUCTION), antigens from a large strobilated worm presented by the enteric route would be expected to stimulate immunity. Should the outcome of this experiment turn in the predicted direction and the vaccinated mice are protected against challenge, then it is certain that:-

(a) the strobilate 8-day-old worm is capable of providing an antigenic stimulus sufficient to induce protective immunity.

(b) the s.c. and i.p. routes are less efficient than the enteric route in the stimulation of this immunity.

7.4.2 Materials & Methods

Seven-week-old female CFLP mice received surgically single 8-day-old worms implanted into the duodenum.

7.4.3 Design

The transplanted worms were eliminated with 'Zanil' on days 1 or 3 of transplantation. This allows for estimation of the magnitude of the protective response induced following short-term antigenic stimulation, as in parenteral vaccination with live strobilate worms (Table 7, Fig. 12-2). In another group, the transplanted worms were eliminated on day 16 (strictly speaking, these are the worms that survived up to this time since natural loss of an 8-day-old H. diminuta transplant from CFLP mice infected for the first time commences on day 8 $\pm$ 1 of transplantation (Hopkins et al., 1976). Sham operations were performed on naive controls. A fifth group was used as an immune control. All mice were challenged on day 21 by surgically transplanting into the duodenum single 8-day-old worms from mouse donors. They were killed on day 27.

7.4.4 Results

The growth and survival patterns of challenge worms (Fig. 14) show that strobilate worms implanted in the intestine and eliminated after 1, 3 or 16 days are almost apparently capable of inducing only a weak protective response (in terms of mean worm weight, the most unreliable criterion in evaluating the immune response to H. diminuta; see Hopkins et al., 1980). The reduction in the growth of challenge

Figure 14.

Growth and survival of 8-day-old H. diminuta single-worm transplants in mice immunized intraduodenally with strobilate worms and in the immune and naive recipient controls.

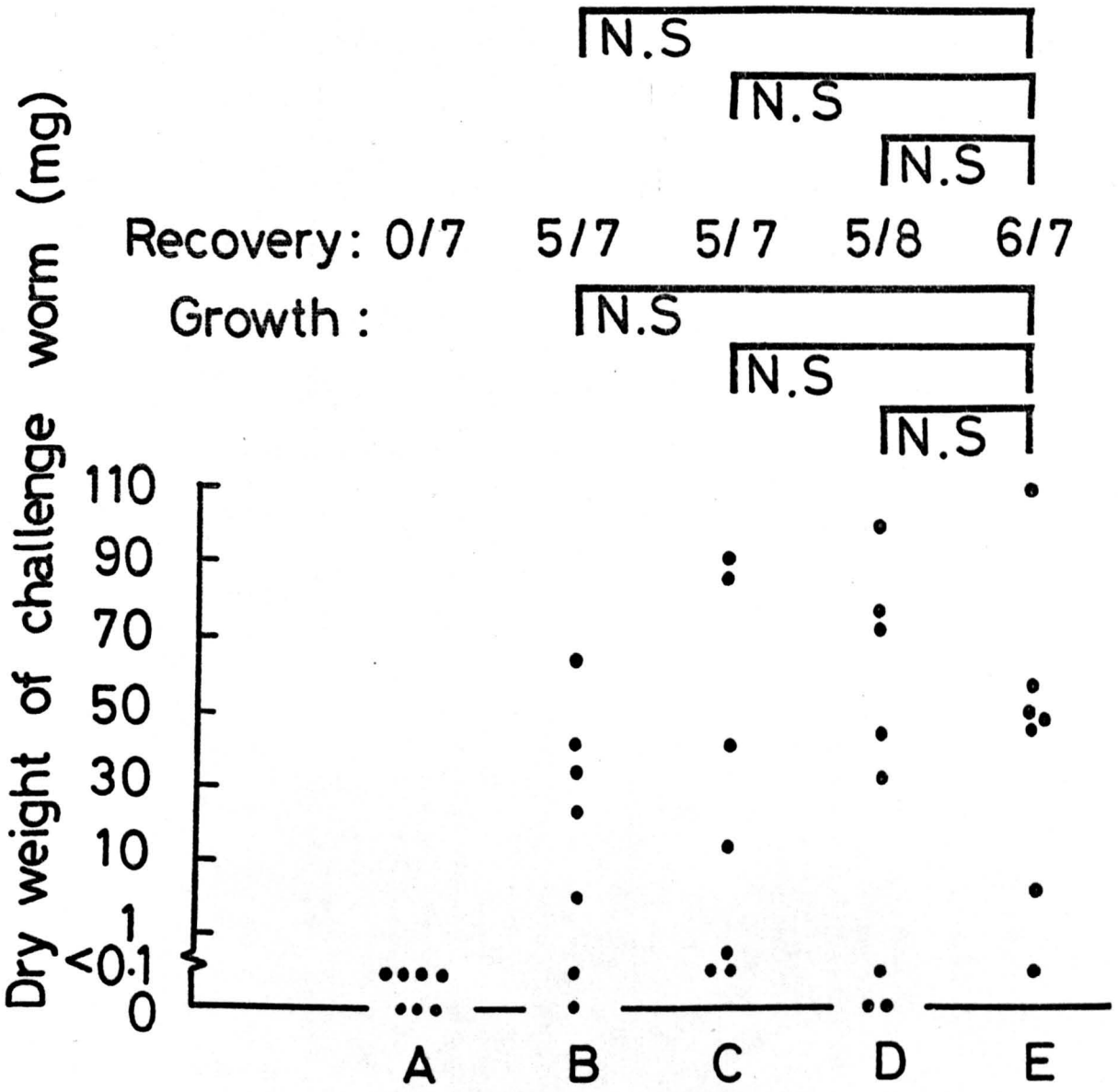
A: Immune controls

B: Immunized with a worm eliminated on day 16.

C: Immunized with a worm eliminated on day 3.

D: Immunized with a worm eliminated on day 1.

E: Naive controls.


N.S
N.S
N.S
Recovery: 0/7 5/7 5/7 5/8 6/7
Growth :
N.S
N.S
N.S
Dry weight of challenge worm (mg)
110
90
70
50
30
10
1
<0.1
0
A
B
C
D
E

worms is not statistically significant (Wilcoxon). This evidence regarding the weak immunogenic potential of the 8-day-old strobilate worm is considered inconclusive because of the following reasons:-

(1) the induction of the immune response or its expression following the enteral sensitization with antigens from the strobilate worm could have been altered by surgical interference since mice in this experiment were operated on twice within 3 weeks.

(2) if an oral infection from cysticercoids undoubtedly stimulates the mouse immune system and there appears to be no low antigenic thresholds in H. diminuta enteric infections (Hopkins, 1980) and that the strobila of the worm is the major source of the functional antigens (Christie, 1978; 1979), it is rather unexpected to obtain only marginal protection (if any) when mice were enterally immunized with a large strobilate 8-day-old worm (this experiment).

(3) if an oral infection starting from cysticercoids stimulates protection against challenge (INTRODUCTION), then immunization by implanting strobilate worms directly into the intestine bypasses the excystation step which could be associated with a release of antigens possibly essential for the induction of protective immunity.

(4) another possibility is that the efficacy in the induction of protective immunity against the tapeworm could be a function of the age of the worm: a young worm growing from a cysticercoid is more immunogenic than a worm introduced as 8-days-old.

The following experiments attempt to find an answer to these queries and to the basic question pertaining to the failure to protect mice by vaccination with live strobilate worm antigens and the failure to induce immunity by the administration of antigens from the killed parasite.

7.5 The Significance of the *in vivo* Process of Excystation in the Induction of Functional Immunity Against *H. diminuta*

7.5.1 Materials & Methods

Mice were female CFLP of 6 weeks of age. Cysticercoids were excysted *in vitro* by treatment at 37°C with pepsin/HCl for 11 min (Appendix) followed by trypsin/sodium tauroglycocholate for 12 min.

7.5.2 Design

Mice received by surgically implanting into the duodenum 12 live worms from cysticercoids excysted *in vitro* immediately after excystation. Another group of mice was given 12 cysticercoids by a stomach tube and sham operations were performed. All mice were challenged surgically on day 21 by transplanting single 8-day-old worms from mice treated with cortisone. They were killed on day 27.

7.5.3 Results

The results shown in Fig. 15 provide no evidence that substance(s) supposedly released during the excystation process of cysticercoids in the intestine is/are essential for the induction of functional immunity against *H. diminuta* in mice; worms that were excysted *in vitro* were as immunogenic as worms from cysticercoids excysting *in vivo*. The results also imply that the subjection of mice to surgery twice within 21 days did not prevent the induction and expression of immunity to the tapeworm.

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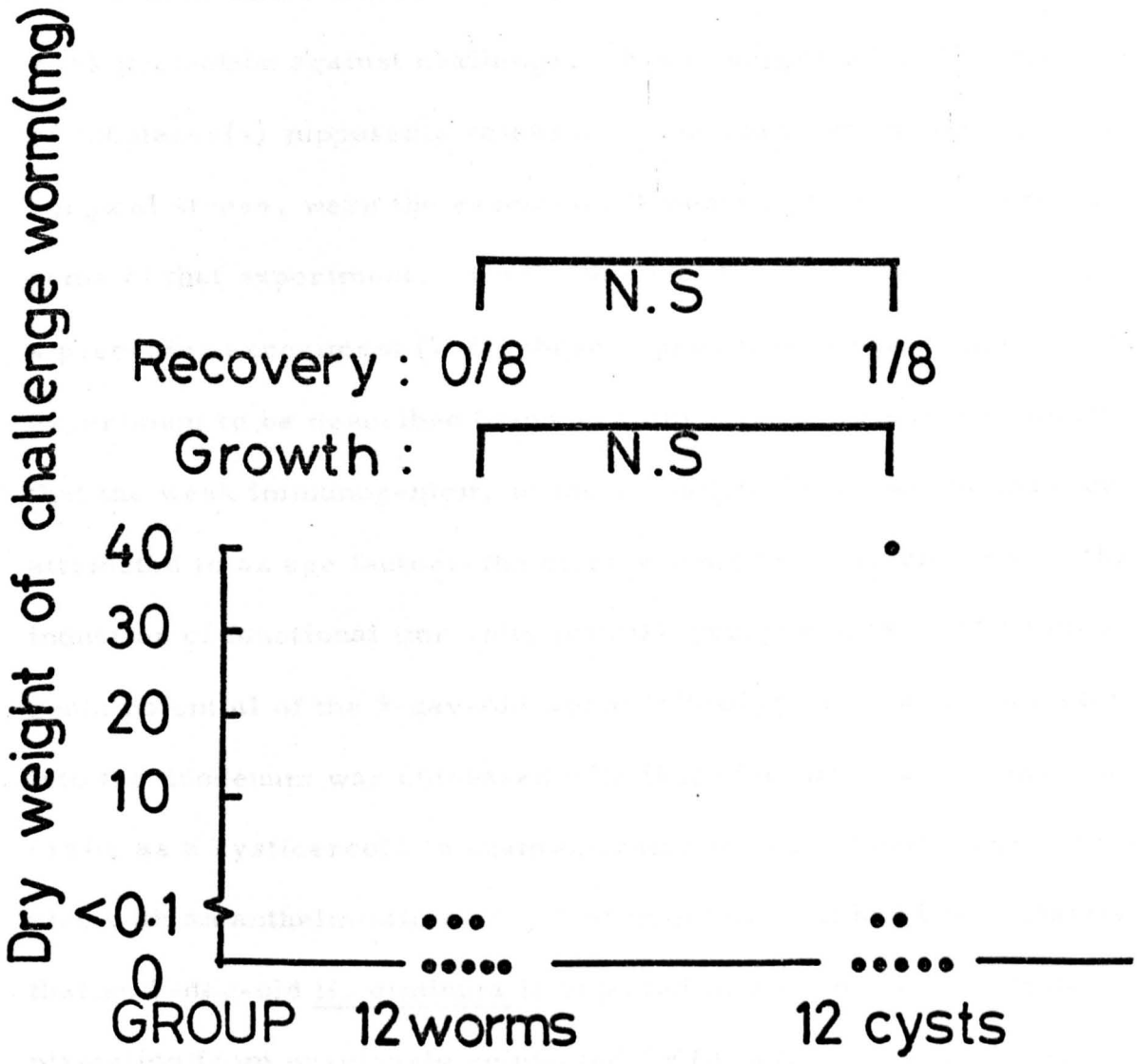
Figure 15.

Growth and survival of 8-day-old H. diminuta single-worm transplants in mice immunized enterally with excysted worms or cysticercoid infections.

WORMS: Excysted worms

Cysts: Cysticercoids.


Dry weight of challenge worm(mg)
N.S
Recovery : 0/8
1/8
Growth :
N.S
40
30
20
10
<0.1
0
GROUP
12 worms
12 cysts

7.6 Immunity to H. diminuta in Mice: Immunogenicity of the Strobilate Worm

7.6.1 Introduction

Results from a previous experiment (7.4) showed that a strobilate worm implanted into the duodenum of mice as 8-days-old and eliminated with an anthelmintic after 1, 3 or 16 days, apparently stimulated weak protection against challenge. It was suggested that an absence of substance(s) supposedly released during excystation, together with surgical stress, were the essential elements that determined the outcome of that experiment. On the basis of the evidence obtained from a preceding experiment (7.5), these suppositions were refuted. The experiment to be described below examines the alternative explanation that the weak immunogenicity of the strobilate 8-day-worm could be attributed to an age factor: the older worms are less efficient in the induction of functional immunity than the younger ones. The immunogenic potential of the 8-day-old worm following surgical implantation into the duodenum was compared with that of a single worm introduced orally as a cysticercoid to sham-operated mice. Worms were eliminated with an anthelmintic on day 7 of infection. It has been established that an 8-day-old H. diminuta is rejected in 8 $\pm$ 1 days after transplantation from previously uninfected CFLP mice (Hopkins et al., 1976). On the other hand, a worm administered as a cysticercoid loses its strobila at about day 10 of infection and it is subsequently expelled (Hopkins et al., 1972 a). On the basis of these criteria, a worm administered as 8-days-old or that growing from a cysticercoid should be present in the gut on day 7 of infection when the anthelmintic

is administered. In this case, mice from both groups will be exposed to single worm infections for the same period of time. The crucial point of difference lies in the mass of strobilar tissue presented to the host in each case within these 7 days of antigenic stimulation and in the age of the worms. Another point of difference is the presence or absence of substance(s) released during the in vivo excystation of the worm. It has already been proven that these substances are not involved in the stimulation of protective immunity against the tapeworm (7.5.3). On day 0 of infection, the host will be initially presented with worm strobilar tissue of approximately 1 mg dry wt, the weight of an 8-day-old mouse worm (Appendix). This is perhaps more than 17,000 fold the dry weight of a worm from a cysticercoid on this day of infection. By day 7, the weight of the transplanted worm would increase to about 50 mg compared to a weight of less than 1 mg dry wt of a worm growing from a cysticercoid (see Appendix; Roberts, 1961; Hopkins et al., 1976).

7.6.2 Materials & Methods

Mice were male NIH of 6 weeks of age. Infection and transplantation of worms has been described earlier.

7.6.3 Design

The schedule of immunization is shown in Table 9. Immunizing worms were eliminated with 'Zanil' on day 7 of infection. Mice were challenged with six cysticercoids on day 21 and they were killed on day 29.

7.6.4 Results

Fig. 16-1 and Fig. 16-2 show the growth and survival patterns of the challenge worms respectively. They indicate:

Table 9. Immunization of NIH mice with orally administered cysticercoids and with strobilate worms introduced surgically

Group	Immunizing infection	Reduction in total biomass of challenge worms **
1. Immunized	6c *	90%
2. Immunized	2c *	82%
3. Immunized	2 8-day-old worms	63%
4. Immunized	1c *	62%
5. Immunized	1 8-day-old worm	46%
6. Naive control	- *	0%

c = cysticercoid

* sham-operated

** compared with naive controls

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Figure 16-1.

Growth of worms from a six-cysticercoid challenge infection in mice immunized enterally with strobilate worms or cysticercoids and in the naive controls.

C: Cysticercoid.

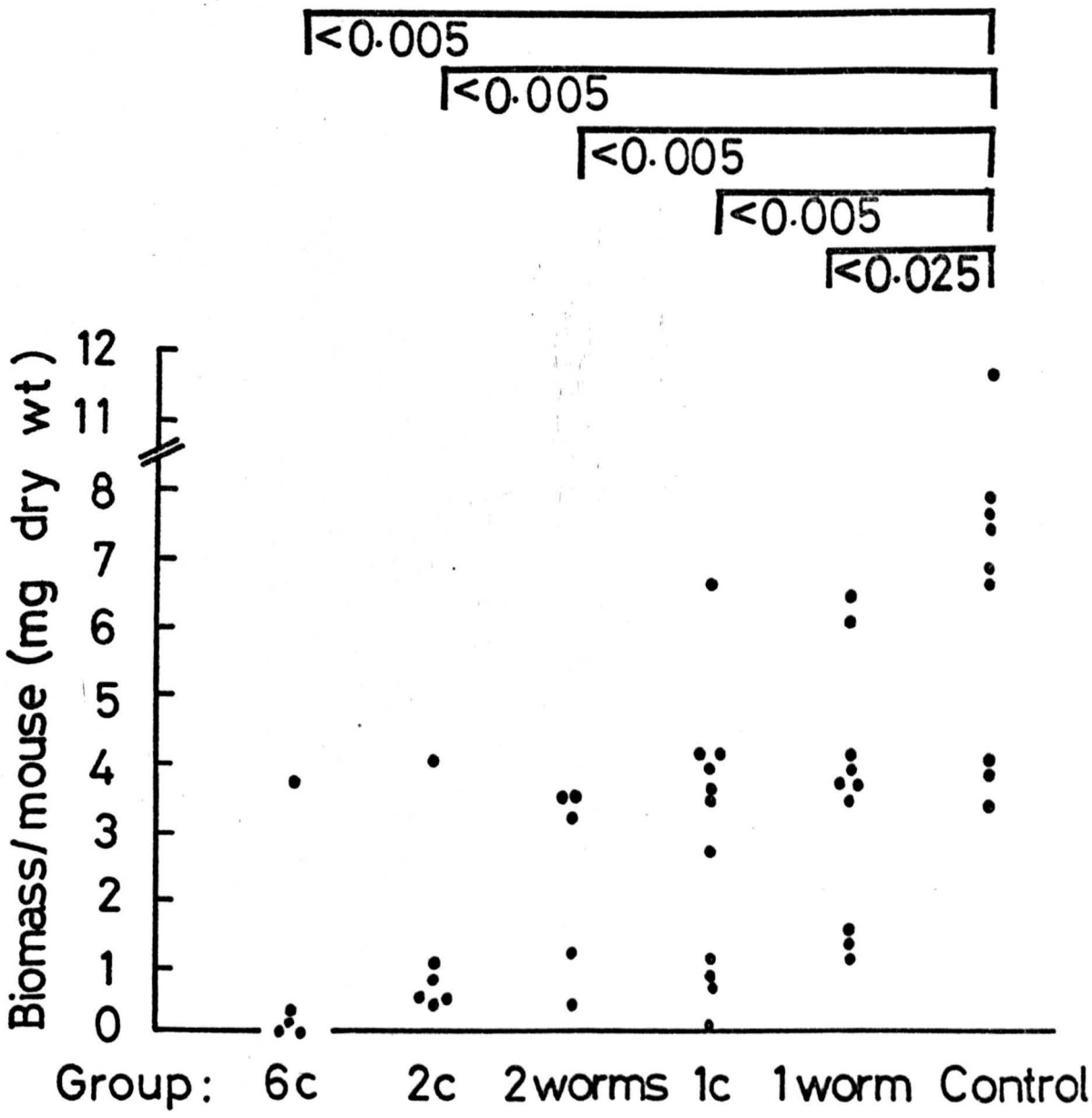

<0.005
<0.005
<0.005
<0.005
<0.025
Biomass/mouse (mg dry wt)
12
11
8
7
6
5
4
3
2
1
0
Group: 6c 2c 2worms 1c 1worm Control

Figure 16-2

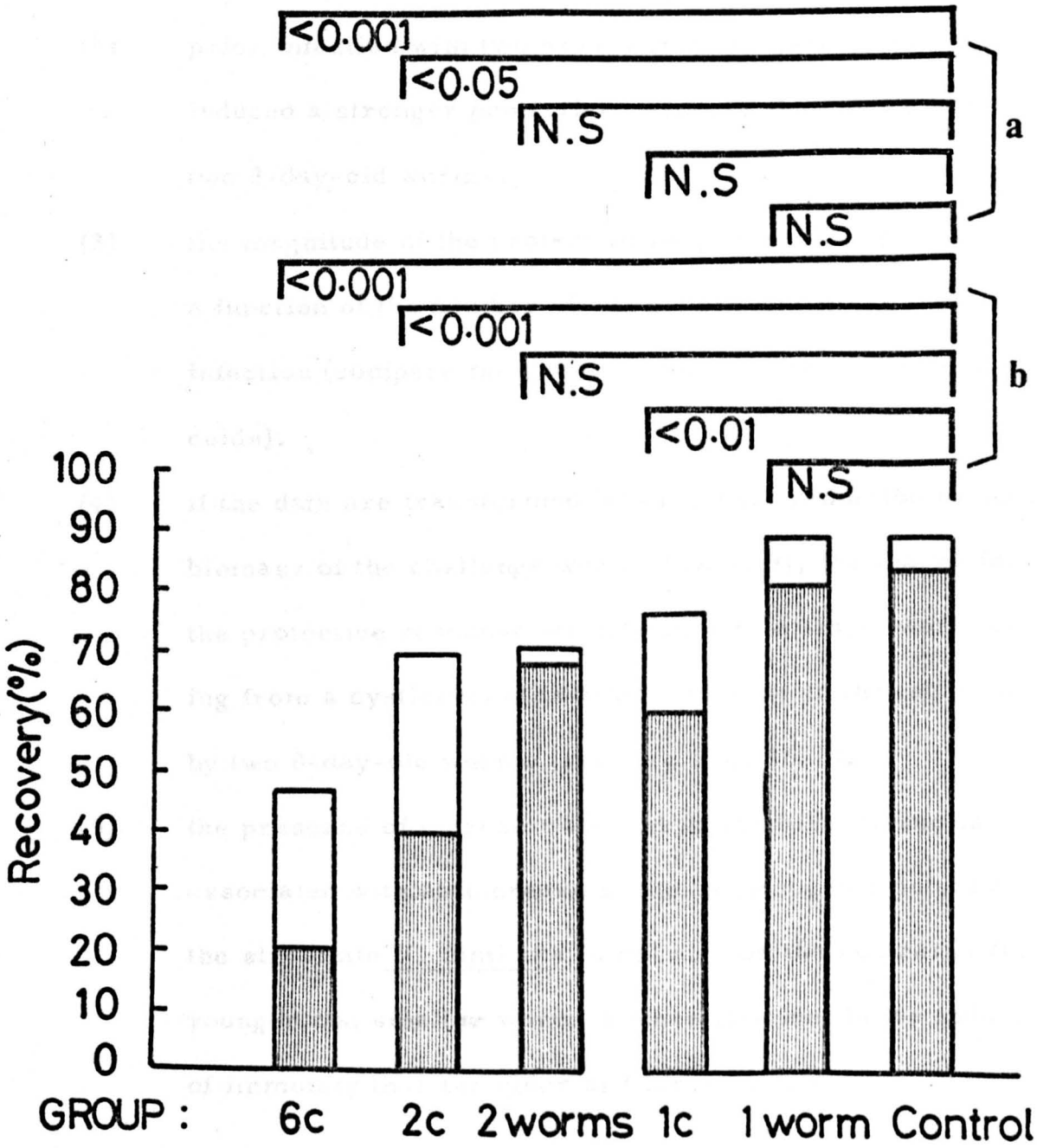
Survival of worms from a six-cysticercoid challenge infection in mice immunized enterally with strobilate worms or cysticercoids and in the naive controls.

Shaded portions of bars represent worms $\geqslant$ 0.1 mg; open portions, worms $<$ 0.1 mg.

a: Level of significance (total recovery)

b: Level of significance (worms $\geqslant$ 0.1 mg)

c: cysticercoid.


<0·001
<0·05
N.S
N.S
N.S
a
<0·001
<0·001
N.S
<0·01
N.S
b
100
90
80
70
60
50
40
30
20
10
0
Recovery(%)
GROUP :
6c
2c
2worms
1c
1worm
Control

(1) prior infection, for the same period (7 days), with a worm growing from a cysticercoid stimulated a stronger protective response than that evoked by a worm introduced as 8-days-old.

(2) prior infection with two worms from a cysticercoid infection induced a stronger protective response than that evoked by two 8-day-old worms.

(3) the magnitude of the protective response against challenge is a function of the number of worms present in the immunizing infection (compare the groups infected with 1, 2 & 6 cysticercoids).

(4) if the data are transformed into per cent reduction in the total biomass of the challenge worms (Table 9), the magnitude of the protective response stimulated with a single worm growing from a cysticercoid is almost similar to that stimulated by two 8-day-old worms (see also Fig. 16-2).

(5) the presence of a greater amount of strobilar tissue is not associated with an increase in the immunogenic potential of the strobilate H. diminuta in mice. On the contrary, the younger and smaller worm is more efficient in the induction of immunity than the older and larger worm.

7.7 Immunity to H. diminuta in Mice: Relation to Age of the Worm

7.7.1 Introduction

The conclusion drawn from the previous experiment (7.6) is that a young tapeworm growing from a cysticercoid is more efficient in the induction of functional immunity against challenge than a worm introduced as 8-days-old. This experiment investigates the immunogenic potentials of the 4- and the 8-day-old worms by measuring their relative efficiency in raising protective immunity against challenge.

7.7.2 Materials & Methods

Mice were male NIH of 6 weeks of age. Four-day-old and 8-day-old worms were obtained from rats each infected with 10 cysticercoids. Transplantation of worms into the duodenum of mice was as described in the previous experiments.

7.7.3 Design

One group of mice received single 8-day-old worms implanted into the duodenum. Mice from another group received single 4-day-old worm transplants. Worms were eliminated with 'Zanil' 7 days after transplantation. By this time worms of the two categories should be present in the gut (see Hopkins et al., 1976). All mice were challenged on day 21 by the oral administration of six cysticercoids. They were killed 7 days after challenge.

7.7.4 Results & Discussion

The growth figures of the challenge worms (Fig. 16-3) show that significantly smaller worms were recovered from the group previously infected with a 4-day-old worm than from the group immunized with an 8-day-old worm.

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Figure 16-3

Growth of worms from a six-cysticercoid challenge infection in mice previously immunized by transplanting into the duodenum strobilate worms of different ages.

A: Immunized with a 4-day-old worm

B: Immunized with an 8-day-old worm

Biomass/mouse(mg dry wt)
2
1
0
<0.05
Group: A
B

To obtain an index of difference between the amount of strobilar tissue (antigens) presented to mice in each case after transplantation of the immunizing worms, worms from the same rat donors were weighed. A 4-day-old worm has a mean dry wt of 0.05 mg. An 8-day-old worm weighs, on average, 5.18 mg, more than a 100 fold the mean dry wt of the 4-day-old worm (Appendix). The results from this experiment indicate that the younger worm presenting the host with the smaller amount of strobilar tissue is more efficient in the stimulation of protective immunity than the older worm presenting the host with the greater amount of this tissue. This is in agreement with the trend established in the previous experiment (7.6) that the younger worm (from a cysticercoid infection) is more efficient than the older 8-day-old worm in the stimulation of immunity against the homologous challenge. Observations from this experiment and experiment (7.6) are at variance with those of Christie (1978) who suggested that the 8-day-old H. diminuta in the mouse is more immunogenic than the 4-day-old worm by virtue of the greater amount of the 'immunogenic' strobilar tissue presented to the host by the older worm. Christie's findings could be attributed to inherent differences between worms of different ages in their susceptibility to immunological attack thus affecting their rate of rejection (which Christie adopted as a measure of the immunogenicity of the strobilate worm).

The evidence provided by the present experiments suggests:

(1) the failure to induce a strong protective response against challenge by implanting a strobilate 8-day-old worm s.c. (7.1), i.p. (7.2 & 7.3) or intraduodenally (7.4) is possibly due to the fact that the 8-day-old worm is, in itself,

inefficient in inducing a pronounced protective response even when presented enterally.

(2) the fact that a weak protective response was induced by the intraduodenal administration of the 8-day-old worm (7.4 & 7.7) and that no protection was induced following the parenteral administration of this worm (7.1, 7.2 & 7.3) suggests that the enteric route is more efficient in the induction of functional immunity to the tapeworm than either the s.c. or the i.p. routes. It is, however, very difficult at this stage to conclude whether this difference is due to an inherent property of the mouse immune system to interact with worm antigens when these were given by the various routes or because of a failure of the immunizing worms themselves to elaborate their antigens when presented to the host by the parenteral route. It could be that the period of effective production of the functional antigens in the subcutis or the peritoneal cavity is not long enough and that a longer period, analogous to that required for the development of immunity from an enteric H. diminuta infection, is needed.

(3) the young worm is more efficient in the induction of protective immunity than older worms. It is unknown whether or not this difference in immunogenicity is due to an actual potential of the younger worm to produce the protective antigens or present these antigens more effectively.

(4) the significance of the worm strobila being the major source of H. diminuta functional antigens (Christie, 1978) is questionable.

(5) these findings could explain the failure reported in this thesis (SECTION 1) to immunize mice by the administration of killed worm antigens. It is pertinent that these antigens were mainly derived from strobilar tissue obtained from worms even older than the 8-day-old worms whose poor immunizing potential was demonstrated above.

8. EXCYSTED WORM ANTIGENS

The evidence obtained from previous experiments implies:

(1) excystation in vivo is not associated with the induction of protective immunity against H. diminuta (7.5).

(2) the enteric route is more efficient than either the s.c. or the i.p. routes in raising protective immunity against this tapeworm (7.1, 7.2, 7.3, 7.4, 7.6, 7.7).

(3) the magnitude of the protective response induced by an enteric infection (starting from cysticercoids or strobilate worms introduced surgically) is dependent upon the number of worms in the immunizing infection (7.6).

(4) the immunizing potential of the tapeworm is related to its stage of development; the protective antigens are produced (or presented) more effectively by the younger worms (7.6, 7.7).

(5) the significance of the strobila as the major source of the functional antigens is debatable (7.6, 7.7).

These observations were suggested to account for the failure to stimulate immunity by vaccinating mice with antigens largely from the strobilae of considerably old worms. It was therefore decided to investigate further the validity of these observations by using antigens from live excysted worms which provide only scolex and neck antigens. This approach provides an advantage since it allows for the immunogenicity of the live young worm to be evaluated in the absence of strobila-specific antigens. It was Andreassen et al. (1978 a) who suggested, citing unpublished results, that the H. diminuta 'functional antigens are related to the scolex and not to the total worm biomass or surface

area'. Moreover, because of their relatively small size (0.175 mm; see Roberts, 1961), a great number of excysted worms can be injected parenterally into mice without the great risk of exposing the host to large amounts of foreign tissue (cf. administration of a strobilate worm). This is of relevance considering the earlier finding that the magnitude of the protective immune response to the tapeworm is a function of the number of the immunizing worms (7.6). In addition, the efficacy of the parenteral route in raising protective immunity can be expressed as an index of the magnitude of the immune response induced when the worms are presented enterally.

8.1 Vaccination of NIH Mice with Excysted Worms Grown in *Tribolium confusum*

8.1.1 Materials & Methods

Six-weeks-old, male NIH mice were used in this experiment. Cysticercoids were obtained from infested specimens of *T. confusum*. The *in vitro* technique of excystation has been described previously. Excysted worms were washed repeatedly in HBSS, thus avoiding the inoculation of the enzymes present in the excystation medium. Worms were counted and administered s.c. in a volume of 0.2 ml HBSS containing 'Crystamycin' (100 units sodium benzylpenicillin & 100 ug streptomycin sulphate/ml) using a 25G needle. Excysted worms were administered not more than 15 min after excystation.

8.1.2 Design

A group of mice was given 36 viable excysted worms as a single s.c. injection. The controls received s.c. injections of 0.2 ml of HBSS containing 'Crystamycin'. A third group was used as an immune control which was given *per os* six cysticercoids. All mice were challenged on day 21 with an oral infection of six cystercoids. They were killed on day 29.

8.1.3 Results

Although immunization with an oral infection from six cysticercoids induced protection against challenge, the s.c. implantation of 36 excysted worms failed to induce such a response (Fig. 17-1 & Fig. 17-2).

Figure 17.

Growth (1) and survival (2) of worms from a six-cysticercoid challenge infection in NIH mice inoculated s.c. with excysted worms from T. confusum and in the immune and naive controls.

Stippled portions of bars represent worms $\geqslant$ 0.1 mg; open portions, worms $<$ 0.1 mg.

a: Level of significance (total recovery)

b: Level of significance (worms $\geqslant$ 0.1 mg)

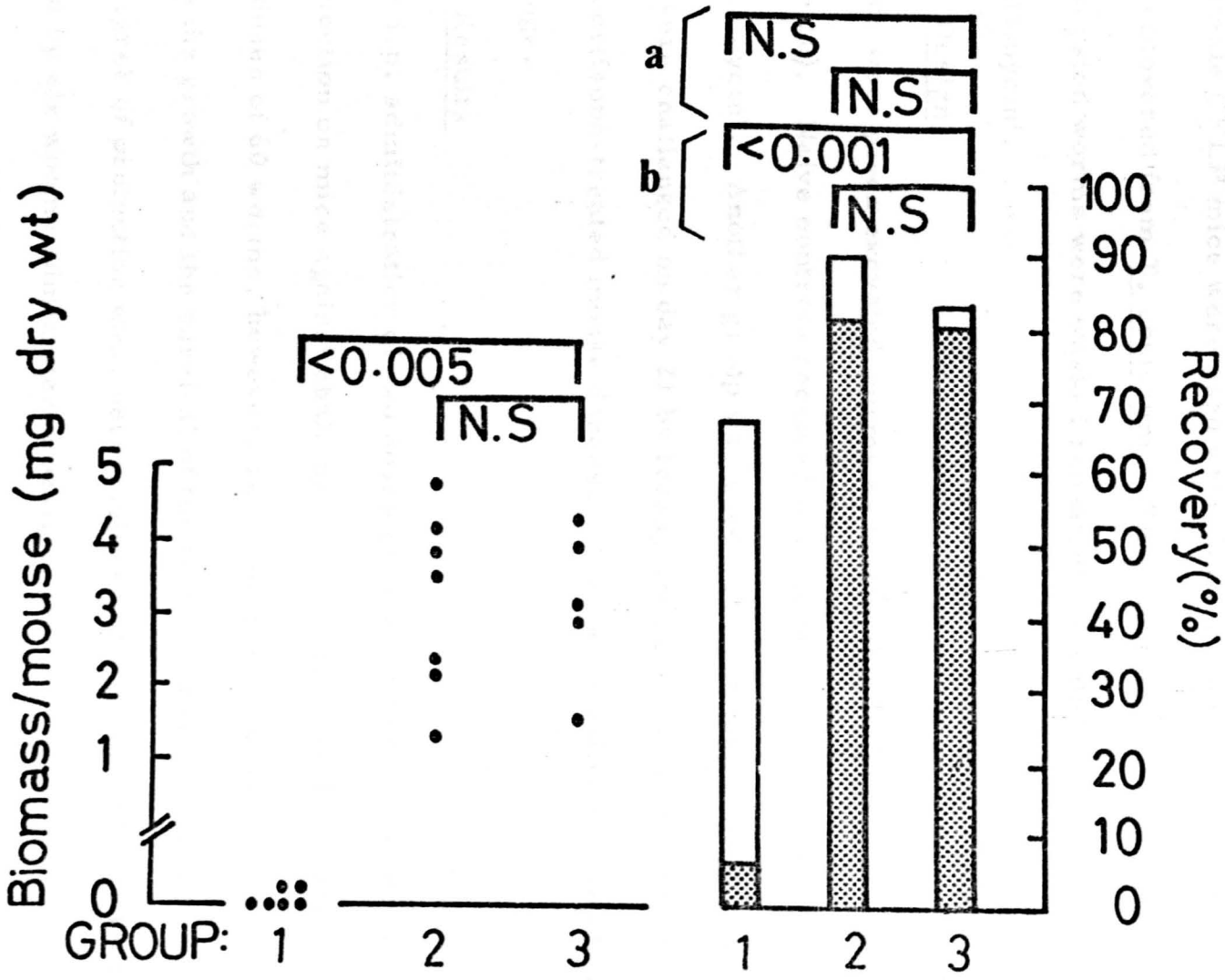
Group 1: Immune controls

2: Inoculated with excysted worms

3: Naive controls

1.

2.


a
N.S
N.S
b
<0·001
N.S
<0·005
N.S
Biomass/mouse (mg dry wt)
5
4
3
2
1
0
GROUP: 1
2
3
1
2
3
100
90
80
70
60
50
40
30
20
10
0
Recovery(%)

8.2 Vaccination of CFLP Mice with Excysted Worms Grown in T. confusum

8.2.1 Materials & Methods

Female CFLP mice were used when 6-weeks-old. Cysticercoids were recovered from T. confusum. They were excysted in vitro and the excysted worms were washed repeatedly in HBSS containing 'Crystamycin'.

8.2.2 Design

Mice were given excysted worms as two or three i.p. injections (Table 10). Naive controls received injections of HBSS containing 'Crystamycin'. Another group was used as an immune control. All mice were challenged on day 21 by transplanting single 8-day-old worms from cortisone-treated mouse donors. They were killed 6 days after challenge.

8.2.3 Results

The i.p. administration of two doses of 60 excysted worms conferred no protection on mice against challenge (Fig. 18). The inoculation of three doses of 60 worms, however, resulted in a significant reduction in both the growth and the survival of the transplanted challenge worms. This degree of protection was, nevertheless, of a lower order to that induced by six worms administered orally.

The longevity of excysted worms in the peritoneal cavity was not investigated in detail because of the difficulty in recovering worms of this size (particularly when there is host tissue reaction). Excysted worms contained in Millipore diffusion chambers (membrane size 0.45 um), however, were capable of surviving for up to 24 h following insertion of the chambers into the peritoneal cavity.

Table 10 Vaccination of CFLP mice with excysted worms from cysticercoids grown in T. confusum

Group	Injections (days) 0	7	11	12
1. Immune control	6c (oral)			
2. Vaccinated	60 EW		60 EW	
3. Naive control	HBSS		HBSS	
4. Vaccinated	60 EW	60 EW		60 EW
5. Naive control	HBSS	HBSS		HBSS

c = cysticercoid; EW = excysted worms; HBSS = Hanks' balanced salt solution.

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Figure 18.

Growth and survival of 8-day-old H. diminuta single-worm transplants in CFLP mice inoculated i.p. with excysted worms from T. confusum and in the immune and naive recipient controls.

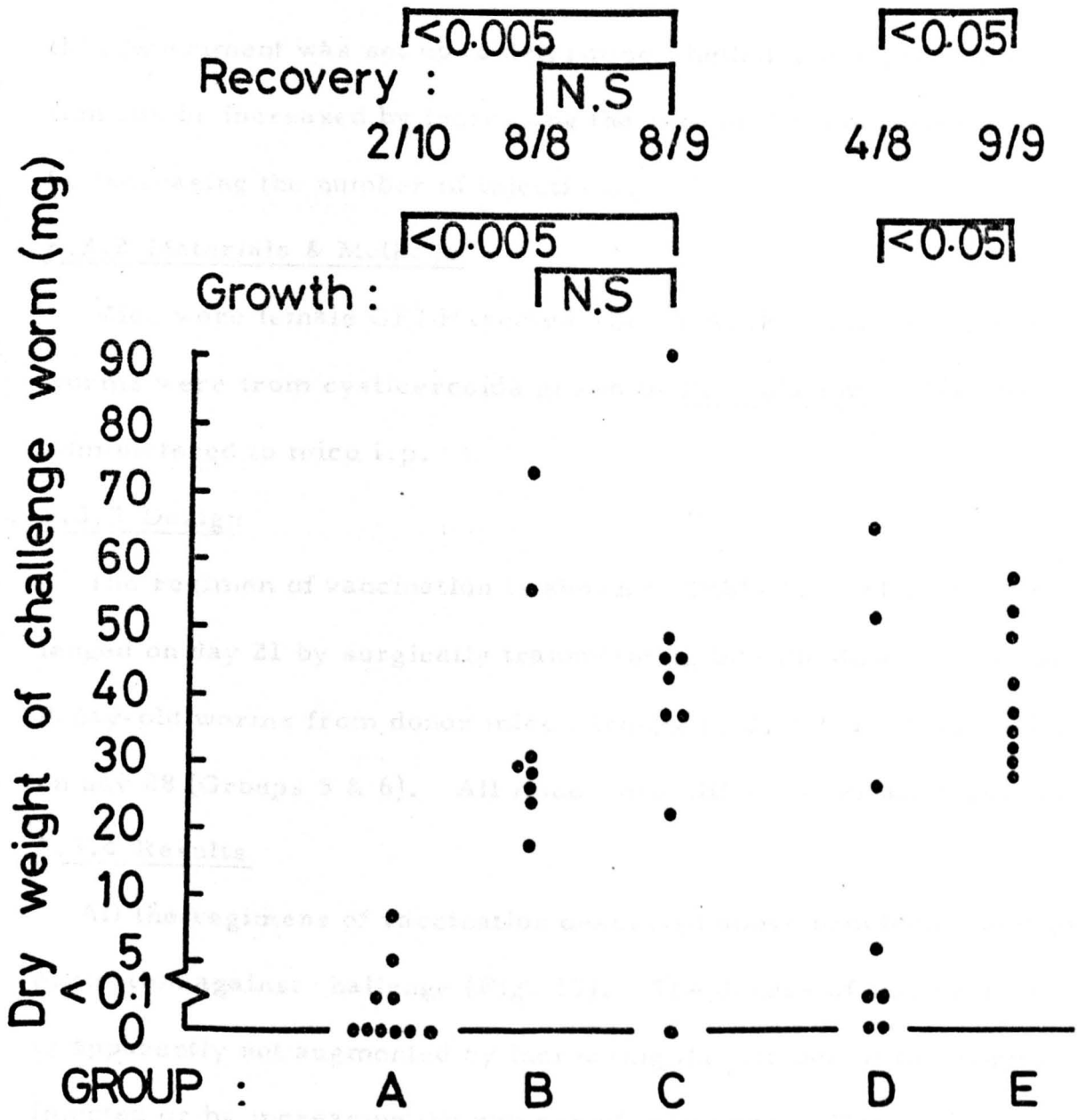
A: Immune controls

B: Inoculated with two doses

C: Naive controls for B

D: Inoculated with three doses

E: Naive controls for D


<0.005
<0.05
Recovery :
N.S
2/10
8/8
8/9
4/8
9/9
<0.005
<0.05
Growth :
N.S
Dry weight of challenge worm (mg)
90
80
70
60
50
40
30
20
10
5
< 0.1
0
GROUP :
A
B
C
D
E

8.3 Vaccination of CFLP Mice with Excysted Worms Grown in T. confusum: Effect of Varying the Regimen of Immunization

8.3.1 Introduction

In view of the results obtained from the previous experiment (8.2), this experiment was set up to determine whether the degree of protection can be increased by increasing the size of the inoculated dose or by increasing the number of injections.

8.3.2 Materials & Methods

Mice were female CFLP treated when 6-weeks-old. Excysted worms were from cysticercoids grown in T. confusum. They were administered to mice i.p.

8.3.3 Design

The regimen of vaccination is shown in Table 11. Mice were challenged on day 21 by surgically transplanting into the duodenum single 8-day-old worms from donor mice (Groups 1, 2, 3 & 4; Table 11) or on day 28 (Groups 5 & 6). All mice were killed 6 days after challenge.

8.3.4 Results

All the regimens of vaccination described above provided significant protection against challenge (Fig. 19). The degree of this protection is apparently not augmented by increasing the number of the worms injected or by increasing the number of injections. These observations confirm earlier findings (8.2) that the i.p. administration of live worms from cysticercoids excysted in vitro evoke protection against challenge albeit in a lower order to that stimulated by the oral administration of cysticercoids.

Table 11 The regimen of vaccination of CFLP mice with excysted worms from cysticercoids grown in T. confusum.

Group	Injections (days) 0	7	12	14	21
1. Immune control	6c (oral)				
2. Vaccinated	60 EW	60 EW	60 EW		
3. Vaccinated	80 EW	80 EW	80 EW		
4. Naive control	HBSS	HBSS	HBSS		
5. Vaccinated	70 EW	70 EW		70 EW	70 EW
6. Naive control	HBSS	HBSS		HBSS	HBSS

c = cysticercoid; EW = excysted worms; HBSS = Hanks' balanced salt solution.

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Figure 19.

Growth and survival of 8-day-old H. diminuta single-worm transplants in CFLP mice injected i.p. with excysted worms from T. confusum and in the immune and naive recipient controls.

A: Immune controls

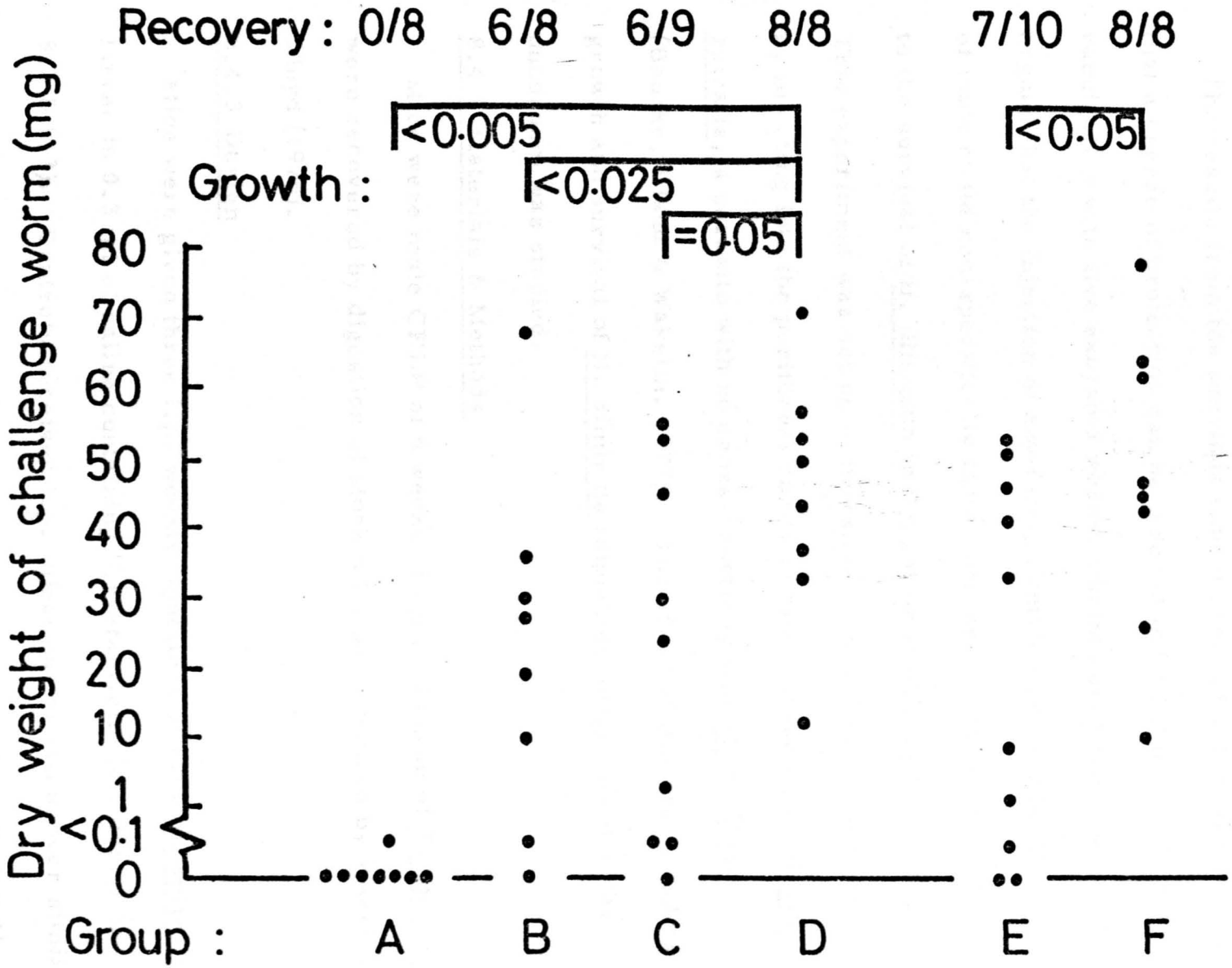
B: Injected with three doses of 60 worms

C: Injected with three doses of 80 worms

D: Naive controls for both B & C

E: Injected with four doses of 70 worms

F: Naive controls for E


Recovery : 0/8 6/8 6/9 8/8 7/10 8/8
<0.005
Growth :
<0.025
=0.05
<0.05
Dry weight of challenge worm (mg)
80
70
60
50
40
30
20
10
1
<0.1
0
Group : A B C D E F

8.4 Specificity of the Protective Response Obtained Following Vaccination with Excysted Worms

8.4.1 Introduction

The results from the previous experiments (8.2 & 8.3) have shown that a degree of protection can be induced in CFLP mice following vaccination with live excysted worms administered i.p. It might be argued that the injection of any foreign antigen into the peritoneal cavity of mice could non-specifically induce a reaction that is detrimental to the survival of H. diminuta in the gut or inhibitory to worm growth. This experiment was set up to investigate the validity of this argument by injecting into the peritoneal cavity of mice larvae of Trichinella spiralis, a parasite with no cross-reactivity with H. diminuta (Behnke, Bland & Wakelin, 1977). The effect of this treatment on the growth and survival of H. diminuta implanted surgically into the duodenum was studied.

8.4.2 Materials & Methods

Mice were male CFLP of 6 weeks of age. Larvae of T. spiralis were recovered by digestion of stock mice as described by Wakelin & Lloyd (1976).

8.4.3 Design

Mice were given three i.p. weekly injections of 2000 T. spiralis larvae in 0.3 ml of saline containing 'Crystamycin' (cf. design in 8.2 & 8.3). Controls received i.p. injections of saline over similar periods of time. Mice from the two groups were given on day 21 single 8-day-old H. diminuta transplants obtained from mice treated with cortisone. All mice were killed on day 27.

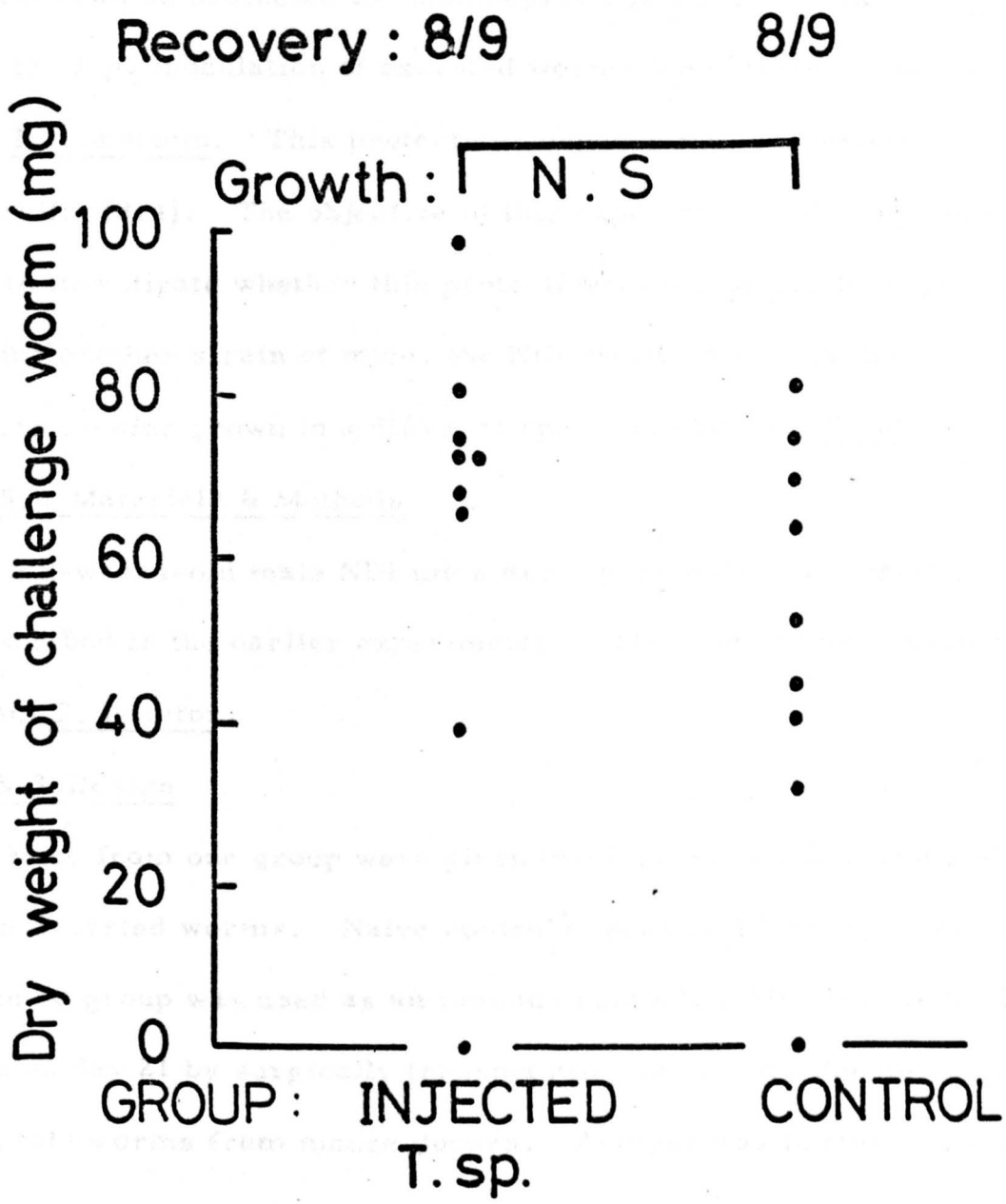
8.4.4 Results

There is no evidence that the induction of a non-specific inflammatory response in the peritoneal cavity of mice affects the growth or the survival of H. diminuta implanted surgically into the duodenum (Fig. 20). This suggests that the response evoked against H. diminuta by the i.p. administration of the homologous excysted worms (8.2 & 8.3) is a specific one.

Facing page 127

Figure 20.

Growth and survival of 8-day-old H. diminuta single-worm transplants in mice previously injected with larvae of Trichinella spiralis (T. sp.) and in the controls.


Recovery : 8/9
8/9
Growth :
N. S
Dry weight of challenge worm (mg)
100
80
60
40
20
0
GROUP :
INJECTED
T. sp.
CONTROL

8.5 Vaccination of NIH Mice with Excysted Worms Grown in Tenebrio molitor

8.5.1 Introduction

Results from experiments 8.2 & 8.3 showed that mice of the CFLP strain can be protected to some degree against infection with H. diminuta by the i.p. inoculation of excysted worms from cysticercoids grown in T. confusum. This protective response was suggested to be specific (8.4). The objective of this experiment and experiment 8.6 is to investigate whether this protective response can be reproduced using another strain of mice, the NIH strain, and excysted worms from cysticercoids grown in a different species of beetle, Tenebrio molitor.

8.5.2 Materials & Methods

Six-weeks-old male NIH mice were given excysted worms i.p. as described in the earlier experiments. The cysticercoids used were from T. molitor.

8.5.3 Design

Mice from one group were given two i.p. weekly injections of 80 live excysted worms. Naive controls received HBSS injections (0.2 ml). A third group was used as an immune control. . All mice were challenged on day 21 by surgically transplanting into the duodenum single 8-day-old worms from mouse donors. Autopsy was performed 5 days later.

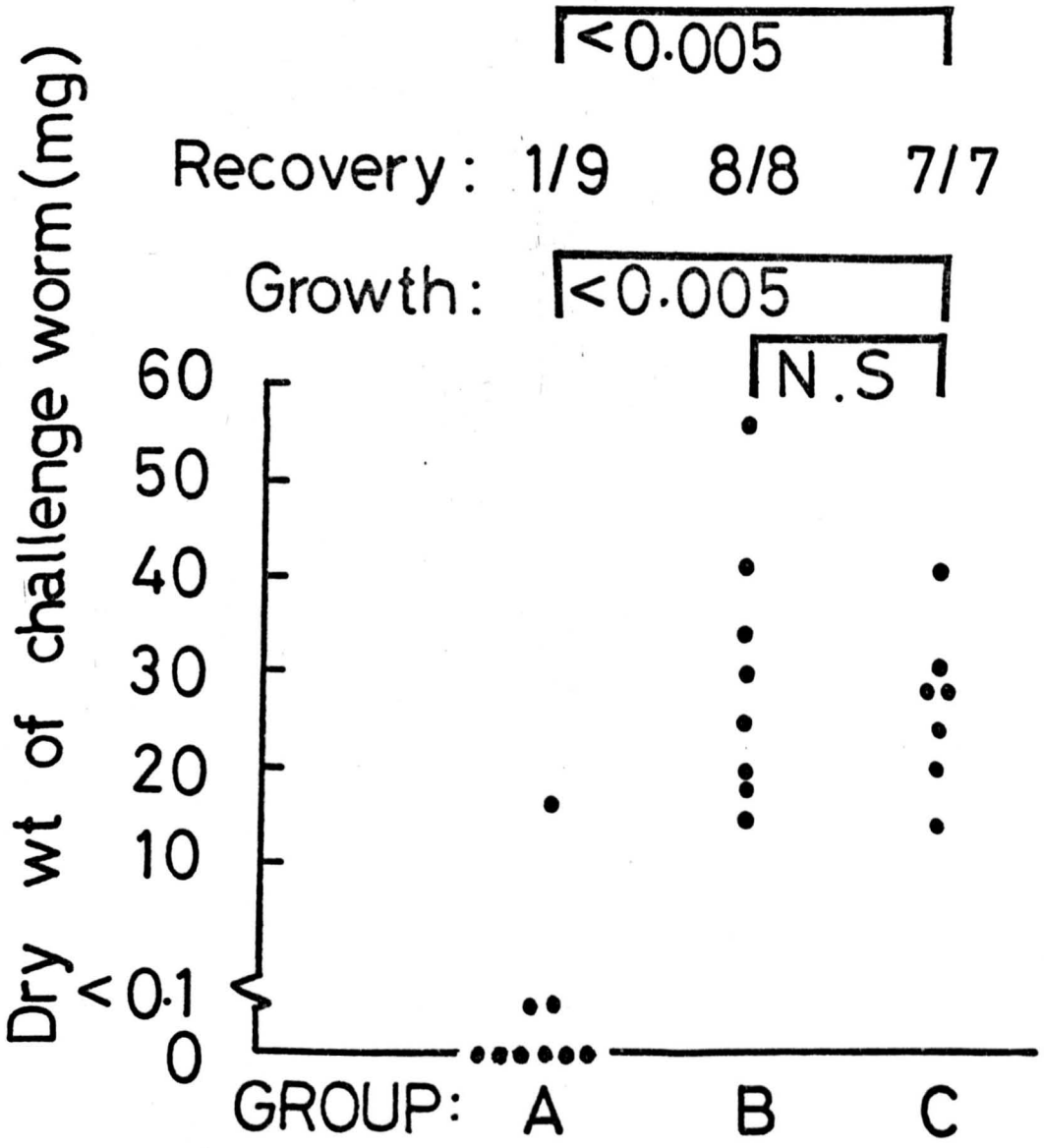
8.5.4 Results

Vaccination of NIH mice with two doses of 80 worms given i.p. resulted in no protection against the challenge transplants (Fig. 21). This is consistent with the previous finding (8.2) that mice (CFLP)

Facing page 129

Figure 21.

Growth and survival of 8-day-old H. diminuta single-worm transplants in NIH mice inoculated i.p. with excysted worms grown in T. molitor (B) and in the immune (A) and naive (C) recipient controls.


<0.005
Recovery: 1/9 8/8 7/7
Growth: <0.005
N.S
Dry wt of challenge worm (mg)
60
50
40
30
20
10
<0.1
0
GROUP: A B C

were not protected against challenge transplants by the inoculation of two doses of excysted worms (grown in T. confusum).

8.6 Vaccination of NIH Mice with Excysted Worms Grown in T. molitor: Effect of Varying the Regimen of Immunization and Challenge

8.6.1 Materials & Methods

Male NIH mice were injected when 6-weeks-old with live excysted worms from cysticercoids grown in T. molitor.

8.6.2 Design

A group of mice was given three i.p. injections (day 0, 7 & 12) each contained 80 live excysted worms. Naive controls received three i.p. injections of 0.2 ml HBSS containing 'Crystamycin'. Mice from a third group were used as an immune control and were given oral infections of six cysticercoids. All mice were challenged on day 21 with six cysticercoid infections administered per os. They were killed on day 29.

8.6.3 Results

The i.p. administration of excysted worms given as three doses of 80 worms did not confer on NIH mice any protection against challenge (Fig. 22-1 & Fig. 22-2). This is at variance with the results obtained from CFLP mice in which this schedule of immunization (with worms from T. confusum) provided an antigenic threshold that was sufficient to sensitize these animals. This discrepancy between results could be attributed to differences between worms grown in different beetle hosts. At least there is the difference of size; the worms grown in T. molitor were the smaller. It is also possible that the antigenic threshold required to induce immunity in the two strains of mice is different; the CFLP is believed to be a better rejector of the tapeworm than the NIH strain (Hopkins, personal communication).

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Figure 22.

Growth (1) and survival (2) of H. diminuta from a six-cysticercoid challenge infection in NIH mice inoculated i.p. with excysted worms grown in T. molitor (B) and in the immune (A) and naive (C) controls.

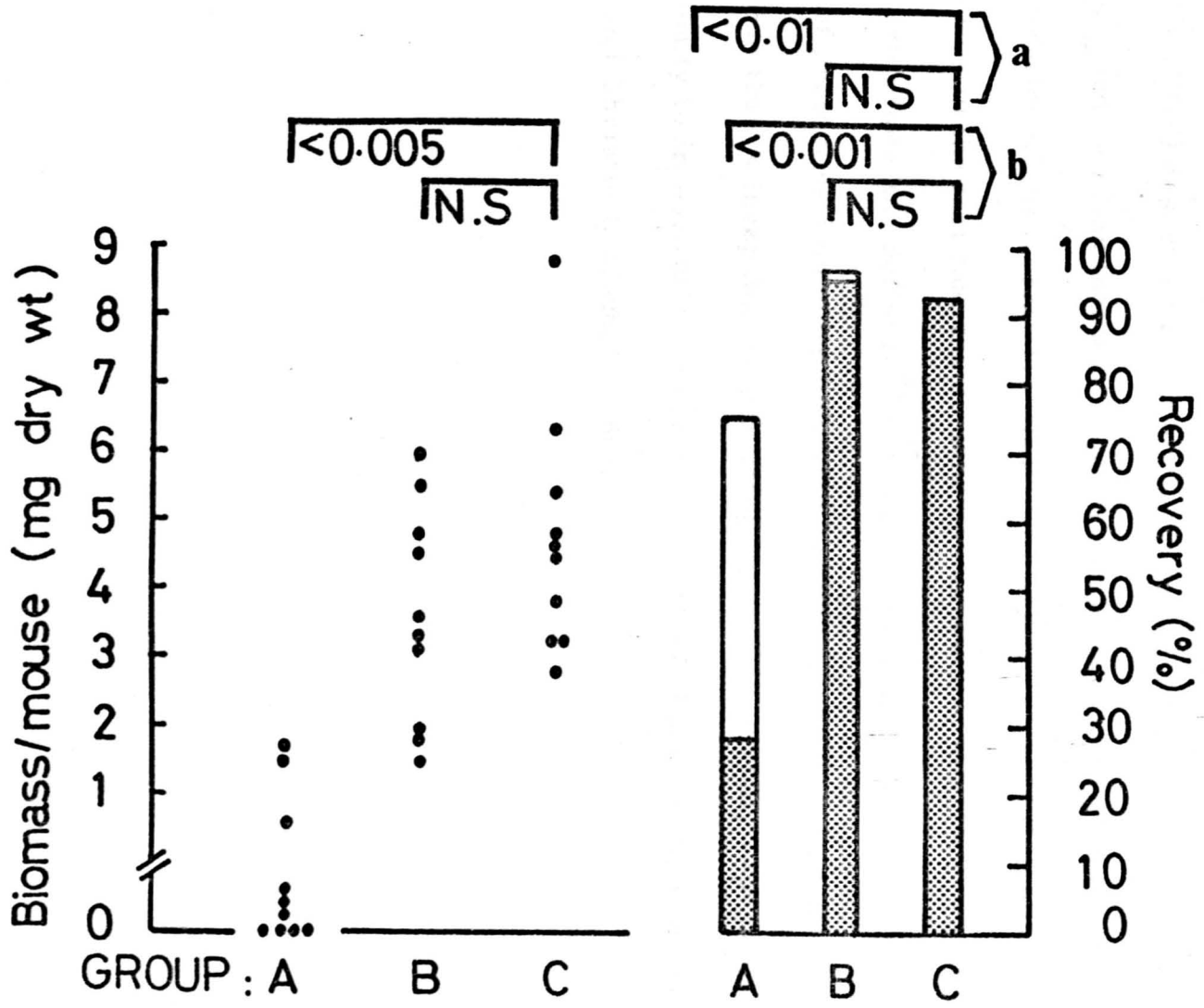
Stippled portions of bars represent worms $\geqslant$ 0.1 mg; open portions, worms $<$ 0.1 mg.

a: Level of significance (total recovery)

b: Level of significance (worms $\geqslant$ 0.1 mg)

1.

2.


<0·01
N.S
a
<0·001
N.S
b
<0·005
N.S
Biomass/mouse (mg dry wt)
0
1
2
3
4
5
6
7
8
9
GROUP : A
B
C
Recovery (%)
0
10
20
30
40
50
60
70
80
90
100
A
B
C

9. IRRADIATED WORM ANTIGENS

The prospects of irradiated vaccines have been outlined in the introduction to this chapter. The experiments described below outline the immunological potential of H. diminuta irradiated with gamma rays. Christie (1979) described the effect of irradiation on the growth of H. diminuta and he found that a dose of 35 Krad almost completely inhibited the formation of the worm strobila. The significance of this observation lies in the fact that the control of a tapeworm infection in both the intermediate and definitive vertebrate hosts can be brought about by the stimulation of protective immunity following upon vaccination with a live tapeworm that is incapable of producing eggs. This study represents a continuity to investigations carried out in this laboratory by Christie (1979) and Christie & Moqbel (1980).

9.1 Vaccination with Irradiated H. diminuta Cysticercoids. I. Immunization Against Challenge with Six Cysticercoids

9.1.1 Materials & Methods

Male NIH mice were vaccinated when 6-weeks-old. Cysticercoids of H. diminuta were irradiated with 35 Krad from a ^{60}Co source as described by Christie (1979) with the exception of the irradiation dose being delivered at 1.0 Krad min^{-1}.

9.1.2 Design

Groups of mice were given by a stomach tube a single dose of six normal or six irradiated cysticercoids. Two other groups were given similar infections but followed by a booster (Table 12). Naive controls were given HBSS and Zanil treatments. All mice were challenged on day 42 with oral infections of six normal cysticercoids. They were killed on day 50.

9.1.3 Results

To check the growth and survival of the immunizing worms and the effects of irradiation on these, 3, 5, 3 & 3 mice were taken at random from groups 1, 2, 4 & 5 (Table 12) respectively and were killed on day 8 of infection. Of the total number of worms from unirradiated cysticercoids administered, 94% were recovered and these were large strobilate worms of an average dry weight of 0.72 mg. This indicates that these worms had grown appreciably. Of the total number of the irradiated worms administered, 42% were recovered. These irradiated worms failed to grow strobilae and resembled destrobilated worms in gross morphology. This indicates that a dose of 35 Krad is completely inhibitory to strobila formation by the tapeworm, an observation

Table 12 Regimen of vaccination with normal and irradiated cysticercoids

Group	Treatment (day) 0	10	20	30
1. Vaccinated; 2 doses	6c, N	Z	6c, N	Z
2. Vaccinated; 2 doses	6c, IR	Z	6c, IR	Z
3. Naive control	HBSS	Z	HBSS	Z
4. Vaccinated; 1 dose	HBSS	Z	6c, N	Z
5. Vaccinated; 1 dose	HBSS	Z	6c, IR	Z

c = cysticercoid; N = normal; IR = irradiated; Z = Zanil; HBSS = Hanks' balanced salt solution.

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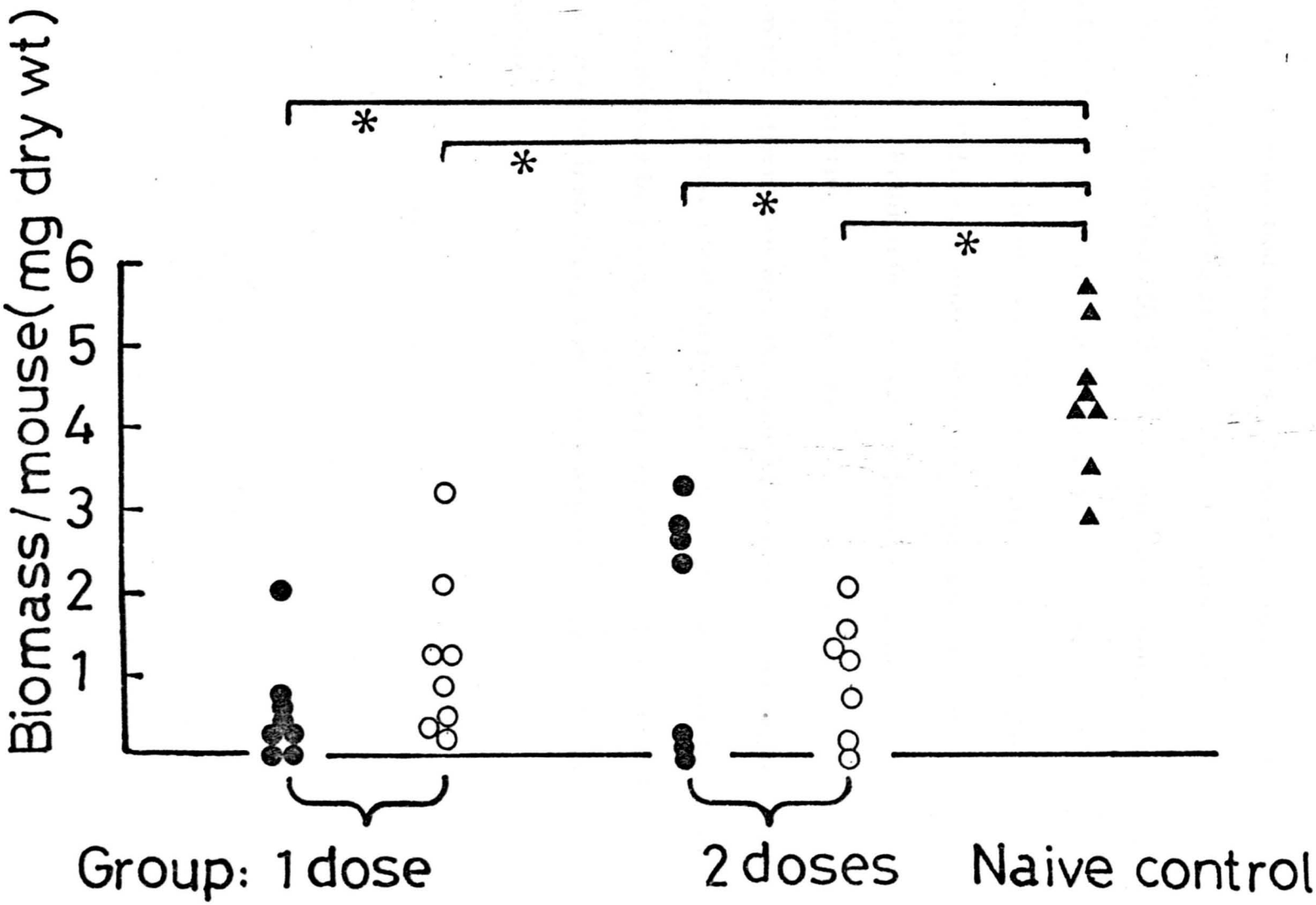
Figure 23.

Growth of H. diminuta from a six-cysticercoid challenge infection in mice vaccinated with normal (●) or irradiated (○) cysticercoids and in the naive controls (▲).

*, $p < 0.005$


Biomass/mouse(mg dry wt)
6
5
4
3
2
1
*
*
*
*
Group: 1 dose
2 doses
Naive control

consistent with that of Christie (1979). This author attributed the low rate of recovery of irradiated worms from infected mice to technical difficulties in finding worms of such a small size in the intestine. He also reported that worms which were irradiated functioned normally with regard to feeding and detection of location signals.

Fig. 23 shows the effect of the immune response on the growth of challenge worms in mice previously infected with normal or irradiated worms. Prior infection with worms from normal cysticercoids, for a period of 10 days, stimulated strong immunity in these animals against challenge. Prior infection with irradiated worms also evoked a strong protective response, the degree of which is indistinguishable from that stimulated with the normal worms. The degree of the immune response obtained following a single exposure to infection was not augmented by giving a booster infection. The immunity induced by normal or irradiated worm primary infections is apparently absolute.

9.2 Vaccination with Irradiated H. diminuta Cysticercoids. II. Effect of Varying the Schedule of Immunization

9.2.1 Materials & Methods

Female NIH mice of 6 weeks of age were used in this experiment. Irradiation and infection were as described in the previous experiment (9.1).

9.2.2 Design

The experimental design is shown in Table 13. All mice were challenged on day 27 with an oral infection of six normal cysticercoids. They were killed on day 35.

9.2.3 Results

A single infection with normal worms for a period of 8 days stimulated a strong protection against challenge (Fig. 24). An exposure for the same period of time to irradiated worms resulted in a similar response. Immunization by exposure twice to infections of 4 days duration from either normal or irradiated worms induced a degree of protection that is almost indistinguishable from that evoked by exposure to single infections of 8 days duration.

Table 13 Schedule of immunization with normal and irradiated cysticercoids

Group	Treatment (day) 0	4	8	9	13
1. Vaccinated, 2 doses	6c, N	Z		6c, N	Z
2. Vaccinated, 2 doses	6c, IR	Z		6c, IR	Z
3. Naive control	HBSS		Z		Z
4. Vaccinated, 1 dose	6c, N		Z		Z
5. Vaccinated, 1 dose	6c, IR		Z		Z

c= cysticercoid; N = normal; IR = irradiated; Z = Zanil; HBSS = Hanks' balanced salt solution

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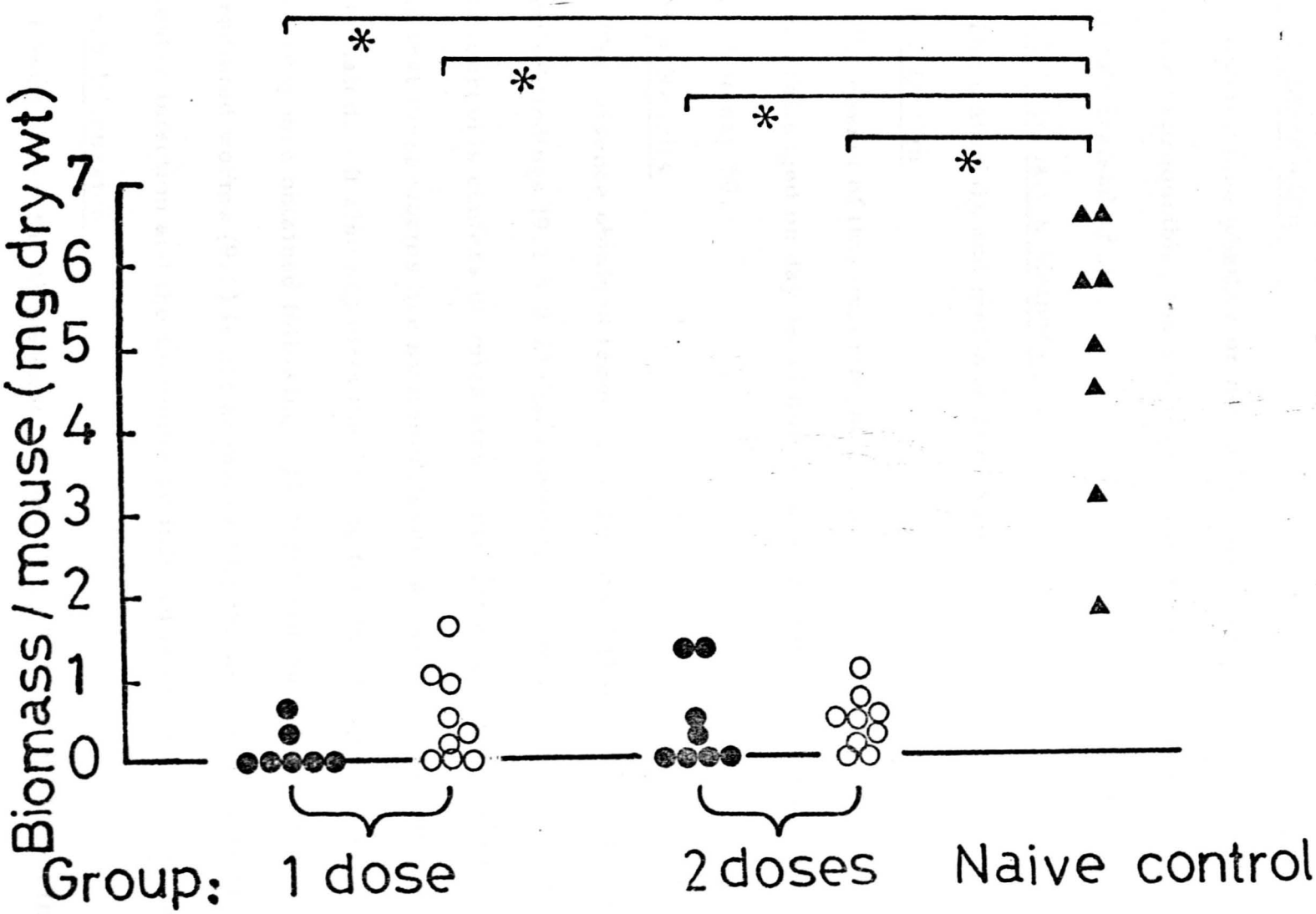
Figure 24.

Growth of H. diminuta from a six-cysticercoid challenge infection in mice previously exposed to infections from normal (●) or irradiated (○) cysticercoids and in the naive controls (▲).

*, $p < 0.005$


Biomass / mouse (mg dry wt)
0
1
2
3
4
5
6
7
*
*
*
*
Group:
1 dose
2 doses
Naive control

9.3 Vaccination with Irradiated H. diminuta Cysticercoids

III. Effect of Varying the Challenge Infection

9.3.1 Introduction

To determine whether or not the results from experiments 9.1 and 9.2 are reproducible, the challenge infection was given as three cysticercoids instead of six.

9.3.2 Materials & Methods

The materials and methods were as in 9.2.1.

9.3.3 Design

The design of this experiment is shown in Table 14. All mice were challenged on day 42 with three cysticercoids. They were killed on day 50.

9.3.4 Results

The evidence obtained from this experiment (Fig. 25) confirms earlier findings (9.1 & 9.2) that exposure to worms from irradiated cysticercoids confers on mice strong protection against challenge and that these worms are as immunogenic as worms which are not irradiated. It also supports the finding that the degree of the protective response obtained following 10 days' exposure to normal or irradiated worms (9.1) is not augmented by the administration of a booster infection and the immunity so induced is apparently absolute.

9.3.5 Discussion

Results from the above experiments (9.1, 9.2 & 9.3) showed that an irradiated vaccine against H. diminuta produced immunity against the homologous challenge comparable to that induced by a normal infection. The merit of immunizing with irradiated worms is that

Table 14 Schedule of vaccination with normal and irradiated cysticercoids

Group	Treatment (day)			
	0	10	21	27
1. Vaccinated, 2 doses	6c, N	Z	6c, N	Z
2. Vaccinated, 2 doses	6c, IR	Z	6c, IR	Z
3. Naive control	HBSS	Z	HBSS	Z
4. Vaccinated, 1 dose	6c, N	Z	HBSS	Z
5. Vaccinated, 1 dose	6c, IR	Z	HBSS	Z

c = cysticercoid; N = normal; IR = irradiated; Z = Zanil; HBSS = Hanks' balanced salt solution.

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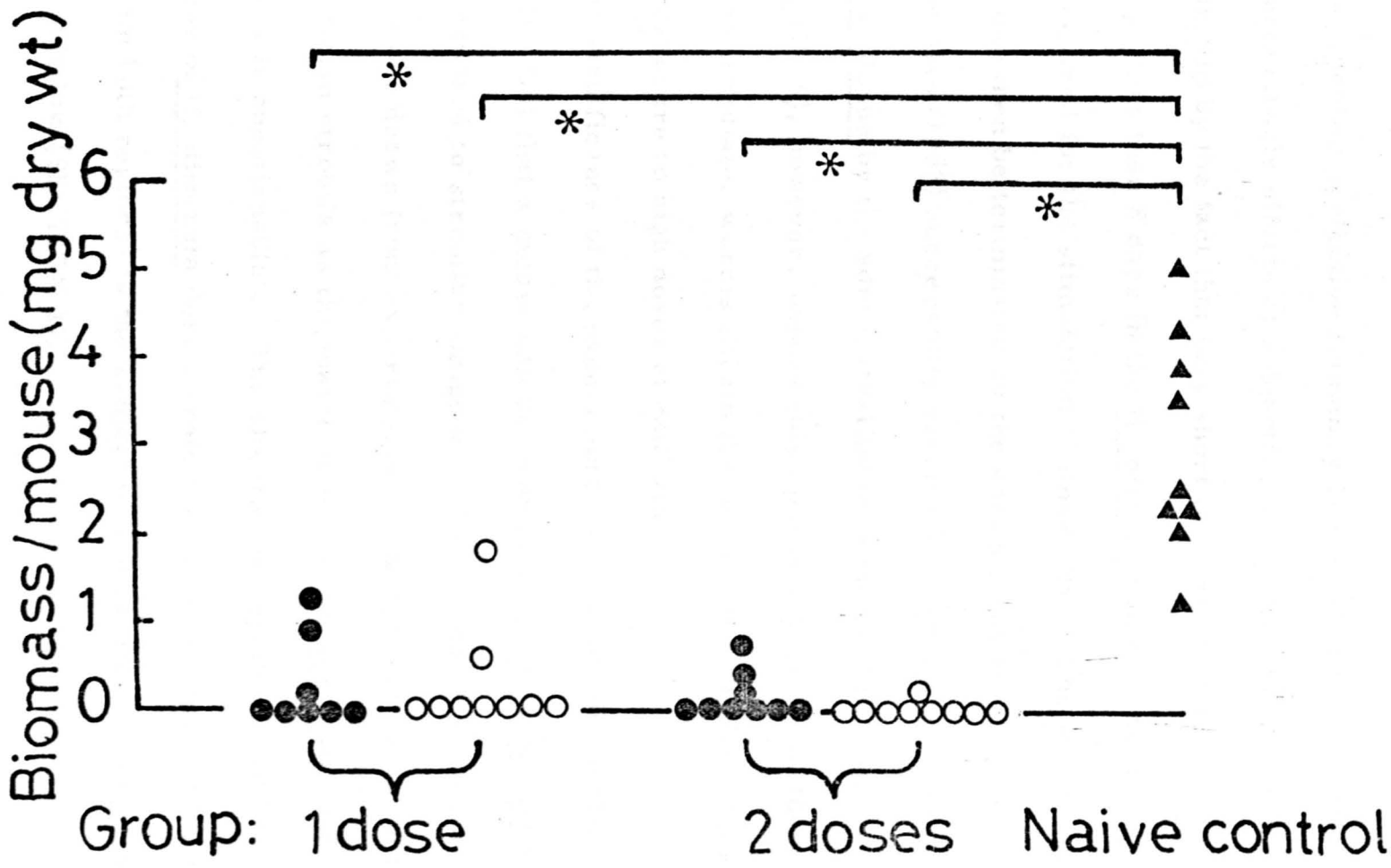
Figure 25.

Growth of H. diminuta from a three-cysticercoid challenge infection in mice previously infected with normal (●) or irradiated (○) cysticercoids and in the naive controls (▲).

*, $p < 0.005$.


Biomass / mouse (mg dry wt)
0
1
2
3
4
5
6
*
*
*
*
Group:
1 dose
2 doses
Naive control

irradiation inhibits strobila formation (and hence the production of eggs). The infection can thus be denoted as self-limiting resulting in the prevention of propagation of the parasite to the intermediate host at a time specific protective immunity is raised in the definitive host. The undesirable effects of subjecting a host to a tapeworm infection is minimized by the fact that only short periods of antigenic stimulation, perhaps less than 8 days in the H. diminuta infections in the mouse, are required for the stimulation of immunity. The immunizing infections can then be terminated by the administration of anthelmintics. Movsesijan (1968) successfully vaccinated dogs against infection with E. granulosus by the administration of irradiated parasites. Herd et al. (1975), however, oppose this approach because of their observation that the irradiated worms regain the potential to produce eggs even after exposure to high doses of radiation.

The significance of the experiments described herein also derives from the fact that a mouse can be immunized to H. diminuta without being exposed to strobilar antigens. This evidence conforms to the conclusions drawn from experiments 7.6 & 7.7 that 'the significance of the worm strobila as the major source of H. diminuta functional antigens is questionable'. The alternative hypothesis that the functional antigens of H. diminuta derive from the scolex or neck of the tapeworm (or from both regions) is the subject of a separate investigation outlined below (SECTION 3).

SECTION 3

THE ORIGIN OF *H. DIMINUTA* PROTECTIVE ANTIGENS

HYMENOLEPIS DIMINUTA: THE ORIGIN OF THE PROTECTIVE ANTIGENS

E. E. ELOWNI

Wellcome Laboratories for Experimental Parasitology,
University of Glasgow, Bearsden Road, Bearsden,
Glasgow, G61 1QH, Scotland

ABSTRACT

Studies were made to locate the origin of the protective antigens of Hymenolepis diminuta. Mice were infected orally with 1, 6 or 30 cysticercoids allowed to develop for different periods of time which exposed the hosts to antigens from the prestrobilate, early strobilate or fully strobilate worms. Other groups were immunized by intraperitoneal (i.p.) implantation of a live strobilate worm or by i.p. implantation of live worms from cysticercoids excysted in vitro. Strong protection against challenge with a surgically-transplanted strobilate worm was achieved by prior infection with 6 or 30 worms eliminated as early as day 3 of infection. By this time these worms would not have strobilated. Conversely, a single worm, strobilating extensively over 16 days, stimulated only weak protection. Parenteral implantation of excysted worms protected mice but parenteral implantation of a strobilate worm had no effect. It is suggested that (i) the tapeworm protective antigens are primarily related to the scolex and/or the germinative region; (ii) the number of worms and the duration of antigenic stimulation in an immunizing infection determine the magnitude of a protective secondary response.

INDEX DESCRIPTORS: Hymenolepis diminuta; Cestode; Protective antigens; Scolex; Germinative region; Immunization; Mice, CFLP.

INTRODUCTION

Results from several investigations (Hopkins et al. 1972 a, b; Befus 1975) indicate that the tapeworm Hymenolepis diminuta is rejected from mice by an immunologically-mediated mechanism. The origin and identity of the antigens that elicit this protective response (protective antigens) are unknown. Christie (1978) found that an 8-day-old worm implanted surgically into the duodenum was rejected earlier than a 4-day-old worm presenting a smaller amount of strobila. He proposed the strobila of the worm as the major source of the protective antigens but he concluded that his experiments did not rule out a role for scolex antigens nor did they exclude a possibility for physiological differences between the worms affecting their rates of rejection. Andreassen et al. (1978) cited unpublished results in which it was found that five H. diminuta were rejected by nude mice at a time when their combined weight was less than that reached by two worms which were not rejected. They suggested that the protective antigens of this tapeworm were "related to the scolex region and not to the total worm biomass or surface area."

This paper describes attempts to locate the origin of H. diminuta protective antigens with an aim to identification. The relative immunogenic potentials of the scolex, neck (germinative region) and strobila were evaluated by comparing the abilities of their antigens to stimulate in mice a protective response against challenge following artificial immunization or exposure to chemically-abbreviated enteric infections.

MATERIALS AND METHODS

Mice - Tapeworm-free, female CFLP mice were used when 7 weeks old. They were maintained under conventional laboratory conditions and supplied with mouse breeding diet (Grain Harvesters Ltd.) and water ad libitum. Oxytetracycline HCl ('Terramycin', Pfizer Ltd.), at a concentration of 165 mg/l, was given in the drinking water for three consecutive days after surgery or following intraperitoneal (i.p.) injection of worms.

Parasite and Infection The strain of H. diminuta used was described by Hopkins et al. (1972 a). Twenty-nine day-old cysticercoids obtained from Tribolium confusum were administered to mice by intragastric intubation. Sham-infected controls received 0.1 ml of balanced salt solution (BSS). Cysticercoids were excysted in vitro by a technique described by Goodall (1973). Immediately after excystation, worms were washed free from the excysting medium enzymes, counted and injected into the peritoneal cavity of mice in 0.2 ml BSS containing 20 units of sodium benzylpenicillin and 20 μg of streptomycin sulphate ('Crystamycin', Glaxo).

Anthelmintic Treatment Praziquantel (Bayer AG, Leverkusen) was prepared as an aqueous suspension with the addition of Cremophor and was administered to mice as a single oral dose of 100 mg/kg body weight. At this concentration, tests against 1, 5, 7 and 9-day-old H. diminuta in mice had shown the compound to be 100% effective (Elowni unpublished). This dose level was reported by

Thomas & Gönnert (1977) to bring about rapid effect on H. diminuta in rats and H. nana and H. microstoma in mice. Oxyclozanide ('Zanil', I.C.I. Ltd.) was given to mice as a single oral dose of 150 mg/kg (Hopkins et al. 1973).

Worm Donors Eight-day-old H. diminuta, weighing approximately 1 mg dry weight, were recovered from mice infected when 6 weeks old with four cysticercoids and treated with 1.25 mg of cortisone acetate ('Cortistab', Boots) injected subcutaneously on days 2, 4 and 6 post-infection.

Surgical Challenge Each mouse was challenged by implanting into the duodenum (Hopkins & Zajac 1976) a single 8-day-old worm on day 21 after the immunizing or sham infections.

Worm Recovery Six days after challenge, worms were recovered by a technique similar to that described by Hopkins et al. (1972 a) whereby very small worms, often < 1 mm in length, can be recovered. Large worms were dried at 95 - 100 °C for 24 h and weighed separately to the nearest one tenth of a mg. Very small worms were not weighed as their weight would have been less than 0.1 mg.

Presentation and Analysis of Data A point on a figure at '0' mg indicates that no worm was recovered from a mouse. A worm of < 0.1 mg is a destrobilated worm; it is in the process of being rejected (Hopkins et al. 1976) and has therefore been excluded from recovery figures. Weights of challenge worms from experimental

and control mice, including destrobilated worms, were compared by the one-tailed Wilcoxon two-sample test (Colquhoun 1971). Recoveries were analysed by the Fisher exact probability test (Siegel 1956). Differences between groups are considered significant when $P \leq 0.05$. For illustrative purposes, differences in growth of the challenge worms between experimental and sham control groups were transformed into percentages and referred to as 'per cent reduction in total worm biomass'.

Experimental Design

Immunization with Cysticercoids Preliminary experiments showed that worms from a six cysticercoids infection in naive female CFLP mice had not formed proglottids (strobila) by day 3 of infection. On gross examination, of 10 worms taken at random from a group of 42 4-day-old worms, only 1 was found to be segmented. At days 1 and 3, all the worms examined (20 and 30 respectively) were merely scoleces with neck regions. Experimental mice were infected with 1, 6 or 30 cysticercoids. Primary infections were eliminated with Praziquantel on days 1 or 3 of infection (prestrobilate stage), on day 5 (early strobilate stage) or on day 16 (the fully strobilate stage). Sham-infected controls received Praziquantel over similar periods of time.

Immunization with Excysted Worms Mice received three weekly i.p. injections of 60 excysted worms. Sham controls were injected with BSS containing 'Crystamycin'. Another group was infected

orally with six cysticercoids on day 0 and the worms were eliminated with 'Zanil' on day 16 (immune control group).

Immunization with a Strobilate Worm Single 8-day-old worms from donor mice were implanted in the peritoneal cavity on days 0, 7 and 14. Sham controls received i.p. injections of BSS containing 'Crystamycin'. An immune control group (see above) was also included.

RESULTS

Immunization with Cysticercoids

Prior infection with one cysticercoid for 16 days had no significant effect (Fig. 1) on either the growth or the survival of the challenge transplants when compared with the sham infected controls (25% reduction in worm total biomass). Conversely, infection with six cysticercoids for the same period resulted in a significant reduction in worm survival and worm growth (89% reduction in biomass). Mice previously infected with six cysticercoids for 5 days harboured significantly fewer worms than the controls and their worms were significantly lighter (76% reduction in biomass). A five-fold increase of the immunizing dose resulted in a stronger protective response (88% reduction in biomass). Both levels of infection, 6 and 30 cysticercoids, eliminated after 3 days, induced a strong immunizing effect equivalent to 76% and 91% reduction in worm biomass respectively. Exposure to infection for 1 day resulted in no statistically significant differences between the sensitized and the control recipients. Reductions of 31% and 38% in worm biomass were encountered in the groups previously infected with 6 and 30 worms for this period of time respectively. In the latter group, 50% of the challenge worms weighed less than the lightest worm from the sham-infected controls.

Immunization with Excysted Worms

Prior immunization with excysted worms significantly reduced the growth (54%) and the survival of challenge worms (Fig. 2).

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Figure 1.

The growth and survival of 8-day-old H. diminuta single-worm transplants in recipients previously infected with cysticercoids and in the sham controls.

*: Significance (the Fisher exact test)

**: Significance (Wilcoxon test)

N.S. : Not significant

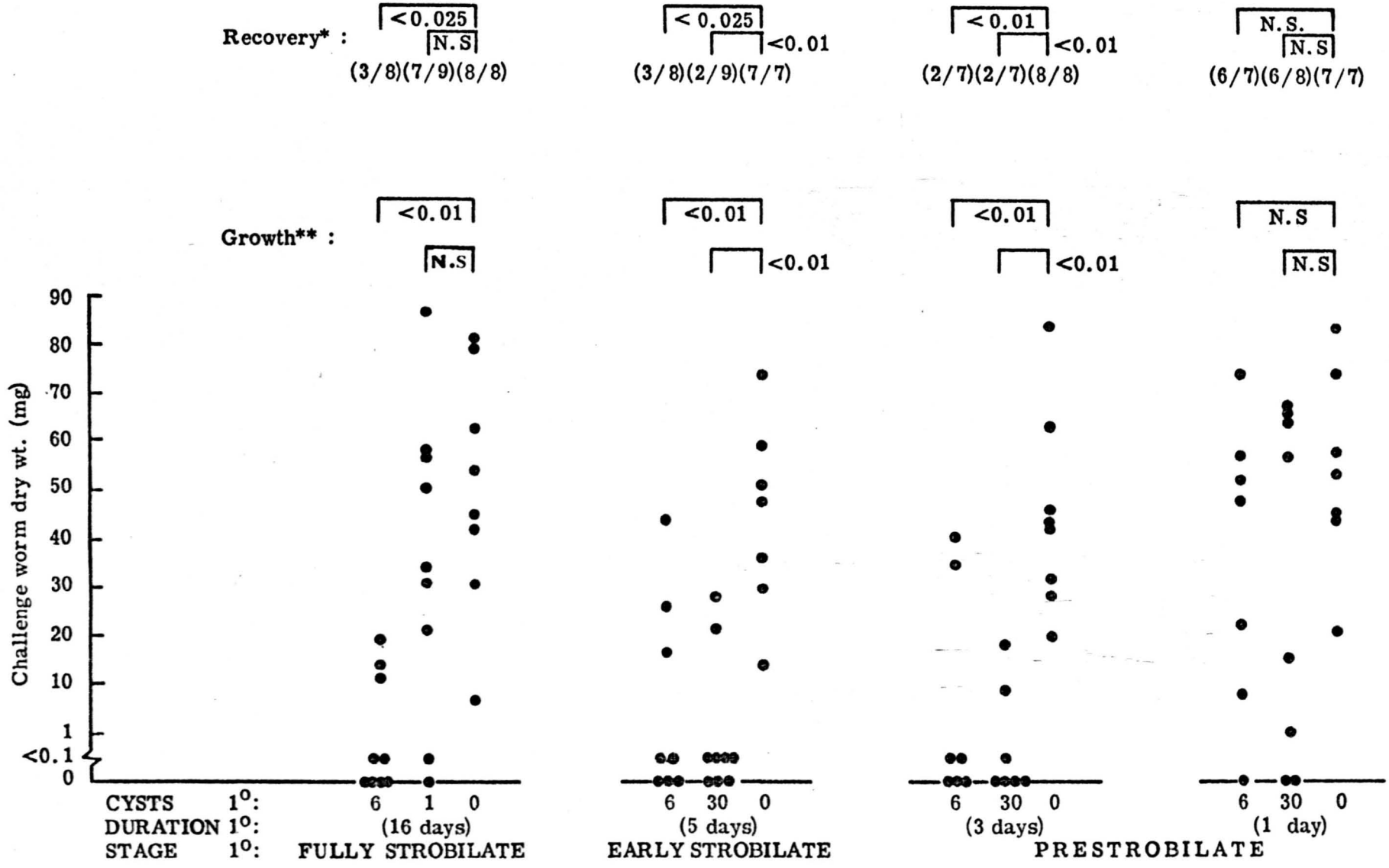

Recovery* :
<0.025
N.S
(3/8)(7/9)(8/8)
<0.025
<0.01
(3/8)(2/9)(7/7)
<0.01
<0.01
(2/7)(2/7)(8/8)
N.S.
N.S
(6/7)(6/8)(7/7)
Growth** :
<0.01
N.S
<0.01
<0.01
<0.01
<0.01
N.S
N.S
Challenge worm dry wt. (mg)
90
80
70
60
50
40
30
20
10
1
<0.1
0
CYSTS 1°:
DURATION 1°:
STAGE 1°:
6 1 0
(16 days)
FULLY STROBILATE
6 30 0
(5 days)
EARLY STROBILATE
6 30 0
(3 days)
6 30 0
(1 day)
PRESTROBILATE

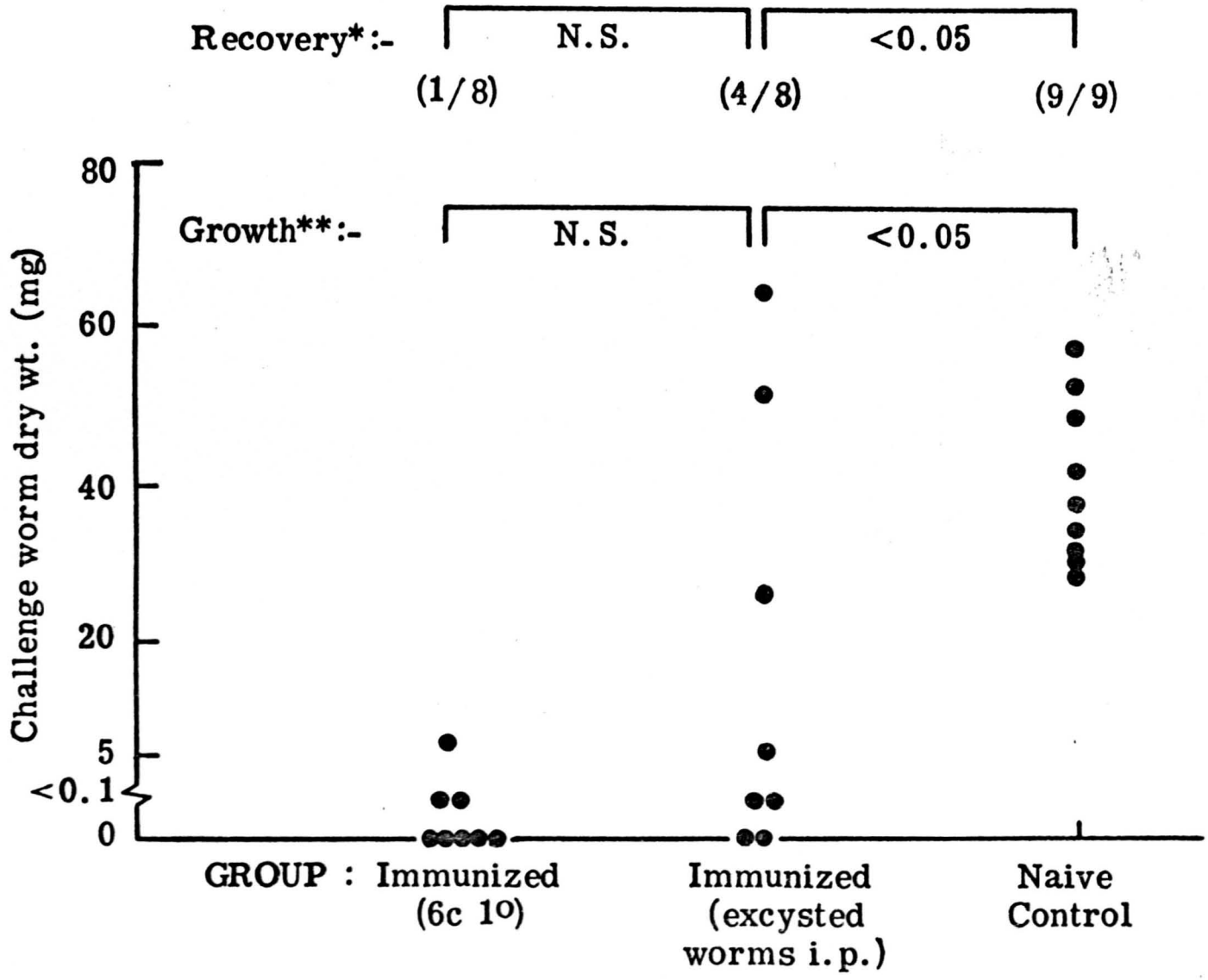
Figure 2.

The growth and survival of 8-day-old H. diminuta single-worm transplants in recipients previously immunized by the i.p. injection of excysted worms and in the controls.

*: Significance (the Fisher exact test)

**: Significance (Wilcoxon test)

N.S.: Not significant


Recovery*:-
N.S.
<0.05
(1/8)
(4/8)
(9/9)
Growth**:-
N.S.
<0.05
Challenge worm dry wt. (mg)
80
60
40
20
5
<0.1
0
GROUP : Immunized (6c 1o)
Immunized (excysted worms i.p.)
Naive Control

Immunization with a Strobilate Worm

Immunization with an 8-day-old worm injected i.p. evoked no protection against challenge (Fig. 3).

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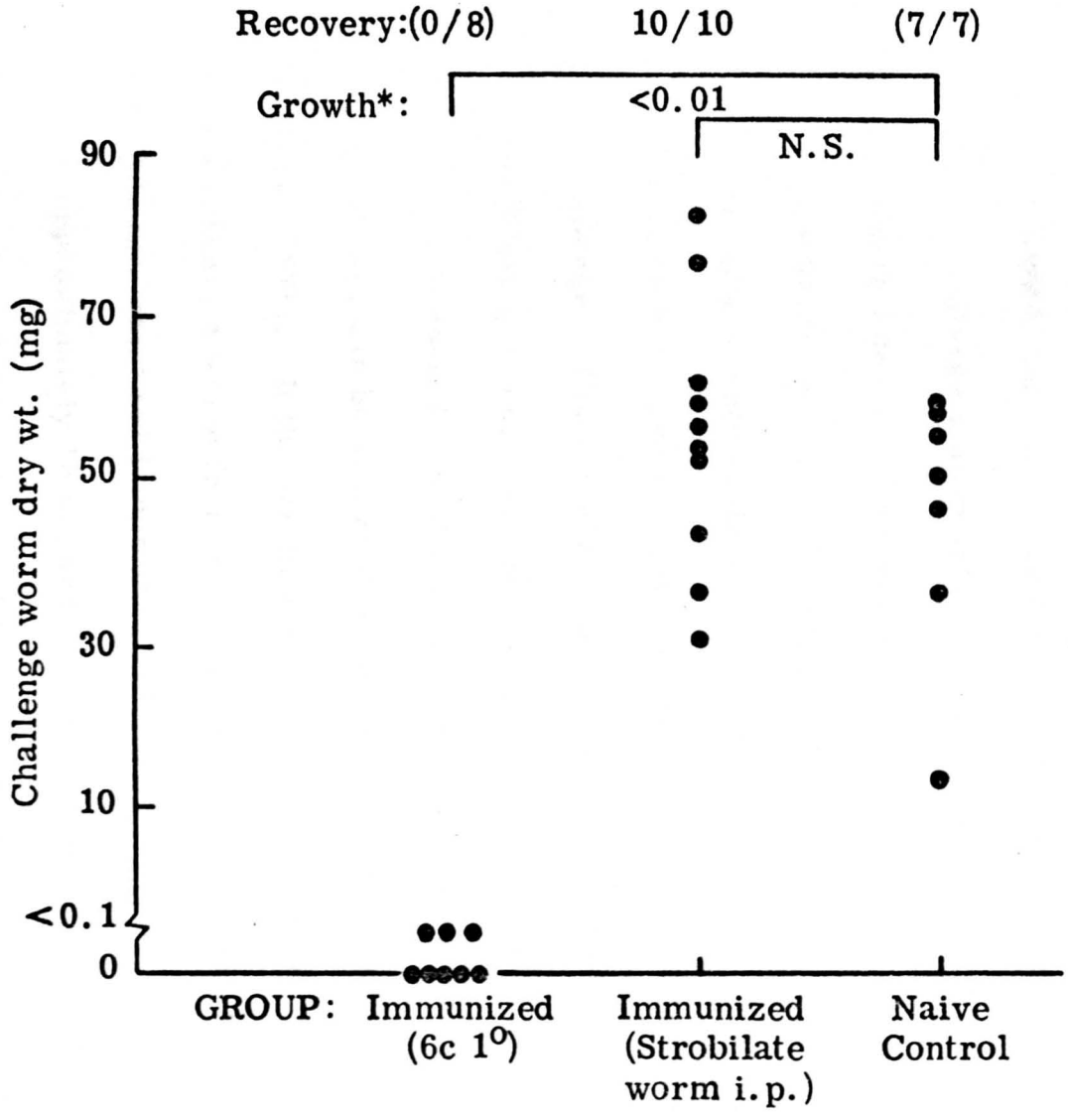
Figure 3

The growth and survival of 8-day-old H. diminuta single-worm transplants in recipients previously immunized by the i.p. injection of strobilate worms and in the controls.

*: Significance (Wilcoxon test)

N.S.: Not significant


Recovery:(0/8)
10/10
(7/7)
Growth*:
<0.01
N.S.
Challenge worm dry wt. (mg)
90
70
50
30
10
<0.1
0
GROUP:
Immunized (6c 1°)
Immunized (Strobilate worm i.p.)
Naive Control

DISCUSSION

A strobilate 8-day-old H. diminuta transplanted surgically into the duodenum of an immune CFLP mouse is rejected within 4 days compared with a period of not less than 7 days in the naive recipient (Hopkins et al. 1976). The distinction therefore between immune, weakly immunized, and unsensitized mice, in terms of growth and survival of a challenge transplant, could be made on day 6 of challenge (see experimental design) if not earlier.

Exposure for only 3 days to H. diminuta antigens from worms consisting of only scoleces and neck regions and weighing not more than a few microgrammes was shown to strongly immunize mice against challenge. Conversely, exposure to infection with a single worm strobilating extensively over 16 days stimulated only weak protection. It is well documented that intact antigenic materials, in immunogenic quantities, can be absorbed by the gut mucosa (reviewed by Hemmings 1978). If the strobila was the major source of the protective antigens, a worm from one cysticercoid infection (Fig. 1), residing in the intestinal lumen for a minimum of 10 days by which time it will be approximately 18 mg and possibly remaining for up to 16 days (Hopkins et al. 1972 a, b), would be expected to present strobilar antigens which may be taken up sufficiently to elicit a strong protective response. The observation that mice could be protected against challenge by the i.p. injection of scolex and neck antigens (from excysted worms) and not by injection of strobilar antigens (from a strobilate worm) casts further doubts on the significance of the strobila as the

major source of the tapeworm protective antigens. The protective response evoked by the inoculation of excysted worms is believed to be specific. The induction of a non-specific inflammatory response in the peritoneal cavity with larvae of Trichinella spiralis, a parasite with no cross-reactivity with H. diminuta (Behnke et al. 1977), was found to have no effect on the growth and survival of H. diminuta implanted surgically into the duodenum of mice (Elowni unpublished).

Protective antigens have been obtained from selected glands or organs of several nematodes (Despommier & Müller 1970; Jenkins & Wakelin 1977). Although scolecial glands were described from several cestodes (Smyth 1969), including some of the hymenolepid species (Baczynska 1914; Fuhrmann 1918; Baer 1956; cited by Smyth 1964), their functions are not yet fully understood. Rothman (1963) observed that the 'cuticle' of the scolex of H. diminuta was made up of dense, osmophilic granules which closely resembled the secretory granules from vertebrate tissue (mammary glands). These granules, though present in the tegument and body of the scolex, were not identified in any structure in the strobila. Smyth (1964) suggested that the granules described by Rothman from the scolex of H. diminuta possibly resembled the secretory granules of the scolex of Echinococcus granulosus. It is significant that the rostellar glands from the scolex of E. granulosus secrete substances capable of reacting with immune canine serum (Smyth 1969) and that dogs can be protected against infection with this tapeworm by vaccination with antigens from protoscoleces (reviewed by Gemmell & Soulsby 1968). The evidence provided by the present investigation indicates that the scolex of

H. diminuta could be the source of the worm protective antigens. Whether such antigens are derived from granules or glandular products of the scolex is, as yet, unknown.

In cyclophyllidean cestodes, strobilization has been attributed to budding from the neck region (Wardle & McLeod 1952). Bolla & Roberts (1971) describe the neck 'germinative region' of H. diminuta as a zone of rapid cell replication and growth; being most active in DNA synthesis and cell division at 2-4 days after infection. This period corresponds to the period of efficient recognition of the tapeworm's protective antigens and the induction of immunity (Fig. 1). This suggests that an active germinative region could be associated with the production of the protective antigens. Moreover, the observations that protective immunity against H. diminuta is predominantly inhibitory to worm growth (see Befus 1975) and that destrobilation, a manifestation of worm rejection (Hopkins et al. 1972 a), not infrequently occurs at the neck (personal observation) support the contention that the protective antigens can be generated from this region.

Worms from six cysticercoids eliminated after 24 h (pre-strobilates) were shown to stimulate weak protection against challenge but 30 worms eliminated after the same period apparently stimulated a stronger protective response. The general trend was that worms from a primary infection induced progressively stronger immune responses with an increase in the duration and intensity of infection. These observations are in agreement with those of Befus (1975), who suggested that the duration of the immunizing infection and the amount of antigen presented could influence the development of the protective immune response against H. diminuta in mice. Studies with

H. microstoma have shown that the degree of the protective response against challenge was also dependent upon both the intensity and the duration of the immunizing infection (Howard 1976).

Additional evidence in favour of the hypothesis that the scolex is the source of H. diminuta protective antigens came from experiments in which active cellular replication in the germinative region and strobilization were completely suppressed by irradiation (Elowni in preparation). Induction of protective immunity to the tapeworm was found to be independent of the presence of strobilar antigens and independent of the presence of the actively dividing cells of the germinative region.

ACKNOWLEDGMENTS

I wish to thank Professor C. A. Hopkins and Dr. D. Wakelin for critically reviewing the manuscript. I am also very grateful to my colleagues in the Wellcome Laboratories for their valuable suggestions and stimulating criticisms.

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HYMENOLEPIS DIMINUTA: SOURCE OF THE PROTECTIVE ANTIGENS AS DETERMINED BY IRRADIATION AND CHEMICAL ELIMINATION OF IMMUNIZING WORMS

E. E. ELOWNI

Wellcome Laboratories for Experimental Parasitology,
University of Glasgow, Bearsden Road, Bearsden,
Glasgow, G61 1QH, Scotland

ABSTRACT

Prior infection with Hymenolepis diminuta, whose strobila-forming capacity was completely or partially suppressed by irradiation, conferred on mice a degree of protection against challenge which was indistinguishable from that stimulated by worms with fully developed strobilae. Irradiated day 2 or day 3 worms (prestrobilates) were as immunogenic as worms of the same age which were not irradiated. Thus neither the presence of the strobila nor the presence of the actively dividing cells of the neck region was necessary for the stimulation of immunity. An increase in the duration of primary infections was associated with a decrease in the rate of growth and survival of challenge worms. These results confirm earlier findings that the induction of functional immunity against H. diminuta in mice is independent of the presence of a strobila: is determined by the duration of an antigenic stimulus deriving from the scolex and/or neck regions.

INDEX DESCRIPTORS: Hymenolepis diminuta; Cestode; Protective antigens; Scolex; Germinative region; Mice, CFLP

INTRODUCTION

The tapeworm Hymenolepis diminuta is rejected from mice by a mechanism that has an immunological basis (Hopkins et al. 1972 a, b; Befus 1975). The origin and nature of the antigens responsible for the stimulation of this protective response (protective antigens) are unknown. Christie (1978) suggested that the strobila of the worm was the major source of protective antigens but he concluded that his experiments did not rule out a role for scolex antigens. Andreassen et al. (1978), however, were of the opinion that the protective antigens "were related to the scolex region and not to the total worm biomass or surface area". In a previous study (Elowni, 1980), the origin of H. diminuta protective antigens was investigated by employing chemical elimination of worms from oral infections and by artificial immunization with strobilate or excysted worms. From this work it appeared that the scolex and/or the neck 'germinative' region were the sources. The present study, using the techniques of irradiation and chemical elimination of immunizing worms, was undertaken to evaluate more critically the immunogenic potentials of the three anatomical regions of the tapeworm.

Irradiation was used to prevent the formation of a strobila and allow immunization with worms consisting only of the scolex and neck. In cyclophyllidean cestodes, strobilization results from budding from the neck region (Wardle & McLeod 1952). In H. diminuta, the neck was defined as a zone of active DNA synthesis and rapid cell replication containing cell types capable of acting as stem cells

(Bolla & Roberts 1971). High levels of irradiation have the effect of almost completely suppressing the formation of a strobila by this species without affecting the ability of the worms to survive (Christie & Moqbel 1980). Immunizing infections were carried out with either normal or irradiated worms and the anthelmintic 'Praziquantel' was used to terminate infections at the prestrobilate or fully strobilate stages (see Elowni 1980). This allows for the immunizing potential of the scolex to be studied in the presence or absence of a germinative region that is active in cell replication. The effect of the duration of antigenic stimulation from the scolex and neck regions can also be evaluated in the presence or absence of antigens from the strobila.

MATERIALS AND METHODS

Mice Tapeworm-free, male CFLP mice (Anglia Laboratory Animals) were used when 6 weeks old. They were maintained under conventional conditions and supplied with mouse breeding diet (Grain Harvesters Ltd.) and water ad libitum.

Parasite, Irradiation and Infection The strain of H. diminuta used has been described by Hopkins et al. (1972 a). Cysticercoids (32 days old) from Tribolium confusum were exposed to ^{60}Co irradiation as described by Christie et al. (1980) at an irradiation dose of 1.05 Krad min^{-1}. Total doses of 17.5 or 35 Krad were given. Six normal or irradiated cysticercoids were administered to each mouse by intragastric intubation while under light ether anaesthesia. Sham-infected controls received 0.15 ml of balanced salt solution.

Experimental Design Six groups of mice (Fig. 1) were infected with normal or irradiated (35 Krad) cysticercoids. The infections were terminated with Praziquantel (100 mg/kg body weight) on day 2 or day 3 of infection (the prestrobilate stage) or on day 16 (after extensive strobilization). An additional group was infected with six cysticercoids irradiated at 17.5 Krad and the worms were eliminated on day 16. The corresponding sham-infected controls received similar anthelmintic treatment. Mice were challenged with six normal cysticercoids 21 days after the initial cysticercoid or sham infections and were killed 8 days later.

Worm Recovery and Computation of Results The technique of worm recovery was described by Hopkins et al. (1972 a). Worms from each mouse were counted, dried at 95-100°C for 24 h and weighed (giving worm biomass/mouse). Worms of < 0.1 mg are very small worms consisting of scoleces and neck regions. It is established that, on day 8 of infection, worms of this size are worms that have grown slowly (see Hopkins et al. 1972 a; Befus & Featherston 1974) having been affected by the immune response. Such worms are not weighed and are shown in Fig. 1 as being 0 mg. Worms of ≥ 0.1 mg were segmented worms. These are worms that have grown and the effect of the immune response on their rate of growth was determined by the one-tailed Wilcoxon two-sample test (Colquhoun 1971).

The total recovery (the proportion of cysticercoids administered to a group of mice and recovered as worms regardless of worm weight) was compared by the χ^2 test, Yates' correction (Siegel 1956). The proportion of cysticercoids administered and recovered as worms ≥ 0.1 mg was also compared by this test. Proportions were transformed into percentages in Fig. 2 but actual recovery figures were used in the test. In both tests, differences were considered significant when $P \leq 0.05$.

RESULTS

Preliminary experiments showed that 76.7% of normal cysticercoids and 77.5% of cysticercoids irradiated at 35 Krad excysted *in vitro*. As a check for establishment and growth of normal and irradiated worms, two mice taken at random from each of three experimental groups were killed on day 8 of infection. All the 12 worms from normal cysticercoids were recovered and these were large strobilate worms of 11.1 mg total dry weight. Worms from cysticercoids irradiated at 35 Krad were merely scoleces and neck regions and resembled destrobilated worms in gross morphology. The total recovery of worms exposed to this dose of radiation was 58%. Worms irradiated at 17.5 Krad were also without strobilae and 50% of these worms were recovered.

Immunization with Normal Cysticercoids

Prior infection with normal worms for 16, 3 or 2 days induced a significant reduction in the growth of challenge worms as compared with the corresponding naive controls (Fig. 1). The total recovery of worms from mice previously infected for 16 or 3 days was significantly lower than that from the controls and the proportions of worms $\geq$ 0.1 mg were significantly smaller (Fig. 2). Although immunization for 2 days had no significant effect on total worm recovery, the proportion of weighable worms was significantly smaller than in the controls. An increase in the duration of primary antigenic stimulation was associated with a decrease in the rate of growth and

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Figure 1.

The growth of H. diminuta from a six-cysticercoid challenge infection in mice previously infected with normal or irradiated (Kr) cysticercoids and in the sham controls (O).

Cyst: Cysticercoids

Kr: Krad

*: Wilcoxon test

N.S.: Not Significant

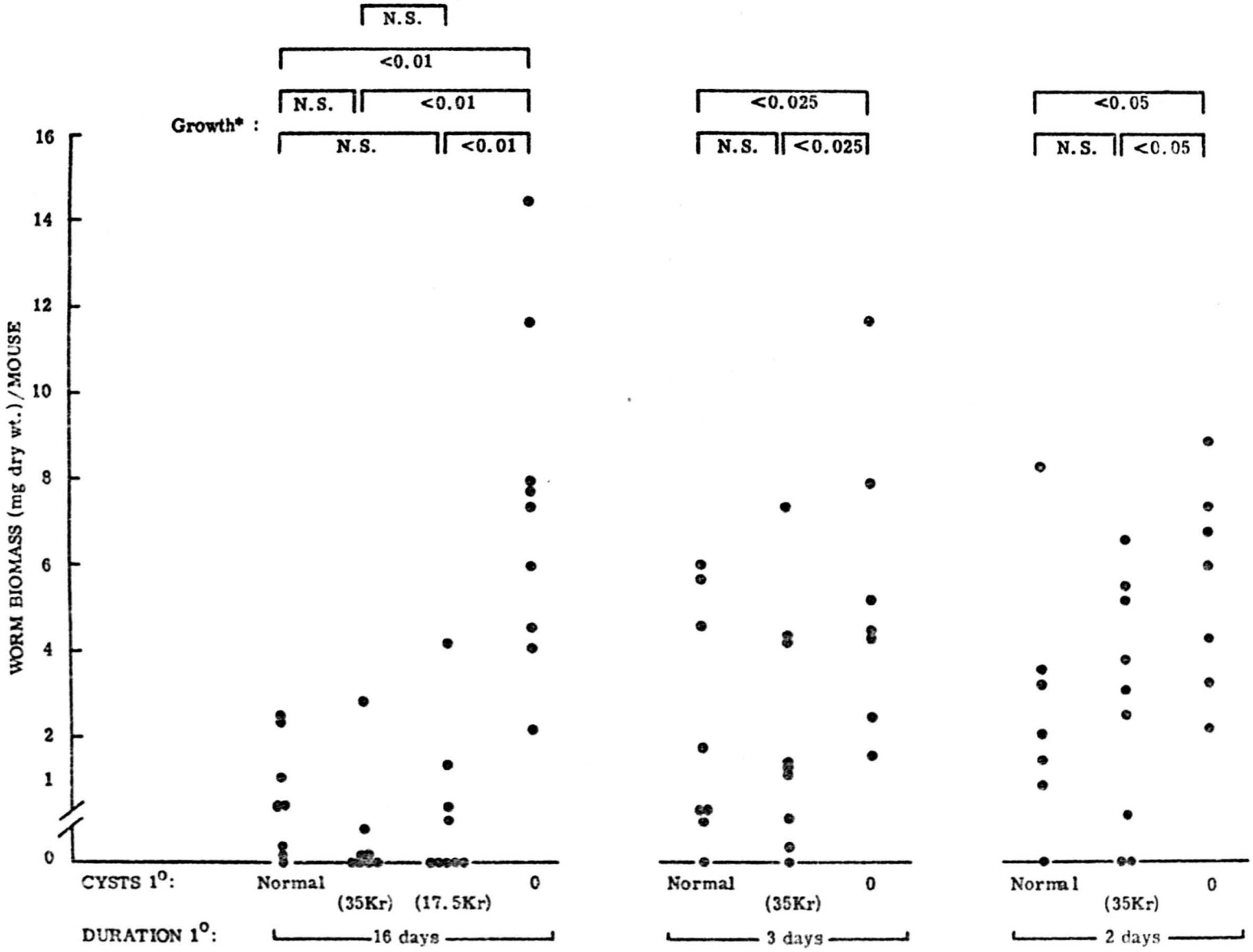
N.S.
<0.01
N.S.
<0.01
Growth* :
N.S.
<0.01
<0.025
N.S.
<0.025
<0.05
N.S.
<0.05
WORM BIOMASS (mg dry wt.)/MOUSE
16
14
12
10
8
6
4
2
1
0
CYSTS 1°:
Normal
(35Kr)
(17.5Kr)
0
Normal
(35Kr)
0
Normal
(35Kr)
0
DURATION 1°:
16 days
3 days
2 days

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Figure 2.

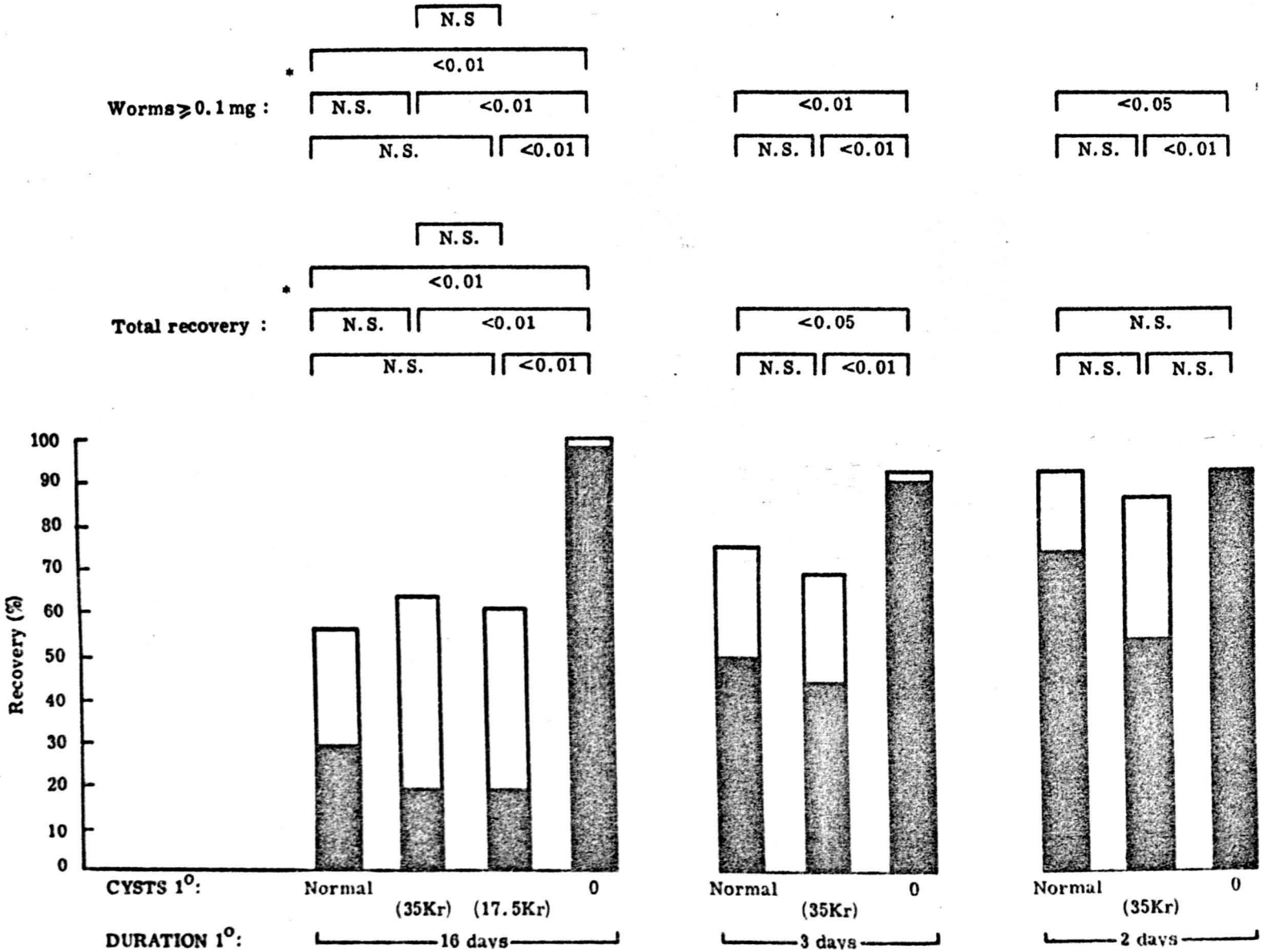
The survival of H. diminuta from a six-cysticercoid challenge infection in mice previously infected with normal or irradiated (Kr) cysticercoids and in the sham controls.

Cyst: Cysticercoids

Kr: Krad

*: The χ^2 test

N.S.: Not significant


N.S
<0.01
Worms ≥ 0.1 mg :
N.S.
<0.01
N.S.
<0.01
<0.01
N.S.
<0.01
<0.05
N.S.
<0.01
N.S.
<0.01
Total recovery :
N.S.
<0.01
N.S.
<0.01
<0.05
N.S.
<0.01
N.S.
N.S.
N.S.
Recovery (%)
100
90
80
70
60
50
40
30
20
10
0
CYSTS 1°:
Normal
(35Kr)
(17.5Kr)
0
DURATION 1°:
16 days
Normal
(35Kr)
0
3 days
Normal
(35Kr)
0
2 days

survival of challenge worms.

Immunization with Irradiated Worms

On all the days studied, prior infection with worms irradiated at 35 or 17.5 Krad conferred on mice degrees of protection (in terms of suppression of worm growth, reduction in total worm recovery and a decrease in the proportion of worms $\geq$ 0.1 mg) which were similar to those stimulated by normal worms eliminated over similar periods of time (Figs. 1 and 2).

DISCUSSION

In the preliminary experiments, examination of the growth patterns of worms irradiated at 35 Krad confirmed the findings of Christie et al. (1980) that this level of radiation completely suppresses the growth of a H. diminuta strobila. This dose of radiation had no effect on the ability of cysticercoids to excyst in vitro. Christie (1979) reported that worms from cysticercoids exposed to radiation 'apparently' function normally regarding such indices as feeding and detecting location signals and he attributed the low rates of recoveries to technical difficulties in finding such small worms in the intestine. Worms irradiated at 17.5 Krad also failed to grow strobilae. Since only two mice were examined, this dose of radiation was assumed, on the basis of parameters established by Christie et al. (1980) not to have caused complete suppression of strobilar growth of worms in all the mice.

Irradiated worms, completely lacking strobilae throughout the inductive phase of the immune response, were shown to be capable of stimulating in mice a degree of protection that was almost identical to that stimulated by normal worms with extensive strobilae. A similar effect was obtained with worms irradiated at 17.5 Krad, a dose partially restrictive to strobilar growth. These observations confirm earlier findings (Elowni 1980) that the induction of functional immunity against H. diminuta in mice is independent of the presence of a strobila and lend support to the hypothesis put forward by

Andreassen et al. (1978) that protective immunity against this tapeworm is not related to the total worm biomass or surface area.

Worms without strobilae were shown to stimulate stronger protective responses in 16 days than in 2 or 3 days. This agrees with previous findings (Elowni 1980), using drug-abbreviated infections, that the duration of antigenic stimulation from the scolex and/or the germinative region in the immunizing infection determines the magnitude of the protective secondary response.

When infections were terminated in the prestrobilate stage, irradiation was found to have no effect on the immunogenicity of the prestrobilate worm. The germinative region of H. diminuta has the properties of active DNA synthesis and rapid cell replication (Bolla et al. 1971) and is a likely target for immunological damage (Befus & Threadgold 1975). It is significant that destrobilation, one of the manifestations of worm rejection (Befus 1975; Hopkins & Zajac 1976), not infrequently occurs at this region (personal observation). For these reasons, it has been suggested that the neck region may well be a source of potential protective antigens (Elowni 1980). Abrogation of its normal function of cell replication with heavy radiation had no effect on the capacity of the worm to evoke a protective response. This suggests that active cell replication in this region is not a prerequisite for the induction of functional immunity. Ionizing radiations have as their most salient effect on mammalian cells a profoundly damaging action on DNA (Nossal 1971) but high doses were required to impair the functions of RNA and protein synthesis. The present study provides no evidence as to the extent to which doses of 35 or

17.5 Krad affect cellular functions other than division in the germinative region. Therefore the possibility of protective antigens being generated from this region cannot be ruled out.

The rejection of H. diminuta from mice is known to be T-cell dependent (Bland 1976) but the precise thymus-dependent components of the mechanism of rejection are ill-defined. Since the protective antigens of this tapeworm are localized within a defined region of the parasite, the utilization of these antigens, e.g. from excysted worms, in an in vitro test for cell-mediated immunity could prove valuable in the elucidation of the mechanisms of protective immunity against the tapeworm.

ACKNOWLEDGMENTS

The manuscript was critically read by Dr. D. Wakelin and Dr. R. Moqbel, to both of whom I am most grateful. I would also like to thank Professor C. A. Hopkins for his valuable suggestions.

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CHAPTER TWO

THE ROLE OF THE BURSA OF FABRICIUS IN THE REJECTION OF *RAILLIETINA CESTICILLUS* BY CHICKENS

INTRODUCTION

INTRODUCTION

(1) RAILLIETINA CESTICILLUS

(a) Classification

Family Davaineidae Fuhrmann, 1907

Subfamily Davaineinae Braun, 1900

Genus Raillietina Fuhrmann, 1920

Raillietina (Skrjabinia) cesticillus (Molin, 1858)

(b) Morphology

R. cesticillus is a large tapeworm with a cylindrical and sometimes spheroid scolex. A broad rostellum is readily recognizable under low magnification. Rostellar hooks are arranged in double rows. The characteristic rostellum and the weak unarmed suckers are diagnostic. The neck is very short. Mature segments are three times as broad as long. Genital pores are irregularly alternating, one in each segment. Testes are numerous and the majority are in the aporous side. Uterine capsules contain only one egg per capsule. The funnel-shaped filaments between the inner and the middle egg membranes are also diagnostic features.

(c) Host Range

The tapeworm infects domestic fowls, turkeys, pheasants and guinea fowls.

(d) Life Cycle

Over 100 species of beetles, belonging to 10 families, may act as intermediate hosts for this tapeworm (Reid, 1962). It takes an oncosphere 2-4 weeks to develop to an infective cysticercoid (Wehr, 1965) depending upon the temperature (Reid, loc. cit.). In T. confusum

for example, cysticercoids develop to the mature stage in 14 days when the beetles are reared at 30°C. Elowni (1977) described the stages of development of R. cesticillus cysticercoids in experimentally infected Tribolium castaneum kept at room temperature (26-30°C/ 33-37°C) and the fully developed cysticercoids were identified on day 15 of infection.

The minimum time required following ingestion of cysticercoids until the detection of gravid proglottids in the droppings of chickens was found to be 13 days (Reid, Ackert & Case, 1938). The prepatent period in male and female White Leghorn chickens infected when 10 weeks of age ranged between 13.5 and 21.5 days (Elowni, loc. cit.).

(e) Acquired Resistance

Gray (1972 a) described the course of infection of the worm in chickens with elaboration on worm distribution in the intestine, worm growth and the dynamics of infection. In birds infected at 3 days of age, worms lose their strobilae progressively. By day 70 of infection all the worms become destrobilated and they are subsequently expelled. The destrobilation process proceeds more rapidly in the female than in the male host. Because of this sex difference, Gray suggested that host physiology has at least some influence on the process. He also observed that in primary infections, the worms grew rapidly until patency (day 14) then followed a slower increase until complete destrobilation at day 70 of infection.

In a carefully designed experiment, Gray (1972 b) described the effect of host age on the course of infection of R. cesticillus in chickens infected at ages ranging from 3 to 224 days. He found that the number

of established worms varied little between the different groups with the exception of a group infected at 224 days where the number of scoleces recovered from male birds decreased and that from the females increased. He concluded that male hosts became resistant to the tapeworm with increasing age and that age resistance developed more rapidly in the female than in the male hosts. This resistance was manifested by an increased rate of worm destrobilation, loss of scoleces and a decreased rate of worm growth.

Worms in secondary infections established normally in infected birds but they failed to grow mature strobilae indicating the development of acquired resistance (Gray, 1973). According to this author, development of this resistance was inhibited by injecting the immunosuppressant and anti-inflammatory drug, dexamethasone which prevented local inflammation around the attached scoleces characteristic of secondary infections of the tapeworm. Mast cell hyperactivity and eosinophil infiltration in intestinal tissues and specific antibodies in the sera were also demonstrated in the infected birds. The above author also showed that destrobilated worms from refractory birds regrew their strobilae on subsequent surgical transferrence to intestines of naive recipients. From these observations, he (Gray) concluded that R. cesticillus evokes a protective immune response in chickens and that the immune response so evoked 'may be partly responsible for age resistance' to the cestode. On the other hand, both Luttermoser (1938) and Sinha and Srivastava (1958) failed to demonstrate resistance to superinfections of R. cesticillus in chickens and Meinkoth (1947) was unable to detect circulating antibodies against the cestode with ring precipitin tests. Gray (1973) suggested that this difference of opinion could be related

to the fact that both Luttermoser and Sinha et al. used the number of established worms as their criterion of resistance which he considered as a poor, short-term measure.

Gray (1976) extended his studies on the R. cesticillus/fowl system and investigated the cellular responses to the tapeworm in primary and secondary infections. He found that infections of this tapeworm caused in the small intestine mononuclear lymphocyte infiltration in the tissues around the scoleces, particularly in secondary infections. Mast cell and pyroninophilic cell numbers increased in the intestinal mucosa of birds with long standing primary infections and in birds with secondary infections. Very little eosinophil infiltration, however, was detected in these birds and the globule leucocyte response was very low. Moreover, he found that infections of the tapeworm had no effect on the numbers of theliolymphocytes and goblet cells. Although the administration of a challenge infection did not result in an increased level of serum antibodies, more of the cells containing globulin 'seemed' to be present in the intestinal mucosa of infected birds (Gray, 1976).

(2) THE LYMPHOID SYSTEM OF THE FOWL

The domestic fowl possesses a thymus, a spleen and an organ unique to birds, the bursa of Fabricius. It has no lymph nodes but small collections of lymphoid tissue, the mural lymphoid nodules, are present in the walls of lymphatic vessels. Lymphoid tissue is also present in the lamina propria and submucosa of the alimentary canal consisting of diffuse masses with germinal centres and a prominent lymphoid structure, the caecal tonsils.

The bursa is an ovoid, sac-like structure located in the extreme

caudodorsal aspect of the abdomen between the cloaca and the coccygeal vertebrae. It is connected by a short narrow stalk to the dorsal cranial part of the proctodeum. Its interior is composed of folds known as the 'plicae' which extend towards the centre of the cavity of the organ. Plicae have an epithelial covering and their parenchyma is composed almost entirely of lymphoid follicles. The size of the bursa varies with the strain and sex of chicken and with the rearing method. The timing of lymphocytopoiesis in the embryonic bursa has been reviewed by Warner et al. (1964). By the fourteenth or fifteenth day of incubation, rapid lymphoblastic transformation occurs in the bursa. The lymphoblast cells proliferate and give rise to developmental series of lymphocytes (Hodges, 1974). At the time of hatching the bursa, and also the thymus, are fully lymphoid. Indeed, they are the only lymphoid tissues at this stage (Warner, 1967). This early lymphoid development is to be carefully considered; the thymus and bursa could, in fact, commence their normal function as central lymphoid organs (see below) even before the time of hatch.

In general, it is demonstrated that embryonic, recently hatched and very young birds are relatively poor synthesizers of antibody (reviewed, McGhee, 1970). Antibody (precipitins)-producing ability develops gradually as the chickens age and between four and five weeks of age there appears to be a rather sudden maturation of antibody-producing ability (Wolfe & Dilks, 1948). Haemagglutinin antibody titres increase most rapidly between the ages of three to five weeks with a levelling off of titres after five weeks (Seto et al., 1968; cited by Gray, 1972 b).

Comprehensive reviews of the literature on the structure of the lymphoid system of the fowl and the ontogeny of immunity in this animal were published by Warner et al. (1964), Cooper, Gabrielsen & Good (1967), Payne (1971) and Loor (1977).

(3) FUNCTIONS OF THE BURSA AND THYMUS

The first indication that the bursa of Fabricius is essential in the development of avian humoral immunogenesis came from a chance discovery by Glick et al. (1956) in which they demonstrated that chickens from which the bursa had been removed surgically became very susceptible to infection with Salmonella typhimurium and that these birds failed to produce detectable antibody. A few years later, it was demonstrated that the removal of the thymus in neonate animals prevented the development of normal immunological functions (reviewed by Cooper et al., 1967). Since these original reports, extensive investigations on the functions of the bursa and thymus were carried out and it has been indicated that both organs have in common the potential for effecting specific adaptive immune responses that are essential to body defence (Cooper, Peterson, South & Good, 1966). The selective suppression of different types of immune responses by bursectomy and thymectomy led to the formulation of a concept of dissociation of immunological responsiveness in birds (Warner et al., 1962 a; Szenberg et al., 1962; Warner et al., 1964; Warner, 1967). In this concept it was proposed that the bursa controls the development of cells of the plasmacytic series responsible for immune reactions mediated by antibody, and the thymus is primarily concerned with the development and maintenance of an immunologically competent population of

cells responsible for reactions denoted as cellular immunities. Although the bursa- and thymus-derived cells have different functions, they are capable of cooperation (Cooper et al., 1966) and, by interacting with other types of cells, they execute a complex series of reactions characterizing the immune response in chickens.

(4) BURSECTOMY

To completely obliterate the immunoglobulin synthesizing system in chickens, it is necessary to ablate both the bursa and the bursa-derived lymphoid cells that migrate to the peripheral tissues before or after hatching. In this respect, several techniques have been described. Although some of these techniques are more effective in the ablation of bursal function than others, this gain in efficiency is frequently obtained at a sacrifice of specificity by inducing secondary effects on other organs including the thymus.

(a) Hormonal Bursectomy

Several investigations described the suppression of bursal development in chick embryos by injection into the egg of gonadal hormones (Aspinall, Meyer & Rao, 1961) or by dipping of eggs into solutions of these hormones (Glick & Sadler, 1961). If the hormone is injected on the 5th or 6th day of incubation, the entire bursal structure fails to develop (Meyer, Rao & Aspinall, 1959). If injection is delayed until the 11th or 13th day, however, lymphocytopoiesis specifically is inhibited in the bursa (Warner & Burnet, 1961), which remains as an epithelial sac (Rao, Aspinall & Meyer, 1962). The most striking change in the lymphoid tissue of birds reared from eggs injected with hormones is a reduction of cells of the plasmacytic series (Warner et al., 1964).

In many cases, the reduction is nearly absolute.

Most hormonally bursectomized chickens fail to give primary antibody responses but progressively greater proportions of treated birds produce specific antibodies with passage of time after primary antigen stimulation or with increased antigen exposure (Warner, Uhr, Thorbecke & Ovary, 1969; Bryant, 1973). The amounts of immunoglobulins in hormonally bursectomized birds also vary from normal to severe depletion, or absence (Pierce, Chubb & Long, 1966; Warner et al., 1969). The effect of hormonal bursectomy on antibody or immunoglobulin formation depends on the completeness of ablation of the bursa; the persistence of only a small amount of residual bursal tissue is known to permit normal antibody production (Warner et al., 1962 a). The effect of hormonal bursectomy is also related to the relative potency of the androgen injected (androgens with a completely reduced steroid nucleus are the most active), androgen form, the dose injected and the route and time of administration (Aspinall et al., 1961; Rao et al., 1962).

In addition to its effect on bursal development, hormonal treatment of chick embryos can also alter the development of the thymus and may result in complete atrophy of the cortex of this organ (Szenberg et al., 1962). Chickens hatching from eggs treated with hormones around the 5th day of incubation show poor viability (Mueller, Wolfe, Meyer & Aspinall, 1962) and anatomical malformations in the rectal complex (Meyer et al., 1959). Injection of hormones around the 12th day of incubation has been reported to cause high mortality but the chickens in this case may look quite healthy during most of their short lives (Warner et al., 1962 a).

(b) Surgical Bursectomy

Chickens surgically bursectomized at hatching show absence or marked reduction of their primary antibody responses (Mueller et al., 1962; Cooper et al., 1966; Arnason & Jankovic, 1967). The levels of immunoglobulins in the sera of these birds may be normal or show a deficiency of IgG and IgA frequently with an increase of IgM (Ortega & Der, 1964; Cooper et al., 1966; Arnason et al., 1967; Perey & Bienenstock, 1973). In the secondary response, bursectomized birds may show normal or nearly normal antibody levels (Jankovic & Isakovic, 1966). Although the titres may be normal in the secondary response, the antibodies formed are of the 19S type in contrast to the 7S antibodies found in secondary responses in intact birds (Claflin, Smithies & Meyer, 1966; Arnason et al., 1967). After surgical bursectomy, as after hormonal bursectomy, higher antibody titres will be attained if the period between bursectomy and antigen challenge is increased (Warner et al., 1964).

The residual humoral immune responses in birds from which the bursa has been resected on the day of hatching have been attributed to the presence of bursal remnants or to bursa-derived lymphoid cells that migrate to the peripheral lymphoid tissue prior to removal of the bursa (Cooper et al., 1966). Because chemical suppression of bursal development with androgens and surgical bursectomy did not lead to total absence of this cell system and circulating immunoglobulins (see above), it has been argued that sites other than the bursa may foster development of the immunoglobulin-producing system. As Bryant (1974) wrote '... suggesting the existence of an alternative pathway of immunogenesis'.

Cooper, Cain, Van Alten & Good (1969) and Warner et al. (1969), however were of the opinion that the bursa is the only site of control of the development of the antibody-forming system.

To destroy the peripheral bursa-dependent tissue responsible for the development of the residual humoral response in birds bursectomized on the day of hatching, Cooper et al. (1966) advocated the use of high sublethal total-body X-irradiation. They found that chickens which had been bursectomized and irradiated failed to develop germinal centres, plasmablasts and plasma cells. The birds remained agammaglobulinaemic and incapable of producing circulating antibodies even after vigorous or repeated antigenic stimulation. Moreover, birds treated in this way exhibited full recovery of the thymus after the irradiation and developed cellular immunities normally. From these studies, Cooper et al. (1966) suggested that the separate roles of the major central lymphoid organs in chickens, the bursa and thymus, can be delineated in birds that were surgically bursectomized at hatching and irradiated. They proposed that this system could be regarded as a model of agammaglobulinaemia. For this reason, bursectomy at hatching and irradiation were used in the present study to investigate the mechanisms of the immunologically-based rejection of R. cesticillus in chickens.

Another approach to eliminating the bursa-dependent system is to extirpate the bursa surgically at specific times during late embryonic life, before the peripheral tissues are populated with bursa-derived lymphocytes. The general finding is that the earlier surgical bursectomy is performed, the greater will be the suppression of potential antibody formation (Mueller et al., 1962; Cooper et al., 1967;

Blythman & White, 1977). IgG levels were found to be normal or elevated following surgical bursectomy at hatching but greatly lowered levels were obtained following in ovo surgical bursectomy on or before the 20th day of incubation (Cooper et al., 1969). Consistent suppression of IgM levels, however, was achieved only in embryos bursectomized as early as the 17th day. Using this technique, Blythman et al. (1977) found that bursectomy on days 18, 19 or 20 of incubation produced very low serum levels of haemagglutinins, a reduction of SRBC rosette-forming cells together with disappearance of germinal centres in the spleen. In ovo surgical bursectomy at 70 h of incubation was reported by Fitzsimmons, Garrod & Garnell (1973) to lead to the formation of small amounts of antibody to BSA and SRBC in few of the birds. These authors also reported that this treatment resulted in an incomplete development of the thymic cortex in the bursectomized birds of which only 10-20% survived up to hatching. Surgical removal of the bursal primordium of chicken embryos at 52-64 h of incubation was found to cause a moderate depletion of bursa-derived lymphocytes, a decrease of IgM-containing cells and impairment of the primary antibody response (Jankovic et al., 1975). Birds treated in this way, however, were found to respond to hyperimmunization. These authors, therefore, proposed that there are non-bursal sites of differentiation of the lymphocytes destined to make immunoglobulins [in a previous investigation, Jankovic and his co-workers (1966) put forward this hypothesis which is in agreement with the opinions of Bryant et al. (1973) and Bryant (1974) but at variance with those of Warner et al. (1967) and Cooper et al. (1969) (see above)] .

(c) -- Other Methods of Bursectomy

Treatment of chicken embryos with antibody specific for the IgM heavy chain (anti-u) followed by surgical bursectomy at hatching was reported to result in long-term suppression of the ability of birds to synthesize IgM, IgG and IgA (Leslie & Martin, 1973 a). The blood leucocytes of these birds, however, may have an enhanced capacity to induce graft-versus-host reaction. The embryonic administration of $F(ab')_2$ fragments of goat anti-u in conjunction with surgical bursectomy at hatch was also found to suppress the formation of immunoglobulins in serum (Leslie & Martin, 1973 b). Chicks receiving anti-u or $F(ab')_2$ anti-u prior to hatch and which were bursectomized at hatch also had a total lack of secretory IgA in the oral secretions (Leslie & Martin, 1974).

Cyclophosphamide is an alkylating agent known to be cytotoxic to all dividing cells (Bruce, Meeker & Valeriote, 1966). Chickens given this agent after hatching failed to produce antibodies for at least 6 weeks (Rouse, 1974). Birds surviving for more than 6 weeks, however, recovered their immunocompetence. Moreover, in one-week-old treated birds allogenic skin grafts were not rejected and in many of the birds chronic degenerative changes were observed in epithelial cells in many of the organs including the thymus. Lerman & Weidanz (1970), on the other hand, claimed that cyclophosphamide was capable of selectively suppressing the ontogeny of the humoral immune response in chickens with little or no effect on cell-mediated responses. Cyclophosphamide treatment combined with surgical bursectomy was reported by Glick (1971) to produce agammaglobulinaemia. Treatment

with this drug in conjunction with hormonal bursectomy was demonstrated to effectively abolish the secretory immunoglobulins normally present in tears as well as serum immunoglobulins (Lerman et al., 1970).

(d) Cellular Immunity and Bursectomy

Neither steroidal nor surgical bursectomy had any effect on the skin homograft reaction (Warner et al., 1962 b; Aspinall, Meyer, Graetzer & Wolfe, 1963) even when cytotoxic antibodies to donor antigens were absent (Perey et al., 1967). Surgical and hormonal bursectomy also have no effect on the delayed hypersensitive reactions to tuberculin, chicken spinal cord lipid (Jankovic et al., 1963; Warner et al., 1967), diphetheria toxoid (Cooper et al., 1966) or on experimental allergic encephalomyelitis (Jankovic et al., 1963). Chickens hormonally bursectomized or surgically bursectomized and irradiated also show a normal graft-versus-host reaction (Warner et al., 1962 a). Cooper et al. (1966) found no evidence of significant impairment of reticuloendothelial function in bursectomized-irradiated chickens as judged by the clearance of colloidal gold or isotope-tagged haemocyanin. Peripheral blood small lymphocytes were reported by Warner et al. (1967) to be present in normal numbers in both surgically and hormonally bursectomized chickens.

GENERAL MATERIALS & METHODS

GENERAL MATERIALS & METHODS

1. Animals

Chickens were B1 Marshall's (D. B. Marshall Ltd., Whitburn), Ross White and Ross 1 (Ross Poultry Great Britain Ltd., Inverurie) or Hubbard Golden Comet (J. R. Todd Ltd., Kilmarnock).

2. Maintenance

Birds were obtained at 1-day-old and reared for about 2 weeks in large polypropylene boxes heated with 60W bulbs. Birds to be kept for more than 2 weeks were transferred to wire floor cages in a conventional animal house. They were fed ad libitum a commercial diet (515 Intensive Growers Crumblets ACS, BOCM SILCOCK, Basingstoke). Birds which were bursectomized or irradiated were given oxytetracycline hydrochloride ('Terramycin', Pfizer Ltd.) in the drinking water at a concentration of 165 mg/l for 7 consecutive days. Controls were also given antibiotic in the drinking water.

3. Bursectomy

(a) Anaesthesia

During the present study four anaesthetics (ether, halothane, thiopentone sodium and sodium pentobarbitone) were tested for their efficiency in inducing deep surgical anaesthesia in 1-day-old chicks. Sodium pentobarbitone ('Sagatal', May & Baker Ltd.) was found to be the most reliable of these agents. The response of birds of the different strains to the barbiturate varied, but a dose of 0.3 ml of a 10% solution of this anaesthetic (made up in HBSS containing 19% ethanol) injected i.p. was effective in inducing and maintaining deep

anaesthesia for at least 2 h in day-old B1 chicks (av. wt 45 g). To avoid puncture of the abdominal air sacs during the administration of the anaesthetic, the needle (25G, $^{5}/_{8}$ in) was inserted in the midline, midway between the cloaca and the sternum, and directed anteriorly almost parallel to the abdominal wall.

(b) Surgery

Bursectomy was performed on newly hatched male chicks (B1 Marshall's) obtained on the morning of the 21st day of incubation. The anaesthetized birds were placed ventral side down with the tail towards the operator. The tail was held upright by an artery forceps hanging from metal clamps fixed to a stand. Feathers between the tail and the cloacal orifice were plucked and the area was swabbed with 70% ethanol. A superficial incision, about 5 mm long, was made on the skin just above the upper lip of the cloacal vent and where the lower edge of the pygostyle vertebra can be felt. The bursa was gently freed from its fascial attachments by blunt dissection through the peritoneum and loose connective tissue. The organ was grasped at the base with dissecting forceps and pulled out gently. The delicate bursal stalk was exposed and excised next to its cloacal attachment. This stage of the operation was performed under a magnifying lens to avoid severing the ureters which lie close to the bursal stalk. Bleeding was usually minimal. In cases where the bursal capsule was damaged, the bursectomy was considered unsatisfactory, and the birds were excluded from the experiment. The abdominal cavity was sprayed with an aerosol mixture of antibiotics ('Rikospray', Riker Laboratories Ltd.) and the incision was closed with two

interrupted stitches using 6/0 sutures ('Mersutures', Ethicon Ltd.). In sham operations, the bursa was exposed but not excised thereby paralleling the surgical stress and potential bacterial contamination of the ablative procedure.

4. Irradiation

Chicks were exposed to total-body X radiation 1 - 2 h after bursectomy and while the birds were still under anaesthesia. They were given 500 rads as a maximum dose (measured approximately 1.3 cm below the upper body surface) from a Dynaray-4, 4MeV linear accelerator (Radiation Dynamics Ltd.) at a dose rate of 260 rads/min in a field of 30 x 30 cm.

5. Parasite

The strain of R. cesticillus used throughout these experiments was obtained from Dr. J. S. Gray (University College, Dublin). It was originally acquired from the University of Georgia, U.S.A.

6. Infection

Beetles (T. confusum), starved for 5 - 6 days, were fed gravid R. cesticillus proglottids obtained from patent worms (15 to 21-day-old) recovered from experimentally-infected chickens. The beetles were exposed to infection on two or three consecutive days. They were then transferred to glass jars containing whole wheat flour and maintained at 25^{o}C in a humid incubator. Mature cysticercoids (over 15-days-old) were recovered by mechanically disintegrating infected beetles using an MSE homogenizer operated at approximately 7000 r.p.m. Chickens were infected by the oral administration of cysticercoids using a dropper as described by Elowni (1977) or by feeding cysticercoids

contained in gelatin capsules (size 4, Farillon Ltd., Romford) reduced to half-size for young birds. Comparisons of the effects of varying the method of infection on the level of parasite establishment in chickens was a subject of an independent investigation (see below).

7. Anthelmintic Treatment

In a pilot experiment, three anthelmintics (oxyclozanide, bunamidine hydrochloride and Praziquantel)were tested for their cestocidal activity against strobilate R. cesticillus in chickens. The last compound was found to be 100% effective when administered as a single oral dose of 100 mg/kg body weight. It was given as an aqueous suspension with the addition of 'Cremophor EL' (4%, BASF, Ludwigshafen).

EXPERIMENTAL

A. INFECTION

12. Level of Parasite Establishment

Ten 2-day-old chicks (Ross White) were each given eight 30-day-old cysticercoids administered by a dropper.

Table 15 shows the level of establishment of the tapeworm 19 days after the administration of cysticercoids. In the immunologically-immature chicken no expulsion of R. cesticillus would be expected to have taken place by this time (see introduction) and thus worm recovery can be considered as a direct measurement of the ability of the worm to establish in the gut. Of the 40 cysticercoids administered to male or female chickens, 37.5% and 42.5% developed to mature worms respectively. The difference in worm establishment between the sexes is not statistically significant.

Table 15. Establishment of R. cesticillus in male and female Ross White chicks

Group	No of chicks	Sex	Worms recovered per bird		Total recovery per group
A	5	♀	2, 3, 3, 4, 5	*	42.5%
B	5	♂	2, 2, 3, 4, 4		37.5%

* Difference is not significant (Wilcoxon, 2-tailed)

13. The Effect of Age on the Infectivity of *R. cesticillus* Cysticercoids

R. cesticillus cysticercoids of different ages were administered by a dropper to 1-day-old chicks of the Ross 1 strain. Table 16 shows the level of establishment of the tapeworm when the birds were autopsied on day 21 of infection. Fewer worms were recovered from infections given with cysticercoids older than 4 months, suggesting that the infectivity of cysticercoids decreased with the increase of age. Cysticercoids aged 4 months were somewhat less infective than the 30-day-old cysticercoids used in experiment 12 (total recovery 28% *vs* 37.5 - 42.5%). By about 7 months of age, the cysticercoids were almost uninfective.

Table 16. The effect of age on the infectivity of R. cesticillus cysticercoids.

Group	No. of birds	No. of cysticercoids given/bird	Age of cysticercoids (months)	Worms recovered per bird	Total recovery %
A	5	15	4	1,4,4,6,6	28
B	6	15	$5\frac{1}{2}$	0,0,0,0,1,1	2.2
C	4	10	$7\frac{1}{2}$	0,0,0,0	0

14. Effect of Varying the Method of Infection on the Level of Parasite Establishment

Three-day-old male B1 chicks were randomly divided into four groups of six birds and were given five 19-day-old cysticercoids each. The cysticercoids were administered either by the dropper technique to birds from group A and group B (Table 17) or contained in gelatin capsules (groups C & D). Food and water were withheld for 5 h from chickens of groups B & D before the cysticercoids were administered. All birds were killed on day 21 after infection.

Table 17 shows worm recoveries from the birds infected by the different procedures. Administration of cysticercoids in gelatin capsules was more effective in establishing higher worm burdens than was the administration of cysticercoids by dropper. When cysticercoids were given by a dropper, prior starvation of birds apparently had no effect on the level of parasite establishment. On the other hand, when cysticercoids were administered in gelatin capsules, more worms established in birds that were previously starved than in those which were not starved.

Data obtained by Elowni (1977) from 23 male and 23 female White Leghorn chickens given 3, 4, 8, 10, 15, 16 or 25 cysticercoids per bird administered by a dropper showed that 24% of these cysticercoids established. This figure closely resembles the 23% level obtained from birds of group A (Table 17) infected by the same procedure but lower than the 37.5% level obtained from Ross White male birds (Table 15). This discrepancy could be attributed to differences in susceptibility

Table 17. Effect of varying the method of infection on the level of establishment of *R. cesticillus* in chickens.

Group	Method of infection	Starved (+) Not starved (-)	Worms recovered per bird	Total recovery (%)
A	Dropper	-	0,1,1,1,2,2	23
B	Dropper	+	0,1,1,2,2,2	27
C	Gelatin capsule	-	1,2,2,3,4,4	53
D	Gelatin capsule	+	1,4,4,4,4,5	73

of birds of different strains to the parasite. Conclusions, however, can only be drawn from a controlled experiment in which birds of different strains but of similar age groups were given equal numbers of cysticercoids.

B. THE IMMUNE RESPONSE OF BURSA-DEFICIENT AND INTACT CHICKENS TO R. CESTICILLUS

15. Secondary Infection

15.1 Materials & Methods

Male chicks (B1 Marshall's) were surgically bursectomized and X-irradiated on the same day of hatching (BX). Another group was sham bursectomized and exposed to radiation (SX). Birds from a third group were not treated and were used as infection controls (C).

BX and SX chickens, which were previously starved for 5 h, were infected when 5-weeks-old (age of serological maturity; Introduction) with 100 32-day-old R. cesticillus cysticercoids given as two successive doses of 50 cysticercoids contained in gelatin capsules (Table 18). Infections were terminated with Praziquantel. All chickens from the three groups were challenged with 50 36-day-old cysticercoids given on the same day as two successive doses of 25 cysticercoids in gelatin capsules. Birds were killed on day 14 after giving the challenge infection. By this time, R. cesticillus normally is strobilate and many of the worms have reached patency (Elowni, 1977) . The intestines (gizzard to Meckel's diverticulum) were taken out, slit open longitudinally and pinned to wax in a trough. Intestinal contents were removed by flushing with water. Worm recoveries were interpreted in terms of the number of worms that were fully strobilated and retained their strobilae. This is because at this stage, and in terms of gross morphology, it is almost impossible to distinguish between a destrobilated worm and a worm that failed to grow a strobila initially. Moreover,

Table 18. Infection of bursectomized and intact chickens with *R. cesticillus* cysticercoids

Group	Day 0	15	19
C			50c
BX	100c	P	50c
SX	100c	P	50c

C, Infection controls

BX, Surgically-bursectomized and X-irradiated

SX, Sham-bursectomized and X-irradiated

c, cysticercoid

P, Praziquantel

the error inherent in counting very large worms is negligible compared to that encountered when attempting to recover and count very small worms, often < 1mm in length, embedded in the intestinal mucosa. Worms from each bird were recovered following scraping of the mucosa, dried at 95-100°C for 24 h and weighed en masse to give 'worm biomass per chicken'. Worms which were very small were not weighed as their weight would have been < 0.1 mg.

In bursectomized chickens, bursal remnants were sought for, both by inspection of the cloacal area and by histologic examination of serial sections of the cloaca. The ability of bursectomized birds to produce specific antibodies against R. cesticillus antigens was tested by the indirect immunofluorescent method. R. cesticillus cysticercoids were excysted in vitro by incubation for 12 min in HBSS containing pepsin and HCl (pH 1.7) at 37°C followed by incubation for 5-7 min in HBSS to which trypsin and sodium tauroglycocholate were added (pH 7.2). The excysted worms were used as the test antigen. These worms were washed 3-4 times in HBSS and incubated at 37°C with sera from BX, SX and C chickens or with PBS (pH 7.2) for 1 h. They were then washed three times in PBS and incubated at the same temperature with rabbit anti-chicken globulin conjugated with fluorescein isothiocyanate ('RACh/FITC', Nordic Immunological Laboratories) for 45 min. The worms were then washed in three changes of PBS and mounted in glycerol/PBS (1:1).

15.2 Results

Gross and histological examination of the cloacal areas of bursectomized chickens showed that bursal tissue had been successfully

ablated (Plates I & II). All of the intact infection control birds (C) and those that were sham-bursectomized and irradiated (SX) formed specific anti-worm antibodies and the fluorescence was predominantly in the rostellar region of the excysted worm's scolex (Plates III & IV). Sera from four out of the five bursectomized birds failed to give positive fluorescence when tested against excysted worm antigens. Excysted worms incubated with PBS were consistently negative when treated with RACh/FITC (Plate V).

Fig. (26) shows that of the cysticercoids administered to previously uninfected chickens (C), 20.3% developed to worms with fully grown strobilae. In BX and SX chickens, which were previously exposed to infection, 9% and 10.8% of the cysticercoids administered were recovered as fully strobilated worms. C chickens also supported a greater mass of worms (mean biomass 37 mg) than either BX or SX chickens (mean biomass 15 and 19.7 mg respectively). The difference between BX and SX chickens, in terms of the mass of worms they supported and the number of worms recovered as > 0.1 mg, is not statistically significant (the Wilcoxon two-sample test, two-tailed).

15.3 Discussion

Chickens surgically bursectomized on the day of hatching and exposed to sublethal total-body X-irradiation have been shown to be incapable of developing germinal centres, plasmablasts and plasma cells (Cooper et al., 1966). Such birds remain agammaglobulinaemic and fail to produce circulating antibodies even after vigorous or repeated antigenic stimulation. In contrast, birds treated in this way develop normal levels of cellular immunity. R. cesticillus has

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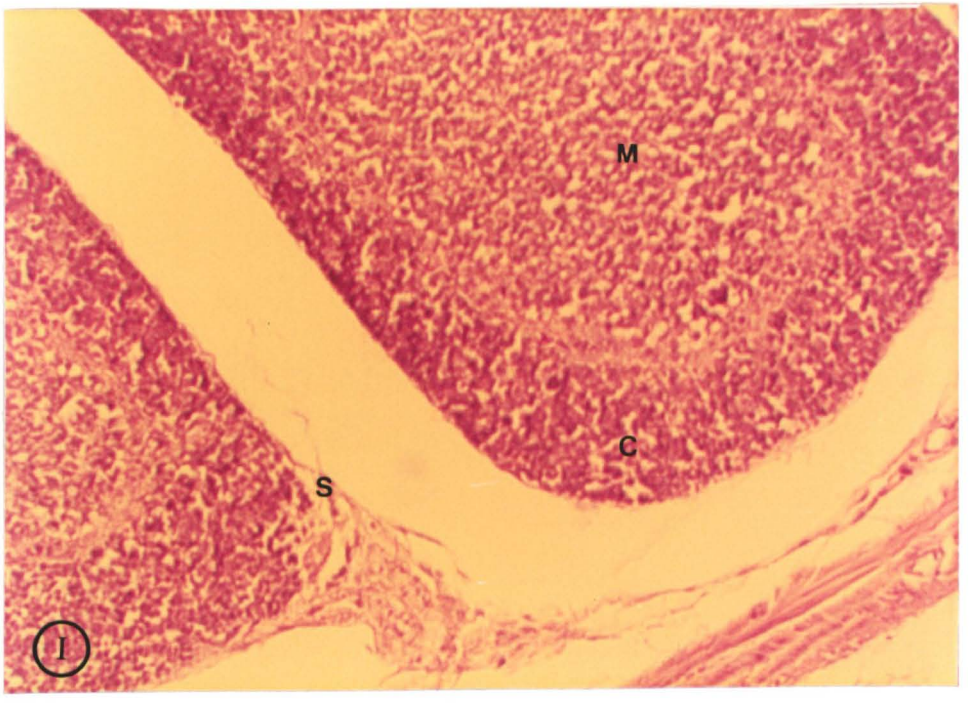
Plate I. Lymphoid follicles in bursa from an SX chicken, showing cortex (C), medulla (M), surface epithelium (S). (H and E, X 100).

Plate II. Transverse section through the wall of the cloaca of a BX chicken: a. villus; b. crypt; c. muscularis mucosa.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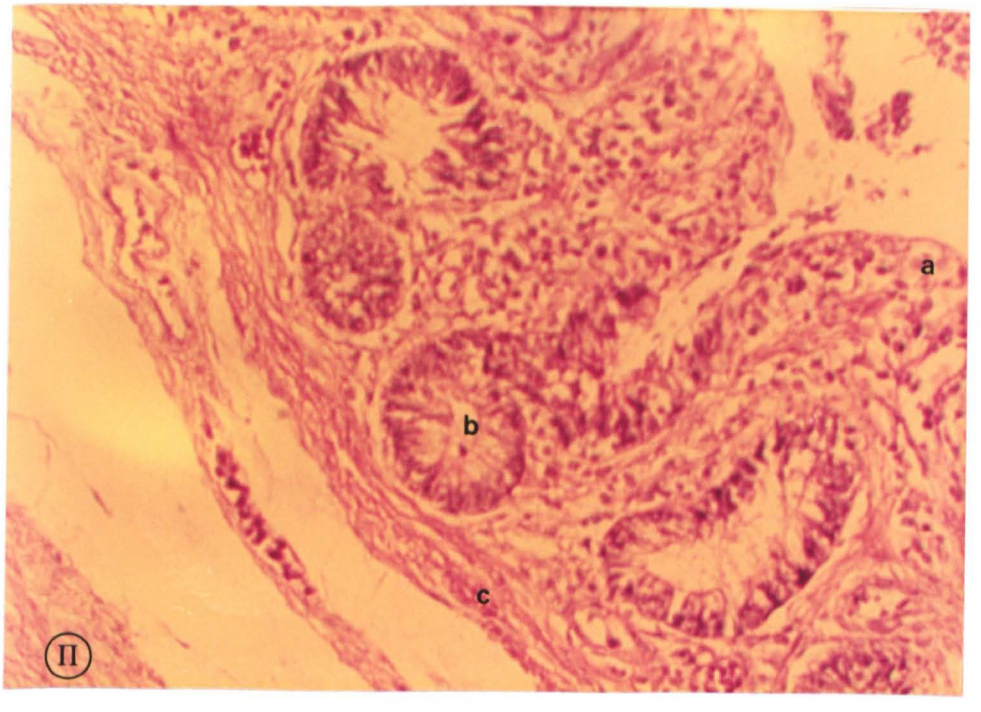
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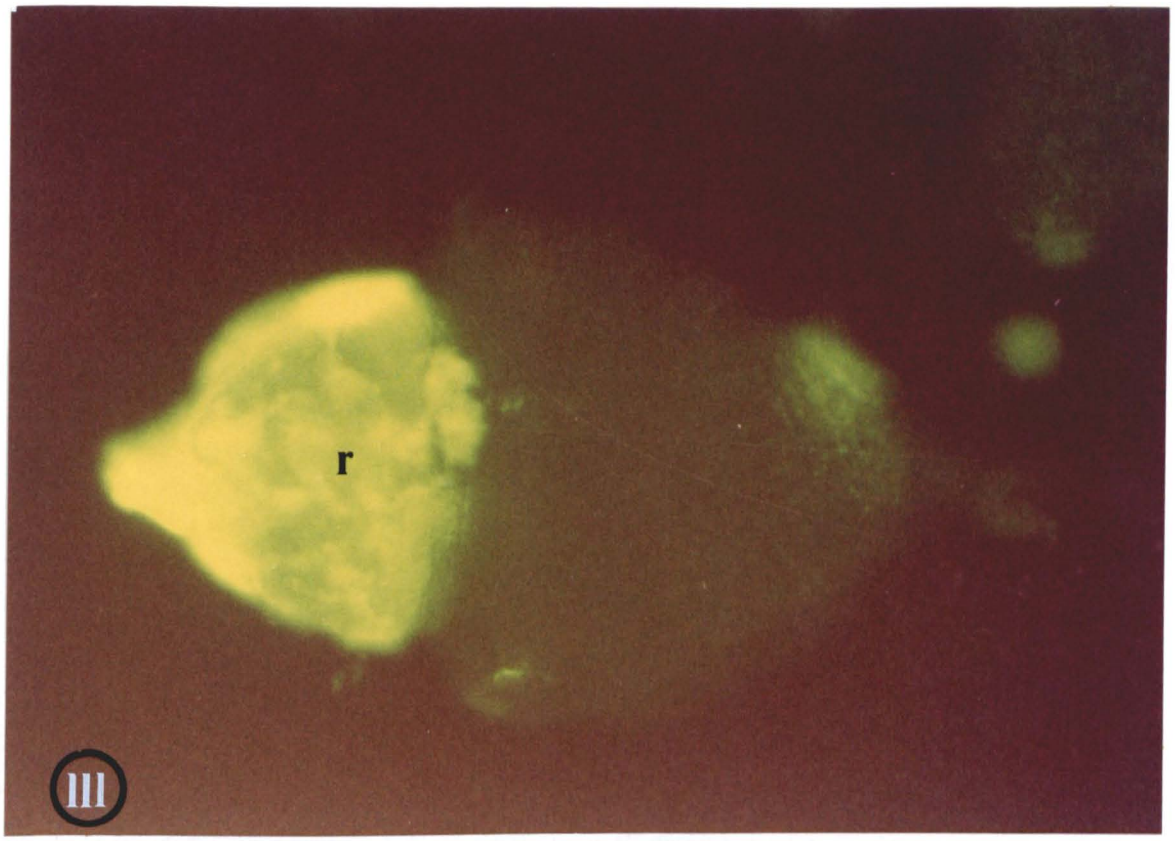
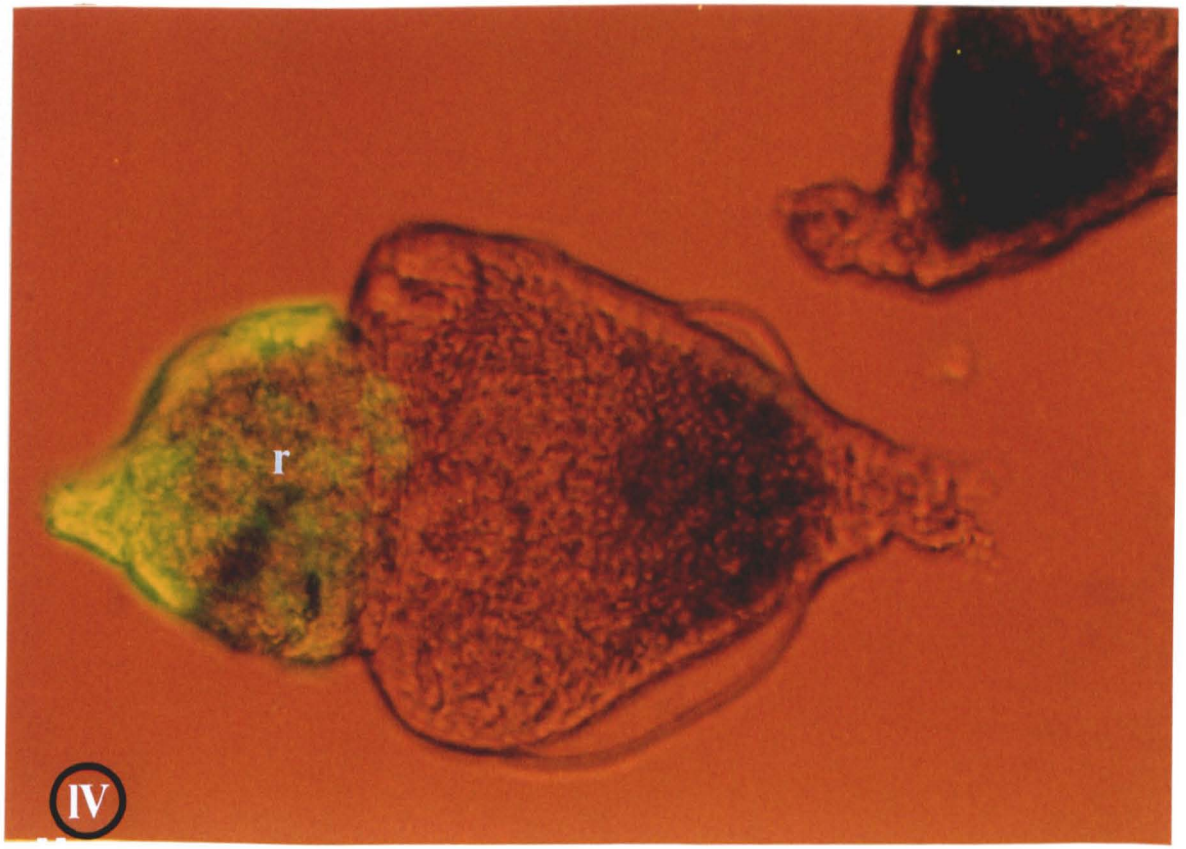
Plates III & IV. Photomicrographs of an excysted worm previously incubated with immune serum from an intact chicken and RACh/FITC. Fluorescence is confined to the rostellar region (r). X 250.


r
III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IV

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Plate V. Photomicrograph of an excysted worm previously incubated with PBS and RACh/FITC.

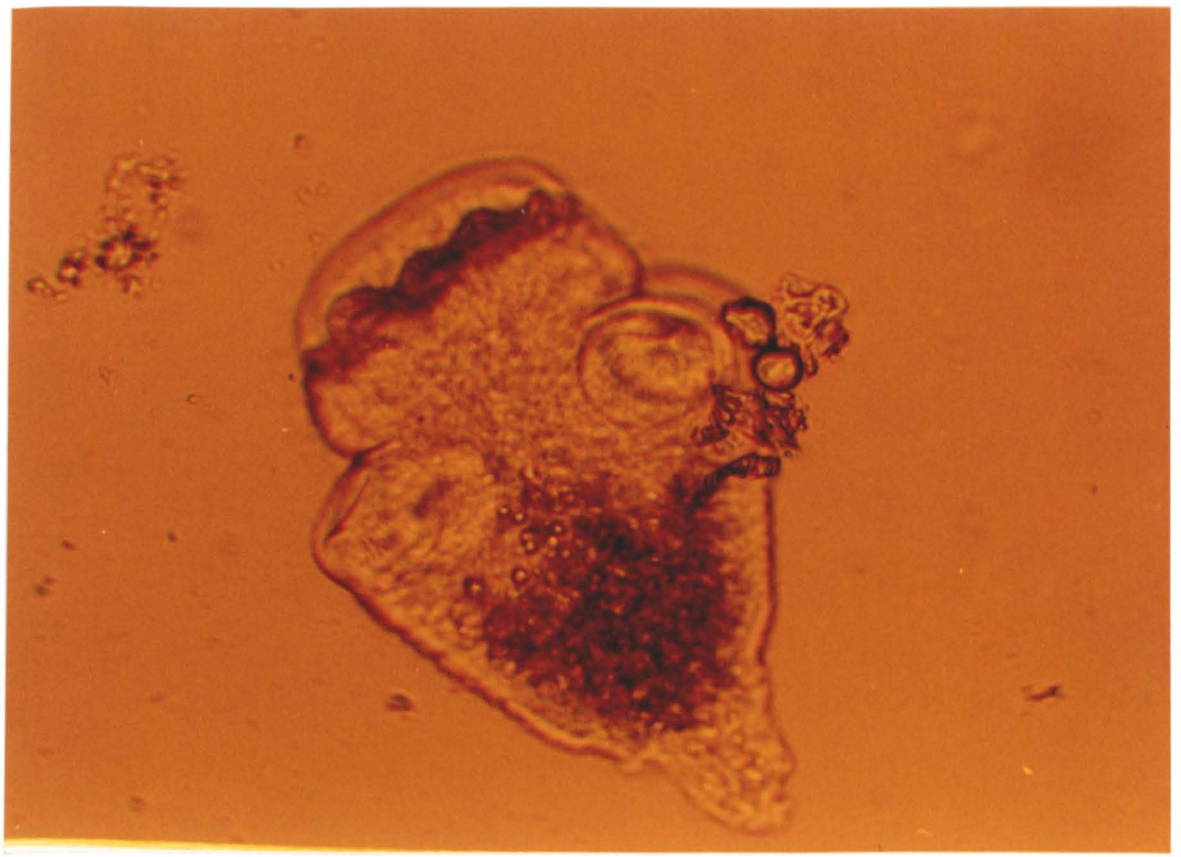

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Figure 26.

Growth (1) and survival (2) of R. cesticillus in bursa-deficient and intact chickens.

* Mean biomass

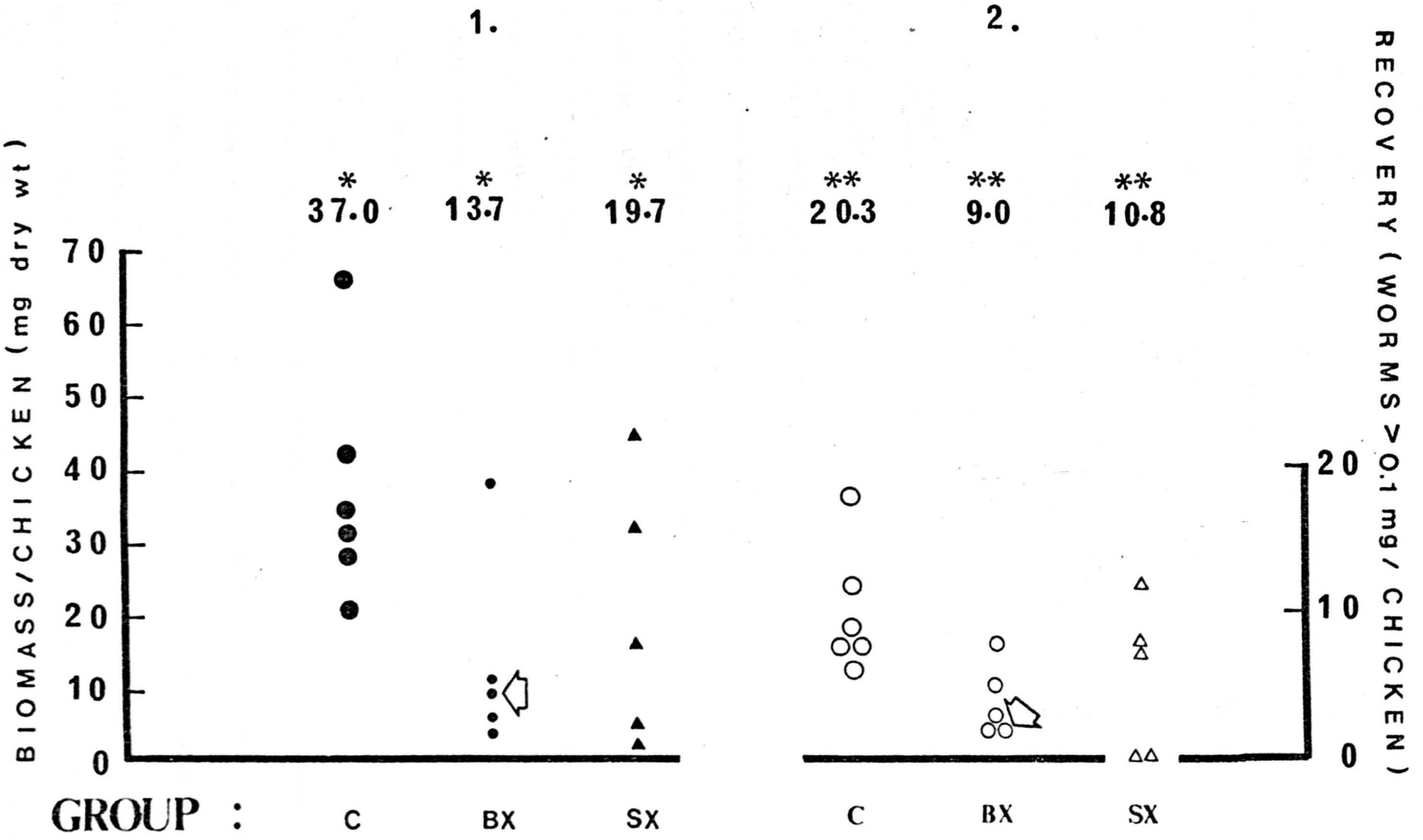
** Recovery per cent

The chicken with specific anti-worm antibodies (arrow) was excluded from growth & recovery figures

C: Infection controls.

BX: Bursectomized and X-irradiated

SX: Sham-bursectomized and X-irradiated


1.
2.
*
37.0
*
13.7
*
19.7
**
20.3
**
9.0
**
10.8
BIOMASS/CHICKEN (mg dry wt)
70
60
50
40
30
20
10
0
RECOVERY (WORMS >0.1 mg/ CHICKEN)
20
10
0
GROUP :
C
BX
SX
C
BX
SX

been shown to evoke a protective immune response in chickens (Gray, 1973). Worms from a primary infection lose their strobilae and they are subsequently expelled. The degree of destrobilation increases significantly in secondary infections. Chickens immune to R. cesticillus form anti-worm humoral antibodies (Gray, 1976) and show an increase in the number of mast cells and pyroninophilic cells in the intestinal mucosa. Attempts to demonstrate the presence of anti-worm antibodies in mucus extracts from intestines of infected chickens, using the indirect immunofluorescent method, however, were unsuccessful (Gray, 1973). The results obtained from the present investigation confirm Gray's findings that R. cesticillus evokesprotective immunity in chickens, as judged by the decrease in the number of fully strobilated worms in the secondary infection and the decrease in growth of secondary worms. Chickens whose ability to produce humoral antibodies was abrogated by bursectomy and irradiation developed protective immunity against the tapeworm as did the controls with specific anti-worm antibodies in their sera. This observation suggests that humoral antibody is not the crucial component of the mechanism affecting the growth and development of R. cesticillus in immune chickens.

Tapeworms inhabiting the intestinal lumen evoke antibody responses in their hosts (Williams, 1979). However, it has proved difficult to correlate the presence of these antibodies with protection since attempts to passively transfer immunity with serum have largely been unsuccessful (Wassom, De Witt & Grundmann, 1974; Andreassen et al., 1978 a; Hopkins, 1980). Mice whose antibody-forming capacity was

suppressed by the inoculation of rabbit anti-mouse IgM expelled an H. diminuta infection as effectively as the unsuppressed controls (Isaak, 1976). This author also found that the incubation of H. diminuta cysticercoids with immune serum and complement did not reduce the infectivity of these cysticercoids. Moreover, in infections with Apatemon gracilis, a trematode inhabiting the gut of ducks, a strong immune response is induced and the parasite is rapidly rejected (Blake, 1974; cited by Ogilvie, 1974). Bursectomy of ducks, however, has no influence on the course of infection. Ogilvie suggested that the immunological control of this trematode might be similar to the response to nematodes of the intestine.

Befus (1977) showed that intestinal immunoglobulins bind to the tegument of H. diminuta. It was suggested that these immunoglobulins were specific anti-worm antibodies bound to antigens on the worm surface and that they might be involved in immunological damage to the tegument (Befus & Threadgold, 1975). Befus (1977), however, was careful to point out that his findings did not exclude the possibility that these immunoglobulins were non-specifically adsorbed to the surface of the worms. It is of interest that these immunoglobulins occur on the surface of H. diminuta in mice, which reject the tapeworm (Hopkins et al., 1972 a, b), and in rats in which the worm can survive for the life of the host (Read, 1967). Befus also reported the presence of immunoglobulins on the surface of the bile duct cestode, H. microstoma which raises in mice high titres of antibodies and it is 'apparently' unaffected by these antibodies (Moss, 1971). The evidence obtained from the present investigation suggests that humoral

antibodies do not apparently play a major role in functional immunity to the davaineid tapeworm, R. cesticillus in chickens. The chicken model offers the potential for separating the roles of the central lymphoid organs, the bursa and thymus, in influencing the development of specific adaptive immune responses mediated by cells or antibodies (Szenberg & Warner, 1962). Indeed, further studies are necessary to evaluate the role of cellular immunity in the rejection of R. cesticillus by chickens.

16. Primary Infections

16.1 Materials and Methods

Bl chicks were surgically bursectomized on the day of hatching and exposed to X-irradiation (BX). Another group of chicks was sham bursectomized and X-irradiated (SX). The chickens received when 5 weeks old a total of 50 17-day-old R. cesticillus cysticercoids administered in two gelatin capsules containing 15 and 35 cysticercoids respectively. The capsules were given within a few minutes of each other. All birds were starved overnight before the cysticercoids were administered.

Autopsy of birds was performed on day 15 of infection. Normally R. cesticillus strobilates extensively within the first 2 weeks of a primary infection and the worms reach patency about day 15.5 - 21.5 (Elowni, 1977). Such worms lose their strobilae progressively and by day 70 of infection, all worms become destrobilated and they are subsequently expelled (Gray, 1972a). Development of a protective response against the parasite in the primary infection is therefore assessed by the presence of destrobilated worms (<0.1 mg dry wt). An important point to be considered is that in secondary R. cesticillus infections, worms of this weight are also

affected by the host immune response but it is almost impossible, in terms of gross morphology, to distinguish between those that had actually strobilated and subsequently lost their strobilae and those that failed to grow strobilae initially (15.1). The difference between the two groups of chickens was assessed by measuring the mass of worms they supported and the number of worms (>0.1 mg or <0.1 mg) they harboured.

16.2 Results

Gross inspection of the cloacal area and histological examination of serial sections of the cloacal wall in bursectomized chickens showed that bursal remnants were absent in these birds. Using the indirect immunofluorescent method (15.1) all SX chickens formed anti-*R. cesticillus* antibodies. Sera from four out of the five BX birds were negative for these antibodies. Immunofluorescence tests were also performed with control serum (Flow Laboratories) from previously uninfected chickens. No fluorescence on excysted worms (the antigen) was obtained following incubation with this control serum indicating specificity of the test. The presence of immunoglobulin-positive cells in the spleens and intestines of BX and SX birds was investigated using the direct immunofluorescent method. Tissues from all SX birds

contained immunoglobulin-positive cells (Ig-cells). From the BX group, however, only the chicken with anti-worm antibodies in its serum had Ig-cells in its intestine and spleen.

The difference between BX and SX chickens, in terms of worms' growth and recovery, is not statistically significant (Wilcoxon test, two-tailed). Transformation of the data into mean biomass and per cent recovery (Fig. 27), however, indicates that BX chickens support less worm mass, less worms of >0.1 mg and a greater number of destrobilated worms than SX chickens.

16.3 Discussion

The results obtained lend support to earlier findings (15.2) that R. cesticillus evokes a protective response in chickens, as judged by the presence of destrobilated worms in intact (SX) chickens. The development of this protective response is not impeded in BX chickens incapable of producing detectable humoral antibody against the tapeworm and with no Ig-cells in their intestines and spleens. These observations suggest that antibody is not the essential component of the immune mechanism by which R. cesticillus is rejected from chickens. The fact that retardation of worm growth and destrobilation are more pronounced in BX than in SX chickens suggest that antibody may even be involved in protection of the parasite against immunological attack.

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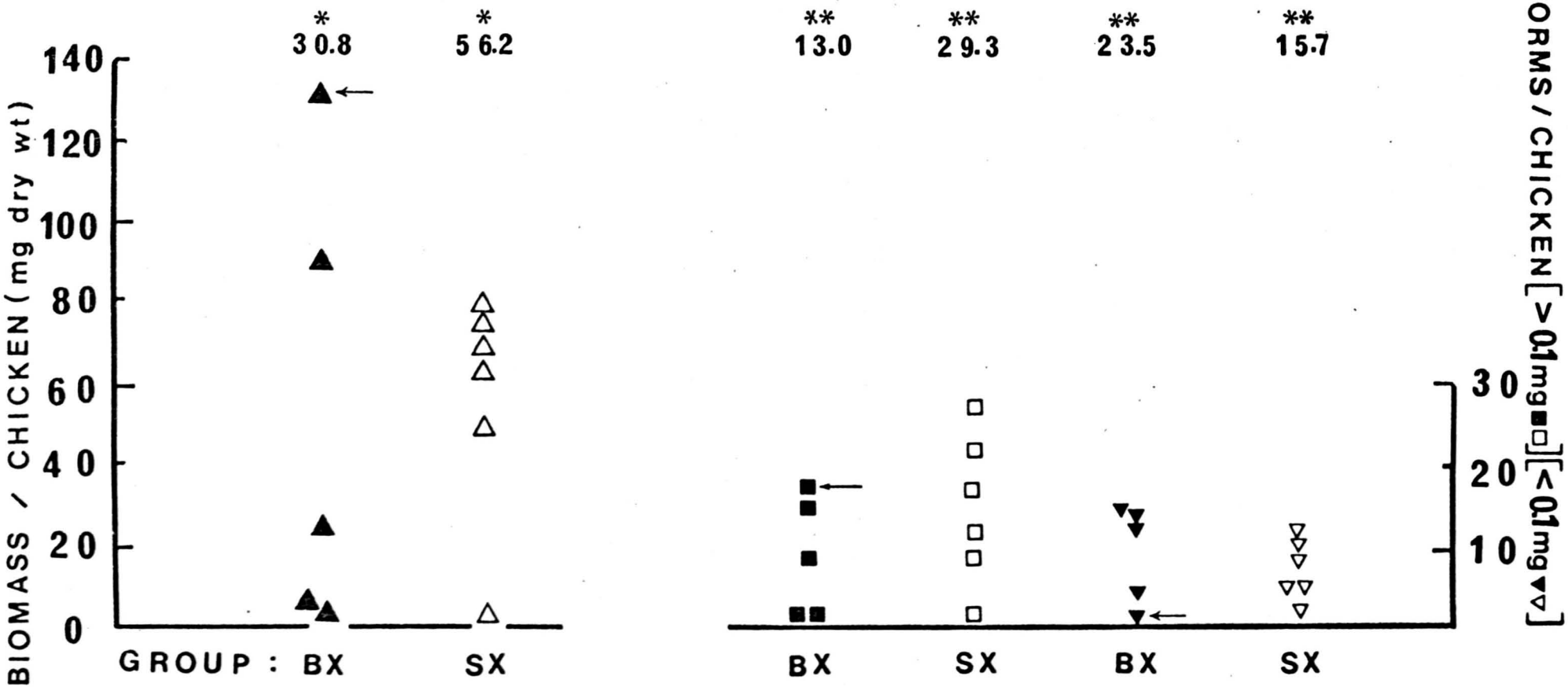
Growth and recovery of R. cesticillus from a 50-cysticercoid primary infection in bursectomized (BX) and intact (SX) chickens.

* Mean biomass

** Cysticercoids administered to a group and recovered as worms (%)

A chicken with anti-worm antibody in its serum and immunoglobulin-positive cells in intestine and spleen (arrow) was excluded


BIOMASS / CHICKEN (mg dry wt)
140
120
100
80
60
40
20
0
GROUP : BX
SX
* 30.8
* 56.2
** 13.0
** 29.3
** 23.5
** 15.7
BX
SX
BX
SX
WORMS / CHICKEN [<0.1mg■□][<0.1mg▲▽]
30
20
10

APPENDIX

APPENDIX

(1) The Efficacy of Praziquantel Against H. diminuta in Mice

Praziquantel (Bayer AG, Leverkusen) is an effective compound against cestodes in sheep, dogs, cats (Thomas & Gonnert, 1978), rats and mice (Thomas et al., 1977). In the present study it was tested against H. diminuta of various ages in mice. Previous statements in the text (SECTION 3) concerning the efficacy of Praziquantel against H. diminuta were based on the results tabulated below.

Groups of 6-weeks-old male CFLP mice were given oral cysticercoid infections. Some of these groups were given cortisone treatment (1.25 mg/mouse) in order to suppress the development of an immune response against the tapeworm (Hopkins et al., 1972 b) which could interfere with the assessment of the actual effect of the anthelmintic on the parasite.

Praziquantel was prepared as an aqueous suspension with the addition of Cremophor EL (4%; BASF, Ludwigshafen) and was administered to mice as a single oral dose of 100 mg/kg body wt. All mice were killed 6 days after anthelmintic treatment. Table 19 shows the design of the experiment and the results. At this dose the compound was 100% effective against H. diminuta of 1, 5, 6, 7 and 9 days of age.

Table 19. The efficacy of Praziquantel against H. diminuta of different ages

Group	No. of mice	Cysticercoids given/mouse	Cortisone (days p.i.)	Age of worms (days)	Treatment	Worms recovered
1	3	15		1	P	0
2	3	15		1	C	38
3	3	4	2, 4, 7	5	P	0
4	2	4	2, 4, 7	5	C	8
5	4	4	2,4,6,8	6	P	0
6	4	4	2,4,6,8	6	C	13
7	6	4	2,4,6,8,11	7	P	0
8	5	4	2,4,6,8,11	7	C	16
9	2	4	2,4,6,8,11	9	P	0
10	2	4	2,4,6,8,11	9	C	7

P, Praziquantel; C, Untreated

(2) Dry Weight of 8-day-old H. diminuta Mouse Worms

Male CFLP mice (6 to 7 weeks old) were given orally four cysticercoids each and they were treated with cortisone acetate (1.25 mg) injected s.c. on days 2, 4 and 6 post-infection. Worms were recovered on day 8 of infection.

Group	No. of worms	Total dry weight (mg)
1	13	11.8
2	9	16.4
3	8	9.8
4	5	4.8
5	5	5.9
Total	40	48.7

Mean worm weight is 1.22 mg.

(3) Dry Weight of 4- and 8-day-old Rat Worms

Rats were given by a stomach tube 10 cysticercoids each. They were autopsied on day 4 or day 8 of infection.

Group	Age of worms	No. of worms	Total dry weight (mg)
1	4 days	6	0.3
2	4 days	14	0.7
3	8 days	6	34.8
4	8 days	8	37.7

Mean dry weight of a 4-day-old rat worm is 0.05 mg.

Mean dry weight of an 8-day-old rat worm is 5.16 mg.

(4) Tris-maleate buffered saline (TMBS)

10X Tris-maleate buffer, pH 7.2 (0.25M) was prepared by dissolving 33.3 g of tris (hydroxymethyl) methylamine (BDH Chemicals Ltd.) and 29.0 g of maleic acid (BDH) in 950 ml distilled water. The pH was adjusted to 7.2 by adding 12M NaOH. Distilled water was added to adjust the final volume to 1 litre.

NaCl solution was made by adding 11.7 g of the salt to 1 litre of distilled water.

TMBS was prepared by adding the following: 5 mg of the tris-maleate buffer, 20 ml of the NaCl solution and 10 ml of distilled water. Osmolarity was approx. 300 mosmol.

(5) Earle's Salt Solution (ESS)

Component	g/litre
NaCl	6.80
KCl	0.40
$NaH_2PO_4.H_2O$	0.13
$MgSO_4.7H_2O$	0.20
Glucose	1.00
$CaCl_2$ (anhydrous)	0.20
Phenol red	0.01
$NaHCO_3$	2.20

(6) Excystation of H. diminuta and R. cesticillus cysticercoids in vitro

Solution 1: 0.8 g pepsin (BDH Chemicals Ltd.) was dissolved in

100 ml of HBSS. The pH was adjusted to 1.7 by the addition of 0.2N-HCl.

Solution 2: 0.2 g sodium tauroglycocholate and 0.3 g trypsin (BDH Chemicals) were dissolved in 100 ml HBSS and pH was adjusted to 7.2 by the addition of 0.2 N-NaOH.

Cysticercoids were incubated at 37°C with solution 1 for 11 min. They were washed in three successive changes of HBSS and reincubated at the same temperature with solution 2 for 12 min (H. diminuta cysticercoids) or 5-7 min (R. cesticillus cysticercoids).

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KEY TO ABBREVIATIONS

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BCG	Bacille Calmette-Guerin
BSA	Bovine serum albumin
cf.	Compare
EA	Egg antigens
ESS	Earle's Salt Solution
EXA	Exoantigens
FCA	Freund's complete adjuvant
Fig.	Figure
g	Gramme
g	Acceleration due to gravity
h	Hour
Ho	The null hypothesis (hypothesis of no difference)
HBSS	Modified Hanks' balanced salt solution
Ig	Immunoglobulin
i.p.	Intraperitoneal; Intraperitoneally
Krad	Kilorad
l	Litre
MDS	Membrane disruption solution
min	Minute
mol. wt	Molecular weight

mosmol	milliosmole
OD	Outer diameter
p	Probability of occurrence as a result under Ho
Pa	Pascal
PBS	Phosphate-buffered saline
p.i.	post-infection
RACh/FITC	Rabbit anti-chicken globulin conjugated with fluorescein isothiocyanate
r.p.m.	Revolution per minute
S	Svedberg unit
SA	Somatic antigens
s.c.	Subcutaneous; subcutaneously
SDA	Sonic disruption antigens
SEA	Saline extract antigens
sec.	Second
SRBC	Sheep red blood cells
TA	Tegument antigens
TMBS	Tris-maleate buffered saline
wt	Weight