VACCINATION AGAINST CANINE HOOKWORM

A Dissertation

submitted for

The Degree of Doctor of Philosophy

in

The Faculty of Veterinary Science

of

The University of Glasgow

by

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October, 1969.

PREFACE

The research work recorded in this thesis was conducted in the Department of Veterinary Medicine, University of Glasgow, and all of the data was obtained by the author.

ACKNOWLEDGEMENTS

It is with pleasure and gratitude that the following are acknowledged:

Professor W.I.M. McIntyre for supervision, advice, encouragement and facilitation of the conduct of the research project.

Doctor G.M. Urquhart for his constant advice and encouragement and for his many helpful suggestions.

Mrs. J. McInnes, Mrs. J. Ledger and Mrs. E. Thompson for their diligent and competent technical assistance.

Miss L. McCracken and the kennel staff of the Department of Veterinary Medicine for maintenance and care of the experimental dogs.

Dr. A.A. Charteris, of the Radiotherapy Department, Western Infirmary, Glasgow and the staff of the Physics Section in that department for the use of calibrated X-ray machines.

Mr. A. Finnie for his help in photographing the text figures.

Miss N. Brown of the Department of Animal Husbandry for the analyses of the constituents of the experimental diet.

Miss L. Lennie for typing the manuscript.

This work was supported by a grant from Allen & Hanburys Ltd., Ware, England.

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SECTION I

INTRODUCTION AND REVIEW OF LITERATURE

Classification of the hookworms

The hookworms, originally so described on account of a dorsad flexion of their anterior end, are parasites of mammals being most frequent in primates, carnivores and ungulates with a few species in other groups including two aquatic mammals. With few exceptions, hookworms are equatorial, tropical or sub-tropical in their natural distribution, this distribution being determined primarily by the temperature requirements for development of their free-living stages. As with most zoological classifications there has been considerable discussion over specific nomenclature (Looss, 1911; Lane, 1916, 1917; Biocca & Le Roux, 1957; Rep, 1963, 1966) and frequent changes have been proposed in the specific names, arrangement and groupings of the species. A recent review (Rep, 1963) proposed that the family Ancylostomidae comprised two sub-families Ancylostominae and Bunostominae. The presence of a well-developed buccal cavity armed with either teeth and/or cutting plates and the possession of a well-developed muscular oesophagus characterises these worms.

All four hookworms of dogs, Ancylostoma caninum (Ercolani, 1859), Ancylostoma braziliense (De Faria, 1910), Ancylostoma ceylanicum (Looss,

1911) and Uncinaria stenocephala (Railliet, 1884) have been classified in the Ancylostominae (Rep, 1963). The morphological details of these four hookworms and their host lists are described in most of the standard text books (Yorke & Maplestone, 1926; Monnig, 1947; Cameron, 1951; Chandler, 1955; Lapage, 1956; Faust & Russell, 1964; Soulsby, 1965) and in the original descriptions and subsequent descriptive publications (Lane, 1916, 1917; Biocca, 1951; Biocca & Le Roux, 1957; Rep, 1963, 1964).

Significance of the hookworms

Hookworm disease, affecting man (Stoll, 1962) and his dog is one of the major scourges of the humid tropical and sub-tropical areas of the world (Erhardt & Schulze, 1961; Faust & Russell, 1964; Soulsby, 1965).

In the dog the 3 species, A. caninum, A. braziliense and U. stenocephala, have usually been considered and discussed together without differentiating their relative pathogenesis (Monnig, 1947; Hatch, 1961; Soulsby, 1965; Bailey et al., 1968); differentiation has been made only on the matter of geographical distribution. The pathogenesis of all 3 species in dogs have usually been considered to be the same, although this has recently been disproven in that the most pathogenic species is A. caninum (Miller, 1968). This is also the most widely distributed species (Bailey et al., 1968). A. braziliense is also widely distributed in dogs and cats in tropical areas, but with its relatively low incidence of infection and discontinuous distribution

(Rep, 1963; Beaver, 1966) and low pathogenicity to dogs (Miller, 1966) it is of relatively minor importance. U. stenocephala, the fox or European dog hookworm, has a very different geographical distribution, being found in temperate areas to the north and south of the tropical hookworm belt (Soulsby, 1965; Bailey et al., 1968), and like A. braziliense is of relatively minor pathogenic significance to dogs compared with A. caninum (Miller, 1968).

The important species affecting man are Ancylostoma duodenale (Dubini, 1843) and Necator americanus (Stiles, 1902), sometimes termed respectively the "old" and "new" world hookworms, although both species are widely distributed and in many tropical areas mixed infections are relatively common (Faust & Russell, 1964). A third hookworm of man, A. ceylanicum, the specific differentiation of which from A. braziliense has been the subject of prolonged contention (Lane, 1922; Darling, 1924; Biocca, 1951; Biocca & Le Roux, 1958; Rep, 1963, 1966), appears to have a rather restricted and discontinuous distribution in man and carnivores (Darling, 1924; Yoshida et al., 1968). This is the only hookworm which parasitises and completes its development in both man and his dogs and cats. A. braziliense, although thought to be incapable of reaching maturity in man (Beaver, 1966), has a considerable nuisance value in man. Creeping eruption or dermal larva migrans, caused by the wandering of 3rd stage hookworm larvae (particularly A. braziliense), the path of which migration is exhibited by inflammatory and allergic reactions, is a well described condition (Dove, 1932; Beaver, 1959, 1966).

The association between anaemia and infection of man with "abdominal worms" (probably hookworms) was reported approximately 3,500 years ago (Ebers Papyrus, translated in 1873 and dated about 1,500 B.C., as quoted by Watson, 1960). Although there has been no doubt that infections with certain species of hookworm in man and dogs induce anaemia, the mechanism by which the hookworms induce disease has been the centre of considerable controversy, since the various spectra of opinions embrace intestinal haemorrhage (Huart, 1929; Foster & Landsberg, 1934), intravascular haemolysis (Liefmann, 1905; Whipple, 1909; Schwartz, 1921: Fulleborn & Kikuth, 1929), myelotoxins with depressed erythropoiesis (Gentile, 1956), malabsorption (Sheehy et al., 1962), intoxications from worm metabolic products (De Langen, 1922; Hall, 1925) and from secondary microbial invasion of the walls of the alimentary canal. Only in the last 10 years has incontrovertible evidence been obtained to show that only one of these factors (i.e., intestinal haemorrhage) merits description as a primary pathogenic mechanism in the induction of hookworm anaemia in man (Roche et al., 1957; Foy et al., 1958; Gilles et al., 1964) and in dogs (Clark et al., 1961). Other factors such as absorption and intoxication by metabolic products of the hookworm may have some importance in the genesis of many of the other primary signs and symptoms seen in man (e.g., upper respiratory catarrh, dyspepsia, abdominal pain). It is, however, difficult to determine whether such symptoms occur in the dog, and consideration of hookworm pathogenesis in this host species has therefore been confined principally to signs associated with anaemia

(Huart, 1929; Sarles, 1929b; Cort, 1933; Foster & Landsberg, 1934; Landsberg & Cross, 1935; Landsberg, 1937, 1939; Rubin & Butler, 1951; Bailey et al., 1968). Nor have distinctions been drawn between the consequences of infection with the different species of hookworm in a given host species. Signs and symptoms associated in man with monospecific infections of A. duodenale (Brumpt, 1952, 1958) have been extrapolated freely to those occurring during infection with N. americanus. As a general principle it has been assumed that the pathogenesis and signs of A. caninum infection in the dog are applicable to all hookworm infections of the dog (Hall, 1925; Monnig, 1947; Hatch, 1961; Soulsby, 1965; Bailey et al., 1968). Thus many of the more expansive and liberal extrapolations are subject to serious criticism.

Life cycle of the hookworms

The life cycle of all species of hookworm is direct. There is no intermediate host and the first two free-living larval stages, derived from worm ova in the host's faeces, give rise to a third free-living but infective stage in the environment of the host. Environmental temperature and humidity control the success and the rate of development of the free-living stages (Stiles, 1921; Okoshi, 1967a) and hence determine the geographical distribution of the parasite. Different species of hookworm have different temperature requirements in their free-living stages (Foster & Daensgsvang, 1932; Gibbs &

Gibbs, 1959; Okoshi, 1967a) and through this factor potential distributions of individual species are further restricted.

The third larval stage commonly infects the host by active skin penetration followed by somatic migration and vascular and lymphatic transport to the lungs (Looss, 1911), whence the larvae reach the intestines via trachea and oesophagus. The third moult (1st parasitic moult) usually occurs after the larvae leave the lungs, fourth stage larvae being found in the intestine. The fourth, or second parasitic moult, occurs in the intestine in which the fifth, or adult stage, remains for the rest of its life. For some hookworms in their proper hosts, certainly A. caninum in the dog (Foster & Cross, 1934; Matsusaki, 1950), A. tubaeformae in the cat (Okoshi, 1967b), U. stenocephala in the dog (Fulleborn, 1929) and probably for the others, the lung migration is not essential and maturation in situ in the alimentary tract may follow oral infection. In the case of U. stenocephala it has been suggested that the normal and certainly the most successful route of infection is per os since few larvae reach the intestine after percutaneous infection (Gibbs, 1958). Necator americanus in man appears to require to undergo somatic migration since oral infection has been shown to be singularly unsuccessful. In an abnormal host (i.e., not proper for that species of hookworm), the great majority of larvae undergo lung migration irrespective of the route of administration, and subsequent worm development is slight or absent.

Whether percutaneous or oral infection is the more common route under natural conditions of exposure is not known. It would seem to be a reasonable

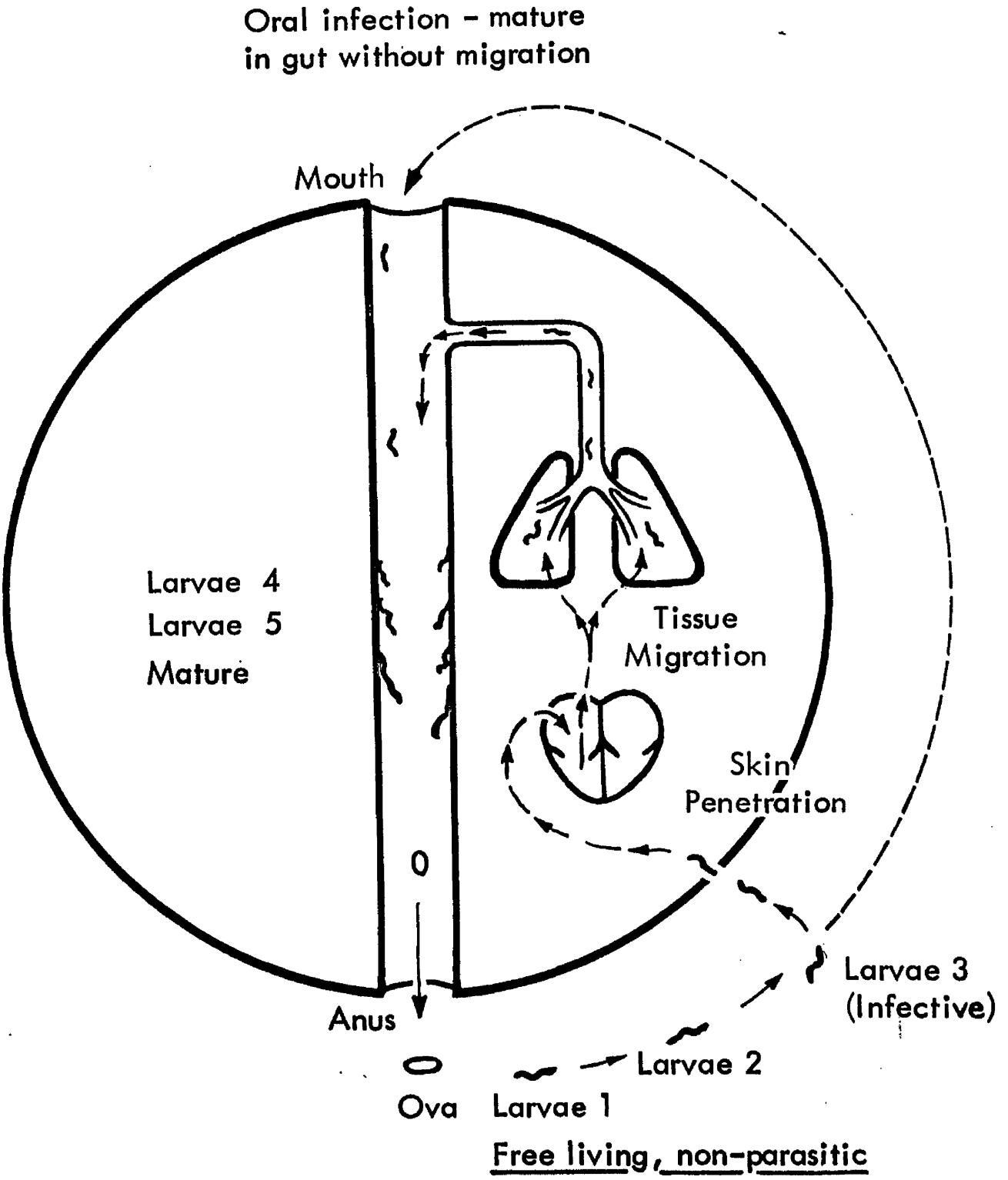

FIGURE 1. Diagramatic representation of the life cycle of A. caninum showing the stages of free-living development and the parasitic stages as they develop in a susceptible pup. (This diagram is similar to that of Lapage, 1956, but with several modifications.)

supposition that where both oral and percutaneous experimental infection have been successful natural infections will occur by both routes, the relative importance of each route being determined by host behaviour patterns and environmental influences.

The life cycle of A. caninum in dogs was investigated intensively by Matsusaki (1950) and was found to conform to the general system as illustrated above (Fig. 1). Infection of pups can be achieved by administering 3rd stage larvae orally by pipetting larvae into the mouth of the pup, by enclosing the larvae in gelatin capsules and administering in this fashion, or by means of a stomach tube. Infection can also be established by inoculating larvae subcutaneously. When infective larvae are placed on the unbroken skin of pups, the larvae penetrate the epithelium and subsequently reach the intestine to establish an infection.

Matsusaki (1950) showed that after infective larvae were administered by placing them on the unbroken skin of susceptible pups, 3rd stage larvae could be recovered from the lungs, larynx and pharynx for the first 24 hours. The 3rd moult (1st parasitic moult) then took place in the lungs, larynx and pharynx and moulting 3rd stage larvae were demonstrated in these organs from 44 to 48 hours after infection. Fourth stage larvae were then seen in the trachea, larynx, pharynx, oesophagus and intestine from 64 to 72 hours. Fourth stage larvae, in numbers representing a large proportion of the total dose of larvae given, were then recovered from the intestine at 4 days. The fourth moult (2nd parasitic moult) occurred in the intestine and all the

hookworms recovered from the intestine by the 6th day were immature adult hookworms. Development of the reproductive systems of these adult hookworms commenced on the 12th day and by the 17th day all worms were mature. Hookworm eggs can usually be demonstrated in the faeces of pups from the 14th day onwards.

Immunity in A. caninum infection of dogs

Approximately 40 years have passed since the first experiments, which showed that dogs could develop resistance to reinfection with A. caninum, were reported (Herrick, 1928). In the subsequent 10 years there were a number of publications on the stimulation of resistance in dogs against infection with A. caninum (Sarles, 1929a; McCoy, 1931; Foster, 1935; Otto & Kerr, 1939; Otto, 1941). This work was reviewed in 1940 by Cort and Otto, and again in 1948 by Otto. In these experiments, various degrees of resistance were stimulated by administering, usually over a prolonged period of time, infective larvae of A. caninum. Resistance was then demonstrated by reduced morbidity and/or mortality of a potentially severe challenge infection and by reduced worm establishment from larvae of the challenge inocula. In some cases worm egg outputs from the challenge hookworms were also recorded as being diminished. In most of the experiments (Sarles, 1929a; McCoy, 1931; Foster, 1935; Otto & Kerr, 1939) at least 100 days, between first vaccinating infection and inoculation of the challenge infection, was allowed for the development of a satisfactory resistance and periods of

up to 7 months (Foster, 1935; Otto & Kerr, 1939) and even 2 years (Sarles, 1929a) were allowed to elapse between commencement of vaccination and administration of challenge inoculations.

Unfortunately, many of the reports describe experiments in which stray or "pound" dogs of unknown age were used after acquisition from hookworm-enzootic areas. Resistance shown to challenge infection in these, often uncontrolled, experiments ascribed by the author to be a result of either age or acquired immunity now appear to have been a combination of both manifestations of resistance. Therefore, factors such as previous exposure to hookworm, unknown age and absence of control dogs tend to render unreliable many of the conclusions drawn by some of the earlier authors.

It has been shown that the stimulation of protective immunity against nematode infection is most efficiently obtained by infection with live larvae. Antigens derived from worm somatic material or from worm metabolic products may stimulate the elaboration of antibodies (Stewart, 1950) and some degree of resistance (Urquhart et al., 1962), but this resistance is usually only significant statistically and is not sufficiently marked as to be of any practical significance in the protection of animals against morbidity and mortality associated with severe challenge infections.

Vaccination with irradiated infective larvae

The damaging effect of ionising radiation on helminth parasites was first discovered about 50 years ago (Tyzzer & Honeij, 1916) but only in the

last 15 years has this discovery been extensively exploited. The impetus to recent expansion of knowledge in the field of irradiated helminth vaccines was provided by the observation that vaccination of calves with X-irradiated larvae of Dictyocaulus viviparus stimulated a high degree of resistance to severe challenge infection. This discovery then led to the development of an effective irradiated vaccine for cattle (Jarrett et al., 1961b), and its subsequent commercial use in the United Kingdom (Poynter, 1964) and Western Europe. An irradiated vaccine for a related parasite of sheep, Dictyocaulus filaria, has been used in Yugoslavia for several years (Sokolic, 1964). Many other host-parasite systems of veterinary interest have also been examined to determine the feasibility of vaccination with irradiated larvae. These include Haemonchus contortus (Jarrett et al., 1959, 1961a) and Trichostrongylus colubriformis in lambs (Jarrett et al., 1960a), Haemonchus placei in cattle (Ross et al., 1959), Trichinella spiralis in pigs (Cabrera & Gould, 1964), Ascaridia galli in chickens (Varga, 1964b) and Syngamus trachea in chickens and pheasants (Varga, 1968). The use of X-irradiation to attenuate helminth larvae and of these attenuated larvae to stimulate immunity in various experimental host-helminth systems have also been investigated. These include Trichostrongylus axei in the rabbit (Ciordia & Bizzell, 1960), Trichinella spiralis (Levin & Evans, 1942; Gould et al., 1955) and Nippostrongylus brasiliensis in rats (Prochazka & Mulligan, 1965). Schistosoma mansoni in mice (Erickson, 1964) and in rhesus monkeys (Smithers, 1962; Sadun et al., 1964), Schistosoma japonicum in rhesus monkeys (Hsu et al., 1963) and Fasciola hepatica

in the rat (Thorpe & Broome, 1962) have been investigated as host-trematode systems. In the cestodes, the systems investigated have been those of the intermediate stage of Taenia taeniaeformis in rats (Dow et al., 1962), and Taenia saginata in calves (Urquhart et al., 1963). Among the parasitic protozoa, ionising radiation has been used to attenuate Plasmodium berghei for infection of mice (Corradetti et al., 1966) and Coccidia in chickens (Waxler, 1941). The first experiments in which X-irradiation was used to attenuate hookworms were those reported by Dow et al. (1959, 1961) in which successful vaccination with X-irradiated Uncinaria stenocephala was described with single (1959) and double (1961) vaccination schedules.

Most of the work in this field has been restricted to parasites of veterinary rather than medical significance. The reasons for this emphasis are mainly that acute experiments involving slaughter and recovery of worm burdens are possible using farm and experimental animals and that the progressive intensification of agricultural practice has resulted in certain parasitic diseases becoming more noticeable and economically more important. The absence or scarcity of experimental animals as suitable alternative hosts for the helminths that produce disease in man have in most cases presented an insurmountable barrier. However, a considerable volume of information has been accumulated on infections of rodents and sub-human primates with irradiated schistosomes and irradiated Trichinella infections of rodents (see above for specific references).

Four sources of ionising radiation have been employed in this type of investigation. The original source was the radium needle (Tyzzer & Honeij, 1916). The most commonly used source has been the radiotherapy machine, or a comparable industrial unit producing 100-250 kv X-rays. Harder or more energetic radiations such as those produced by the radioactive isotope ^{60}cobalt (1.1 - 1.3 Me V) and by the linear accelerator (4 Me V), have also been used. Comparison of the relative biological effectiveness (RBE) of X-rays and γ-rays have given variable results. Sometimes X-rays were more damaging to the parasite (Gould et al., 1957; Villella et al., 1958; Kassai et al., 1966), while in another experiment the reverse was shown (Jovanovic, 1964). Hard radiation (4 Me V) from the linear accelerator has not been used sufficiently to be certain of its RBE, although the results obtained by Fitzpatrick (personal communication) suggest that it is not very different from X-rays and γ-rays.

Physical factors, such as the geometry of the source of radiation related to position, environment, and substance of the dish containing larvae, filtration employed to remove low energy radiation from X-rays, depth of liquid in the radiation dish, and method of calibration of radiation output, have important influences on the apparent effect of irradiation. These factors should be kept constant to achieve a standard dose effect, and should be exactly specified in reports of experiments (Mulligan, 1964). The concentration of larvae in the medium during irradiation (Jennings et al., 1963), temperature (Fitzpatrick, personal

communication), and infectivity of the raw material (i.e., unirradiated larvae) are also important and must be specified. Most biological systems are more sensitive to radiation in the presence of oxygen, although the significance of an oxygen effect has yet to be investigated in the preparation of irradiated helminth vaccines. Within certain limits, radiation dose rate has been shown not to be an important factor in the efficacy of inactivation (Jennings et al., 1963; Fitzpatrick, personal communication).

Biological factors concerning the parasite, such as its zoological classification (i.e., phylum, e.g., Protozoa or Metazoa; class, e.g., Platyhelminthes or Nemathelminthes; order, e.g., Trematoda or Cestoda; and family, e.g., Strongylidae, Ancylostomidae, Metastrongylidae or Ascarididae) appear to have an important influence on radiosensitivity. The stage of the parasite that is subjected to irradiation has a bearing on the effect produced by, and on the optimal attenuating dose of radiation. Trematode cercariae and metacercariae require radiation doses in the range of 2 - 3 kiloroentgens (kr) of X-rays or γ-rays to achieve suitable attenuation (Villella et al., 1961; Hsu et al., 1962; Smithers, 1962; Thorpe & Broome, 1962; Erickson, 1964; Sadun et al., 1964; Perlowagora-Szumlewicz, 1966); while radiation doses in excess of 5 kr were lethal (i.e., rendered uninfective) to F. hepatica (Thorpe & Broome, 1962) and to Schistosoma mansoni (Villella et al., 1961) and more than 24 kr proved lethal to Opisthorchis metacercariae encysted in fish meat (Mitrokhin, 1959).

Third stage infective nematode larvae of the Trichostrongylidae, Metastrongylidae and Ancylostomidae have been found to be attenuated by radiation doses in the range of 40 - 60 kr of X-rays or γ-rays (Dow et al., 1959, 1961, 1962; Jarrett et al., 1959, 1960a, 1960b, 1961a, 1961b; Gordon et al., 1960; Prochazka & Mulligan, 1965; Sokolic et al., 1965), while doses near or in excess of 100 kr were required to render the nematode larvae non-infective in that adult worms could not be recovered from their proper location (Giordia & Bizzell, 1960; Gordon et al., 1960; Prochazka & Mulligan, 1965). However, not all nematode infective stages have the same susceptibility to radiation. Infective eggs of Ascaris suum have been shown to require about 100 kr of γ-radiation to reduce their pathogenesis during lung migration in guinea pigs (Villella et al., 1958) while a closely related parasite, Ascaridia galli, was shown to be suitably attenuated by only 1/5th to 1/10th of this radiation dose (Varga, 1964a). Encysted Trichinae in muscle have been shown to be attenuated by a wide range of radiation doses from 1.2 to 12 kr (Semrad, 1937; Zalman et al., 1961). Variation in the results obtained by different workers using the same species of parasite and host (Semrad, 1937; Zalman et al., 1961) may be attributable to the different sources of radiation used (i.e., X-rays or ^{60}Co) although, with few exceptions, reports of most experiments using this or other parasites have not included sufficient data on the techniques of irradiation to permit more than a guess at the source of variability in the results.

SECTION II

STATEMENT OF PROBLEM

In spite of the many deficiencies in the reports describing experiments in which immunity to canine hookworm infection was investigated and claimed, as reviewed above, there is a considerable volume of evidence that an acquired resistance to reinfection occurs in dogs after previous infection with *A. caninum*, and that this acquired immunity or resistance may protect dogs against severe and potentially lethal challenge infections (Otto, 1941). Similarly, there is a large amount of data which shows that after exposure to X-irradiation, nematode and other helminth larvae retain their immunogenic potency while their pathogenicity is usually diminished and their reproductive fecundity either diminished or abolished (Urquhart *et al.*, 1962). Two experiments reported by Dow *et al.* (1959, 1961) showed that larvae of the European hookworm of carnivores, *U. stenocephala*, could be attenuated by exposure to 40 kr of X-rays and that single (1959) and double vaccination (1961) with such X-ray-attenuated larvae conferred partial protection on pups against the establishment of a subsequent challenge infection of normal *U. stenocephala*. These observations and the success recorded in the widespread practical use of an irradiated *Dictyocaulus viviparus* vaccine (Poynter, 1964) stimulated the initiation of a research project to investigate the possibilities of attenuating the larvae of *A. caninum*, and of using X-ray-

attenuated A. caninum larvae as an immunising agent against the canine hookworm disease caused by infection with this hookworm.

The principal questions, and their sequential arrangement, to which answers were sought were:-

1. Is the infectivity of larvae given orally the same as when given by subcutaneous inoculation?
2. What is the effect of various doses of X-rays on the infective larvae of A. caninum, particularly with regard to the pathogenesis of the hookworms which develop in the intestine of pups after infection with irradiated larvae?
3. How effective is a single infection of X-irradiated larvae in preventing establishment of adult hookworms from a subsequent challenge infection of normal larvae?
4. Is there an advantage to be gained in giving pups more than one vaccination with X-irradiated larvae?
5. Since A. caninum can establish infection after subcutaneous inoculation and after oral administration of larvae, are there differences in safety or efficacy between oral and subcutaneous vaccination?
6. Since there is already a large volume of evidence that infection with normal A. caninum larvae can stimulate a strong immunity, what advantages may be gained by using X-irradiated rather than normal larvae as vaccine?
7. Since enzootic canine ancylostomiasis is primarily a disease of the young pup, how early can immunity be stimulated in pups and what are the comparative efficacies of vaccinating pups and dogs at different ages?

8. Although it is unlikely that pups in hookworm-enzootic areas would escape infection and reinfection for any appreciable length of time, it would be useful to know how long immunity persists after vaccination.

9. To what extent is the immunity following vaccination enhanced by repeated low grade infection?

SECTION III

MATERIALS AND METHODS

Dogs

Dogs of mixed and usually unidentifiable ancestry were purchased from dealers as newly weaned litters at the age of 6 weeks. Pups to be used on long-term experiments (i.e., extending beyond 3 weeks) were immediately isolated in small groups, each group consisting of at most 3 litters or 20 pups. Pups to be used on short experiments (i.e., vaccine radiation-attenuation controls and other controls to determine infectivity of normal larvae) were placed in the experimental kennels immediately after purchase and thereafter were used for experiments within 5-7 days.

Isolation premises were distant from the centres of dog population (i.e., on isolated farms or smallholdings). Isolation was continued until the pups were approximately 3-months-old. During isolation the pups were vaccinated against canine distemper, contagious canine hepatitis, and leptospiroses canicola and icterohaemorrhagie (Canilep® - Glaxo Laboratories Ltd., Greenford, England). The usual procedure for vaccination was to inoculate the mixed hepatitis/leptospira vaccine first when the pups were between 8 and 9-weeks-old, and to complete the vaccination schedule using the trivalent vaccine (i.e., including canine distemper) when 11-weeks-old. During

the isolation period, the pups were also treated twice with piperazine adipate (Coopane ® - Cooper, McDougall & Robertson Ltd., Berkhamsted, England) to remove ascarids (Toxocara and Toxascaris).

Examination of the faeces of the pups after purchase and prior to anthelmintic medication demonstrated the absence of hookworm eggs, and hence precluded current infection with hookworm (including U. stenocephala). Since neither A. caninum nor A. braziliense have been shown to occur naturally in indigenous dogs in Scotland and since the climate, particularly with respect to environmental temperature, is generally unsuitable for development of the free-living stages of these 2 parasites, it was confidently assumed that accidental previous or inter-current infections were extremely unlikely. This was further confirmed by the observation that at no time did uninfected and susceptible pups become accidentally infected with any hookworm (including U. stenocephala) while in the isolation premises or experimental kennels. The possibility of prior infection with U. stenocephala, the indigenous canine hookworm of the United Kingdom, was also excluded since at no time were untreated pups found to be excreting hookworm ova, and it was considered unlikely that this hookworm would have been acquired and then naturally expelled within the first 6 or 8 weeks of life (i.e., before purchase of the pups).

Housing

In the isolation premises, the pups were maintained in loose-boxes or sheds with concrete or stone floors and with adequate light and ventilation. The floors were littered with clean straw or wood shavings. In winter, artificial heat was provided by means of suspended area heating lamps.

In the experimental kennels, the cages were incorporated in the structure of the building and were constructed of galvanised sheet metal partitions with weld-mesh door and roof, and with an impervious composition asphalt floor laid on concrete. The cages were cleaned, scrubbed, and scalded with hot water daily, and were thoroughly scrubbed, disinfected and left to dry between each experiment. The pups were confined in the cages in specific experimental groups, the number of pups in each cage being determined by the age and size of the pups and by the experimental segregation. The pups were exercised in open concrete pens, each pen being reserved for pups on a particular experimental treatment. The exercise pens were cleaned and hosed daily.

Nutrition

While under experiment, the pups were fed once daily *ad lib.* with solid food consisting of a mixture of compounded dehydrated dog biscuit containing some dehydrated meat (Digestive terrier meal, Sterling Foods Ltd.,

Oswaldtwhistle, England) and canned cooked meat (Vealox, Warrington Canneries Ltd., Warrington, England). The solid diet, in the proportions offered, had the approximate composition expressed as percentage of the dry matter of: 1% fibre, 43% protein (based on Kjeldahl nitrogen estimation), and 19% oil. Cod liver oil (1-2 ml per pup per day) and sterilised bone flour (2-3 g per pup per day) were added to this food. Each pup, depending on size, was also offered 0.5 to 1 pint of reconstituted full cream dried milk (Glaxo Laboratories Ltd.).

Hookworm species and strain

The culture of *A. caninum* was obtained from the Wellcome Veterinary Research Laboratories, Tunbridge Wells, England, where it had been maintained by subculture in dogs since importation to the United Kingdom following isolation in 1960 at the Wellcome Veterinary Research Laboratories, Nairobi, Kenya, East Africa (W.H.O., 1966). On repeated microscopic examination of adult worms of this culture, their specific morphological statistics were found to conform to that described first by Ercolani (1859), as repeated by Rep (1963).

Culture technique

Each hookworm culture was maintained in previously uninfected pups by means of heavy, yet sub-lethal, infections. The pups were aged 12 to 14 weeks when first infected and were often reinfected when 5 or 6-months-old to ensure continued high worm egg outputs in their faeces. The usual primary culture infection was 1,000 larvae with subsequent reinfection of 2,000 larvae. Larvae were injected by subcutaneous inoculation. Faeces were collected from approximately the 20th day after inoculation of larvae, by which time worm egg counts had usually reached suitable levels to permit culturing for infective larvae (e.g., more than 5,000 e.p.g.).

Faeces were cultured in the dark at 26 or 30°C for 8 or 5 days, respectively, using a modification of the method of Barakat (1951) as described by Jennings et al. (1963). Approximately 0.5 g of faeces were spread on a 4 cm diameter area in the centre of a dry 7 cm Whatman No. 1 filter paper. This was then placed on top of a piece of soft wet plastic foam sheeting, which formed a platform on the base of a 10 cm plastic disposable Petri dish. The diameter of the plastic foam disc was approximately 5 cm. The filter paper was then moistened by spraying with water, the lid was placed on the Petri dish and the dishes were stacked and bound together by cellulose adhesive tape.

Harvest of larvae

To harvest the infective larvae, the contents of the Petri dishes (i.e., paper and plastic discs) and the bases of the dishes were immersed and then rinsed in tepid water (at about 30°C). This fluid was then filtered through a sieve (20 meshes/cm) to remove coarse particulate material. The fluid was then held in tall glass cylinders and the larvae permitted to sediment, after which the supernatant was removed and clean water added. Larvae were washed several times by this method of sedimentation and decantation before being further cleaned by subjecting them to Baermann filtration (Baermann, 1917; Cort et al., 1922). The filter in the Baermann treatment was again a 20-mesh sieve but this was covered by a thin layer of cellulose tissue (Kleenex Medical Wipes®, Kimberly-Clark, England). The Baermann funnel was filled to the level of the sieve with water at about 26-30°C and the concentrated suspension of washed larvae poured carefully onto the surface of the cellulose. About 2-4 hours later, clean larvae were collected as a concentrated suspension from the outlet of the funnel. (The number of larvae that were recovered terminally was equivalent to between 10 and 20% of the number of eggs which had been cultured). During the washing and filtration procedures, the preparation of larvae had been subjected to dilution factors of the vehicle in excess of 10^{-12}.

Counting of larvae

The larval suspension was agitated by pouring several times from one beaker to another and small samples taken immediately. Repeat samples were taken after repeated agitation. The samples were taken with auto-zero pipettes of capacities in the range of 0.02-0.1 ml. Samples were spread immediately on microscope slides and the larvae counted using 10 X magnification through a projection microscope (Projectina Co., Heerbrugg, Switzerland). Motile and non-motile (dead) larvae were counted separately. Sampling was repeated until at least 400 motile larvae had been counted. The concentration of the suspension of larvae was then adjusted to desired values by either adding more fluid or by removing supernatant after sedimentation. After the first count, adjustment in concentration was made where required to permit repetition of the count on the preparation at which time the concentration of live larvae had been set to about 1,000 per ml (900-1,100). From the results of this final count, further readjustments of the volume of vehicle were made to give the desired final preparation and concentration.

For irradiation, the concentration was set in the range of 10,000 to 50,000 motile larvae per ml of deionised water, while for infection the concentration was usually set in the range of 500 to 1,500 motile larvae per ml, in physiological saline for subcutaneous inoculation, or in water for oral administration.

Irradiation

The technique of X-irradiation has been described by Jennings et al. (1963) and Mulligan (1964). The radiation source was a Siemens Stabilipan (Siemens Reiniger-Werke, A.G., Erlangen, W. Germany) operated at 140 kv and 20 ma with external filtration of 0.1 mm copper and 1 mm aluminium producing X-rays of half value layer 8 mm aluminium after filtration. The geometrical arrangements of the irradiation dish containing larvae and the X-ray beam were similar to that described by Mulligan (1964) and were on all occasions the same. During irradiation the larvae were at a concentration of between 10,000 and 50,000 live larvae per ml of deionised water. The temperature during irradiation was the same as the environmental laboratory temperature (range 18-23°C). X-ray dose rate was noted and was always within the range of 650-750 roengtens per minute.

Before commencing irradiation the output of the X-ray unit was calibrated as described elsewhere (Mulligan, 1964) using a Baldwin-Farmer substandard dose meter (Baldwin Instrument Co., Dartford, England), and the time of exposure calculated for the desired total dose of X-rays.

Storage, preparation and inoculation of larvae

Larvae were inoculated subcutaneously, usually at a site behind the shoulder, or were administered orally. Oral administration comprised the

expulsion of the dose of larvae in 1 to 2 ml of water, from a syringe to the anterior pharynx of the dog, followed by a few ml (i.e., less than 5) of water to stimulate further swallowing.

During storage and irradiation, the larvae were maintained as far as possible at room temperature (i.e., in the range of 18-23°C) and all infections were performed with larvae less than 10-days-old (i.e., since harvest from culture).

Faecal examination

Samples of faeces were collected from the floor of the cages and were examined by a salt (saturated sodium chloride) flotation method based on the "McMaster" technique (Gordon & Whitlock, 1939). When hookworm eggs were detected by this method, a modified McMaster technique (Armour, 1967) was used to count the number of hookworm eggs per gram of faeces. In this latter method, samples of the fluid preparation were taken immediately after agitating the suspension in which the eggs would be randomly distributed, while in the flotation method the suspension of filtered faecal material in saturated salt solution was allowed to stand for 5 minutes, after which the surface layer of fluid containing the eggs was removed for examination.

Haematology

Blood samples were obtained by puncture of the cephalic vein with a 20 gauge needle, after shaving the hair and disinfecting the skin with tincture of cetrimide, B.P. (Cetavlon®, Imperial Chemical Industries, Cheshire, England). Blood was collected in glass tubes which contained diamino-ethane tetra-acetic acid (di-potassium salt) as anticoagulant. Packed cell volume or microhaematocrit was measured after centrifuging capillary tubes of blood at 12,000 G. for 6 minutes in a micro-haematocrit centrifuge (Hawksley, London) by a standard technique (Dacie & Lewis, 1966). Haemoglobin concentration was measured colorimetrically (Evans Electroselenium, Harlow, England) after converting to oxyhaemoglobin by a 1 in 200 dilution with 0.04% ammonium hydroxide solution (Dacie & Lewis, 1966). Light absorption was measured at a wavelength of approximately 545 mμ using an Ilford 625 blue-green filter. The colorimeter was first calibrated by the cyanmethaemoglobin method (Dacie & Lewis, 1966) using a commercial cyanmethaemoglobin standard solution (G. Davis Keeler, London, England). For this calibration a series of dilutions of blood in saline were prepared. The haemoglobin concentrations of these dilutions were measured by both alkaline haematin and cyanmethaemoglobin methods. A graph was then prepared, from which the haemoglobin concentrations, corresponding to optical density of alkaline haematin could be measured.

Biochemistry

After determining haematocrit and haemoglobin levels, the remaining blood sample was centrifuged at approximately 4,000 r.p.m. for 10 to 15 minutes. Plasma samples were then used to measure total plasma protein concentration by the Biuret method with colorimetric measurement (Weischelbaum, 1946).

Necropsy and recovery of hookworms

Pups were killed by intravenous injection of pentobarbitone sodium (3 gr/ml). The intestine was removed from pylorus to caudal rectum, opened into a bucket and cooled in a refrigerator for at least 30 minutes before washing with water. The gut was discarded after careful examination for the complete removal of all macroscopic hookworms, and the contents of the bucket were washed over a 20-mesh sieve. The debris remaining on the sieve was examined in small aliquots over a black background using a head-band stereoscopic magnifier (X 2), and the worms were sexed and counted. All worms were also examined at a magnification of at least 10 X to determine the stage of development of males and females and fertility of the female hookworm population. No attempt was made to recover microscopic (i.e., 3rd stage) hookworms, since the pups were killed not less than 14 days after infection by which time A. caninum develops to the adult stage in susceptible pups (Matsusaki, 1950).

Measurements, terminology and statistical analyses

The following terms were used to facilitate comparisons in results between different experiments and between different pups and different groups of pups in each experiment.

Percent take of larvae inoculated measured the infectivity of the larvae and was calculated as,

$$\frac{\text{No. of adult worms recovered}}{\text{No. of motile larvae inoculated}} \times 100$$

Relative infectivity of irradiated larvae measured the degree of attenuation of these larvae compared with normal or unirradiated larvae, the infectivity of the latter being taken as equivalent to 100%. It was calculated as,

$$\frac{\text{\% take of irradiated larvae}}{\text{\% take of unirradiated larvae}} \times 100$$

Protection (%) measured the efficacy of vaccination by comparing worm burdens in vaccinated and control pups after challenge of immunity. It was calculated as,

$$\frac{\text{\% take in controls} - \text{\% take in vaccinates}}{\text{\% take in controls}} \times 100$$

Growth rates

Since breed and ancestry of the experimental pups were indeterminable, the weight data of the pups when the experiments were commenced spread over a wide range. The heavier pups had a potential for realising greater increases of bodyweight in absolute (e.g., lbs/unit time) although not in relative values (i.e., % increase in bodyweight/unit time). Therefore, the pups in each experiment in which weight changes were to be recorded were allocated to experimental groups such that mean group weights were comparable at the start of the experiment (see Appendix III). However, further analysis of the experimental results on the basis of mean group weights failed in most cases to show significant treatment-associated effects. This was a consequence partly of the large range of weights in each group which resulted in excessive standard deviations of the means. In addition, at the individual level the deleterious effects of a standard challenge within groups of pups having widely varying weights was weight-related. This latter factor further extended the range of the group weight data. Accordingly, comparisons based on mean weights (± standard deviation) were utilised only for ensuring uniformity of experimental groups at the start of each experiment, while growth rates were utilised for statistical analysis to determine the consequence of the experimental treatment.

Growth rates of individual pups were calculated as the difference in weight (gain or loss) over a period expressed as percent of the pup's weight

at the beginning of the period. Mean group growth rates (± standard deviation) were then calculated by the algebraic summation of the individual percent change in weight over the appropriate period.

Statistical analyses were based on Student's "t" test (Snedecor & Cochrane, 1967).

SECTION IV

INFECTIVITY OF A. CANINUM AND EFFECT OF ROUTE OF INFECTION ON THE SUCCESS OF ESTABLISHMENT OF THE HOOKWORM

Introduction

Many of the hookworms can establish infection by either the percutaneous or oral routes. In absence of exact quantitative data on the relative success of establishment of the larvae of A. caninum comparing infection by oral administration and subcutaneous inoculation, a preliminary experiment was designed to supply this information.

Experimental Design

Ten uninfected pups were randomised into 2 groups when 8-weeks-old and were each infected with 350 normal A. caninum larvae. To the 5 pups in one group, larvae suspended in physiological saline were inoculated subcutaneously, while to the 5 pups in the other group, larvae from the same preparation were administered orally. Nineteen days after infection, the pups were killed and necropsy worm burdens enumerated.

Results

The success with which the larvae established infection was high (60-80%) and was similar in the 2 groups of pups (Table 1). There was no significant difference between the infections when group mean percent takes (± standard deviation) were compared by Student's "t" test ($P > 0.4$).

Discussion

In spite of the differences in behaviour of larvae, with respect to migration in the body of the dog (i.e., between those given parenterally and orally) the success with which the larvae reached the intestine and maturity was similar. Although this finding has been anticipated by implication in the general statements of the standard textbooks, it has not been properly tested heretofore.

The infectivity of the larvae in this experiment was similar to (Okada, 1931; Okoshi & Murata, 1968), or was at the upper extreme of reported limits (Foster & Cort, 1937; Otto, 1940, 1941), or was greater (Herrick, 1928; Sarles, 1929b; Scott, 1929; Foster & Daensvang, 1932; Foster & Cross, 1934; Krupp, 1961) than that found by previous workers who investigated infections with A. caninum in dogs. The explanation of the lower infectivity of larvae as reported in the prior literature may, as noted above (Section I), be related to the prior infection with hookworm

Table 1. Infectivity, as measured by the number of adult hookworms in the intestine, of *A. caninum* larvae given by subcutaneous inoculation and oral administration to 8-weeks-old pups. Each pup was given 350 larvae and worm burdens were enumerated 19 days after infection.

No. of pups	Route of infection	No. of worms	Mean % take ± s.d.
5	Subcutaneous	173 246 248 262 263	68.1 ± 10.7
5	Oral	214 237 265 281 288	73.4 ± 8.9

of pups and young dogs obtained from ensootic areas, and to the indeterminate age of many of these experimental subjects. In one case (Krupp, 1961) and probably also in others, a delay in the time of termination of the experiment (i.e., more than 50 days) compared with the present procedure (i.e., 19 days) may also have been responsible for apparently lower infectivity figures in the previous literature.

Summary

The infectivity of normal A. caninum larvae, as measured by necropsy worm burdens of adult hookworms on the 19th day after infection, was high (60-80%). There was no significant difference in the infectivities of larvae between those given either orally or by subcutaneous inoculation.

SECTION V

INVESTIGATION OF THE EFFECT OF X-IRRADIATION ON THE INFECTIVE LARVAE OF *A. CANINUM* TO ENABLE SELECTION OF A SUITABLE ATTENUATING DOSE OF RADIATION FOR VACCINE PREPARATION

Introduction

The first step in such investigations is that of determining the effects of various doses of X-rays on the infectivity, pathogenicity and fecundity of the parasite. With consideration to the type of parasite and its life cycle and by analogy with available data from experiments with other nematode parasites (see review of literature), a series of radiation doses was arbitrally selected.

In each of 3 experiments, infective larvae from the same cultures were subjected to irradiation while keeping all other physical and biological factors constant. In this, physical factors were constant within the range that was known, from other sources (Jennings *et al.*, 1963), likely to be compatible with uniformity of results. It was intended that the only variable between different batches of irradiated larvae within each experiment would be that of total dose of X-rays.

In the first 2 of the 3 experiments, irradiated and normal larvae were administered by 2 routes (i.e., subcutaneously or orally) to confirm the findings of the previous section (Section IV) and to determine if there was

any difference in infectivity of irradiated larvae when given by either route. In the third experiment, only the subcutaneous route of infection was employed.

Experimental Design

Fifty-five uninfected pups randomised into 13 groups were infected orally or by subcutaneous inoculation in 3 separate experiments (Table 2) with A. caninum infective larvae after exposure to X-rays at 4 different dose levels (i.e., 20, 30, 40 and 60 kr). In each experiment pups in control groups were infected either orally or by subcutaneous inoculation with unirradiated larvae to determine the normal infectivity of the larvae in each batch before exposure to radiation. In all 3 experiments the pups were 3-months-old at the beginning. They were infected with 1,000 larvae and necropsy worm burdens were enumerated 24-30 days later. Samples of blood and faeces were obtained during this time and their clinical appearances were also observed.

The assessment of X-ray attenuation was made primarily by comparing necropsy worm burdens, the results of haematologic, coprologic and clinical examinations being regarded as confirmatory data.

Results

Data on necropsy worm burdens are recorded in Tables 3, 4 and 5, while mean group haematologic changes are shown in Fig. 3, and mean group faecal

Table 2. Plan of 3 consecutive experiments to investigate the effects of various doses of X-rays on the infective larvae of *A. caninum* and to enable the selection of a suitable attenuating dose for vaccine preparation

Experiment	No. of pups	Radiation dose (kr)	Route of infection
1	4	0	Subcutaneous
	4	0	Oral
	4	20	Subcutaneous
	3	20	Oral
	4	30	Subcutaneous
	4	30	Oral
	4	40	Oral
2	6	0	Subcutaneous
	2	0	Oral
	5	40	Subcutaneous
	4	40	Oral
3	4	0	Subcutaneous
	7	60	Subcutaneous

All infections were made with 1,000 larvae.

Table 3. The effect of irradiating A. caninum larvae with 20 - 40 kr of X-rays and of route of infection on the infectivity of the larvae measured by the number of hookworms which developed in 3-months-old pups. Necropsy worm burdens were enumerated 24 to 30 days after infection.

FIRST EXPERIMENT

Radiation dose (kr)	Route of infection	No. of pups	Mean % take ± s.d.	Relative infectivity (%)
0	Subcutaneous	4	37.6 ± 3.7	100
0	Oral	4	48.2 ± 23.2	100
20	Subcutaneous	4	29.3 ± 1.4	78
20	Oral	3	20.5 ± 2.4	43
30	Subcutaneous	4	15.5 ± 4.2	41
30	Oral	4	18.0 ± 3.1	37
40	Oral	4	6.7 ± 1.9	14

Individual necropsy worm burdens are given in Appendix II, Table 39.

Table 4. The effect of irradiating A. caninum larvae with 40 kr of X-rays and of route of infection on the infectivity of the larvae measured by the number of hookworms which developed in 3-months-old pups. Necropsy worm burdens were enumerated 24 to 30 days after infection.

SECOND EXPERIMENT

Radiation dose (kr)	Route of infection	No. of pups	Mean % take ± s.d.	Relative infectivity (%)
0	Subcutaneous	6	64.2 ± 10.1	100
0	Oral	2	70.2	-
40	Subcutaneous	5	16.2 ± 5.0	25
40	Oral	4	16.2 ± 4.7	23

Individual necropsy worm burdens are given in Appendix II, Table 40.

Table 5. The effect of 60 kr X-irradiation of A. caninum infective larvae on their infectivity measured by subsequent establishment of adult worms in 3-months-old pups. Each pup was given 1,000 larvae by subcutaneous inoculation and necropsy worm burdens were enumerated 25 days after infection.

THIRD EXPERIMENT

Radiation dose (kr)	No. of pups	Mean % take ± s.d.	Relative infectivity (%)
0	4	82.9 ± 12.3	100
60	7	4.7 ± 1.9	6

Individual necropsy worm burdens are given in Appendix II, Table 41.

egg counts are listed in Table 6. The results of the 3 experiments reported under sub-headings were:-

Route of infection

Comparison of the necropsy worm burdens between the pups that were infected orally or by subcutaneous inoculation in each experiment showed that, with one exception, there was no significant difference between these two routes ($P > 0.1$). The exception, and this was based on a marginal number of only 3 observations in one of the 2 comparable groups, was in the first experiment (Table 3) in the burdens of pups given 20 kr-irradiated larvae ($P < 0.01$).

Level of irradiation and worm burdens

As the total dose of X-rays to which the larvae were exposed increased, the size of the necropsy hookworm burdens and the figures for percent take were progressively reduced. When percent take figures were corrected to compensate for variation in normal infectivity of unirradiated larvae between the 3 experiments (i.e., to give a figure for relative infectivity) there was apparently a direct relationship between total X-ray dose and infectivity throughout all three experiments. The mean infectivity (relative) of unirradiated larvae was taken as 100 (for reason see Section III) and this figure was progressively reduced to approximately 60, 40, 20 and 6 as total dose of X-rays was increased through 20, 30, 40 and 60 kr, respectively.

Table 6. Mean group worm egg counts in the faeces of pups infected with X-irradiated A. caninum larvae (experiments 1, 2 and 3).

Radiation dose (kr)	Flotation test positive = days after infection	Mean faecal egg counts (thousands/g)			
		15	20	25	27
0	14	1.8	16	30	40
20	20	-	0.05	0.18	0.2
30	24	-	-	0.15	0.15
40	-	-	-	-	-
60	-	-	-	-	-

Dose of radiation and fertility of worm burdens

Male larvae were more sensitive to radiation than were female larvae, and this disparity between the sexes was progressively accentuated at higher levels of radiation (Appendix II, Tables 39-41). After 60 kr-irradiation of larvae, very few irradiated male worms were recovered at necropsy. At radiation doses of 40 kr and greater the female worms in the resulting population were invariably sterile (Fig. 2). Some sterile female worms were also noted in worm populations from larvae irradiated with 20 and 30 kr of X-rays, while all the female hookworms recovered from the control pups infected with normal larvae were fertile and mature (Fig. 2).

Faeces were collected from the pups at 14, 15, 20, 25 and 27 days after infection. The results of these examinations (Table 6) illustrated the reduced fertility of irradiated worms and the sterility of all female hookworms that had developed from larvae irradiated with 40 and 60 kr of X-rays. Despite the recovery of a considerable number of fertile female worms at necropsy of pups given 20 and 30 kr-irradiated larvae, the reproductive capacity of these worms appear to have been considerably reduced and the length of their pre-patent period, as measured by first detection of hookworm eggs, was extended.

Haematology and clinical signs

Mean group haematocrit and haemoglobin levels (Fig. 3) showed that the pathogenicity of the infections was diminished as the level of irradiation was

FIGURE 2. The normal mature fertile A. caninum female compared with the female worm sterilized by prior irradiation of the larvae with 40 kr of X-rays. (The fertile worm is in the superior position)

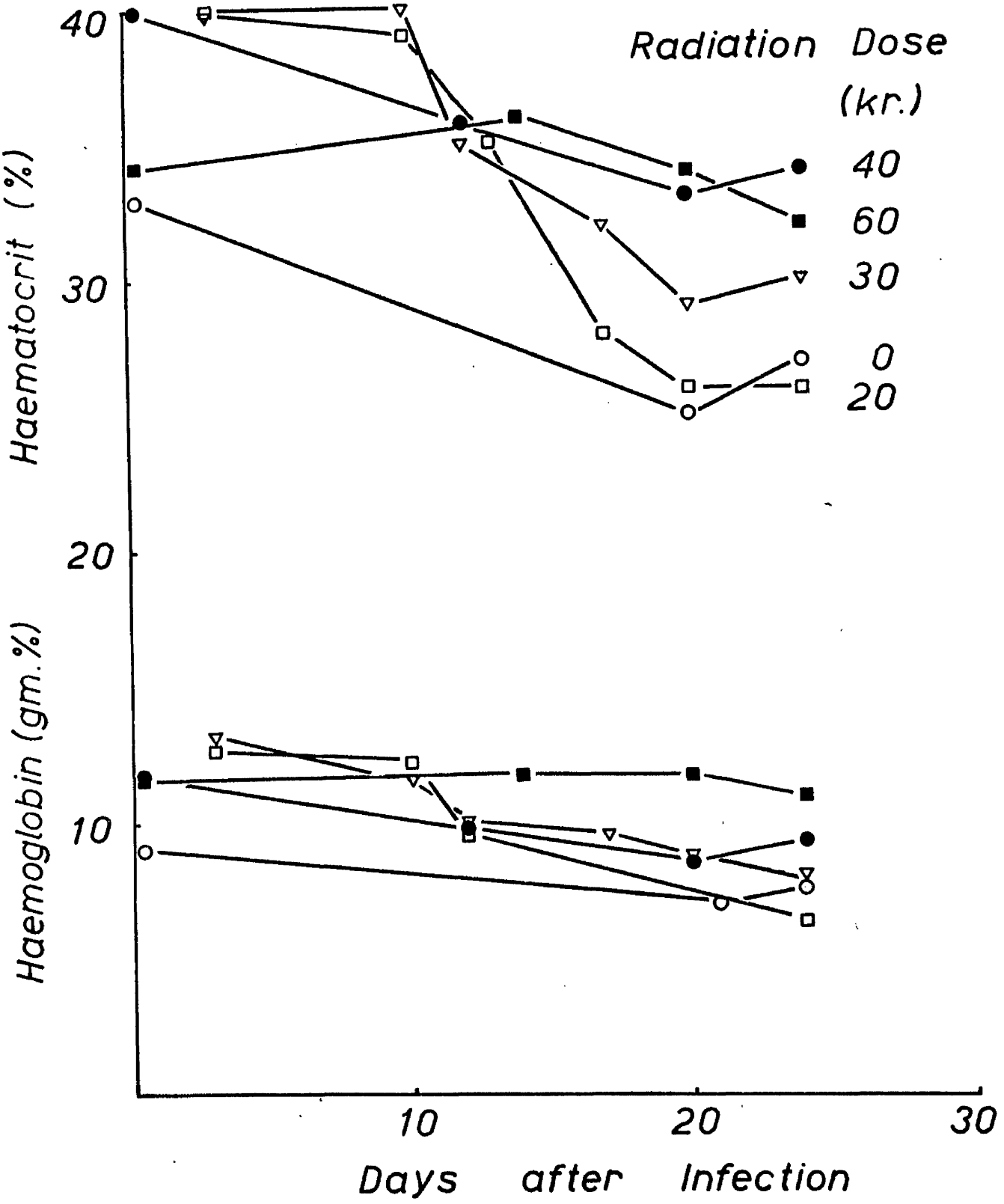

FIGURE 3. Pathogenicity, as measured by mean group haematocrit and haemoglobin values, of the worm burdens established following infection of pups, aged 3 to 4 months, with 1,000 A. caninum larvae exposed to various doses of X-rays.

increased. When pups were infected with larvae irradiated at 20 kr, the haematologic values showed that such hookworms were as pathogenic as were normal hookworms. The pathogenic effects of the worms in pups given 40 and 60 kr-irradiated larvae were mild, being reflected in only negligible reductions in haematocrit and haemoglobin values.

It was extremely difficult to detect adverse changes in appearance, activity and health of the pups infected with larvae irradiated at 40 and 60 kr. Only 3 of 4 pups given oral infections with 20 kr-irradiated larvae survived to the end of the experiment and all the pups infected with 20 kr-irradiated and those infected with normal larvae were severely affected and exhibited the signs of severe acute hookworm disease. These were loss of weight, lassitude, intermittent to continuous blood-stained liquid or semi-liquid diarrhoea, and an extreme pallor of the visible buccal and conjunctival epithelia.

Discussion

The progressive reduction in the success of establishment of the infection, as measured by adult worm burdens, with increase in the dose of X-rays to which the larvae were exposed, was to be expected and was in line with prior reports in the literature (Jarrett *et al.*, 1959, 1960a; Ciordia & Bizzell, 1960; Gordon *et al.*, 1960; Dow *et al.*, 1961; Villella *et al.*,

1961; Hsu et al., 1963; Varga, 1964c; Prochazka & Mulligan, 1965; Kassai et al., 1966). Although the choice of X-ray dose for the attenuation of larvae for vaccine preparation depends principally on the reduction in number of adult worms which would develop, in a parasite such as A. caninum where the primary pathogenic effect is the blood-sucking activity of adult worms, haematologic and clinical measures must also be considered in this choice. The above results indicate that this choice should include 40 kr, 60 kr and possibly even greater quantities of X-rays, since radiation treatment with 40 kr and 60 kr of X-rays reduced the size of the resultant hookworm burdens to apparently safe and satisfactory levels.

The relative pathogenesis of infections with irradiated A. caninum, as measured by changes in haematocrit and haemoglobin values, was diminished as the total X-ray dose was increased above 20 kr in step with the corresponding reductions in worm burdens. Negligible reductions in haematocrit and haemoglobin values were recorded in pups given 40 and 60 kr-irradiated larvae so that a suitable level of radiation selected by these criteria would also be 40 kr or greater. The clinical observations further supported this choice.

Larvae irradiated with 40 kr and greater doses of X-rays gave rise only to sterile female worms so that such a product could be safely used without the possibility of the geographic dissemination of hookworm infection by pups after inoculation with these larvae as vaccine. From the standpoint of practical usage and acceptibility this safety factor is of prime importance.

Sexual sterility of adult worm populations derived from infections with irradiated larvae has been recorded previously (Semrad, 1937; Evans et al., 1941; Katz, 1960; Rick & Keith, 1960; Mulligan et al., 1961; Varga, 1964c; Sokolic et al., 1965; Kassai et al., 1966), although the sterilising dose of radiation has been found to vary from one parasite to another depending on species and the radiosensitive or radiation-treated stage in the life cycle. The increased radiosensitivity of male larvae compared with female larvae noted in the present results has also been recorded in other systems (Ciordia & Bizzell, 1960; Katz, 1960; Mulligan et al., 1961; Ruff et al., 1965; Sokolic et al., 1965).

Results, perhaps more closely related to the present experiments, which Dow et al. reported (1959, 1961) with U. stenocephala showed that this hookworm of dogs was similarly susceptible to X-irradiation as has been demonstrated above in the case of A. caninum. Similar reductions in worm establishment figures at radiation doses of 20 and 40 kr were reported although the sterilising effect of 40 kr of radiation was not observed.

Summary

X-irradiation of the infective larvae of A. caninum reduced the infectivity of the larvae, as measured by subsequent intestinal establishment of adult hookworms. As the dose of radiation was increased, the infectivity of the larvae was decreased and the pathogenicity to the host of the resulting

burden of irradiated hookworms was reduced, as exhibited in the haematologic measurements and clinical findings. Male larvae were more sensitive to the effect of X-irradiation than were female larvae, particularly at the higher levels of radiation. At radiation doses of 40 kr and greater, the female worms in the resulting population were invariably sterile. From these results it was concluded that larvae irradiated with 40 kr or greater doses of X-rays would be more suitable for vaccination experiments.

SECTION VI

THE EFFECT OF SINGLE VACCINATION OF PUPS IN CONFERRING PROTECTION AGAINST CHALLENGE OF IMMUNITY

Introduction

Having determined what dose of X-rays would be likely to induce such a degree of attenuation that pathogenicity during vaccination would be acceptable, a single vaccination experiment was then conducted. This experiment was designed to determine the resistance of singly-vaccinated pups against a severe challenge of immunity.

Experimental Design

Six 3-months-old uninfected pups were vaccinated by subcutaneous inoculation with 1,000 40 kr-irradiated A. caninum larvae. Twenty-eight days later their immunity was challenged by subcutaneous inoculation of 1,100 normal A. caninum larvae. At the same time, the infectivity and pathogenicity of the larvae used for challenge were determined by subcutaneous inoculation of 1,100 larvae to 12 uninfected and unvaccinated control pups.

To control infectivity and degree of radiation attenuation of the irradiated larvae used for vaccination, nine 6 to 8-weeks-old pups in two

groups were inoculated subcutaneously with either irradiated or normal larvae at the time that irradiated larvae were inoculated to the vaccinates. Five pups in one group were inoculated with 1,000 irradiated larvae while the remaining four pups received 500 normal larvae. The reason for using different numbers of larvae to infect the pups in these 2 groups was that 1,000 irradiated larvae of diminished infectivity were necessary to permit recovery of sufficient sterile worms for accurate assessment of attenuation; while 1,000 unirradiated larvae of normal infectivity constituted a lethal infection for 6 to 8-weeks-old pups. These nine pups were killed 21 days after infection and their necropsy worm burdens recorded.

Vaccinated and control pups were observed clinically and their haematocrit and haemoglobin values determined throughout the experiment. Plasma protein values were also recorded. At suitable intervals after inoculation of challenge larvae, samples of faeces were obtained from vaccinated and control pups and were examined firstly by the flotation method. When hookworm eggs were found, the number of hookworm eggs were then counted by the McMaster method. The weights of the vaccinated and control pups were recorded at the time that irradiated larvae were inoculated to the vaccinates, when challenge larvae were inoculated to the pups of both groups and when the vaccinated and surviving control pups were killed at the end of the experiment, or at earlier death of unvaccinated control pups (Appendix III, Table 53). Mean group growth rates of vaccinated and control pups over each period of the experiment were calculated, as specified in

Materials and Methods, and the significance of apparent differences in growth rates were ascertained.

Results

Attenuation

Infectivity and radiation-attenuation control figures (Appendix I, Table 34), calculated from the necropsy worm burdens (Appendix II, Table 42), showed that exposure to 40 kr of X-rays suitably attenuated the larvae which constituted the vaccine (mean takes of normal and irradiated larvae were 48 and 4%, respectively, equivalent to a relative infectivity of the vaccine of 8%). Every female hookworm recovered from the control pups that had been infected with irradiated larvae was sterile, and hookworm eggs were not detected in the faeces of vaccinated pups at any time before inoculation of the challenge infection.

Protection against infection

Single vaccination conferred a highly significant protection ($P < 0.001$) on the vaccinated pups when measured by their ability to resist establishment and development of adult hookworms from the challenge inoculum of normal *A. caninum* (Table 7). At necropsy the 6 vaccinated pups were found to harbour 596 ± 164 worms while the 12 controls had 951 ± 97 worms. As a residue from the single vaccine inocula, a total of 106 sterile irradiated

Table 7. Single subcutaneous vaccination of 3-months-old pups with 1,000 40 kr-irradiated A. caninum larvae with challenge of immunity of the vaccinated and of unvaccinated control pups by subcutaneous inoculation of 1,100 normal A. caninum larvae 28 days later. Necropsy worm burdens were enumerated 22 days after inoculation of the challenge larvae.

Treatment	No. of pups	Mean % take (± s.d.)	Vaccine protection (%)
Control	12	86.5 ± 8.8	0
Vaccinated	6	54.2 ± 14.9	37

Individual necropsy worm burdens are given in Appendix II, Table 43.

hookworms persisted in the intestine of vaccinated pups until the time of necropsy.

Haematology

After vaccination, the vaccinated pups suffered slight depressions of haematocrit and haemoglobin values compared with the, as yet uninfected, controls (Fig. 4). These depressions showed as an interruption of the normal growth rise in haematologic values rather than an absolute depression and, compared with mean contemporary control values, were equivalent to about 10% of the normal values. After inoculation of the challenge larvae, the values of the vaccinates showed further depressions equivalent to 20% of the levels at the time of inoculation of the challenge. This depression was first apparent in the haematologic values of samples taken at 10 days after challenge infection. By comparison, control unvaccinated pups suffered severe depressions of haematocrit and haemoglobin values, the reductions exceeding 50% of the measurement at the time of challenge infection.

Biochemistry

The plasma protein values also changed during the experiment, with depressions after challenge in the values of both vaccinated and control pups being of similar size and proportion to the haematologic changes (Fig. 5).

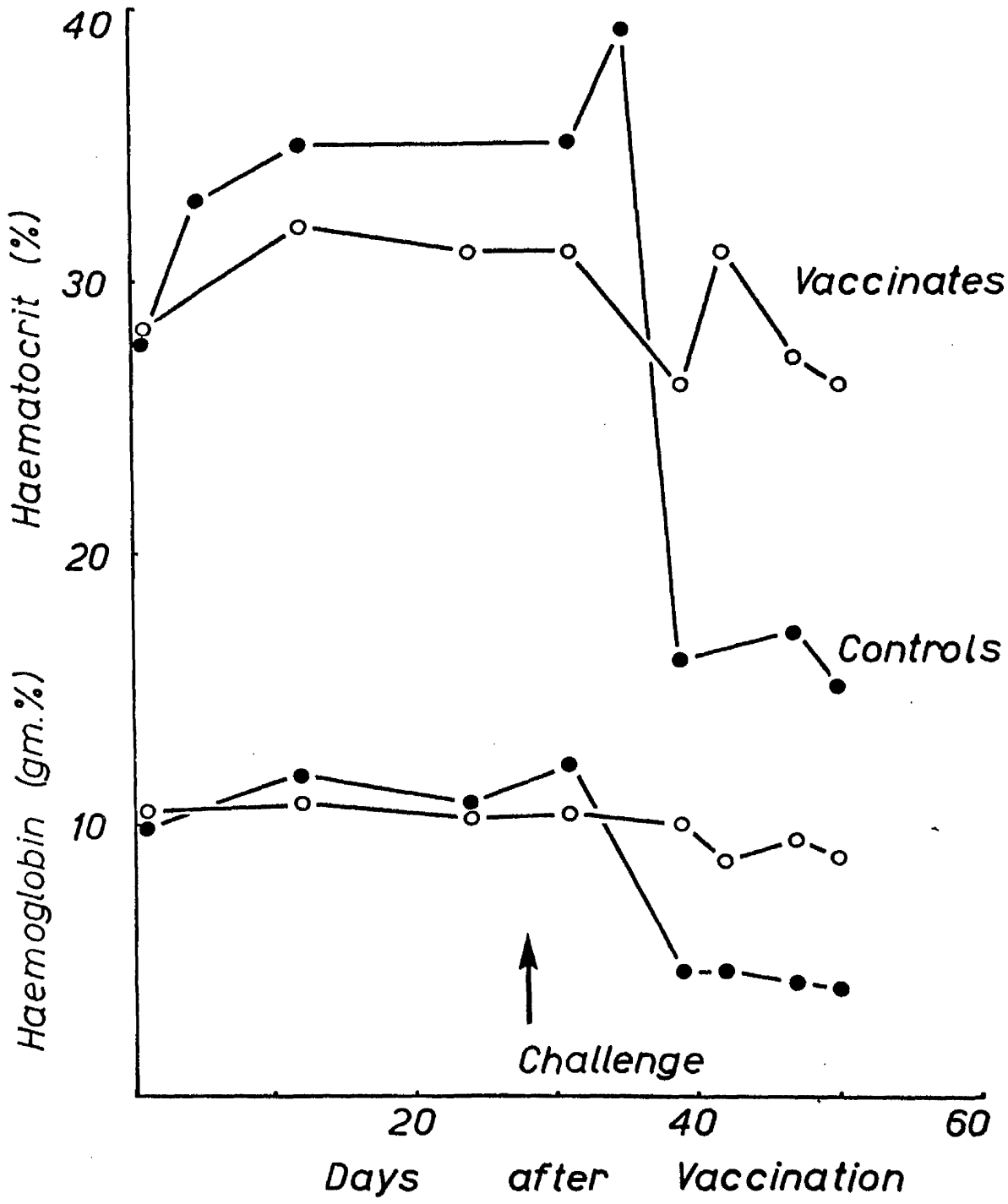

FIGURE 4. Mean group haematologic values of single-vaccinated pups and of unvaccinated control pups.

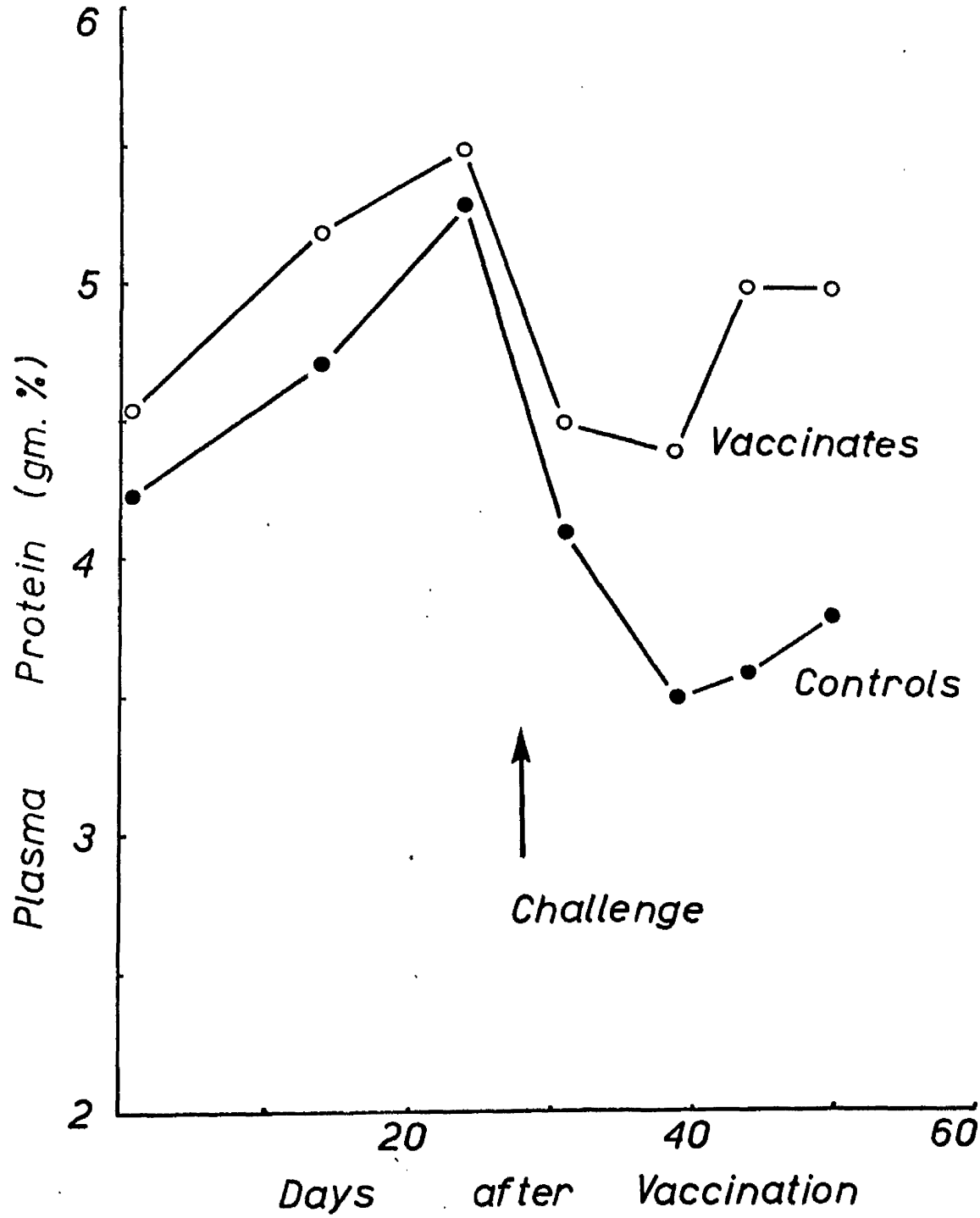

FIGURE 5. Mean group plasma protein values of single-vaccinated and of unvaccinated control pups.

Clinical findings

As a consequence of the severe pathogenesis of the challenge, 5 of the 12 control pups died between the 12th and 22nd days after infection. The surviving control pups and those which died were thus severely affected by the challenge infection and showed severe clinical signs of acute ancylostomiasis. In contrast, all the vaccinated pups survived. They were clinically unaffected before challenge infection and thereafter the only sign of ancylostomiasis was that of occasional diarrhoea since their relatively mild anaemia was undetectable in their clinical appearances.

Weight changes

At the start of the experiment, there was no significant difference in the group body weights of the pups between pups that were to be vaccinated and the controls (Appendix III, Table 53). A significant difference was apparent between the growth rates of vaccinated and control pups only after inoculation of the challenge (Table 8). The vaccination procedure, involving the presence in the vaccinates of a small number of sterile hookworms, did not adversely affect the growth of these pups compared with the uninfected control pups. After inoculation of the challenge infection, the growth rate of the vaccinated pups was reduced compared with their growth rate before challenge, although their post-challenge growth rate was significantly and highly favourable compared with that of the unvaccinated control pups. The pups in this latter group either failed to grow or lost weight in association with their acute ancylostomiasis.

Table 8. Mean group growth rates of single-vaccinated and of control pups were calculated as percent increase or decrease* in weight over each period of the experiment. Statistical analyses of the apparent differences between vaccinated and control pups were by Student's "t" test; the probabilities (P) refer to comparisons of data immediately above and below each statement.

	No. of pups	Growth rate (% ± s.d.) from	
		Vaccination to challenge	Challenge to termination
Controls	12	75 ± 47	* 3 ± 22
		0.3 < P	P < 0.02
Vaccinates	6	53 ± 16	24 ± 9

* Loss of weight

Faecal egg counts

Mean group hookworm egg counts showed large and disproportionate reductions (i.e., compared with relative challenge worm burdens) in the faeces of vaccinated compared with unvaccinated control pups (Table 9). While protection against the establishment of challenge hookworms in vaccinated compared with the control pups was equivalent to 37% (Table 7), relative or per cent reduction in worm egg count was of the order of 60-90%.

Discussion

By all criteria of measurement, it was shown that single vaccination of 3-months-old pups by subcutaneous inoculation of 1,000 40 kr-irradiated larvae conferred on the vaccinated pups a satisfactory resistance to a severe challenge of immunity. The most striking consequence of their resistance was the protection against potential morbidity and mortality of the challenge infection which proved to be an LD_{40} to the controls.

Earlier work, in which the immunity of pups was challenged with normal *U. stenocephala* after single vaccination with 40 kr-irradiated *U. stenocephala* larvae (Dow *et al.*, 1959), furnished results showing a greater level of resistance to establishment and maturation of challenge hookworms than did the present experiment of single vaccination and challenge with *A. caninum*. However, the challenge of immunity in the *Uncinaria* experiment was delayed until 4 months after single vaccination, compared with a delay of only 1 month

Table 9. Mean group hookworm egg counts (thousands per gram) in the faeces of single-vaccinated and of control pups after challenge infection.

Days after challenge	3	11	16	21
Controls	0	0	4.6	123
Vaccinates	0	0	1.7	11

in the present experiment. In view of the epizootiology of canine ancylostomiasis, early experimental challenge was considered to be essential in the present investigation. Clinical ancylostomiasis, including death, is a common problem in young pups so that a prime requirement for any vaccination schedule is that immunity be established at an early age and that this immunity be effective as soon after vaccination as possible.

In addition to the present results and those of Dow et al. (1959), single vaccination with irradiated helminth larvae has been previously shown to be effective in provoking immunity against subsequent challenge infection with normal larvae in some experiments (Jarrett et al., 1959, 1960b; Poynter et al., 1960; Villella et al., 1961; Cabrera & Gould, 1964; Sokolic et al., 1965) but not in others (Smithers, 1962; Miller, 1963).

Summary

A single subcutaneous vaccination of pups when 3-months-old with 1,000 40 kr-irradiated A. caninum larvae conferred a highly significant resistance against the establishment as adult hookworms of normal larvae from a challenge infection given when 4-months-old. Vaccination conferred highly satisfactory resistance also to the potential morbidity and mortality of such a challenge, when measured in terms of haematologic and clinical findings. The most spectacular aspect of the resistance conferred by vaccination was in protecting vaccinated pups against the lethality of the challenge since 5 of the 12

unvaccinated control pups died, while all vaccinated pups survived and were relatively unaffected by their challenge infection.

SECTION VII

THE EFFECT OF DOUBLE VACCINATION AND OF ROUTE OF VACCINATION AND CHALLENGE INFECTION IN CONFERRING PROTECTION AGAINST CHALLENGE OF IMMUNITY

Introduction

Having shown (Section VI) that single vaccination of pups by subcutaneous inoculation of X-irradiated A. caninum larvae was successful in conferring protection against the potential morbidity and mortality of a severe challenge infection, the next logical step was to investigate the efficacy of double vaccination schedules. Since A. caninum larvae can establish infection equally by the subcutaneous and oral routes and since it was considered almost impossible to remove microbial contamination of faecal origin from the larvae before subcutaneous inoculation, it would be preferable that irradiated larvae should be given by the oral route. Also, restriction of the method of challenge infection to the subcutaneous route to the exclusion of the oral route might be considered to be unnatural since the route of challenge under natural conditions is probably both percutaneous and oral. Thus the following 2 experiments were designed primarily to determine the efficacy of a double vaccination schedule by subcutaneous inoculation of irradiated larvae against subsequent subcutaneous challenge of immunity; and secondarily to compare subcutaneous and oral vaccination against challenges of immunity in which larvae were given by either route (i.e., these 2 routes of vaccination and challenge infection were permutated in 4 ways).

Experimental design

This section contains results of two separate experiments in which 1,000 40 kr-irradiated A. caninum larvae were inoculated subcutaneously or were administered orally in double vaccination schedules to pups when 3 and 4-months-old. When the vaccinated pups were 5-months-old, their immunities were challenged by infection, either subcutaneously or orally, with 1,000 normal A. caninum larvae. To control the challenge in each experiment, 5-months-old unvaccinated pups were also infected with 1,000 normal larvae administered by the appropriate method. Oral infection was accomplished by the method described previously (Section III). The plan of the experiments is shown in Table 10.

On each occasion that vaccine was prepared, the infectivities of the normal and of irradiated larvae were determined by enumeration of worm burdens 21 days after infection of 3-months-old pups by subcutaneous inoculation of 1,000 larvae.

In both experiments, haematologic values (i.e., haematocrit and haemoglobin) of the vaccinated pups were recorded at intervals throughout the experiments while those of the unvaccinated control pups were recorded after administration of the challenge infection. The clinical appearances of the pups were also observed throughout both experiments. After subcutaneous inoculation of the challenge infection in the first experiment, clinical observations also included daily measurement of pulse rate over the femoral arteries of pups vaccinated by the subcutaneous route and of their respective controls.

Table 10. Plan of experiments to determine the efficacy of a double vaccination schedule by subcutaneous inoculation of 40 kr-irradiated *A. caninum* larvae against subcutaneous challenge of immunity; and to compare the subcutaneous and oral routes of double vaccination when the challenge of immunity was by either route.

No. of pups	First vaccination	Second vaccination	Challenge
EXPERIMENT 1			
6	S/c	S/c	S/c
5	Oral	Oral	Oral
6	-	-	S/c
6	-	-	Oral
EXPERIMENT 2			
5	S/c	S/c	S/c
5	S/c	S/c	Oral
6	Oral	Oral	S/c
3	-	-	Oral
3	-	-	S/c

S/c = by subcutaneous inoculation

Oral = larvae administered by pipette to the anterior pharynx, as specified in Materials and Methods (Section III).

In the first experiment, the weights of vaccinated and of control pups were recorded at the time of administering the first dose of vaccine, when challenge larvae were given, and finally when the experiment was terminated (Appendix III, Table 54). As previously, growth rates were analysed over the periods from first vaccination to challenge and from challenge to termination, and the significance of apparent differences were determined. Samples of faeces from vaccinated and from control pups were collected at various times after first vaccination, and after challenge, respectively, and were examined by the flotation and quantitative techniques described earlier. Vaccinated and unvaccinated control pups were killed on the 25th day after administering the challenge infections, necropsies performed and their intestinal infections with adult hookworms enumerated. Student's "t" test was employed to determine the significance of apparent differences in worm burdens (expressed as % take of challenge larvae) between the various treatments.

Results

Attenuation

The necropsy worm burdens of the vaccine radiation-attenuation control pups (Appendix II, Table 44) showed that the larvae used to prepare vaccine for both experiments were of high infectivity (mean group percent takes of 59 - 84%) before exposure to X-rays (Appendix I, Table 35). After irradiation

with 40 kr of X-rays, their infectivities were reduced to that previously associated with irradiation at this level (15 - 17% mean group takes, equivalent to 18 - 27% relative infectivity), except for the larvae used for first vaccination in the second experiment (36% mean group take equivalent to 55% relative infectivity). The reason for the greater resistance of these larvae to the effects of radiation was not at that time apparent but may have been related to the concentration of the larval suspension (i.e., number of larvae/ml) during the period of exposure to X-rays. The apparently radiation resistant larvae (relative infectivity 55%) were irradiated at a concentration of 45,000 larvae per ml of suspending fluid, while previous irradiation procedures were performed on larvae at concentrations of 21,000 - 35,000/ml. Irrespective of the relative infectivities of different batches of irradiated larvae (i.e., 18 - 55%), all female worms recovered at necropsy on the 21st day after infection were sterile and at no time were hookworm eggs seen in the faeces of vaccinated pups before expiry of the normal prepatent period after challenge infection with normal larvae (Table 15).

First experiment: Protection against infection

All vaccinated pups exhibited significant protection against challenge infection compared with the respective unvaccinated controls ($P < 0.01$). Comparison of the necropsy worm burdens of vaccinated pups, expressed as percent take (Table 11), showed that subcutaneous vaccination protected pups more effectively against subcutaneous challenge of immunity (88% protection)

Table 11. First experiment to compare subcutaneous vaccination and challenge with oral vaccination and challenge. The pups were vaccinated when 3 and 4-months-old with 1,000 40 kr-irradiated A. caninum larvae. Challenge of immunities of the vaccinated and of unvaccinated control pups was by infection with 1,000 normal A. caninum larvae when they were 5-months-old. Protection from vaccination was measured by necropsy worm burdens which were enumerated 25 days after challenge infection.

Route of administration		No. of pups	Mean % take (± s.d.)	Vaccine protection (%)
Vaccine	Challenge			
Control	S/c	6	77.9 ± 9.7	0
S/c	S/c	6	9.7 ± 6.0	88
Control	Oral	5	86 ± 7.9	0
Oral	Oral	5	33.6 ± 22.1	61

Individual necropsy worm burdens are given in Appendix II, Table 45.

than did oral vaccination against oral challenge (61% protection). This difference in challenge worm burdens between these 2 groups of pups was statistically significant ($P < 0.05$), while there was no difference in challenge worm burdens in unvaccinated control pups between subcutaneous and oral infection ($P < 0.1$).

It is worth noting also that in the pups of the subcutaneous-vaccination-and-challenge group all challenge worm burdens were small (97 ± 60 worms), and hence protection associated with this procedure was uniform and of a high order. In contrast (Appendix II, Table 45), the numbers of challenge hookworms (336 ± 221 worms) in vaccinated pups of the oral-vaccination-and-challenge group varied widely since only 2 of these 5 vaccinated pups harboured small numbers of hookworms, while the other 3 had large challenge worm burdens (i.e., worm burdens that were individually equivalent to less than 50% protection). Oral vaccination thus conferred a significantly inferior level of immunity against oral challenge and this immunity varied widely between individual orally-vaccinated pups.

First experiment: Haematology

The haematologic results (Fig. 6) showed that although pups of both groups exhibited variations in mean haematocrit and haemoglobin levels after first vaccination, reductions in these values were small. After challenge the haematologic values of the control pups, with a mean worm burden of 820 normal hookworms, were reduced to less than half their

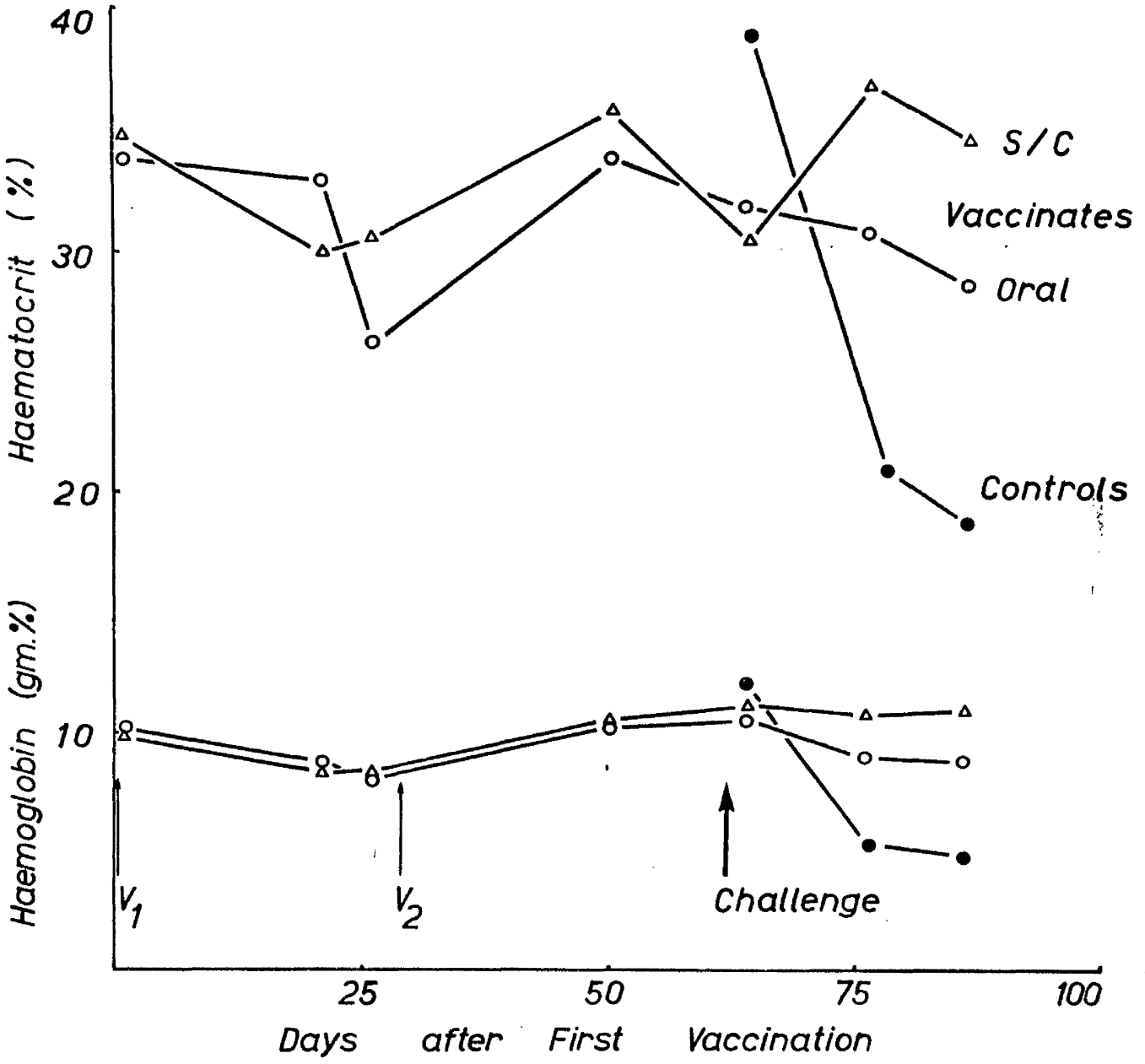

FIGURE 6. Mean group haematologic values during vaccination and challenge of double vaccinated pups (routes of administration of larvae shown), and of unvaccinated controls after challenge of immunity in the first experiment.

previous levels. After challenge the pups of the subcutaneous vaccine group, with means of 97 normal and 18 sterile hookworms, experienced a rise in haematologic values while pups of the oral vaccine group, with means of 336 normal and 16 sterile hookworms, experienced only slight and insignificant reductions in their haematologic values (less than 10% of at-challenge levels). Thus while unvaccinated pups in the control groups were severely affected by their infection, as reflected in the haematologic results, the vaccinated pups were for all practical purposes unaffected. Even pups that were vaccinated and to which challenge larvae were given by the oral route, and which harboured large challenge worm burdens, suffered only mild and disproportionate reductions in haematocrit and haemoglobin values.

First experiment: Clinical findings

The clinical findings of the vaccinated pups during the period from first vaccination to inoculation of the challenge did not deviate from normal except for the passage of an occasional semi-fluid stool, the significance of which event was not apparent although it may have been associated with the presence of irradiated hookworms. After challenge infection, clinical differences between vaccinated and control pups were striking, as exemplified by the data in Table 12. The control pups had signs of severe acute ancylostomiasis, whereas the vaccinated pups were practically unaffected.

Table 12. Clinical findings after subcutaneous inoculation of the challenge infection to double-vaccinated and unvaccinated control pups of the first experiment. (Only vaccinates that received irradiated larvae by subcutaneous inoculation were included.)

	Activity	Lassitude	Diarrhoea	Dysentery	*Epithelia	**Pulse rate at rest (mean ± s.d.)
Controls	Depressed	Profound from 10th day	Severe, fluid	Severe from 10th day	White	138 ± 12.7
Vaccinates	Normal	-	Slight, occasional	-	Pink	118 ± 3.6

* Epithelial colour observed in conjunctival and in unpigmented buccal areas (gums, excluding tongue).

** Calculation based on twice daily measurements of pulse rate at rest during the period 10-20 days after challenge.

As regards pulse rate measurements taken at rest, the vaccinated and control pups in these experiments were used also in an isotope experiment and quickly became accustomed to daily withdrawal of blood samples in connection with isotope measurements. In these circumstances and due to frequent handling of the pups, counts of pulse rate over the femoral artery could be made with reasonable repeatability of result. Although these measurements in the controls were taken over the 10-20th day, initial measurements were closer to normal, becoming increasingly erratic, with the rate accelerating towards the end of this time as their anaemia developed. In spite of the severe signs of ancylostomiasis in the 5 to 6-months-old unvaccinated controls, all these pups survived to the end of the experiment.

Although many of the orally-vaccinated pups were found at necropsy to be harbouring much larger worm burdens from their orally-administered challenge larvae, there was no appreciable divergence in clinical findings between these pups and those that were vaccinated and to which challenge larvae were given by subcutaneous inoculation. Thus the haematologic findings following challenge of vaccinated pups in these 2 groups were reflected in the clinical data, although the detailed clinical measurements (Table 12) were derived from observation of only the subcutaneously-vaccinated pups.

First experiment: Weight changes

Growth rates were calculated for each period of the experiment (i.e., from first vaccination to challenge, from challenge to termination)

Table 13. Mean group growth rates of double vaccinated and of control pups in the first experiment which compared the efficacies of subcutaneous vaccination and challenge with oral vaccination and challenge. Growth rates were calculated as percent increase or decrease* in weight over each period of the experiment. Statistical analysis of apparent differences were by Student's "t" test; the probabilities (P) refer to comparisons of data immediately above and below each statement.

Route of infection		No. of pups	Growth rate (% ± s.d.) from	
Vaccine	Challenge		First vaccination to challenge	Challenge to termination
Control	S/c	6	111 ± 41	8 ± 21
			0.5 < P	0.6 < P
S/c	S/c	6	67 ± 48	19 ± 13
			0.3 < P	0.05 < P
Oral	Oral	5	98 ± 49	* 3 ± 20
			0.7 < P	0.6 < P
Control	Oral	5	131 ± 104	8 ± 13
			0.6 < P	0.4 < P
Control	S/c	6	111 ± 41	8 ± 21

* Loss of weight

For ease of comparison the first group of results have been repeated at the foot of the table.

and the significance of apparent differences were determined (Table 13). There were no significant differences in growth rates between any of the groups. Thus vaccination did not interfere with growth; while the challenge infection, in spite of inducing relatively severe depressions in haematologic values and adverse clinical signs, failed to reduce the growth rates of the controls.

Second experiment

In the second experiment in which routes of vaccination and challenge were permutated between subcutaneous and oral infection there were no significant differences either in necropsy worm burdens (Table 14, $P > 0.1$), in haematologic findings (Fig. 7), or in clinical observations between any of the 3 groups of vaccinated pups. The normal larvae used for challenge infection were not of such high infectivity as in the first experiment, and there was no significant difference in infectivity between larvae given by either route to the control pups ($P > 0.9$). The differences between challenge worm burdens in the vaccinated pups of all three groups (mean group worm burdens of 15 - 88) and those in the appropriate control pups (539 - 549 mean worm burdens) were highly significant ($P < 0.001$). Protections within each of the 3 groups of vaccinated pups were also quite uniform, even within the group in which the pups were vaccinated orally and immunity was challenged by subcutaneous inoculation.

Even though the infectivity of the challenge larvae was diminished, the reductions in haematologic values of the unvaccinated control pups were

Table 14. Second experiment to compare vaccination and challenge by permutations of the oral and subcutaneous routes of infection. The pups were double vaccinated when 3 and 4-months-old with 1,000 40 kr-irradiated A. caninum larvae. Challenge of immunity of the vaccinated and of unvaccinated control pups was by infection with 1,000 normal A. caninum larvae when they were 5-months-old. Protection from vaccination was measured by necropsy worm burdens which were enumerated 25 days after challenge infection.

Route of administration		No. of pups	Mean % take (± s.d.)	Vaccine protection (%)
Vaccine	Challenge			
Control	S/c	3	54.9 ± 1.6	0
S/c	S/c	5	4.1 ± 4.8	93
Oral	S/c	6	8.8 ± 9.8	84
Control	Oral	3	53.9 ± 13.3	0
S/c	Oral	5	1.5 ± 0.5	97

Individual necropsy worm burdens are listed in Appendix II, Table 46.

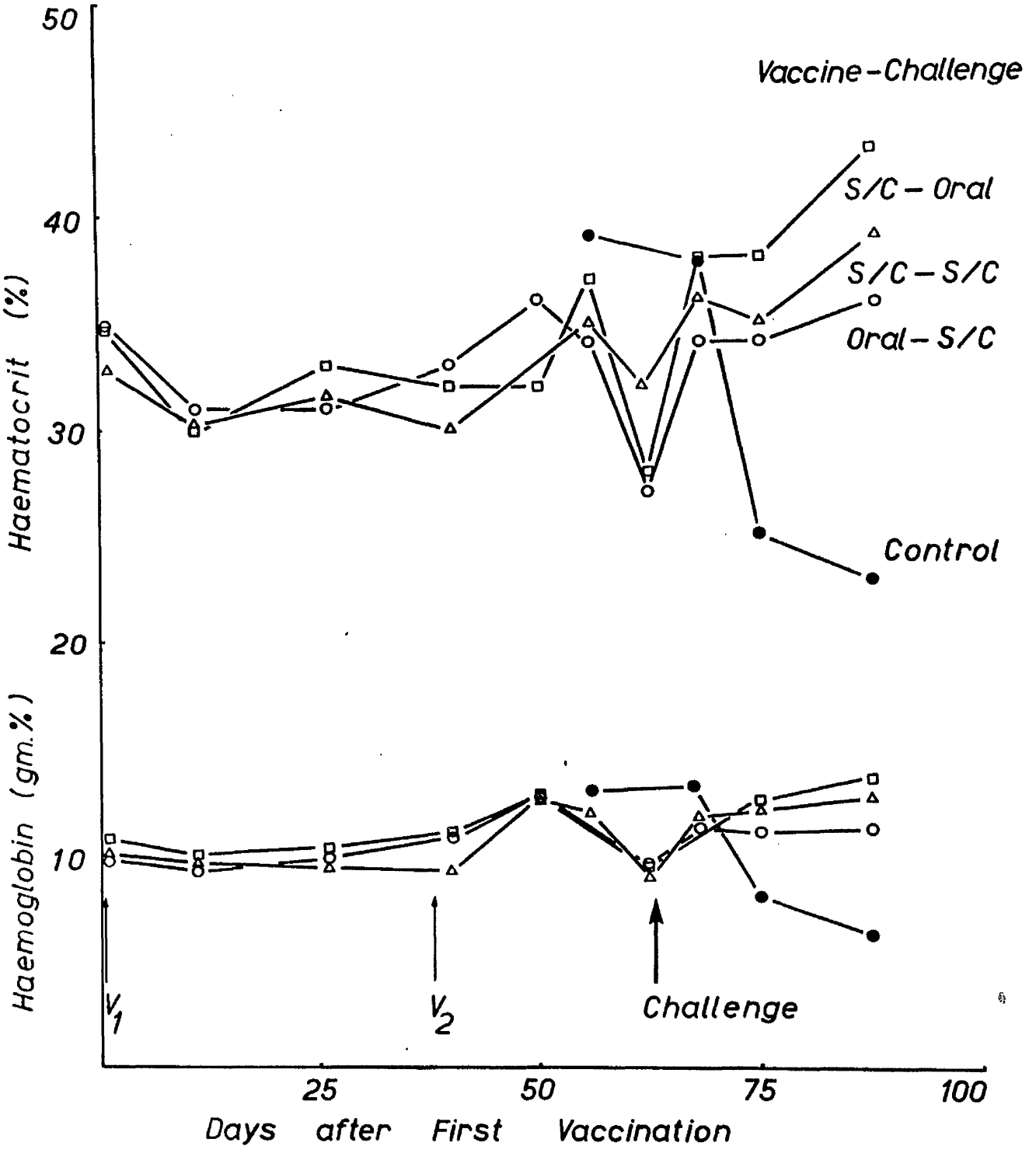

FIGURE 7. Mean group haematologic values during vaccination and challenge of double vaccinated pups (routes of administration of larvae shown), and of unvaccinated controls after challenge in the second experiment.

considerable (Fig. 7) and were associated with clinical signs of acute ancylostomiasis, while the vaccinated pups of all three groups were unaffected in both haematologic values and in clinical appearances. The weights of the pups were not recorded in this experiment.

Both experiments: Faecal egg counts

In both experiments the results of faecal examinations (Table 15) showed that mean group hookworm egg counts reflected, in an approximate fashion, the numbers of fertile hookworms in pups of each group. The onset of patency (i.e., first detection of hookworm eggs in the faeces) in vaccinated pups of the second experiment was delayed. In the second experiment eggs were not detected (even by the flotation method) at any time in the faeces from pups that had been vaccinated by the subcutaneous route and then challenged orally, although these pups each harboured 15 (mean) normal mature A. caninum, of which 10 (mean) were fertile female worms.

Discussion

Attenuation

A number of the observations recorded above require discussion to a fuller degree than given to them so far. One of these is variation in the degree of radiation-induced attenuation of larvae, particularly the high

Table 15. Mean group hookworm egg counts (thousands per gram) in the faeces of vaccinated and of control pups after challenge infection.

Days after challenge	6	14	19	25
EXPERIMENT 1				
Challenge controls - S/c	0	0	17.2	24.7
Challenge controls - Oral	0	0	4.9	47.4
S/c vaccine - S/c challenge	0	0	3.2	0.9
Oral vaccine - oral challenge	0	0	0.7	9.0
EXPERIMENT 2				
Challenge controls - combined	0	0	21	40
S/c vaccine - S/c challenge	0	0	0	1.7
S/c vaccine - Oral challenge	0	0	0	0
Oral vaccine - S/c challenge	0	0	0	2.4

infectivity of the larvae used for first vaccination in the second experiment. It seemed possible that the low attenuation or high infectivity of these irradiated larvae was associated with the concentration at which they were suspended in water during the time that they were exposed to X-rays, since larval concentration is likely to influence oxygen tension and the latter has been shown to be important in determining radiosensitivity of biological tissues (Bacq & Alexander, 1961). Thus at higher larval concentrations the amount of available oxygen in solution is likely to be diminished so reducing the susceptibility of the larvae to the effects of ionising radiation. Increase in temperature, presumably by increasing the respiratory function in poikilothermic nematode larvae and by depleting the available oxygen, has a similar effect. These factors would serve to increase the infectivity of attenuated nematode larvae (Fitzpatrick, personal communication).

Jennings et al. (1963) showed that variation in larval concentration did not affect radiosensitivity of N. brasiliensis larvae until the concentration of larvae was reduced below 1,500 per ml. They also showed that at concentrations between 9,000 and 50,000 per ml a uniform attenuation was produced by a standard dose of X-rays. Therefore in the present series of experiments with A. caninum, in absence of more exact information at the time that the experiments were planned and executed, this range of 9,000 to 50,000 was selected in expectation that standard attenuation would be achieved. However, it seemed that the findings from experiments with N. brasiliensis larvae may not be extrapolated freely to A. caninum larvae. This has been

further confirmed and retrospective explanations are now available from work done subsequent to the series of experiments reported in this dissertation (Miller, unpublished data) to account for some of the earlier variations in radiosensitivity of A. caninum larvae.

Single or double vaccination

The results showed that subcutaneous vaccination with 2 doses of 40 kr-irradiated A. caninum larvae was more than twice as effective as was single subcutaneous vaccination (Section VI). Measured as protection against the establishment of adult hookworms from the inoculation of normal challenge larvae, double subcutaneous vaccination prevented 88 to 97% of these larvae from reaching maturity (Tables 11, 14) while in single-vaccinated pups only 37% of the challenge larvae were prevented from maturing (Table 7). However, this advantage of double over single subcutaneous vaccination was not reflected in the comparison of post-challenge weight gains (Tables 9, 13), nor was the advantage so striking in the haematologic data (Figs. 4, 6, 7) since all singly and doubly vaccinated pups resisted equally the potential pathogenesis of their challenge infection. Since hookworms do not multiply within the host, this superiority of double compared with single vaccination was not entirely unexpected, particularly as the most important immunogenic phase of hookworm infection may be brief and be associated with migrating larvae (see below).

Superior efficacy of double compared with single vaccination has been a frequent finding in previous vaccination experiments in which X or γ-ray-attenuated helminth larvae have been employed to stimulate immunity (Jarrett et al., 1958, 1961b; Dow et al., 1959, 1961; Poynter et al., 1960; Villella et al., 1961; Smithers, 1962; Cabrera & Gould, 1964; Sokolic et al., 1965). Whether this superior immunogenic efficacy was associated with increased antigenic stimulation or with the longer time interval between first vaccination and challenge infection (Villella et al., 1961) was not in most cases apparent, although in experiments for which protocols permit adequate comparison (Dow et al., 1959, 1961; Sokolic et al., 1965) it seemed that both factors contributed.

Route of vaccination and of challenge infection

Yokagawa and Oiso (1926), Foster and Cross (1934) and Matsusaki (1950) showed that following administration of A. caninum infective larvae to dogs by stomach tube or by oesophageal fistula, the majority of larvae matured in the alimentary tract without undertaking the somatic migration via lungs, trachea and oesophagus. Garoian (1957) showed that infective larvae of A. caninum would mature in a Thiry fistula and that, following this method of infection, hookworms were not recovered elsewhere in the intestines (i.e., indicating that the larvae did not leave the intestinal pouch, or undergo somatic migration). Shirai (1926) reported experiments which showed that less than 0.35% of larvae administered by stomach tube

could be recovered from the lungs. He also claimed that following oral infection, as constituted by pipetting larvae into the mouth, some of these larvae could be demonstrated histologically in the buccal, pharyngeal and oesophageal sub-epithelial tissues at 3 hours after infection, and also in the lungs at 24 hours. Fulleborn (1925, 1929) and Nagoya (1931), however, suggested that lung migration following oral infection was obligatory. It seems likely that confusion as to what each author meant by "oral" or "per os" infection was responsible for much of the initial controversy as to whether lung migration was necessary for development of A. caninum in dogs following these methods of infection. In the present experiments with oral infection in which larvae were first exposed to the pup in the buccal cavity, anterior pharynx and oesophagus, it is likely that a variable proportion of the dose of larvae would penetrate the epithelia of these cavities and undergo somatic migration. Variation in the proportion of larvae following each pathway may serve to explain some of the variations in the results, particularly in the oral vaccine - oral challenge group (see Table 16 and discussion in the next paragraph).

A review of the results of the 2 experiments (Table 16) indicated that the most uniform and effective protection, in terms of resistance to establishment of hookworms from the challenge infection, was exhibited by pups that had been vaccinated by subcutaneous inoculation of irradiated larvae, while oral vaccination was less efficacious. Subcutaneous vaccination was highly effective in controlling the challenge when this was administered by either route, while orally vaccinated pups were apparently more resistant to subcutaneous than to oral challenge infection. It is

Table 16. Summary of the degree of protection against challenge hookworm infection, correlated with the amount of somatic migration by the larvae of vaccine and challenge depending on the route of administration of the larvae.

Route of infection		Vaccine protection	Somatic exposure	
Vaccine	Challenge	(%)	Vaccine	Challenge
S/c	S/c	88	Entire	Entire
S/c	Oral	97	Entire	Variable/ minimal
Oral	S/c	84	Variable/ minimal	Entire
Oral	Oral	60	Variable/ minimal	Variable/ minimal

interesting to speculate that the presence or absence of somatic migration (i.e., via the lungs) may be correlated with the relative efficacies of immunogenesis following vaccination by the different routes (Table 16). Where total dose lung migration occurred (i.e., following subcutaneous vaccination) immunogenesis was maximal. When only a small, undetermined, and variable amount of migration occurred (i.e., following oral vaccination) immunity tended not to be so effective. This was true only when the challenge larvae were also administered orally.

When 40 kr-irradiated A. caninum larvae were administered by subcutaneous inoculation, between 75 and 90% of the larvae appeared to have become arrested or to have died before they reached the intestine. This arrest or death of larvae may have occurred in the lungs, as was shown to be the case when irradiated larvae of N. brasiliensis were given to rats (Jennings et al., 1963). Therefore it seemed probable that arrest and/or death of immature irradiated larvae at the migratory stage in a somatic location was responsible for stimulating a maximal immunogenic response (i.e., after subcutaneous inoculation of vaccine). This hypothesis was further supported by the finding that subcutaneous vaccination with normal infective larvae failed to stimulate as high a level of resistance as did subcutaneous vaccination with irradiated larvae (Section VIII). Following subcutaneous inoculation of normal larvae, a very large proportion of the larval dose (60-70%) reached the intestine in what would appear to be the shortest possible time, with a relatively small proportion being delayed or lost on route through the lungs.

The difference in immunity following oral vaccination with irradiated larvae between pups whose immunities were challenged by the oral compared with the subcutaneous route (the latter pups were more resistant to challenge worm establishment) may indicate that the apparently inferior immune response following oral vaccination was more effective in destroying challenge larvae that were migrating through the pups' tissues (i.e., after subcutaneous inoculation) than when these larvae were maturing in the alimentary tract. The irregularity of the individual results (i.e., challenge worm burdens) in some of the pups that were vaccinated and given a challenge infection by the oral route may have been a function of the proportion of vaccine and/or challenge larvae which, following oral inoculation, succeeded in penetrating the epithelia of the mouth, pharynx and oesophagus thence to undergo lung migration in the individual pup. In the first experiment within the orally-vaccinated and orally-challenged group, 2 of the 5 pups were protected against challenge as effectively as were pups that received both vaccine and challenge larvae by subcutaneous inoculation, while the other 3 pups were almost as susceptible to establishment of the challenge hookworms as were unvaccinated pups in the control group.

Summary

Double vaccination was more than twice as effective as was single vaccination when vaccine and challenge larvae were administered by subcutaneous

inoculation, when efficacy was measured by resistance to the establishment of adult hookworms from the challenge infection. Double subcutaneous vaccination conferred equal protection against subcutaneous and oral challenge while double oral vaccination, although not so effective against oral challenge, conferred satisfactory protection against subcutaneous challenge. However, in terms of resistance to the potential morbidity of the challenge infection, both methods of vaccination were equally effective compared with the effects of challenge infection on unvaccinated control pups. The results would support the hypothesis that the arrest of X-irradiated larvae in some somatic location on their migratory route, following subcutaneous inoculation, produced a better immunogenic stimulation of the host, and that when the larvae of the challenge infection underwent this somatic migration they were more adequately exposed to the immune response of the host.

SECTION VIII

COMPARISON OF THE IMMUNOGENIC EFFICACIES OF IRRADIATED AND OF NORMAL *A. CANINUM* LARVAE

Introduction

It has been shown that vaccination of pups with 40 kr-irradiated *A. caninum* larvae stimulated an effective immunity against the establishment of adult hookworms from a challenge infection, the best protection being that conferred by two doses of vaccine inoculated by the subcutaneous route. There is also a considerable amount of evidence that infection with unirradiated or normal larvae may stimulate a high degree of immunity (see Section I). In other host-parasite systems in which comparisons have been made between vaccination with irradiated and with normal larvae, it has been a frequent finding that the former procedure induced a superior resistance against challenge infection (see discussion of this section for review of published work).

This section reports an experiment in which double vaccination by the subcutaneous and oral routes with irradiated larvae was compared with similar regimens of vaccination with normal *A. caninum* larvae. Since this experiment was conducted at the same time as experiment 1 of the previous section (VII), the data from challenge control pups and those vaccinated with irradiated larvae were common to both experiments and recur in the tables in this section.

Experimental design

The plan of experiment is illustrated in Table 17. Thirty-eight 3-months-old pups were segregated into 2 primary groups. To the 20 pups in one primary group all infections were made by subcutaneous inoculation while to the 18 pups in the other group all infections were by the oral route.

Each of the 2 primary groups were further subdivided to 4 subgroups. The pups of the first subgroup were retained to control the challenge infections. The pups of the second subgroup were twice vaccinated with irradiated larvae, while those of the third subgroup were twice vaccinated with normal larvae. The immunities of these vaccinated pups were then challenged by infection with normal larvae.

The pups in the last subgroup were twice vaccinated with normal larvae but were not given a challenge infection, their purpose being to control the efficacy of the anthelmintic treatment which was given to all pups 27 and 28 days, respectively, after first and second vaccination with normal larvae. The anthelmintic was thenium p-chlorobenzene sulphonate (Ancaris®, Burroughs Wellcome & Co., London). The treatment schedule comprised 2 doses, each of 250 mg thenium base administered as recommended by the manufacturers. Since "Ancaris" is a compound tablet, the pups also received 1 g piperazine hexahydrate at each double treatment. The pups that were vaccinated with irradiated larvae were not treated with anthelmintic.

Table 17. Plan of experiment designed to compare the immunogenicity of irradiated with normal A. caninum larvae by double vaccination of pups via the subcutaneous and oral routes. All vaccine and challenge infections comprised 1,000 larvae. Pups vaccinated with normal larvae were treated with anthelmintic 27 and 28 days after first and second vaccinations, respectively.

No. of pups	Double vaccination		Anthelmintic treatment	Route of challenge
	Larvae	Route		
6	Control			S/c
6	Irradiated	S/c		S/c
6	Normal	S/c	+	S/c
2	Normal	S/c	+	Control
5	Control			Oral
5	Irradiated	Oral		Oral
6	Normal	Oral	+	Oral
2	Normal	Oral	+	Control

The pups were vaccinated with 1,000 normal or irradiated larvae when 3 and 4-months-old; and when 5-months-old the immunities of the surviving vaccinated pups and of the control pups were challenged by infection with 1,000 normal larvae. Necropsy worm burdens were determined 25 days after challenge infection.

The numbers of sterile female hookworms from the irradiated vaccine, which persisted in pups that were vaccinated with irradiated larvae, were determined by microscopic examination and were then discounted from total worm burdens to derive a figure for challenge worm burdens in these pups. The numbers of normal hookworms which had resisted anthelmintic treatment were recovered at necropsy of the pups of the fourth subgroups and were also discounted to give a figure for challenge worm burdens in the pups that were vaccinated with normal larvae. The challenge worm burdens of vaccinated and of control pups, expressed as mean group percent take, were compared and the significance of apparent differences were determined.

On each of the two occasions that vaccines (i.e., X-irradiated and normal larvae) were administered, 3-months-old uninfected pups in 2 further groups were infected by subcutaneous inoculation. The pups of one group were infected with irradiated and those of the other with normal infective larvae. At necropsy of these pups on the 21st day after infection, their worm burdens, expressed as mean group percent take and as relative infectivity, served to control the infectivities of the normal and irradiated larvae that were used as vaccine.

Haematologic values of the vaccinated pups were recorded at suitable intervals throughout the experiment while the values of the control pups were recorded after challenge infection. Weights of vaccinated and of challenge control pups were recorded when the first dose of irradiated or unirradiated vaccine was administered, when challenge larvae were given, and finally when the experiment was terminated (Appendix III, Table 55). As previously, growth rates were analysed over the vaccination and challenge periods and the significance of apparent differences determined. Samples of faeces from vaccinated and from control pups were examined at various times after first vaccination. The clinical appearances of the pups were observed throughout the experiment.

Results

Attenuation

The infectivities of the normal and irradiated larvae used as vaccine (Appendix I, Table 36) were satisfactory. Before irradiation, the larvae were of high infectivity and attenuation by 40 kr of X-rays was similar to that recorded previously. At no stage, during vaccination and before the appropriate prepatent period after the challenge infection, were hookworm eggs detected in faeces of the pups being vaccinated with irradiated larvae (Table 18), and all female hookworms recovered from vaccine control pups that were infected with irradiated larvae were sterile. Pups being vaccinated with normal larvae passed large numbers of hookworm eggs in their faeces after

Table 18. Mean group hookworm egg counts (in thousands per gram) in the faeces of vaccinated pups after first vaccination and before challenge infection. The results of pups that were vaccinated with the same preparation have been combined, irrespective of the route of vaccination.

Vaccine	Days after first vaccination						
	21	26	30(V_2)	36	53	56	62(C)
Irradiated	0	0	0	0	0	0	0
Unirradiated	33	48	0.7	0.2	25	14	1.4

V_2 = Second vaccination; C = Challenge infection

first infection, and the effect of anthelmintic treatment on their egg counts was apparent in the samples examined on the 30th, 36th and 62nd days after first infection.

Protection against infection

The necropsy worm burdens, expressed as percent take, from the challenge infection of normal A. caninum larvae and statistical analyses of the apparent differences between these values are listed in Tables 19 and 20, respectively. The worm burdens in all groups of vaccinated pups were significantly smaller than were those in the controls. However, only the pups that were vaccinated by subcutaneous inoculation of X-irradiated larvae were uniformly and highly resistant to the establishment of adult hookworms from the challenge infection (for individual worm burdens, see Appendix II, Table 47). The resistance of individual pups in the other 3 groups of vaccinates (i.e., that were vaccinated orally with irradiated larvae or by either route with normal larvae) varied widely; and the mean group protection figures (57-61%) were lower than the mean figure for protection in the pups that were vaccinated by subcutaneous inoculation with irradiated larvae (88%). Within each of the 3 groups, some of the vaccinated pups were highly resistant (i.e., protection of about 90%), some were partially resistant (about 60% protection) while others had challenge burdens that were almost as large as were those in the unvaccinated control pups.

Table 19. Double vaccination of pups when 3 and 4-months-old with 1,000 *A. caninum* larvae, to compare the immunogenic efficacies of 40 kr-irradiated and of normal larvae against challenge infection with 1,000 normal larvae when 5-months-old. Protection from vaccination was measured by necropsy worm burdens which were enumerated 25 days after challenge infection.

Vaccine larvae	Route of vaccination & challenge	No. of pups	Mean % take (± s.d.)	Vaccine protection (%)
Control	Subcutaneous	6	77.9 ± 9.7	0
Irradiated	Subcutaneous	6	9.7 ± 6.0	88
Normal	Subcutaneous	3	33.2 ± 19.7	57
Control	Oral	5	86.0 ± 7.9	0
Irradiated	Oral	5	33.6 ± 22.1	61
Normal	Oral	4	30.0 ± 30.2	65

Discrepancies in the number of pups in the groups that were vaccinated with normal larvae, compared with the numbers in Table 17, were a consequence of mortality from the vaccination before the time of challenge infection.

Individual necropsy worm burdens, including those of the anthelmintic control pups, are given in Appendix II, Table 47.

Table 20. Results of statistical analysis (Student's "t" test) to determine the significance of apparent differences in challenge worm burdens between the different groups of vaccinates and between vaccinated and control pups.

Treatment	Vaccinates (X S/c)	Vaccinates (X Oral)	Vaccinates (N S/c)	Vaccinates (N Oral)
Vaccinates (X S/c)	-	$P < 0.05$	$P < 0.05$	$P > 0.1$
Vaccinates (X Oral)	$P < 0.05$	-	$P > 0.8$	$P > 0.8$
* Controls (S/c)	$P < 0.001$	-	$P < 0.005$	-
* Controls (Oral)	-	$P < 0.005$	-	$P < 0.01$

* Comparing those 2 routes of infection in unvaccinated control pups, $P > 0.5$.

Symbols "X", "N", refer to irradiated and normal "vaccine", respectively.

Haematology

Since there were found to be no differences in the haematologic findings between pups that received irradiated larvae by either the oral or subcutaneous routes (Section VII), the figures for these 2 groups were combined. Similarly, the figures for all pups that were vaccinated with normal larvae were combined, as were the haematologic values of all the challenge control pups (Fig. 8).

After first vaccination there were depressions in the figures of both groups of vaccinates. The depressions in the haematologic values of the 11 pups that received irradiated vaccine were slight, while those of the 14 pups given unirradiated vaccine were large, exceeding 40% of the initial values. At this time 5 of these 14 pups died of acute ancylostomiasis. At the time of second vaccination the haematologic values of the surviving 9 pups of this group exhibited rapid improvement consequent to anthelmintic treatment. After second vaccination the haematologic figures of both groups of vaccinated pups were slightly depressed and anthelmintic treatment again appeared to have stimulated a rebound response in the figures of pups vaccinated with normal larvae. After challenge infection the haematologic values of the vaccinated pups were not significantly altered and there were no differences between any of the 4 groups of vaccinates. In contrast the haematologic values of the challenge control pups were severely depressed.

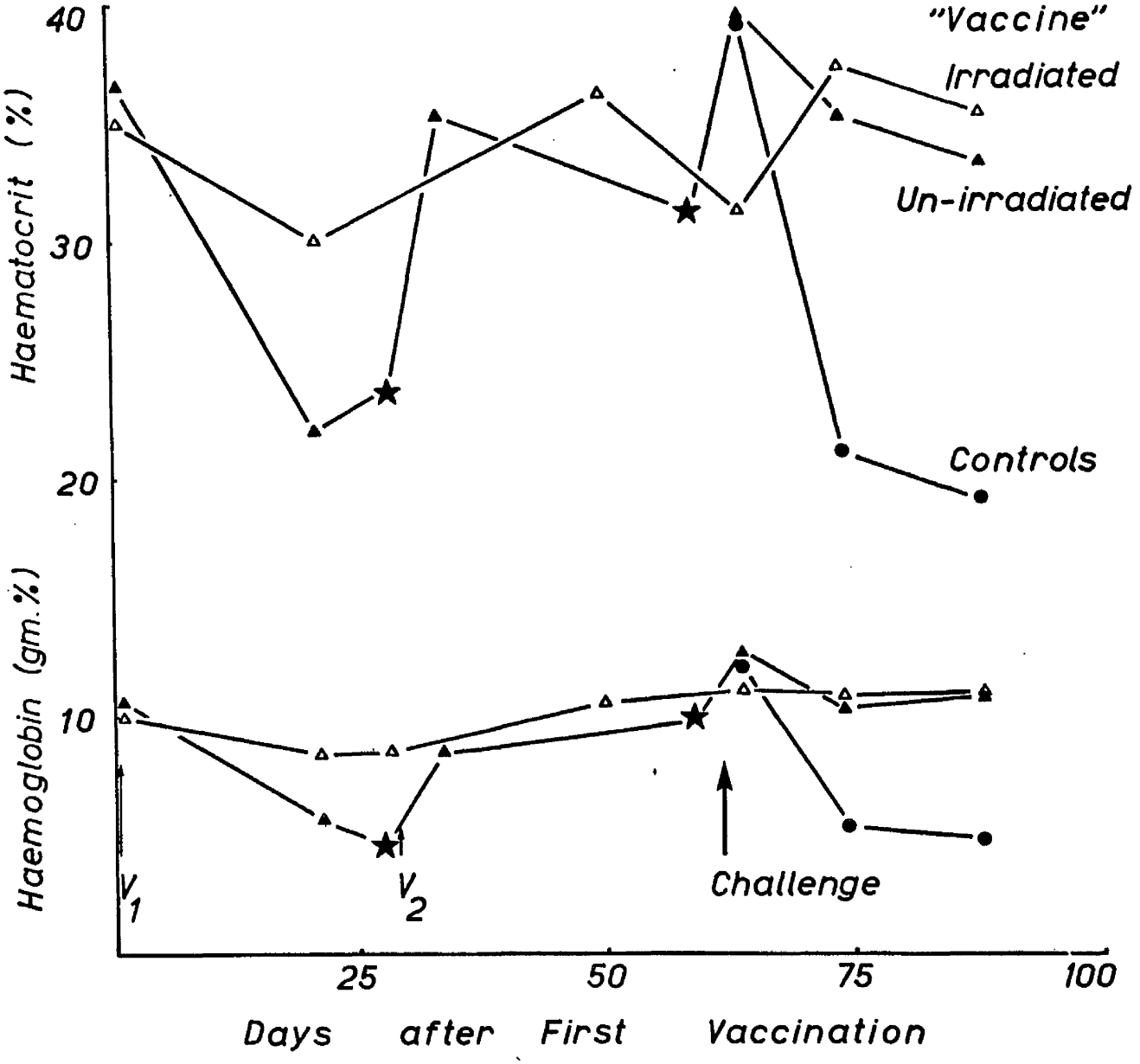

FIGURE 8. Mean group haematologic values after vaccination with normal and X-irradiated A. caninum larvae. Results of groups vaccinated by the oral and subcutaneous routes have been combined. The times of anthelmintic treatment to pups being vaccinated with normal larvae are indicated thus - *.

Clinical findings

At no stage during the experiment were any of the pups that were vaccinated with irradiated larvae affected clinically, either by their irradiated hookworms before challenge infection or afterwards by their normal hookworms. The pups that were vaccinated with normal larvae were severely affected by their vaccine, the first dose of which proved to be an $L.D._{35}$. The surviving pups in these two groups, after recovery following the first anthelmintic treatment, showed no further signs of ancylostomiasis, even when heavily parasitised by more normal hookworms from their challenge infection. The unvaccinated pups in the challenge control groups were all affected clinically by this, their primary infection, and showed signs of acute ancylostomiasis from the 12th day after inoculation of challenge larvae.

Weight changes

Statistical analyses of growth rates over each period of the experiment (Table 21) showed that during the vaccination period, the pups that were being vaccinated with irradiated larvae gained weight at a similar rate to the, as yet uninfected, controls. In marked contrast, the pups that were vaccinated with normal A. caninum larvae, and which survivied to the end of the vaccination period, were severely affected by their vaccine burdens of normal hookworms, since several of them failed to gain weight and the growth rates of the others were depressed. After challenge infection, all groups gained weight at the same rate, despite large variations in hookworm burdens between the different groups of vaccinated pups and between some of the vaccinates and the control pups.

Table 21. Mean group growth rates of pups that were vaccinated with irradiated or with normal A. caninum larvae and of challenge control pups. Growth rates were calculated as percent increase in weight over each period of the experiment. Statistical analysis of apparent differences were by Student's "t" test; the probabilities (P) refer to comparisons of data immediately above and below each statement.

Vaccine larvae	No. of pups	Growth rate (%, ± s.d.) from	
		First vaccination to challenge	Challenge to termination
Irradiated	11	81 ± 46	9 ± 16
		0.2 < P	0.8 < P
Control	11	120 ± 72	8 ± 17
		P < 0.01	0.7 < P
Normal	7	2 ± 34	6 ± 12
		P < 0.01	0.3 < P
Irradiated	11	81 ± 46	9 ± 16

For analysis, the results of all pups that were vaccinated with the same preparation and of all control pups, irrespective of route of infection, have been compared.

For ease of comparison the first group of results have been repeated at the foot of the table.

Faecal egg counts

After inoculation of the challenge infection the reductions in hookworm egg counts in the faeces of vaccinated compared with control pups were of the order that reflected approximately (i.e., as accurately as might be expected from the technique used with single group faecal examples) the differences in their respective challenge burdens of fertile hookworms (Table 22).

Discussion

In the previous section (VII) it was shown that double subcutaneous vaccination of pups with X-irradiated A. caninum larvae conferred a more uniform and significantly greater resistance to establishment of hookworms from a challenge infection than did double oral vaccination with irradiated larvae. For ease of comparison, and because part of the previous experiment and the current experiment were conducted simultaneously, some of the data listed in this section is a repetition of the previous results. However, the prime conclusion from the present data is that when the vaccine comprised normal instead of irradiated larvae, both subcutaneous and oral vaccination schedules proved to be inferior to subcutaneous vaccination with irradiated larvae. There was also no difference between the efficacies of oral and subcutaneous vaccination with normal larvae. Vaccination with normal larvae, in addition to being less immunogenic, was an extremely hazardous procedure since it was accompanied by considerable morbidity and mortality. The

Table 22. Mean group hookworm egg counts (in thousands per gram) in the faeces of vaccinated and of control pups after challenge infection.

Vaccine (Route of infection)	Days after challenge infection			
	0	6	19	25
Control (S/c & Oral)	0	0	10	35
Irradiated (S/c)	0	0	3.2	0.9
Irradiated (Oral)	0	0	0.6	9.0
Unirradiated (S/c & Oral)	1.4	0.4	31.2	13.5

apparently greater immunogenicity of irradiated compared with normal larvae has also been recorded with U. stenocephala in dogs (Dow et al., 1959) and Haemonchus contortus in sheep (Jarrett et al., 1959).

The results of the present experiment furnish further support for the hypothesis advanced in the discussion of the previous section (VII) that the arrestment or death of irradiated larvae in some somatic location after subcutaneous inoculation, with prolonged and intimate exposure of these larvae and their antigens to the tissues of the host, was responsible for the superior resistance to challenge infection after vaccination by this method. In contrast, more than half of the normal larvae completed their migration to the intestine as rapidly as possible after subcutaneous inoculation and after oral administration, normal larvae do not usually undergo migration but mature directly in the intestine.

The similarity of haematologic and clinical findings after challenge infection, even in the heavily parasitised pups that had been vaccinated by either route with normal larvae or orally with irradiated larvae, may be a consequence of prior stimulation of their erythropoetic tissues by haemorrhagic blood loss caused by the burdens of sterile worms from the vaccine infections. This consequent state of erythropoetic preparedness would conceivably permit pups on adequate diets to respond immediately and effectively to blood loss associated with challenge infection, such that their infection was clinically and haematologically inapparent. The failure of such large numbers of normal hookworms of the challenge infection, as were present in unvaccinated pups of the control groups and in

some of the pups that had been vaccinated with normal larvae, to adversely affect growth has been a frequent finding in pups infected with 1,000 normal larvae when they were 5-months-old. Weight loss or failure to grow at an adequate rate has been a regular observation only in unvaccinated pups that were infected when 4-months-old or less (Section VI, VII, IX, X).

Much of the earlier work (Herrick, 1928; Sarles, 1929a; McCoy, 1931; Foster, 1935; Otto & Kerr, 1939; Cort & Otto, 1940; Otto, 1941), showed that an effective immunity against challenge infection could be stimulated by repeated infections of normal A. caninum larvae by either oral administration, subcutaneous inoculation or by permitting natural skin penetration. In most of these experiments (Sarles, 1929a; McCoy, 1931; Foster, 1935; Otto & Kerr, 1939) at least 100 days between first vaccinating infection and the administration of challenge larvae were made to elapse before demonstration of satisfactory resistance of hookworms from the challenge infection. Periods of up to 7 months (Foster, 1935; Otto & Kerr, 1939) or even 2 years (Sarles, 1929a) were allowed to elapse between commencing vaccination and administering the challenge infection. At this time dogs are naturally highly resistant to the establishment of a primary infection (Section IX). Natural age resistance in the absence of prior exposure to hookworm begins to operate, particularly in bitches, as early as 8 months after birth (Section X). Often the ages of the dogs were unknown at the start of the experiment (Herrick, 1928) and the dogs were likely to have been adult at the time challenge larvae were given (Herrick, 1928; Foster, 1935; Otto & Kerr, 1939). Previous exposure

to hookworm had probably occurred (Herrick, 1928; Foster, 1935) suggesting that the dogs were at least partially immune to hookworm infection before the start of the experimental vaccination schedule.

The most rapid immunogenesis shown (Otto, 1941), which was comparable with the present experiments, followed the administration of multiple doses of normal larvae (110 times the number of larvae used to vaccinate the dogs of this experiment) over a period of 7 weeks and with weekly anthelmintic treatment to prevent the pathogenic effects of such massive infections from killing the dogs that were being vaccinated. The immunity developed in these dogs, that were of similar age and history to those used for the present experiment, was of a very high order (99% protection) and there was no difference in resistance to challenge infection following either oral or subcutaneous vaccination. It seems likely that a great excess of larvae was used as vaccine when these results (Otto, 1941) are compared with the immunity shown in the present experiment following inoculation of 1/110th of the dose of larvae, when these larvae were irradiated. Were it not for the anthelmintics used in Otto's experiments the dosage of normal infective larvae used for vaccination would represent approximately 100 $L.D._{100}$ for each dog. Otto's results also showed that larval stages alone appeared to be able to stimulate a satisfactory resistence. This is supported by the present finding of optimal immunogenesis following vaccination by subcutaneous inoculation of the X-irradiated larvae since this procedure resulted in only a small number of adult hookworms reaching the intestine.

Summary

Comparison of the immunogenic efficacies of X-irradiated and of normal A. caninum larvae by double vaccination of 3 and 4-months-old pups revealed that subcutaneous vaccination with irradiated larvae was more uniformly effective than were either subcutaneous or oral vaccination with normal larvae. Oral vaccination with irradiated larvae conferred similar protection to vaccination with normal larvae by either route. Anthelmintic treatment was a necessary adjunct of vaccination with normal larvae, and such vaccination proved to be extremely hazardous for the health and survival of the pups since 5 of 14 pups died after first vaccination. In spite of these differences in safety and immunogenic potential between irradiated and normal larvae, all vaccinated pups resisted equally the potential morbidity of the challenge infection, compared with the unvaccinated control pups which experienced severe depressions of haematologic values and exhibited severe clinical signs of ancylostomiasis.

SECTION IX

EFFECT OF AGE OF THE DOG ON IMMUNOGENIC EFFICACY OF VACCINATION WITH IRRADIATED A. CANINUM LARVAE

Introduction

The results of the previous experiments showed that the administration of 40 kr X-irradiated A. caninum larvae, preferably by two subcutaneous inoculations spaced one month apart, stimulated a high degree of resistance to the establishment of adult hookworms and to the potential morbidity and mortality of a challenge infection with normal A. caninum larvae. In all previous experiments the pups were 3-months-old at the time that the first inoculation of vaccine was made. The purpose of the next 2 experiments, was to determine the efficacy of vaccination in pups of various ages and in adult dogs.

Experimental Design

The plan of the experiments is illustrated in Table 23. In the first experiment, vaccination was commenced when pups were 3-days-old or when 4-weeks-old, second vaccination was given 4 weeks later, and immunity was challenged 4 weeks after second vaccination. To reduce the potential dangers of vaccination in these very young pups, the dose of vaccine was reduced from

Table 23. Plan of 2 experiments to compare the efficacies of double vaccination by subcutaneous inoculation of 40 kr-irradiated A. caninum larvae in pups of various ages and in adult dogs.

No. of pups /dogs	Age (weeks) at		Vaccine - No. of larvae (total or no./lb)	Challenge infection	
	First vaccination	Second vaccination		Age (months)	No. of larvae
EXPERIMENT 1					
4	3/7	5	100/lb	2	1,000
7	"	"	"	2	1,000
9	4	8	100/lb	3	1,000
11	"	"	"	3	1,000
EXPERIMENT 2					
6	12	16	1,000	5	1,000
6	"	"	"	5	1,000
5	Adult	Adult	1,000	Adult	50/lb
5	"	"	"	Adult	50/lb

the previous standard dose of 1,000 to a dose-rate of 100 irradiated larvae per lb bodyweight. Thus mean group vaccine dosage for first and second vaccinations, respectively, was 100 and 325 larvae to pups vaccinated when 3 days and 5-weeks-old, and 160 and 355 larvae to pups vaccinated when 4 and 8-weeks-old. In the second experiment, pups were double-vaccinated by subcutaneous inoculation of 1,000 irradiated larvae when 3 and 4-months-old, and adult dogs were similarly twice vaccinated, their two vaccine inocula being separated by one month.

To challenge immunity, the vaccinated pups in all three groups and similar but unvaccinated pups in three control groups were infected by subcutaneous inoculation of 1,000 normal larvae one month after second vaccination. Vaccinated adult dogs and their controls were infected by subcutaneous inoculation of 1,000 normal A. caninum larvae for each 20 lbs bodyweight one month after the time of second vaccination. Increase in the size of the challenge infection to adult dogs was designed to compensate for increased bodyweight and the natural age resistance to primary infection (Foster & Daensvang, 1932) of adult dogs and was also designed to induce clinical and haematologic signs of ancylostomiasis in control dogs.

On each occasion that vaccine was prepared, the infectivities of normal and of irradiated larvae were determined by enumeration of worm burdens 21 days after infection of 12-weeks-old pups in 8 groups by subcutaneous inoculation of 1,000 larvae.

Haematologic values of pups that were first vaccinated when 1 and 3-months-old and of vaccinated adult dogs were recorded at suitable intervals throughout the experiments, while the values of control pups and adult dogs, and of pups first vaccinated when 3-days-old were recorded after inoculation of the challenge infection. Clinical appearances were also observed. The weights of pups first vaccinated when 3-months-old and of their respective controls were recorded at the time of inoculating the first dose of vaccine, when challenge larvae were given, and finally when the experiments were terminated (Appendix III, Table 56). The weights of all the other vaccinated and control pups in these 2 experiments were recorded when the challenge larvae were given and when the experiments were terminated. Growth rates were analysed as previously and the significance of apparent differences determined. After inoculation of challenge larvae group faecal samples were examined.

The vaccinated and control pups and adult dogs were killed 22 - 26 days after challenge infection, their intestinal infections of adult hookworm were enumerated, and the significances of apparent differences were determined.

Results

Attenuation

The normal larvae used to prepare vaccine for both experiments were of high infectivity (mean take 59 - 76%) before exposure to X-rays (Appendix I,

Table 37). After irradiation their infectivities were reduced to levels usually associated with 40 kr-irradiation (mean takes 3 - 17%, relative infectivities 4 - 27%), with the second vaccine of the first experiment being at the lowest extreme of the range of relative infectivities so far recorded after 40 kr-irradiation (mean take 2.9%, relative infectivity 4%). All hookworms recovered from the control pups infected with 40 kr-irradiated larvae were sterile, and hookworm eggs were not detected in the faeces of vaccinated pups or adult dogs before inoculation of normal larvae of the challenge infection, and before expiry of the usual pre-patent period of 13-14 days after this infection.

Protection against infection

Vaccination conferred highly significant protection against the establishment of hookworms from challenge infections in all the pups and in adult dogs (Tables 24, 25). Differences in challenge worm burdens between the vaccinates and their respective controls were highly significant ($P < 0.001$). The protections (85 - 90%) conferred on pups vaccinated first when 4-weeks-old, 3-months-old and on vaccinated adult dogs were not significantly different. In pups first vaccinated when 3-days-old, protection against challenge infection (51%) was of a lower degree (Table 24) but was still highly significant. Control adult dogs exhibited a natural age resistance to primary infection. This age resistance was responsible for an additional protection in vaccinated adults that was additive to their actively acquired resistances from vaccination (Table 25).

Table 24. First experiment to measure the immunogenic efficacy of double subcutaneous vaccination of pups at 2 different ages. The pups in 2 groups were given their first inoculation of vaccine when 3 days or 4-weeks-old, with a second vaccination 4 weeks later. Vaccine of 40 kr-irradiated larvae was given at a rate of 100 per lb bodyweight in each inoculum. One month after second vaccination the immunities of vaccinated and of similar but unvaccinated control pups in 2 groups were challenged by subcutaneous inoculation of 1,000 normal A. caninum larvae. Protection from vaccination was measured by necropsy worm burdens enumerated 22 to 26 days after challenge infection.

Age when first vaccinated	Age when immunity challenged	No. of pups	Mean % take (± s.d.)	Vaccine protection (%)
Controls	8 weeks	7	72.5 ± 4.0	0
3 days	8 weeks	4	35.7 ± 8.1	51
Controls	3 months	11	67.5 ± 8.7	0
1 month	3 months	9	10.2 ± 8.4	85

Individual necropsy worm burdens are given in Appendix II, Table 49.

Table 25. Second experiment to measure the immunogenic efficacy of double vaccination of pups when 3 and 4-months-old and of adult dogs, by subcutaneous inoculation of 1,000 40 kr-irradiated A. caninum larvae. One month after second vaccination, the immunities of the vaccinated pups and adult dogs, and of similar but unvaccinated control pups and dogs, were challenged by subcutaneous inoculation of 1,000 (1,000 per 20 lb bodyweight to adults) normal A. caninum larvae. Protection from vaccination was measured by necropsy worm burdens enumerated 25 days after challenge infection.

Age when vaccinated	No. of pups/dogs	Mean % take (± s.d.)	Vaccine protection (%)
Controls	6	77.9 ± 9.7	0
3 months	6	9.7 ± 6.0	88
Controls	5	22.9 ± 9.2	0
Adult	5	2.4 ± 2.9	90

Individual necropsy worm burdens are given in Appendix II, Table 50.

Haematology and clinical findings

(i) In vaccinates before challenge infection

After first vaccination in the second experiment, the 3-months-old pups and adult dogs experienced slight, temporary and clinically insignificant reductions in haematocrit values (Figs. 9, 10). Haematologic values of the other vaccinates were not measured until after challenge infection. Adverse clinical signs were not observed in any of the pups and adult dogs during the vaccination period.

(ii) In controls

The severity of haematologic changes and associated adverse clinical signs in control pups and dogs after inoculation of the challenge infection were related to the age of the pups and dogs at the time that these larvae were inoculated. Control pups infected when 2-months-old were severely affected (Fig. 11). Six of these 7 pups died 12 to 14 days after infection at which time mean group haematocrit and haemoglobin values had decreased by 60 and 35%, respectively, compared with the values at challenge.

The challenge infection was relatively less severe to the older control pups since while the challenge proved to be an $L.D._{85}$ to 2-months-old controls, it was equivalent to an $L.D._{55}$ in 3-months-old controls. Six of these 11 pups died between 14 and 20 days after infection. The mean haematologic values of the surviving five showed reductions on the 20th day (Fig. 12) that were of similar proportions to the reductions in the values of the surviving

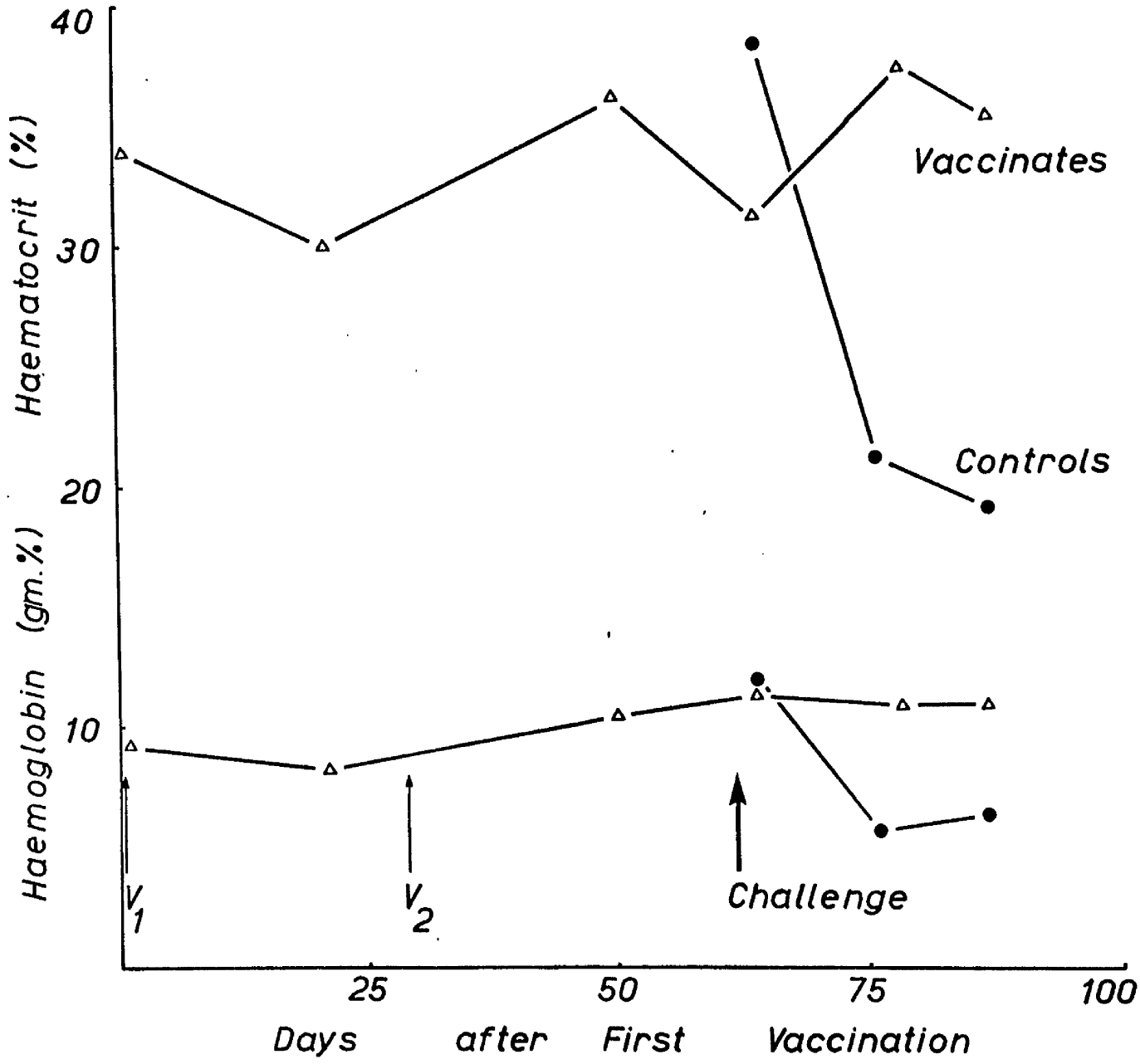

FIGURE 9. Mean group haematologic values during double subcutaneous vaccination of 3-months-old pups, and of unvaccinated controls after challenge infection.

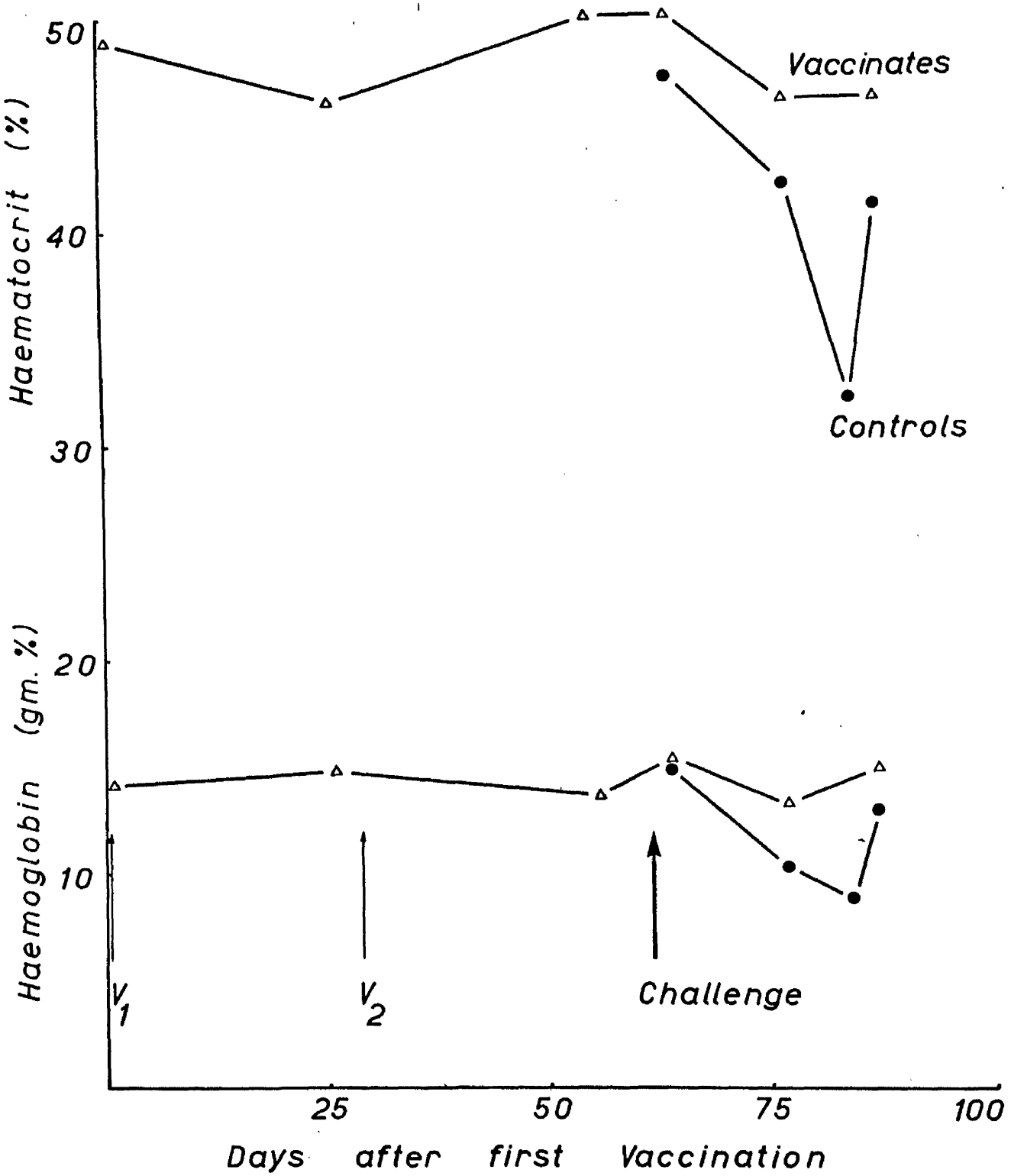

FIGURE 10. Mean group haematologic values during double subcutaneous vaccination of adult dogs, and of unvaccinated adult controls after challenge infection.

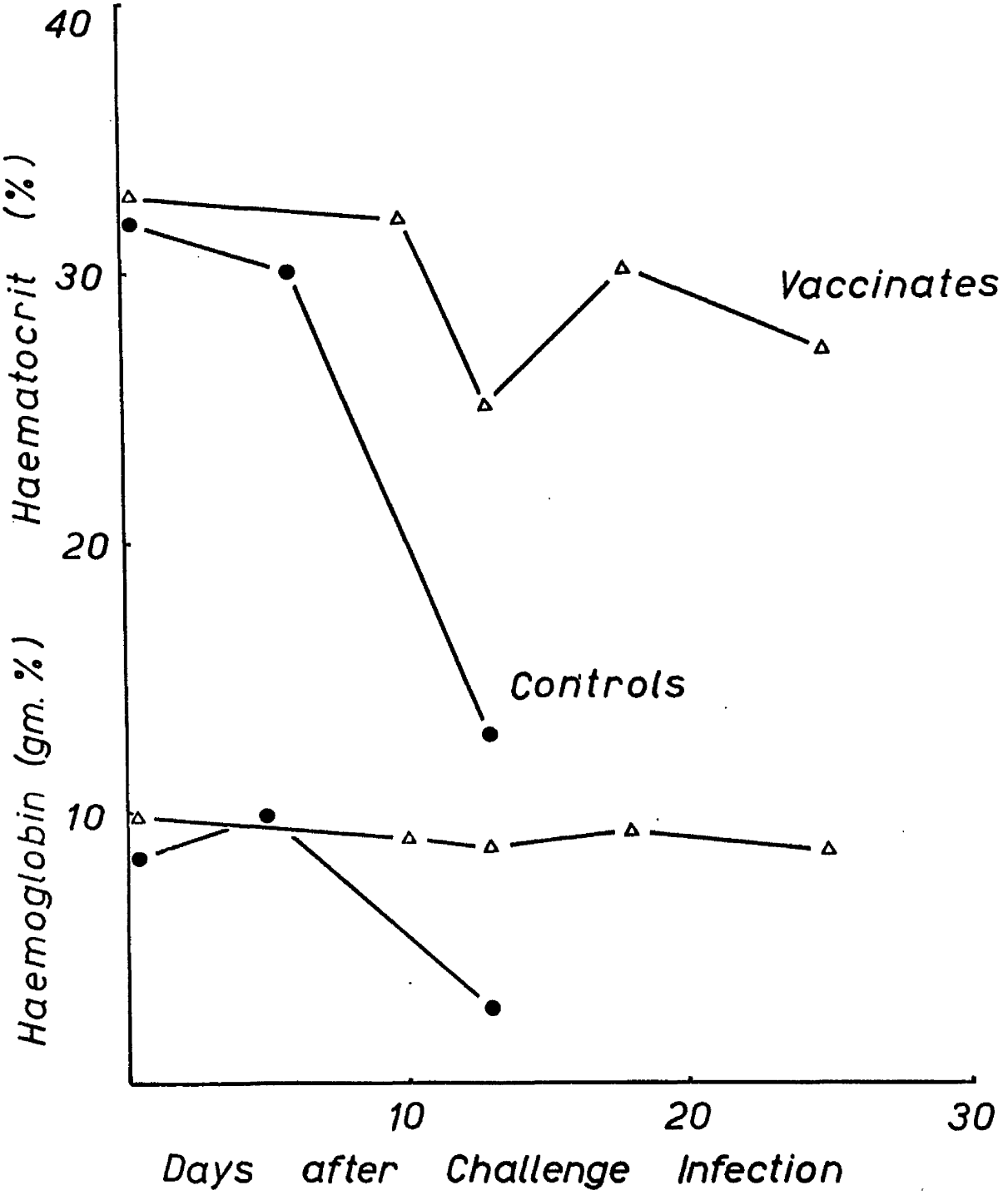

FIGURE 11. Mean group haematologic values of double-vaccinated and of unvaccinated 2-months-old pups after challenge of immunity. (Vaccination was commenced when 3-days-old).

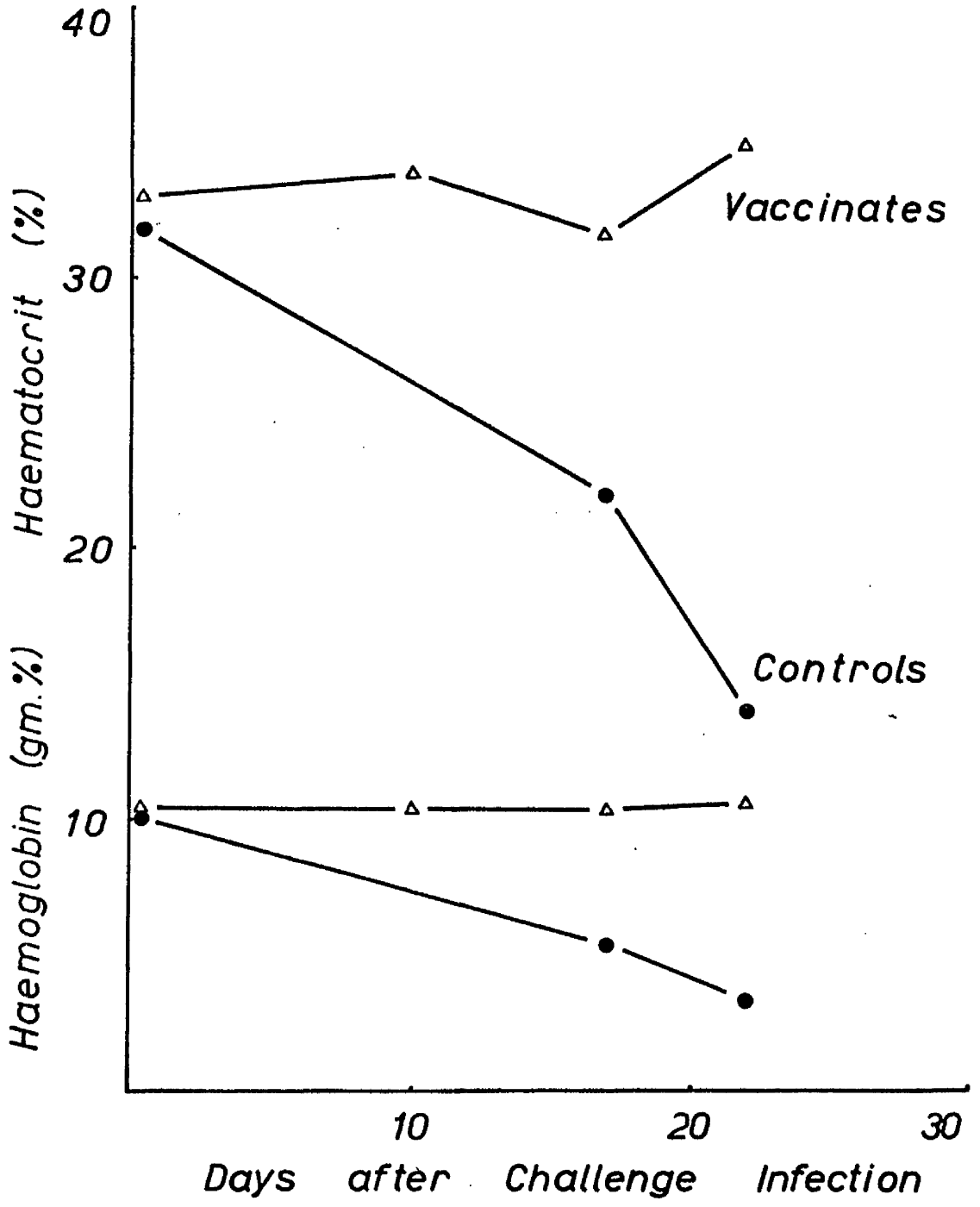

FIGURE 12. Mean group haematologic values of double-vaccinated and of unvaccinated 3-months-old pups after challenge of immunity. (Vaccination was commenced when 4-weeks-old)

2-months-old controls on the 14th day (Fig. 11). The surviving 3-months-old controls also exhibited very severe signs of ancylostomiasis.

Although they all survived to the end of the experiment, the controls that were infected when 5-months-old also experienced severe reductions in haematologic values (approximately 50%). Maximum depressions of these values were recorded at 20 - 22 days after infection (Fig. 9). After the 22nd day, their haematologic values showed signs of recovery (an observation not recorded in younger unvaccinated pups).

The control adult dogs exhibited transient depressions of haematocrit and haemoglobin values (Fig. 10). These reductions were accompanied by a brief period of listless ness and some diarrhoea.

(iii) In vaccinates after challenge infection

The vaccinated pups that received their challenge infections when they were 3 and 5-months-old experienced neither changes in haematologic values (Figs. 9, 12) nor did they show adverse clinical signs. The youngest vaccinated pups that were given their challenge infection when 2-months-old (Fig. 11) experienced reductions in haematocrit (25%) and haemoglobin values (10%, relative to the values at challenge). Unlike their controls, they all survived and showed only mild clinical signs of ancylostomiasis (occasional diarrhoea). The haematologic values of the adult vaccinates were temporarily depressed between the 15th and 20th days after challenge infection although there were no associated adverse clinical signs.

Weight changes

The only significant difference in growth rates after challenge infection between vaccinates and controls was in the pups given their challenge infection when 3-months-old (Table 26). Although there was no difference in growth rates between vaccinated and control adult dogs, they all lost some weight after challenge infection. Since 6 of the 7 control pups given a challenge infection when 2-months-old died within 14 days, it was not appropriate that their growth data over this short period be compared with the figures of their respective vaccinates. However, the growth rate of the latter pups (10% increase over 3 weeks post-challenge) was depressed compared with the normal rapid growth of uninfected pups in their third month (approximately 50 - 75% over 4 weeks).

Faecal egg counts

Depressions of hookworm egg outputs in the faeces of vaccinates after challenge infection were of a similar order as were reductions in hookworm establishment, except in adult dogs (Table 27).

Discussion

Double-vaccination of pups and adult dogs by subcutaneous inoculation of 40 kr-irradiated *A. caninum* larvae stimulated resistances that enabled them to withstand severe and potentially lethal challenges of immunity with

Table 26. Mean group growth rates of double-vaccinated and of control pups and adult dogs in the 2 experiments to determine the efficacies of vaccination of pups of various ages and of adult dogs. Growth rates were calculated as percent increase or *decrease in weight from challenge infection to termination, and the significance of apparent differences were determined. The probabilities (P) refer to comparisons of data immediately above and below each statement.

Age when first vaccinated	Age when immunity challenged	No. of pups /dogs	Growth rate (%, ± s.d.) from challenge to termination
Controls	3 months	5**	10 ± 23
			P < 0.01
1 month	3 months	9	40 ± 14
Controls	5 months	6	8 ± 21
			0.3 < P
3 months	5 months	6	19 ± 13
Controls	Adult	5	*7 ± 14
			0.6 < P
Adult	Adult	5	*6 ± 3

* Loss of weight.

** Only 5 of the 11 pups in this group (see Table 23) survived to the end of the experiment.

Table 27. Mean group hookworm egg counts (in thousands per gram) in the faeces of vaccinated and of control pups and adult dogs after inoculation of the challenge infection.

Age when first vaccinated (weeks)	Age when immunity challenged (months)	Days after challenge infection									
		0	5	10	14	18	19	21	23	25	26
Controls	2	0	-	0	1.5	66	-	-	-	-	147
3/7	2	0	-	0	0	3.5	-	-	-	-	67
Controls	3	0	-	0	-	1.5	-	-	41	-	69
4	3	0	-	0	-	0.3	-	-	3.3	-	9
Controls	5	-	0	-	0	-	17	-	-	25	-
12	5	-	0	-	0	-	3.2	-	-	0.9	-
Controls	Adult	-	0	-	0	-	-	1.3	-	4.0	-
Adult	Adult	-	0	-	0	-	-	0.8	-	1.2	-

normal A. caninum larvae. Vaccination was effective even when the first inoculation of irradiated larvae was made within 72 hours after birth. Vaccination of 72-hour-old pups was not so effective in protecting them against some of the adverse consequences of the challenge infection when they were 8-weeks-old (i.e., in haematologic changes, growth rate and clinical signs), compared with the protection from vaccination of older pups; although in terms of resistance to the most important and dramatic effect of challenge (i.e., mortality) vaccination at any age was satisfactory and was comparatively most satisfactory in the youngest vaccinated pups.

The haematologic results of the adult dogs revealed that in unvaccinated dogs small burdens of A. caninum caused sufficient blood loss to induce depressions of their haematologic values with concomitant loss of weight. Although the pathogenic effects of small burdens of A. caninum from the challenge infection of vaccinated adults were not apparent in their haematologic values, some morbidity was exhibited by a slight loss of bodyweight during the 3 weeks after challenge. In younger rapidly growing pups these two consequences of infection would tend to be obscured by the rapid growth and associated increase in haematologic values. Depression in haematologic values and reduction in growth rates from small worm burdens were therefore exhibited more clearly in adult dogs which had otherwise static weights and haematologic values and had relatively smaller amounts (if compared with younger dogs) of active erythropoetic tissue. In spite of relatively severe depressions in haematologic values after primary infection of 5-months-old

controls with 1,000 normal larvae, there was no significant depression of growth, any tendency in this direction presumably having been obscured by their potential for rapid growth at this age.

Controls given a primary infection of 1,000 normal larvae when they were 4-months-old (Section VI) or when younger, exhibited significant depression in growth only when they survived long enough after infection. Depression of growth or loss of weight appeared therefore to be a consequence more of chronic (i.e., more than about 16 days after infection) rather than of acute canine ancylostomiasis, and was related apparently to both the size of the infection and the age of the pup. Deaths that were classifiable as from acute ancylostomiasis were recorded only in pups that received 1,000 normal A. caninum larvae when they were less than 5-months-old. With this level of infection, 5-months-old pups survived and suffered only from some of the consequences of chronic ancylostomiasis.

The reduction in apparent infectivity of normal A. caninum larvae in control adult dogs, compared with the infectivity recorded from the same batch of larvae in control pups, indicated the presence of an age resistance to primary infection. This is discussed more fully in the next section (X). Age resistance also served to reduce further the susceptibility of vaccinated adult dogs to infection (i.e., in addition to their resistance from vaccination).

Summary

Subcutaneous vaccination with two doses of 40 kr-irradiated A. caninum larvae protected pups of different ages and adult dogs against the establishment of adult hookworms and consequently against the potential morbidity and mortality of challenge infection. Vaccination was effective when commenced as early as 72 hours after birth of the pups. Although protection against establishment of adult hookworms in these young pups was inferior to the protection in older vaccinates, the comparative protection against the potential mortality of challenge was more striking, since morbidity and mortality from the challenge infection was relatively more severe in younger controls. Death of control pups occurred with regularity only in those given a challenge infection of 1,000 normal larvae when less than 5-months-old.

Adult dogs were also shown to benefit from vaccination, although age per se conferred a considerable additional resistance to primary infection and to its morbidity in unvaccinated control adults. The influence of age resistance to primary infection was also operative in the vaccinated dogs, since age resistance was additive to the acquired resistance from vaccination.

SECTION X

PERSISTENCE OF IMMUNITY AFTER VACCINATION AND EFFECT OF REPEATED INFECTION WITH NORMAL LARVAE ON THIS IMMUNITY

Introduction

The necessity of vaccinating pups as early as possible after birth in hookworm-enzootic areas is self-evident, since exposure to infection early in the pup's life is a serious problem. It is unlikely that pups would be separated from environmental infection for any great length of time in most areas and under normal systems of management. However, where there is a marked seasonal variation in climate, infection from the environment is probably seasonal. At the northern extremes of distribution of A. caninum (e.g., Canada, Italy), as a consequence of low winter temperatures, the environmental challenge is seasonal. It would therefore be useful if the immunity following vaccination were to persist throughout the season during which natural reinfection, with its consequent amnestic recall, is absent. It is unlikely that such a season would exceed 6 months. To determine how long immunity would persist and if immunity was altered by repeated low-grade challenge infections, the following experiments were conducted.

Experimental design

The plan of experiment is illustrated in Table 28. Forty-eight pups were randomly distributed as to litter origin, but were selected on sex and bodyweight into 8 similar groups. When 3 and 4-months-old, 21 pups in 4 of these groups were vaccinated twice by subcutaneous inoculation of 1,000 40 kr-irradiated A. caninum larvae, the other 27 pups remaining uninfected as future challenge controls. When 5-months-old, the immunities of the 5 vaccinated and of 6 similar but unvaccinated control pups were challenged by subcutaneous inoculation of 1,000 normal A. caninum larvae. Six 3-months-old control pups were also infected by subcutaneous inoculation of 1,000 normal larvae at that time and were killed 22 - 24 days later. Their necropsy worm burdens served as an additional control for the infectivity of the normal larvae in the challenge infections. The prime purpose of this additional infectivity control was to measure the effect of natural age immunity to primary infection in older controls, and to determine if age immunity was present in the 5-months-old controls.

When 8-months-old the immunities of pups in 2 further groups (8 vaccinated and 6 control pups) were challenged, these pups being killed 21 days later. Five 3-months-old control pups were also infected by subcutaneous inoculation of 1,000 normal larvae and were killed 21 days later, their necropsy worm burdens to serve as additional control for infectivity of the larvae used to challenge immunity (i.e., to measure age resistance in the 8-months-old controls).

Table 28. Plan of experiments to investigate the persistence of immunity after double vaccination of pups when 3 and 4-months-old, and to determine the effect on this immunity of repeated infections with normal larvae ("trickle infection") after completion of the vaccination schedule and before inoculation of the challenge infection.

No. of pups	Treatment	Age when immunity challenged (months)	Purpose of experiment
6	Control	3	Infectivity-age resistance controls
6	Control	5	To control results of 5-months-old vaccinates
5	Vaccinated	5	To measure persistence of immunity 2 months after first vaccination
5	Control	3	Infectivity-age resistance controls
6	Control	8	To control results of 8-months-old vaccinates
5	Vaccinated	8	To measure persistence of immunity 5 months after first vaccination
7	Control	3	Infectivity-age resistance controls
9	Control	11	To control results of 11-months-old vaccinates
6	Vaccinated	11	To measure persistence of immunity 8 months after first vaccination
6	Control (+ trickle inf.)	11	To control results of 11-months-old vaccinates that received the "trickle infection" after vaccination
5	Vaccinated (+ trickle inf.)	11	To measure the effect on vaccinal immunity of the "trickle infection" when immunity was challenged 8 months after first vaccination

When 11-months-old, the immunities of 6 more vaccinated and of 9 control pups were challenged and the pups killed 21 days later. At the time of challenge infection, seven 3-months-old controls were also infected by subcutaneous inoculation of 1,000 normal larvae for the same purpose as previously (i.e., to monitor age resistance in the 11-months-old controls). Pups in the 2 remaining groups (i.e., 5 vaccinates and 6 controls) were similarly given their challenge infections when 11-months-old, but these 11 pups differed from all others in having been exposed between the ages of 6 and 11 months to low-grade "trickle" infection with normal larvae. The "trickle" infections were made in an attempt to simulate field conditions in hookworm-enzootic areas. These 11 pups received 1,400 larvae by subcutaneous inoculation over a period of 5 months, each infection being spaced 10 - 14 days apart and comprising 100 larvae.

At each of the 2 vaccinations additional uninfected 3-months-old pups in 4 groups were inoculated with irradiated or normal A. caninum larvae prepared from the same cultures. The pups in these 4 groups were killed 21 days after infection when their necropsy worm burdens served to control infectivity of normal larvae from which vaccine was prepared and radiation-attenuation of the larvae in the vaccine.

Haematological and coprologic examinations were performed throughout the experiment and the pups were observed for clinical signs of ancylostomiasis. Weights and growth rates were not recorded since it had been shown earlier that vaccination with 40 kr-irradiated larvae did not regularly interfere with the

normal growth of vaccinated pups, and that after challenge of immunity with 1,000 normal larvae significant differential growth rates between vaccinated and unvaccinated control pups were recorded only in pups that were less than 5-months-old at the time of the challenge infection (Section IX).

At necropsy of vaccinated pups, the numbers of hookworms that persisted from the vaccine inocula of irradiated larvae were determined by microscopic examination. From the 11-months-old vaccinated and control pups of the fourth groups, the hookworms derived from the "trickle" infections of normal larvae were discounted on size. Old adult worms from previous inocula of normal larvae were distinctly and macroscopically larger than worms derived from the challenge infection when the pups were killed 21 days after this infection. After discounting adult hookworms of vaccine and/or "trickle" infection origin, challenge worm burdens were recorded and expressed as per cent takes. Where sufficient observations were available, mean group per cent takes were compared to determine the significance of apparent differences.

Per cent protection figures were calculated for each appropriate comparison. These comparisons were between vaccinated and challenge control pups, between older challenge controls and 3-months-old infectivity controls, and between challenge control pups to compare those given the "trickle" infection and the respective controls given only a single challenge infection when 11-months-old. Protection figures were calculated only when significant differences in worm burdens were recorded between the two groups under consideration. It was assumed, for the purpose of comparison, that vaccinated pups had the same age resistance and/or trickle-acquired resistance as did

their corresponding control pups. It was also assumed, when immunity was challenged in 11-months-old vaccinated pups that had received the trickle infection, that these pups had the same protection due to age and to prior vaccination for the purpose of determining the effect of the third variable (i.e., "trickle" infection) on their resistances to challenge infection. Similarly, 11-months-old unvaccinated control pups given their challenge infection after "trickle" infection were credited with the age resistance that was exhibited by previously uninfected 11-months-old controls, to permit separation of protection due to age and to the "trickle" infection.

Results

Attenuation

The infectivity and radiation-attenuation control results (Appendix I, Table 38) showed that before irradiation the larvae were of high normal infectivity (mean takes 64 - 71%) and that after exposure to 40 kr of X-rays they were suitably attenuated (mean takes 4 - 22%, relative infectivities 6 - 34%). As previously, the female hookworms recovered at necropsy from the vaccine control pups that had been infected with 40 kr-irradiated larvae were sterile and hookworm eggs were not detected in the faeces of vaccinated pups until the appropriate pre-patent period had expired after inoculation of the normal larvae of the challenge infection.

Protection against infection

(i) Challenge 2 months after first vaccination

Necropsy worm burdens of vaccinated and of unvaccinated control pups, expressed as per cent take of challenge larvae and calculated as protection in vaccinates (91%), showed that double subcutaneous vaccination was as effective in stimulating immunity against challenge when 5-months-old (Table 29) as had been observed previously. The difference between vaccinate and control worm burdens was highly significant ($P < 0.001$).

(ii) Challenge 5 months after first vaccination

This level of immunity was fully maintained three months later when the 8-months-old vaccinated and control pups were inoculated with their challenge infection (Table 30). At this age there was a divergence between dog and bitch controls in their susceptibility to primary infection. Age *per se* had conferred a significant resistance to primary infection of 8-months-old unvaccinated bitches ($P < 0.01$, comparing worm burdens of four 8-months-old and five 3-months-old bitches). However, the two 8-months-old control dogs were still fully susceptible to infection and there was no difference in challenge worm burdens between vaccinated dogs and bitches. The three vaccinated 8-months-old bitches had derived a significant protection (88%) from vaccination (comparing the challenge worm burdens of vaccinated and control bitches, $P < 0.001$) which was additive to the common age resistance of all seven 8-months-old bitches.

Table 29. Double vaccination of pups when 3 and 4-months-old by subcutaneous inoculation of 1,000 40 kr-irradiated A. caninum larvae, with challenge of immunity when 5-months-old by subcutaneous inoculation of 1,000 normal larvae. Protection from vaccination was measured by necropsy worm burdens which were enumerated 22 to 24 days after challenge infection.

Treatment	No. of pups	Necropsy worm burdens		Mean % take (± s.d.)	Vaccine protection (%)
		Dogs	Bitches		
Control	6	830 82 899	853 885 971	88.7 ± 4.8	0
Vaccinated	5	24 70	14 18 287	8.3 ± 11.6	91
*Control (3-months-old)	6	(Appendix II, Table 52)		84.3 ± 16.2	0

* These pups were infected as controls for infectivity of the larvae used to challenge immunity in the 5-months-old vaccinates and controls (i.e., infectivity - age resistance controls).

In addition to normal hookworms from the challenge, there was a group total of 80 sterile irradiated female hookworms in the vaccinated pups.

Table 30. Persistence of immunity, after double subcutaneous vaccination of pups when 3 and 4-months-old by subcutaneous inoculation of 1,000 40 kr-irradiated A. caninum larvae, until challenge infection when 8-months-old by subcutaneous inoculation of 1,000 normal larvae. Persistence of immunity was determined by the protection figures, the latter being calculated from necropsy worm burdens which were enumerated 21 days after challenge infection.

Treatment	No. of pups	Necropsy worm burdens		Mean % take (± s.d.)	Protection (%) from	
		Dogs	Bitches		Vaccine	Age
Control	2	748 846		79.7	0	0
Control	4		338 390 456 559	43.6 ± 9.5	0	51
Combined dog and bitch controls				55.6	0	-
Vaccinated	2	6 48		2.7	97	0
Vaccinated	3		16 52 95	5.3 ± 3.9	88	51
Combined dog and bitch vaccinates				4.3 ± 3.5	92	-
*Control (3-months-old)	5	(Appendix II, Table 52)		88.7 ± 10.2	0	0

* These pups were infected as controls for infectivity of the larvae used to challenge immunity in the 8-months-old vaccinates and controls.

In addition to normal hookworms from the challenge, there was a group total of 15 sterile irradiated female hookworms in the vaccinated pups.

(iii) Challenge 8 months after first vaccination without intervening (i.e., "trickle") infection

The challenge worm burdens of vaccinated dogs and bitches in the control group (Table 31) showed that all these 11-months-old pups had a significant age resistance to primary infection compared with the 3-months-old dogs and bitches in the infectivity control group ($P < 0.01$ comparing dog, and $P < 0.001$ comparing bitch worm burdens). The apparent difference in susceptibility to primary infection shown in the worm burdens of these 11-months-old controls, as between dogs and bitches, was also significant ($P < 0.01$). However, there was no significant difference ($P > 0.2$) in burdens of challenge hookworms between vaccinated dogs and bitches. The worm burdens of the vaccinates were significantly smaller than those in their controls when dog and bitch results in each group were combined ($P < 0.025$). The difference in necropsy worm burdens was also significant between vaccinated and control dogs ($P < 0.02$) and between vaccinated and control bitches ($P < 0.05$). Thus vaccinated dogs and bitches which received their challenge infection when 11-months-old had an acquired immunity from vaccination (80% protection) which was additional and was similar to their resistances attributable to age *per se* (i.e., age resistance as exhibited in the 11-months-old controls).

Immunity had thus persisted at a highly satisfactory level for seven months in absence of further exposure to infection after completion of the vaccination schedule when the pups were 4-months-old. The persistence of small numbers of sterile female hookworms during these seven months appeared

Table 31. Persistence of immunity after double subcutaneous vaccination of pups when 3 and 4-months-old by subcutaneous inoculation of 1,000 40 kr-irradiated A. caninum larvae, until challenge of immunity when 11-months-old by subcutaneous inoculation of 1,000 normal larvae. Challenge worm burdens were enumerated at necropsy 21 days after inoculation of challenge larvae.

Treatment	No. of pups	Necropsy worm burdens		Mean % take (± s.d.)	Protection (%) from	
		Dogs	Bitches		Vaccine	Age
Control	3	230 267 353		28.3 ± 6.3	0	53
Control	6		36 38 134 150 152 185	11.6 ± 6.3	0	81
	Combined dog and bitch controls			17.2 ± 10.2	0	-
Vaccinated	3	17 36 125		5.9 ± 5.7	79	53
Vaccinated	3		0 5 24	1.0 ± 1.3	91	81
	Combined dog and bitch controls			3.5 ± 4.6	80	-
*Control (3-months-old)	7	(Appendix II, Table 52)		60.2 ± 3.9	0	0

* These pups were infected as controls for infectivity of the larvae used to challenge immunity in 11-months-old vaccinates and controls.

In addition to normal hookworms from the challenge, there was a group total of 11 sterile irradiated female hookworms in the vaccinated pups.

to have no influence on the degree of immunity since there was no relationship either between the presence or the numbers of these sterile worms in individual vaccinates and the immunity of each animal. This observation would tend to exclude the probability that a premune phenomenon, related to persistence of adult worms from the vaccine, was operating in the maintenance of immunity.

(iv) Challenge 8 months after first vaccination with intervening "trickle" infection

There were no significant differences ($P > 0.5$) in challenge burdens of adult hookworms between vaccinates and controls nor between the dogs and bitches when they had been exposed to a series of trickle infections with normal larvae before challenge infection. The trickle infection had conferred a significant acquired resistance ($P < 0.01$, protection 93%) to reinfection of unvaccinated controls (Table 32) compared with controls to which their challenge was a primary infection (Table 31). Therefore, the immunity stimulated by trickle infection had rendered these controls (Table 32) as highly resistant to challenge infection ($P > 0.5$) as were the vaccinated dogs and bitches that did not experience trickle infection (Table 31).

Haematology

The haematologic results (Fig. 13) showed that, compared with the uninfected controls, the vaccinates experienced slight but insignificant reductions in haematocrit and haemoglobin values after inoculation of the

Table 32. Persistence of immunity, after double subcutaneous vaccination of pups when 3 and 4-months-old by subcutaneous inoculation of 1,000 40 kr-irradiated *A. caninum* larvae, and after a series of 14 infections with 100 normal *A. caninum* larvae ("trickle" infection), until challenge of immunity when 11-months-old by subcutaneous inoculation of 1,000 normal larvae. Challenge worm burdens were enumerated at necropsy 21 days after inoculation of challenge larvae.

Treatment	No. of pups	Necropsy worm burdens		Mean % take (± s.d.)	Protection (%) from		
		Dogs	Bitches		Trickle	Vaccine	Age
Control	2	10 12		1.1	96	0	53
Control	4		3 4 6 39	1.3 ± 1.7	89	0	81
	Combined dog and bitch controls			1.2 ± 1.4	93	0	-
Vaccinated	2	7 16		1.2	70	79	53
Vaccinated	3		10 22 26	1.9 ± 0.8	0	91	81
	Combined dog and bitch vaccinates			1.6 ± 0.8	-	80	-
* Control (3-months-old)	7	(Appendix II, Table 52)		60.2 ± 3.9	0	0	0

*These pups were infected as controls for infectivity of the larvae used to challenge immunity in 11-months-old vaccinates and controls.

In addition to normal hookworms from the challenge there was a group total of 13 normal old (i.e., large) hookworms from the "trickle" infections in the unvaccinated controls and 14 sterile irradiated female hookworms (but no old worms) in the vaccinated pups.

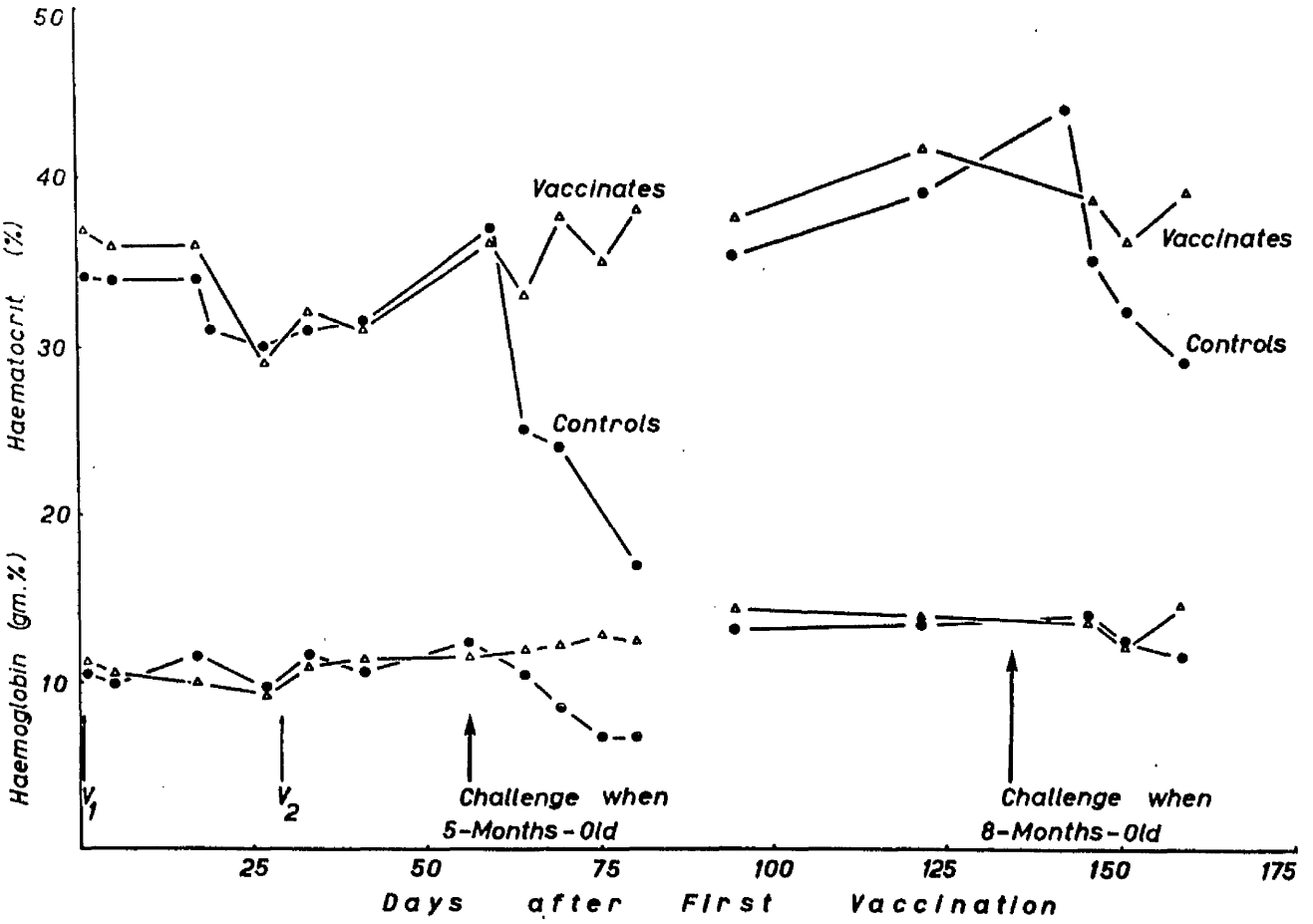

FIGURE 13. Mean group haematologic values of vaccinated and unvaccinated control pups following double-vaccination when 3 and 4-months-old with challenge of immunity when 5 or 8-months-old.

first dose of irradiated larvae. For some unknown reason control pups also suffered a fall in haematologic values at that time, so it seems unlikely that reductions in the vaccinates were associated with vaccination. After these initial reductions, the haematologic status of the vaccinates improved at a steady rate until maximum haematocrit of 37 - 42% and haemoglobin of 12 - 16g% had been reached. These values were maintained in spite of the establishment of normal hookworms in pups that received their challenge infections when they were 5, 8 and 11-months-old. Similarly, the trickle infections did not cause any change in haematologic values either in vaccinates or in controls.

Control pups also showed similar increments in haematologic values with intermediate and at-challenge values being similar to those of the vaccinated pups. After challenge infection when 5 and 8-months-old, the haematologic values of controls and vaccinates diverged. Control pups that received their challenge infection when 5-months-old suffered severe depressions in haematocrit and haemoglobin that were equivalent to half of their at-challenge values, while reductions in controls given the challenge infection when 8-months-old were equivalent to 25% (haematocrit) and 15% (haemoglobin).

Depressions in haematologic values and significant differences between controls and vaccinates were not observed in pups that were given a challenge infection with normal larvae when 11-months-old. Age immunity *per se* in 11-months-old control dogs and bitches had thus conferred a resistance to the potential morbidity of challenge infection with normal *A. caninum* consequent to their resistance to the establishment of adult hookworms.

Clinical findings

The clinical findings followed closely the haematologic changes. The only adverse clinical signs observed were in control pups that received their challenge infections when 5 or 8-months-old. The controls infected when 5-months-old showed signs of severe acute ancylostomiasis and of those infected when 8-months-old, a few showed mild and intermittent signs of the disease. The controls that were infected when 11-months-old, and all vaccinated pups remained normal and without clinical evidence of hookworm infection throughout the experiment.

Faecal examination

The coprologic findings (Table 33) showed that after challenge infection with normal A. caninum larvae, mean group output of hookworm eggs in the faeces of vaccinated and control pups reflected approximately the sizes of their infections with adult hookworms. The onset of patency after challenge infection was delayed in vaccinated compared with control pups.

Discussion

The results showed that the immune response, measured by resistance to challenge infection with normal larvae consequent to double vaccination of pups when 3 and 4-months-old, persisted at a highly satisfactory and significant level for at least 7 months after second vaccination. This immunity persisted,

Table 33. Mean group hookworm egg counts (in thousands per gram) in the faeces of vaccinated and of control pups.

Treatment	Age at challenge (months)	Days after challenge infection				
		3	14	17	20	24
Controls	5	0	-	3.2	-	25.9
Vaccinates	5	0	-	0	-	2.9
Controls	8	0	0.7	-	14	17
Vaccinates	8	0	0	-	0.8	1.8
Controls	11	0	-	0.2	-	1.7
Vaccinates	11	0	-	0	-	0.2
With "trickle" infection						
Controls	11	0.15	0.05	-	-	0.8
Vaccinates	11	0	0	-	-	0.4

moreover, in absence of further exposure to hookworm infection. It is unlikely that in a hookworm-enzootic area, dogs would be maintained in such conditions after vaccination to preclude further infection for this length of time. Such a period of protection is therefore unlikely to be generally necessary in the application of the vaccine. However, in areas in which there are marked seasonal temperature variations, reinfection from the environment might not occur for periods of up to six months. In addition, 11-months-old control pups were shown to develop an age resistance, both to infection and to the pathogenic effects of infection. Age resistance to infection appeared to develop earlier in bitch pups (i.e., when 8-months-old) than in dogs. In vaccinated age-resistant pups, the vaccinal immunity appeared to be additive to the age resistance.

It has been shown that the immunity following vaccination with normal larvae could be demonstrated at 7 months (McCoy, 1931; Foster, 1935; Otto & Kerr, 1939) and as long as 2 years (Sarles, 1929a) after commencing the vaccination schedule, although in all these experiments there was a continuous stimulation of immunity by large infections during these intervals. Therefore, these previous results are comparable only to the trickle infection experiments of the present report.

The failure of the trickle infections of normal larvae to enhance the immune response from prior vaccination suggested that vaccination had induced what could be considered to be the maximum potential acquired immune response of which pups were capable. It should also be noted that the trickle infections

of 1,400 normal larvae to the control dogs and bitches stimulated as good a resistance to challenge infection as did vaccination. This resistance was comparable to the immunity reported elsewhere (McCoy, 1931; Otto & Kerr, 1939) in which experiments many times this number of normal larvae were used to produce this level of immunity (in excess of 20,000 larvae).

Summary

The resistance to both the establishment and potential morbidity of challenge infections, consequent to double subcutaneous vaccination of pups when 3 and 4-months-old, persisted in absence of further exposure to hookworm for at least 7 months after completion of the vaccination schedule. Towards the end of this period age resistance, as exhibited by control pups, augmented the immunity of vaccinated pups. Vaccinated pups at all ages were completely immune to the potential morbidity of the challenge infection, while older control pups were partially (i.e., when 8-months-old) or completely (at 11 months) protected against these effects by age resistance *per se*. The control pups infected when 5-months-old were severely affected in terms of adverse clinical and haematologic changes. Vaccination with X-irradiated larvae appeared to have stimulated a maximal immunity which was not altered by numerous small infections of normal larvae during the period between vaccination when 3 and 4-months-old and challenge of immunity when 11-months-old.

SECTION XI

GENERAL SUMMARY

Hookworm disease affecting man and his dog is one of the major scourges of the humid tropical and sub-tropical areas of the world. In dogs the most important hookworm is Ancylostoma caninum, the prime pathogenesis of which resides in the ability of the adult worms to cause blood loss from the intestine of susceptible pups. Although it has been apparent for some time that dogs acquire an active immunity to reinfection with A. caninum, vaccination procedures using normal hookworm larvae have been shown to be lengthy and hazardous. The inactivating or attenuating effect of ionising radiations on helminth parasites has been recognised for a considerable length of time, but only comparatively recently has this discovery been exploited in a practical fashion in the development of irradiated helminth vaccines. In the light of this information, a series of experiments were planned to investigate the possibility of using X-ray attenuated A. caninum larvae to stimulate immunity in susceptible pups against subsequent infection.

There was no significant difference in the infectivities of larvae between those given either orally or by subcutaneous inoculation.

Exposure of A. caninum infective larvae to various doses of X-rays reduced the infectivity of the larvae as measured by subsequent intestinal establishment of adult hookworms. As the dose of radiation was increased,

infectivity was progressively decreased and the pathogenicity of the hookworm burdens were reduced. Male larvae were more sensitive to the effects of X-irradiation than were female larvae, particularly at the higher levels of radiation. At X-ray doses of 40 kr and greater, the female worms in the resulting population were invariably sexually sterile. From these conclusions it appeared that larvae irradiated with 40 kr or greater doses of X-rays would be suitable for vaccination experiments.

A single vaccination of pups when 3-months-old by subcutaneous inoculation of 1,000 40 kr-irradiated larvae conferred a highly significant resistance when 4-months-old against the establishment and potential morbidity and mortality of a subcutaneous challenge infection with normal larvae. Although the protective effect of vaccination was exhibited in such measures of pathogenesis as haematologic and clinical values, the most spectacular advantage was the survival of all vaccinates while 5 of 12 controls died.

Compared with single vaccination, double vaccination of pups conferred superior resistance to the establishment of normal larvae from a challenge infection. Complete protection against the potential morbidity of the challenge infection was exhibited by double vaccinated compared with control pups, although the challenge infection was relatively less pathogenic to 5-months-old controls.

Double subcutaneous vaccination of 3 and 4-months-old pups with 1,000 40 kr-irradiated larvae was more effective against a subcutaneous challenge than was double vaccination by the oral route against an oral challenge,

when resistance was measured by the establishment of adult hookworms after challenge infection with normal larvae. Subcutaneous vaccination conferred equal protection against subcutaneous and oral challenge, while oral vaccination conferred a satisfactory protection only against subcutaneous challenge. However, in terms of resistance to the potential morbidity of the challenge infection, both methods of vaccination were equally effective compared with the effects of challenge on control pups.

Comparison of the immunogenic efficacies of X-irradiated and of normal A. caninum larvae by double vaccination of 3 and 4-months-old pups, revealed that subcutaneous vaccination with irradiated larvae was more uniformly effective than were either subcutaneous or oral vaccination with normal larvae. Oral vaccination with irradiated larvae conferred similar protection to that following vaccination by either route with normal larvae. Anthelmintic treatment was a necessary adjunct of vaccination with normal larvae, and this method of vaccination proved to be extremely hazardous for the health and survival of the pups since 5 of 14 pups died after vaccination.

Subcutaneous vaccination with two inocula of 40 kr-irradiated A. caninum larvae protected pups at different ages and adult dogs against the establishment and potential morbidity of challenge infection. Vaccination was effective when commenced as early as 72 hours after birth of the pups. Although protection against establishment of adult hookworms in these pups was of a lower level than in older vaccinated pups, the relative degree of

protection against the potential morbidity and mortality of challenge infection in such young pups was more striking than in the older pups. Death of unvaccinated control pups occurred with regularity only in those given a challenge infection of 1,000 normal larvae when less than 5-months-old. Adult dogs were also shown to benefit from vaccination, although age per se conferred a considerable additional resistance to primary infection in adult controls. Age resistance in the vaccinated dogs was additive to their acquired resistance from vaccination.

The resistance to the establishment and potential morbidity of the challenge infection that was stimulated by double subcutaneous vaccination of pups when 3 and 4-months-old, persisted in the absence of further exposure to hookworm for at least 7 months after completion of the vaccination schedule. Towards the end of the 7-month period age resistance, which was exhibited in previously uninfected control pups, augmented the immunity of vaccinated pups against challenge hookworm infection. Vaccinated pups at all ages were completely immune to the pathogenic effects of the challenge while older controls were partially (at 8 months) or completely (at 11 months) protected against these effects by age resistance per se. Vaccination with X-irradiated larvae stimulated an apparently maximal immunity which was not further improved by numerous small infections of normal larvae during the period between vaccination and challenge infection when the vaccinated pups were 11-months-old.

SECTION XII

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SECTION XIII

APPENDICES

APPENDIX I

INFECTIVITY FIGURES OF NORMAL AND OF IRRADIATED LARVAE TO CONTROL VACCINE ATTENUATION

Table 34. (Section VI, p 45 refers)

Infectivity of larvae, calculated from necropsy worm burdens enumerated 21 days after subcutaneous inoculation of 6 to 8-weeks-old pups with 500 normal or 1,000 40 kr-irradiated A. caninum larvae. This data records the degree of attenuation of the 40 kr-irradiated larvae used as vaccine in the single vaccination experiment.

Treatment of larvae	No. of pups	Mean % take (± s.d.)	Relative infectivity (%)
Normal	4	48.1 ± 18.2	100
Irradiated	5	4.0 ± 1.0	8

Individual necropsy worm burdens are given in Appendix II, Table 42.

Table 35. (Section VII, p 53 refers)

Infectivity of larvae calculated from necropsy worm burdens enumerated 21 days after subcutaneous inoculation of 1,000 normal or 40 kr-irradiated *A. caninum* larvae to 12-weeks-old pups. This data measures the attenuation of vaccine used in the 2 experiments in which the subcutaneous and oral routes of vaccination and of challenge infection were permutated.

Treatment of larvae	No. of pups	Mean % take (± s.d.)		Relative infectivity (%)
		First vaccination	Second vaccination	
EXPERIMENT 1				
Normal	4	71.9 ± 4.0		100
Irradiated	3	17.2 ± 4.7		24
Normal	4		59.3 ± 8.8	100
Irradiated	6		16.0 ± 4.8	27
EXPERIMENT 2				
Normal	3	66.6 ± 4.2		100
Irradiated	3	36.4 ± 4.7		55
Normal	5		84.1 ± 11.0	100
Irradiated	3		15.1 ± 6.0	18

Individual necropsy worm burdens are given in Appendix II, Table 44.

Table 36. (Section VIII, p 70 refers)

Infectivity of larvae calculated from necropsy worm burdens enumerated 21 days after subcutaneous inoculation of 1,000 normal or 40 kr-irradiated *A. caninum* larvae to 3-months-old pups. This data measures the attenuation of vaccine used in the experiment in which the immunogenicities of irradiated and of normal larvae were compared.

Treatment of larvae	No. of pups	Mean % take (± s.d.)		Relative infectivity (%)
		First vaccination	Second vaccination	
Normal	4	71.9 ± 4.0	-	100
Irradiated	3	17.2 ± 4.7	-	24
Normal	4	-	59.3 ± 8.8	100
Irradiated	6	-	16.0 ± 4.8	27

Individual necropsy worm burdens are given in Appendix II, Table 44 (Experiment 1).

Table 37. (Section IX, p 82 refers)

Infectivity of larvae calculated from necropsy worm burdens enumerated 21 days after subcutaneous inoculation of 1,000 normal or 40 kr-irradiated *A. caninum* larvae to 3-months-old pups. This data measured the attenuation of vaccine used in the 2 experiments in which the efficacies of vaccination were determined in pups of various ages and in adult dogs.

Treatment of larvae	No. of pups	Mean % take (± s.d.) First vaccination	Mean % take (± s.d.) Second vaccination	Relative infectivity (%)
EXPERIMENT 1				
Normal	8	55.0 ± 9.5		100
Irradiated	10	6.1 ± 2.8		11
Normal	5		76.1 ± 15.7	100
Irradiated	5		2.9 ± 2.5	4
EXPERIMENT 2*				
Normal	4	71.9 ± 4.0		100
Irradiated	3	17.2 ± 4.7		24
Normal	4		59.3 ± 8.8	100
Irradiated	6		16.0 ± 4.8	27

* The same vaccine was used for this experiment and Experiment 1 of Section VII (Appendix I, Table 35).

Individual necropsy worm burdens are given in Appendix II, Tables 43 (Expt. 1) and 44 (Expt. 2).

Table 38. (Section X, p 93 refers)

Infectivity of larvae, calculated from necropsy worm burdens enumerated 21 days after subcutaneous inoculation of 3-months-old pups with 500 normal or 1,000 40 kr-irradiated A. caninum larvae. This data records the degree of attenuation of the irradiated larvae used as vaccine in the "persistence of immunity" experiment.

Treatment of larvae	No. of pups	Mean % take (± s.d.)		Relative infectivity (%)
		First vaccination	Second vaccination	
Normal	5	64.4 ± 25.3		100
Irradiated	4	21.8 ± 10.7		34
Normal	4		71.2 ± 8.6	100
Irradiated	5		4.0 ± 1.0	6

Individual necropsy worm burdens are given in Appendix II, Table 51.

APPENDIX II

INDIVIDUAL NECROPSY WORM BURDENS FROM WHICH INFECTIVITY AND PERCENT TAKE FIGURES WERE CALCULATED

Table 39. (Section V, Table 3 refers)

Necropsy worm burdens enumerated 24 to 30 days after infection of 3-months-old pups with 1,000 *A. caninum* larvae.

Radiation dose (kr)	Route of infection	No. of pups	Necropsy worm burdens	Mean group worm sex ratio, M/F
0	S/c	4	323, 382, 399, 401	1/1.1
0	Oral	4	147, 534, 565, 680	1/1.2
20	S/c	4	214, 293, 302, 304	1/3
20	Oral	3	179, 210, 227	1/3
30	S/c	4	93, 164, 180, 181	1/5.2
30	Oral	4	13, 180, 193, 211	1/5.3
40	S/c	4	42, 63, 73, 88	1/4.7

Table 40. (Section V, Table 4 refers)

Necropsy worm burdens enumerated 24 to 30 days after infection of 3-months-old pups with 1,000 *A. caninum* larvae.

Radiation dose (kr)	Route of infection	No. of pups	Necropsy worm burdens	Mean group worm sex ratio, M/F
0	S/c	6	490, 583, 597, 702, 728, 748	1/1
0	Oral	2	664, 740	1/1.1
40	S/c	5	120, 131, 148, 167, 245	1/5
40	Oral	4	105, 151, 171, 220	1/6

Table 41. (Section V, Table 5 refers)

Necropsy worm burdens enumerated 24 to 30 days after subcutaneous inoculation of 3-months-old pups with 1,000 A. caninum larvae.

Radiation dose (kr)	No. of pups	Necropsy worm burdens	Mean group worm sex ratio, M/F
0	4	676, 828, 836, 978	1/1.2
60	7	29, 31, 40, 45, 47, 52, 87	1/34

Table 42. (Section VI, Appendix I, Table 34 refers)

Necropsy worm burdens determined 21 days after subcutaneous inoculation of 6 to 8-weeks-old pups with normal or irradiated A. caninum larvae. This data measured the infectivity of the normal larvae used to prepare vaccine and attenuation induced in these larvae by 40 kr of X-rays.

Treatment of larvae	No. of larvae inoculated	No. of pups	Necropsy worm burdens	Mean group worm sex ratio, M/F
Normal	500	4	189, 190, 207, 376	1/1.2
Irradiated	1,000	5	28, 32, 43, 44, 53	1/52

Table 43. (Section VI, Table 7 refers)

Necropsy worm burdens of single vaccinated and of unvaccinated control pups enumerated 22 days after challenge infection with 1,100 normal A. caninum larvae.

Treatment	No. of pups	Necropsy worm burdens (fertile worms)	Sterile female worms (group total)	Mean group worm sex ratio, M/F
Control	12	741, 782, 913, 937, 951, 970, 974, 989, 998, 1015, 1036, 1109	-	1/1.1
Vaccinated	6	432, 448, 464, 725, 733, 775	106	1/1.1

Table 44. (Section VII, Appendix I, Table 35; and Section VIII, Appendix I, Table 36 refer)

Necropsy worm burdens enumerated 21 days after subcutaneous inoculation of 1,000 normal or 40 kr-irradiated *A. caninum* larvae to 3-months-old pups.

Vaccination	Treatment of larvae	No. of pups	Necropsy worm burdens	Mean group worm sex ratio, M/F
EXPERIMENT 1				
1	Normal	4	661, 728, 735, 753	1/1
1	Irradiated	3	131, 161, 224	1/8
2	Normal	4	490, 583, 597, 704	1/1
2	Irradiated	6	111, 120, 148, 167, 171, 245	1/4.3
EXPERIMENT 2				
1	Normal	3	626, 666, 708	1/1.4
1	Irradiated	3	319, 370, 403	1/4.3
2	Normal	5	676, 828, 836, 886, 978	1/1.2
2	Irradiated	3	112, 120, 221	1/8.1

Table 45. (Section VII, Table 11 refers)

Necropsy worm burdens of double vaccinated and of unvaccinated control pups enumerated 25 days after challenge infection with 1,000 normal A. caninum larvae. First experiment to compare subcutaneous vaccination and challenge with oral vaccination and challenge.

Route of administration		No. of pups	Necropsy worm burdens (fertile worms)	Sterile female worms (group total)	Mean group worm sex ratio, M/F
Vaccination	Challenge				
Control	S/c	6	615, 711, 801, 845, 850, 852	-	1/1.1
S/c	S/c	6	32, 46, 65, 113, 139, 188	110	1/1.6
Control	Oral	5	778, 795, 842, 940, 946	-	1/1.8
Oral	Oral	5	108, 109, 412, 439 610	82	1/1.7

Table 46. (Section VII, Table 14 refers)

Necropsy worm burdens of double vaccinated and of unvaccinated control pups enumerated 25 days after challenge infection with 1,000 normal A. caninum larvae. Second experiment to compare vaccination and challenge by permutations of the oral and subcutaneous routes of infection.

Route of administration		No. of pups	Necropsy worm burdens (fertile worms)	Sterile female worms (group total)	Mean group worm sex ratio, M/F
Vaccination	Challenge				
Control	S/c	3	535, 546, 566	-	1/1.5
S/c	S/c	5	0, 1, 19, 77, 107	110	1/1.4
Oral	S/c	6	11, 17, 54, 66, 83, 299	197	1/1.1
Control	Oral	3	437, 491, 690	-	1/1.7
S/c	Oral	5	10, 11, 14, 20, 22	85	1/1.9

Table 47. (Section VIII, Table 19 refers)

Necropsy worm burdens of double vaccinated pups, of anthelmintic control pups and of unvaccinated challenge control pups, 25 days after the time of challenge infection with 1,000 normal A. caninum larvae.

Vaccine larvae	Route of vaccination & challenge	No. of pups	Necropsy worm burdens (fertile worms)	Sterile female worms (group total)	Mean group worm sex ratio, M/F
Control	S/c	6	615, 711, 801, 845, 850, 852	-	1/1.1
Irradiated	S/c	6	32, 46, 65, 113, 139, 188	110	1/1.6
Normal	S/c	3	126, 466, 470	-	1/1.2
Normal	S/c	2	16, 28 (Anthelmintic control)	-	1/2
Control	Oral	5	778, 795, 842, 940, 945	-	1/1.8
Irradiated	Oral	5	108, 109, 412, 439, 610	82	1/1.7
Normal	Oral	4	54, 146, 307, 737	-	1/1.3
Normal	Oral	2	10, 12 (Anthelmintic control)	-	1/4

Table 48. (Section IX, Appendix I, Table 37 refers)

Necropsy worm burdens enumerated 21 days after subcutaneous inoculation of 1,000 normal or 40 kr-irradiated *A. caninum* larvae to 3-months-old pups.

Vaccination	Treatment of larvae	No. of pups	Necropsy worm burdens	Mean group worm sex ratio, M/F
EXPERIMENT 1				
1	Normal	8	414, 433, 469, 590, 599, 620, 634, 642	1/1.3
1	Irradiated	10	20, 33, 33, 35, 61, 73, 76, 83, 87, 104	1/76
2	Normal	5	524, 683, 828, 863, 908	1/1.2
2	Irradiated	5	3, 17, 19, 43, 65	1/29

EXPERIMENT 2

Since the same vaccine was used for the second experiment of this section (IX) as was used in experiment 1 of Section VII, the relevant vaccine attenuation control data and necropsy worm burdens are listed in Table 44 of this Appendix (II).

Table 49. (Section IX, Table 24 refers)

Necropsy worm burdens of pups that were double vaccinated at different ages, and of unvaccinated control pups, enumerated 22 to 26 days after challenge infection with 1,000 normal *A. caninum* larvae.

Age when first vaccinated	Age when immunity challenged	No. of pups	Necropsy worm burdens (fertile worms)	Sterile female worms (group total)	Mean group worm sex ratio, M/F
Controls	8 weeks	7	649, 706, 723, 726, 748, 753, 771	-	1/1.1
3 days	8 weeks	4	278, 300, 403, 448	13	1/1.2
Controls	3 months	11	516, 541, 619, 645, 667, 714, 720, 728, 741, 761, 768	-	1/1.1
1 month	3 months	9	20, 24, 38, 48, 74, 99, 187, 196, 238	82	1/1.2

Table 50. (Section IX, Table 25 refers)

Necropsy worm burdens of vaccinated and of unvaccinated pups and adult dogs enumerated 25 days after challenge infection with normal A. caninum larvae.

Age when vaccinated	No. of pups /dogs	No. of larvae in challenge inocula	Necropsy worm burdens (fertile worms)	Sterile female worms (group total)	Mean group worm sex ratio, M/F
Controls	6	1,000	615, 711, 801, 845, 850, 852	-	1/1.1
3 months	6	1,000	32, 46, 65, 113, 139, 188	110	
Controls (adult)	5	(1,000 (2,500 (1,000 (3,500 (3,500	180) 360) 369) 627) 949)	-	1/1.2
Adult	5	(1,000 (3,500 (3,500 (1,500 (3,000	3) 13) 26) 61) 201)	52	1/1.6

Table 51. (Section X, Appendix I, Table 38 refers)

Necropsy worm burdens enumerated 21 days after subcutaneous inoculation of 500 normal or 1,000 40 kr-irradiated *A. caninum* larvae to 3-months-old pups.

Vaccination	Treatment of larvae	No. inoculated	No. of pups	Necropsy worm burdens	Mean group worm sex ratio, M/F
1	Normal	500	5	163, 229, 327, 438, 452	1/1.1
1	Irradiated	1,000	4	114, 186, 205, 368	1/3
2	Normal	500	4	302, 344, 380, 399	1/1.2
2	Irradiated	1,000	5	28, 32, 43, 44, 53	1/52

Table 5a (Appendix II) has been displaced during binding to Appendix III

APPENDIX III

ANALYSES OF WEIGHT DATA ON THE BASIS OF MEAN GROUP WEIGHTS

Table 52. (Section X, Tables 29 - 32 refer)

Necropsy worm burdens of 3-months-old control pups 21-24 days after infection with 1,000 normal A. caninum larvae by subcutaneous inoculation. The size of these worm burdens served to control the infectivity of the larvae that were inoculated to challenge immunity in older, age-resistant control pups in the "persistence of immunity" experiment (i.e., infectivity-age resistance controls).

Appendix to Table No.	No. of pups	Necropsy worm burdens	
		Dog pups	Bitch pups
29	6	606, 865, 907	692, 967, 1023
30	5	895, 984	714, 917, 926
31/32	7	567, 581, 637	545, 599, 640, 644

Table 53. (Section VI refers)

Mean group weights of single-vaccinated and of control pups at the times of vaccination, inoculation of the challenge and termination of the experiment (when the pups died or were killed). Statistical analyses of the apparent differences between vaccinated and control pups were by Student's "t" test; the probabilities (P) refer to comparisons of data immediately above and beneath each statement.

Group	No. of pups	Mean weight in lb (± s.d.) at time of		
		Vaccination	Challenge	Kill
Controls	12	10.8 ± 5.5	14.8 ± 5.2	15.2 ± 8.4
		0.4 < P	0.7 < P	0.5 < P
Vaccinates	6	9.0 ± 3.1	14.0 ± 2.6	17.3 ± 4.2

Table 54. (Section VII refers)

Mean group weights of double vaccinated and of control pups at the time of first vaccination, at inoculation of the challenge and at termination of the experiment when the pups were killed. This data refers to the first experiment in which pups were vaccinated and their immunity challenged by the same route (i.e., subcutaneous vaccination - subcutaneous challenge, or oral vaccination - oral challenge). Statistical analyses of the apparent differences were by Student's "t" test; the probabilities (P) refer to comparisons of data immediately above and below each statement.

Route of infection		No. of pups	Mean weight in lb (± s.d.) at time of		
Vaccine	Challenge		First vaccination	Challenge	Kill
Controls	S/c	6	7.3 ± 2.6	14.8 ± 2.6	16.0 ± 3.9
			0.7 < P	0.7 < P	0.8 < P
S/c	S/c	6	8.4 ± 1.9	13.5 ± 2.7	15.1 ± 3.5
			0.05 < P	0.7 < P	0.3 < P
Oral	Oral	5	5.5 ± 2.7	12.8 ± 3.9	12.9 ± 3.4
			0.6 < P	0.5 < P	0.4 < P
Controls	Oral	5	7.7 ± 3.1	15.7 ± 3.3	16.9 ± 4.1
			0.8 < P	0.6 < P	0.7 < P
Controls	S/c	6	7.3 ± 2.6	14.8 ± 2.6	16.0 ± 3.9

For ease of comparison the first group of results have been repeated at the foot of the table.

Table 55. (Section VIII refers)

Mean group weights of pups that were vaccinated with irradiated or normal A. caninum larvae and of unvaccinated control pups at the time of first vaccination, at challenge infection with normal larvae, and at termination when the pups were killed. Statistical analyses of the apparent differences were made by Student's "t" test; the probabilities (P) refer to comparisons of data immediately above and below each statement.

Vaccine	No. of pups	Mean weight in lb (± s.d.) at time of		
		First vaccination	Challenge	Kill
X-irradiated	11	7.1 ± 2.2	13.2 ± 3.1	14.1 ± 3.3
		0.7 < P	0.1 < P	0.1 < P
Controls	11	7.5 ± 2.7	15.2 ± 2.8	16.4 ± 3.8
		P < 0.01	0.9 < P	0.7 < P
Unirradiated	7	12.4 ± 2.7	15.3 ± 4.9	15.8 ± 4.8
		P < 0.01	0.2 < P	0.3 < P
X-irradiated	11	7.1 ± 2.2	13.2 ± 3.1	14.1 ± 3.3

For the purpose of analyses the results of all pups that were vaccinated with the same preparation (i.e., irradiated or normal larvae) and of all control pups, irrespective of the route of vaccination and challenge infection, were grouped together.

For ease of comparison the first group of results have been repeated at the foot of the table.

Footnote to Table 55 (continued)

It should be noted that at the time of first vaccination, the mean group bodyweight of the pups that were vaccinated with normal larvae was approximately 50% greater than the mean bodyweights of all other vaccinated and control pups. This was the result of the deliberate selection at the start of the experiment of heavier pups in each litter and of their allocation to the group to be vaccinated with normal larvae.

Since fatalities from experimental ancylostomiasis appeared to be related to bodyweight (i.e., pups infected with the greatest number of adult hookworms per lb of bodyweight tend to die first), the pups to be vaccinated with normal larvae should as a result of the above selection have had a greater ability to survive until the time of challenge infection. This was designed to give sufficient observations at each stage of the experiment to permit statistical analyses. As a consequence of this selection and of the failure of the surviving pups after vaccination with normal larvae to gain weight during the vaccination period, all the groups had similar weight statistics at the time challenge larvae were inoculated.

Table 56. (Section IX refers)

Mean group weights of double vaccinated and of unvaccinated control pups and adult dogs in 2 experiments to compare the efficacy of vaccination of pups of various ages, and of adult dogs. Statistical analyses of the apparent differences were by Student's "t" test; the probabilities (P) refer to comparisons of data immediately above and below each statement.

Age when 1st vaccinated (months)	Age when immunity challenged (months)	No. of dogs	Mean weight in lb (± s.d.) at time of		
			First vaccination	Challenge	Kill
Controls	3	5*	-	9.2 ± 4.4	9.6 ± 3.3
				0.05 < P	0.6 < P
1	3	9	-	6.3 ± 0.9	9.1 ± 1.5
Controls	5	6	7.3 ± 2.6	14.8 ± 2.6	16.0 ± 3.9
			0.4 < P	0.4 < P	0.6 < P
3	5	6	8.4 ± 1.9	13.5 ± 2.7	15.1 ± 3.5
Controls	Adult	5	-	45.8 ± 21.0	42.6 ± 19.1
				0.8 < P	0.7 < P
Adult	Adult	5	-	49.1 ± 27.0	46.7 ± 24.0

* Only 5 of 11 (Table 23) 3-months-old controls survived to the end of the experiment.

VACCINATION AGAINST CANINE HOOKWORM

Summary of a thesis submitted for the degree of Doctor of Philosophy of the University of Glasgow by Thomas A. Miller, B.Sc., B.V.M.S., M.R.C.V.S.

The work reported in this thesis constituted some of the first steps in the invention and development of an irradiated vaccine for canine hookworm disease. The thesis is divided into 7 sections of experimental results.

There was no significant difference in the infectivities of A. caninum larvae between those given either orally or by subcutaneous inoculation. Exposure of the infective larvae to various doses of X-rays reduced their infectivity, as measured by subsequent intestinal establishment of adult hookworms. As the dose of radiation was increased, infectivity was progressively decreased and the pathogenicity of the hookworm burdens were reduced. At X-ray doses of 40 kr and greater, the female worms in the resulting population were invariably sexually sterile. It appeared that larvae irradiated with 40 kr or greater doses of X-rays would be suitable for vaccination experiments.

Single vaccination of pups when 3-months-old by subcutaneous inoculati of 1,000 40 kr-irradiated larvae conferred a highly significant resistance when 4-months-old against the establishment and potential morbidity and mortality of a subcutaneous challenge infection with normal larvae.

Compared with single vaccination, double vaccination of pups conferred a superior resistance to the establishment of a challenge infection. Double subcutaneous vaccination of 3 and 4-months-old pups with 1,000 40 kr-irradiated larvae was more effective against subcutaneous challenge than was double vaccination by the oral route against an oral challenge. Subcutaneous vaccination conferred equal protection against subcutaneous and oral challenge, while oral vaccination conferred a highly satisfactory protection only against subcutaneous challenge. Complete protection against the potential morbidity of the challenge infection was exhibited by all double vaccinated pups.

The immunogenic efficacies of X-irradiated and of normal A. caninum larvae were compared by double vaccination of 3 and 4-months-old pups. Subcutaneous vaccination with irradiated larvae was more uniformly effective than were either subcutaneous or oral vaccination with normal larvae. Anthelmintic treatment was a necessary adjunct of vaccination with normal larvae; and this method of vaccination proved to be extremely hazardous for the health and survival of the pups, since 5 of 14 pups died after vaccination.

Subcutaneous vaccination protected pups of different ages and adult dogs against the establishment and potential morbidity of challenge infection Vaccination was effective when commenced as early as 72 hours after birth of the pups. Adult dogs were also shown to benefit from vaccination,

although age per se conferred a considerable additional resistance to primary infection in adult controls. Age resistance in the vaccinated dogs was additive to their acquired resistance from vaccination.

The resistance to both establishment and potential morbidity of challenge infection persisted in the absence of further exposure to hookworm for at least 7 months after completion of the vaccination schedule. Towards the end of the 7 month period age resistance, as exhibited in previously uninfected challenge control pups, augmented the immunity of vaccinated pups against challenge hookworm infection. Vaccinated pups at all ages were completely immune to the pathogenic effects of challenge while older controls were partially (at 8 months) or completely (at 11 months) protected against these effects by age resistance per se. Vaccination with X-irradiated larvae stimulated an apparently maximal immunity which was not further improved by numerous small infection of normal larvae during the period between second vaccination when 4-months-old and challenge infection when 11-months-old.