A STUDY OF CELLULAR RESPONSES TO *SCHISTOSOMA MANSONI* IN THE MOUSE

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Summary

Mice percutaneously infected with 50 cercariae of Schistosoma mansoni showed an increase in the total number of leukocytes and in the concentration of protein in the peritoneal cavity 7 weeks later. The leukocytosis was enriched with eosinophils. The peritoneal IgG against antigens prepared from adult worms and eggs increased simultaneously with the IgG in serum. A good correlation was found between the level of IgG in peritoneal fluids and the corresponding serum.

Mice, normal and infected, were intraperitoneally challenged with mechanically transformed schistosomula and the host cell reactions in these two groups were compared. Two parameters were used to quantitate the host response; one was the cellular infiltration and the other the cellular adherence to schistosomula. In normal mice an acute and transient infiltration of neutrophils occurred. The number of neutrophils reached a peak at 2-6 hr and fell to control level by 24 hr. In contrast, the infected mice mounted a continuous peritoneal activity up to 4 days. This was characterized by a biphasic cellular infiltration- initially with an acute neutrophil infiltration comparable to that in challenged normal mice, followed by an infiltration of eosinophils and macrophages. The secondary macrophage infiltration ceased by 4 days but the eosinophilic infiltration continued. The magnitude of the infiltration and the nature of the responding cells depend on several factors: (1) the intensity

of primary infection; an increase in the proportion of eosinophils was observed in mice previously infected with 20-100 cercariae, whereas the induction of macrophage increase required a relatively heavy infection (50-100 cercariae each mouse), (2) the length of the primary infection period before challenge; the increase in eosinophils occurred in mice infected for 1 week or longer, whereas the increase in macrophages took place only in mice in which the worms from the primary infection had started laying eggs, (3) the size of challenge dose of schistosomula; the challenge doses of 450-900 schistosomula could elicit the increase in eosinophils, but a larger dose (900 schistosomula each mouse) was required to induce the increase in the proportion of macrophages, and (4) the sex of cercariae in the primary infection; the increase in eosinophils could be induced in mice infected with cercariae of either a bisexual or unisexual population, but the increase in macrophages took place only in mice with a bisexual infection. A challenge with Escherichia coli, Trichinella spiralis or Toxocara canis failed to alter significantly the proportions of eosinophils and macrophages. Intravenous injection of serum from chronically infected mice resulted in an increase in peritoneal IgG in the normal mouse recipients. These mice responded with an eosinophilic infiltration when intraperitoneally challenged with schistosomula. A crude extract prepared from adult worms was found to induce an infiltration of eosinophils

in infected mice and of neutrophils in normal mice when intraperitoneally injected. The crude worm extract was subsequently separated by anion exchange chromatography. Three fractions (S1, S2 and S3) were eluted using two salt gradients. The eosinophilic activity was detected in fractions S1 and S2, and the neutrophilic activity in S3.

The cellular adherence was scored on the basis of the number of cells adhering to schistosomula. A prompt and marked cellular adherence to schistosomula developed in both normal and infected mice by 30 min. However, in contrast to the continued cellular binding to schistosomula in infected mice, the displacement of cells from schistosomula was observed in normal mice by 24 hr. The prolonged cellular binding could be achieved by an intravenous injection of immune serum into normal mice 20 hr before challenge with schistosomula. An increase in cellular adherence to schistosomula was also observed in mice previously infected with T. spiralis for 4 weeks.

The identity of cells involved in the adherence was determined by four methods. It was shown that macrophages dominated (averaging 80%) the early phagocytic activity in all mice regardless of their immune status. In infected mice, the proportion of eosinophils increased to 45% of the active cell population and then equalled the macrophages in number by 24 hr. In contrast, most schistosomula recovered from normal mice attracted few cells at this time, and the phagocytic cells were predominantly macrophages. A few neutrophils were also involved

in both early and late adherence. When schistosomula labelled with fluorescent wheat germ agglutinin were used, loss of fluorescence was observed at 24 hr. The schistosomula recovered from the normal peritoneal cavity no longer attracted cellular binding when incubated with immune cells in the presence of antibodies.

The value of using the peritoneal cavity as the site for the investigation of cellular activity is discussed in relation to three major aspects: (1) chemotaxis in S. mansoni infection, (2) parasitic attrition mediated by leukocytes, and (3) parasitic evasion of host cellular activity.

INTRODUCTION

Schistosomiasis, a world health problem of immense proportion, affects an estimated 200-300 million individuals and is currently increasing both in prevalence and distribution as man continues to produce ecological environments suitable for disease transmission (Farooq, 1969; Jordan, 1972; Warren, 1973b).

One of the important schistosome species that infects man and other mammals is Schistosoma mansoni. This species which causes intestinal schistosomiasis is initiated by free swimming cercariae, released from infected snails of the genus Biomphalaria which penetrate the skin of the mammalian host and transform into larval forms known as schistosomula. From the skin the schistosomula travel to the lung where they reside for about a week before finishing their migration. They reach the hepatic portal system and mature into egg-producing adult worms 5-6 weeks after entry as cercariae. The host is therefore exposed to all these various growth stages of the parasites during the course of infection and is offered the opportunity to act against them in various ways involving cellular factors.

During infections with S. mansoni, most experimental hosts acquire partial resistance to subsequent challenge. The host immune response is directed largely against the early migrating worm from the challenge infection rather than against adult worms which have already established in the portal system-hence the term 'concomitant immunity'. Many investigators have conducted both in vivo and in vitro experiments to elucidate the mechanisms underlying the cellular infiltration that is triggered by the invading parasites and also the parasite attrition induced by the newly arriving host cells at various tissue sites.

1. Experimental models in studies of cell-mediated immunity

The early investigations of cell-mediated parasite attrition were carried out in the tissue through which the parasites migrate in whole living experimental animals. Biopsies and histochemical techniques were employed to study the sequence of cellular responses to cercariae during dermal penetration, to schistosomula during pulmonary migration and around degenerating adult worms and eggs in the liver (*in vivo* system). The parasiticidal efficacy is assessed by the severity and duration of the cellular infiltration into the site and the frequency of cell-worm contact.

There have, in addition, been several techniques described that utilize suspensions of various types of isolated effector cells with humoral factors in various combinations (*in vitro* system). In these models.the index of schistosomacidal activity is cellular adherence, cessation of motility of worms or release of label from worms.

1.1. In vivo system

Large numbers of studies on the immune status of a variety of animal species have been performed. Reviews of the reported gross and microscopic observations reveal the differences in the manner of the cellular responses, in skin and lung,against the invading parasites during the primary and challenge infections when different animal species were used. The variations in cellular reactions in the previously unexposed animals have been regarded as contributory to the variation of innate immunity exhibited by each host species. The responses, in humoral and cellular levels, of the infected host

to superinfecting cercariae may be important aspects of acquired resistance to reinfection.

1.1.1. Skin

The first host tissue the parasites contact during their passage is the skin where the cercariae penetrate. High proportions of invading cercariae die in the skin of naive and infected animals (Clegg and Smithers, 1968; Smithers and Gammage, 1980). The death may attributed to exhaustion of the energy reserves of the parasites during cutaneous penetration (Rai and Clegg, 1968; Ghandour and Ibrahim, 1978) as well as to specific or non-specific host defense mechanisms.

The epidermal/dermal barrier may not causally relate to the variation in innate resistance since the number of cercariae penetrating the skin of the mouse, guinea pig, hamster and rat are similar though the susceptibility of these hosts to infection with S. mansoni varies considerably (Warren and Peters, 1967). On the other hand, the primed skin proves a formidable barrier and provides a site for destruction of cercariae from the challenge infection and the degree of the effectiveness of the skin immunity appears to be host species- dependent (Phillips and Colley, 1978).

The evolution of cutaneous reactions to the invading schistosomula was generally investigated, with the exception of rhesus monkey model, at 10-30 mins, 4-8 hrs and 24-72 hrs after exposure to cercariae, for immediate (Type I), Arthus-like (Type III) and delayed (Type IV) reactions, respectively. The three types of hypersensitivities also could be induced by various soluble antigens of schistosome origin in experimental animals or man infected with schistosomes (Phillips and Colley, 1978).

1.1.1.1. Histological findings on skin immunity

The mouse is a permissive host for S. mansoni infection and acquires a partial resistance against reinfection which reaches its peak at 12 weeks after the initial infection.

Smithers and Gammage (1980) observed an early phase of parasite attrition operating in the abdominal skin of mice. Approximately 65% of the cercariae died during migration through normal skin. This is probably the major, if not sole mechanism responsible for the innate immunity since no further significant loss of the infection was detected after the skin stage. In the infected, resistant mice, a further killing of the challenge infection in the skin took place and accounted for the death of approximately 30% of the remaining parasites that survived in the normal animals. The skin immunity develops slowly and can only be demonstrated late in the infection after the egg laying has been commenced (Smithers and Gammage, 1980). There is also evidence that the migration of schistosomula from the skin to lungs is delayed in the infected animals (Sher et al., 1974; Smithers and Gammage, 1980).

It is not known whether the degree of skin immunity demonstrated in reexposed animals depends on the skin architecture since the identities of the infiltrating cells in the cutaneous inflammation induced by cercarial exposure vary when different skin sites are used, Colley et al (1972) reported a biphasic cutaneous reactivity, exhibiting the classic Arthus and delayed-type hypersensitivities, in the abdominal skin where cercariae penetrated. On the other hand, the delayed mononuclear infiltration was not detected when the mouse

ear pinna skin was exposed to cercariae (Lichtenberg et al., 1976). On the contrary, the indurated granulocytic infiltration enriched with eosinophils was observed throughout the 48 hr experiment, though an early, transient neutrophil infiltration occurred in either tested skin sites in normal controls. The discrepancy between the reported cellular reactions was not discussed in either paper. It may be that it is due to the microvasculature of ear pinna allowing diapedesis of more eosinophils and neutrophils to the tested ear pinna skin site, or that the mast cells, particularly rich in the ear pinna, are the primary factor responsible for the difference in the cellular responses.

The quantitative differences in the overall intensity of cellular contact to the invading schistosomula in the normal and immune mice were established in both abdominal and ear pinna skin models (Colley et al., 1972; Lichtenberg et al., 1976). The frequency of cellular contact was significantly greater in the immune mice than in the controls. However, neither paper supplied information on the quantitative differences in the numbers of damaged schistosomula in the skin of immune and non-immune groups.

The cutaneous granulocytic infiltration could be reproduced when skin-schistosomula, metamorphosed in vitro, were intradermally injected into the mouse ear pinna (Lichtenberg et al., 1976). This indicates that the concentric migrating patterns of leukocytes observed in the cutaneous reaction to cercarial penetration were largely against schistosomula themselves rather than attributable to cercarial antigenic or enzymatic products. It is interesting to note that local injection in the ear pinna of soluble crude saline extracts of cercariae and eggs triggered a different cutaneous response compared to that of whole worms. The infiltration pattern is characteristic

of progressive skin test to PPD in sensitized animals in which a sequential cellular pattern initiated with neutrophil infiltration, accumulation of mixed cell types, ultimately terminated with mononuclear predominance during the first 30 hrs after injection was demonstrated (Colley et al., 1972).

Mice immunized percutaneously with highly irradiated live cercariae were shown to develop resistance to reinfection that was equivalent to that seen in infected mice 12 weeks after exposure to untreated cercariae (Minard et al., 1978a; Hsu et al., 1981; Miller and Smithers, 1980). Interestingly, the site of the major attrition of challenge parasites varies in the two systems using different modes of immunization. The early skin-phase attrition is greatly enhanced in mice immunized with irradiated cercariae and it is the major contribution to parasitic death (Miller and Smithers, 1980). In contrast, in mice exposed to untreated cercariae, the majority of parasite attrition occurs after the lung phase of migration (Smithers and Gammage, 1980).

In a study using hamsters, Smith (1975) reported neutrophil infiltration in the abdominal skin which could be detected at 5 hrs and which persisted throughout the 24 hr-study. At 5 and 24 hrs after cercarial exposure, schistosomula could be detected in the epidermis and dermis of normal and immune hamsters. While distinct neutrophil aggregation occurred near the schistosomula in the skin of immune animals, the schistosomula in the deeper dermal tissue did not appear to elicit any cellular reaction, though intense neutrophil infiltration took place in the epidermis of the normal skin. This observation led to the interpretation that two separate mechanisms may operate in the skin, the unspecific neutrophil

infiltration in response to schistosomula which died shortly after penetration (Clegg and Smithers, 1968) and the Arthus reaction against the living schistosomula which had penetrated further into the dermal connective tissue (Smith 1975).

Similar observations of the neutrophil infiltration to those of hamster were reported in the skin of Macaca mulata monkeys (Lichtenberg and Ritchie, 1961). This reaction was mostly confined to the epidermis. Although a marked increase in the duration and severity of the dermal reaction to challenge cercariae was observed in immune monkeys, the differential studies of the number of schistosomula in the horny and prickle cell layers and dermis could not give clear proof that the migration speed and retention rate of challenge schistosomula in the dermal stage of immune monkey is any greater than in normal controls. This indicates that the skin barrier may play only a minor role in the high degree of resistance to reinfection (Smithers and Terry,1965a; Ritchie et al., 1966; Maddison et al.,1971; Lichtenberg and Ritchie, 1961).

However, in several more extensive studies using another schistosome species, S. japonicum, the sequential cutaneous response, reaginic whealing reaction, Arthus-like reaction and delayed-type hypersensitivity, were triggered by challenge cercariae in the S. japonicum infected monkeys (Hsu et al., 1975). The association of cell infiltration with destruction of migrating schistosomula was evident by the frequent observations of degenerating schistosomula amid eosinophil-enriched cellular aggregates (Hsu et al., 1974; Davis et al., 1963).

Dead schistosomula within the dermis were also observed in the

rhesus monkeys immunized with large number of cercariae of S. japonicum previously exposed to high dose of X-irradiation (Hsu et al.,1971; 1977). As observed in the mouse model, monkeys immunized with irradiated cercariae develop a strong immune response which is largely effective in the skin. On the other hand, only a few schistosomula originating from the challenge infection perished in the skin of animals infected with normal cercariae.

The guinea pig proved to be a unique host in its cutaneous response to reinfection. While there was no discernible alteration in the number of basophils in mice (Lichtenberg et al., 1976) and while decrease in basophil numbers was detected in rhesus monkeys (Maddison et al., 1973), guinea pigs previously infected for 4-12 weeks were found capable of mounting a delayed reaction characterized by basophil accumulation (30-40% of the total infiltration) concomitant with mononuclear infiltration (55-60%) upon reexposure to cercariae (Askenase et al., 1976).

The erythematous basophil-rich cutaneous response became perceptible at about 6 hrs and reached its peak at 24 hrs, it subsequently waned at 48 hrs. This transient reaction was delimited within the size of ring used to apply the challenge cercarial suspension and thus resembled the contact allergy (Dvorak and Mihm, 1972; Dvorak et al.,1971). This contact dermatitis appears to be immune-status- dependent since the cercarial penetration did not cause reaction macroscopically discernible in the skin of normal controls.

The basophil-enriched cellular condensations were most abundant near the schistosomula residing in the uppermost dermis.

The direct cellular adherence to schistosomula was rarely seen. That the basophils were active around the schistosomula was suggested by the presence of discoloured granules indicating the loss of granule substance of basophils nearest the schistosomula. Prominent oedema focally surrounded some schistosomula and a few were seen in a degenerating state. Askenase et al. (1976) did not indicate whether the histologic features observed in challenged immune animals, focal oedema and schistosomula degranulation were also found in normal animals. The guinea pig, like the rat and rhesus monkey, seems to reject an initial infection by some kind of 'self cure' which may or may not have an immunological basis.

The expression of cutaneous basophil hypersensitivity unique in the guinea pig appears to be dependent upon the mode of sensitization of animals. The basophil response could be demonstrated in animals immunized with live or dead cercariae. In contrast, a tuberculin-like delayed hypersensitivity characterized by predominant mononuclear infiltration was elicited in animals sensitized with whole viable eggs or soluble egg extract (Boros et al., 1973).

In conclusion, the cutaneous response to reinfection varies when different animal models are used. The reasons for the variation in cellular components involved in the delayed (48 hrs after cercarial exposure) reactions in the skin of mouse (eosinophil), rhesus monkey (mononuclear cells) and guinea pig (basophil) upon reexposure to cercariae are not known. Substances produced by immune inflammatory responses, or local factors, may regulate the transit of different leukocyte types from circulation into inflammation sites.

1.1.1.2. Host factors involved in the mechanism of skin immunity

The findings regarding the cutaneous reaction must be considered in any attempt to explain the mechanisms of protective immunity.

Studies of passive sensitization showed that humoral antibody is involved in the acquired immunity to schistosomiasis. The transfer of immune serum prior to the cercarial challenge could significantly reduce the number of adult worms recovered in mice (Sher et al., 1975b). The fact that the protective effect is lost if the serum is administered 3 days after cercarial challenge led to the speculation that the adopted resistance conveyed by immune serum may be operative in the skin of the serum-recipients.

IgG1 was identified as the factor presented in the immune serum which was responsible for the transfer of protection in the mouse recipients (Sher et al., 1977). Intradermal injection of immune serum containing IgE at the tested site prior to cercarial exposure resulted in reduction of worm burden in rat (Ogilvie et al., 1966). Similar observations have been reported using the mouse model (Doenhoff and Long, 1979). Colley et al. (1972) showed that transfer of immune serum by intravenous injection route resulted in an augmented early neutrophil infiltration in the abdominal skin at 2 hrs after exposure to cercariae.

These results indicated that the cellular infiltration was antibody dependent. The mechanism underlying the cellular infiltration is likely to involve the interaction between antigen and specific antibodies, either in soluble form or bound to cell membrane via receptors for Fc moiety of cytophilic antibodies (Ishizaka et al., 1970a; 1970b; Lehrer, 1976; Ovary et al., 1975; Parish, 1972).

The killing mediated by lethal antibody (Clegg and Smithers, 1972; Sher et al., 1974; Capron et al., 1977a; Smith and Webbe, 1974; Murrell and Clay, 1972) and that in collaboration with newly infiltrated leukocytes may explain the augmented mortality of the challenge schistosomula in the skin of immune mice. Elevated levels of homocytotropic and precipitating antibodies closely related to the two mechanisms mentioned above, anaphylaxis and Arthus-like reaction, have been reported in man and animals infected with S. mansoni (Sher et al., 1977; Rousseaux-Prevost et al., 1977; Camus et al., 1977).

Passive transfer of immunity to normal rats could be achieved by intravenous injection of the second peak obtained from fractionation of immune serum on a Sephadex-QAE- column (Perez 1974). It was postulated that the second fraction peak containing opsonizing antibody, which was observed to enhance the macrophage adherence to schistosomula in vitro(Perez and Smithers, 1977) might be also operative in vivo. However, the site of macrophage-mediated parasite attrition was not described in the above mentioned papers.

The concept that the acquired immunity to S. mansoni might depend upon the existence of an intact complement system was introduced by the studies in mice congenitally deficient in complement components and mice previously treated with cobra venom factor which deplete complement (Sher et al.,1975; Tavares et al., 1978). Although these groups made no histological study, it is conceivable that decomplementation may abrogate the cutaneous reaction to challenge cercariae. Activation of complement via

classic or alternative pathway yields fragments C3a and C5a (anaphylatoxins) and complexes of components ($C\overline{567}$) with chemotactic activity for neutrophils (Lepow et al., 1970; Ward et al.,1966; Snyderman et al., 1971; Ward and Newman, 1969) and macrophages (Snyderman et al.,1972; 1975).Anaphylatoxins also cause the degranulation of mast cells, releasing histamine which is selectively chemotactic for eosinophils (Mota, 1959). A number of in vitro studies also have yielded indirect evidence that might be interpreted as indicating that complement is utilized in chemotaxis and in killing mechanisms in schistosomiasis which will be discussed later.

It appears that the innate immunity, mainly operative in the skin (Smithers and Gammage, 1980), of mice is not thymic cell dependent since the worm burden of congenitally athymic mice (Nu/Nu) was found not significantly different from their heterozygous littermates (Nu/+) (Phillip et al., 1977). The peripheral blood eosinophilia normally associated with schistosomiasis was not developed in the athymic mice, and the egg granuloma, a manifestation of hypersensitivity, was also reduced. Similar findings were made in mice deprived of T cells by thymectomy and administration of anti-thymocyte serum (Pepys et al., 1980).

Conversely, the presence of T cells is a prerequisite for the acquired immunity. Studies employing athymic mice and the lung model of Lichtenberg et al.(1977), which bypass the skin penetration stage of parasites, showed an elimination of inflammatory foci which normally form around schistosomula injected into the lung of immune mice (Sher et al., 1977). The depletion of T cells by thymectomy and anti-thymocyte serum is also effective in ablation of the acquired resistance to reinfection if the depletion procedure

is applied before initial infection (Mahmoud et al., 1975a; Doenhoff and Long, 1979). However, anti-thymocyte serum applied after an active infection has been established does not suppress the resistance in thymectomized animals. In fact, application of anti-lymphocyte serum does not affect the acquired resistance in immune intact mice (Mahmoud et al., 1975a). It therefore seems that the effector mechanisms responsible for resisting challenge had already been induced and were no longer sensitive to anti-lymphocyte serum administered just before cercarial challenge.

The function of T cells in immunity to schistosomiasis is probably manifested through biologically active molecules (lymphokines) released by sensitized lymphocytes when stimulated by specific antigen. Among the wide range of lymphokines are substances which regulate the antibody formation by B cells (Kishimoto and Ishizak, 1973; 1975; Tada et al., 1973), mediators which are chemotactic for leukocytes (David, 1966), factors which inhibit the migration of macrophages (David & David, 1972; Bloom & Bennet, 1966) and activate macrophages which render them more effective in tumoricidal (Fidler, 1975; Piessen et al., 1978; Churchill et al., 1975; Hibbs et al., 1978) and in bactericidal activity (Mackaness, 1970; 1972; Hahn, 1974). Direct T cell mediated killing of schistosomula does not take place in vitro (Butterworth et al., 1979a).

Depletion of bursal equivalent lymphocytes (B cells) by intraperitoneal injection of anti-mouse μ chain (Lawton et al., 1972) from the day of birth for ten days did not alter significantly the innate susceptibility of mice to S. mansoni and, in contrast to the observations made on athymic mice, the

granuloma formations in B cell-deficient mice are similar to those of intact mice (Maddison et al., 1978). The role of B cells in the acquired resistance to reinfection has not been studied. Nevertheless, their role in specific antibody production indicates their likely importance.

The development of acquired immunity is certainly dependent on the presence of one or several types of radiosensitive cells since a total elimination of immunity assessed by lung recovery technique was found if the immune mice were irradiated 5 days prior to intravenous challenge of schistosomula prepared by in vitro skin passage (Sher, 1977). Miller and Smithers also found that the early skin phase of parasite attrition described by Smithers and Gammage (1980) is abolished if immune mice are irradiated before challenge (unpublished data, see McLaren, 1980).

The transfer of immune lymphoid cells developed a delayed mononuclear infiltration in the skin of mouse recipients against the challenge cercariae (Colley et al., 1972). However, attempts to transfer resistance to recipients using immune lymphoid cells together with immune serum gave equivocal results. Immune serum recipients who developed significant resistance did not give superior performance when immune lymphoid cells were administered concomitantly (Sher et al.,1975b). Conversely, Maddison and Kagan (1979) found that passive transfer of immune serum alone in the mouse did not convey protection while combination of lymphoid cells from immune donor and immune serum did result in a significant reduction in the worm burden of the challenge infections.

Studies of cutaneous response to reinfection in immune rhesus monkeys showed an induced neutrophil infiltration (Lichtenberg and Ritchie, 1961). Nevertheless, an early eosinophil and late mononuclear infiltration pattern was demonstrated when immune skin was tested with adult worm extract (Maddison et al., 1973). The delayed hypersensitivity could be reproduced in monkeys receiving, 3 weeks prior to skin test, transfer factor, the lysate of lymphoid cells or peripheral leukocytes prepared from infected donors (Maddison et al., 1972).

However, injection of transfer factor alone failed to convey protection in the monkeys receiving it (Maddison et al., 1976). When immune serum was introduced together with transfer factor, significant protection was conveyed to recipients (Maddison et al., 1976). The mechanism whereby transfer factor acts as an immunopotentiator in the rhesus monkeys has not yet been elucidated. The function of transfer factor does not depend on the immune status of the cell donors since material prepared from normal animals was equally efficacious.

Maddison and coworkers (1972; 1976) did not provide information as to whether the viable immune lymphoid cells could also achieve the protection in the monkey recipients. However, combination of viable lymphoid cells and immune serum failed to give protection against schistosomiasis in rat (Maddison et al., 1970). Immune serum in conjunction with viable cells did not confer greater protection than that achieved by immune serum alone in mouse recipients (Sher et al.,1975b). The apparent success of transfer factor should encourage further research in its effect using mouse and rat models.

Lichtenberg et al.(1976) reported the occurence of free mast cell granules accompaning the granulocytic infiltration in both normal and immune mice when mast cell-rich ear pinna was exposed to cercariae. The notion that the inflammatory reaction is an effect of mast cell degranulation is supported by the following findings: (1) cellular infiltration is greatly reduced in mice pretreated with reserpine which depletes the serotonin, the principal vasoamine stored in the granules of mast cells (Askenase, 1977) (2) there is abolition of cutaneous inflammatory responses accompanying an initial infection in mice depleted of mast cells by chronic treatment with compound 48/80. Additionally, the treated mice could no longer reject challenge infection (Dean et al., 1976). The granule-associated mediators have been reported to function either as vasoactive or chemotactic factors or both (Clark et al., 1975; Goetz and Austen, 1975; Wasserman et al., 1974; Schwartz et al., 1977).

The role of mast cell in the skin immunity is also evident in the guinea pig model. The guinea pig is unique in demonstrating basophil hypersensitivity when reexposed to cercariae (Askenase et al., 1976). The delayed infiltration may be induced by chemotactic factors released from sensitized lymphocytes triggered by the surfaces of schistosomula as in the case of classic tuberculin type hypersensitivity. Degranulation of mast cells was evident when the skin was examined by light and electron microscopy. The extensive intra-epidermal and dermal oedema surrounding the parasites is likely to be due to vasoactive mediators from the newly arrived mast cells. At present, it is not known whether the newly arrived basophils have a direct cytotoxic effect on schistosomula.

In conclusion, the histological examinations of skin where the cercariae penetrated demonstrate the phenomena of oedema and cellular infiltration. The role of humoral and cellular factors in the change of vascular permeability and distribution of leukocytes has been reviewed. The results of these approaches indicate that the skin immunity is a system activated by the interaction of schistosome antigens with their specific antibodies. This antigen and antibody interaction results in the generation of biologically active fragments from complement (anaphylatoxins), the vasoactive amines (histamine and serotonin) from mast cells and the chemotactic lymphokines from lymphocytes.

1.1.2. Lung

Lung is the second host tissue where the migrating schistosomes contact. There they reside for an unknown period before continuing their migration course.

The presence of schistosomula in lungs causes tissue injury due to mechanical damage of capillaries by the migrating parasites or else to the host immunological responses in which the neutrophil may be responsible for the haemorrhage (Movat, 1971).

In general, schistosomula in previously unexposed animals evoke only minor lung reactions, while in the immune hosts, the inflammatory responses vary greatly among a variety of animal species. The mechanisms underlying the pulmonary infiltration in immune host may be analogous to those postulated for the skin immunity. The cellular response is associated with decreased number of parasites recovered from minced lung tissue.

1.1.2.1. Histological findings in lung immunity

Lichtenberg et al. (1976) described a negligible host reaction to schistosomula from challenge infection which had survived the skin immunity and reached the lungs of mice. In contrast, Magalhaes-Filho (1959) reported a marked cellular infiltration in the lungs following a challenge in immune mice. It is not clear whether this early report was in fact an observation of egg granuloma or that the cellular reactivity in lungs varies when different strain of mice are used.

The strain differences in the cellular response to challenge schistosome have been recorded when hamster model is used (Smith et al., 1975). It appears that a correlation exists between the occurence of the pulmonary reaction and the level of acquired resistance assessed by either lung recovery or portal perfusion assays. Extensive haemorrhage was visible macroscopically on the surface of the lungs of the high responder (WO strain) hamsters. Histological examination of the lungs revealed neutrophil infiltration of the pulmonary interstitium, occlusion of capillaries, pulmonary venules with neutrophils, and evidence of vasculitis. However, the aggregation of neutrophils around the schistosomula was not seen ;the neutrophils were well distributed through the lung tissue.

There was no significant alteration histologically except some minute haemorrhages due to mechanical damage to the capillaries by the migrating schistosomula from the challenge infection in the lungs of normal controls or in the low responder (LGN strain) hamsters previously infected with S. mansoni. The proportions of

eosinophils and macrophages were not significantly different from their respective prechallenge levels.

The massive neutrophil infiltration was also recorded in immune rhesus monkeys 6-8 days after reexposure to cercariae (Lichtenberg and Ritchie, 1961), but unlike that described in the studies using hamster model (Smith, 1975), the infiltrate was surrounding the invading schistosomula in 'tuft-like' fashion. Degeneration of schistosomula within the foci of inflammatory cells was evident by their morphological changes. The 'tuft-like' cellular aggregations were not observed around schistosomula passing through the normal lungs.

From the studies using mouse, hamster and rhesus monkey models, it appears that the neutrophil, the predominant infiltrating cell type, plays the major role in the lung immunity.

1.1.2.2. Host components involved in the lung immunity

Comparison of the number of schistosomula recovered from skin, lung and liver of immune and normal mice suggests that lung may not be the location for the destruction of challenge schistosomula in mice (Smithers and Gammage, 1980). This suggestion is supported by the observation of negligible pulmonary reaction against the challenge schistosomula in immune mice (Lichtenberg et al., 1976). These results led to the hypothesis that schistosomula become progressively less easily recognizable or susceptible to the killing by host leukocytes. There is evidence that numerous tegumental changes occur during early development and the mechanisms by which the tegument avoids damage by the effector

cells of the immune mice have been suggested by several groups

The tegumental modulation of the schistosomula apparently is a prerequisite for their survival. The *in vitro* skin-penetrated schistosomula intravenously injected could, in contrast to the minimal pulmonary reaction resulting from a percutaneous challenge, induced massive cellular infiltration which resulted in the killing of schistosomula in the lungs. The magnitude of the cellular reaction was significantly reduced if the schistosomula had been cultured *in vitro* for up to 44 hr in the presence of mouse erythrocytes prior to injection. The cultured parasites also survived better in immune mice than do newly transformed schistosomula (Lichtenberg *et al.*, 1977). It was also found that pulmonary inflammation was elicited if the cercariae were X- ray attenuated before their percutaneous exposure in mice (Lichtenberg and Sadun, 1963). This may indicate that the capacity of the schistosomula to undergo the tegumental modulation is impaired by the irradiation and present as relatively 'foreign bodies' in lungs hence the increment in cellular responses.

The inflammatory reaction to the intravenously administered schistosomula in the lungs appears dependent on a T cell-mechanisms since it did not occur in the lungs of preinfected athymic mice (Sher *et al.*, 1977). The serum obtained from chronically infected nude mice cannot adoptively transfer the reaction though serum produced by infected heterozygous litter-mates can transfer a modicum of lung reactivity to normal nude mice.

However, since little cellular response to schistosomula occurs in the lungs of the immune mice *in vivo* (Lichtenberg *et al.*, 1976), the lung model (Lichtenberg *et al.*, 1977) in which mechanically transformed schistosomula are used may not represent the *in vivo* host response to the migrating schistosomula. The cellular responses to migrating schistosomula in the lungs of the other kinds of animals appear to be different from that occurring in the mouse.

The pulmonary inflammation in the immune hamster after reinfection (Smith *et al.*, 1975) macroscopically and histologically resembles interstitial pneumonitis induced by repeated injection of foreign proteins (Brentjen *et al.*, 1974). Passive transfer of immune serum into the normal animals on the day of infection also leads to an increased inflammatory reaction (Smith *et al.*, 1975). This suggests that humoral factors may be responsible for this response. It is intriguing to note that the presence of schistosomula is not the obligatory condition for the response. The pulmonary inflammation may occur as a consequence of skin penetration. This was suggested by the finding that the neutrophil response in lungs took place within 24 hrs after reexposure before the schistosomula have migrated out of the skin. The removal of the skin area containing the challenge cercariae did not prevent the onset of neutrophil infiltration in the lungs in immune hamsters though the presence of schistosomula augmented the magnitude of the inflammatory response.

In the study of pulmonary response using a BSA-rabbit model, a bilateral inflammation was also demonstrated by Kaplan *et al.* (1980). Both pneumonitis and pleuritis were elicited in immunized rabbits

though specific BSA challenge was conducted only intrapleurally. The mechanisms invoked by Kaplan et al.(1980) may also be involved in the schistosome-hamster model (Smith, 1975). The antigens secreted from the challenge schistosomula in the dermis may enter the circulation. The combination of antigens with specific anti-schistosome antibodies circulating in the blood of immune hamsters may cause infiltration of neutrophils into the lungs where the immune complexes have been deposited.

It has been long known that intravascular administration of immune complexes elicites the pulmonary infiltration (Movat et al., 1968). As in the cutaneous Arthus-like reaction induced by immune complexes, the sequestration of neutrophils in the lungs may involve the activation of complement cascade. The accumulating neutrophils ingest the immune complexes. The phagocytosis results in the secretion of their granule content with enzymatic activities into the surrounding tissue and mainly the wall of venules hence the intense haemorrhage (Cochrane and Aikin, 1966). The phagocytosis of antigen also is known to cause enhanced metabolic activity and enzyme synthesis in the neutrophils (Wilkinson, 1974). Smith (1975) did not quantitate the effectiveness of the infiltrating neutrophils in the schistosomacidal activity in the lungs of hamsters. But these granule-associated lysosomal proteases released from phagocytosing neutrophils may be responsible for the death of schistosomula entrapped in the 'tuft-like' neutrophil aggregations in the lungs of immune rhesus monkeys described by Lichtenberg and Ritchie (1961).

The assumption that the pulmonary infiltration may be the cause of the reduction of schistosomula recovered from the lungs of immune hosts (Smith, 1975; Lichtenberg and Ritchie, 1961; Magalhaes-Filho, 1959) was derived from the studies of heterologous resistance. Intravascular administration of Gram-negative microorganisms and antigen-antibody precipitates and other aggregated antigens have been known to cause lung inflammation (Scherza and Ward,1978). The stimulation of protection against infection with S. mansoni in normal mice and hamsters pretreated with formalised E. coli, heated-aggregated human serum albumin (HSA),or Spider crab (Maia squinado) hemocyanin (MSH) complexed with rabbit anti-MSH antibody, were recorded (Smith, 1975). The E. coli themselves and the products of activation of the complement cascade by immune complexes have been previously demonstrated to induce accumulation of neutrophils (Schiffman et al., 1974; Brentjens et al., 1974). Interestingly, the last two inflammation-inducing agents, HSA and MSH, in soluble forms also could stimulate lung inflammation and protection against schistosome infection (Smith, 1975). The reasons for the unexpected results are not known.

Pretreatment of mice with high dose of viable Mycobacteria bovis, Bacillus Calmette-Guerin strain (BCG), given intravenously, also conferred significant protection against subsequent infection with S. mansoni (Bout et al., 1977; Civil and Mahmoud, 1977; Civil et al., 1978; Maddison et al., 1978). To determine the site of parasite attrition in the mice pretreated with BCG

proved confusing. While Maddison et al. (1978) reported a post-lung attrition phase and minor interstitial pneumonitis elicited by BCG, Civil et al. (1978) observed profound infiltration of macrophages in the lungs and earlier phase attrition, probably in the lungs of the BCG-treated mice. This discrepancy may be due to the use of different doses of preparation of BCG, or to the differences between mouse strain in 'responsiveness' to BCG.

The mechanisms whereby BCG suppresses a schistosome infection has not been elucidated. The BCG has been shown to act as an immunopotentiator on the production of antibody-formation cells (Miller et al., 1973). However, protection induced by pretreatment with BCG was not significantly different in intact and B cell-deficient mice (Maddison et al., 1978). In fact, the high dose BCG which afforded protection suppressed the antibody production detectable by Cercarienhüllenreaktion. These results led to the conclusion that B cells do not participate in the mechanism of protection afforded by BCG treatment.

Since BCG suppresses eosinophilopoeisis (Maddison et al., 1978), the eosinophil may not be the cell type which kills schistosomula in these experiments. Without any definitive evidence one can speculate that the mode of action of BCG in the resistance to S. mansoni may be analogous to that observed in the studies of tumour immunity. The intralesional injection of BCG caused the regression of local tumor nodules in experimental animals (Zbar et al., 1971; Baldwin and Pimm, 1972). It has been widely accepted that the anti-tumour effects of BCG are principally mediated through the host macrophages activated by the immunostimulant (Alexander,

1973; Hibbs, 1973; Germain et al., 1975).

The lung immunity appears to depend on the mode of immunization. In the mice vaccinated with crude worm extract, though the level of circulating antibodies was raised and infiltration of both neutrophils and macrophages did take place when the challenge schistosomula entered the lungs of hamsters (Smith, 1975), the vaccination was unable to confer resistance to cercarial challenge. Such inability of inflammatory cells to destroy schistosomula was also demonstrated in mice vaccinated with crude cercarial extract (Colley et al., 1977). Though the vaccinated mice were capable of producing eosinophil infiltration in skin tested with the same cercarial preparation, they were unable to mount resistance to S. mansoni.

These observations suggest that cellular infiltration does not necessarily result in the killing of schistosomula during their migration in the dermal and lung tissues, and the 'activation' of inflammatory cells may be stimulated specifically with an an active schistosome infection or unspecifically with some materials unrelated to S. mansoni.

1.2 In vitro system

Although the cellular responses occurring in skin and lung against the invading parasites supply evidence for the importance of effector cells in the immunity to schistosome infection, the limitation of interpreting histologic findings in terms of functional interactions between cells, and between cells and humoral components, which ultimately result in the killing effect,

has prompted many groups of investigators to conduct *in vitro* studies.

Cell suspensions enriched in one type of leukocytes of interest have been prepared from either peripheral blood or peritoneal exudates collected from a variety of animal species. The capacity of leukocytes to kill schistosomula, either mechanically transformed (Ramalho-Pinto *et al.*, 1974) or *in vitro* skin-penetrated (Clegg&Smithers, 1972), was determined using three experimental systems: (1) cell + complement (complement-alone system) (2) cell + antibody (antibody-alone system) and (3) cell + complement + antibody system. The third system may be more relevant to the *in vivo* situation. The sources of antibody and complement used in cell-mediated activity were generally autologous in respect to the leukocyte donors.

The damage or destruction of schistosomula was determined by one or more of the following criteria; (1) the loss of motility of the target schistosomula (2) failure in dye exclusion by schistosomula (3) ultrastructural studies of schistosome tegument (4) ^{51}Chromium release from prelabelled parasites (5) infectivity of schistosomula after being intravenously injected. The effectiveness of killing of schistosomula mediated by host leukocytes *in vitro* appears to be affected by the concentration of antibody and complement and the effector cell:worm ratio (McLaren, 1980).

1.2.1. Complement

It has been suggested that complement components are involved in more than one aspect of mechanisms underlying the attrition of

schistosomula of S. mansoni, both in vivo and in vitro. Therefore, the role of complement in the killing mechanisms mediated by antibodies and/or leukocytes will be considered before a discussion of assay systems consisting of the complement and various types of leukocytes.

The concept that intact complement system is the prerequisite condition for an effective resistance to schistosome infection is suggested by the in vivo studies using strains of mice congenitally deficient in complement components or mice pretreated with anti-complementary cobra venom factor (Waksman and Cook, 1975; Sher et al., 1975a;Tavares et al., 1978a).These mice exhibited increased susceptibility to cercariae from an initial or challenge infection.

A number of in vitro experiements have yielded indirect evidence that might be interpreted as indicating that complement may participate in the innate and acquired immunity to S. mansoni.

The rapid killing of cercariae in the normal serum suggests that the components of glycocalyx may act as a C3-convertase which activates the complement system independent of C1, C4 and C2 (Machado et al., 1975). A glycoprotein prepared from cercarial extracts generated anaphylatoxic activity in normal serum with effect biologically similar to C5a (Gazzinelli et al., 1969).

Several groups of investigators, using similar experimental protocols to those described by Machado et al. (1975), demonstrated that some of the newly prepared schistosomula, both mechanically transformed and in vitro skin-penetrated forms, were killed after incubation with normal chicken, guinea pig, human,

monkey, rat and mouse serum (Santoro et al.,1979; Silva and Kazatchkine, 1980). The activation of complement by the residual glycocalyx on the schistosomula is believed to be responsible for the complement-mediated killing. The following data conclusively indicate that such complement-mediated killing of schistosomula is dependent on the activation of the alternative pathway of complement; (1) the full killing effect of C4-deficient guinea pig and C2-deficient human serum, (2) the inactivity of serum depleted of factor B or of factor D and serum treated with zymosan against schistosomula, (3) the consumption of components of alternative pathway or factor B during the incubation of normal serum with schistosomula in the serum-Mg^{++} -EGTA assay system, (4) the detection of fluid-phase breakdown products of C3 (C3d) during the contact C2-deficient human serum with schistosomula by immunoprecipitation test, (5) conversion of native C3 into a component with a more anodal electrophoretic mobility (Santoro et al., 1979; Silva and Kazatchkine, 1980).

Therefore, the activation of the alternative pathway by schistosomula appeared to result in assembly of the cytolytic membrane attack complex(Kolb and Muller-Eberhard, 1973) which damage schistosomula as evident by ultrastructural studies (Ouaissi et al., 1980). The question as to whether the full expression of the late reacting sequence from the C3 through C9 components or whether only a partial activation of this sequence is necessary for the killing is not known. Nevertheless, the activation of the alternative pathway of complement is therefore envisaged as a natural and nonspecific defense mechanism against an initial schistosome infection.

The activation of classic pathway of complement by interaction with antibody on the surface of schistosomula appears to be the major mechanism responsible for the killing observed when schistosomula were incubated in the presence of antibody and complement (Tavares et al., 1978a). The use of C4-deficient guinea pig serum as the source of complement significantly reduced the schistosomacidal activity, whereas serum treated with zymosan at 17°C to deplete the terminal complement components (Pillemer et al., 1954) or heated at 50°C for 30 min to inactivate the factor B (Gookofsky and Lepow, 1971) only partially inhibited the killing effect mediated by antibody (Tavares et al., 1978a).

It has been demonstrated that the Fc region of IgG could initiate the activation of classic pathway of complement either via the recognizing site of C1 for IgG-Fc (Kehoe and Fougereau, 1969; Allan and Isliker, 1974a, b; Johnson and Thames, 1976) or at the site of receptors for IgG-Fc or for C1q on the surface of schistosomula (Torpier et al., 1979; Santoro et al., 1979; 1980).

The components of complement may also be involved in the immune adherence of host leukocytes to the target parasites. The presence of C3 on the surface of schistosomula was confirmed by indirect immunofluorescent studies using fluorescein-conjugates of anti-C3 antibodies (Santoro et al., 1979; Ouaissi et al.,1980). Examination of reaction supernatant of schistosomula and complement and rosette formation with sensitized sheep erythorcytes coated with C1-C3b indicated that the cleavage product was C3b (Ouaissi et al.,1980; Santoro et al.,1979; Silva and Kazatchkine, 1980). The opsonically active C3b was postulated to be associated with antibody-independent adherence of mast cells (Sher, 1976;

Sher and McIntyre, 1977), eosinophils (Ramalho-Pinto et al.,1978), neutrophils (Dean et al., 1974; 1975; McLaren, 1980) and of macrophages (Mahmoud et al., 1979a) to the schistosomula. This adherence is mediated by the membrane receptors for C3b of these leukocytes (Sher and McIntyre, 1977; Henson, 1971; Scribner and Fahrney, 1976; Gupta et al., 1976; Butterworth et al.,1976). The presence of opsonically active C3b on the surface of schistosomula also enhanced the antibody-dependent parasiticidal function mediated by various types of host leukocytes in vitro. This will be discussed in their respective section later.

However, the consequence of complement activation are not only to bring about the damage or destruction of the target worms, but probably as important, the elaboration of by-products which are mediators of the inflammatory responses. The activation of complement cascade by the residual glycocalyx as well as by immune complexes (Santoro et al., 1979; Silva and Kazatchkine, 1980; Torpier et al., 1979; Santoro et al., 1980) results in the release of small fragments, C3a and C5a, from C3 and C5. These fragments possesses anaphylatoxin properties and have been demonstrated to cause increased capillary permeability when injected intradermally (Lepow et al., 1970) and to release histamine from mast cells in vitro (Mota, 1959). The anaphylatoxins also have been shown to act as chemoattractants for neutrophils and monocytes (Lepow, 1971; Ward et al., 1966; Snyderman et al., 1971; 1975). Though the extent to which the anaphylatoxin is a significant chemotactic factor is not known, it is conceivable

that the induced infiltration of these effector cells to the site of inflammatory responses would permit efficient adherence and the ultimate killing of schistosomula opsonized with C3b.

1.2.2. Mast cell

It is perhaps surprising that little work has been done to investigate the role of mast cells in the immunity to S. mansoni, either in vivo or in vitro systems, in view of the vast number of granule-associated substances released from mast cells that are capable of inducing the vascular reaction and chemotaxis (Lagunoff, 1976).

At present, their function in schistosome immunity is not clear. Evidence suggesting the mediation of mast cells in the acquired resistance have been provided by in vivo studies using reserpine and compound 48/80, specific antagonists of granule-associated histamine and serotonin (Askenase, 1977). However, the following two criteria: (1) the death of schistosomula resulting from direct adherence of mast cells, and (2) the isolation of mediators such as histamine released at the time of its proposed action during the inflammatory response, which would suggest the involvement of mast cells in the schistosome immunity have not been met.

Sher (1976.) demonstrated the adherence of rat mast cells to in vitro skin-penetrated schistosomula in the presence of normal

and immune serum. This reaction appears to depend mainly on the complement rather than antibody since heated immune serum failed to inhibit the adherence if fresh normal serum was added in the assay system.

The findings that the adherence reaction was reduced if the serum was pretreated with zymosan or cobra venom factor or addition of EDTA to the culture, and that the treatment with EGTA failed to inhibit rosette formations . of mast cells to schistosomula, indicate that the adherence may depend on the activation of the alternative pathway of complement (Sher, 1976). The cercarial glycocalyx retained on the surface of schistosomula has been demonstrated to be capable of activating the complement cascade via the alternative pathway (Santoro et al., 1979; Ouassie et al., 1980; Silva and Kazatchkine, 1980). Rat mast cells have been shown to possess membrane receptors for C3 (Sher and McIntyre, 1977).

Although the degranulation of mast cells after contact with antibody-opsonized schistosomula in vitro has not been demonstrated, anaphylactic degranulation of cutaneous mast cells in the animals previously infected with S. mansoni was evident in the histologic studies (Lichtenberg et al., 1976; Askenase et al., 1976). Some of the well characterized mediators released from degranulated mast cells have been shown to chemoattract effector cells and to increase vascular permeability for antibodies and complement (Kaliner et al., 1973; Lewis et al., 1975). In addition, soluble products released by nonimmunologic or immunologic activation of mast cells were shown to augment the killing of schistosomula mediated by eosinophils in vitro (Capron et al., 1978b).

Therefore, while the schistosomacidal effect of mast cells resulting from direct contact has not been demonstrated, certain elements of the inflammatory process contributing to immunity to S. mansoni may be contingent on the functions of the mediators released from mast cells by the interaction of schistosome antigens and the cell-bound anaphylactic antibodies.

1.2.3. Neutrophil

The neutrophil is generally the first major type of cell to arrive at the skin site where cercariae from the initial infection penetrate (Colley et al., 1972; Lichtenberg et al., 1976; Smith, 1975; Lichtenberg and Ritchie, 1961). To date, there has been no in vitro experiment conducted to elucidate the mechanisms underlying chemotaxis for neutrophils as a consequence of an initial S. mansoni infection.

Some indirect evidence led to the postulation that the anaphylatoxins (C3a and C5a), the by-products of activation of complement cascade via either classic or alternative pathways or both which have been demonstrated to be chemotactic toward neutrophils in vitro (Ward, 1967; Hill and Ward, 1969; Bokisch et al., 1969; Shin et al., 1968; Snyderman et al., 1971) may be the major chemotactic substances responsible for the neutrophil infiltration observed in vivo.

The destruction of schistosomula, of both mechanically transformed and skin transformed forms, mediated by the accumulated neutrophils, has been studied using in vitro assay systems of various combinations of cellular and humoral factors (Anwar et al., 1979; McLaren, 1980: Vadas et al., 1979).

The schistosomacidal mechanism of neutrophils is to adhere and to release lysosomal enzymes to destroy the targets. Using rat and guinea pig models, the cellular adherence was observed to take place when the schistosomula were incubated in the presence of opsonizing IgG and complement in vitro. This ultimately resulted in the death of the parasites (Dean et al., 1974; 1975). These findings were confirmed by Vadas et al. (1979) and Anwar et al. (1979) using human neutrophils.

Several groups of investigators have detailed the two phases in interaction with schistosomula:(1) adherence to and (2) killing, of the parasites. The cellular adherence could be induced by antibody alone or by complement alone (Dean et al., 1974; 1975; Anwar et al., 1979; Vadas et al., 1979; McLaren, 1980; McKean et al., 1981), presumably via receptors for Fc domains of antibody (Lay and Nussenzweig, 1968; Messner and Jelinek, 1970; Ishizaka et al.,1970b) and C3b (Lay and Nussenzweig, 1968; Eden et al., 1973; Dietrich et al., 1974; Welleck et al., 1975), respectively, on the membrane of neutrophils.

However, the adherence does not invariably result in the death of entrapped schistosomula. The inactivity of neutrophils adherent to the schistosomula in the antibody-alone system was described by Dean et al. (1974; 1975) and McLaren (1980) using rat neutrophils. In their systems, the presence of complement appeared a prerequisite for the ultimate killing of target. Extracellular release of lysosomal enzymes from neutrophils stimulated by the interaction of immune complexes either phagocytosable (Movat et al.,1964; Uriuhara and Movat, 1964 ; 1966) or nonphagocytosable (Hawkins,

1971; Henson, 1971.) forms have been demonstrated in vitro. The latter mode of neutrophil degranulation seems analogous to the killing effect to the multicellular schistosomula mediated by human neutrophils in the antibody-alone assay systems reported by Anwar et al. (1979) and Vadas et al. (1979).

On the other hand, the killing effect of neutrophils in the complement-alone system also proves confusing. Dean et al. (1974; 1975) were unable to induce killing effect of neutrophil under such conditions, whereas McLaren (1980) and Anwar et al. (1979) demonstrated a complement-dependent schistosomacidal function of neutrophils. In the absence of antibody, the neutrophils did not release granule enzymes though the adherence had taken place via the C3b receptors (Dean et al., 1974; 1975; McLaren, 1980). Thus, there may be a synergistic action of neutrophils and complement in which there is osmotic damage caused by complement, and mechanical damage by neutrophils as postulated by McLaren (1980).

The release of lysosomal enzymes from neutrophils reacting with immune complexes on nonphagocytosable surfaces was demonstrated to be enhanced by the presence of C3b, presumably because of the increase in immune adherence made possible through C3b-receptors (Henson, 1971). The irreversible adherence of neutrophils to schistosomula mediated by complement has been demonstrated (McLaren, 1980). The intimate binding with the subsequent release of lysosomal enzymes from the degranulated neutrophils triggered by the immune complexes may be attributable to the augmented killing of schistosomula in the assay systems consisting of neutrophils, antibody (IgG) and complement (Dean et al., 1974;

1975; Anwar et al., 1979; Vadas et al., 1979; McLaren, 1980). Furthermore, the cleavage products of complement components, C3a, C5a and $\overline{C567}$ induce the release of lysosomal enzymes of neutrophils in vitro (Becker et al., 1974).

Direct evidence of the release of lysosomal enzymes by the adherent-neutrophils was demonstrated by histochemical study of worm-cell foci formed in vitro (Dean et al., 1974). The ensheathed schistosomula showed patches of black stain after treatment with nitroblue tetrazolium dye. This observation indicates that release of oxidative enzymes by neutrophils had taken place after adherence. The nature of the enzymes released by the adherent neutrophils and the mechanisms that control their release have not been established. Neutrophils, having a short life span of mere hours (Cronkite and Vincet, 1970), may disintegrate at the surface of schistosomula to which they adhere and release materials injurious to the parasites, or that the release is an active process akin to secretion triggered by immune complexes (Hawkins, 1971; Henson, 1971).

In conclusion, the role of neutrophils in the defense mechanism against S. mansoni is mainly attributable to the release of schistosomacidal enzymes onto the surface of the invading schistosomula. Fixation of opsonic antibodies and complement to the parasite targets enhances both adherence and degranulation of neutrophils. The elevation of both IgG (Sher et al., 1977b) and C3 (Pepys et al., 1980) were observed in S. mansoni infected animals. Thus this neutrophil-IgG-C3 mechanism may explain the augmented parasite attrition in the skin of immune host.

1.2.4. Macrophage

A tuberculin-type delayed hypersensitivity in mouse and mononuclear infiltration accompanied by skin-contact reaction in guinea pig was observed in the skin at the regions of penetration of the cercariae from a challenge infection (Colley et al., 1972; Askenase et al., 1976).

The mononuclear cell infiltration could be transferred by injection of lymphoid cells from immune donors to normal recipients (Colley et al., 1972). The responsible subpopulation of the sensitized lymphocytes for the cutaneous cellular infiltration may contain thymus-derived cells (T-cell) (Arnason and Waksman, 1963; Parrott and Sousa, 1969). . . . In support of the concept of the recruitment of cells to the site of challenge, in vitro studies have shown that lymphocytes isolated from S. mansoni infected mice responded to culture with schistosomula or schistosome antigen by the production of lymphokines which induced the migration of mononuclear cells (James and Sher, 1980; Chen and Dean, 1977) and which arrested the migration of co-cultured macrophages (Boros et al., 1973).

The lymphoid cells of S. mansoni infected mice can transfer the cutaneous mononuclear infiltration response to normal recipients (Colley et al., 1972), but do not confer resistance to schistosome infection (Sher et al.,1975b). However, macrophages isolated from S. mansoni infected mice demonstrated not only schistosomacidal activity but also tumoricidal activity (James et al., 1982). These findings indicate that the expression of delayed hypersensitivity is due to the presence of sensitized lymphocytes,

whereas enhanced anti-schistosomula resistance is a separate phenomenon and required activated macrophages.

The mechanisms whereby the macrophages might be activated to become effective in mediating resistance to a challenge infection have been dissected by several *in vitro* studies using either peripheral monocytes or peritoneal macrophages (Capron *et al.*, 1975; 1977b; Joseph *et al.*, 1977; Ellner and Mahmoud, 1981). The adherence of monocytes and macrophages to target worms, as was found in other types of effector cells, functions primarily through their membrane receptors. Two Fc receptors have been demonstrated that bind IgG (Berken and Benacerraf, 1966) or IgE (Capron *et al.*, 1975; Joseph *et al.*, 1977), either free or in the forms of antigen-antibody complexes.

The adherence and schistosomacidal activity mediated by rat macrophages via either type of cytophilic immunoglobulin receptors has been described independently by two groups of investigators (Perez and Smithers, 1977: Capron *et al.*, 1977b). Both groups incubated the peritoneal cell monolayer rich in macrophages with immune serum. This procedure subsequently caused damage to the tegument of schistosomula demonstrable at ultrastructural level. However, when the assay systems used heat-inactivated immune serum, the two groups reported different results in the capacity of cells to adhere. Perez and Smithers (1977) found that the heat inactivation process did not affect the macrophage adherence, whereas Capron *et al.* (1977b) observed the loss of the adherence of macrophages to the targets. Neither group has rationalised the differences concerning the immune adherence mediated by IgG or IgE.

Nevertheless, both groups found that the development and decline of macrophage sensitizing or activating activity in the immune serum parallels the development and decline of schistosome immunity in rat (Capron et al.,1977b; Perez and Smithers, 1977).

The role of complement in macrophage-mediated killing appears to be minor. The surface of the macrophage possesses receptors for C3 (Lay and Nussenzweig, 1968). However, the addition of complement source or depletion of complement components did not alter the activity of macrophages, both in adherence and killing, observed in either IgG- or IgE-macrophage systems (Capron et al., 1977b; Joseph et al., 1977; Perez and Smithers, 1977; Kassis et al., 1979).

Based on the finding from a series of in vitro experiments using (1) immune serum depleted of individual immunoglobulin classes, and (2) adsorption of schistosome antigen by heterologous antiserum during the sensitization of macrophages and the subsequent incubation with target parasites, Capron et al. (1977) postulated that, in vivo, macrophages were activated by interaction with schistosome antigen-IgE complexes which could subsequently kill the IgE-opsonized schistosomula from challenge infection.

The activation of macrophages by incubation with immune serum was evident by the significant increase of the protein content, the lysosomal β glucuronidase and of the cytoplasmic leucine-aminopeptidase activity (Joseph et al., 1977). Similar observations were also made with normal human and baboon peripheral monocytes and baboon peritoneal macrophages (Capron et al., 1975). None of these mentioned reports has described how the schistosome antigen-IgE

complexes function in rendering macrophages active in the killing of schistosomula.

Under no condition had significant schistosomacidal activity been elicited by normal macrophages when simultaneously incubated with normal or immune serum together with the target parasites (Capron et al.,1975; 1977b; Joseph et al., 1977; James et al., 1982). Conversely, the peritoneal macrophages isolated from S. mansoni infected mice demonstrated direct schistosomacidal effect in the absence of immune serum (James et al., 1982). The killing of the parasites was verified by their inability to mature to adult worms upon injection into normal mice. The simultaneous addition of immune serum to the culture of macrophages either activated the hitherto inert macrophages isolated from normal mice (Kassis et al., 1979) or increased the effectiveness of active macrophages from S. mansoni-infected mice in both adherence and killing of schistosomula (James et al., 1982).

The mechanism underlying the activation of macrophages stimulated by an active schistosome infection remains to be defined. Immune complexes of IgE suggested by Capron et al.(1975; 1977b) may be involved. A lymphokine generated from spleen cells stimulated by mitogen (Con A) could activate normal macrophages to kill S. mansoni schistosomula in vitro (Bout et al., 1981). It is not yet known whether lymphocytes specifically challenged by schistosome antigens or schistosomula could be equally effective in activation of macrophages.

The correlation between the onset of the activation of macrophages and the development of acquired immunity to S. mansoni has not been

investigated. The schistosomacidal activity elicited by the S. mansoni activated macrophages may not be specific since the same cell preparation also demonstrated tumoricidal activity in vitro (James et al., 1982). This capacity of antibody-independent killing by S. mansoni-activated macrophages seems to be analogous to that demonstrated by macrophages from BCG-treated mice. The BCG-activated macrophages have been shown to produce marked resistance against a variety of parasitic infection including S. mansoni and tumors both in vivo and in vitro (Ortiz-Ortiz et al., 1975; Clark et al., 1976; Mahmoud et al., 1979a). It is not yet known if infection with S. mansoni activates macrophages to show an enhanced functional capacity in a comparable fashion to the enhancement of macrophage activity by BCG.

The mode of action of the specifically activated macrophages in schistosomacidal activity has not been clarified. Direct evidence of exocytosis of enzymes by the adherent macrophages onto the surface of parasites has not been demonstrated. Additionally, it is not yet known whether the effector cell-target parasite contact is a prerequisite for the killing effect. Using similar assay systems, the adherence and killing of S. mansoni schistosomula by peritoneal macrophages isolated from mice pretreated with BCG or Corynebacterium parvum was demonstrated (Mahmoud et al., 1979a). However, the cell-free culture conditioned by the prior incubation of BCG-activated macrophages with schistosomula was shown to be also toxic to the new schistosomula subsequently added to it, although to a lesser extent compared to that exhibited by direct cellular contact (Mahmoud et al., 1979a). On the other hand, though direct

contact was artificially induced between normal mouse macrophages and Con A-coated schistosomula or untreated parasites in the presence of immune serum, the mortality of schistosomula was not significantly higher than their respective controls (James et al., 1982).

From the current data in relation to the macrophage reactions, it seems reasonable to hypothesize that one of the mechanisms for resistance to S. mansoni by immune animals involves an immunologically specific interaction between schistosome antigens and host lymphocytes which triggers mononuclear infiltration and arrests local macrophages from emigration. Destruction of the schistosomula from challenge infection would be then be nonspecific killing mediated by the accumulated macrophages which are already in activated states as a consequence of an initial schistosome infection at the site of delayed hypersensitivity reaction.

1.2.5. Eosinophil

Two important observations obtained from in vivo experiments provide evidence for a specific effector function for eosinophils. One is the histological observation of cutaneous granulocytic infiltration enriched in eosinophils and the close contact of eosinophils with degenerated schistosomula from the challenge infection (Lichtenberg et al., 1976). The other is the abrogation of both actively acquired immunity and the passive acquired resistance conferred by immune serum in the mice treated with anti-eosinophil serum at a time when the challenge cercariae were in their skin-migration phase (Mahmoud et al., 1975a).

Several factors have been postulated to be involved in the

induction of eosinophil infiltration to the site of cercarial challenge. The demonstration of activation of complement by the IgG coated schistosomula or by the residual glycocalyx on the surface of schistosomula in vitro led to the speculation that the eosinophils may be induced to migrate in responding to anaphylatoxins derived from components C3 and C5 in mice and rat (Machado et al., 1975; Ramalho-Pinto et al., 1978; James and Sher, 1980).

The importance of vasoactive amine in the expression of cutaneous reaction was implicated in view of the abrogation of formation of eosinophil-rich lesions in animals following the administration of serotonin-depleting drug reserpine before challenge (Sher, 1976). The local deposition of IgE at the cercarial challenge site of rhesus monkeys indicated that chemoattractants for eosinophils released following specific challenge of actively sensitized mast cells might participate in the triggering of eosinophil response (Hsu and Hsu, 1976). This hypothesis may be applicable to that of eosinophil infiltration observed in the mast cell-rich mouse ear-pinna skin but not at abdominal skin where few mast cells were found to be presented (Colley et al., 1972; Lichtenberg et al., 1976).

The involvement of lymphokines in the localization of eosinophils was suggested by the in vitro detection of chemotactic factor for eosinophils along with macrophage migration inhibitor factor in the supernatant of culture of schistosomula with lymphocytes from mice previously infected with S. mansoni (Phillips and Colley, 1978; James and Sher, 1980). Similar speculation concerning the participation of sensitized lymphocytes was also made

by Hsu et al. (1975) based on the histological finding that eosinophils were associated with macrophages and lymphocytes in the delayed hypersensitivity reaction in rhesus monkeys.

In summary, several immunologic reactions involving cytophilic and complement-fixing antibodies, complement, mast cells and lymphocytes may lead to the generation of factors responsible for the accumulation of eosinophils at the site of cercarial challenge observed in vivo. However, the factors that are selectively chemotactic for eosinophils generated from these various processes, at present, have not been isolated nor identified, at the physicochemical and functional levels, in terms of well characterized mediators, such as eosinophil stimulation promotor (Colley, 1973; Lewis et al., 1977; Greene and Colley, 1976); C3a, C5a, $\overline{C567}$ (Kay et al., 1973; Lachmann et al., 1970); eosinophil chemotactic factor of anaphylaxis (Kay et al., 1971; Kay and Austen, 1971) and histamine (Clark et al., 1975) each has been suggested as being involved in the responses to reinfection.

Additional data obtained from in vitro experiments have been collected in support of the participation of eosinophils in the immunity to S. mansoni. As shown by other types of leukocytes, eosinophils possess membrane recognition units for immunological reactants, immunoglobulins and complement components, that facilitate the adherence and enhance the associated degranulation which appear to result in the death of the schistosomula. Both antibody-dependent (Anwar et al., 1979; Butterworth et al., 1977a;b; Vadas et al., 1979; Mackenzie et al., 1977) or complement-dependent (Ramalho-Pinto et al., 1978) schistosomacidal activity mediated by

eosinophils collected from normal human or animals were demonstrated using either antibody-alone or complement-alone experimental systems.

The class of adherence promoting antibody is apparently animal species-dependent. IgG1 and IgG2a have been identified, as involved in the adherence of, respectively, mouse and rat eosinophils to schistosomula *in vitro* (Ramalho-Pinto *et al.*, 1979; Capron *et al.*,1978a). Although the release of lysosomal enzymes by human eosinophils that have been incubated with immune complexes composed IgE and anti-human IgE was demonstrated (Takenaka et al., 1977), a role for IgE receptors in the rat eosinophil-mediated schistosomacidal activity *in vitro* could not be detected (McLaren, 1980).

It is intriguing to note that, in contrast to the killing effect demonstrated by normal eosinophils in the presence of immune serum, eosinophils isolated from patients or animals previously infected with *S. mansoni* were ineffective in the killing of antibody-opsonized schistosomula though adherence had taken place (Butterworth *et al.*, 1977b ;James *et al.*, 1982; Capron *et al.*, 1979). It is believed that the inactivity of eosinophils was due to the competive binding of Fc receptors by circulating immune complexes *in vivo*, since the schistosomacidal activity was inhibited by the addition of immune complexes *in vitro* (Butterworth *et al*; 1977b).

As shown by neutrophils, the eosinophils also adhere to schistosomula via C3-C3 receptor interaction (Ramalho-Pinto *et al.*, 1978). The relative functional role of IgG-Fc and C3b receptors on

the membrane of eosinophils has been assessed in terms of the cellular adherence and killing of schistosomula (Ramalho-Pinto et al., 1978). It appears that at least in the rat model, the eosinophil adherence and killing is more efficient and occurs more rapidly when the cells adhere through C3b receptors Criticism that eosinophils may serve merely to enhance damage mediated by complement (Vadas et al., 1979) has been refuted by Anwar et al. (1979) who introduced purified complements, serially reacted up to but not beyond C3, into their in vitro assay systems. Comparable levels of schistosomular killing were recorded despite the absence of the lytic terminal components of the complement cascade.

The augmented schistosomacidal activity mediated by rat eosinophils was detected when mast cells were added into the assay system. The mast cell potentiation could be reproduced by substitution of the intact cells by soluble substances released from mast cells either specifically or unspecifically (Capron et al., 1978b). The mechanism underlying the synergistic action of mast cells or their products and eosinophils is not known.Two types of mast cell-associated mediators, histamine and ECF-A, has been shown to increase the density of C3b receptors on human eosinophils (Anwar and Kay, 1977b). Therefore, the modulation of expression of eosinophil receptors was suggested to account for the increased killing effect exhibited by rat eosinophils in the culture containing mast cells (Capron et al., 1978b).

Electron microscopic studies of the interaction of rat

eosinophils with schistosomula, either via IgG-Fc or C3b receptors, indicates that intimate contact between effector cells and target worms was followed by eosinophil degranulation (McLaren et al., 1977;1979). The deposition of peroxidase-positive granule material resulted from eosinophil degranulation was demonstrated by cytochemical technique at the ultrastructural level (McLaren et al., 1977). Major basic protein has also been detected on the surface of schistosomula during the antibody-dependent interaction of human eosinophils and schistosomula in vitro (Butterworth et al., 1979b).The damage to the tegument of schistosomula detected both microscopically and by the release of ^{51}Cr from labelled parasites could be achieved by incubation in the cell-free culture containing the major basic protein isolated from human or guinea pig eosinophils (Butterworth et al.,1979b). Therefore, the mode of action of eosinophils is apparently analogous to that demonstrated by neutrophils, namely, the release of granule enzymes that damage the tegument of schistosomula they adhere to via the IgG-Fc or C3b receptors or both.

Aim of the thesis

As is evident from the above discussion, many experiments of the last decade have shown the existence of a local cellular immunity operative in the skin, and the recruitment of cells to the invaded site appears to be a critical step in the defence against schistosome infection in mice. However, the majority of work concerning cellular immunity has been focussed on the mechanisms underlying the killing of schistosomula by leukocytes. Relatively little attention has been given to the concept of chemotaxis triggered by the invading parasites.

The infiltration of cells into the skin site exposed or re-exposed to cercariae of S. mansoni is a complex antigen-dependent event that may require the participation of cells such as lymphocytes and mast cells, and humoral factors such as antibodies and complement. The exact nature of the cooperation between cells and humoral factors in vivo is little known. Several in vivo studies of chemotaxis in the skin have been devised, such as the skin window method (Otani and Hugli, 1977) and air enclave method (Larson, 1969; Tempel et al., 1970). However, either method carries the risk of introducing artifacts during the manipulation necessary to prepare the tissue for study. Also, it is difficult to obtain biological fluids in sufficient quantities from the reactive skin (Hirashima and Hayashi, 1976; Cohen et al., 1973) to identify the chemotactic mediator released

in vivo and responsible for the innate or acquired immunity occuring in the skin. Most of the knowledge about chemotaxis has been obtained from studies carried out *in vitro*, using experimental systems consisting, either of suspensions of mediator-producer cells and specific antigen, or of responding cells and chemotactic factors. The chief advantage of this approach has been the control and limitation of variables. However, such model systems arbitrarily exclude a major part of the natural setting in which chemotaxis takes place.

Hopper and Nelson (1979) reported an accumulation of macrophages in the peritoneal cavity of sarcoma tumor-immune mice following an intraperitoneal challenge with homologous tumor. This specific response depends on the immune status of the host. The infiltrating cells eliminated the intraperitoneally implanted tumor. This paper prompted us to investigate the feasibility of employing the peritoneal cavity as the site in which to study the cellular activity induced by schistosomula of *S. mansoni*.

In this study, the characteristics of cellular infiltration into the peritoneal cavity in response to injected schistosomula was studied. The suitability of this model system for the study of chemotactic responses in natural infections will be discussed. The following were investigated:

(1) Humoral and cellular constituents in the peritoneal cavity of mice infected with *S. mansoni*.

(2) Identities of cells infiltrating into the peritoneal cavity in response to schistosomula intraperitoneally inoculated in normal and schistosome infected mice.

(i) effect of the length of primary infection period on types of cells infiltrating in response to schistosomula.

(ii) effect of the cercarial dose used in the primary infection on the expression of primary and secondary inflammations.

(iii) comparison of the secondary cellular infiltration in mice previously infected with unisexual and bisexual cercariae.

(3) Identities of cells infiltrating into the peritoneal cavity in response to non-schistosome organisms intraperitoneally inoculated in normal and schistosome infected mice.

(4) Role of antibodies in the induction of cellular infiltration.

(5) Cellular adherence to schistosomula in the normal and schistosome infected mice.

(i) role of antibodies in the cellular adherence in the peritoneal cavity.

(ii) identities of cells adhering to schistosomula in the peritoneal cavity.

(6) Cellular adherence to schistosomula in mice infected with non-schistosome organisms.

(7) Isolation of worm antigen fractions responsible for the induction of cellular infiltration.

The results are divided into eight chapters. An appendix concerning the quantitative analysis of cellular adherence to schistosomula *in vitro* is also added. In each chapter, descriptions of experimental protocols, the results and the immediate conclusions are presented. In the final chapter (Chapter 9), the results are discussed in the light of their possible relevance to the host inflammatory response triggered by invading schistosomula. Since there were few published studies concerning mechanisms underlying the inflammation in the peritoneal cavity, a complete understanding of the results is not possible. Instead, the present discussion intends: (1) to record and interrelate the present observations with those in the literature; (2) to look for evidence that the mechanisms postulated from *in vitro* experiments are active in the peritoneal cavity, and (3) to offer suggstions for future lines of research.

MATERIALS AND METHODS; RESULTS; CONCLUSIONS

1. Quantitative and qualitative changes in peritoneal cells during the course of S. mansoni infection

The fact that there is leukocytosis within the peritoneum of mice due to a percutaneous schistosome infection had been appreciated for some time in our laboratory. Since no report has been made on the cellular content of the peritoneal cavity of infected mice, the first series of experiments in this study was to investigate the quantitative and qualitative changes in numbers of peritoneal cells and their profiles following an infection with S. mansoni.

1.1. Experimental design

1.1.1. Animals

Age- and weight-matched male BALB/c mice (7-8 weeks old, weight range 18-23 g) were used throughout. They were bred in the Animal Breeding Unit of the University of Glasgow, and maintained in a temperature-controlled enviroment. They received rat and mouse breeding diet (RHM Algriculture Ltd. Dorset).

1.1.2. Infection with S. mansoni

Groups of mice exposed to 40-50 cercariae of S. mansoni and uninfected controls were caged at the same time and maintained under identical conditions.

The larvae for each infection were shed from 20-30 infected snails under an artificial light and were used within 2 hrs.

For percutaneous infection, the method described by Smithers and Terry (1965) was used. Mice were anaesthetized with sodium pentobarbital (BDH, Dorset) : ethanol : H_2O (1:1:9) solution via intraperitoneal injection route at 0.1 ml/10 g body weight. This anaesthetic dose immobilized the mice for 1-2 hr. The abdomen was shaved with an electric clipper and a stainless steel ring of 1 cm diameter was laid on the shaved abdomen providing a shallow well. Known numbers of cercariae were applied to this well in a total volume of 100-200 µl and allowed to penetrate for 15-25 min.

1.1.3. Collection and processing of peritoneal fluids

All the samples were collected under sterile conditions and kept on ice until use or storage.

Mice were starved overnight. Groups of mice were killed by cervical dislocation under mild ether anaesthetic. Two sets of samples were collected from each animal; serum and lavaged peritoneal fluid.

The carcass was pinned on a dissecting board. An incision was made through the skin from the chin to the root of the tail. The skin was reflected to expose the full length of the ventral surface. The serum was subsequently collected by heart puncture after cutting through the ribs on the left side of the sternum. Care was taken to preserve the diaphram intact to prevent the spilling of blood into the peritoneum.

After the serum was taken, the peritoneal lavage was

immediately carried out. A volume of 2 ml of phosphate buffered saline (pH 7.0±0.1) containing 5 U/ml heparin (Vestric Co.) was injected into the peritoneal cavity with a 25 G x 3/8" needle. The abdomen was gently kneaded for 1 min and the cell-rich fluid was removed by inserting a 21 G x 1½" needle in the flanks. The sample collection procedure from heart puncture to the completion of peritoneal lavage required approximately 3-5 min.

Blood was allowed to clot at 4°C overnight and the serum was centrifuged in an Eppendorf centrifuge (type No. 5412) for 3 min to remove the residue red cells and subsequently stored in several small portions at -20C.

A small portion of the lavaged peritoneal sample was taken and the total cell count and differential count were performed. The lavaged fluid was subsequently freed of cells by centrifugation at 1,000 r.p.m. for 5 min in a bench centrifuge at room temperature. The cell-free supernatant was either immediately frozen at -20° C in several portions or pooled and concentrated by using Aquacide II (Calbiochem. Ltd.) depending on the nature of the subsequent studies.

1.1.4. Cellular compositions

1.1.4.1. Total peritoneal cell counts

The total cell count was performed using a haemocytometer chamber, a leukocyte pipette at the standard dilution of 1 in 20 and leukocyte diluent containing 0.01% methylene blue in 2% acetic acid. Two counts were made for each sample,

but repeated if the value of X^2 test for the two counts exceeded 3.84. The mean value of the four corner-squares of the chamber multiplied by 2×10^5 represented the total leukocytes per ml of the individual sample.

1.1.4.2. Differential cell counts

The peritoneal cell smears were prepared by cytocentrifugation at 750 r.p.m. for 5 min in a Cytospin (Shandon Southern Instruments, Sewickley. Pa., U.S.A.). The smears were dried in the air and then fixed and stained with May-Grunwald-Giemsa stain.

The smears were fixed by immersing in a jar containing May-Grunwald stain diluted with an equal part of neutralised buffer (pH 6.8) (Sigma Chemical Co. Poole; 1 tablet/l 1 H_2O). After the smears had been stained for 5 min, they were transfered without washing to a jar containing Giemsa stain (BDH) with nine parts of buffer. After being counterstained for 10-15 min, the slides were transferred to a jar containing fresh buffer and rapidly washed in three or four changes and allowed to stand undisturbed for 1-2 min for differentiation to take place. The stained slides were then washed under tap water and then stood upright to dry. Duplicated cytocentrifuged smears were prepared from each sample and differentially counted. The mean of the two determinations was taken. Stained smears can be stored after wiping clean of microscopic immersion oil with xylene.

Cells were differentially counted under oil immersion with a x 100 objective and a x 7 eye piece with the aid of a reticulocytic disc. A microscopic field normally contained 50-100 cells, and for each count five or six fields were examined. For mast cells below 1%, usually occurring in chronically infected mice, 10-15 fields were examined using x 45 objective lens to obtain more accurate mast cell proportions.

1.2. Results

1.2.1. Total number of peritoneal cells

Fig. 1.1. illustrates the total peritoneal leukocyte counts determined prior to infection and on weeks 1-19 postinfection. It showed a steady increase at 7 week until 11 week postinfection at which time, the total number reached a peak. It remained at the plateau level throughout the 19 weeks of this experiment. In a control experiment, groups of age-matched uninfected mice were also sacrificed and their peritoneal lavaged fluids collected. No significant increase in the total leukocyte numbers in these mice was observed.

1.2.2. Cellular composition of the peritoneal exudates

1.2.2.1. Macrophage

There was a significant increase in the total number of macrophages in the peritoneal cavity of infected mice from week 7 onward (Fig.1.2a). However, the increase reflected elevations in the peritoneal exudate cell count rather than

an increase in the relative proportion of macrophages in the infected mice.

1.2.2.2. Lymphocyte

The lymphocyte is the major cell type of peritoneal leukocytes. An increase in the number of lymphocytes occurred on week 7 postinfection and onward (Fig. 1.2.b.). However, as described in the case of macrophage (see Section 1.2.2.1.), the increase reflected a generalized leukocytosis, as the percentage of lymphocyte in infected and normal controls was not significantly different.

1.2.2.3. Eosinophil

An increase in eosinophils of infected mice occurred from week 7 postinfection as evidenced by both total cell counts and percentages of these cells (Fig. 1.2.c.). Total counts and percentage of eosinophils in infected mice were significantly higher than those in uninfected controls. The percentages of eosinophils declined slightly toward the end of this experiment.

A slight increase in the eosinophil number in normal controls occurred toward the end of the experiment, probably as a result of the older age or unspecific infection. At no time during the experiment did eosinophil levels in normal controls exceed 3%, whereas in the infected mice, eosinophils accounted for as much as 13% (15-week infected group) of the

total peritoneal leukocytes.

1.2.2.4. Neutrophil

Though found in the peripheral blood, the neutrophils were rarely seen in the peritoneal exudates of normal mice. A few mice previously infected for 7 weeks or longer demonstrated slightly elevated neutrophil levels (Fig. 1.2.d.). But these never exceeded 0.8% of the total peritoneal leukocytes.

1.2.2.5. Mast cell

Mast cells in the infected mice did not respond like all the other leukocyte types reported above. No significant increase in the number of mast cells was observed during the generalized leukocytosis (Fig. 1.2.e.). The percentage of mast cells declined on week 7 postinfection and remained low until the termination of the experiment. The percentage of these cells at week 19 was about 1/10 of the normal controls.

1.2.3. Variation

While the peritoneal leukocyte composition in normal mice was relatively constant, that of infected mice, particularly in relation to total leukocyte counts and eosinophil percentages, showed rather larger variation from group to group. Table 1.1. showed the variation of leukocyte composition in three groups of mice infected with 40-50 cercariae,

at different occasions for 8 weeks (54-57 days). Their age-matched uninfected controls were also examined at the same time. Generally, in agreement with the observations described above (see Section 1.2.1. and Section 1.2.2.), the infected mice had significantly elevated total peritoneal leukocyte counts and eosinophil percentages, whereas the levels of mast cells decreased compared with normal controls. There were no apparent changes in the percentages in other leukocyte types.

1.3. Conclusion

The most marked features of the cellular reactivity in the peritoneal cavity in responding to a S. mansoni infection were the leukocytosis (Section 1.2.1.), selective increase in the proportion of eosinophils (Section 1.2.2.3.) and the corresponding decrease in mast cells (Section 1.2.2.5.). Neutrophils were rarely observed in samples collected from normal and infected mice.

Table 1.1.

Cellular profiles of mice infected with 40-50 S. mansoni cercariae for 8 weeks (independent tests)

Group	Mouse status	Total number per ml ($\times 10^{-6}$)	Cell content (mean±S.D.)%				
			Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
A)	Infected (7)	17.1±8.2	22.2±6.3	58.7±7.1	18.3±4.5	0.4±0.6	0.3±0.1
	Normal (4)	3.8±1.3	19.3±9.2	74.0±8.7	4.1±1.7	0.5±1.0	1.9±0.6
B)	Infected (5)	9.8±2.7	25.9±6.8	69.2±9.7	4.2±2.2	0.2±0.3	0.5±0.2
	Normal (5)	2.1±0.3	18.1±1.2	78.1±3.7	1.4±1.1	0.3±0.3	2.1±0.7
C)	Infected (6)	19.6±5.2	15.6±7.6	77.8±7.9	6.2±2.1	0.2±0.5	0.3±0.9
	Normal (5)	3.0±0.4	16.7±2.8	78.5±4.1	2.7±1.1	0.9±0.6	1.2±0.6

Fig. 1.1.

Time course of total cell counts of peritoneal exudates of normal mice and mice infected with S. mansoni

Mice were percutaneously infected with 40-50 cercariae of S. mansoni . Groups of mice were killed weekly, and their cell-rich peritoneal fluids were lavaged by an intraperitoneal injection of 2 ml of PBS/heparin. The total number of cells in one ml of the lavaged sample was determined (●—●). Peritoneal samples collected from age-matched uninfected mice were also counted in the same manner (O- -O). Each value represents the mean ± S.D.. Numbers in parentheses refer to the numbers of mice examined.

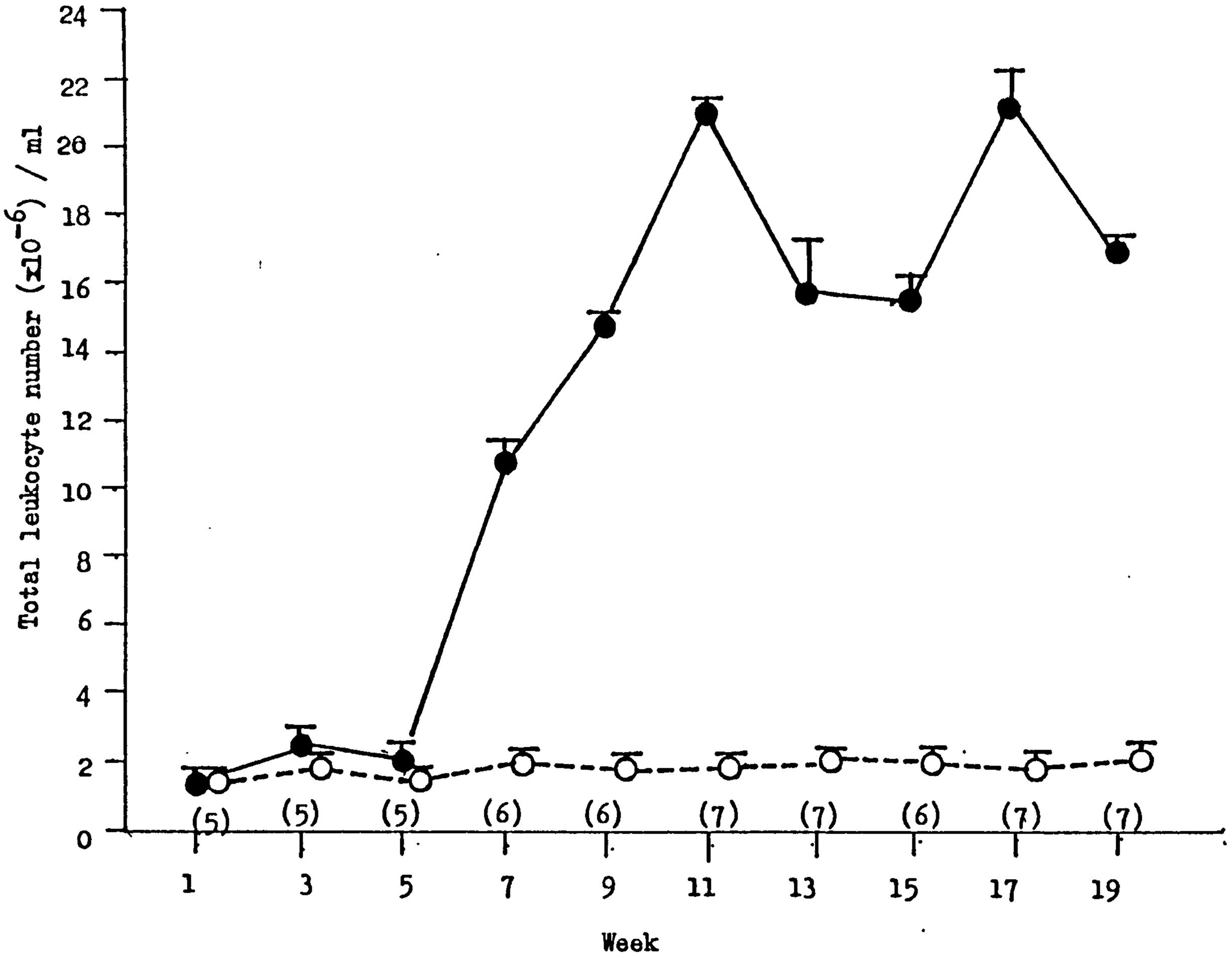
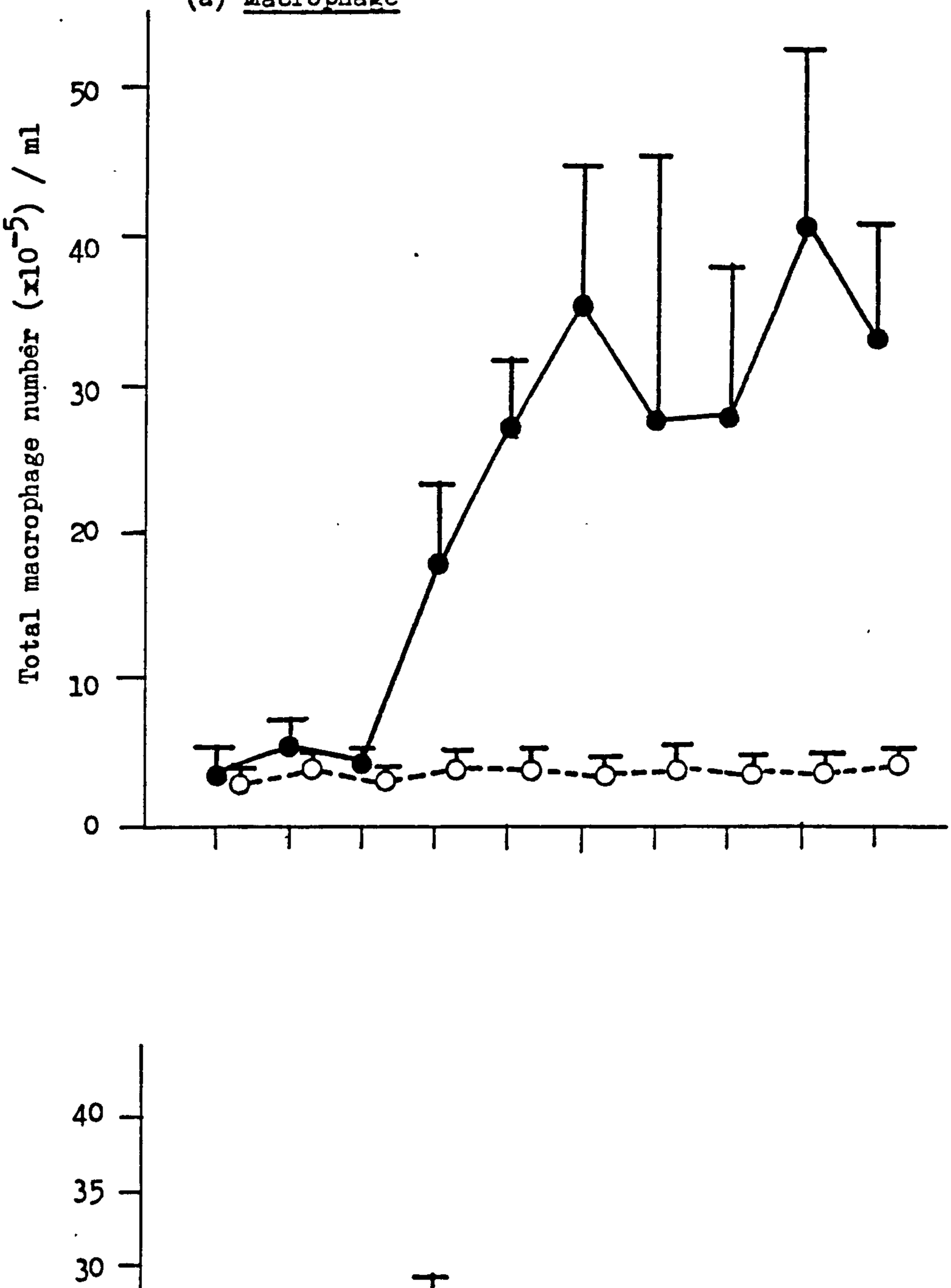

Total leukocyte number ($\times 10^{-6}$) / ml
0
2
4
6
8
10
12
14
16
18
20
22
24
(5)
(5)
(5)
(6)
(6)
(7)
(7)
(6)
(7)
(7)
1
3
5
7
9
11
13
15
17
19
Week

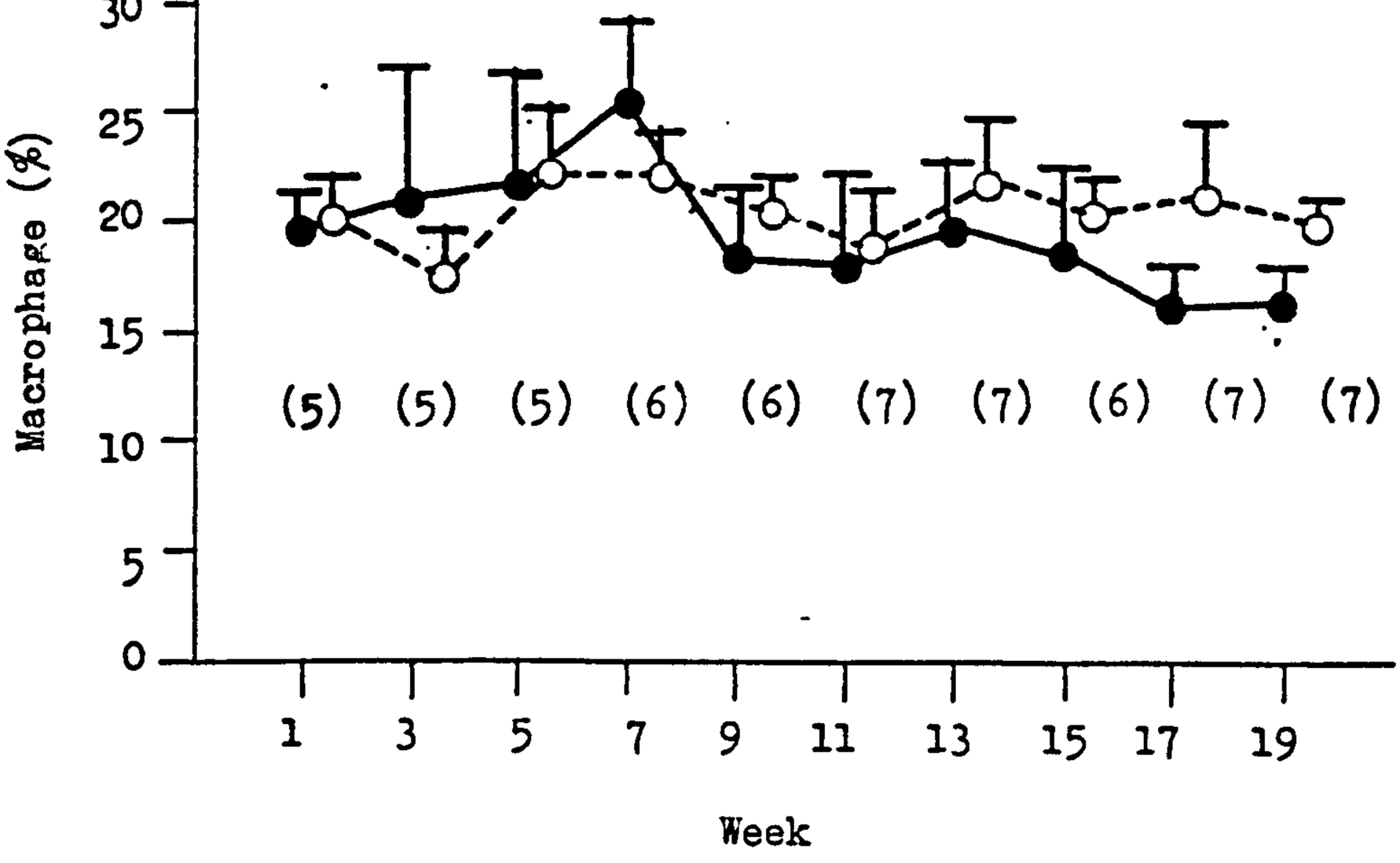
Fig. 1.2.

Cellular profiles of peritoneal fluids of normal mice and mice infected with S. mansoni

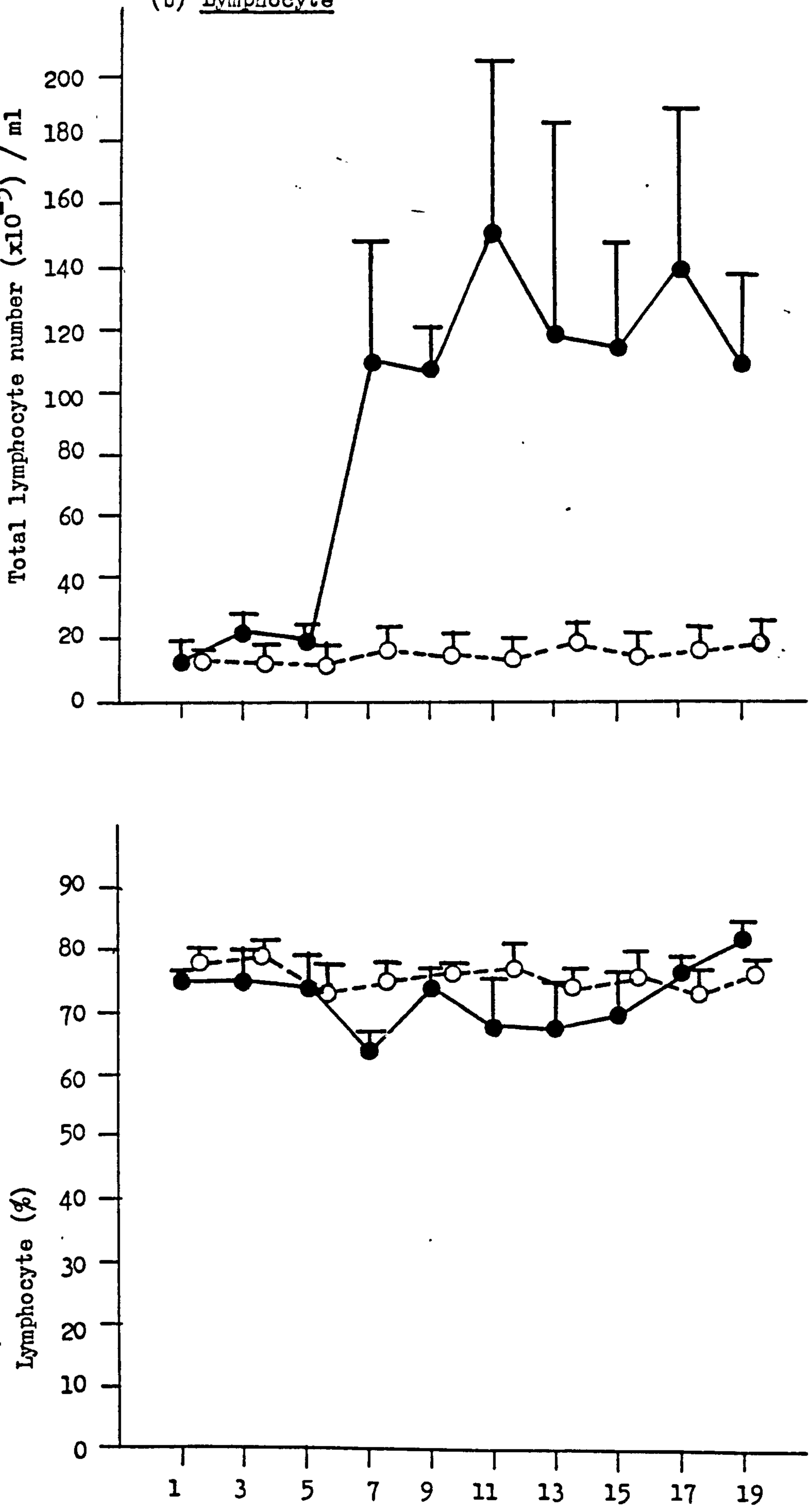
Mice were percutaneously infected with 40-50 cercariae of S. mansoni. Groups of mice were killed every two weeks after infection, and their cell-rich peritoneal fluids were lavaged by intraperitoneal injection of 2 ml of PBS/heparin. The percentages of (a) macrophages (b) lymphocytes (c) eosinophils (d) neutrophils and (e) mast cells were determined by examining the May-Grunwald-Giemsa stained cell smears. The total number of each cell type in one ml of lavaged peritoneal sample was obtained by multiplying its respective percentage by the total number of peritoneal leukocytes. Peritoneal samples collected from age-matched uninfected mice were also counted in the same manner. Open symbols (⊙-○) show the values obtained from uninfected mice and the closed symbols (●—●) from infected mice. Each value represents the mean± S.D.. Numbers in parentheses refer to the numbers of mice used in each group.

(a) Macrophage

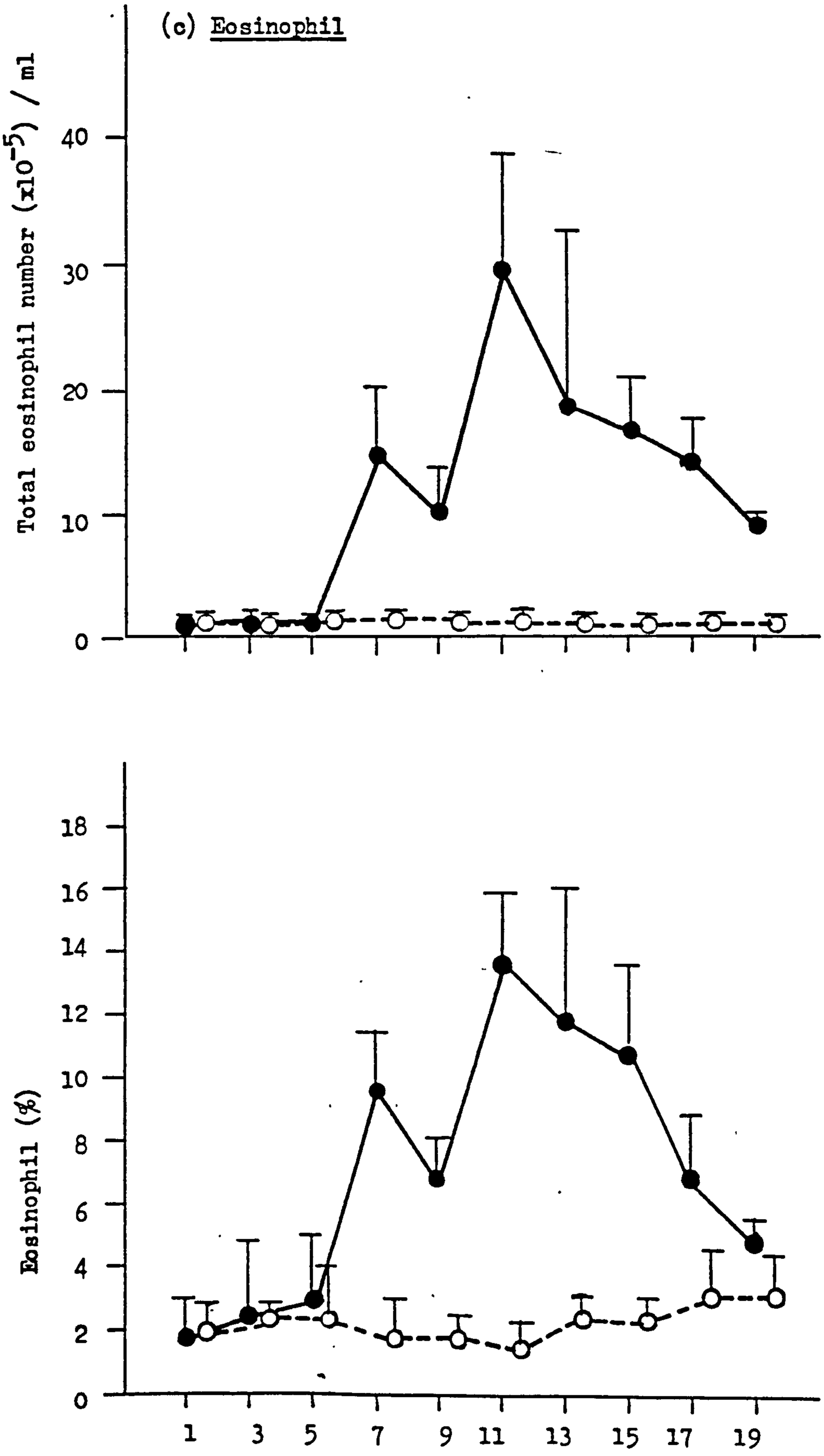




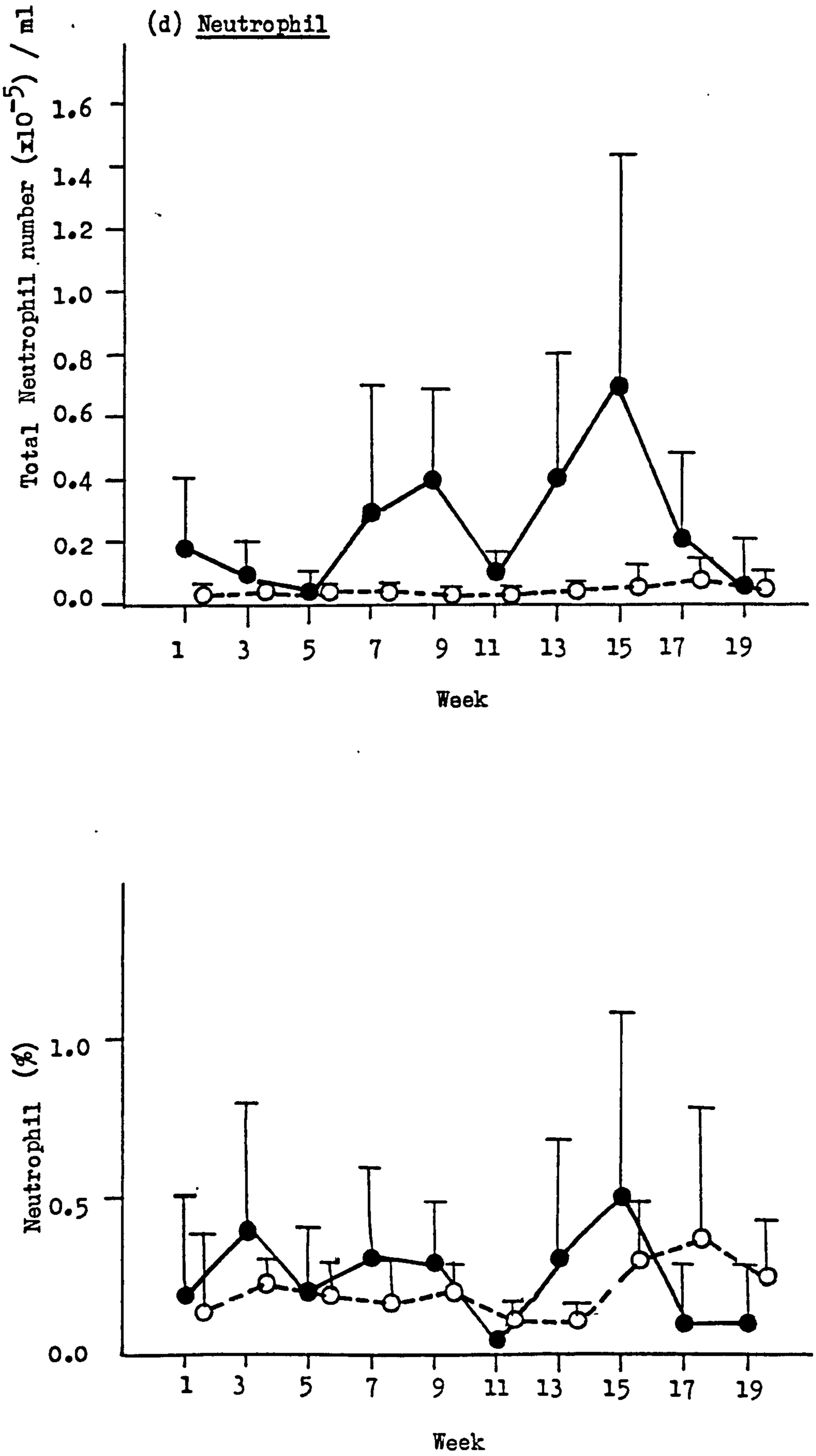
(b) Lymphocyte



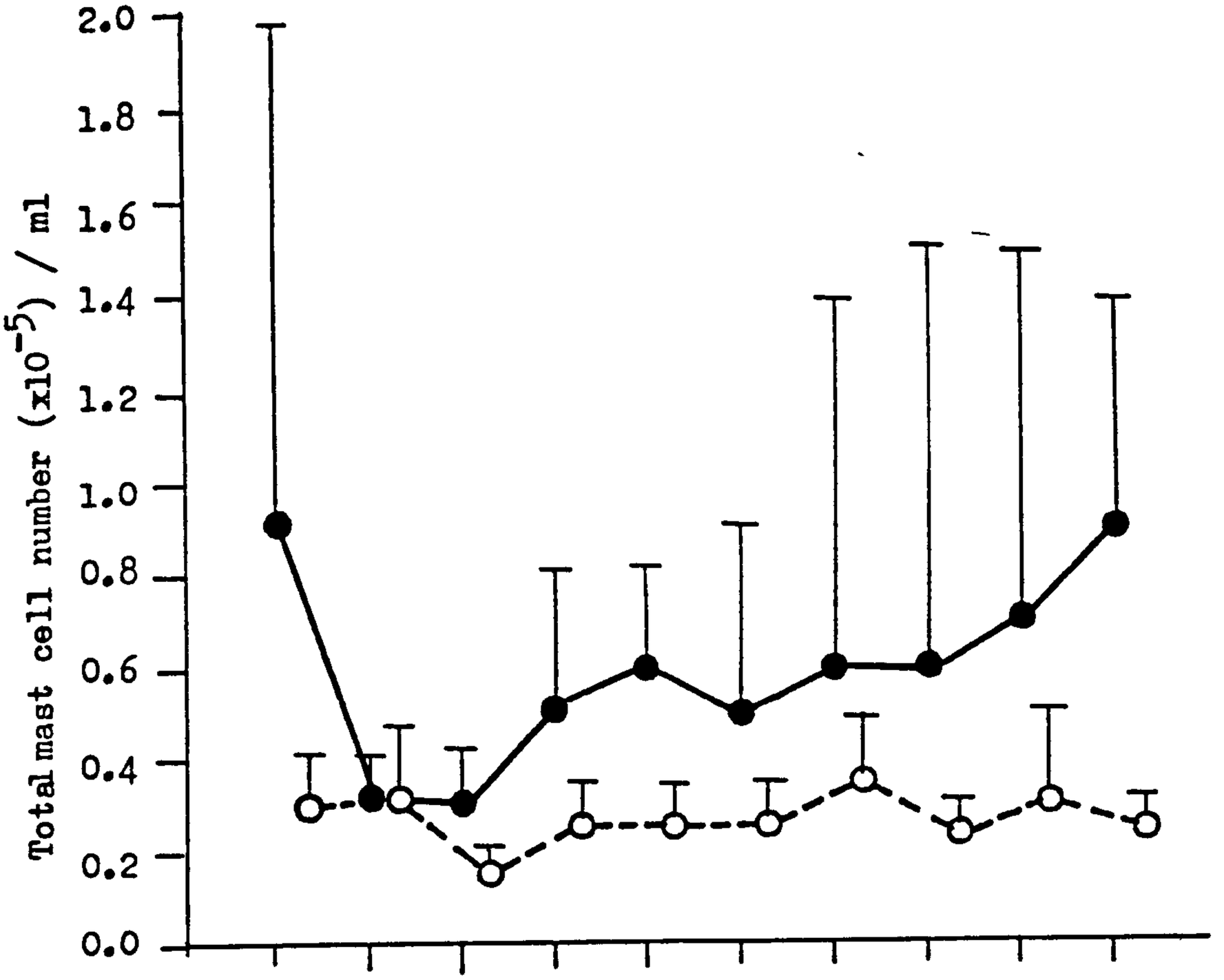
(c) Eosinophil

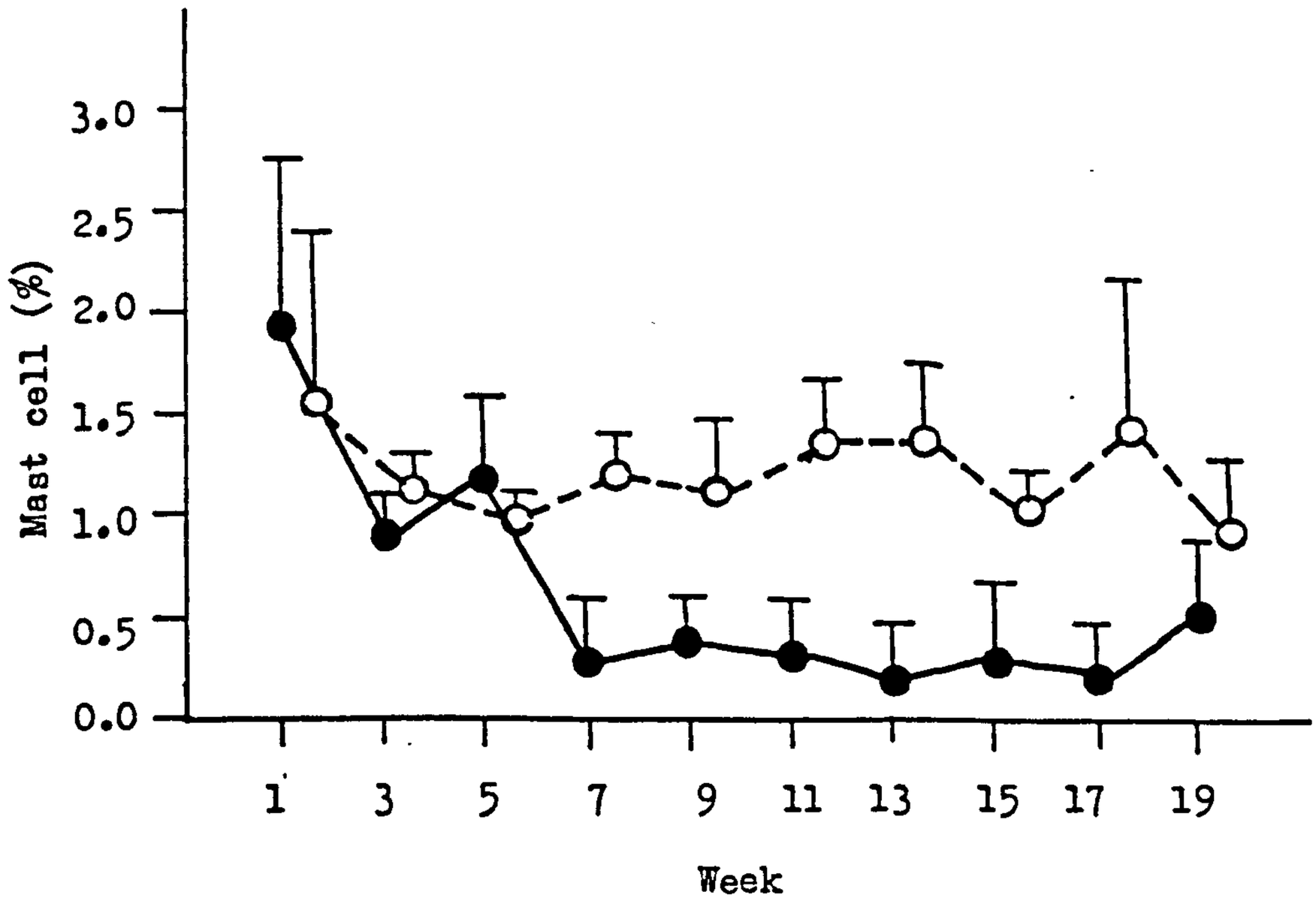


(d) Neutrophil



(e) Mast cell


Total mast cell number ($\times 10^{-5}$) / ml
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0


Mast cell (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
3
5
7
9
11
13
15
17
19
Week

2. Quantitative and qualitative changes in the protein content in peritoneal fluids during the course of S. mansoni infection

The second series of experiments was designed to obtain information related to the effect of a schistosome infection on protein composition in the peritoneal fluid and sera.

2.1. Experimental design

The non-cellular samples were collected from the cell donors used in the first series of experiments (see Section 1.1.3.). Three biochemical techniques, protein determination, immunoelectrophoresis and SDS-polyacrylamide gel electrophoresis, were employed to analyse the protein content of peritoneal fluids and their corresponding sera.

2.1.1. Protein determination

The total concentration was assessed by the method described by Lowry *et al* (1951) using bovin albumin (SIGMA, Dorset) as a standard.

2.1.2. Immunoelectrophoretic analysis

Immunoelectrophoresis was carried out essentially as described by Scheidegger (1955). A solution of 1% w/v agarose in 1/4 strength of 0.075 M Veronal buffer (pH 8.6) was pipetted onto microscopic slides and allowed to cool. Wells and troughs were cut in the agarose gel using a cutting template. The wells were filled with the samples to be

tested (5 µl) and the gels electrophoresed at 6 V/cm for 1 hr. After electrophoresis the troughs were filled with rabbit antiserum against mouse serum (10-20 µl). The gels were then incubated in a humid chamber at 4°C overnight. After the precipitin arcs developed, the gels were washed in three changes of PBS in two days. The precipitin arcs were stained with 0.5% Amido black (BDH Chemicals Lt., Dorset) in 5% acetic acid for 1 hr and then destained with 2% acetic acid at room temperature until the background was cleared.

<u>0.075 M veronal buffer (pH 8.6)</u>

Diethylbarbituric acid	2.75 g
Sodium diethylbarbiturate	15.45 g
Dist. H_2O	1000 ml

<u>0.01 M phosphate-buffered saline (pH 7.1) (PBS)</u>

Sodium chloride	8.5 g
Disodium hydrogen phosphate . $2H_2O$	1.3 g
Sodium dihydrogen phosphage. $2H_2O$	0.4 g
Dist. H_2O	1000 ml

<u>Raising of antiserum against mouse whole serum</u>

Mouse serum (0.5- 1 ml) was mixed with an equal volume of complete Freund's adjuvant (Difco Lab., Surrey) and the mixture emulsified by sonication. Rabbits were given two primary injections intramuscularly at times separated by one week. From 0.2 to 1.0 mg of protein antigen was used each injection.

Booster injections were given every four weeks, begining

four weeks after the last primary injection. Approximately 0.2 mg of antigen was used and the injections given at multiple sites subcutaneously. Rabbits were bled from the ear vein, beginning seven weeks after the last primary injection, and thereafter at 2 week intervals. Twenty to 50 ml of blood was taken at each bleeding, and allowed to clot at room temperature for 30 min and overnight at 4 C. The serum was stored in several portions at -20 C.

2.1.3. SDS-polyacrylamide gel electrophoresis

Discontinuous SDS-PAGE using a Tris-glycine buffer system was done according to the method of Laemmli (1970). Slab gels of 65 x 80 x 1 mm size were prepared as follows.

Sealing gel preparation

Acrylamide/N,N'-Methylene bisacrylamide (30:0.8, w/v) (BDH Chemicals Ltd., Dorset)	2.5 ml
0.15 M Tris-HCl (pH 8.8) (Sigma Chemicals Ltd., Dorset)	7.5 ml
N,N,N',N'-Tetramethyl ethylenediamine (BDH Chemicals Ltd., Dorset)	0.25 ml
10 % Ammonium persulfate (BDH Chemicals Ltd., Dorset)	0.25 ml

Separation gel preparation

The following table was used to prepare 20 ml separating gel solution of the required percentage acrylamide:

Constituent	ml used	Final concentration
Acrylamide/bis (30:0.8)	5.0	7.5 %
	6.7	10.0 %
	8.4	12.5%
1.5 M Tris-HCl (pH 8.8)	5.0	0.375 M
Dist. H_2O (bring volume to)	19.6 ml	
TEMED	0.01	0.05 %
10 % SDS	0.2	0.1 %
Degas:		
10 % Ammonium persulfate	0.2	0.1 %
Final volume	20 ml	

The gel was immediately poured and filled the casting mould up to 15 mm from top of glass, layered with 1 mm H_2O and left to polymerize at room temperature for 1 hr.

Stacking gel preparation

Gel solution for 3 % polyacrylamide stacking gel was prepared as follows.

Constituent	ml used	Final concentration
Acrylamide/bis (30:0.8)	1.0	3 %
0.5 M Tris-HCl (pH 6.8)	2.5	0.125 M
10 % SDS	0.1	0.1 %
TEMED	0.005	0.05 %
Dist. H_2O	6.2	
Degas		
10 % Ammonium persulfate	0.15	0.15 %

The gel solution was poured on top of the separating gel and left to polymerize around a 'well' template with 7 sample application wells.

Running buffer preparation

Constituent	Quantity	Final concentration
Tris	3.0 g	0.0124 M
Glycine	28.8 g	0.192 M
(BDH Chemicals Ltd., Dorset)		
10 % Sodium dodecyl sulphate	20.0 ml	0.1 %
Dist. H_2O (bring volume to)	2000 ml	

Sample preparation and electrophoresis

Protein samples to be electrophoresed were dissolved in an equal volume of sample buffer, and heated at 100 C for 2 min.

Sample buffer

Constituent	ml used	Final concentration
10% SDS	2.0	6.7 %
Mercaptoethanol	0.5	16.7 %
(Koch-Light Lab.Ltd., Bucks)		
Glycerol	0.5	16.7 %
Bromophenol blue		trace
(BDH Chemicals Ltd., Dorset)		

Electrophoresis was carried out with 15-20 mA/gel constant current for about 1-2 hr until the bromophenol blue front reached 5 mm above the sealing gel.

Fixation and staining of gels

Gels were removed from the glass and fixed and stained in a solution containing 50 % methanol, 7 % acetic acid and 0.2 % Coomassie brillient blue R250 (BDH Chemicals Ltd., Dorset) at 60°C for 30 min or room temperature overnight. Gels were destained by diffusion in a solution containing 5 % methanol and 7 % acetic acid for 2-3 hr at 60 C. The gels were then left in the destaining solution at room temperature until the background became colourless.

Conservation of gels

Slab gels were washed with three changes of 20 % methanol and then dried on a piece of Whatman 3 MM paper under vaccum in refrigeration conditions. The drying device should be allowed to warm to room temperature before taking out the gel, otherwise it might curl and break when flattened.

2.2. Results

2.2.1. Total protein concentrations in the peritoneal fluids during S. mansoni infection

The data of analysis of protein concentrations during the infection course of S. mansoni is presented in Fig. 2.1.. Total protein concentrations in the peritoneal fluids were normal during the time between the cercarial exposure and

week 5. On week 7, a sharp rise in protein concentration was detected and reached a peak at week 15 after infection. The protein concentration remained at about three times the normal value until the experiment was terminated on week 19. In contrast, no fluctuation in the protein concentration in the normal peritoneal fluids occurred through out the 19-week experiment.

2.2.2. Immunoelectrophoresis of peritoneal fluids

Comparative immunoelectrophoresis in agarose gels using rabbit antiserum against mouse serum showed that there was no detectable difference in the precipitin lines given by peritoneal fluids, both normal (Plate 2.1a) and infected (Plate 2.1b) compared to that developed by serum

This may indicate an immunological identity of the lavaged peritoneal fluids and serum.

2.2.3. SDS-polyacrylamide gel electrophoresis of peritoneal fluids

Electrophoresis of peritoneal fluids in 7.5 % and 10 % polyacrylamide gels containing 0.1 % SDS yielded a pattern in which seven well-resolved major bands, arbitrarily labelled I-VII predominated. Molecular weight estimates for components were obtained by calibration of gels with molecular weight markers. The calibration curve was extrapolated to yield a rough estimate of 96 ± 2 K, 81 ± 6 K,

63±2 K, 52±1 K and 50±1 K for components III, IV, V, VI and VII, respectively. Components I and II had molecular weights larger than 100 K. (Plate 2.2.). The mean values and their respective standard deviations were calculated from three 7.5 % and one 10 % gels.

There were two striking features of the electrophoretic comparisons of normal and infected peritoneal fluids and their corresponding sera. One was the strong similarities of the electrophoretic patterns between the peritoneal fluid and serum (Plate 2.2.) There may be additional minor components which are specific to peritoneal fluid or serum that have not been seen because the amount of sample applied was kept low (approximately 20 ug each sample) to prevent the distortion that accompanied overloading of the major band, component V (MW 63K), presumably albumin. Higher polyacrylamide concentration gels gave poorly resolved banding patterns and thus were not further used.

The other notable feature of the comparative study of electrophoresis was the variation in the Coomassie blue stain intensity. The difference in electrophoretic patterns between the normal and infected samples, both serum (Plate 2.3.) and peritoneal fluid (Plate 2.4.)was the relatively heavy-stained components III (MW 96K) and VII (MW 50K), exhibited by infected peritoneal fluids and sera. The MW 50K component became proportionally more prominent when the intensity of MW 96K band was reduced (Plate 2.3.) or vice versa (Plate 2.2.).

It is suspected that the MW 50K was a breakdown product of the larger MW 96K component or that the MW 96K may be a dimer of the MW 50K component. Furthermore, the length of infection periods apparently influenced the electrophoretic patterns of the lavaged peritoneal fluids. Changes in amounts of the MW 96K and MW 50K proteins during the development of S. mansoni infection course are shown in Plate 2.5.It is clear from the electrophorogram that the staining density of the two relevant protein bands increased in samples from mice infected for 8 weeks or longer.

Electrophoretic comparison of the proteins of peritoneal fluids and supernatant of mouse red blood cell homogenate (Plate 2.4.) did not detect corresponding components from the peritoneal fluids and the soluble proteins released from red cells. The released haemoglobin was seen migrating with the tracking dye. Thus, it is fairly certain that the heavier stained bands observed in the infected fluids were not due to the substantial contamination of red cells which were occasionally observed in the peritoneal fluids lavaged from chronically infected animals.

2.3. Conclusion

An increase in the total protein concentration in the peritoneal fluid was detected on week 7 after infection (Section 2.2.1.). The present study also showed that the peritoneal fluid and serum were identical in composition.

This is shown by the following findings; (1) serum-associated major proteins that were recognized by rabbit antiserum against mouse serum were also present in the peritoneal fluid (Section 2.2.2.). (2) the comparative SDS-polyacrylamide gel electrophoretic analysis gave similar patterns for peritoneal fluids and serum (Section 2.2.3.). Both possess seven major proteins with MW 96K, 81K, 63K, 52K, 50K and two with molecular weights larger than 100K. (3) simultaneous increase in the concentrations of protein components with MW 96K and 50K in the peritoneal fluid and its serum counterpart collected from schistosome infected mice (Section 2.2.3).

Fig. 2.1.

Total protein concentration in peritoneal fluids during the infection course of S. mansoni

At every two weeks, groups of mice were killed, the peritoneal fluids were lavaged by injection of PBS/heparin. The protein concentration was measured using Lowry's method (1951). Each point was the mean of the values of at least five infected (●) and five uninfected (O) mice, and the vertical bar its standard deviation.

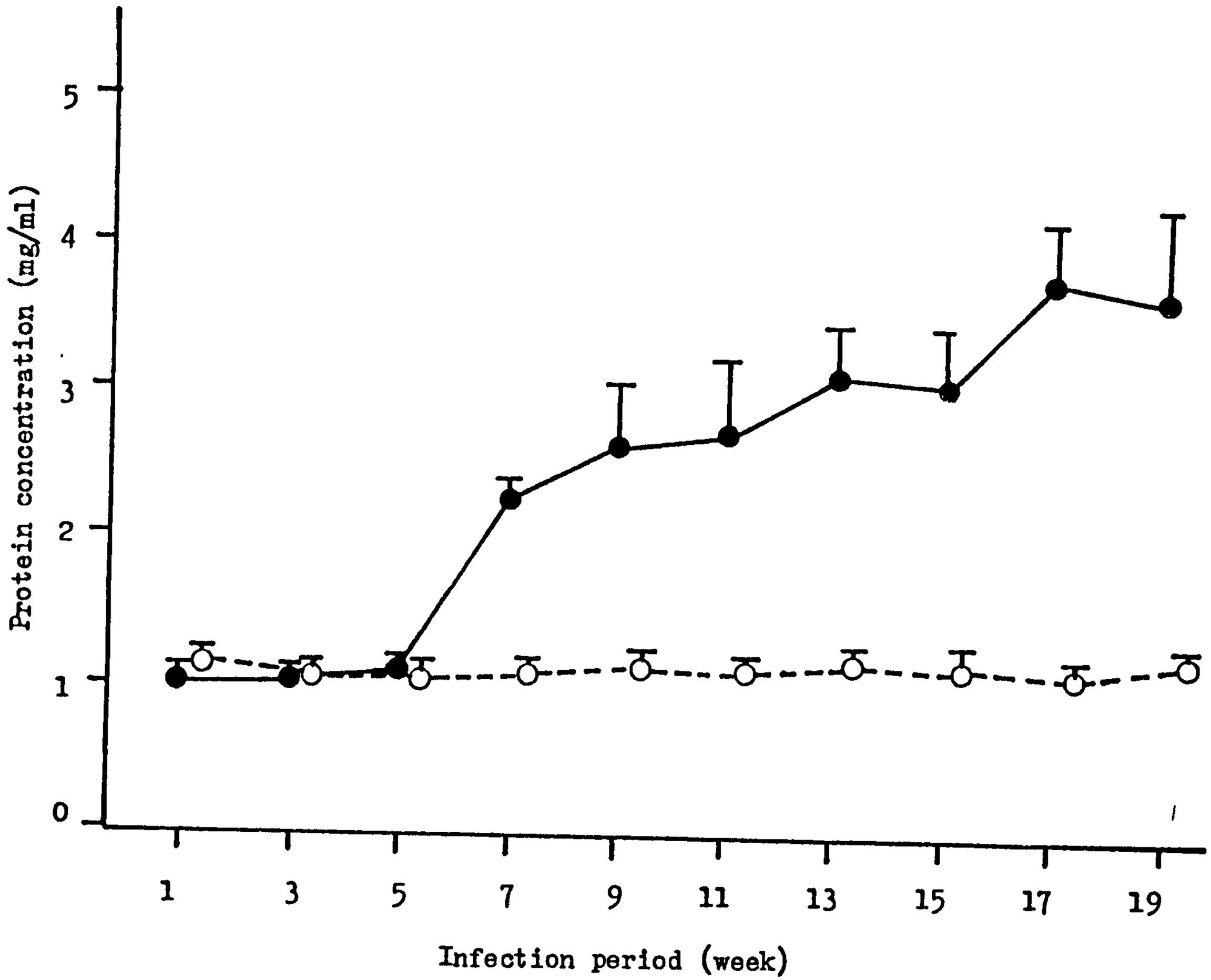
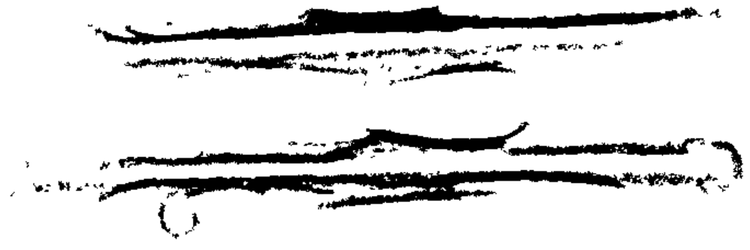

Protein concentration (mg/ml)
0
1
2
3
4
5
1
3
5
7
9
11
13
15
17
19
Infection period (week)

Plate 2.1.

Immunoelectrophoretic analysis of peritoneal fluid and serum

Normal peritoneal fluids and peritoneal fluids lavaged from chronically infected mice (infection periods ranged from 10 to 23 weeks) were concentrated using Aquacide II. The immunoelectrophoretic pattern was developed using rabbit antiserum against mouse serum. The anode was at the right hand end of the plate and the cathode the left. Top well: concentrated peritoneal fluid; middle well: mouse serum; lower well: concentrated peritoneal fluid.

12

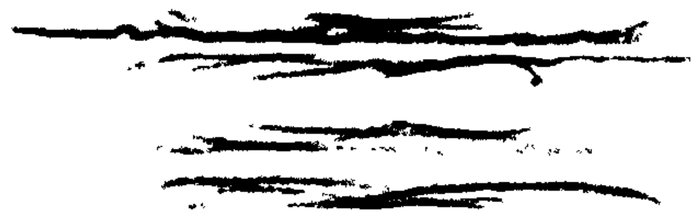


(a)

•	Normal peritoneal fluid
•	Serum
•	Normal peritoneal fluid

All longitudinal basins contained rabbit antiserum against mouse whole serum.

14



(b)

•	Infected peritoneal fluid
•	Serum
•	Infected peritoneal fluid

Plate 2.2.

Representative electrophoretogram of the peritoneal fluid and serum of normal and S. mansoni infected mice

The peritoneal fluids and sera were collected from normal mice and mice infected with 50 cercariae for 28 weeks Electrophoresis was carried out in Tris-buffered slab gel of 7.5% polyacrylamide at pH 8.8. A mixture of molecular weight markers (Bio-Rad Labs., Richmond, Calif., U.S.A.) containing phosphorylase B (94K), Bovine serum albumin (68K), ovalbumin (43K) and carbonic anhydrase (30K) was applied in parallel. Anode is at the bottom of the gel. Protein bands were stained with Coomassie brilliant blue R250.

Samples were: (a) molecular weight markers

(b) normal peritoneal fluid

(c) infected peritoneal fluid

(d) normal serum

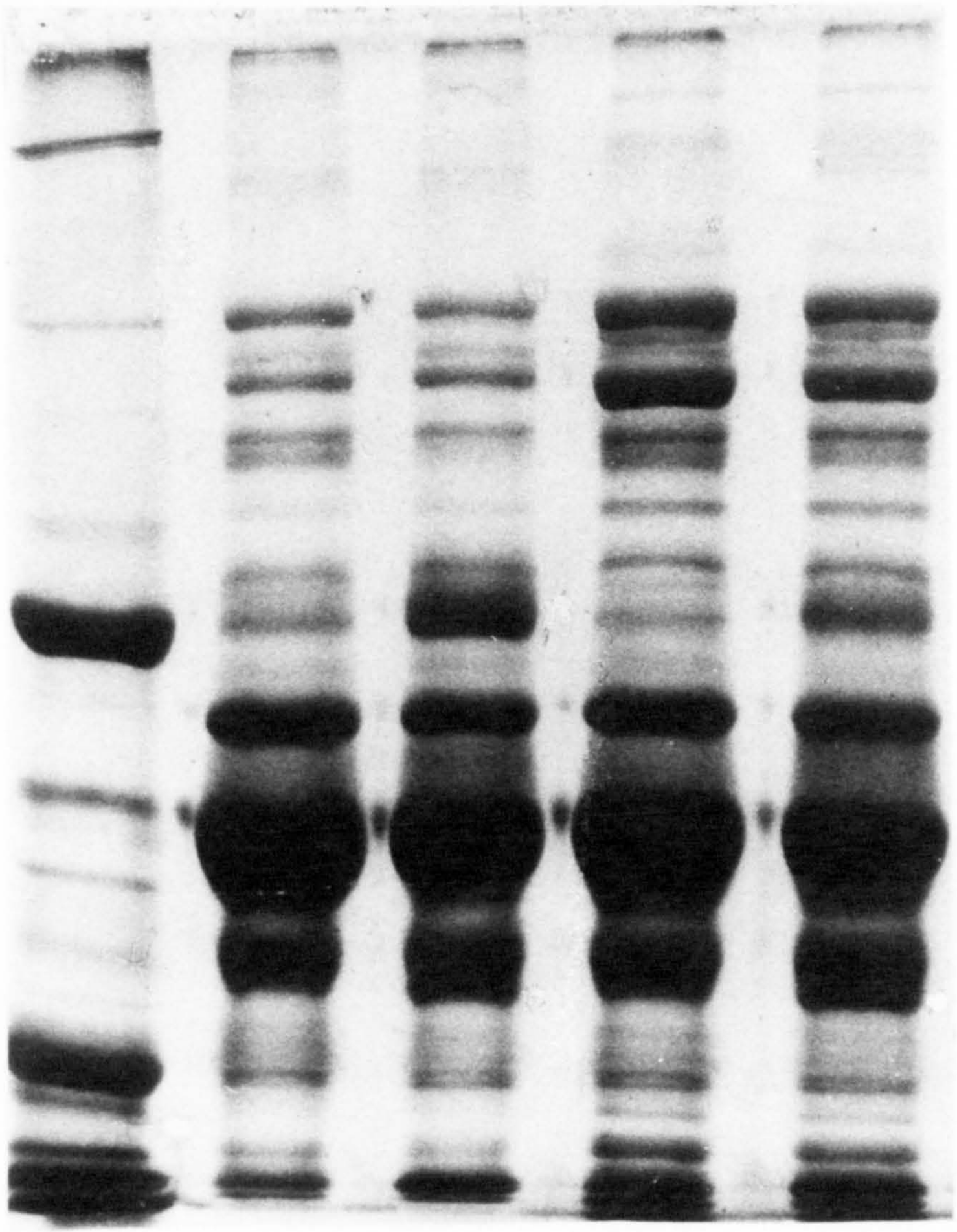
(e) infected serum

The molecular weight estimates calibrated from this and three other gels yield estimates of 96±2 K, 81±6 K, 63±2 K, 52±1 K and 50±1 K for components III, IV, V, VI and VII, respectively. Components I and II had molecular weights larger than 100 K.

(a) (b) (c) (d) (e)

MW of markers

MW of tested samples



94 K

68 K

43 K

30 K

I

II

larger than 100K

III 96 K

IV 81 K

V 63 K

VI 52 K

VII 50 K

Plate 2.3.

Representative electrophoretogram of sera from normal and 32-week infected mice

The sera were collected from three normal mice and three mice infected with 50 cercariae for 32 weeks. Electrophoresis was carried out in Triz-buffer slab gel of 7.5% polyacrylamide at pH 8.8. Anode is at the bottom of the gel. Protein bandings were stained with Coomassie brilliant blue R250.

Samples were: (a) normal serum 1

(b) normal serum 2

(c) normal serum 3

(d) infected serum 1

(e) infected serum 2

(f) infected serum 3

(g) supernatant of mouse red cell homogenate

Approximately same protein amounts (33-38 µg) of samples were added. See plate 2.2. for the components I-VII.

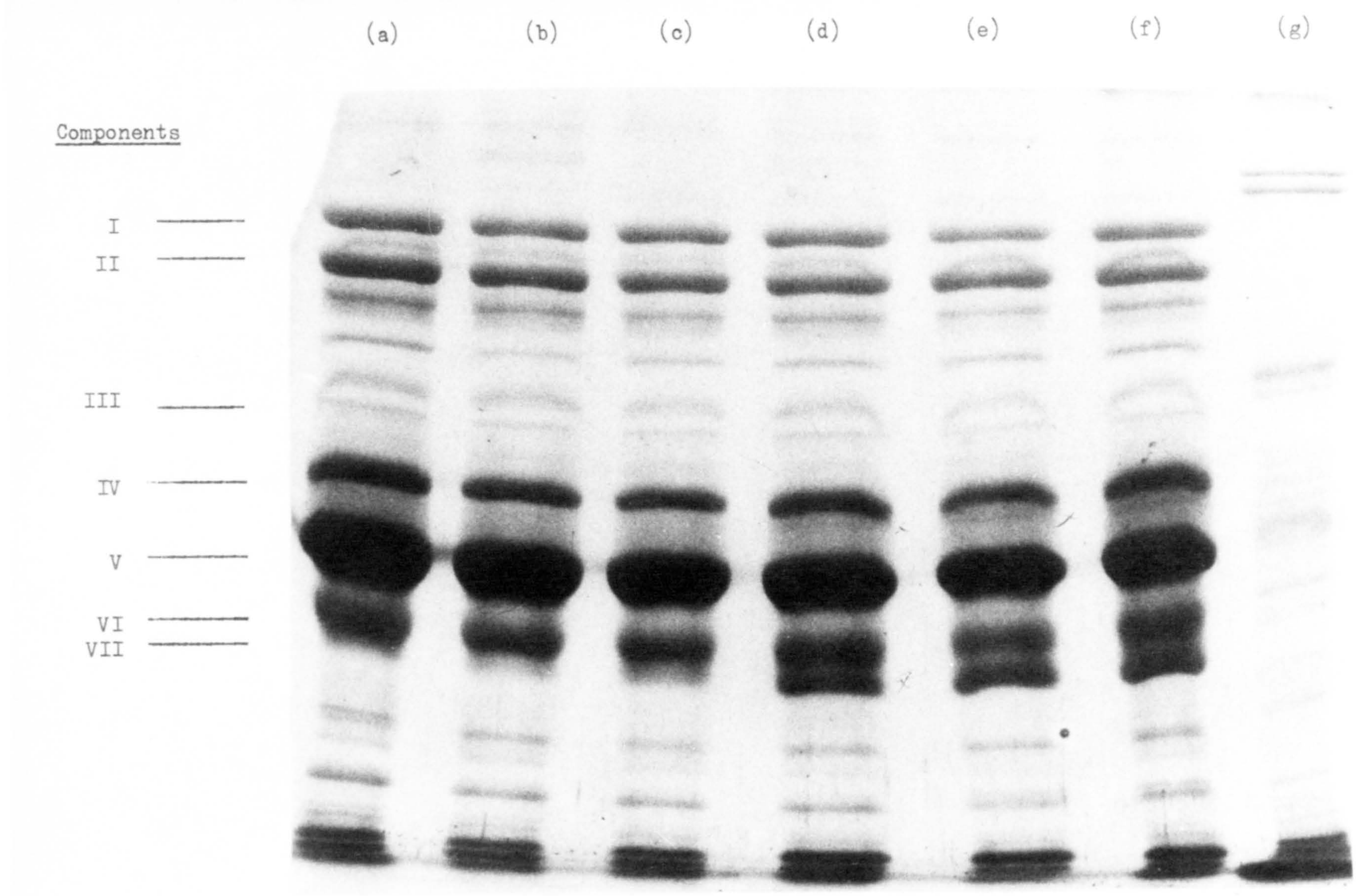

(a)
(b)
(c)
(d)
(e)
(f)
(g)
Components
I
II
III
IV
V
VI
VII

Plate 2.4.

Representative electrophoretogram of peritoneal fluids of normal and 32-week infected mice

The peritoneal fluids were collected from normal mice and mice infected with 50 cercariae for 32 weeks. Samples were concentrated using Aquacide II. Electrophoresis was carried out in Triz-buffer(slab gel of 7.5% polyacrylamide at pH 8.8. Anode is at the bottom of the gel. Protein bandings were stained with Coomassie brilliant blue R250.

Samples were: (a) normal peritoneal fluid

(b) infected peritoneal fluid

(c) supernatant of mouse red cell homogenate

Approximately same protein amounts (27-35 µg) of peritoneal fluids were added. See Plate 2.2. for the components I-VII.

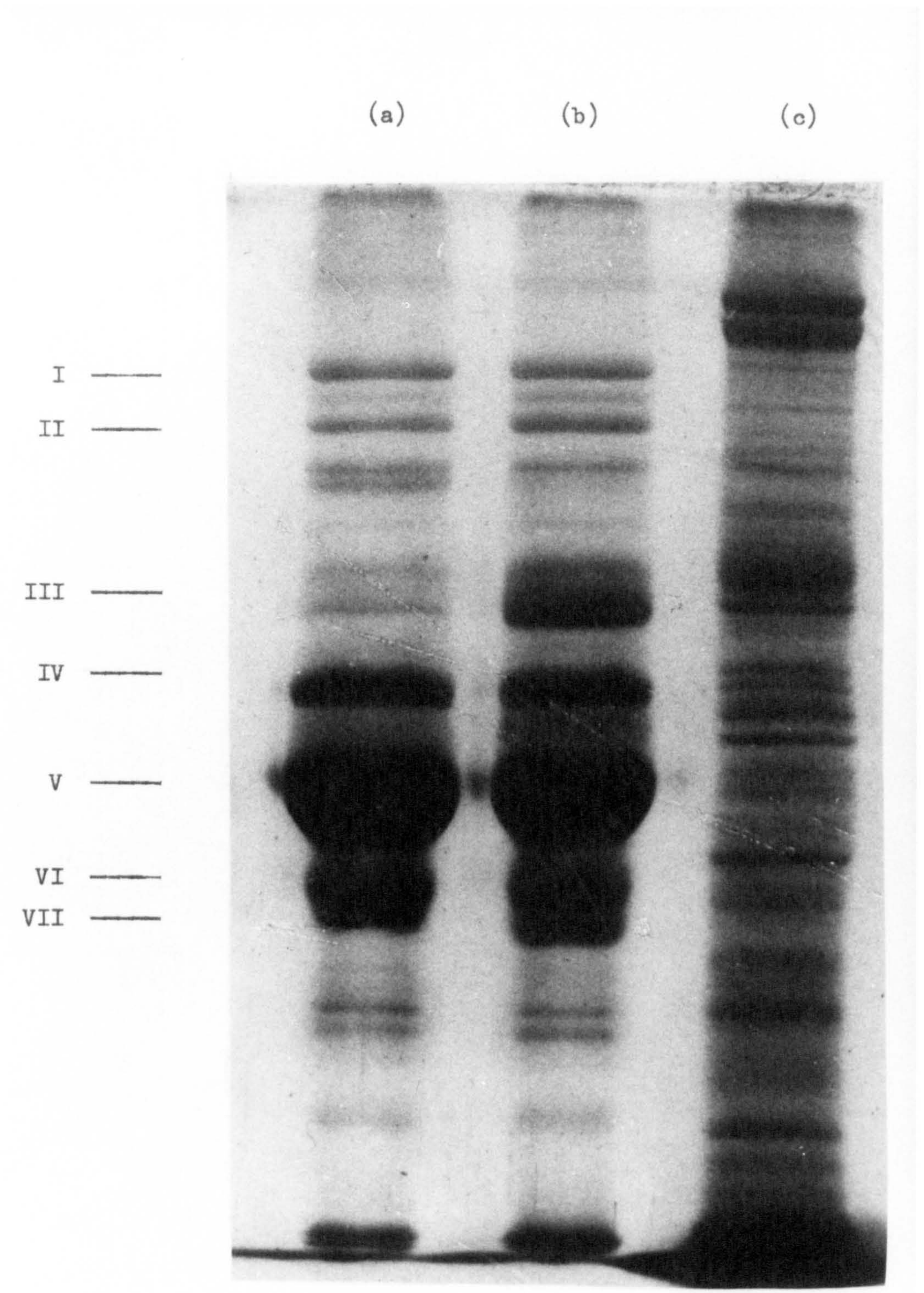

(a)
(b)
(c)
I
II
III
IV
V
VI
VII

Plate 2.5.

Changes in amounts of MW 96K and 50K protein bands during the course of S. mansoni infection

The peritoneal fluids were collected from normal mice and mice infected with 50 cercariae for 1, 3, 8 and 14 weeks. Samples were concentrated using Aquacide II. Electrophoresis was carried out in Triz-buffered slab gel of 7.5% polyacrylamide at pH 8.8. Anode is at the bottom of the gel. Protein bands were stained with Coomassie brilliant blue R250. Samples were: (a) normal peritoneal fluid

(b) 1-week infected peritoneal fluid

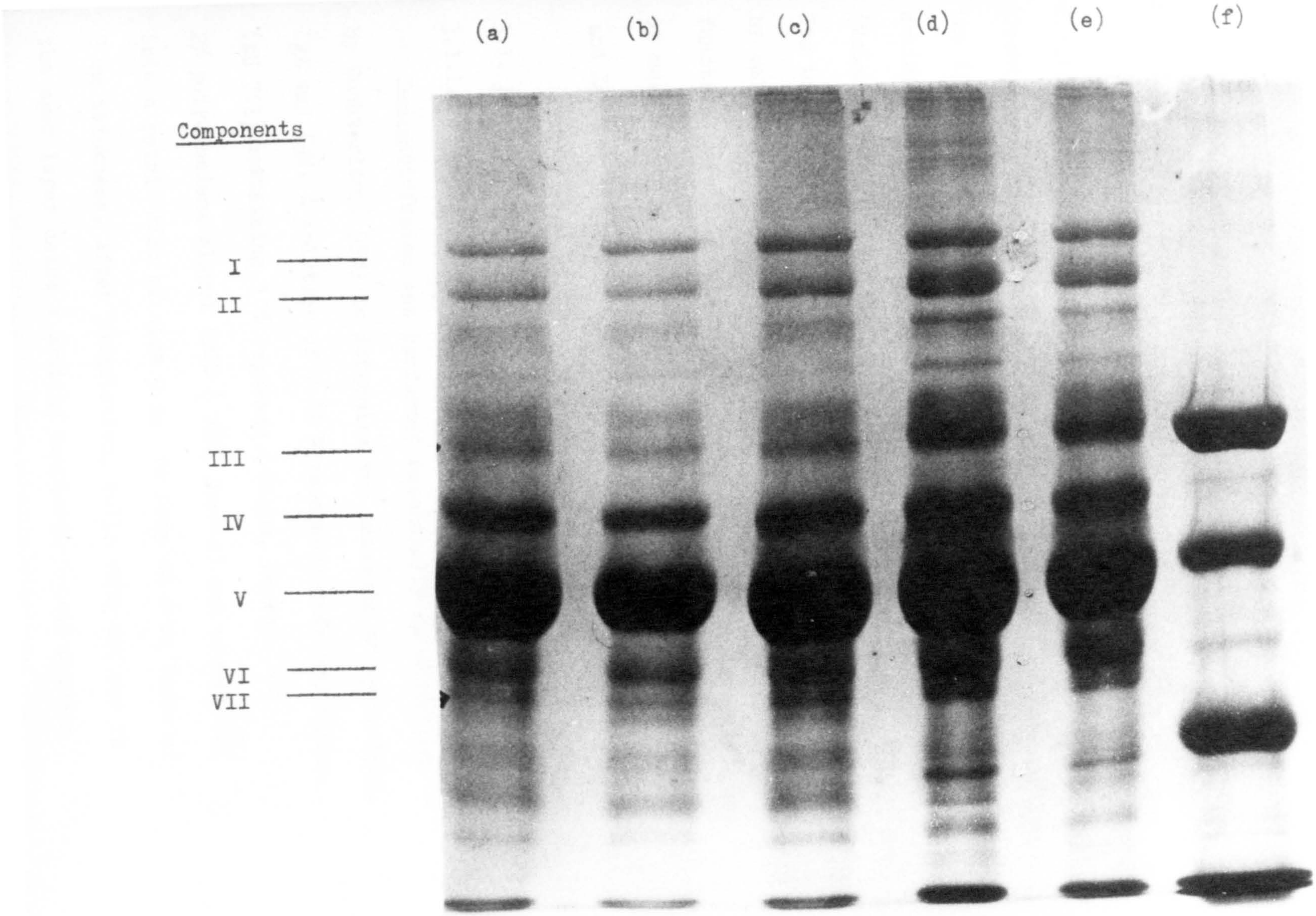
(c) 3-week infected peritoneal fluid

(d) 8-week infected peritoneal fluid

(e) 14-week infected peritoneal fluid

(f) molecular weight markers

Approximately same protein amounts (25-33 µg) of peritoneal fluids were added. See Plate 2.2. for the components I-VII. The molecular weights of components III and VII were 96K and 50K, respectively.


(a)
(b)
(c)
(d)
(e)
(f)
Components
I
II
III
IV
V
VI
VII

3. Analysis of immunoglobulins and anti-S. mansoni IgG in the peritoneal fluids

The immunoglobulin profile in the peritoneal fluid was firstly determined by the microdouble diffusion method (Ouchterlony, 1958). Studies were next undertaken to determine the time course of the production of specific antibody against schistosome antigens by enzyme-linked immunosorbent method (McLaren, 1978). IgG was selected for the quantitative study. Its importance in the schistosomacidal activity mediated by cells is well documented (see Introduction). IgG also functions as cytotropic antibody and causes the degranulation of mast cells in the presence of specific antigens (Schwartz and Levine, 1973)

3.1. Experimental design

3.1.1. Immunodiffusion analysis

Immunodiffusion was performed essentially as described by Ouchterlony(1958) to determine the presence of IgG1, IgG3, IgA and IgM. A solution of 0.01 M phosphate-buffered saline (pH 7.1) comtaining 1 % agarose (Sigma, Dorset) and 2% polyethelene glycol 6000 (BDH, Dorset) was poured hot into a petri dish of size 9 cm to form an even layer of 2 mm thickness. After congelation, wells were cut out in the agar layer using a cutting template. Graded amounts of rabbit serum against mouse IgG1, IgG3 (Miles Ltd.,Stoke Poges) IgA and IgM (gifts from Dr. W. Cushley 1982) were tested

with either normal or infected peritoneal fluids that had been concentrated 5-fold and 10-fold, respectively. Plates were placed in a humid atmosphere at 4°C until the precipitation patterns developed (usually 15 hr).

<u>0.01 M phosphate-buffered saline (pH 7.1) (PBS)</u>

Sodium chloride	8.5 g
Disodium hydrogen phosphate . $2H_2O$	1.3 g
Sodium dihydrogen phosphate . $2H_2O$	0.4 g
Dist. H_2O	1000 ml

<u>Washing, drying, staining and destaining of precipitation plates</u>

After the precipitation patterns had developed, the plates were washed with three changes of PBS for one day, following by three washings in dist. H_2O for 5 hr and then dried at 37°C.

The plates were stained with 0.2% Coomassie blue (BDH, Dorset) in a solution of 50% methanol and 7% acetic acid for 1 hr and then destained in a solution of 5% methanol and 7% acetic acid at room temperature until the background was cleared.

3.1.2. <u>Enzyme-linked immunosorbant assay (ELISA)</u>

To avoid the variability which may occur from test to test due to the minor technical changes, the samples used in the comparison studies were tested simultaneously.

The ELISA was based on the microplate method of

McLaren (1978) with the following modifications. Two crude soluble antigens were prepared from adult worms and eggs of S. mansoni according to the method described by McLaren et al (1978).

Preparation of antigens for ELISA

Worm antigens

Crude worm extract was prepared from freeze-dried adult worms obtained from WHO worm bank (Lot 6790). A lot of 4.5 mg of dried worms were suspended in 1 ml of PBS and homogenized by hand. After adding another 2 ml of PBS, the suspension underwent two cycles of rapid freeze-thawing and then stored at -20°C overnight. The homogenate was thawed at 37°C and allowed to stand at 4°C for 2 hr. The suspension was then subjected to ultracentrifugation at 40,000 r.p.m. for 1 hr at 4°C in a Beckman (USA) L-5-50B Ultracentrifuge using a 50 TI rotor. The supernatant was then stored in several portions at -20 C until use.

Egg antigens

A crude soluble egg extract was obtained through the courtesy of Dr. M. Doenhoff (Winches Farm Field Station, Herts, England). Suspensions of live eggs were extracted from livers and guts of infected mice by trypsinization (1-2 mg trypsin per gut or liver). The eggs were homogenized by hand in 5 ml of PBS per ml of packed eggs. The homogenate was then subjected to ultracentrifugation as described in the preparation of worm antigens. The supernatant was then stored in several portions at -20 C until use.

Technical details of ELISA

Step 1: Coating of microtitre plates

The wells of the polyvinyl chloride microtitre plates (Dynatec Lab. Ltd. Sussex) were coated by adding 150 ul of soluble antigens diluted in carbonate-bicarbonate coating buffer. The plates were then covered and left overnight at room temperature. Next day, the plates were washed and used immediately.

Coating buffer

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Dist. H_2O	1000 ml

Step 2: Washing

This step consisted in emptying the plates, refilling the wells with saline/tween 20 washing buffer from a wash bottle. The fluid was left in the wells for three min each time. The procedure was repeated three times. After the final wash the plates were tapped dry on a pad of tissue papers

Washing buffer

NaCl	9.0 g
Tween 20	0.5 ml
Dist. H_2O	1000 ml

Step 3: Addition of tested sera /peritoneal fluid

This step was performed immediately after the plates were tapped dried, volumes of 150 ul of PBS/Tween 20 incubation buffer was added to the wells. The serum/peritoneal fluids

at suitable starting concentrations were added to the first row of wells of the plates and doubling dilutions made with a Microtiter multidiluter. The plates were incubated at room temperature for 2 hr.

Incubation buffer

PBS (pH 7.6)	900 ml
Tween 20	0.45 ml

PBS (pH 7.6)

NaCl	8.5 g
Na_2HPO_4	1.28 g
$NaHCO_3$	0.16 g
Dist. H_2O	1000 ml

Step 4: Addition of horse-raddish peroxidase conjugates

After repeating the washing procedure described in Step 2, volumes of 150 ul of anti-mouse IgG conjugated with peroxidase (Cappel Lab., Cochranville, Pa., USA) diluted in PBS/Tween 20 incubation buffer were added to the antigen-antibody mixture. The plates were further incubated at room temperature for 3 hr.

Step 5: Addition of enzyme substrate

After a further washing as described in Step 2, volumes of the freshly prepared substrate for peroxidase, orthophenylene diamine (1 mg/ml), were added.

Enzyme substrate

Stock OPD	1.0 ml
0.1 M citric acid	24.7 ml
0.2 M Na_2HPO_4	25.3 ml
30% H_2O_2	10.0 ul
Dist. H_2O	50 ml

Stock OPD

orthophenylene diamine	10 mg
absolute methanol	1 ml

Mixed and stored in dark at 4 C and used within 2-3 days.

Step 6: Termination of the enzyme reaction

The reaction was stopped after incubation for 20 min at room temperature by addition of 150 ul of 8 N H_2SO_4 into each well.

8 N H_2SO_4 solution

H_2SO_4	21.3 ml
Dist. H_2O	100 ml

Step 7: Reading of ELISA value

The absorbance value of the incubation mixture of each well was read spectrophotometrically at 492 nm in a ELISA microplate reader (Titertek Multiskan).

3.2. Results

3.2.1. Immunoglobulin profiles of the peritoneal fluids

The immunodiffusion test revealed that IgG1, IgG3, IgA and IgM were presented in the peritoneal fluids lavaged from either normal mice or mice infected with S. mansoni (Fig. 3.1.).

3.2.2. Measurement of titre of antibodies against schistosome antigens in the peritoneal fluids

After having established that immunoglobulins exist in the peritoneal fluids, studies were next undertaken to investigate the kinetics of antibody production against antigenic materials prepared from adult worms and eggs of S. mansoni.

3.2.2.1. Optimal conditions for ELISA

The optimal dilutions for antigen, peritoneal fluid, sera and peroxidase labelled with anti-mouse IgG conjugate were determined by checker-board titrations.

3.2.2.1.1. Antigen concentration

Three concentrations of worm or egg extracts were titrated against two positive (infected) and one negative (uninfected) lavaged peritoneal fluids. It showed that the optimal antigen concentrations for coating the ELISA trays were approximately 10 ug protein/ml for worm antigens (Fig. 3.2.a.) and 2 ug protein/ml for egg antigens (Fig. 3.2.b.)

Lower antigen concentrations gave significantly lower absorbance readings while the higher concentrations did not increase appreciably the maximum differences in readings between the known positive and negative fluids.

3.2.2.1.2. Conjugate concentration

The checker-board titration of conjugate using one positive peritoneal fluid at the optimal concentration of worm antigens showed that the differences in absorbance readings produced by conjugate at 1:800, 1:1000 and 1:1200 dilution were negligible (Fig. 3.3.). In this study, 1:1000 dilution of conjugate was used for the convenience of manipulation.

3.2.2.1.3. Titration of peritoneal fluids and sera

When ELISA was performed by testing at one dilution only, such as in the study of kinetics of antibody production during the course of infection, the 'appropriate dilution' was determined by carrying out a preliminary test with positive and negative reference fluids. Logically, the dilution which gave the maximum differences in absorbance values between the known positive and negative fluid samples should be used. In this study, it was observed that, in both cases of worm egg antigens, this 'appropriate dilution' usually occurred at lesser dilutions. However, the absorbance values of some fluids particularly those of serum collected from chronically infected mice could not be obtained when

the reading was above the maximum absorbance range of ELISA microplate reader (range up to 2.9 at 492 nm). Typical titration curves using serum or peritoneal fluids of varying antibody titre are presented in Fig. 3.4 and Fig. 3.5..

In the study of kinetics of antibody production to S. mansoni, dilution of 1:300 and 1:2 for serum and peritoneal fluids, respectively, were selected for the following reasons: (1) the ease of dilution manipulation (2) the absorbance values of strong positive fluids at these dilutions fell within range 2.9 at 492 nm and thus could be read in the ELISA microplate reader (3) the values should fall within the upper- or mid-portions rather than the flattened phase of the titration curves where the absorbance values did not decrease correspondingly with the further dilutions of tested fluids.

3.2.2.2. Determination of the positive/negative discrimination level

The discrimination level between positive and negative is a crucial element in any serological test, particularly in the study of kinetics of antibody production during the course of infection. This discrimination level should allow an early indication of increase in antibody level without the risk of giving a false positive.

In this study, to establish the discrimination level, the distribution and limits of the S. mansoni ELISA

'negative' values in lavaged peritoneal fluids and serum were investigated by surveys of a group of 28 normal uninfected mice with age ranged from 7-29 weeks and were examined in a single test. The possible association between the values and age was disregarded since the numbers of samples in each age group were too small to justify a statistical analysis. The result of one of such surveys is presented in Fig. 3.6..

The distribution of negative values of either peritoneal fluids or serum appeared log normal and had median ELISA values of 0.15 for peritoneal fluids and 0.25 for serum when tested against worm antigen (Fig. 3.6.a.). The 'negative' values of peritoneal fluids distributed within the range from 0.11 to 0.20, and 10 in 28 samples tested gave the median value. The 'negative' values of serum scattered over somewhat wider range which cover from 0.22 to 0.35, and 6 of the tested samples gave the median value. Based on the probability plot of the distribution, it seems reasonable to set the minimum 'positive' ELISA values at 0.20 and 0.35 for peritoneal fluids and serum, respectively. These values which covered upper limits of the 'negative' values were approximately the mean of their respective 'negative' values plus three times of their respective standard deviations (S.D.).

Similar observations were obtained when egg antigen was used in determining the positive/negative discrimination

level (Fig. 3.6.b.). Eight of the 28 tested samples gave median ELISA 'negative' values at 0.07 and 0.23 in the peritoneal fluids and serum, respectively. The 'negative' values distributed from 0.02 to 0.08 in the case of peritoneal fluids, and 0.21 to 0.30 in the case of serum. As in the survey using worm antigens, the mean of their 'negative' values plus three times of their respective standard deviations also covered the upper limit of the 'negative' values.

However, these figures obtained from the two surveys described above could not be used as 'absolute' values. When replicated tests of smaller scale (6-10 samples) were made using the same group of normal fluids on different occasions, some gave similar ranges of extinction values whereas others showed rather large variations from those shown above (Table 3.1.). This variation may be due to the minor technical changes. Since the distribution of individual values was not dissimilar to that shown in Fig. 3.6., the ELISA of pathological fluids were always performed together with 8-12 randomly selected normal controls and set the minimum positive response value at the mean absorbance values of the negative groups plus three times of their respective S.D. of the mean.

3.2.2.3. <u>General observations on ELISA of peritoneal fluids and sera.</u>

In this study, the results of ELISA were reported as

absorbance values read photometrically at 492 nm (E_{492}) after incubation with o-phenylene-diamine substrate. The double-serial titration was expressed arithmetically after transformation into Log_2 scale so that a broader range of titration could be covered in a single graph.

The titration curves, the absorbance values (E_{492}) vs dilution (Log_2), flattened toward the titration end-point where the absorbance values did not drop correspondingly at higher dilutions. The flattened phases, in general, began at E_{492} values of 0.10-0.15 for peritoneal fluids, and 0.15-0.20 for serum. The titration curves produced were not parallel among individual fluids. An example of peritoneal fluids and their corresponding serum against worm antigen is given in Fig. 3.7.. The curves produced by peritoneal fluids appeared more uniform than those by serum. The non-parallelism may be be inherited from this particular assay system which involves two antigen-antibody reactions, or due to technical errors (e.g., dilution), or the interferences by the presence of other classes of immunoglobulins in the fluids as described for anti-Toxocara in human serum (Savigny and Voller, 1980).

The patterns of the titration curves produced using worm antigens were different from those using egg antigens. A sharp drop in absorbance values at one further Log_2 dilution of the tested fluid was observed in ELISA using

worm antigens compared with those using egg antigens. The observation was true in both serum and peritoneal fluids (Fig. 3.4. and Fig. 3.5.).

A linear relationship between the absorbance values and the antibody titre in the peritoneal fluids emerged when the raw absorbance values excluding the flattened phases were multiplied by 100 followed by transformation into Log_2 scale, and the new data were subsequently plotted against Log_2 dilution. As had been expected the Log_2-Log_2 lines were not parallel among individual tested fluids. It also showed that the reduction in the absorbance values at a further Log_2 dilution was less with egg antigen than with worm antigen (Fig. 3.8.). In one single ELISA of 15 peritoneal samples collected from mice infected for 7-19 weeks, the Log_2-Log_2 lines produced gave slopes within 0.456 ± 0.054 for worm antigen and 0.154 ± 0.033 for egg antigen. The lines rarely spanned more than two Log_2 of absorbance values through 5-8 Log_2 dilutions.(Fig. 3.8.). The linear relationship was not obtained in the ELISA of serum with either antigen. The reason for the differences in the Log_2-Log_2 expression between peritoneal fluids and serum is not known.

Since the range of Log_2 absorbance values was narrow, there is little potential for creating a standard model using this linear relationship for extrapolation and calibration of antibody titre in a tested sample. Therefore, in the study of kinetics of antibody production during

the course of S. mansoni, the raw absorbance values were used in the data.

3.2.2.4. Development of specific IgG to schistosome antigens in peritoneal fluids and serum

Variability may occur from test to test due to minor technical changes, different batches of antigen preparations and to differences in the ambient temperature in ELISA determinations. Hence, peritoneal fluids and serum of normal and infected mice were used simultaneously in each test using either antigen. Anti-S. mansoni IgG in serum and lavaged peritoneal fluids collected from mice infected for periods ranging from 1 to 19 weeks against worm and egg antigens are presented in Fig. 3.9 and 3.10, respectively.

Antibodies against egg antigen in both serum and peritoneal fluids could be detected at week 7 after infection (Fig.3.10). The level of antibody titre reached a peak at week 15 and remained high until the termination of the 19-week experiment.

The elevation of anti-worm antigen antibody level started earlier than that of egg antigen. It was detected at week 5 and reached a peak at week 15. This was observed in both serum and their corresponding peritoneal fluids (Fig. 3.9). The increase in antibodies to egg antigen was more rapid than to worm antigen. The ELISA values against worm and egg antigens in the peritoneal fluids collected from 15-week infected mice were 6- and 10- fold

of those found in uninfected controls, respectively. The equivalent factors for serum, for the same mice, were 3-fold and 6-fold.

During the study of the kinetics of antibody production, it was noted that some peritoneal fluids collected from mice infected for 3 weeks had ELISA values above the range of negative values. To investigate whether the positive value was a 'by chance'occurrence, a trial of a larger scale was conducted using 15 peritoneal samples collected from mice infected for 3 weeks together with 27 normal controls. The distributions of ELISA values of the two tested groups are presented in Fig. 3.11.

Five of the 15 samples from 3-week infected group had ELISA values above the positive/negative discrimination level (Fig. 3.11.a.). The elevated values ranged from 2 to 4 times the mean of negative values. In contrast, the ELISA values of their corresponding serum were within the range found for normal controls (Fig. 3.11.b.).

The next series of experiments was designed to compare the antibody reactivities to worm and egg antigens in individual samples. The peritoneal fluids and serum were collected from 16 chronically infected mice (infection periods ranged from 10 to 19 weeks) and 8 normal controls. Each sample was diluted (1:2 dilution for peritoneal fluids and 1:300 dilution for serum) and divided to two portions. They were then tested against worm and egg antigens

simultaneously. It showed that the egg antigen was more reactive than the worm antigen, giving higher ELISA values if compared on the basis of coating antigen concentration criterion (Fig. 3.12). This was observed in both peritoneal fluids and serum.

3.2.2.5. Correlation of antibody activities between sera and peritoneal fluids

It was noted that the sera with high ELISA value also exhibited high antibody reactivity in the peritoneal fluid from the same animal, whereas those with low values in serum had low reactivity in their corresponding peritoneal fluids. In the light of this observation, the relationship between the antibody levels in serum and their corresponding peritoneal fluids was examined. A scatter diagram shows the individual ELISA values of peritoneal fluids plotted against those of sera .(Fig. 3.13.). As the antibody level increased in the serum, there was a corresponding increase in the peritoneal fluids. It was observed when tested against either worm (Fig. 3.13.a.) or egg (Fig. 3.131b.) antigens.

Furthermore, when the antibody titre to worm antigen was plotted against egg antigen in the peritoneal fluids, the positive correlation between these two groups of ELISA values was also observed, though a few samples with high ELISA values against worm antigen had low values against egg antigen, and vice versa.

3.2.2.6. Effect of cercarial exposure dose on the response of anti-S. mansoni IgG in peritoneal fluids

This series of experiments was designed to investigate the effect of the number of worms carried on the onset and magnitude of peritoneal IgG. Three groups of mice were percutaneously infected with approximate 20, 50 or 100 cercariae each mouse. At week 7 after infection, the peritoneal fluids were lavaged and their specific IgG against worm antigen were measured by ELISA.

Fig. 3.15. shows that the onset of IgG increase was regulated by the size of cercarial dose used for the infection. At 7 weeks after infection, the increase in peritoneal IgG occurred in mice given the larger cercarial doses (50-100 cercariae), whereas only 2 of the 8 mice exposed to 20 cercariae gave a significant increase in the ELISA value against worm antigen. The reason for the dissynchrony was not known. The ELISA values given by mice infected with 100 cercariae were not significantly different from those by mice infected with 50 cercariae. The infection of all the mice was confirmed by microscopic examination of squashed liver samples.

3.3. Conclusion

Immunoglobulins of four classes, IgG1, IgG3, IgA and IgM, were detected in the peritoneal fluids lavaged from normal mice and mice infected with S. mansoni (Section 3.2.1.).

The optimal conditions for ELISA of peritoneal fluids and serum, the coating concentrations of worm and egg antigens, the dilutions of peritoneal fluids and serum, the concentration of peroxidase-tagged anti-mouse IgG, were defined by chacker-board titrations (Section 3.2.2.1.). The ELISA of individual peritoneal fluids showed that the egg antigen was more active in binding antibodies in vitro than the worm antigen, giving higher ELISA values, when compared on the basis of coating antigen concentration (Section 3.2.2.4.). a steeper drop in ELISA value against worm antigen than egg antigen was obtained when the peritoneal fluid or sera was further diluted.

In this study, the positive/negative discrimination levels for specific IgG in peritoneal fluids and serum were set at the mean ± 3.S.D. of the negative values (Section 3.2.2.2.). Based on this discrimination criterion, the increase in IgG against worm and egg antigens was detected in the serum and peritoneal fluids of mice infected for 5 and 7 weeks, respectively (Section 3.2.2.4.). The specific IgG against worm antigen could be detected in some of the peritoneal fluids collected from mice infected for 3 weeks (Section 3.2.2.4.). The antibody titre against either antigen reached its peak in serum and peritoneal fluids 15 weeks after the infection (Section 3.2.2.4.).The time of the onset of the increase in IgG against worm antigen appeared to be regulated by the

size of infection, whereas the magnitude was not when measured at week 7 (Section 3.2.2.6.). Several interesting features of the data were observed; the simultaneous increase in the specific IgG in peritoneal fluids and serum (Section 3.2.2.5.), the positive correlation of magnitude of antibody titre in the paired peritoneal fluids and serum (Section 3.2.2.5.), and the positive correlation of antibody titre between those against worm antigen and egg antigen in peritoneal fluids (Section 3.2.2.5.). The significance of these features are discussed in the Discussion.

Table 3.1.

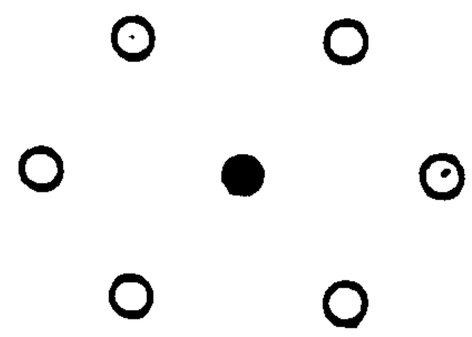
Variation of 'negative' ELISA values against worm antigen. Six tests using peritoneal fluids and sera from normal mice were performed on different occasions. The donors of peritoneal fluids and of sera were not the same mice.

Group	Individual values	Mean ± S.D.
A) Peritoneal fluids (tested at 1:2 dilution)		
1.	0.151, 0.127, 0.131, 0.122, 0.106, 0.128, 0.115, 0.123, 0.117, 0.114	0.123±0.011
2.	0.196, 0.205, 0.234, 0.201, 0.193, 0.212, 0.204	0.206±0.013
3.	0.012, 0.011, 0.028, 0.015, 0.014, 0.012, 0.022, 0.009	0.015±0.006
B) Sera (tested at 1:300 dilution)		
1.	0.123, 0.113, 0.125, 0.108, 0.135, 0.128, 0.148	0.126±0.013
2.	0.265, 0.234, 0.263, 0.277, 0.271, 0.278, 0.251, 0.308, 0.311, 0.283, 0.261	0.275±0.026
3.	0.104, 0.124, 0.121, 0.124, 0.118, 0.111, 0.138, 0.125, 0.154	0.124±0.015

Fig. 3.1.

Comparative immunodiffusion analysis of sera and peritoneal fluids

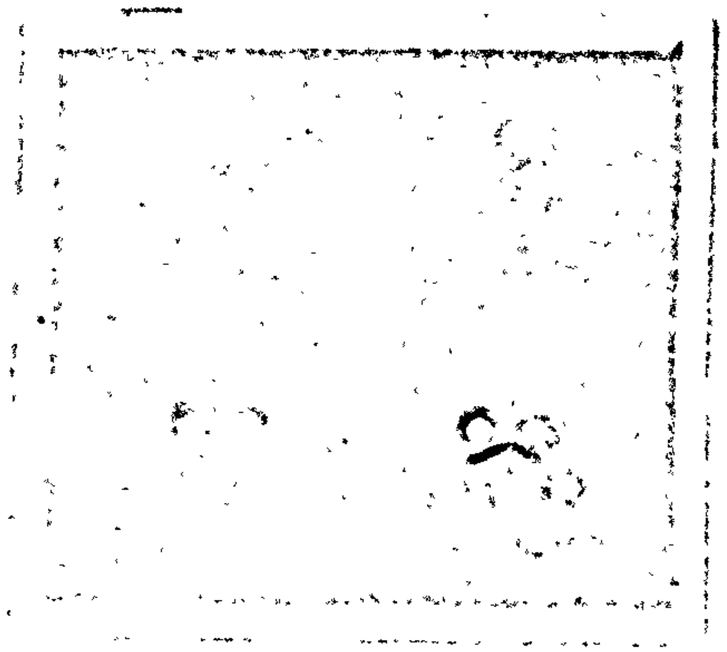
A solution of 0.01 M phosphate-buffer saline (pH 7.1) containing 1% agarose and 2% polyethelene glycol 6000 was poured hot into a petri dish. After congelation, seven wells were cut out in the agar layer using a cutting template. Sera and peritoneal fluids were collected from normal mice and mice previously infected with 50 cercariae for 23 weeks. Infected and normal peritoneal fluids were concentrated 5- and 10- fold, respectively. The tested (a) normal serum (b) infected serum (c) normal peritoneal fluid (d) infected peritoneal fluid were added into the central well and the graded amounts of rabbit serum against mouse IgG1, IgG3, Ig and IgM into the rest six wells. Plates were placed in a humid atmosphere at 4 C for 15 hr. The precipitin arcs were stained with 0.2% Coomassie blue and then destained in a solution of 5% methanol and 7% acetic acid.



○ : Rabbit antiserum against mouse Igs in serial dilutions

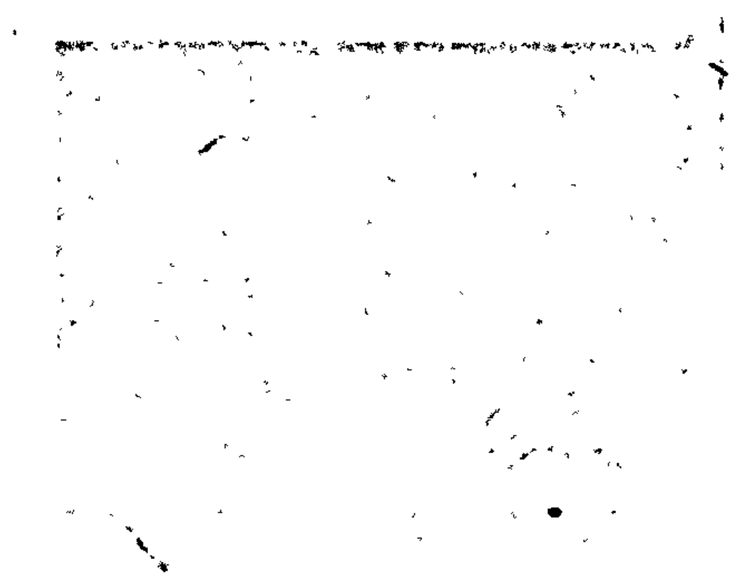
● : Mouse sera or peritoneal fluids

(a) Normal serum



(b) Infected serum

(c) Normal p. fluid



(d) Infected p. fluid

Fig. 3.2

Determination of optimal coating concentrations of antigens for ELISA of peritoneal fluids

a) Assay condition for worm antigen

Worm antigen: Three coating concentrations; 2.2, 10 and 22 ug/ml, were tested.

Peritoneal fluids: Fluid samples were collected from 1 normal mouse (○), and mice infected with 50 cercariae for 10 (●) and 19 (▲) weeks. The starting concentration was 1:2 dilution and 7 doubly dilutions were made.

Peroxidase conjugate: 1:1000 dilution was used.

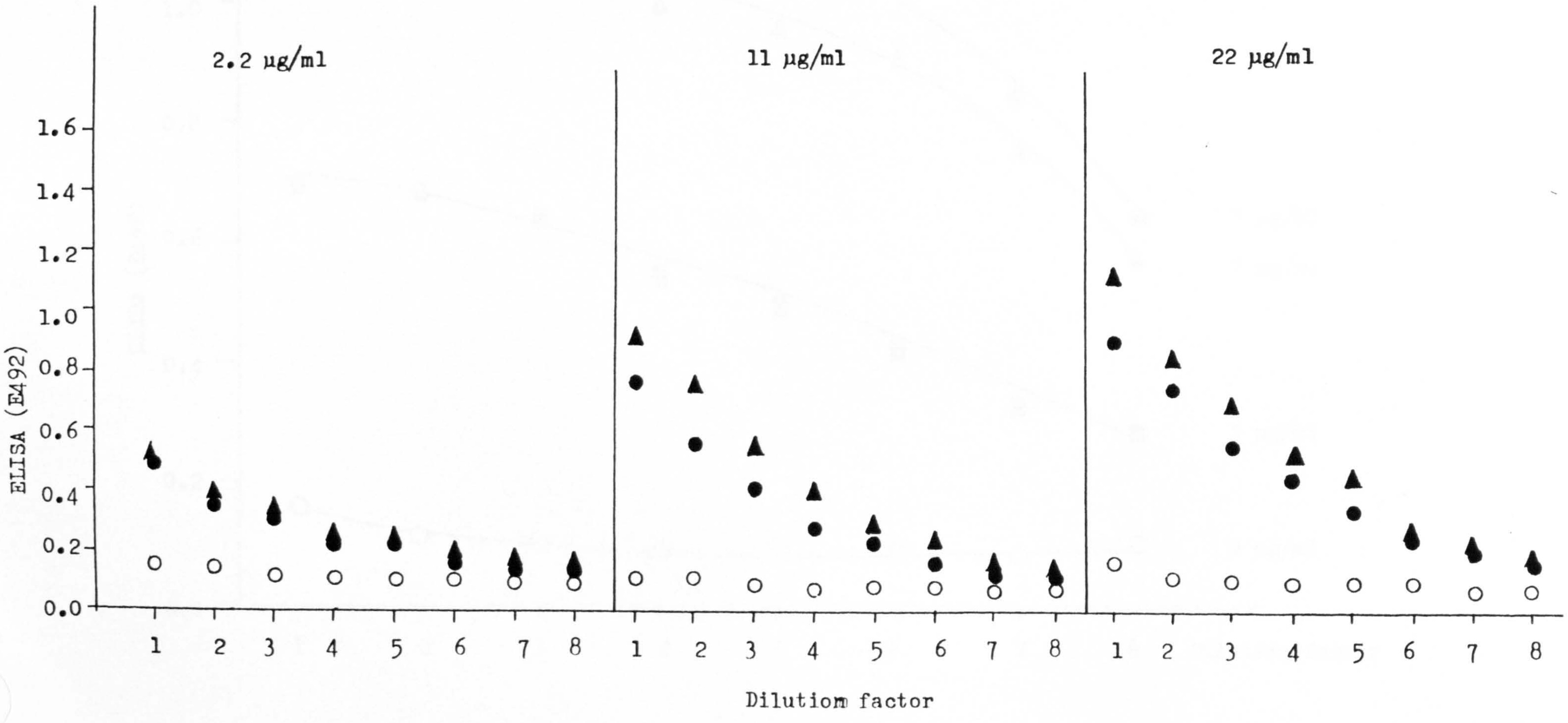
b) Assay condition for egg antigen

Egg antigen: Three coating concentrations: 1 (■), 2 (▲) and 5 (●) µg/ml were tested.

Peritoneal fluids: Fluid samples were collected from 1 normal mouse (○) and mouse infected with 50 cercariae for 16 weeks (●, ▲, ■). The starting concentration was 1:2 dilution and 7 doubly dilutions were made.

Peroxidase conjugate: 1:1000 dilution was used.

a) Worm antigen



b) Egg antigen

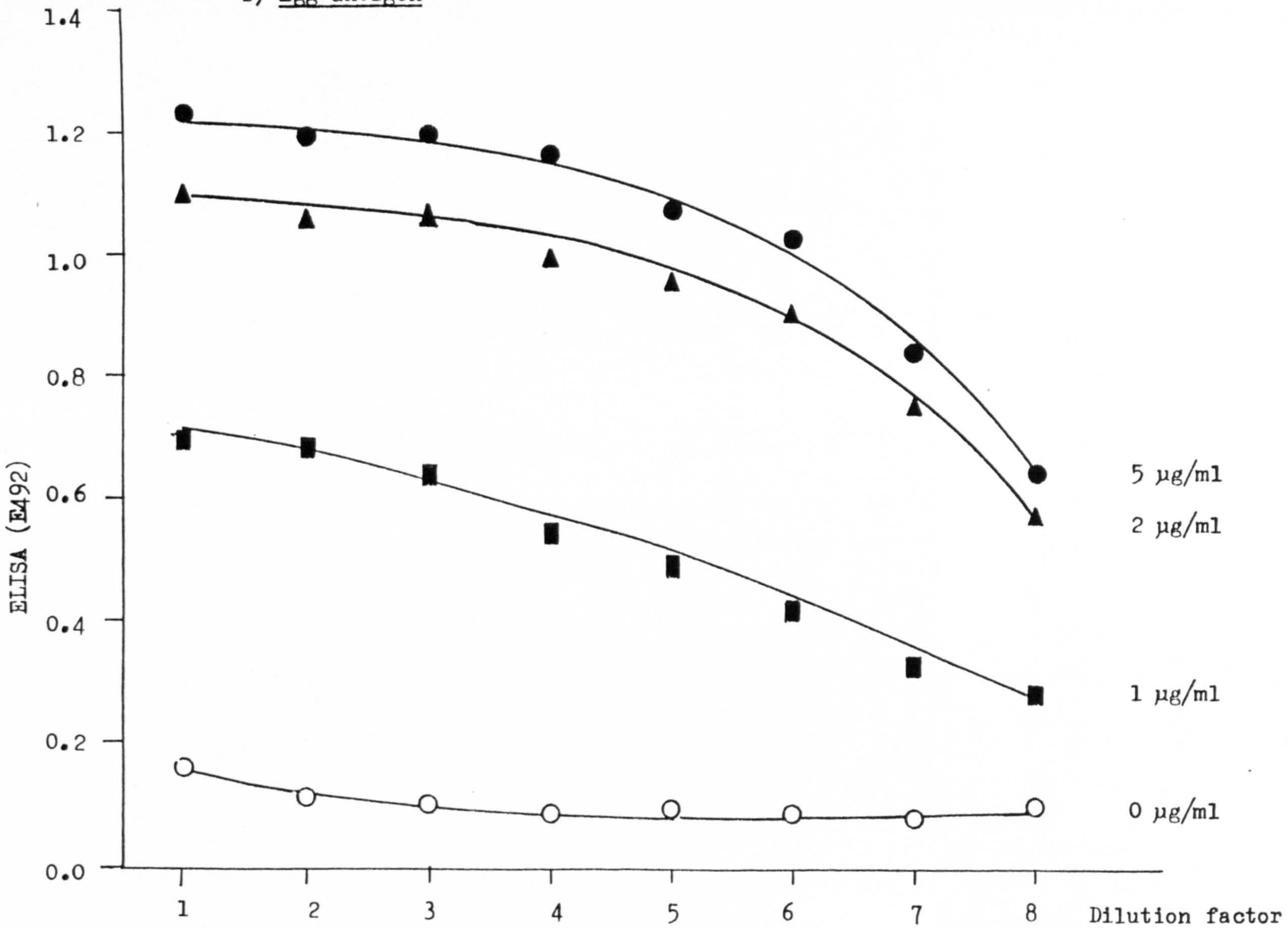


Fig. 3.3.

Determination of optimal concentration for horse radish peroxidase-labelled anti-mouse IgG conjugate for ELISA of peritoneal fluids

Assay condition for peroxidase conjugate

Peroxidase conjugate: Three dilutions; 1:800 (●), 1:1000 (▲) and 1:1200 (■), were tested.

Worm antigen: The coating concentration, 11 µg/ml, was used.

Peritoneal fluid: Fluid sample collected from 1 mouse infected with 50 cercariae for 10 weeks was used. The starting concentration was 1:2 and 7 doubling dilutions were made.

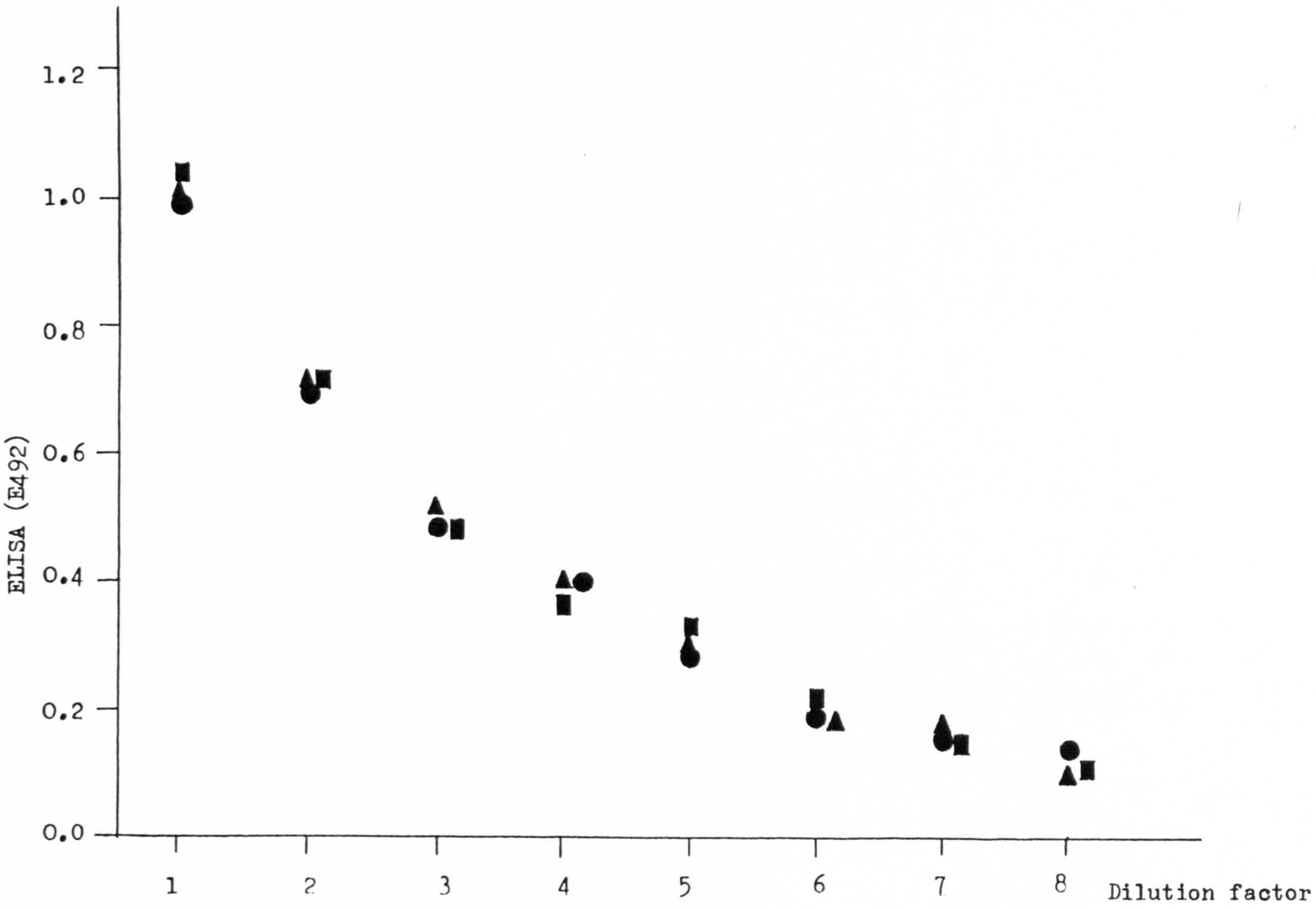

1.2
1.0
0.8
0.6
0.4
0.2
0.0
ELISA (E492)
1
2
3
4
5
6
7
8
Dilution factor

Fig. 3.4.

ELISA titration curves of peritoneal fluids of various antibody titres against worm and egg antigens

a) Assay condition for worm antigen

Worm antigen: The coating concentration was 11 µg/ml.

Peritoneal fluids: Fluid samples were collected from 1 normal mouse (○) and mice infected with 50 cercariae for 5 (▲) and 19 (●) weeks. The starting concentration was 1:2 dilution, and 7 doubling dilutions were made.

Peroxidase conjugate: 1:1000 dilution was used.

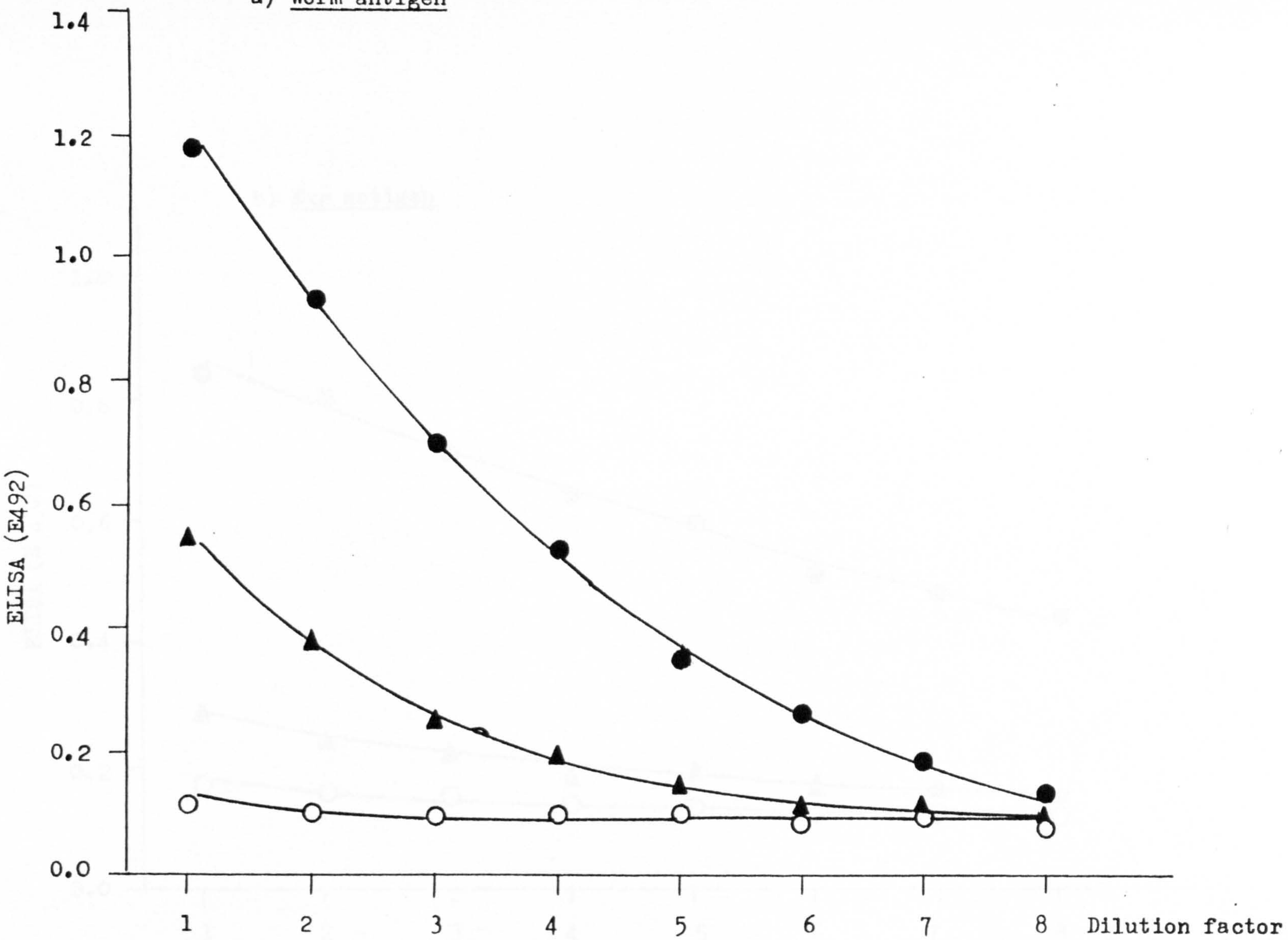
b) Assay condition for egg antigen

Egg antigen: The coating concentration was 2 µg/ml.

Peritoneal fluids: Fluid samples were collected from 1 normal mouse (○) and mice infected with 50 cercariae for 7 (▲) and 14 (●) weeks. The starting concentration was 1:2 dilution, and 7 doubling dilutions were made.

Peroxidase conjugate: 1:1000 dilution was used.

a) Worm antigen



b) Egg antigen

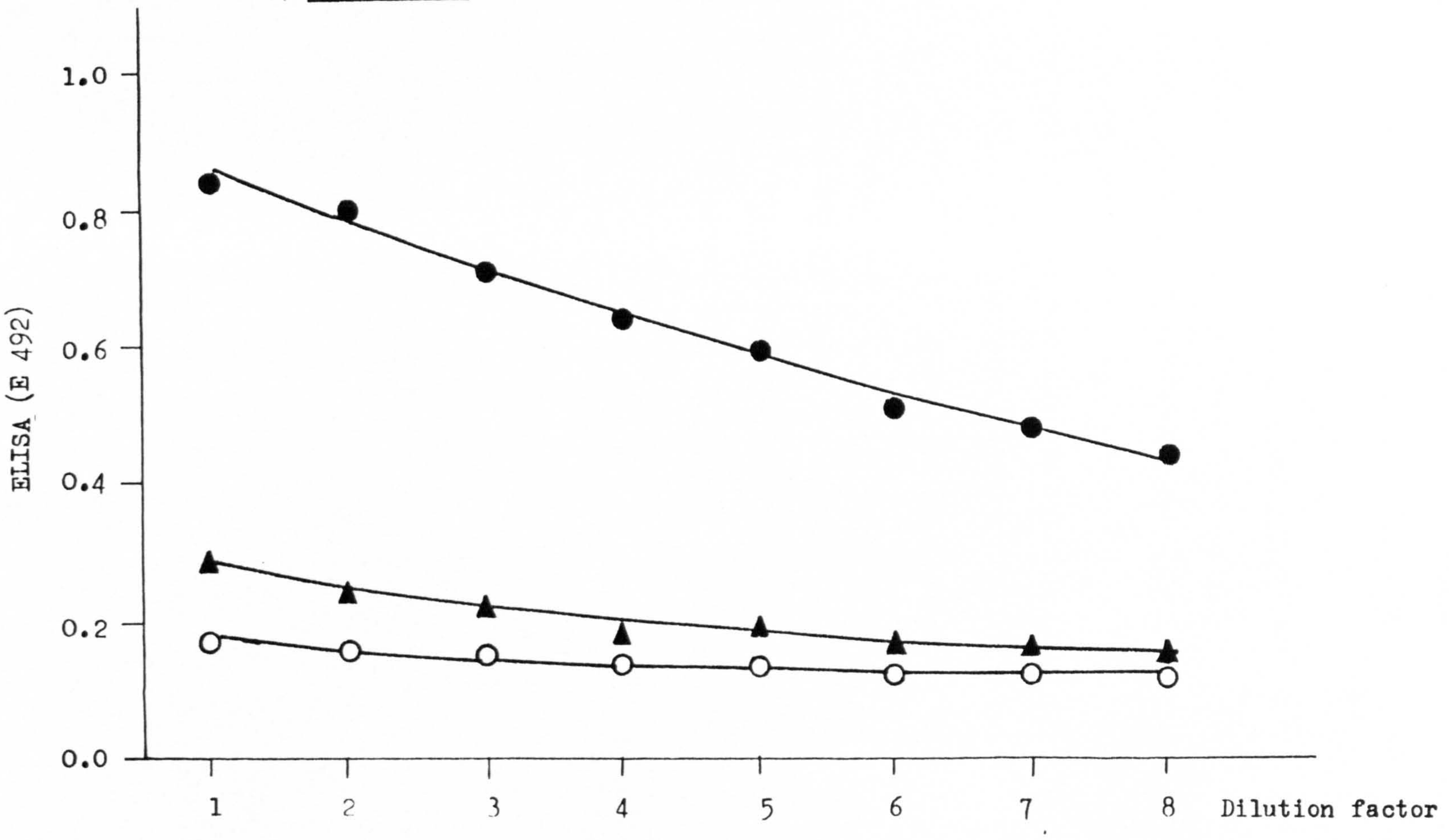


Fig. 3.5.

ELISA titration curves of sera of various antibody titres against worm and egg antigens

a) Assay condition for worm antigen

Worm antigen: The coating concentration was 11 µg/ml

Serum: Samples were collected from 1 normal (○) and mice infected with 50 cercariae for 7 (▲) and 19 (●) weeks. The starting concentration was 1:2 dilution, and 7 doubling dilutions were made.

Peroxidase conjugate: 1:1000 dilution was used

b) Assay condition for egg antigen

Egg antigen: The coating concentration was 2 ug/ml.

Serum: Samples were collected from 1 normal (○) and mice infected with 50 cercariae for 7 (▲) and 11 (●) weeks. The starting concentration was 1:2 dilution, and 7 doubling dilutions were made.

Peroxidase conjugate: 1:1000 dilution was used.

a) Worm antigen

ELISA (E 492)

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

1 2 3 4 5 6 7 8 9 10 11 12 Dilution factor

b) Egg antigen

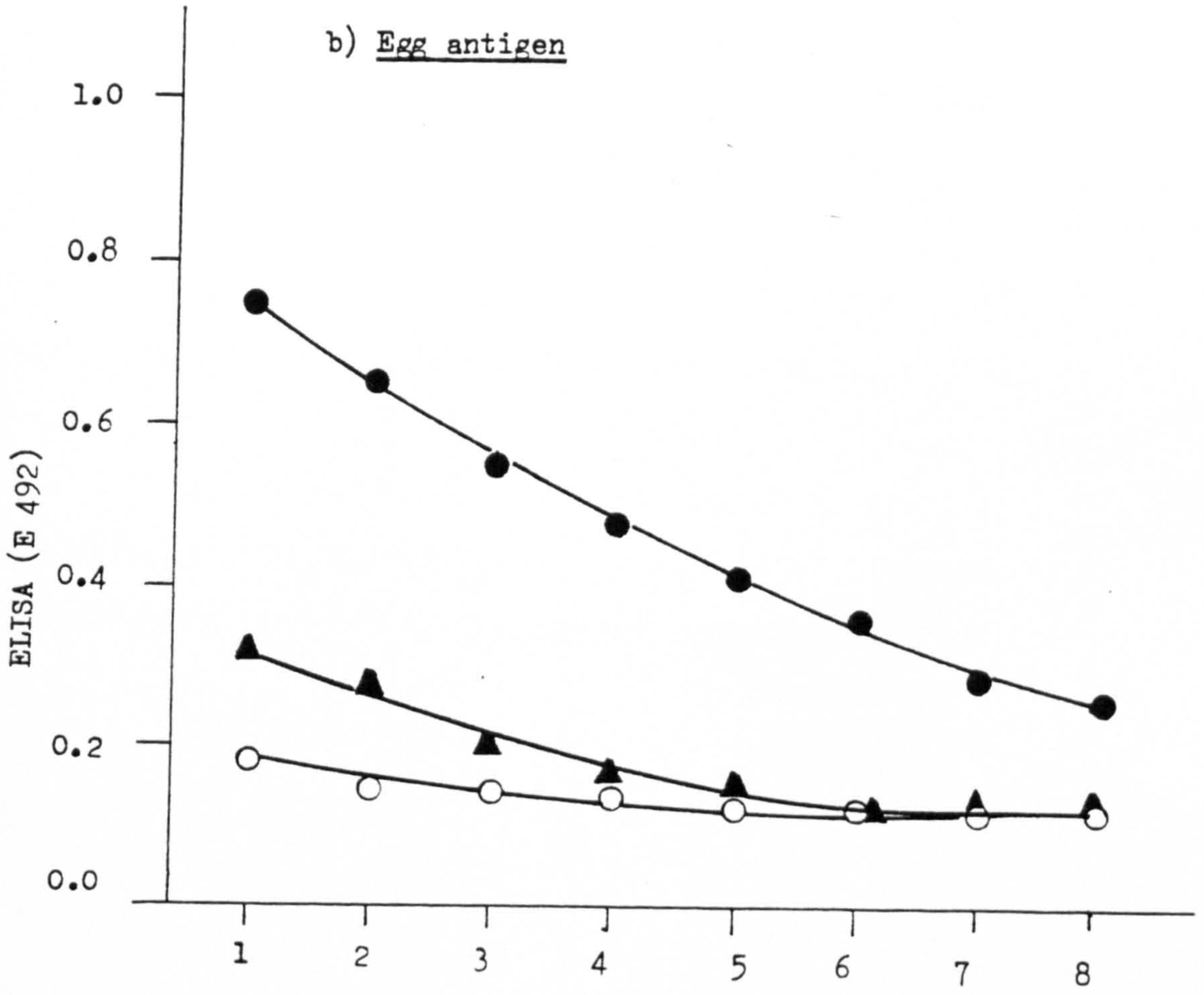


Fig. 3.6.

Distribution of S. mansoni ELISA 'negative' values of peritoneal fluids and sera collected from 28 normal mice

Assay conditions

Coating concentrations of antigens: (a) Worm antigen: 11µg/ml

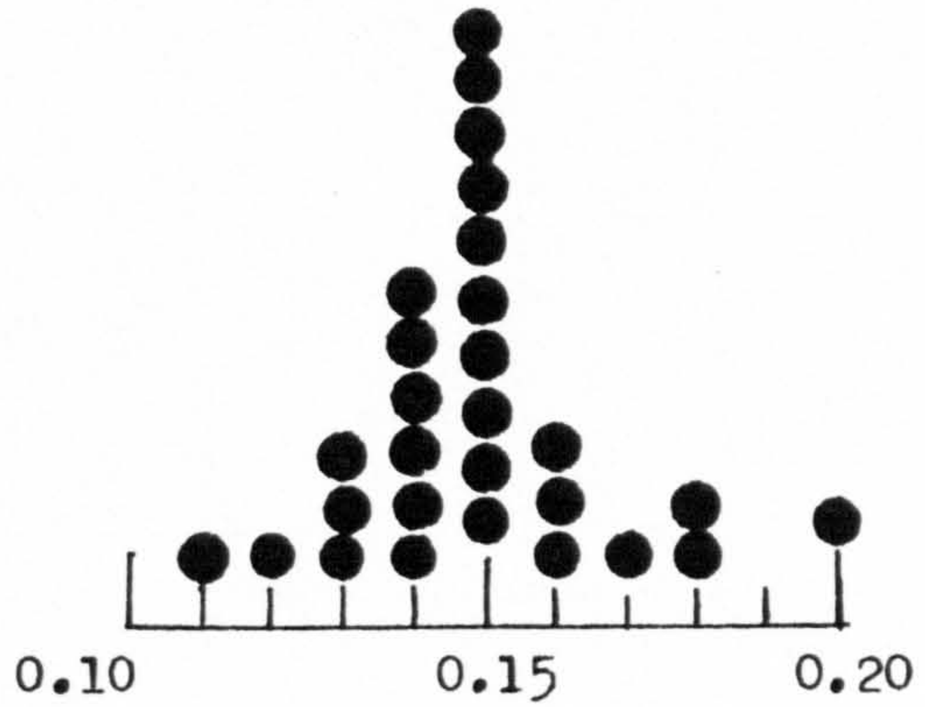
(b) Egg antigen: 2 µg/ml

Dilutions of tested fluids: Peritoneal fluids tested at 1:2 dilution and sera at 1:300 dilution.

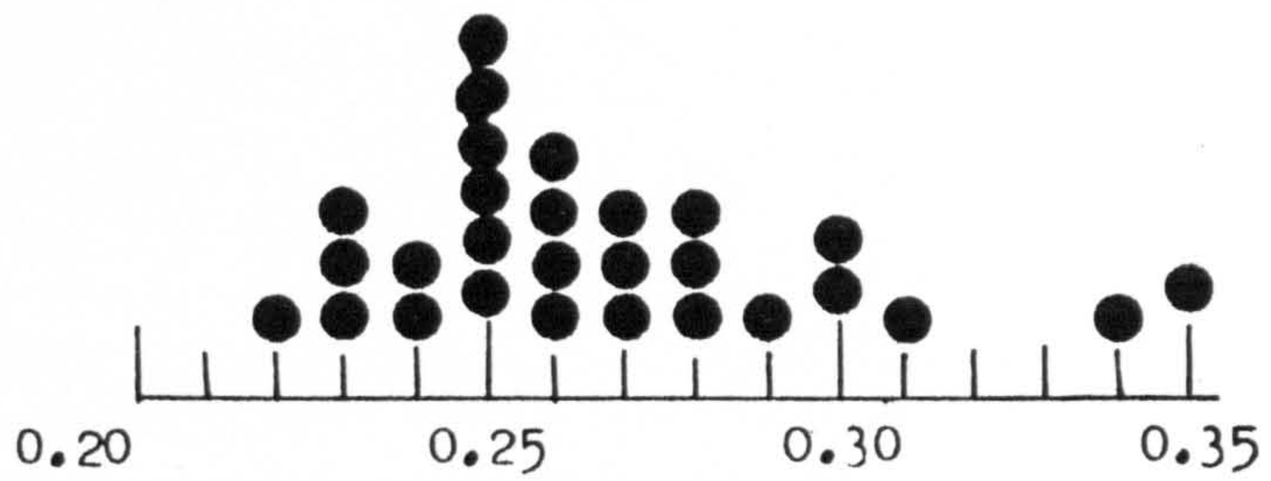
Peroxidase conjugate concentration: 1:1000 dilution was used.

a) Worm antigen

Peritoneal fluid

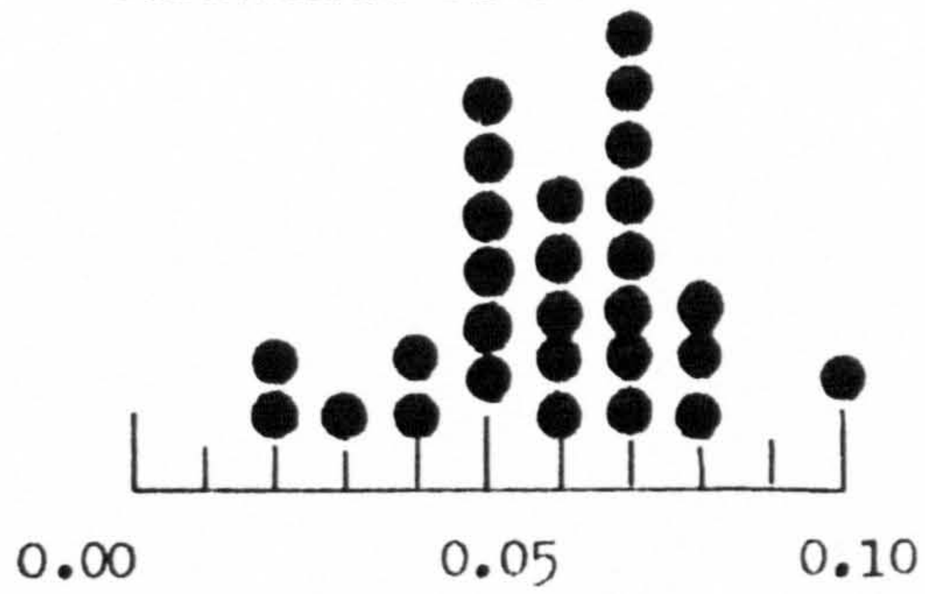


Serum



b) Egg antigen

Peritoneal fluid



Serum

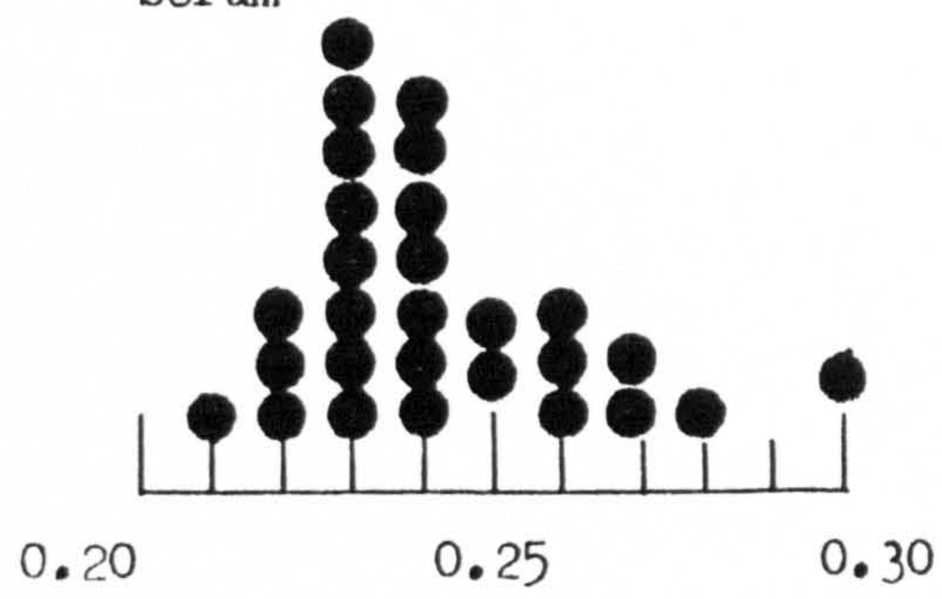


Fig. 3.7.

ELISA titration curves of peritoneal fluids and sera against worm antigen

Assay condition

Peritoneal fluid and serum:

Samples were collected from mice infected with 50 cercariae for 12 weeks. Starting concentration for peritoneal fluids and sera were 1:2 and 1:160 dilutions, respectively. Seven doubling dilutions were made.

Worm antigen:

The coating concentration was 11 µg/ml.

Peroxidase conjugate:

1:1000 dilution was used.

The open symbols (○ , △ , □) show the ELISA values given by peritoneal fluids and the closed symbols (● , ▲ , ■) by sera when titrated against worm antigen.

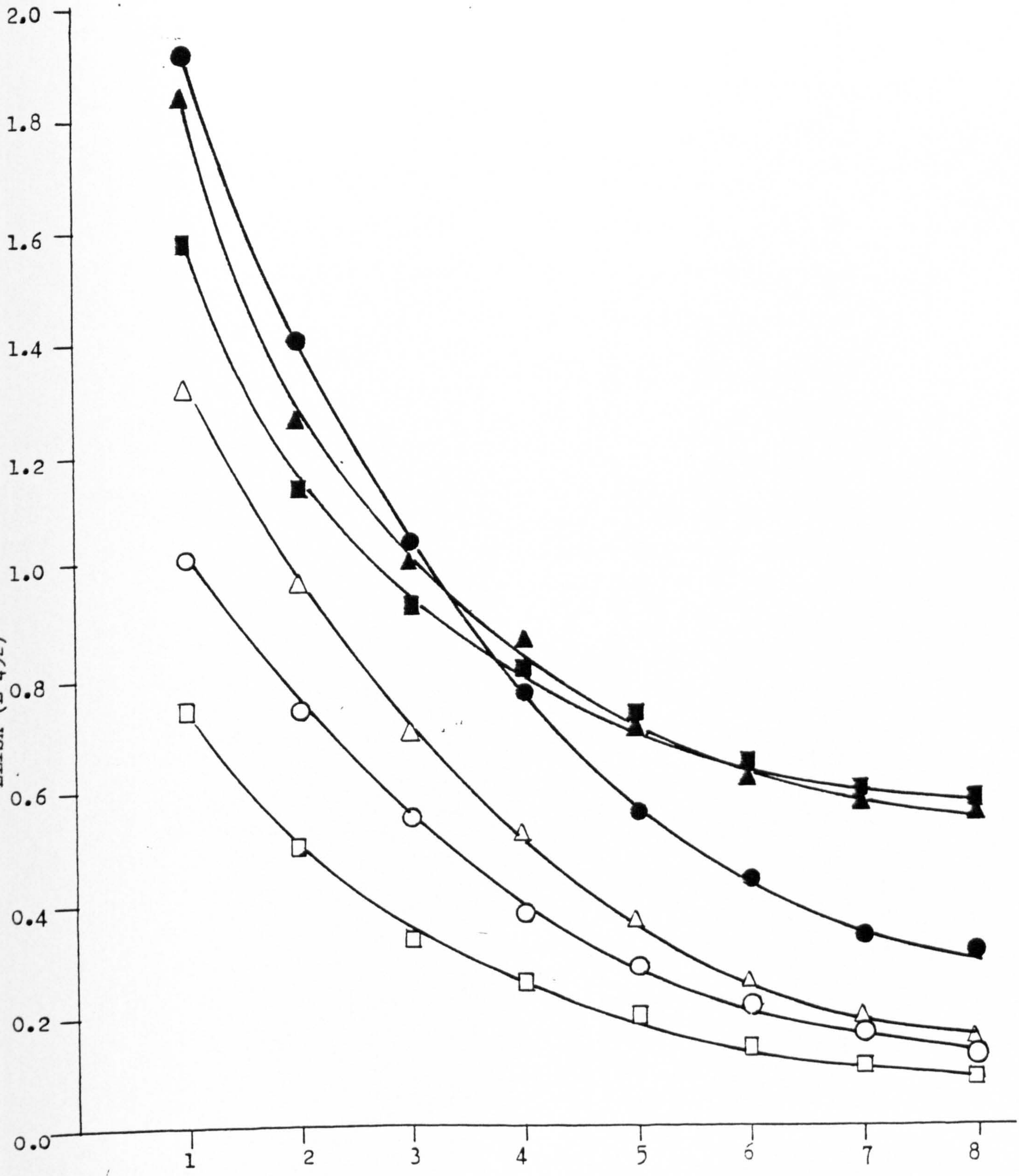

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ELISA (E 492)
1
2
3
4
5
6
7
8
Dilution factor

Fig. 3.8.

ELISA titration curves of peritoneal fluids against worm and egg antigens

Assay condition

Peritoneal fluids:

Samples were collected from mice infected with 50 cercariae for 9 (△ , ▲) and 11 (○ , ●) weeks. The starting concentration for titration was 1:2 dilution of peritoneal fluids. Seven doubling dilutions were made.

Coating concentrations of antigens:

Worm antigen: 11 µg/ml

Egg antigen: 2 µg/ml

Peroxidase conjugate:

1:1000 dilution was used.

The open symbols (△ , ○) show the ELISA values given by titration against worm antigen, and the closed symbols (▲ , ●) against egg antigen. On the abscissa, the Log_2 of the dilution factors of peritoneal fluids. On the ordinate, the ELISA values which had been converted to Log_2 after multiplication by 100.

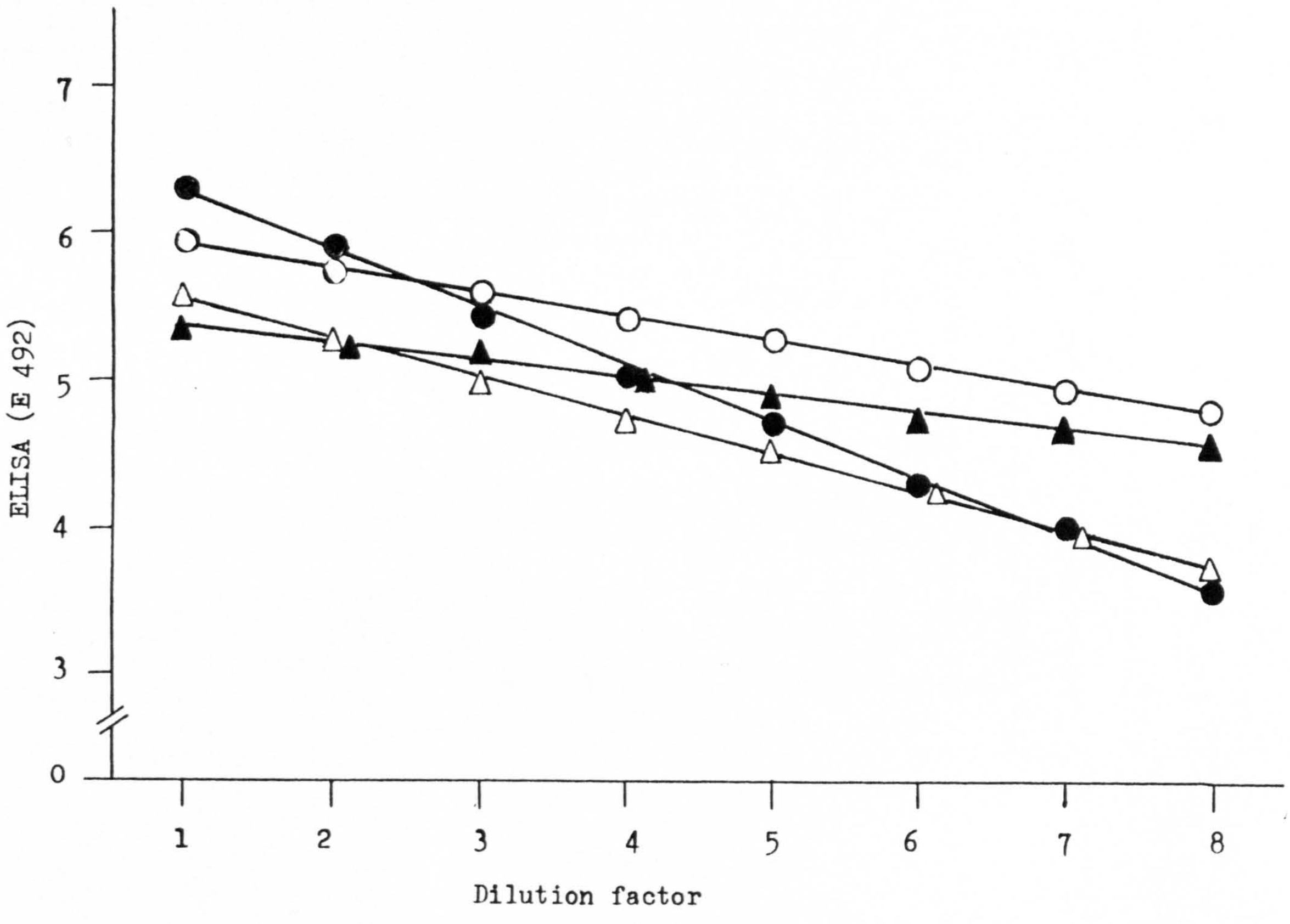

7
6
5
4
3
0
ELISA (E 492)
1
2
3
4
5
6
7
8
Dilution factor

Fig. 3.9 .

Kinetics of specific IgG antibodies to S. mansoni worm antigen in peritoneal fluids and sera during the course of infection

At every two weeks, groups of mice were killed, the peritoneal fluids were lavaged by injection of PBS/heparin, and the sera were also collected by cardiac puncture. The specific IgG activity to S. mansoni worm antigen was estimated by ELISA using the assay conditions described below. Open symbols (○) show the ELISA values in the peritoneal fluids, and closed symbols (●) the sera . Each point is the mean of the values and the vertical bar its standard deviation. Numbers in parentheses refer to the mice in each group. The dashed (--) and solid (——) lines indicate the positive/negative discrimination levels determined for the peritoneal fluids and serum, respectively.

Assay condition

Peritoneal fluids: tested at 1:2 dilution

Sera : tested at 1:300 dilution

Worm antigen: coating concentration at 11 µg/ml

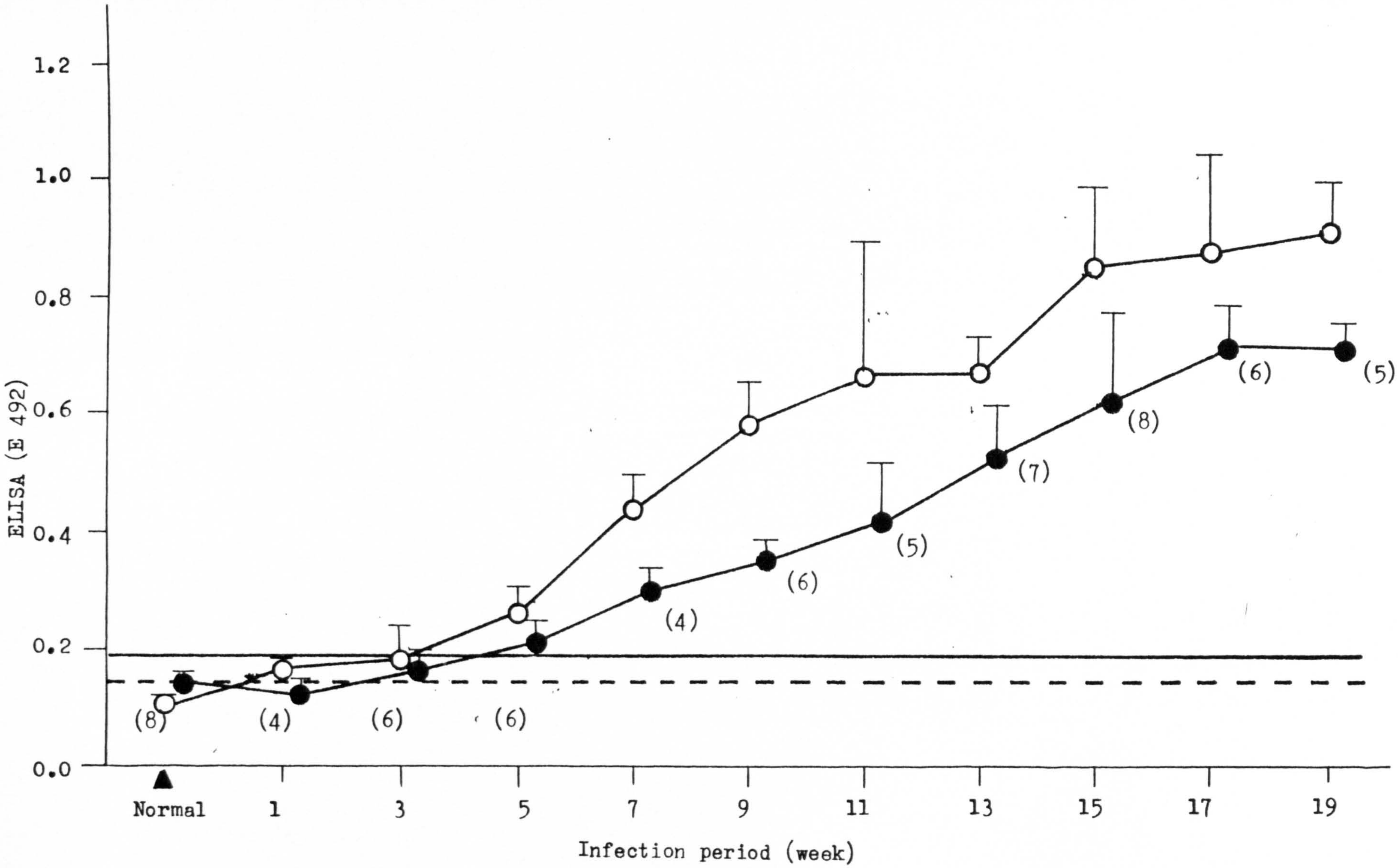

1.2
1.0
0.8
0.6
0.4
0.2
0.0
ELISA (E 492)
(8)
(4)
(6)
(6)
(4)
(6)
(5)
(7)
(8)
(6)
(5)
Normal
1
3
5
7
9
11
13
15
17
19
Infection period (week)

Fig. 3.10.

Kinetics of specific IgG antibodies to S. mansoni egg antigen in peritoneal fluids and sera during the course of infection

Samples and symbols are as described in Fig. 3.9.

Assay condition

Peritoneal fluids: tested at 1:2 dilution

Sera :tested at 1:300 dilution

Egg antigen: coating concentration at 2 µg/ml

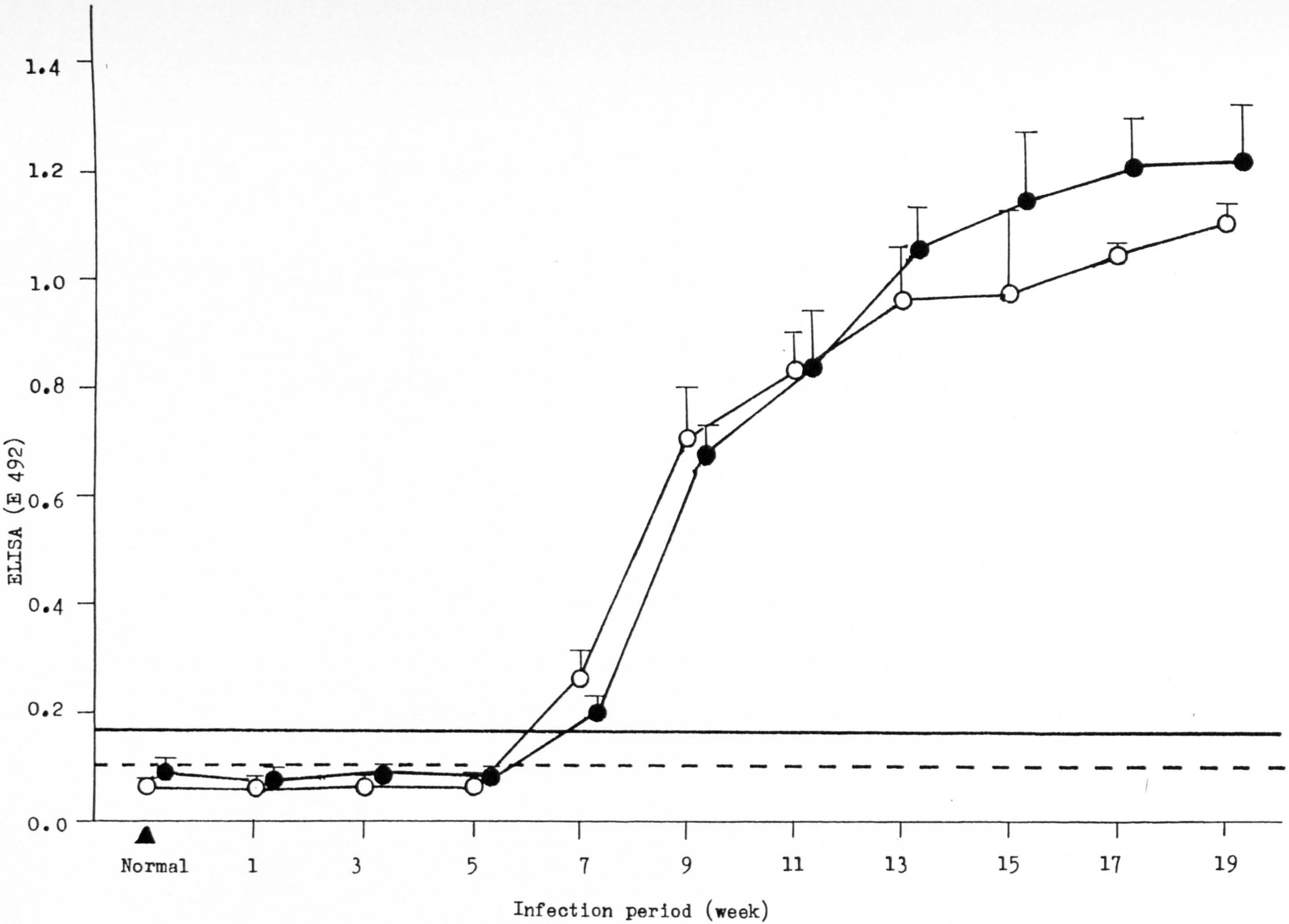

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ELISA (E 492)
Normal
1
3
5
7
9
11
13
15
17
19
Infection period (week)

Fig. 3.11.

Comparison of ELISA values against worm antigen in the peritoneal fluids of normal controls with those of mice infected for 3 weeks

Assay condition

Peritoneal fluid and serum

Samples were collected from normal mice and mice infected with 50 cercariae for 3 weeks. Samples were tested at 1:2 and 1:300 dilutions for peritoneal fluids and sera , respectively.

Worm antigen: coating concentration at 11 μg/ml

Open symbols (○) show the ELISA values in the normal and closed symbols (●) in the infected fluids. Each point was individual value. Numbers in parentheses refer to the number of mice used in each group. The dashed lines indicate the positive/negative discrimination level determined for peritoneal fluids and sera.

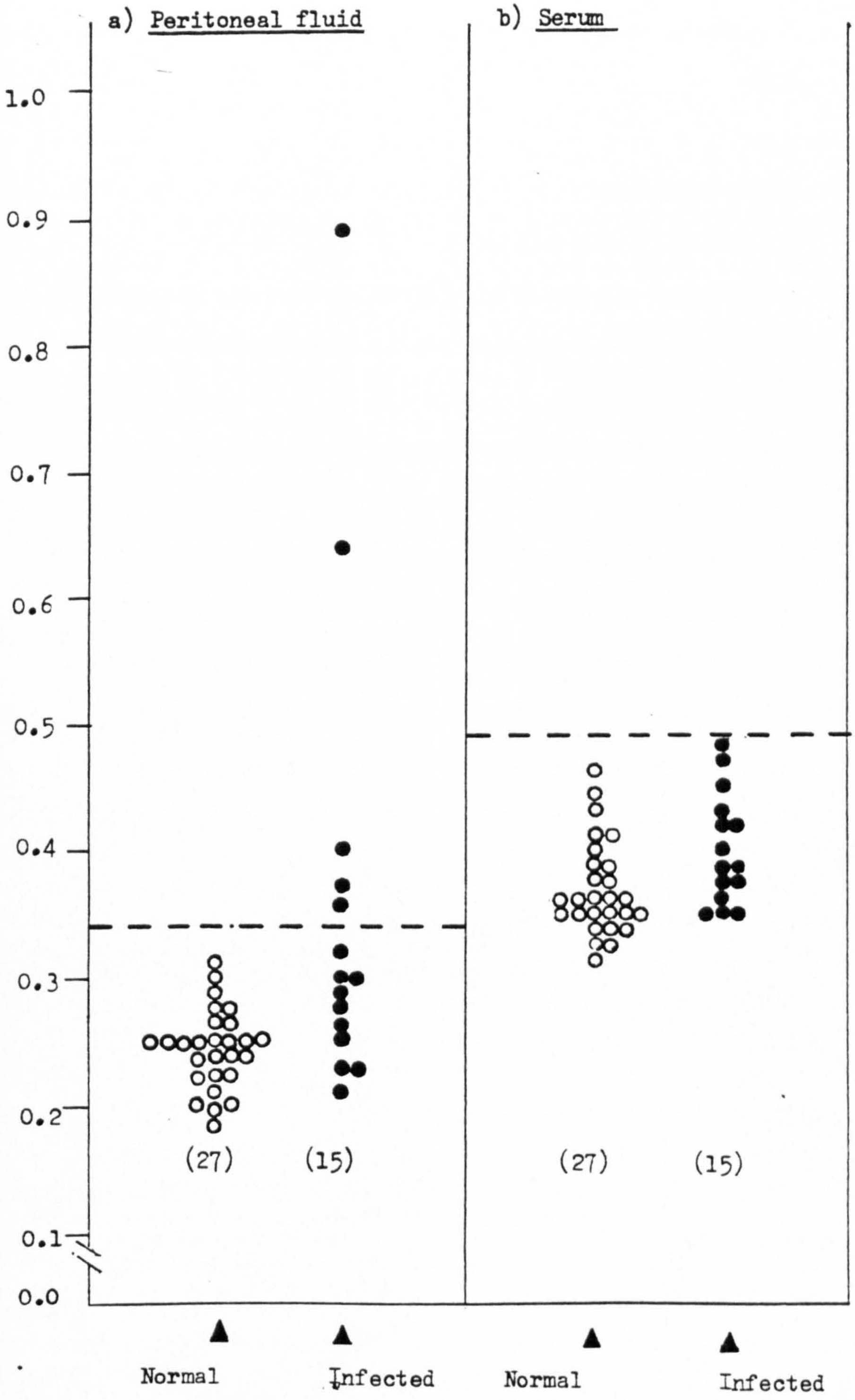

a) Peritoneal fluid
b) Serum
ELISA (E 492)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
(27)
(15)
(27)
(15)
Normal
Infected
Normal
Infected

Fig. 3.12.

Comparison of ELISA values against worm and egg antigens in peritoneal fluids and sera of normal and S. mansoni infected mice

Assay condition :

Peritoneal fluids and sera

Samples were collected from normal mice and mice infected with 50 cercariae for periods ranging from 10 to 19 weeks. Samples were tested at 1:2 and 1:300 dilutions for peritoneal fluids and sera, respectively.

Worm antigen: coating concentration at 11 µg/ml

Egg antigen: coating concentration at 2 µg/ml

Open symbols (○) show the ELISA values in the normal and closed symbols (●) in the infected fluids. Each point was individual value. Numbers in parentheses refer to the numbers used in each group.

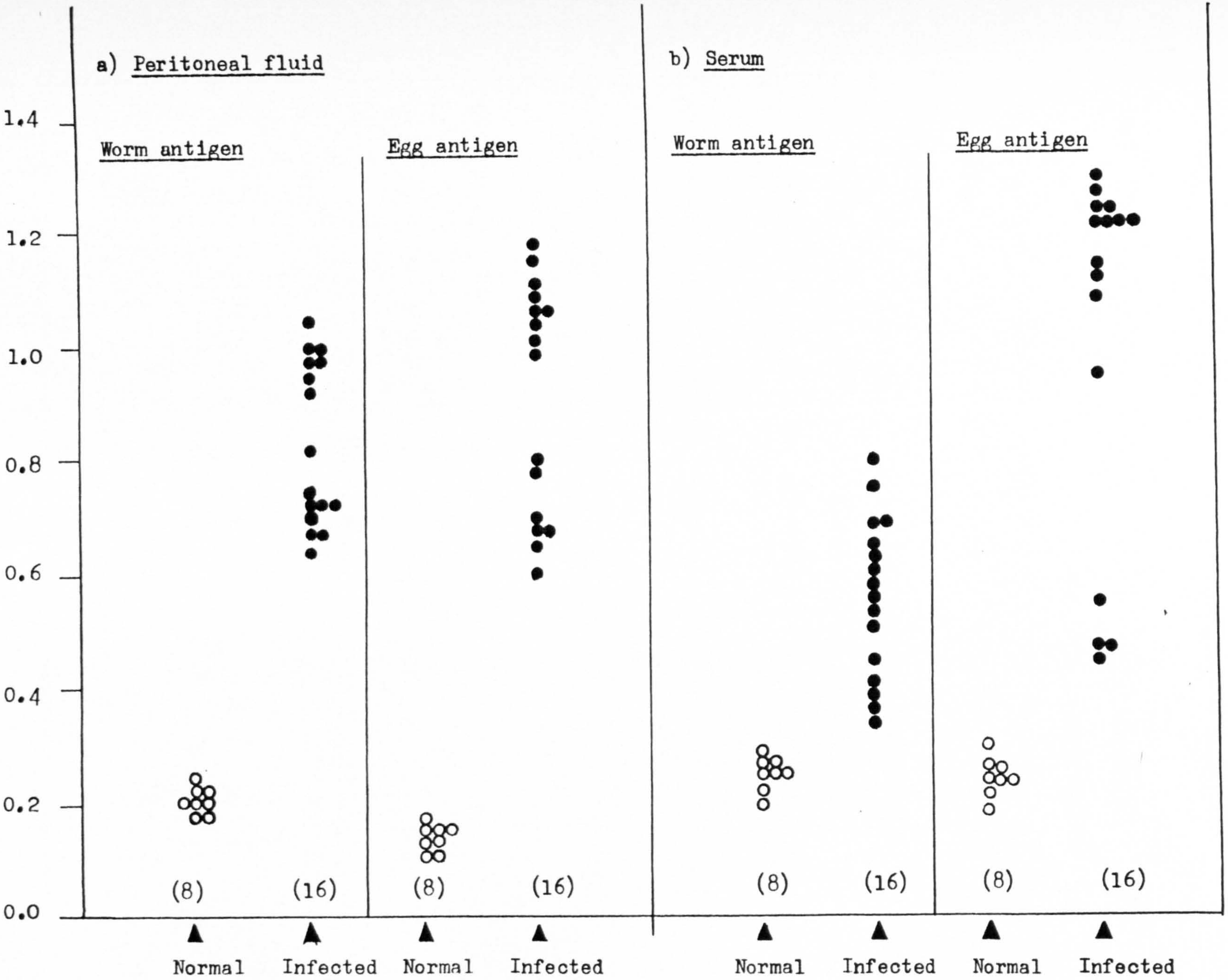

a) Peritoneal fluid
b) Serum
Worm antigen
Egg antigen
Worm antigen
Egg antigen
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
(8)
(16)
(8)
(16)
(8)
(16)
(8)
(16)
Normal
Infected
Normal
Infected
Normal
Infected
Normal
Infected

Fig. 3.13.

Relationship between antibody titres against worm and egg antigens

Assay condition

Peritoneal fluids and sera

Samples were collected from normal mice and mice infected with 50 cercariae for periods ranging from 5 to 19 weeks. Samples were tested at 1:2 and 1:300 dilutions for peritoneal fluids and sera , respectively.

Worm antigen: coating concentration at 11 µg/ml

Egg antigen: coating concentration at 2 µg/ml

Each point (●) represents the value from an infected mouse without regard to the week it was obtained. Eight normal mice were used and the mean ± S.D. (◔) of the negative ELISA values is presented.

a) Worm antigen

b) Egg antigen

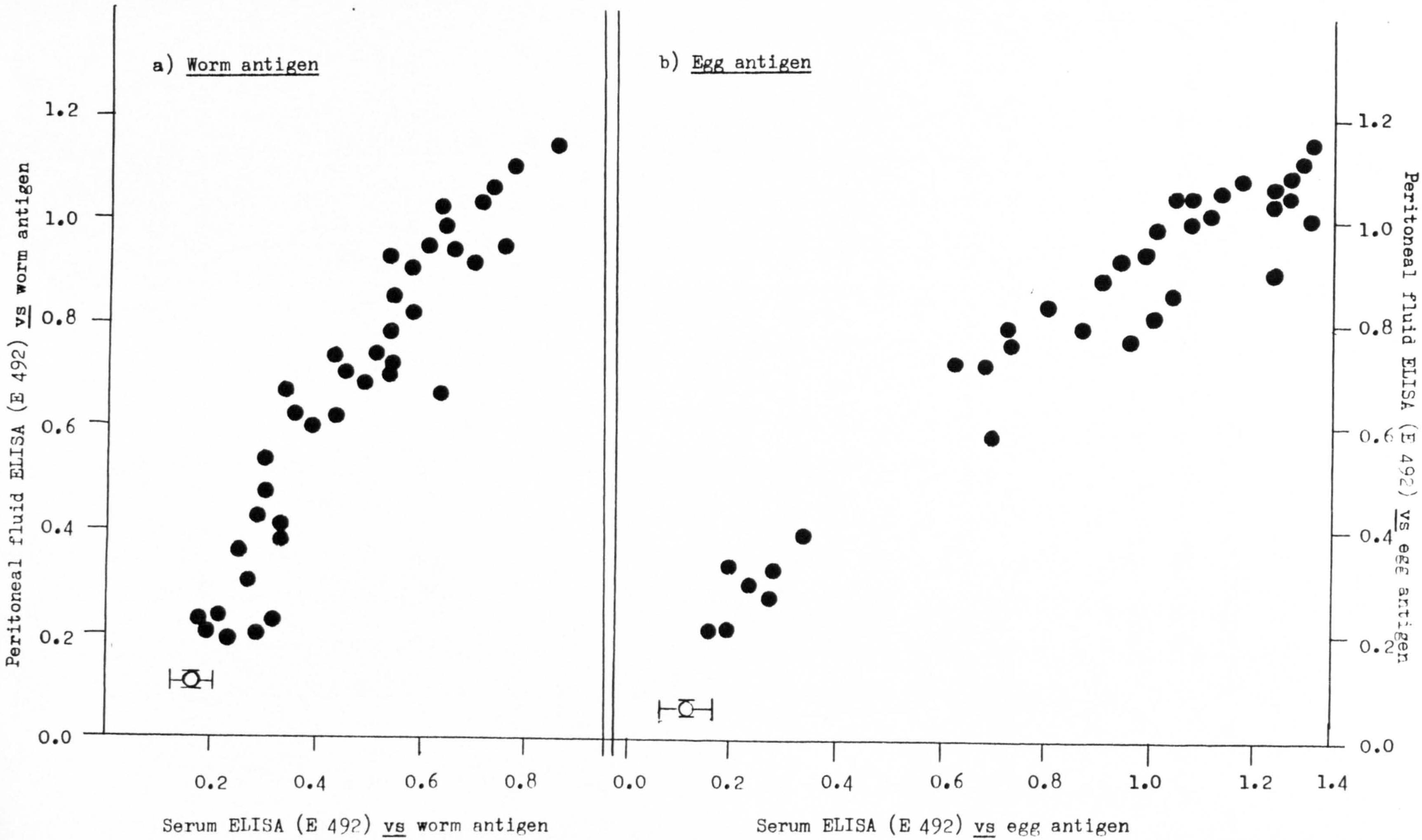


Fig. 3.14.

Relationship between antibody titres against worm and egg antigens in peritoneal fluids

Samples and symbols are as described in Fig. 3.13.

Assay condition

Peritoneal fluids: tested at 1:2 dilution

Worm antigen: coating concentration at 11 µg/ml

Egg antigen: coating concentration at 2 µg/ml

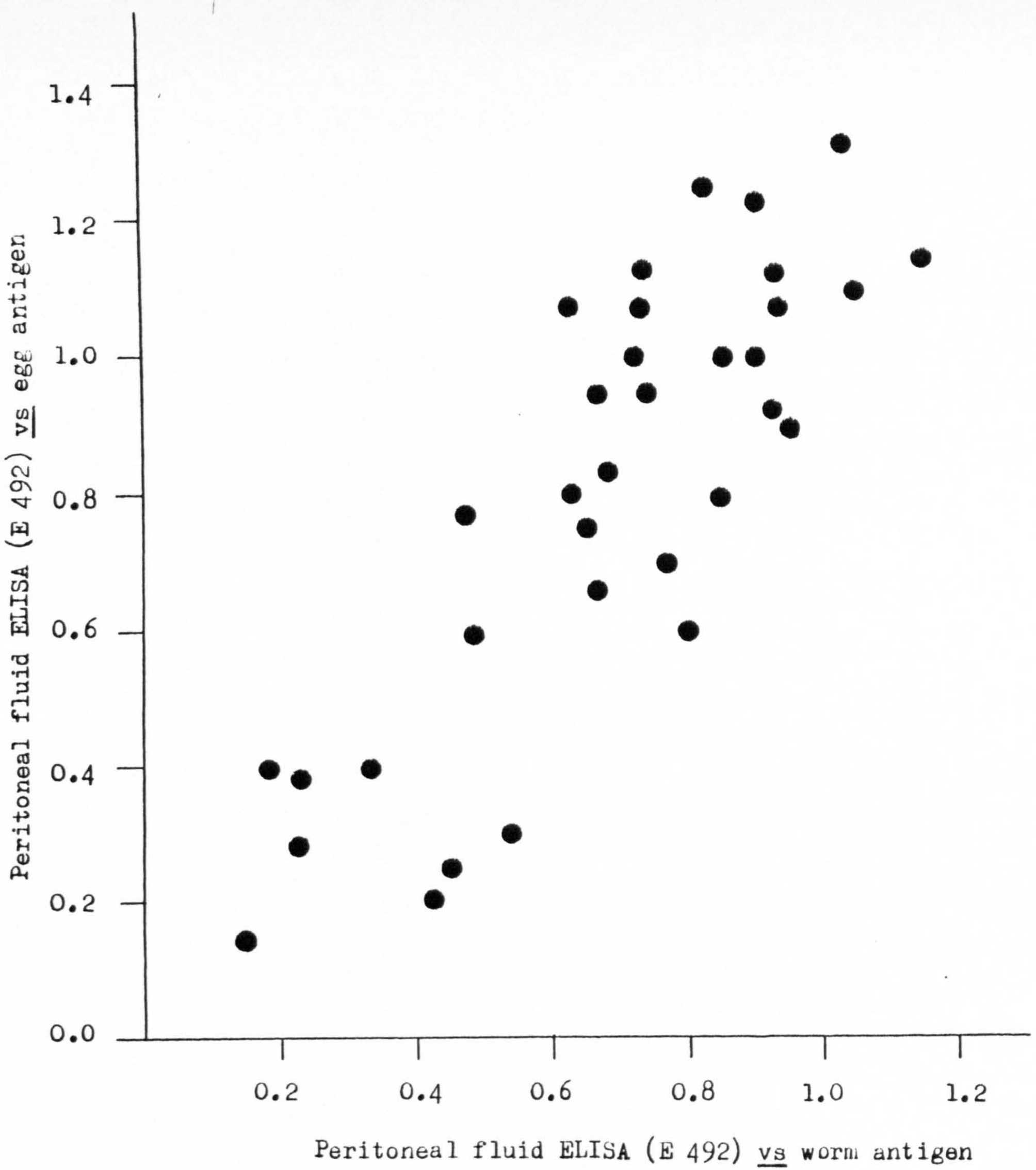

Peritoneal fluid ELISA (E 492) vs egg antigen
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0.2
0.4
0.6
0.8
1.0
1.2
Peritoneal fluid ELISA (E 492) vs worm antigen

Fig. 3.15.

Effect of cercarial exposure dose on the response of anti-S. mansoni IgG in peritoneal fluids

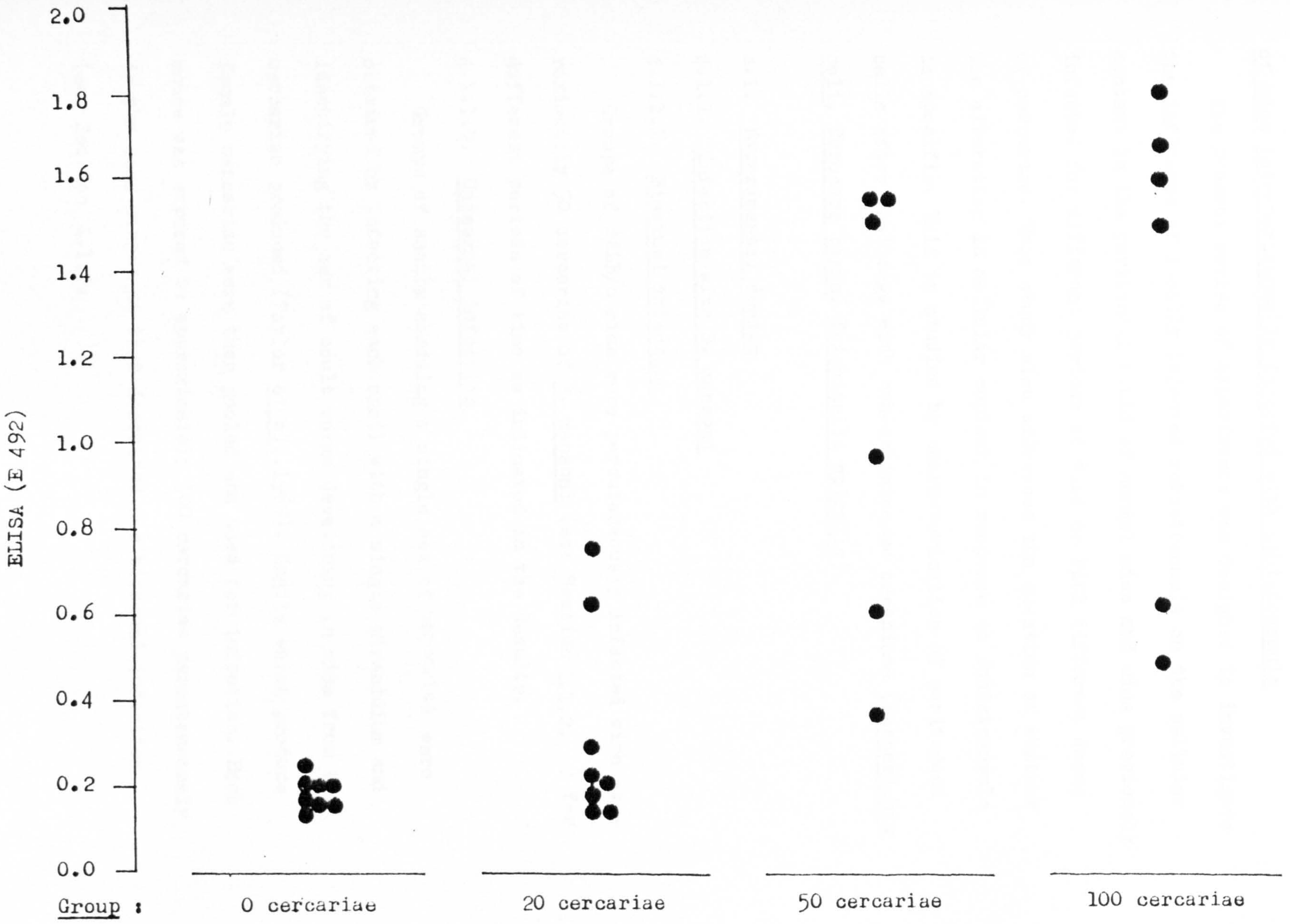
Assay condition

Peritoneal fluids:

Samples were collected from three groups of mice infected with 20, 50 and 100 cercariae each mouse for 7 weeks. Normal fluids were also collected in the same manner. Peritoneal fluids were tested at 1:8 dilution.

Worm antigen: coating concentration at 11 µg/ml

Each point was individual value. The dashed lines indicate the positive/negative discrimination level determined from ELISA values obtained from the uninfected group.


ELISA (E 492)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Group :
0 cercariae
20 cercariae
50 cercariae
100 cercariae

4. Quantitative and qualitative analysis of peritoneal cells of mice intraperitoneally injected with schistosomula

The present series of experiments was designed to investigate the influence of locally injected schistosomula on the cellular content in the peritoneal fluid of normal mice and mice previously infected for different periods of time or with different doses of cercariae. This study also addresses the question of whether the alteration in cellular content in response to schistosomula is specific. This is studied by characterization of peritoneal cells after challenge with non-schistosome organisms (Escherichia coli, Toxocara canis, Trichinella spiralis).

4.1. Experimental design

4.1.1. Infection with S. mansoni

4.1.1.1. Bisexual infection

Groups of BALB/c mice were percutaneously infected wirh approximately 50 cercariae of S. mansoni (see Section 1.1.2.) for different periods of time as indicated in the Results.

4.1.1.2. Unisexual infection

Groups of snails shedding a single sex of cercariae were obtained by infecting each snail with a single miracidium and identifying the sex of adult worms developing in mice from the cercariae produced (Taylor et al.,1969). Snails which produce female cercariae were then pooled and used for infection. Each mouse was exposed to approximately 100 cercariae percutaneously in the same manner as that described for bisexual infection (see Section 1.1.2.).

4.1.2. Preparation of parasite/organism for intraperitoneal challenge

4.1.2.1. Schistosomula of S. mansoni (living larvae)

Schistosomula were prepared by the mechanical transformation method described by Ramalho-Pinto et al (1974). A pooled cercarial suspension was cooled on ice for 20 min to reduce motility of the organisms. The supernatant was decanted and the sedimented cercariae were resuspended in 10 ml of Eagle's medium (see Section 4.1.3.) prewarmed at 37°C. Cercariae were transformed into schistosomula by passing through a 21G x 1½ " needle fitted to a 10 ml syringe five or six times. The transformed schistosomula were then allowed to sediment at 1 x G. The tail-rich supernatant was decanted and the sedimented bodies were washed with three changes of sterile medium and sedimentation. The resultant mechanically transformed schistosomular suspension was then adjusted to the desired concentration for peritoneal challenge experiments and used within 2 hr after transformation. The schistosomular preparation usually contained 2-4 % broken tails.

4.1.2.2. Escherichia coli (heat-killed organisms)

E. coli (strain NCTC 8623, Dept. of Microbiology, Univ. of Glasgow) were grown in Tryptose phosphate broth(DIFCO Labs., Detroit U.S.A.) overnight at 37°C. After determining the number of bacteria by pour-plate colony counts, the organisms were washed in three changes of saline. The suspension was placed in a boiling-water bath for 5 min, cooled and centrifuged. The bacteria were resuspended in Eagle's medium (see Section 4.1.3.) to a desired concentration for intraperitoneal challenge experiments.

4.1.2.3. T. spiralis (living larvae)

Mice were orally infected with 450 larvae of T. spiralis. After one month, mice were killed by cervical dislocation, skinned and eviscerated. The carcasses were then homogenized 1 min by means of a blender in digestive fluid (1 l per mouse) containing 0.85% (w/v) NaCl, 0.5% (v/v) HCl and 0.5% (w/v) pepsin (BDH Chemicals Ltd., Dorset). The homogenate was then incubated at 38°C for 2½ hr. The undigested sediment was filtered off on a coarse sieve of mesh size 250 µm and the rest was allowed to stand at 20°C in a graduated cylinder. The larvae were then washed three times and resuspended in Eagle's medium (see Section 4.1.3.) to a desired concentration for intraperitoneal challenge experiments.

4.1.2.4. T. canis (living larvae)

Adult female worms were obtained from infected dogs at autopsy. The eggs were dissected from the uterus, pooled and stored in 4% formalin at 20°C. After one month, the eggs were washed free of formalin by centrifugation in PBS, and then decoated in 6% sodium hypochlorite solution for 20 min at 39°C. Decoated eggs were washed in PBS and then CO_2 was bubbled through the suspension (5 Cu.ft/h) at 39°C until the larvae hatched (15-20 min). The larvae were maintained in culture in Eagle's essential medium with Hank's salt (Grand Island Biological Co., New York, U.S.A.). After 5 weeks, the larvae were washed and resuspended in Eagle's medium to a desired concentration for intraperitoneal challenge experiments.

4.1.3. Preparation of Eagle's medium for suspending challenge organisms

Glasgow modification of Eagle's essential medium (Flow Lab., Irvine)	12.5 g
$NaHCO_3$	2.5 g
phenol red	0.1 ml
Dist. H_2O	1000 ml

4.1.4. Protocol of intraperitoneal challenge experiments

Four groups of mice were generally used in each experiment. A protocol of the experiment is summarized in Table 4.1. Group A were given the primary infection and intraperitoneal challenge, group B were given a primary infection but challenged with suspending medium only. Group C were normal mice given the challenge and group D were challenged with medium. Primary infection was percutaneous with the indicated number of cercariae. In the challenge experiments, two systems, homologous and heterologous, were adopted by intraperitoneal injection with either schistosomula or other organisms unrelated to schistosome infection (*E. coli*, *T. canis* and *T. spiralis*), respectively. In each experiment, the solution used to wash and dilute challenge organisms to a determined working concentration prior to injection were Eagle's medium (Section 4.1.3.). The challenge was performed using a 18G x 1½ " needle and the volume of injection was adjusted to 0.2 ml. At indicated time after challenge, mice were killed and the cell-rich peritoneal fluids were lavaged by injection of 2 ml of PBS (see Section 1.1.3.). The cellular constituents of the lavaged samples were determined by examining the May-Grunwald-Giemsa stained cytocentrifuged cell smears (see Section 1.1.4.2.).

4.1.5. Statistics and calculations

The results in this thesis were generally expressed as the mean and standard deviation for each experimental group. Except where specifically indicated the data of following paired experimental groups were compared and the level of significance between paired groups was assessed by the Student's t-test (Table 4.2). The Student's t-test was performed using computer program 2976 provided by the Computer Service Center, Univ. of Glasgow. Values of $P < 0.05$ were considered to be significant.

Table 4.2.

Table 4.2. Paired experimental groups for statistics

	Primary infection	Intraperitoneal challenge	
Paired group	+	+	t-test
	+	-	
Paired group	-	+	t-test
	-	-	

Symbols: +, -; see Table 4.1.

4.2. Results

4.2.1. The identity of cells present in the peritoneal fluid at intervals after intraperitoneal inoculation of schistosomula

4.2.1.1 Early stage of cellular responses (within 24 hr after challenge) in normal and infected mice

Cellular content of the peritoneal fluid was determined before (0 hr) and at various intervals (30 min, and 2, 6 and

24 hr) after intraperitoneal challenge with 1,000 mechanically transformed schistosomula in normal and mice infected with 50 cercariae for 11 or 16 weeks. The intraperitoneal inoculation of schistosomula induced a marked alteration in the peritoneal cell composition (Fig. 4.1a; Table 4.3.).

In normal mice, the population of the peritoneal cells consisted mainly of lymphocytes, in addition to 18-25 % macrophages, 0.5-4% eosinophils and 1-5 % mast cells. Neutrophils were rarely seen. The appearance of neutrophils, at 30 min after inoculation of schistosomula, was the first sign of peritoneal inflammation. The number increased rapidly and reached its peak at 6 hr. The magnitude of increase in neutrophil numbers varied from experiment to experiment, and among individuals within the same experimental group. The neutrophil numbers subsequently declined but still remained elevated above the medium-induced level after 24 hr. The percentages of macrophages dropped temporarily at 30 min and rose to near prechallenge level after 24 hr. No significant change was observed in the number of eosinophils over the 24 hrs of the experiments.

In the schistosome infected mice, the residential peritoneal cell content differed from those of normal controls in having elevated eosinophil and decreased mast cell levels (see Section 1.2.2.). As observed in challenged normal mice, neutrophils made their appearance within 30 min and reached a peak after 6 hr, and decreased rapidly to the medium-induced level at 24 hr. An initial 'lag' in the macrophage level at 30 min was also observed. However, this was followed by steady increase and

eventually reached significantly higher than medium-induced level after 24 hr. This increase appeared to depend on the immune status of the tested mice. The proportion of eosinophils remained unchanged in the first 6 hr but subsequently increased compared to that of prechallenge levels by 24 hr. The number of mast cells did not appear to differ from their respective prechallenge levels in either normal or infected mice.

4.2.1.2. Late stage of cellular responses (1-4 days after challenge) in normal and infected mice

The later stage of the cellular reactivity in response to intraperitoneally injected schistosomula was also investigated using normal mice and mice previously infected for 10 weeks. Groups of mice were sacrificed 1, 2 and 4 days after challenge and the peritoneal cellular contents were examined (Fig. 4.1.b.)

In infected mice, increases in the proportion of macrophages and eosinophils were detected at 1 day which were consistent with the observations reported above (see Section 4.2.1.1.) However, the macrophage level declined by day 4 to prechallenge level. In contrast, the proportion of eosinophils remained significantly higher than that of prechallenge level. In normal mice, no significant alteration in the percentages of macrophages and eosinophils was observed from day 1 to day 4 after challenge. Elevation of neutrophil level was observed on day 1 which subsequently declined to medium-induced level by day 4 in both normal and infected mice.

4.2.2. Cellular responses induced by medium and by needle-trauma

Since the challenge schistosomula or other organisms were suspended in Eagle's essential medium (see Section 4.1.2.) the possible synergistic effect exerted by medium in the response was investigated. Medium alone (0.2 ml) was intraperitoneally injected into mice previously infected for 13 weeks. Mice injected with schistosomula were also set up at the same time. The peritoneal cell contents were examined at 2, 6, 12 and 24 hr after injection (Table 4.4.).

In mice injected with medium alone, no apparent change occurred in the percentages of macrophages, eosinophils and mast cells. However, the number of neutrophils increased and reached a peak at 6 hr, declining to near prechallenge level by 24 hr. In mice injected with schistosomula, increases in the proportions of macrophages and eosinophils occurred by 24 hr. As observed in mice injected with medium alone, an increase in the number of neutrophils was observed. However, the neutrophil numbers were invariably higher in the schistosomula-injected mice compared to those injected with medium alone when examined at its peak at 6 hr after inoculation of either agent.

The involvement of needle-trauma in the increase of neutrophil numbers could not be excluded, since elevation of neutrophil level was observed in some normal mice 24 hr after wounding by a needle of size 18 G x 1½" without administration of agent (Table 4.5.).

4.2.3. The effect of the size of the primary infection on cellular responses to peritoneal challenge

This series of experiments was designed to investigate the effect of the number of worms of the primary infection on the cellular response to intraperitoneal challenge with schistosomula. Three groups of mice were given a percutaneous infection with approximate 20, 50 or 100 cercariae. One portion of each group was intraperitoneally challenged with 1,000 schistosomula at 7 weeks after the primary infection. The period of 7 weeks was chosen, since the alteration in both cellular and humoral contents in the peritoneal fluids due to an infection with 50 cercariae was observed at this time (see Section 1.2.1. and 1.2.2.) The rest of each group were injected with medium and served as controls. At 24 hr after challenge, the peritoneal cell contents were examined. Fig. 4.2. depicts four aspects of cellular reactivity in response to intraperitoneal challenge; the total number of cells, percentages of eosinophils, neutrophils and mast cells.

4.2.3.1. Primary cellular responses to percutaneous infection with cercariae

When examined at 7 weeks after infection, all mice in the three groups exposed to varying numbers of cercariae were infected and the presence of adult worms in the portal system was confirmed by microscopical examination of squashed liver samples. However, their responses were dissimilar. Leukocytosis characterized by raised eosinophil percentage occurred in mice given larger cercarial doses (50-100 cercariae groups) but not in those

exposed to 20 cercariae. The eosinophilia was accompanied by a significant decrease in the percentages of mast cells. In mice infected with 20 cercariae, no significant change was observed in the proportion of mast cells compared to those in uninfected mice. Mild neutrophilia was seen in some of mice infected with 100 cercariae.

4.2.3.2. Secondary cellular responses to intraperitoneal injection of schistosomula

Neutrophilia occurred in all the mice injected with schistosomula compared with their medium-injected controls. The magnitude of neutrophilia triggered by injected schistosomula appeared to be unrelated to the intensity of primary infection. The mast cell levels were not significantly altered compared with their respective prechallenge levels. Only mice previously exposed to 50-100 cercariae mounted a secondary eosinophil response upon intraperitoneal challenge with schistosomula.

4.2.4. The effect of interval between primary infection and intraperitoneal challenge on cellular responses

This investigation used three groups of mice previously infected with 50 cercariae for 18, 13 and 6 weeks. They were simultaneously challenged with 1,000 schistosomula per mouse prepared from one pool of cercariae. Cellular responses were assessed 24 hr after challenge.(Table 4.6.).

It was shown that there was a relationship between the magnitude and types of reactive cells and the length of time

elapsing between the percutaneous infection and the intraperitoneal challenge. Mice previously infected for 13 to 18 weeks showed an increase in the percentages of both macrophages and eosinophils after challenge, whereas in mice of shorter primary infection period (6 weeks), only eosinophils increased. No alteration in the numbers of either macrophages or eosinophils was observed in challenged normal mice. A second experiment gave a similar pattern of results (Table 4.7.): C3H/He mice previously infected for 14 and 8 weeks demonstrated an increase in the proportions of macrophages and eosinophils, whereas newly infected (1 and 3 weeks) mice, only eosinophils increased. Neutrophils was seen in all mice injected with either schistosomula or medium alone compared to unchallenged controls.

4.2.5. Comparison of the cellular response in mice with unisexual or bisexual infections

This series of experiments addresses the question of whether the increase in the proportions of macrophages and eosinophils that takes place in mice previously infected with bisexual populations of cercariae after challenge also takes place in mice infected with unisexual population of cercariae. One group of mice were percutaneously infected with 100 cercariae from a female clone. Twenty one weeks later thet were intraperitoneally challenged with 1,000 schistosomula. The cellular reactivity in response to injected schistosomula were examined at 24 hr (Table 4.8.). A group of mice infected with 50 cercariae from a bisexual population 14 weeks previously was also challenged at the same time and used as a positive controls (Table 4.8.).

As reported earlier (see Section 4.2.1.1.), the proportions of macrophages and eosinophils increased in the mice infected with bisexual cercariae when challenged. However, the mice infected with female worms responded with an increase in the proportion of eosinophils but not of macrophages.

4.2.6. The effect of challenge dose of schistosomula on cellular responses

The effect of different schistosomular challenge doses on the peritoneal cell profile was investigated. A portion of a group of mice previously infected with 50 cercariae for 9 weeks were intraperitoneally challenged with 900 schistosomula. The rest of the group were challenged with 450 schistosomula per mouse. A group of uninfected mice were similarly challenged. The peritoneal cells were collected and examined 24 hr after challenge (Table 4.9.).

It was shown that the increase in the proportions of neutrophils and eosinophils could be induced by both challenge doses tested in infected mice. The magnitude of the secondary increase did not appear to depend on the size of challenge dose. The lower challenge dose did not induce a significant increase in the percentage of macrophages whereas the higher dose did. In normal mice, there was no apparent changes in the levels of macrophages and eosinophils. A mild neutrophilia was observed in all the mice intraperitoneally injected with schistosomula.

4.2.7. The specificity of cellular response induced by intraperitoneal challenge with schistosomula

This series of experiments addressed the question of whether the increase in the proportion of eosinophils and macrophages in the chronically infected mice upon challenge is specific to schistosomula. To investigate the specificity, the effect of intraperitoneal injection of larval stages of T. canis and T. spiralis and bacteria E. coli on the cellular content of peritoneal fluids was tested. Homologous challenge with schistosomula of S. mansoni was also carried out at the same time as a positive control. The intraperitoneal injection of schistosome infected mice with any of the three heterologous agents or schistosomula was followed at 24 hr (Table 4.10.).

Within 24 hr after administration of E. coli, there was a drastic increase in the number of neutrophils in the peritoneal exudates. Macrophages with numerous vacuoles in their cytoplasm were frequently seen. Many of the phagocytosing macrophages were ruptured during the standard cytocentrifuge procedure. These features were observed in mice injected with E. coli regardless of their immune status. In contrast, the other two tested organisms, T. canis, and T. spiralis, appeared to be comparatively 'inert'. There was no significant alteration in the cellular constitution of the peritoneal fluids in mice challenged with Toxocara and Trichella compared with controls injected with medium. As observed earlier, mice from the same infected groups demonstrated an increase in the proportions of eosinophils and macrophages when challenged with schistosomula.

4.3. Conclusion

The most marked differences in cellular reactivity in the peritoneal cavity in response to intraperitoneally injected schistosomula in the normal and the schistosome infected mice within 24 hr were the selective increase in the proportions of eosinophils (Section 4.2.1.1.) and macrophages (Section 4.2.2.1.) in the infected mice. An increase in the number of neutrophils was observed in all challenged mice regardless of their immune status (Section 4.2.1.1.). The induction of the increase by schistosomula depends on (1) the intensity of primary infection (Section 4.2.3.); an increase in the proportion of eosinophils was observed in mice previously infected with 20-100 cercariae, whereas the increase in macrophages occurred in relatively heavily infected (50-100 cercariae) mice when intraperitoneally challenged with 1,000 schistosomula (2) the length of primary infection period before challenge (Section 4.2.4.); the increase in eosinophils occurred in mice infected for 1 week or longer. However, the increase in macrophages took place only in mice where the egg laying by worms from the primary infection had commenced (3) the size of challenge schistosomula dose (Section 4.2.6.); the challenge doses of 450-900 schistosomula could elicit the increase in eosinophils, but a larger dose (900 schistosomula) was required to induce the

the increase in the proportion of macrophages (4) the sex of cercariae used in the primary infection (Section 4.2.5.); the increase in eosinophils could be induced in mice infected with either bisexual or unisexual cercariae, but the increase in macrophages took place only in mice with a bisexual infection.

It is also evident that the increase induced by schistosomula is specific (Section 4.2.7.). A challenge with E. coli, T. canis or T. spiralis failed to alter significantly the proportions of eosinophils and macrophages in mice previously infected with S. mansoni, whereas a homologous challenge elicited the increase in the percentages of these two types of cells in the peritoneal fluids.

Table 4.1. Protocol of intraperitoneal challenge experiments

Group	Primary infection		Intraperitoneal injection	
	with *S. mansoni*	+	with parasite/organism	+
	none	-	medium	-
A	+		+	
B	+		-	
C	-		+	
D	-		-	

Table 4.3.

Cellular profiles of peritoneal exudates lavaged from S. mansoni infected (16-week duration) or normal mice after intraperitoneal injection of schistosomula or medium. Lavaged samples were collected at 2, 6, 12 and 24 hr after challenge.

Mouse status & treatment	Total number per ml ($\times 10^{-6}$)	Cell content (mean ± S. D.) %				
		Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
A) Normal group						
Prechallenge (4)	3.1±0.5	19.6±1.3	73.3±1.9	3.6±1.3	0.1±0.1	3.2±0.5
After challenge with schistosomula						
2 hr (3)	2.2±0.7	19.8±8.4	31.5±14.6	1.8±2.0	43.0±20.5	3.6±0.3
6 hr (3)	2.9±1.0	7.8±3.0	10.7±7.0	4.9±1.9	74.4±12.0	1.1±0.2
12 hr (4)	2.7±0.5	12.8±3.3	46.2±15.1	0.8±0.4	38.6±18.3	1.4±0.4
24 hr (5)	3.4±0.6	20.0±1.7	66.2±2.9	4.4±1.5	6.4±2.6	2.9±0.3
After injection with medium alone						
24 hr (3)	2.1±0.5	19.9±1.6	65.8±0.9	4.6±0.3	5.1±0.5	3.8±1.5
B) Infected group						
Prechallenge (5)	9.1±1.8	20.0±2.1	72.4±1.3	6.7±1.1	0.5±0.2	0.3±0.1
After challenge with schistosomula						
2 hr (3)	12.6±3.3	18.8±4.0	58.5±1.0	9.0±3.3	13.2±4.5	0.2±0.2
6 hr (4)	13.9±5.4	16.2±9.7	26.5±20.0	4.3±3.7	50.9±28.4	0.3±0.4
12 hr (4)	8.2±2.4	24.2±0.5	40.8±7.2	10.6±7.1	24.2±14.6	0.1±0.1
24 hr (5)	16.0±3.4	29.9±4.9	45.9±11.9	17.4±3.1	6.6±7.2	0.1±0.2
After injection with medium alone.						
24 hr (3)	7.6±1.7	18.5±0.7	67.1±3.9	6.3±0.4	7.8±3.9	0.1±0.1

Table 4.4

Cellular profiles of peritoneal exudates lavaged from S. mansoni infected (13-week duration) after intraperitoneal injection of schistosomula or medium. Lavaged samples were collected before and at 6 and 24 hr after injection

Treatment	Cell content (mean ± S. D.)%				
	Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
A) Before injection (5)	22.4±2.7	65.6±1.6	11.6±3.2	0.1±0.1	0.3±0.1
B) After injection with medium					
6 hr (6)	18.7±2.2	55.8±5.2	10.3±2.2	14.9±5.8	0.4±0.3
24 hr (5)	21.8±2.1	63.6±5.6	9.3±3.2	4.8±2.4	0.6±0.4
C) After injection with schistosomula					
6 hr (5)	13.1±3.7	32.4±10.3	16.0±3.9	38.3±10.4	0.3±0.2
24 hr (5)	35.0±4.7	36.7±5.8	24.8±4.3	3.3±0.9	0.3±0.1

Table 4.5.

The effect of needle-trauma on the cellular content of peritoneal cavity of normal mice. Lavaged samples were collected at 24 hr after wounding with a needle.

Treatment	Total number per ml ($\times 10^{-6}$)	Cell content (mean ± S. D.) %				
		Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
A) Untreated (6)	2.4±0.4	22.3±4.9	71.5±5.2	3.2±1.3	0.4±0.5	2.4±0.4
B) Needle-wounding (7)	2.5±1.1	24.3±2.5	62.4±7.5	3.9±3.1	6.8±5.0	1.3±1.1

Table 4.6.

Cellular profiles of peritoneal exudates lavaged from normal and mice infected with S. mansoni for 6, 13 and 18 weeks after intraperitoneal injection with schistosomula or medium. Lavaged samples were collected at 24 hr after challenge.

Primary infection	Intraperitoneal challenge	Total number per ml ($\times 10^{-6}$)	Cell content (mean ± S.D.)% Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
A) 18-week group							
S. mansoni	Schistosomula(5)	18.5±6.6	34.1±3.5	40.5±3.6	22.2±4.6	3.1±3.6	0.1±0.1
S. mansoni	Medium (5)	5.8±2.9	16.5±2.9	69.5±10.9	7.6±4.7	5.6±5.8	0.8±1.0
B) 13-week group							
S. mansoni	Schistosomula(6)	13.8±3.1	30.6±3.6	41.7±9.2	21.1±10.7	6.3±5.0	0.2±0.3
S. mansoni	Medium (5)	9.6±3.5	17.7±1.9	59.1±10.2	8.9±2.7	15.6±9.4	0.1±0.1
C) 6-week group							
S. mansoni	Schistosomula(6)	6.8±2.3	22.6±3.6	41.7±7.9	17.6±3.8	17.3±8.1	0.2±0.4
S. mansoni	Medium (6)	1.7±0.7	18.4±3.5	64.1±10.8	4.5±1.4	11.8±9.3	1.0±0.6
D) Uninfected group							
None	Schistosomula(6)	2.7±0.6	17.5±3.0	46.2±13.9	4.7±1.8	28.9±16.2	2.5±2.7
None	Medium (6)	2.6±1.4	18.7±2.5	60.4±7.7	4.9±2.6	11.4±7.3	4.2±3.6

Table 4.7.

Cellular profiles of peritoneal exudates lavaged from normal C3H/He mice and mice infected with S. mansoni for 1, 3, 8 and 14 weeks after intraperitoneal injection with schistosomula or medium. Lavaged samples were collected at 24 hr after challenge.

Primary infection	Intraperitoneal challenge	Total number per ml($\times 10^{-6}$)	Cell content (mean ± S.D.)%			
			Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
A) 14-week group							
S. mansoni	Schistosomula(6)	11.8±6.1	24.8±12.5	47.6±9.9	26.0±13.3	0.3±0.3	1.4±1.2
S. mansoni	Medium (6)	10.6±2.9	16.9±5.9	64.9±8.4	17.4±6.2	0.5±0.6	0.3±0.2
S. mansoni	None (5)	9.5±2.9	12.9±9.9	75.5±13.5	9.4±6.8	0.4±0.8	1.4±1.6
B) 8-week group							
S. mansoni	Schistosomula(5)	18.4±6.7	37.6±11.5	57.3±12.2	4.4±1.5	0.2±0.2	0.4±0.3
S. mansoni	Medium (4)	11.9±1.8	17.6±5.9	79.6±7.9	2.6±2.1	0.1±0.1	0.4±0.3
S. mansoni	None (4)	14.7±4.4	20.5±9.5	76.8±12.0	2.6±1.8	0.3±0.6	0.5±0.4

Table 4.7. (cont'd)

Primary infection	Intraperitoneal challenge	Total number per ml ($x10^{-6}$)	Cell content (mean ± S.D.)%				
			Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
C) 3-week group							
S. mansoni	Schistosomula(6)	1.8±0.3	18.6±2.9	73.9±7.4	1.5±1.0	4.1±5.8	1.8±1.1
S. mansoni	Medium (6)	1.8±0.4	15.1±3.9	77.6±4.5	0.4±0.4	2.5±3.0	4.3±1.9
S. mansoni	None (7)	1.5±0.4	19.1±6.2	76.4±6.8	0.3±0.2	0.2±0.3	3.6±1.3
D) 1-week group							
S. mansoni	Schistosomula(5)	1.8±0.5	17.6±2.5	75.6±4.1	4.7±2.2	0.6±0.4	1.3±0.7
S. mansoni	Medium (4)	1.7±0.2	20.1±4.4	76.8±4.8	0.5±0.3	0.5±0.3	1.8±1.3
S. mansoni	None (4)	1.4±0.4	17.5±5.7	79.2±6.8	0.1±0.1	0.1±0.2	3.1±2.1
E) Uninfected group							
None	Schistosomula(6)	2.1±0.7	20.2±5.7	73.9±7.1	0.5±0.4	2.0±2.2	3.3±1.9
None	Medium (6)	1.7±0.5	16.4±4.9	75.6±4.8	0.9±1.5	1.9±1.5	5.1±1.1
None	None (6)	1.9±0.4	12.7±8.8	83.1±7.8	0.5±0.5	0.1±0.1	3.3±1.6

Table 4.8.

Cellular profiles of peritoneal exudates lavaged from mice with unisexual and bisexual infection after intraperitoneal injection with schistosomula or medium. Lavaged samples were collected at 24 hr after challenge.

Infection & challenge	Total number per ml ($\times 10^{-6}$)	Cell content (mean $\pm$ S.D.)%				
		Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
A) Infection with bisexual population(50 cercariae/mouse) for 11 weeks						
After challenge with schistosomula (6)	15.3±4.3	35.1±5.3	44.9±6.5	16.1±2.3	3.8±1.0	0.3±0.1
After injection with medium alone (5)	12.1±2.9	21.6±3.3	67.2±6.6	9.4±3.2	1.6±0.5	0.3±0.2
B) Infection with unisexual population(100 cercariae/mouse) for 24 weeks						
After challenge with schistosomula (7)	5.5±2.4	25.9±2.0	62.0±5.1	9.0±4.1	2.5±1.3	0.7±0.2
After injection with medium alone (5)	4.1±2.8	21.7±4.6	70.3±10.6	2.3±1.5	1.3±0.4	0.7±0.5

Table 4.9.

The effect of schistosomula challenge dose on peritoneal cell profiles of normal mice and mice infected with S. mansoni for 9 weeks.

Mouse status & treatment	Total number per ml ($x10^{-6}$)	Cell content (mean ± S.D.)%				
		Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
A) Infected group						
Unchallenged (5)	11.8±2.3	20.3±3.2	69.7±7.1	9.4±3.6	0.1±0.1	0.4±0.2
Challenged						
900 schistosomula(6)	12.2±6.9	39.6±5.8	37.0±10.1	20.4±2.9	2.8±0.7	0.5±0.3
450 schistosomula(5)	10.6±1.9	18.9±2.2	56.2±7.7	22.3±5.3	1.6±0.4	0.5±0.2
B) Uninfected group						
Unchallenged (4)	3.2±0.3	19.6±1.4	74.5±3.5	4.5±1.9	0.2±0.1	1.2±0.1
Challenged						
900 schistosomula(5)	3.4±0.9	23.1±2.2	63.5±3.5	5.4±1.4	5.8±0.7	1.6±0.3
450 schistosomula(5)	4.6±1.7	17.9±2.2	67.8±6.4	5.3±0.7	7.7±3.4	1.3±0.2

Table 4.10.

Cellular profiles of peritoneal exudates after intraperitoneal challenge with schistosomula, *E. coli*, *T. canis* or *T. spiralis*. Lavaged samples were collected at 24 hr after challenge.

Mouse status & treatment	Total number per ml ($\times 10^{-6}$)	Cell content (mean ± S.D.)%				
		Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
A) *E. coli* (heat-killed)						
a. 21-week *S. mansoni* infected mice challenged with:						
E. coli at 1.5×10^8 organisms/mouse (7)						
	2.1±1.1	11.1±4.1	27.1±5.9	3.9±1.8	57.1±1.5	0.6±0.4
Medium alone at 0.2 ml/mouse (5)						
	3.5±1.3	24.5±3.6	68.2±6.4	5.9±2.4	1.5±1.4	0.3±0.3
Schistosomula at 1,000 worms/mouse (6)						
	4.2±0.9	31.9±3.9	43.1±9.6	17.7±7.2	6.8±3.6	0.5±0.3
b. Uninfected mice challenged with:						
E. coli at 1.5×10^8 organisms/mouse (5)						
	0.8±0.2	3.6±1.5	7.0±2.1	2.6±2.4	86.8±2.3	0.7±0.8
Medium alone at 0.2 ml/mouse (4)						
	1.6±0.1	18.7±3.9	66.9±6.5	1.4±0.5	4.1±3.8	8.5±1.3

Table 4.10. (cont'd)

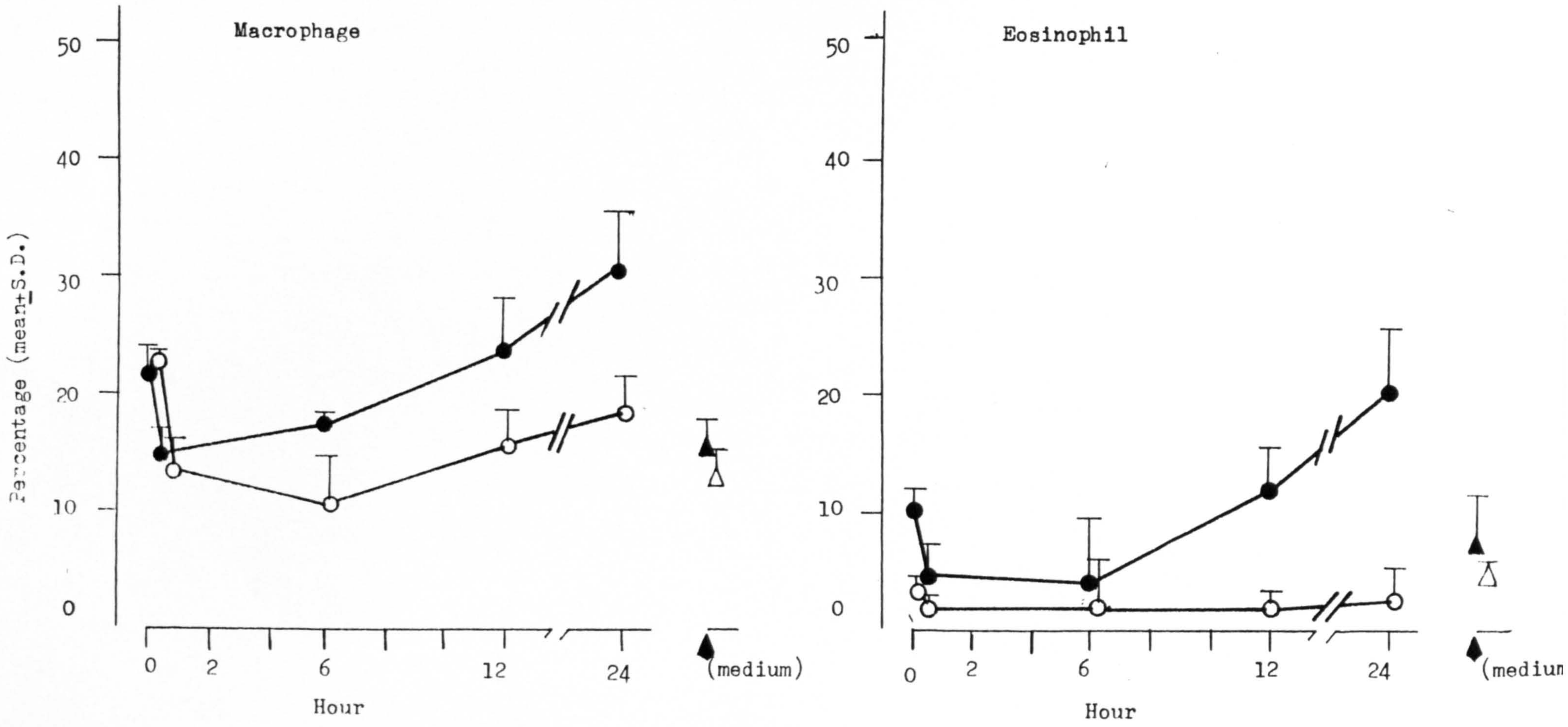
Mouse status & treatment	Total number per ml ($x10^{-6}$)	Cell content (mean ± S.D.)%				
		Macrophage	Lymphocyte	Eosinophil	Neutrophil	Mast cell
B) *T. canis* (living larvae)						
a. 9-week *S. mansoni* infected mice challenged with:						
T. canis at 800 worms/mouse (7)	11.5±2.6	19.2±3.2	67.7±3.7	8.7±4.3	4.1±1.7	0.1±0.1
Medium alone at 0.2 ml/mouse (5)	9.1±5.4	21.4±1.3	56.1±5.7	14.5±3.4	6.1±3.5	0.1±0.1
Schistosomula at 1,000 worms/mouse (5)	12.1±4.8	29.9±3.3	43.9±17.3	25.8±4.8	6.7±2.7	0.2±0.1
C) *T. spiralis* (living larvae)						
a. 13-week *S. mansoni* infected mice challenged with:						
T. spiralis at 600 worms/mouse (5)	12.0±3.6	19.5±1.7	68.5±3.5	7.6±2.2	4.8±1.7	0.1±0.1
1,200 worms/mouse (5)	10.8±3.6	21.6±3.5	48.5±8.1	14.8±7.7	10.6±6.4	0.1±0.2
1,800 worms/mouse (5)	13.8±3.3	17.4±2.7	56.0±11.5	19.1±7.4	8.2±3.4	0.05±0.01
Medium alone at 0.2 ml/mouse (4)	9.7±4.2	19.5±2.0	67.5±4.5	10.9±4.7	1.8±1.7	0.2±0.1
Schistosomula at 1,000 worms/mouse (6)	10.1±3.9	34.5±4.8	32.4±7.4	23.9±5.4	9.1±2.8	0.1±0.1

Fig. 4.1.

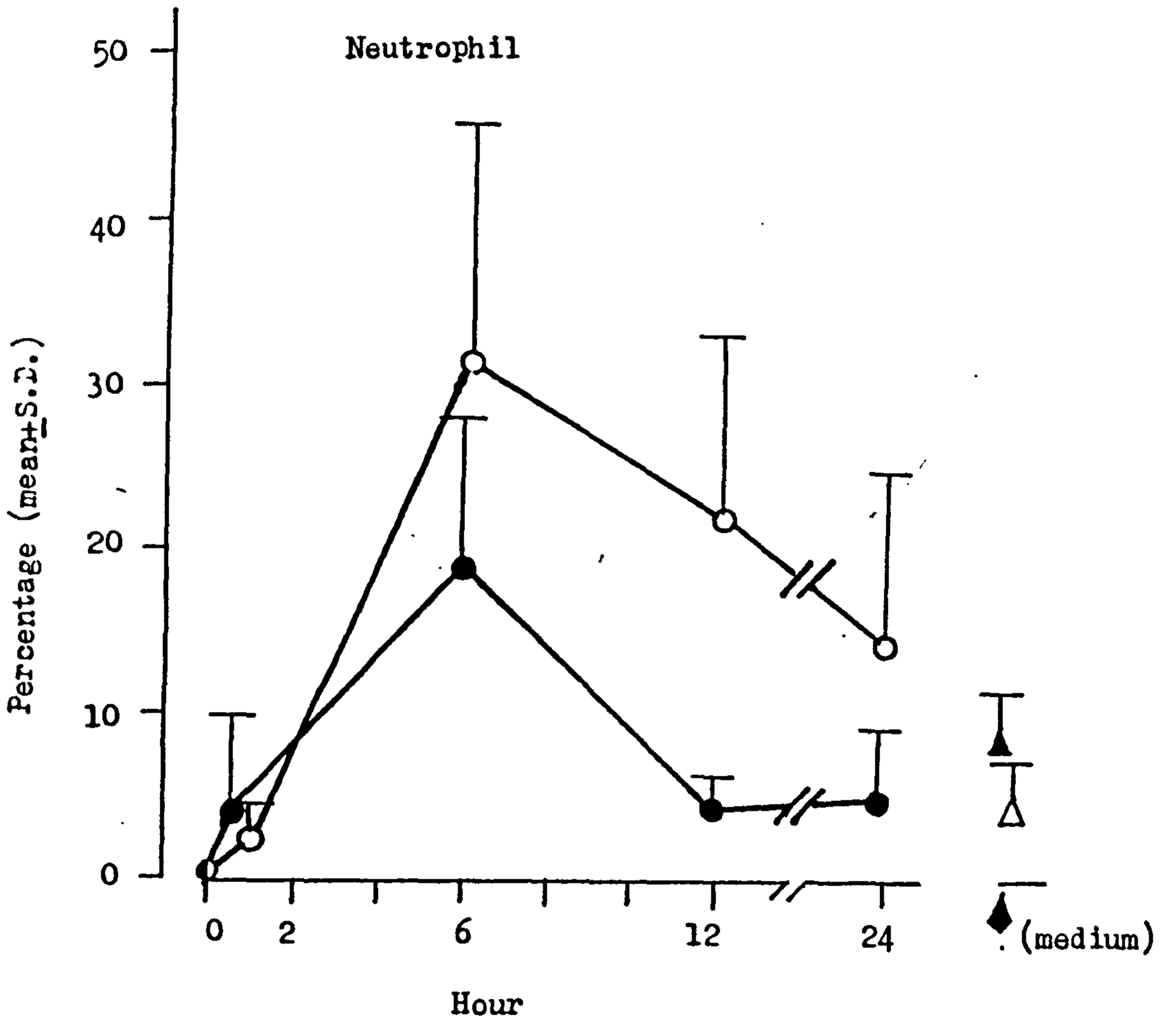
Development of cellular reactivity induced in the peritoneal cavity by schistosomula intraperitoneally inoculated

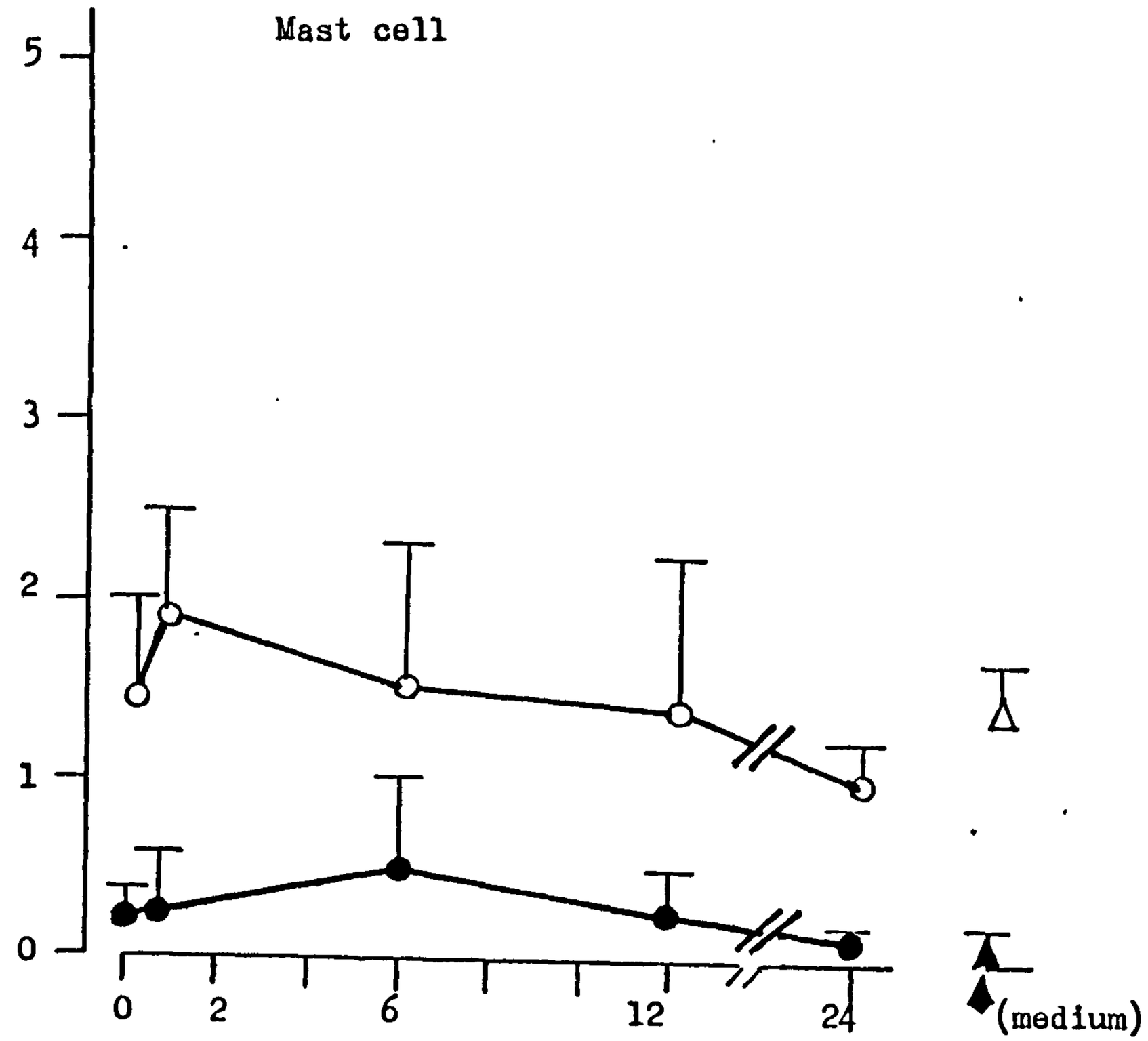
Approximately 1,000 mechanically transformed schistosomula per mouse were intraperitoneally injected into normal mice and mice previously infected with S. mansoni. At intervals, (a) early stage: 30 min and 6, 12 and 24 hr (b) late stage: 1, 2 and 4 days, the cell-rich peritoneal fluids were lavaged by intraperitoneal injection of 2 ml of PBS/heparin. The percentages of macrophages, eosinophils, neutrophils and mast cells were determined by examining the May-Grunward-Giemsa stained cytocentrifuged cell smears. Peritoneal samples were also collected from mice injected with medium alone at (a) 24 hr and (b) 4 days. Open symbols (○, △) show the cellular response in uninfected mice. Closed symbols (●, ▲) shows the responses in mice previously infected with 50 cercariae for (a) 11 weeks and (b) 10 weeks. The symbols (◭, ◭) on the right hand show responses to injection of medium. Each value represents the mean ± S.D.. Numbers in parentheses refer to the numbers of mice used in each test.

(a) Early stage



(a) Early stage (cont'd)





(b) Late stage

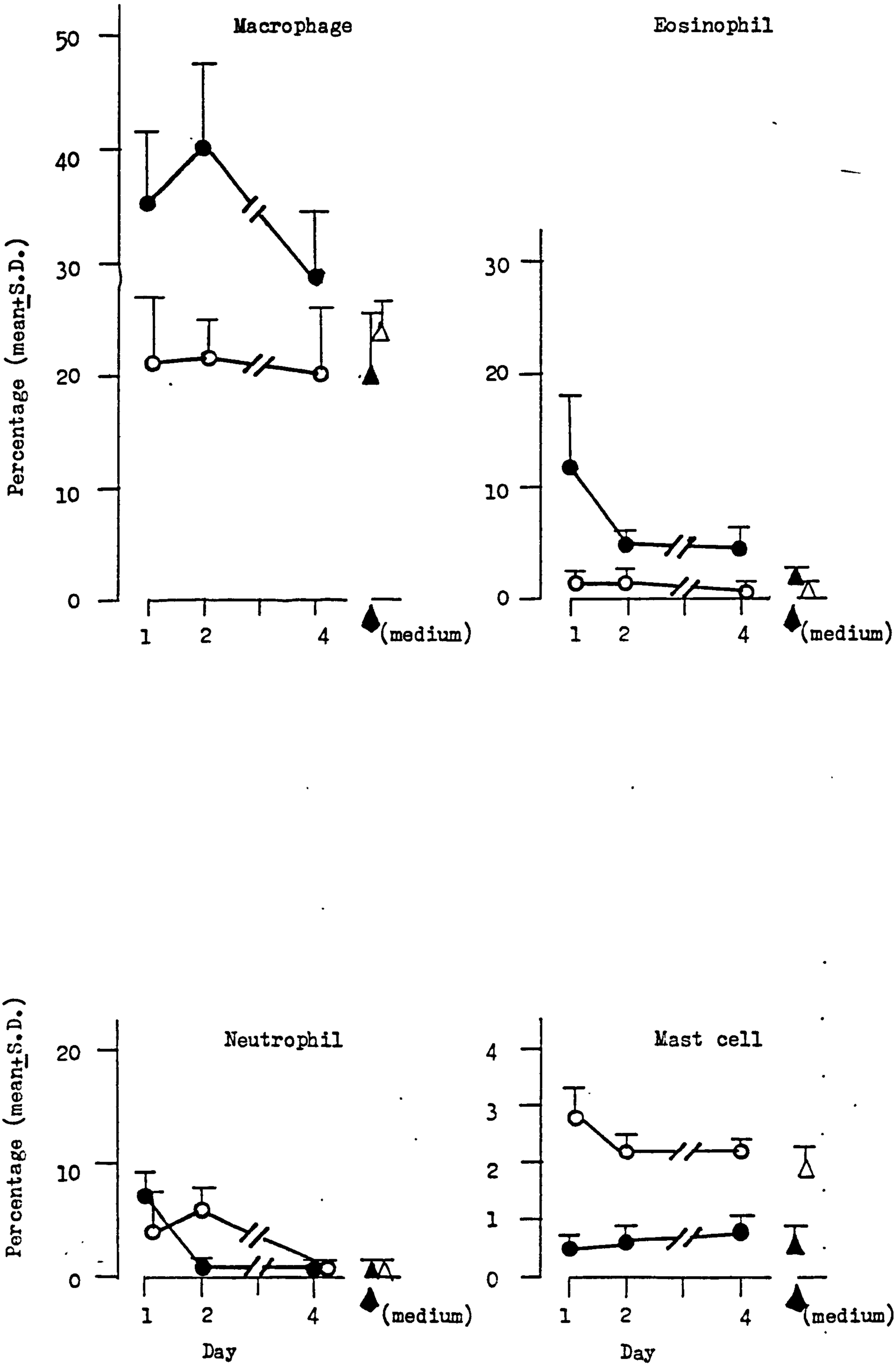
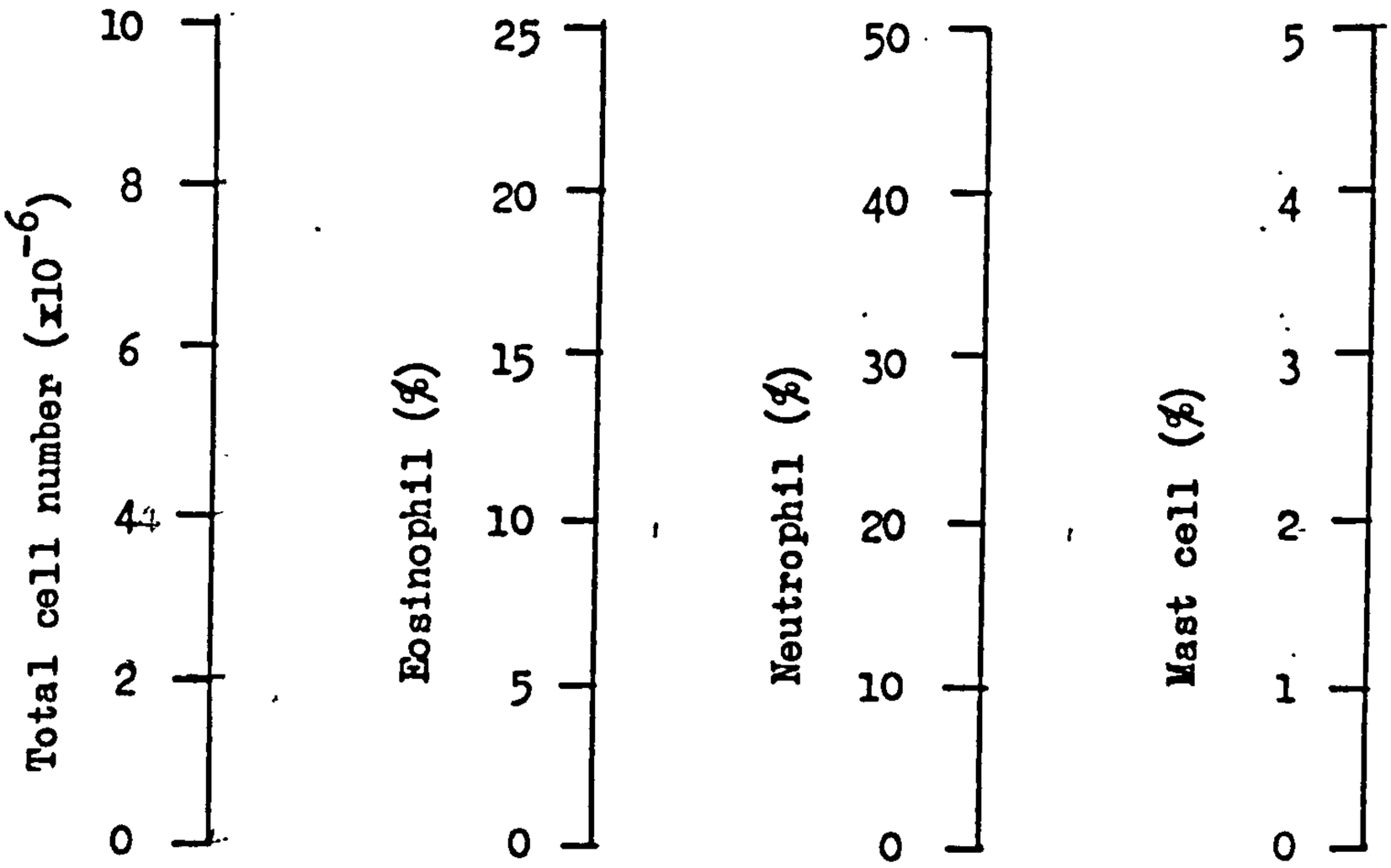

Macrophage
Eosinophil
Neutrophil
Mast cell
Percentage (mean±S.D.)
50
40
30
20
10
0
4
3
2
1
1
2
4
(medium)
Day

Fig. 4.2.

<u>The effect of cercarial exposure dose on the primary and secondary cellular responses</u>

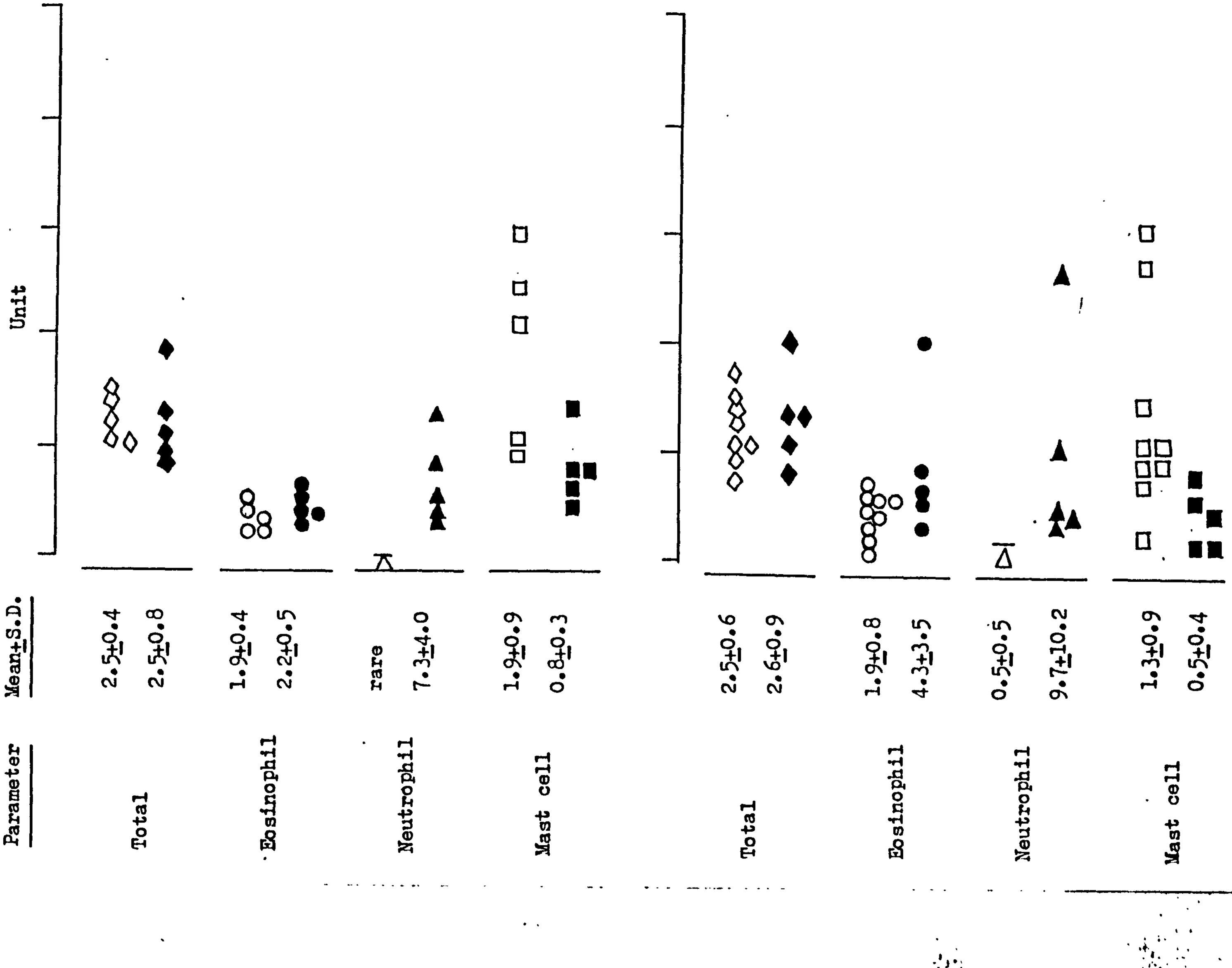
Three groups of mice were percutaneously infected with (a) 20 (b) 50 and (c) 100 cercariae per mouse simultaneously. After 7 weeks, a portion of each group were intraperitoneally challenged with 1,000 schistosomula, and the rest untreated. At 24 hr after challenge, the cell-rich peritoneal fluids were lavaged by injection of 2 ml of PBS/heparin, and the cellular contents were examined. Total cell numbers (◇,◆); percentages of eosinophils (○,●), neutrophils (△,▲) and mast cells (□,■) were the parameters employed to assess the cellular responses. Open symbols (◇,○,△,□) indicate the responses in unchallenged and closed symbols (◆,●,▲,■) the responses in schistosomula-challenged mice. Each point represents an individual value. One unit represents 2×10^6 cells, 5% eosinophils, 10% neutrophils and 1% mast cells (see the simplified diagram below). The mean ± S.D. for each group and the significance of differences between paired groups (S: signicant, NS: not significant) are also presented.

<u>Units:</u>



(a) 0 cercariae/mouse

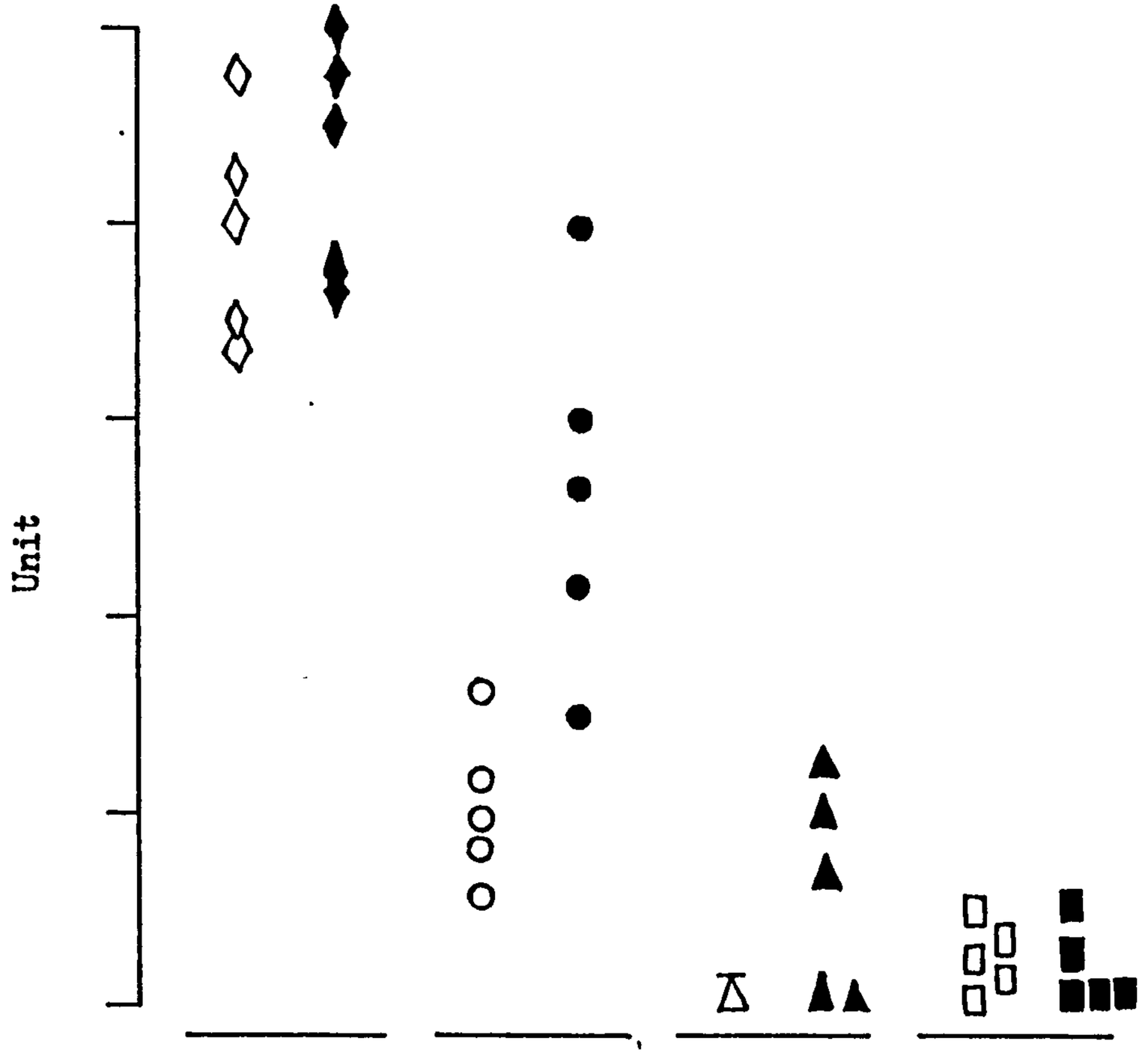
(b) 20 cercariae/mouse



Parameter	Mean±S.D.	Mean±S.D.
Total	2.5±0.4	2.5±0.6
	2.5±0.8	2.6±0.9
Eosinophil	1.9±0.4	1.9±0.8
	2.2±0.5	4.3±3.5
Neutrophil	rare	0.5±0.5
	7.3±4.0	9.7±10.2
Mast cell	1.9±0.9	1.3±0.9
	0.8±0.3	0.5±0.4

(c) 50 cercariae/mouse

(d) 100 cercariae/mouse



Parameter	Mean±S.D.		Mean±S.D.
Total	8.0±1.1		7.2±0.9
	8.7±1.1		8.2±1.3
Eosinophil	4.9±2.1		5.0±0.7
	13.2±4.6		11.7±6.5
Neutrophil	0.4±0.5		1.0±0.5
	6.7±5.6		2.6±3.1
Mast cell	0.3±0.2		0.1±0.2
	0.1±0.2		0.1±0.1

5. The role of anti-schistosome antibody in the peritoneal eosinophilia

The elevation of specific antibody to schistosome antigens occurred concomitantly with the onset of eosinophilia in the peritoneal cavity of schistosome infected mice (see Section 1.2;3.2). The mice exhibiting hypergammaglobulinemia also responded with a secondary increase in the proportion of eosinophils when intraperitoneally injected with schistosomula (see Section 4.2.1.) Thus, to study the possibility of the involvement of humoral factor(s) in the eosinophilic response, mice were passively sensitized by the injection of immune serum via the intraperitoneal or intravenous route.

5.1. Experimental design

5.1.1. Collection of immune serum

Immune serum donors were mice infected with 40-50 cercariae of S. mansoni for 12-24 weeks. Mice were killed and the blood collected by cardiac puncture. Blood, usually pooled from 30-40 mice, was taken aseptically and allowed to clot for 15 min at room temperature and then at 4°C for 2 hr or overnight. The serum collected after centrifugation in a bench centrifuge at 500 x G for 10 min was stored at -20°C until use.

5.1.2. Transfer procedure and schedule

A simplified experimental schedule with dosages of serum used and numbers of challenge schistosomula is described in Table 5.1.. Serum was injected intraperitoneally into normal

recipients 20–22 hr before intraperitoneal challenge with schistosomula. Each injection consisted of stated volumes of serum or diluted serum in Eagle's essential medium. Alternatively, the serum was administered intravenously into the tail veins. Control animals received equivalent amounts of serum from normal donors.

The cellular reactivity in the peritoneal cavities of recipient mice was monitored at 24 hr after schistosomula challenge using the standard procedures described earlier (see Section 1.1.4.) Student's t test was used to evaluate the statistical significance of eosinophil percentages in challenged and unchallenged groups. The titre of IgG against schistosome worm antigens in the peritoneal fluids of recipient mice was measured by ELISA (see Section 3.1.2.). The present study provides evidence for the role of a humoral component in mechanisms underlying the peritoneal eosinophilia.

5.2. Results

5.2.1. Transfer of serum via intraperitoneal route of injection

The first series of the investigations involved sensitization employing the intraperitoneal route of injection The donors of immune serum were three groups of mice previously infected for 12, 20 or 24 weeks. Each recipient was given 0.1 ml of serum of various dilutions in medium. Control groups included mice receiving normal serum or medium alone. Percentages of macrophages eosinophils and neutrophils were the parameters employed to assess the cellular responses occurring in the peritoneal cavity of recipient mice.

As seen in the results of the three groups of transfer experiments summarized in Table 5.2. there was no significant difference in the levels of eosinophils, macrophages and neutrophils in the schistosomula-challenged recipients of normal or immune serum compared with those of medium-injected controls. The increase in the number of neutrophils was observed in all the mice injected with either serum or medium compared with untreated normal mice where neutrophils were rarely seen. The magnitude of increases appeared to bear no apparent relationship to the nature and quantity of material injected.

5.2.2. Transfer of serum via intravenous route of injection

The second series of experiments was to explore the alternative route of sensitization, intravenous injection. Immune serum collected from two groups of donor mice infected for 22 or 25 weeks was injected into normal mice via the tail vein. Each mouse was given 0.3 or 0.4 ml of serum.

As shown in Table 5.3., a significant increase in the proportion of eosinophils occurred in the immune serum recipients upon challenge with schistosomula. Intravenous injection of serum alone did not stimulate the increase compared with untreated controls. The ability of serum to induce peritoneal eosinophilia depends on the immune status of the donors since there was no appreciable change in the normal serum recipients after challenge. Neutrophilia occurred in all the mice intraperitoneally injected with either medium or schistosomula regardless of the

immune status of the serum they received. No significant differences in the total peritoneal leukocytes or in the percentages of the other types of cells, macrophages and mast cells, were noted in the groups treated differently (data not shown). It appeared that IgG specific to schistosome antigens was able to pass from circulating blood to the tissue site as evidenced by the significant high ELISA values against worm antigens in peritoneal fluids lavaged from all groups of mice receiving immune serum by intravenous injection (Table 5.3.).

5.3. Conclusion

The passive sensitization experiments showed that intravenous injection of immune serum resulted in a increase in the percentage of eosinophils in the peritoneal cavity of recipients when subsequently challenged with schistosomula (Section 5.2.2.). In contrast, the injection of schistosomula did not induce significant alteration in the proportion of eosinophils in mice receiving immune serum intraperitoneally under the experimental conditions described (Section 5.2.1.). It was also demonstrated that the intravenous injection of immune serum resulted in the increase in IgG specific to worm antigens in the peritoneal fluids in the recipient mice (Section 5.2.2.).

Table 5.1.

Experimental procedures of passive sensitization and schistosomula challenge

A) Schedule:

Day 1: Sensitization via intraperitoneal or intravenous routes of injection

Day 2: Challenge via intraperitoneal route of injection

Day 3: Peritoneal lavage and examination of lavaged samples

B) Treatment and dosages of transfer and challenge reagents:

Sensitization

Injection route	Dosage of serum transferred	Dosage of challenge schistosomula/medium
Intraperitoneal	0.1 ml of medium alone or 0.1 ml of normal serum of varying concentrations (e.g. neat, 1/2, 1/4, 1/8 dil.) or 0.1 ml of immune serum of varying concentrations (e.g. neat, 1/2, 1/4, 1/8 dil.)	Intraperitoneal injection of 0.2 ml medium or approximate 1,000 schistosomula in 0.2 ml of medium
Intravenous	0.3 ml (Exp. 1) or 0.4 ml of neat normal serum or immune serum	as above

Table 5.2.

The eosinophil and neutrophil responses in the peritoneal cavity of mice intraperitoneally injected with serum after challenge with schistosomula or medium

Group	Transfer agent	Challenge agent	Exp. 1.		Exp. 2.		Exp. 3.	
			Eosinophil	Neutrophil	Eosinophil	Neutrophil	Eosinophil	Neutrophil
A)	None	None	-	-	-	-	2.5±1.9	0.01±0.01(7)
B)	Medium	Medium	-	-	3.5±1.5	17.6±17.0(5)	4.8±2.2	3.6±2.4 (5)
	Medium	Schistosomula	-	-	8.6±6.0	15.5±21.8(6)	6.3±3.0	13.2±8.4 (5)
C)	Normal serum (0.1 ml/mouse)			-				
	Neat	Medium	-	-	-	-	-	-
	Neat	Schistosomula	-	-	-	-	-	-
	1/2 dil	Medium	-	-	2.8±1.5	15.1±15.9(5)	4,8±2.3	13.7±11.8 (5)
	1/2 dil	Schistosomula	-	-	3.7±1.0	10.8±5.6 (4)	4.2±2.0	13.4±4.1 (5)
	1/4 dil	Medium	-	-	3.8±1.5	7.0±3.3 (5)	6.4±3.4	14.2±17.8 (6)
	1/4 dil	Schistosomula	-	-	1.7±0.9	7.4±9.5 (6)	4.0±3.5	21.5±8.4 (6)
	1/8 dil	Medium	-	-	-	-	5.4±1.9	16.9±6.1 (6)
	1/8 dil	Schistosomula	-	-	-	-	3.3±1.2	13.2±10.1 (6)
D)	Immune serum (0.1 ml/mouse)		24-week		12-week		20-week	
	Neat	Medium	4.6±1.9	15.9±11.4(5)	-	-	-	-
	Neat	Schistosomula	5.9±2.1	22.2±14.6(5)	-	-	-	-
	1/2 dil	Medium	-	-	4.0±2.9	3.0±2.1 (6)	4.9±1.8	8.6±5.1 (6)
	1/2 dil	Schistosomula	-	-	8.6±5.2	8.1±5.5 (6)	7.5±3.8	18.5±9.3 (6)
	1/4 dil	Medium	-	-	2.4±0.8	23.2±23.9(5)	3.5±2.7	13.5±8.9 (6)
	1/4 dil	Schistosomula	-	-	5.2±1.1	5.4±4.9 (6)	4.6±1.7	9.6±4.1 (6)
	1/8 dil	Medium	-	-	-	-	4.6±2.4	14.2±10.4(7)
	1/8 dil	Schistosomula	-	-	-	-	4.3±2.6	10.6±4.9 (7)

Table 5.3.

The eosinophil and neutrophil responses in the peritoneal cavity of mice intravenously injected with serum after challenge with schistosomula or medium

Group	Transfer agent	Challenge agent	Exp. 1. Eosinophil	Exp. 1. Neutrophil	Exp. 1. ELISA (E492)	Exp. 2. Eosinophil	Exp. 2. Neutrophil	Exp. 2. ELISA (E492)
A)	None	None	3.1±0.5	0.01±0.01	0.279±0.096(5)	2.2±1.2	0.01±0.01	0.261±0.108(7)
B) Normal serum			(0.3 ml/mouse)			(0.4 ml/mouse)		
	Neat	Medium	1.9±1.7	13.3±7.9	- (4)	1.8±1.4	13.3±10.1	0.215±0.017(6)
	Neat	Schistosomula	2.6±0.9	19.9±12.7	- (4)	2.0±1.6	15.3±7.9	- (6)
C) Immune serum			25-week (0.3 ml/mouse)			22-week (0.4 ml/mouse)		
	Neat	Medium	2.7±1.2	14.9±8.9	0.634±0.071(4)	2.2±0.9	11.9±12.0	0.788±0.041(7)
	Neat	Schistosomula	11.5±6.3	9.1±7.9	-	7.1±2.5	8.1±6.9	- (7)

6. Quantitative analysis of cellular adherence to intraperitoneally injected schistosomula in normal and infected mice

Various *in vitro* studies had led to the suggestion that the presence of antibodies is the prerequisite for effective adherence and cell-mediated killing schistosomula (see Introduction). The presence of IgG antibody against schistosome antigens was detected in the peritoneal fluid of mice infected for 7 weeks or longer (see Section 3.2.2.4). It has also been demonstrated that IgG specific to worm antigens increased in the peritoneal fluids in mice intravenously injected with immune serum (see Section 5.2.2.) Therefore, experiments were designed to compare the cellular adherence to intraperitoneally injected schistosomula in actively or passively sensitized mice and in normal mice. Additionally, the cellular adherence to schistosomula occurring in the peritoneal fluids of mice infected with *T. spiralis* was also quantitatively analysed.

6.1. Experimental design

6.1.1. Infection with S. mansoni or T. spiralis

Groups of mice had been infected either 11-14 weeks previously with 50 cercariae of *S. mansoni* given percutaneously or 4 weeks previously with 300 larvae of *T. spiralis* orally.

6.1.2. Passive sensitization by intravenous injection of immune serum

Immune sera were collected by cardiac puncture from mice previously infected with 50 cercariae for periods ranging

from 8 to 15 weeks. Its titre against worm antigens was measured by ELISA. Normal sera were also collected in the same manner. Volumes of 0.3 ml of the pooled serum were injected into the tail veins of normal mice. The intravenous injection of immune serum resulted in an increase in the concentration of IgG against worm antigens in the peritoneal fluids of the recipients (See Section 5.2.2.). The cellular adherence to intraperitoneally injected schistosomula in the passively sensitized mice was investigated at 20 hr after sensitization.

6.1.3. Harvesting of intraperitoneally inoculated schistosomula

Two thousand mechanically transformed schistosomula were intraperitoneally injected into normal or infected mice. At 1/2, 2 and 24 hr after schistosomula challenge, groups of 4-5 mice were killed and 2 ml of PBS/heparin solution was injected into the peritoneal cavity and withdrawn immediately to remove most of the free unbound cells so as to facilate the location of worms in the gut washing later. The peritoneal wall was slit open and the cavities and viscera were washed in Eagle's medium containing 5 U heparin/ml to collect the worms with their attached cells. The peritoneal samples collected from individuals in each experimental group were pooled. The worms were allowed to sediment at 1 x G. The concentrated worm suspensions were immediately examined microscopically and scored for bound cells without further manipulation to avoid dissociation of cells from worms.

6.1.4. Scoring of cellular adherence to schistosomula

Scoring of the cellular reactivities to intraperitoneally inoculated schistosomula was based on the numbers of cells adhering to schistosomula. The magnitude of the reaction was scored on a scale of ± to +++ (R± to R+++) (Fig 6.1.). None or one or two cells loosely attached to a single schistosomula was scored R±. Up to 10 cells attached to a worm was scored R+ and one or two layers of cells (<50 cells) surrounding a worm that formed a rosette like worm-cell aggregate, R++. Denser cell aggregates consisting of more than two layers surrounding a schistosomula were scored R+++ and the larger aggregates (>0.2 mm), with or without remnants of schistosomula, that were visible without microscopic aid, were scored R++++. Parasitic integrity was not used as a scoring criterion in parallel because of difficulty in reliably recognizing subtle damage under the experimental conditions described. One hundred or more determinations were made for each pooled sample.

6.2. Results

6.2.1. Cellular adherence to schistosomula in the peritoneal cavity of mice previously infected with S. mansoni

Only about 2-8% of the inoculated schistosomula could be recovered from the peritoneal cavities of challenged mice by washing the viscera in the medium at all three time intervals. No schistosomula were found on the gastro-splenic ligament nor on the ligaments connecting the liver and diaphram.

No schistosomula were encountered on the mesenteric folds when the tissues were stretched out onto a glass slide and examined microscopically. Quantitative differences could not be established between normal and infected groups in the recovery rates of challenge schistosomula due to sampling problems. However, distinctive differences in the patterns of the host reactions to schistosomula in the normal and infected mice were observed. Fig.6.2. compares the time course of the magnitude of cellular adherence to schistosomula in the peritoneal cavities of normal and infected mice.

6.2.1.1 Early stage adherence (1/2 to 2 hr after inoculation)

In both normal and infected groups, cellular reactions to schistosomula were instantaneous. Adherence to schistosomula occurred as early as 10 min after inoculation. The ranges of adherence intensity between 30 min and 2 hr were generally similar in all challenged mice irrespective of their immune status (Fig. 6.2.a.). The cellular reaction ranged from nil (R±) to marked cell aggregation around the schistosomula (R++++). The majority of the schistosomula were embedded in a sheath of cells (R+++ to R++++). The entire aggregate assumed the shape of a rounded or enlongated, somewhat flattened focus. The rest of the recovered schistosomula elicited minor cellular reactions (R+ to R++). Cells in the aggregates with or without schistosomula appeared larger in size compared with those form unchallenged mice.

Most of schistosomula with few cells adhering recovered from either challenged normal or infected mice appeared morphologically intact and motile during the early stage. A few more severly damaged schistosomula showed focal tegumental blebbing with cells attached to peeled membrane. The tegumental damage of schistosomula in the foci with R+++ to R++++ cellular reactions could not be judged because the tegument was obscured by cells. Schistosomula in such foci remained alive and motile.

6.2.1.2. Late stage adherence (24 hr after inoculation)

It has been shown that there was no apparent immune status-dependent quantitative variation in intensity of early adherence in normal and infected mice (Fig. 6.2.a.). However, at 24 hr after inoculation, there was a dramatic difference in the cellular reactions to schistosomula between the two challenged groups (Fig. 6.2.b.).

In the normal mice, the worm-cell aggregates were fewer and smaller compared to the earlier responses. The number of large worm foci (R+++ to R++++) seen earlier (Fig. 6.2.a.) was significantly decreased, well over 50% of the schistosomula recovered at 24 hr were motile and free or with only a few cells attached (R+ to R++) (Fig. 6.2.b.).

In the infected mice, in contrast to the observations obtained in challenged normal controls, the overall intensity of cellular adherence to schistosomula was not significantly altered from those at 2 hr (Fig. 6.2.a.) The majority of schistosomula elicited

major cellular reactions (R+++ to R++++). The schistosomula within the foci increased in size and assumed a rounded shape suggesting an alteration in surface membrane permeability. The schistosomula in the aggregates with R+++ to R++++ cellular reactions were no longer motile. The changes in the size and shape, and the lack of motility indicated the death of the ensheathed schistosomula.

6.2.2. Cellular adherence to schistosomula in the peritoneal cavity of mice previously infected with T. spiralis

Data comparing the adherence to schistosomula at 24 hr after inoculation in the peritoneal cavities of mice previously infected with either S. mansoni or T. spiralis are shown in Fig. 6.3. In the homologous challenge group, major focal responses developed in the S. mansoni infected group of a similar order of magnitude of cellular adherence to that reported earlier (see Section 6.2.1.2.). In contrast, the overall intensity of adherence to schistosomula in the T. spiralis infected mice was mild. The majority of schistosomula attracted only R+ to R++ cellular reactions and were generally motile. Few of the larger foci of R+++ to R++++ reactions observed in the homologous challenge group were seen in the heterologous challenge group. Nevertheless, there was significant decrease in the number of schistosomula attracting R± cellular adherence compared to those recovered from normal peritoneal cavities. This indicates that the capacity of cells to adhere to schistosomula was enhanced by a previous infection with T. spiralis.

6.2.3. Cellular adherence to schistosomula in the peritoneal cavity of passively sensitized mice

Data comparing adherence to schistosomula at 24 hr after inoculation in the peritoneal cavity of normal mice and mice previously sensitized by intravenous injection of immune serum are shown in Fig 6.4. The majority of injected schistosomula attracted only R± to R+ cellular adherence in the peritoneal cavities of normal mice. In contrast, the overall intensity in the cellular adherence, particularly at the levels of R++ to R+++, to schistosomula in the passively sensitized mice was greatly increased compared to that in untreated normal mice.

6.3. Conclusion

A prompt and marked cellular adherence to intraperitoneally injected schistosomula developed in both challenged normal mice and mice previously infected with S. mansoni (Section 6.2.1.1.). However, the continued cellular binding apparently depends on the immune status of tested mice (Section 6.2.1.2.). It was observed in schistosome infected mice, whereas most schistosomula injected into the normal mice were eventually free or attracted only mild cellular reactivities at 24 hr even though an early extensive adherence had occurred in these mice. The prolonged cellular binding could be achieved by injecting

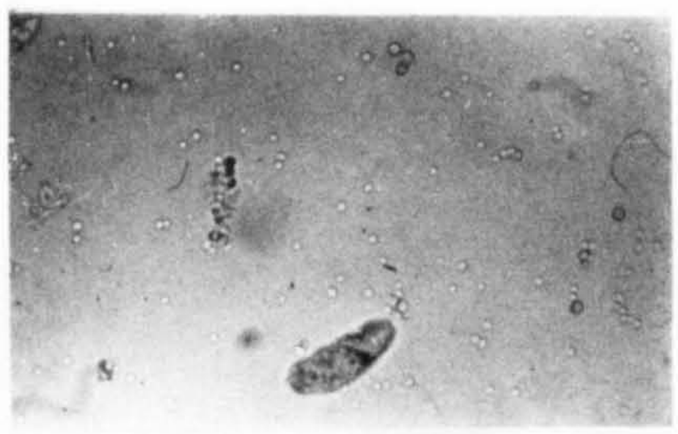
immune serum intravenously into normal mice 20 hr before challenge with schistosomula (Section 6.2.3.). An increase in cellular adherence to schistosomula was also observed in mice previously infected with T. spiralis compared to that occurring in the challenged normal mice. Nevertheless, the intensity of this non-specific cellular adherence was relatively mild compared to that demonstrated in mice infected with S. mansoni (Section 6.2.2.). Few large foci of R+++ and R++++ reactions observed in the homologous challenge groups were seen in the heterologous when examined 24 hr after inoculation of schistosomula.

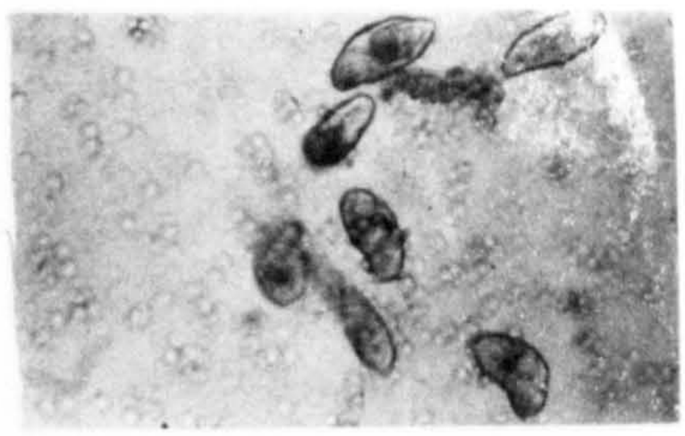
Fig. 6.1.

Plus system for scoring cellular adherence to schistosomula of S. mansoni in the peritoneal cavity

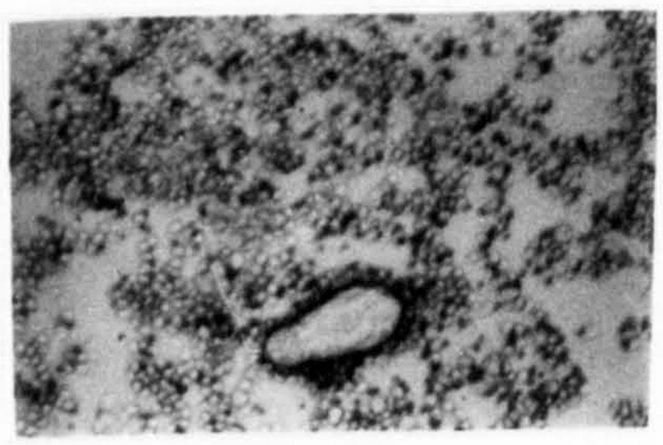
R:

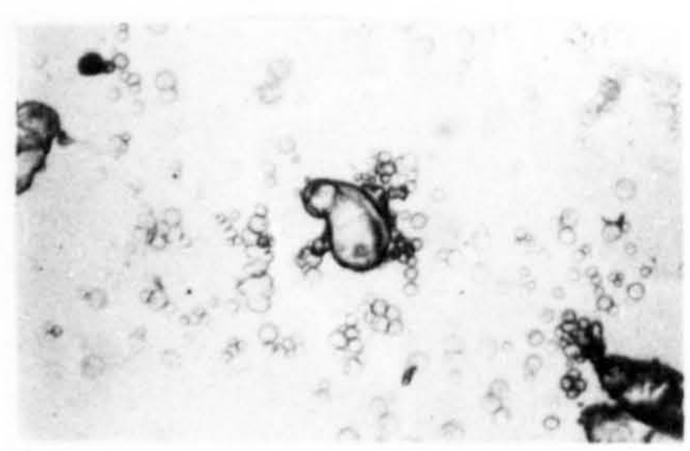
±



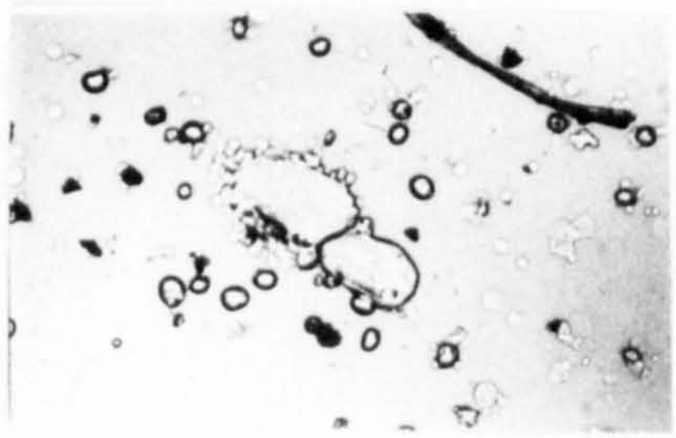


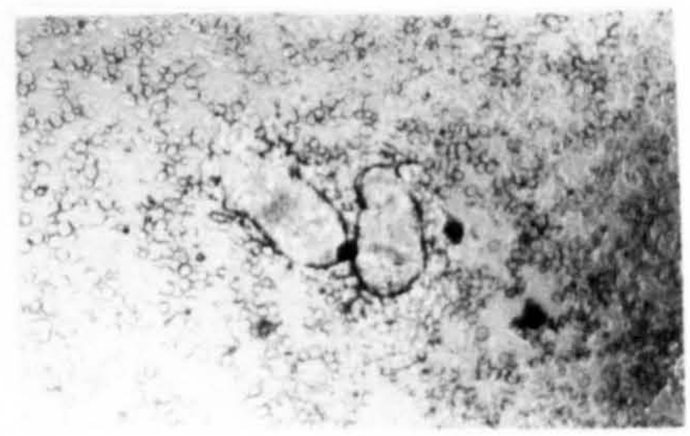
++



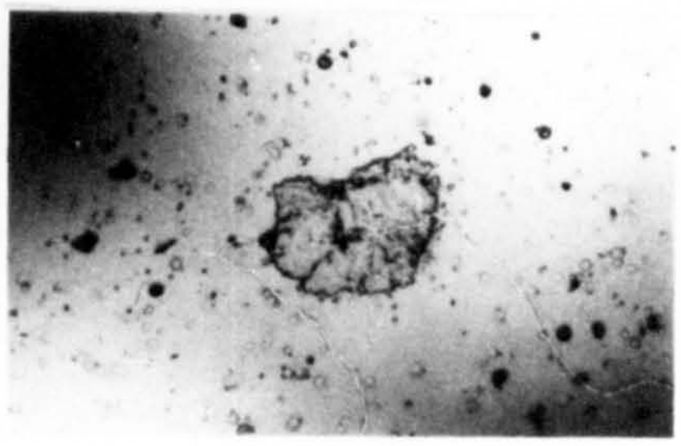


+++





++++



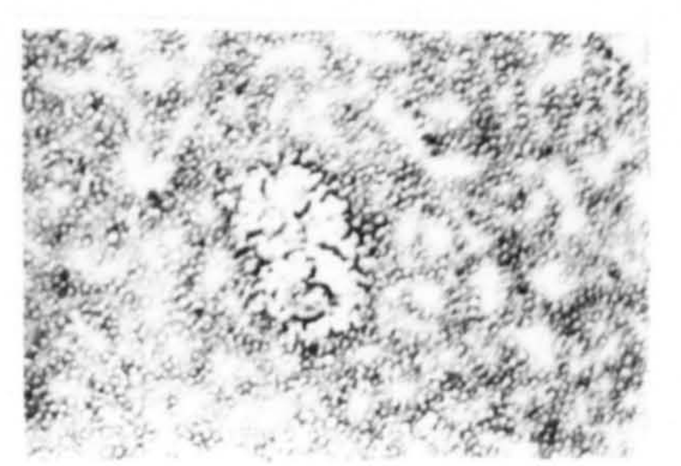
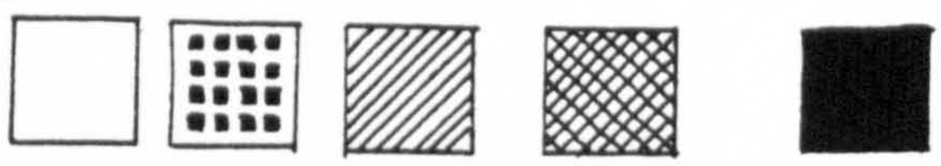


Fig. 6.2.

Time course of magnitude of cellular adherence to schistosomula of S. mansoni in the peritoneal cavities of normal and infected mice

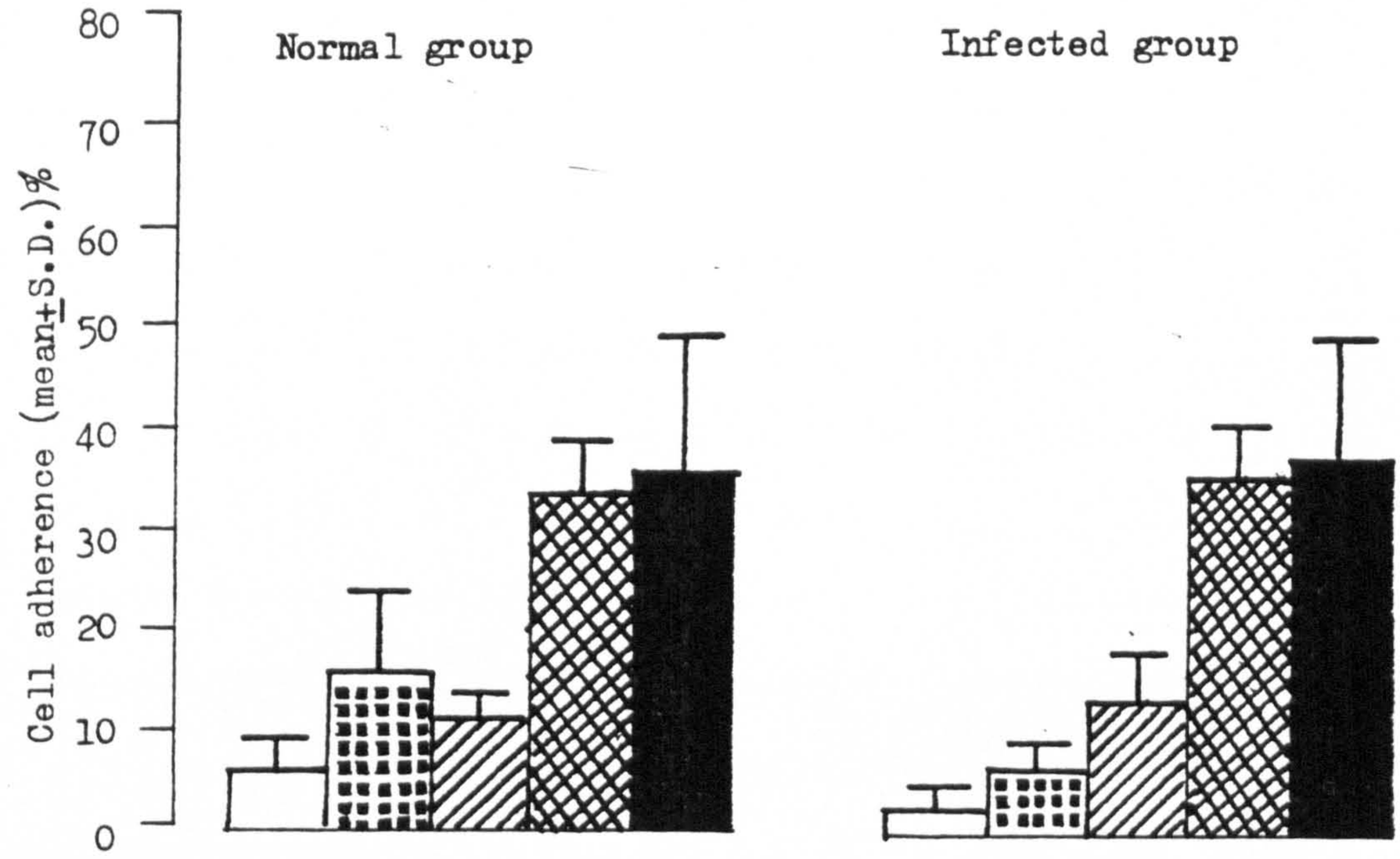
Approximately 2,000 mechanically transformed schistosomula per mouse were intraperitoneally injected into normal mice and mice infected with 50 cercariae of S. mansoni for 11-14 weeks At (a) 2 hr (b) 24 hr after challenge, groups of 4-6 mice were killed and the schistosomula together with their adhering cells were harvested by washing the cavities and viscera in the medium/heparin. Individual washings in each group were pooled and the cell reactivities to schistosomula were scored.

Each column represents mean value of cellular adherence obtained from three experiments on a scale of R± to R++++, and the vertical bars their respective standard deviations.

Column symbol: 

Adherence scale: R ± + ++ +++ ++++

(a) 2 hr


Normal group
Infected group
Cell adherence (mean±S.D.)%
80
70
60
50
40
30
20
10
0

(b) 24 hr

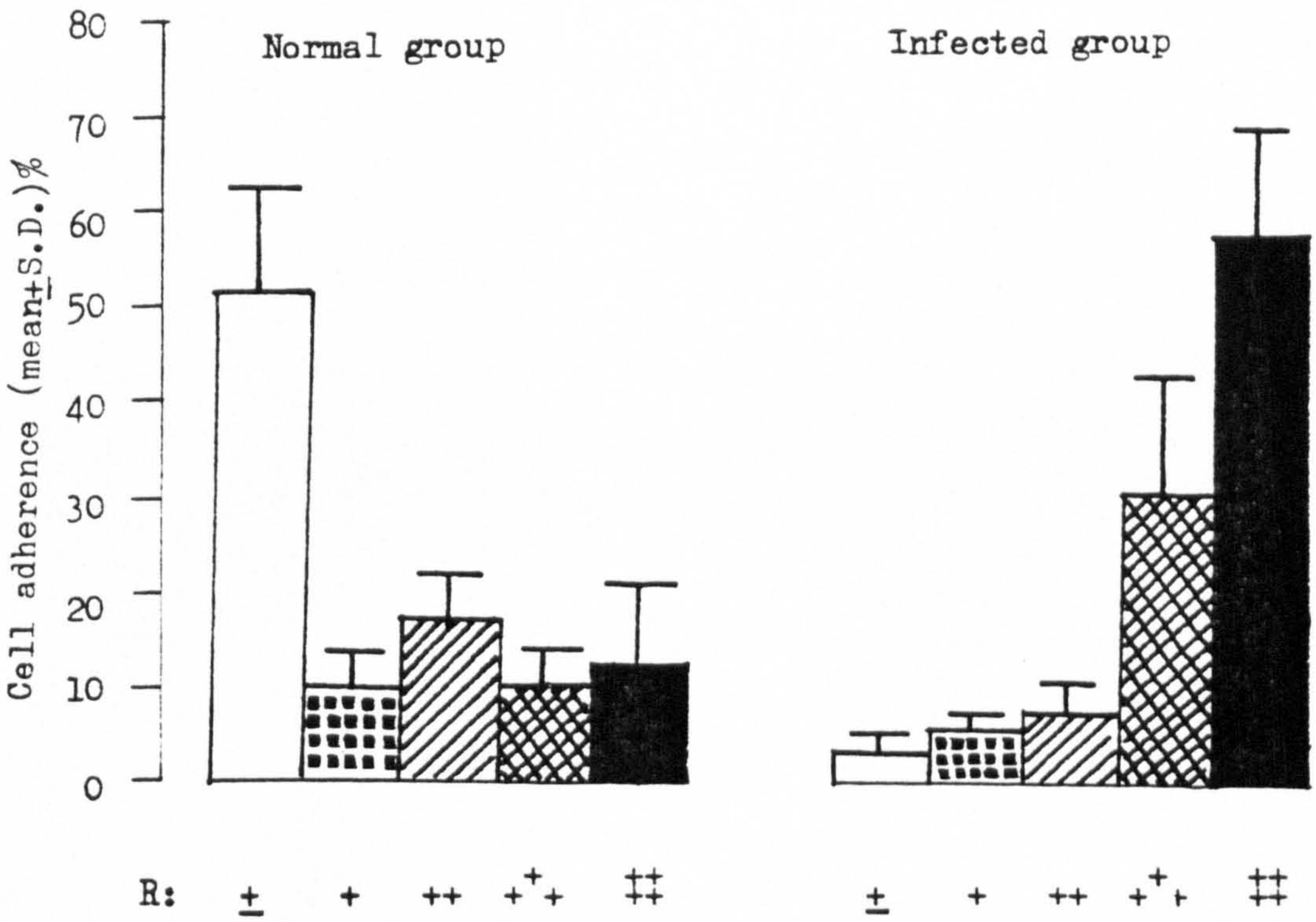

Normal group
Infected group
Cell adherence (mean±S.D.)%
80
70
60
50
40
30
20
10
0
R: ± + ++ +++ ++++
± + ++ +++ ++++

Fig. 6.3.

Cellular adherence to schistosomula in the peritoneal cavities of normal mice and mice infected with S. mansoni or T. spiralis

Approximately 2,000 mechanically transformed schistosomula per mouse were intraperitoneally injected into (a) normal mice and mice infected with (b) S. mansoni for 12 weeks, and with (c) T. spiralis for 4 weeks. At 24 hr after challenge, mice were killed and the schistosomula together with their adhering cells were harvested by washing the cavities and viscera in the medium/heparin. Individual washings in each group were pooled and the cellular adherence to schistosomula was scored.

Each column represents value of cellular adherence obtained from a single experiment on a scale of R± to R++++. Numbers in the parenthesis refer to the number of foci examined. See Fig. 6.2 for column symbols.

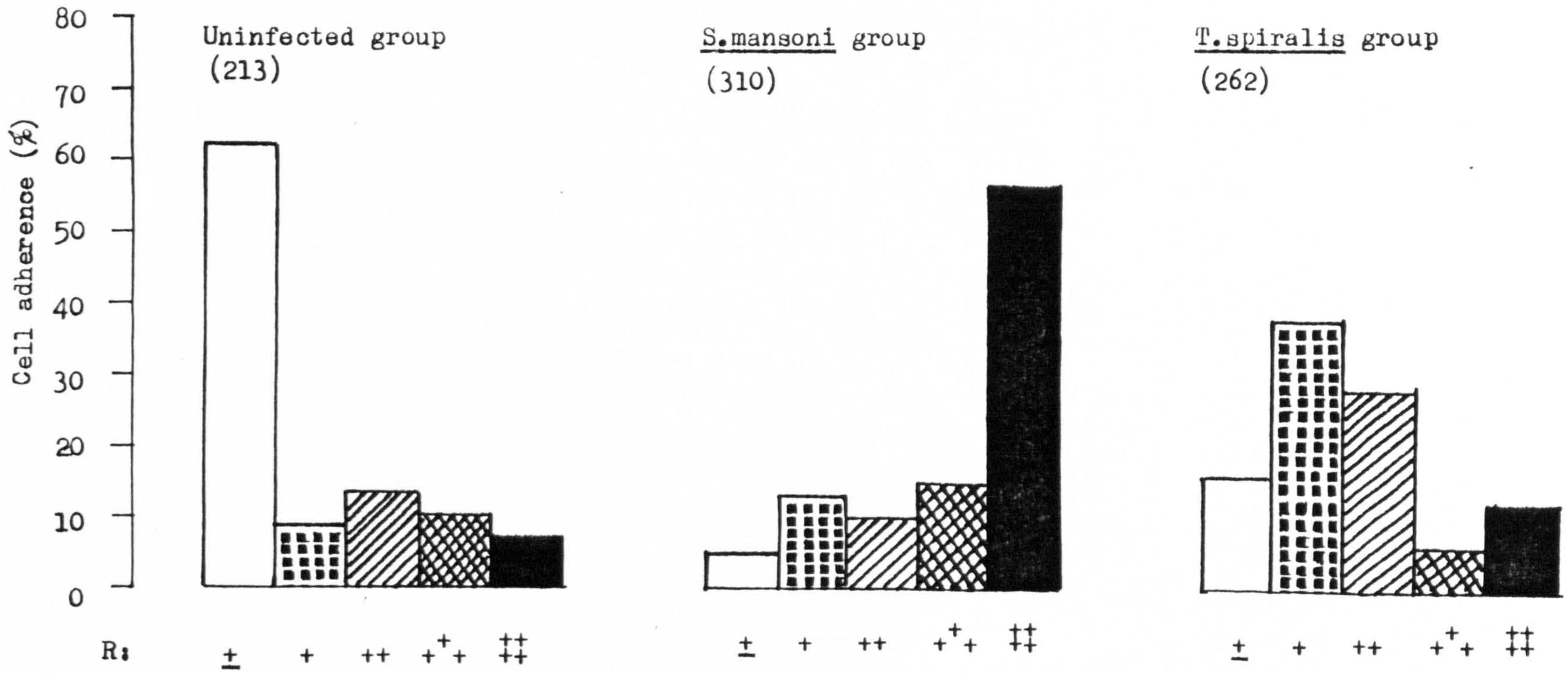
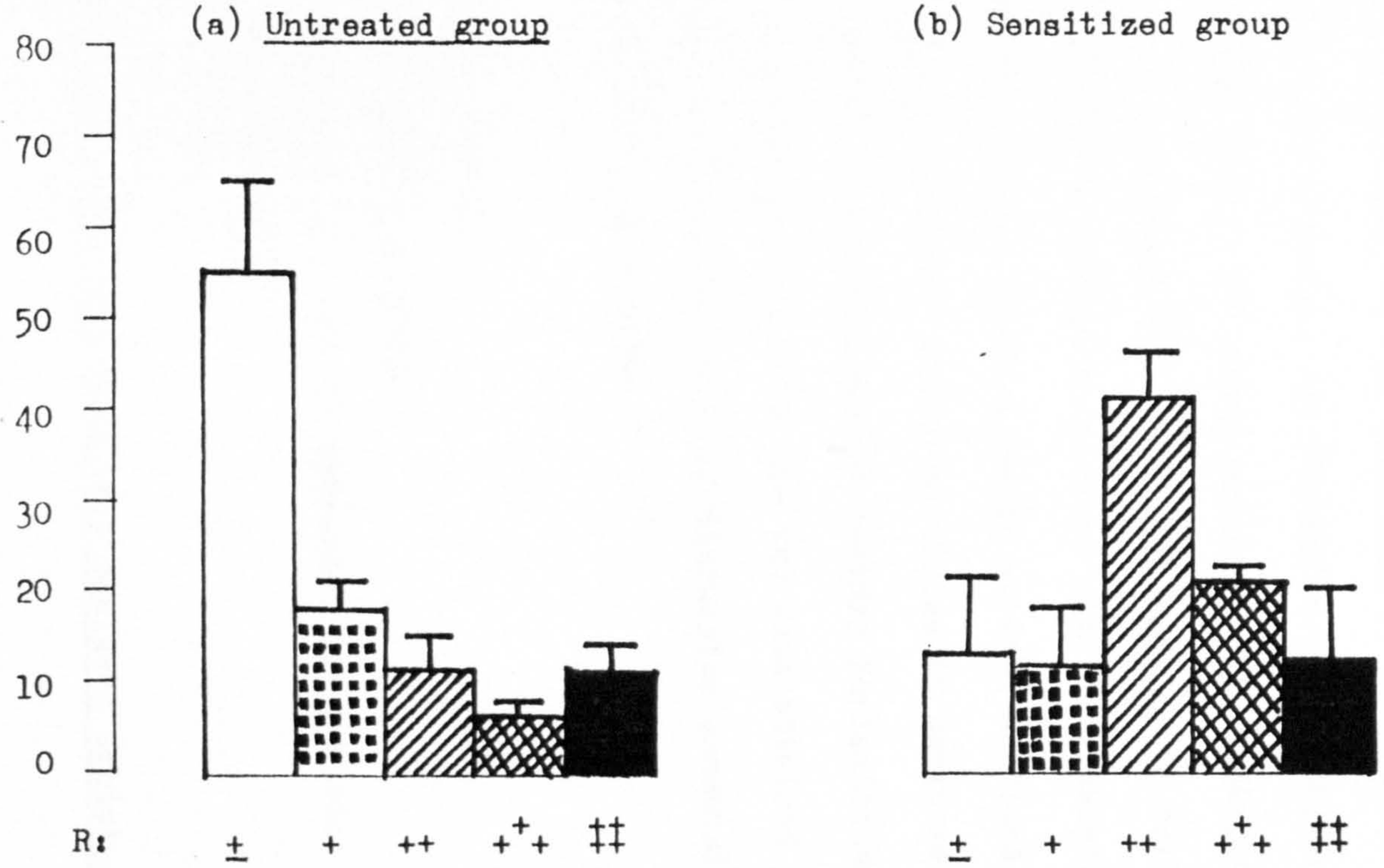

Uninfected group
(213)
S.mansoni group
(310)
T.spiralis group
(262)
Cell adherence (%)
80
70
60
50
40
30
20
10
0
R: ± + ++ +⁺+ ++

Fig.6.4.

<u>Cellular adherence to schistosomula in the peritoneal cavities of normal mice and mice passively sensitized with immune serum</u>

Approximately 2, 000 mechanically transformed schistosomula per mouse were intraperitoneally injected into (a) normal and (b) passively sensitized mice. At 24 hr after challenge, mice were killed and the schistosomula together with their adhering cells were harvested and scored.

Each column represents the mean value of cellular adherence obtained from two experiments on a scale of R± to R++++. and the vertical bars their respective standard deviations. See Fig. 6.2. for the column symbols


(a) Untreated group
(b) Sensitized group
80
70
60
50
40
30
20
10
0
R:
±
+
++
+++
‡‡

7. Identification of cells adhering to intraperitoneally injected schistosomula

The present series of experiments was directed toward defining the cells involved in the adherence to intraperitoneally injected schistosomula. The identification of adherent cells were performed by the following methods; (1) reading of cells enzymatically dislodged from worms (2) reading of cells phagocytosing schistosome antigens labelled with fluorescein. General observations of the histologically stained sections of worm foci and the uptake of carbon particles by peritoneal cells were also made. After each method devised for isolation and identification of adhering cells and the data obtained from each method are described, a brief discussion concerning the technique aspect is presented.

7.1. Experimental design

7.1.1. Infection with S. mansoni

Groups of mice infected with 50 cercariae for periods ranging from 11 to 18 weeks.

7.1.2. Harvesting of intraperitoneally inoculated schistosomula

See Section 6.1.3.

7.1.3. Preparation and photography of worm-cell foci

Both wet and cytocentrifuged smears of the peritoneal samples were prepared. Wet mounts were prepared by transfer of a few drops of worm suspension onto a glass slide and covered by a coverslip. Cytocentrifuged smears were air dried, fixed in methanol and stained with May-Grunwald-Giemsa stain in the usual way (see Section 1.1.4.2.). They were photographed with a Leitz Orthomat photomicroscope fitted with plan optics.

7.1.4. Uptake of carbon particles by cells in the peritoneal cavity

Mice were intraperitoneally injected with 10 ug/g body wt. Indian ink in PBS solution at 15 min before the harvesting of worm-cell aggregates.

7.1.5. Trypsinization of worm-cell foci

At designated times, the worm-foci were recovered from the peritoneal cavities of challenged mice. After the worms had settled under unit gravity, the free unbound cells were removed by suction using a Pasteur pipette. The sedimented pellet containing worm foci together with large cell clusters were resuspended in 0.5 ml medium and carefully layered over 2 ml of 20 % Ficoll (Sigma Chemicals Co., Dorset) in medium and centrifuged at 1,000 r.p.m. for 2-4 min depending on the size of foci in a bench centrifuge.

Under the optimal condition, the residual unbound cells from the first sedimentation at 1 x G were seen lying within the interfacial layer or upper column. The pellet containing large worm foci were collected and gently washed twice with medium and resuspended in 1 ml of medium. A trypsin solution stock (Difco Lab. Mich. U.S.A.) prewarmed to 37° C was then added which gave a final concentration of 0.13 % (w/v) after mixing with the worm foci suspension. The mixture was incubated in a 37 C water bath with gentle agitation. After a series of pilot tests, a standard incubation time of 10 min was adopted. The trypsinization was stopped by the addition of heat-inactivated (56°C/30 min) calf serum at the end of incubation. The worms together with the enzymatically removed cells were then washed twice with fresh medium. Cytocentrifuged cell smears were prepared and stained with May-Grunwald-Giemsa stain (see Section 1.1.4.2.). In order to obtain substantial numbers of cells for smear preparations, worm foci collected from individuals of each group were pooled and treated with trypsin followed by differential counts of stained cell smears.

7.1.6. <u>Preparation of wax embedded sections of worm-cell foci</u>

The large, visible worm-cell foci were picked up carefully by a Pasteur pipette. The foci were fixed in formol

saline, a mixture of saline (0.85% NaCl) and formaldehyde at 100: 1 ratio by volume, for 24 hr. The fixed specimen were then dehydrated by increasing concentration of ethanol from 30% to 90% , cleared in xylene, and embedded in paraffin wax. Sections of 4 micron (in some instances, 6 micron) thickness were cut with a Leitz stainless steel knife. After dewaxing, the sections were stained with Haematoxylin and eosin according to the method described by Culling (1963),and mounted under a coverslip using DPX mounting medium. The stained sections were examined under oil immersion.

7.1.7. Preparation of schistosomula labelled with fluorescein-wheat germ agglutinin conjugate

7.1.7.1. Preparation of wheat germ agglutinin -fluorescein conjugate

Conjugation of wheat germ agglutinin with fluorescein isothiocynate was performed according to the method described by Johnson and Holborow (1973) . Dry isothiocyanate compound (Sigma Chemicals Co., Dorset) was added to a solution containing 10 mg of wheat germ agglutinin (Sigma Chemicals Co., Dorset) per ml in 0.5 M carbonate-bicarbonate buffer (pH 9.0). The dye: protein ratio was set at 0.2: 1. The mixture was gently rotated on a magnetic stirrer for 6 hr at 20°C. This was followed by dialysis against PBS (see Section 3.1.2.) for about 18 hr at 4°C.

0.5 M carbonate-bicarbonate buffer (pH 9.0)

$NaHCO_3$	37 g
Na_2CO_3	6 g
Dist. H_2O	100 ml

7.1.7.2. Labelling of schistosomula with wheat germ agglutinin-fluorescein conjugate

One ml of freshly prepared mechanically transformed schistosomula (see Section 4.1.2.1.) suspension containing 10^5 viable parasites was incubated with 1 ml of fluorescent wheat germ agglutinin conjugate preparation for 1 hr at 37°C. The schistosomula were washed with three-changes of Eagle's essential medium (see Section 4.1.3.), and then resuspended at a desirable concentration for intraperitoneal challenge experiments. A portion of the preparation was examined immediately before experiments for fluorescence using a Leitz photomicroscope.

7.1.8. Identification of cells with cytoplasmic fluorescent inclusions

Groups of normal and chronically infected mice (infection periods ranged from 9 to 22 weeks) were intraperitoneally injected with 2,000 schistosomula labelled with fluorescent wheat germ-agglutinin conjugate. At designated times,

groups of five mice were killed. Most of the free cells were removed followed by harvesting of worm foci and cellular aggregates as described earlier (see Section 6.1.3.). Concentration was achieved by centrifugation at 1,000 r.p.m. for 2 min in a bench centrifuge. Both wet mounts and cytocentrifuged smears of the concentrated worm-cell foci were prepared. It was essential that thin smears were prepared to minimize the overlapping of unreactive cells with reactive cells.

All the preparations were examined using a Leitz photomicroscope equipped with FITC excitor and barrier filters and incident illumination from an HBO-50 mercury vapour lamp. The U.V. light microscopy was performed with 250x magnification to pinpoint the location of positive fluorescent cells or clusters. The numbers of graticule divisions over which the objects for identification fell in the centre point of the microscopic field were recorded.The brief discriptions of the clusters themselves and the particular features of the cells and worms in the same field were noted to ensure the selected targets for identification could be sought out readily after staining. After location of fluorescent cells, the smears were fixed in methanol and stained with May-Grunward-Giemsa stain (see Section 1.1.4.2.). The light microscopy was performed with 500x magnification to define the fluorescent cells in the corresponding

stained smears. Representative fields were photographed using Ektachrome 160 film (Kodak).

7.2. Results

7.2.1 General observations of peritoneal samples lavaged from mice intraperitoneally injected with schistosomula

8.2.1.1. Wet mounts of lavaged peritoneal samples

When the peritoneal samples collected at 2 or 24 hr after challenge were examined in wet mounts, cells in the aggregates with or without schistosomula appeared larger in size compared with those from challenged mice. When lavaged samples containing schistosomula were incubated at 37°C for 30 min, the larger foci with R+++ and R++++ adhered to the surface of the petri dish. This indicates the presence of macrophages in the foci. This feature was later utilised in the experiments of cellular adherence to peritoneal schistosomula *in vitro* (see Section 7.1.3.)

7.2.1.2. Stained preparations of lavaged peritoneal samples

Additional features of the cellular aggregates were detected when the wet mounts were air dried, fixed and stained. The visible large worm foci exhibiting R++++ cellular adherence consisted of two or more schistosomula ensheathed together by numerous cells. This may not

depend on the immune status of the tested mice, since the large foci were also observed in challenged normal mice.

When stained air-dried wet mounts were examined, the cells, free or bound to schistosomula, appeared as small, round and intensely coloured which resulted in the obliteration of the nuclear structure. Cytocentrifugation was found to improve their resolution. It was shown that the cells in the larger worm foci (R++ to R++++) were compacted into pavement fashion which rendered the nuclear structure and stain character difficult to discern. (Plate 7.1.). The worm foci exhibiting R+ also were not the suitable targets for direct identification of adherent cells since those cells directly encroaching on the tegument though appearing swollen when examined in wet mounts, became small or flattened and intensely coloured after fixation and staining (Plate 7.2.).

Many smalI cell aggregates without the suggestive presence of schistosomula were seen in the lavaged peritoneal samples (Plate 7.3).Often many of these clusters consisted of 5-30 cells and their cytocentrifuged preparation displayed identifiable cell compositions which were more easily defined than those presented by the worm foci after staining with May-Grunwald-Giemsa stain. These aggregates exhibited prominent mononuclear cells and a few granulocytes: (Plate 7.4.). Macrophage activity was indicated by

marked cytoplasmic vacuolation and by having remnants of ingested materials. Occasionally, ingested whole erythrocytes and neutrophils could be recognized . The nuclei of macrophages varied in size and shape , and multinucleated forms were not uncommon in peritoneal samples regardless of the immune status of the tested mice. Occasionally, free metachromatic granules were found interspersed among the aggregates or within inclusions in the cytoplasm of macrophages (Plate 7.5.) in either challenge group . Such evident mast cell degranulation was more often observed in normal mice where the number of mast cells was larger (see Section 1.2.2.5.). The free mast cell granules were never detected in unchallenged mice.

Some of the cell-ensheathed schistosomula appeared swollen. This may indicate the altered permeability of parasitic membrane. These worms adopted eosinophilic staining instead of basophilic staining character demonstrated by untreated worms. A few apparently intact worms free of bound cells were observed in peritoneal samples recovered at any time (2, 12 and 24 hr) after challenge in normal and infected mice.

7.2.2. Composition of enzymatically dislodged cells from worms

7.2.2.1. Morphology of the dislodged cells

The adherence of normal cells to worms appeared to be

less intimate than that achieved by cells of infected mice. In general, most of the adhering normal cells (>90%) could be removed easily by treating the worm foci with 0.13% (w/v) trypsin and incubated at 37 C for 10 min. The enzymatic removal of adhering cells from previously immobilized worms resulted in free and rapid thrashing schistosomula. In contrast, the cells could not be removed from worms recovered from infected mice at 30 min by the trypsin treatment described. The prolonged incubation up to 40 min or at increased trypsin concentration to 0.25% (w/v) achieved only marginally superior results and the dislodged cells appeared to be swamped in fibrin-like mesh (Plate 7.6.). Aggregates collected at 2 or 24 hr failed to respond even to the harsher enzymatic treatment described above; most cells still adhered to worms or dislodged as cell clusters.

The binding of cells to worms appeared to cause the loss of integrity of cells since an enhanced disruption rate of the enzymatically dislodged cells was observed during the cytocentrifugation at 750 r.p.m. for 5 min, a standard precedure which normally caused no or little mechanical damage to the peritoneal cells. Most of the survivors on the cell smears had increased cell size as well as the cytoplasm/nucleus ratio, a hypoosmotic-shock-like phenomenon, though most of the cells still retained their respective nuclear shapes and stain characteristics.

The increased fragility of cells after binding to the schistosomula could be due to a direct injurious action of parasitic tegument constituents on the cell membrane or result from a consequence of parasiticidal activities of cells themselves, for example, the external cell membrane that adheres to the parasite tegument could have been substituted for another one with different permeability properties, due to the merging of digestive vacuoles with the outer cell membrane or during cell granule secretion.

The possibility of enzymatic digestion of cell membrane structure and hence the cell rupture is ruled out, since negligible variation in cell size was obtained in cytocentrifuged preparations of cells pretreated with 0.13% (w/v) trypsin for 10 min at 37°C, a standard procedure to strip cells from schistosomula to which they adhered, compared with untreated cells. Thus, the fragility of bound cells may reflect the <u>in vivo</u> change during the contact with schistosomula rather than enzymatic injury to cells <u>in vitro</u>.

7.2.2.2. <u>Identities of the dislodged cells</u>

The method of recovering adherent cells from schistosomula enzymatically proved unsatisfactory. Although, with care, pure worm foci with negligible contaminating unbound cells could be achieved by the one-step Ficoll centrifugation, the dissociation of cells from schistosomula was suscessful only

with the aggregates harvested within 30 min after inoculation of schistosomula in both challenged groups and with limited success with 6 hr old aggregates from normal mice.

At 30 min, macrophages ranging from 74% to 86% were the predominant cell type in the cell population dislodged from schistosomula recovered from normal or infected mice (three experiments) (Fig. 7.1.a).

The analysis of granulocytes showed a quantitative difference in the proportions of eosinophils and neutrophils involved in the adherence in normal and infected groups. Eosinophils averaged 6% in the normal to 13% in the infected mice, whereas neutrophils averaged 8% in the normal to 3% in the infected mice.

The trend of neutrophil-active response in the normal mice continued and increased at 2 hr reaching 19% of the reactive cell population (Fig. 7.1.b.). This augmentation occurred correspondingly with the initiation of the increase in neutrophils (see Section 4.2.1.1.). However, the aggregates collected at 6 hr did not show a further increase in the magnitude of the neutrophil activity in parallel, giving 14% (Fig. 7.1.c.), when the maximal increase in neutrophils occurred and accounted for 32-64% of the unbound cell population (see Section 4.2.1.1.). Due to the limited number of aggregates available at 24 hr after inoculation of schistosomula (see Section 6.2.1.2.), the

identity of cells adherent to schistosomula at that time in the normal mice was not investigated.

7.2.3. Histological observations of worm-cell foci

The simple histological technique using haematoxylin and eosin stains allowed semi-quantitative analysis of cellular composition in paraffin embedded sections of worm foci.

In general, the schistosome section area was demarcated from the surrounding fibrocellular stroma by an intensively stained band (Plate 7.7.). The orientation of the stained cells in relation to the entrapped worms was obvious.

The overall cellular architecture progressed from the loosely arranged cells in the peripheral to the compact in the center of the foci. Some of the surrounding cells were seen in vacuoles (Plate 7.7.). Whether this vacuolation represented a walling off effect of parasitic membrane due to the cellular activity, or merely an artifact due to the fixation procedure is not clear. At 2 and 24 hr after inoculation, the foci were composed of mostly mononuclear cells and a few granulocytes in both normal and infected mice (Plate 7.8.; 7.9.).

7.24. Uptake of carbon particles by cell aggregates

Cell smears prepared from peritoneal samples collected from normal mice at 15 min after injection of carbon

particles showed that macrophages were the most avid phagocytic cells whereas no more than a dozen carbon particles were seen in the neutrophils. Intracellular carbon particles were rarely seen in the eosinophils.

When the worms were recovered at 2 hr after inoculation from mice injected with carbon particles 15 min previously, the majority of worms collected from either challenge group were enmeshed in carbon-containing cells (Plate 7.10.) Some were so heavily ensheathed that the light reflected from the carbon mass when photographed.. This supports the suggestion of involvement of macrophages in the adherence to schistosomula. Apparently, the carbon particles have a toxic effect to macrophages and/or other types of phagocytic cells. This may result in the rupture of cells hence accounting for the carbon deposit around the schistosomula and the loss of the cellular integrity (Plate 7.11.).

It is not known whether the carbon- phagocytosis by cells occurred before or after their adherence to worms. It is also suspected that alteration of surface properties of phagocytosing cells have taken place. The The clumping of neutrophils was observed in the peritoneal samples harvested from normal mice after injection of both carbon particles and schistosomula (Plate 7.12.). This appeared

to be an artifact, since this feature was never observed in the peritoneal samples collected from mice challenged with schistosomula without coinjection with carbon particles. Therefore, the carbon injection method should serve only as a guide to identity and was not routinly used in quantitative assays regarding the involvement of macrophages in adherence to schistosomula.

7.2.5. Composition of cells phagocytosing fluorescent schistosomal surface materials

Small aggregates consisting of 5-50 cells without the suggestive presence of schistosomula were often seen in the peritoneal samples. It has been suspected that the cells originally adhered to but subsequently dislodged from schistosomula. This dislodgement may be caused by either lavage manipulation or that the cells dislodged themselves together with their bound worm membrane. Therefore, the demonstration of intracellular worm materials would establish their roles in the adherence and phagocytic activity. To do this, schistosomula were coated with fluorescein-tagged wheat germ agglutinin prior to the inoculation into the peritoneal cavity of mice.

7.2.5.1. Qualitative findings of fluorescent schistosomula and cells

Schistosomula in wet mounts were immobilized by chilling

the samples at 4°C for 15 min to facilitate the microscopic examination and photography. It was shown that schistosomula reacting with fluorescein conjugated wheat germ agglutinin for 1 hr at 37°C exhibited uniform stained membrane and bright fluorescent areas around the mouth and ventral suckers (Plate 7.13.). Dead schistosomula obtained from old cultures assumed an intense patchy staining character after the fluorescent labelling procedure, and were thereby distinguishable from the homogenous staining shown by the live schistosomula presented in the same preparation (Plate 7.14.). The worm materials within phagocytosing cells were seen as fluorescent grains which rendered the active cells distinguishable from the autofluorescent control cells (cells collected from unchallenged mice) which gave a faint yellow glow under the U. V. light.

The number of fluorescent grains, ranging from a few to many could be seen in the phagocytosing cells.

Fluorescent microscopic examinations of cytocentrifuged smears revealed that little loss of the fluorescent label from the schistosomula and cells had been induced by cytocentrifugation, thus representative films of cyto-centrifuged smears were taken and used to illustrate the qualitative and quatitative findings described here.

By 30 min after inoculation of the labelled schisto-somula, cellular adherence occurred in all the mice. It was clear that the cells adhering to the worms were

phagocytosing as indicated by their intracytoplasmic fluorescent inclusions (Plate 7.15ab.). Cellular aggregates of various sizes with or without the presence of schistosomula in the center and some free single cells had taken up the labelled worm surface materials. Some were so loaded that the ; ruptured when fixed with methanol and became unstainable. Large globule-like objects exhibiting fluorescence were presented in all the washings (Plate 7.16.). Their nature was not determined.

At 2 hr, the elimination of bound fluorescent lectin from the surface of some entrapped or free schistosomula was observed in peritoneal samples collected from normal and infected mice. A few worm foci well advanced in the process of killing activity were observed at the time (Plate 7.17a.). The fluorescent worm debris and cells appeared to concentrate in the centre of the whole body while few cells at the periphery of the foci were fluorescent (Plate 7.17b.).

After 24 hr, all the free schistosomula recovered from challenged mice had lost their fluorescent label and were therefore barely visible in the U. V. light microscope. The denuded schistosomula constituted the majority of the population recovered from normal mice, and few cells had intracytoplasmic fluorescent grains. The observation of reduction in cellular reactivity in normal mice was in

aggreement to the discriptioms already presented (see Section 6.2.1.2.). In contrast, the dispersed fluorescent single cells and small cellular aggregates were the major feature of samples from the infected group. These cells yielded only faint fluorescent dots . As observed earlier, the denuded schistosomula free of bound cells were rarely seen in the samples collected from infected mice (see Section 6.2.1.2.).

7.2.5.2. Quantitative findings of fluorescent cells

The comparative analysis of the compositions of reactive cells to intraperitoneally injected schistosomula in normal and infected mice was performed using small fluorescent aggregates of 5 to 30 cells. Since it is technically difficult to obtain substantial numbers of identifiable aggregates for individual value determinations, several aggregates were selected from each sample and approximately total 100-600 cells from either challenged group were identified.

From the results of two experiments presented in Fig.7.2., it is discerned that the host reaction to 2-hr old schistosomula was basically similar in both normal and infected mice (Fig. 7.2a.). Macrophages were the predominant cell type and accounted for 70-80 % of the reactive cells.

At 24 hr after inoculation, most schistosomula recovered from normal mice were free of bound cells. The cells which took up fluorescent materials were mostly macrophages

and accounted for 70% of the population (Fig.7.2 b.). In contrast, in the 24 hr-old cellular aggregates recovered from infected mice, the number of eosinophils was greatly increased sharing equal proportion with macrophages (Fig. 7.2 b.). At this time, the secondary increase in eosinophils occurred in the challenged infected mice (see Section 4.2.1.1.). It was also noted that fewer neutrophils were involved in the phagocytic activity compared with that demonstrated at 2 hr in both groups (Fig. 7.2b.). Many cells seen in the aggregates appeared to be spent or degenerating and were thus difficult to classify.

7.3. Conclusion and discussion

Differential analysis of cells involved in the adherence and phagocytosis in the peritoneal cavity has been performed using two methods: readings of enzymatically dislodged cells and of cells phagocytosing fluorescent labelled schistosomula surface materials. The advantages and disadvantages of each method are described here.

(1) Enzyme method (Section 7.2.2.); this method included differential gradient centrifugation to recover worm foci followed by enzymatic removal of adhering cells from schistosomula and their identification. This procedure was proved to be of limited success. One of the major problems was the poor yield of cells due to the recovery of limited numbers of worm-cell foci. The relatively high Ficoll

concentration (20%) used in the gradient separation recovered only larger foci with R+++ and R++++ cellular adherence. The intermediate or smaller sizes of aggregates were excluded. The multi-layer discontinuous density gradient may be applied to improve the yield of aggregates. The second problem was the limited success in dislodging adhering cells from schistosomula by trypsin. Only cells of young foci (30 min) could be dislodged under the conditions described.

(2) Fluorescent worm materials uptake method (Section 7.2.5.); this method included the labelling of schistosomula with fluorescein-wheat germ agglutinin conjugate, intraperitoneal injection of labelled schistosomula, followed by identification of cells taking up fluorescent worm materials. The smears of fluorescent cells were stable and could be stored in the dark for at least 3 days without appreciable decrease in the fluorescence intensity, thus obviating the need of immediate staining and photography. The intracytoplasmic fluorescent grains did not affect the staining characteristics of the phagocytic cells by the routine method, they appeared as dirty green smudge inclusions when stained. However, the operations of location and identification of phagocytic cells are inevitably time consuming.

The data obtained from the two methods showed that macrophages were the predominant cell type involved in

the adherence and phagocytosis during the early hours after inoculation of schistosomula in both challenge groups. The marked macrophage activity was also evident in the study using histological method (Section 7.2.3.) and carbon particle uptake probe (Section 7.2.4.). However, at 24 hr after challenge, the intensive adherence and phagocytosis were observed only in the mice previously infected with S. mansoni. In these mice, the eosinophils were active and shared approximately equal proportions with macrophages in the active cell populations. In contrast, most schistosomula recovered from normal mice were free or attracted only a few cells hence few cells exhibiting fluorescent inclusions. The cells involved in phagocytosis were mainly macrophages. An additional interesting feature was the loss of fluorescent label from the surface of schistosomula recovered at 24 hr after inoculation (Section 7.2.5.1.). This will be discussed later.

Fig. 7 .1.

Time course of preferential adherence to schistosomula by various cell types in the peritoneal cavity of normal mice and mice infected with S. mansoni (the cells were enzymatically dislodged from schistosomula before identification)

Approximately 2,000 schistosomula were intraperitoneally injected into normal mice and mice infected with 50 cercariae for 9, 11 or 14 weeks (three experiments). At (a) 30 min, (b) 2 hr and (c) 6 hr after challenge, mice were killed and the peritoneal cavities were lavaged. Individual washings in each group were pooled and the large foci were collected by the one-step Ficoll centrifugation. The cells were then removed from the schistosomula they adhere to by treating the foci with trypsin. The cyto-centrifuged smears of the dislodged cells were subsequently prepared, stained and identified.

Each column represents mean value of cell compositions obtained from three experiments, and the vertical bars their respective standard deviations. Open column shows the values obtained from uninfected mice and the closed column from infected mice. M: macrophages; E: eosinophils; N: neutrophils; Ot: unidentified degenerating cells.

ND: not done due to lack of materials.

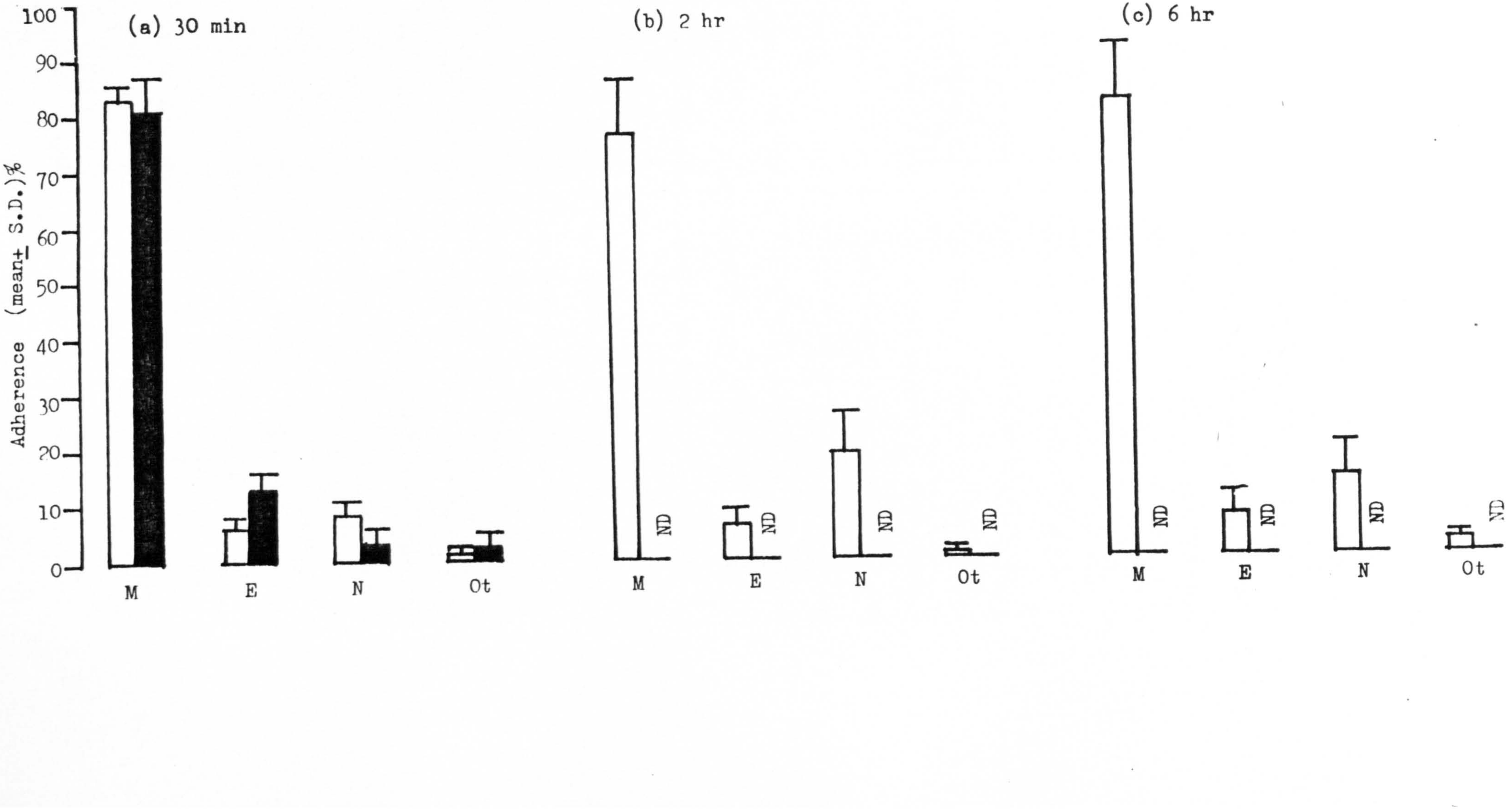
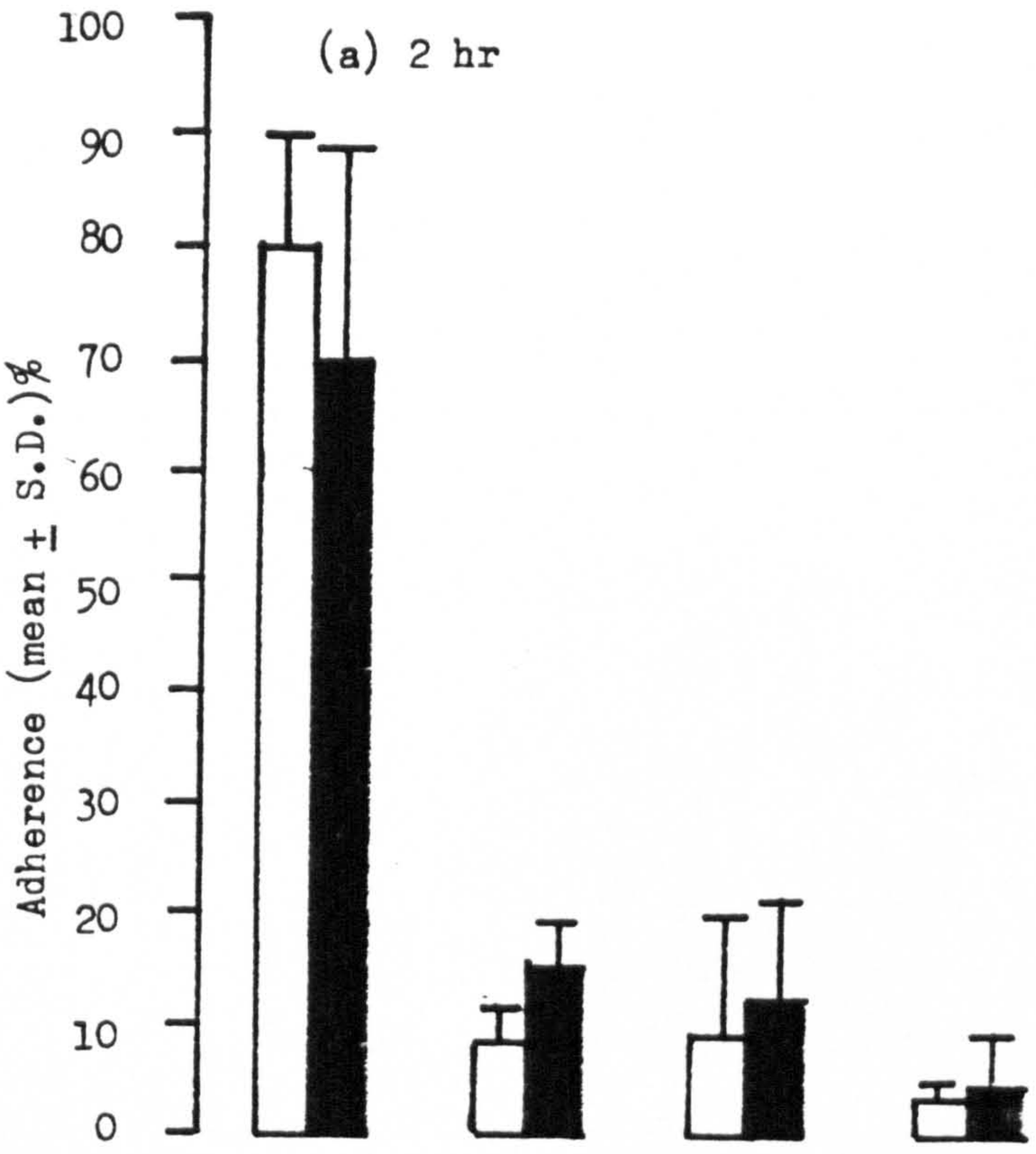

(a) 30 min
(b) 2 hr
(c) 6 hr
Adherence (mean± S.D.)%
100
90
80
70
60
50
40
30
20
10
0
M
E
N
Ot
ND

Fig. 7.2.

Time course of preferential adherence to schistosomula by various cell types in the peritoneal cavity of normal mice and mice infected with S. mansoni (Identity of cells determined on the basis of cytoplasmic fluorescent inclusions)

Approximately 2,000 schistosomula labelled with fluorescein tagged wheat germ agglutinin were intraperitoneally injected into normal mice and mice infected with 50 cercariae for 8 or 18 weeks (two experiments). At (a) 2 hr and (b) 24 hr after challenge, mice were killed and the fluorescein-uptake cells were harvested by peritoneal lavage. Individual washings in each group were pooled and the cells containing fluorescent inclusions were identified.

Each column represents mean value of cell compositions obtained from two experiments, and the vertical bars their respective standard deviations. Open column shows the values obtained from uninfected mice and the closed column from infected mice. M: macrophages; E: eosinophils; N: neutrophils; Ot: unidentified degenerating cells and non-phagocytosing cells (lymphocytes, plasma cells) which were either within or superimposed on the fluorescent cell clusters.


(a) 2 hr
100
90
80
70
60
50
40
30
20
10
0
Adherence (mean ± S.D.)%

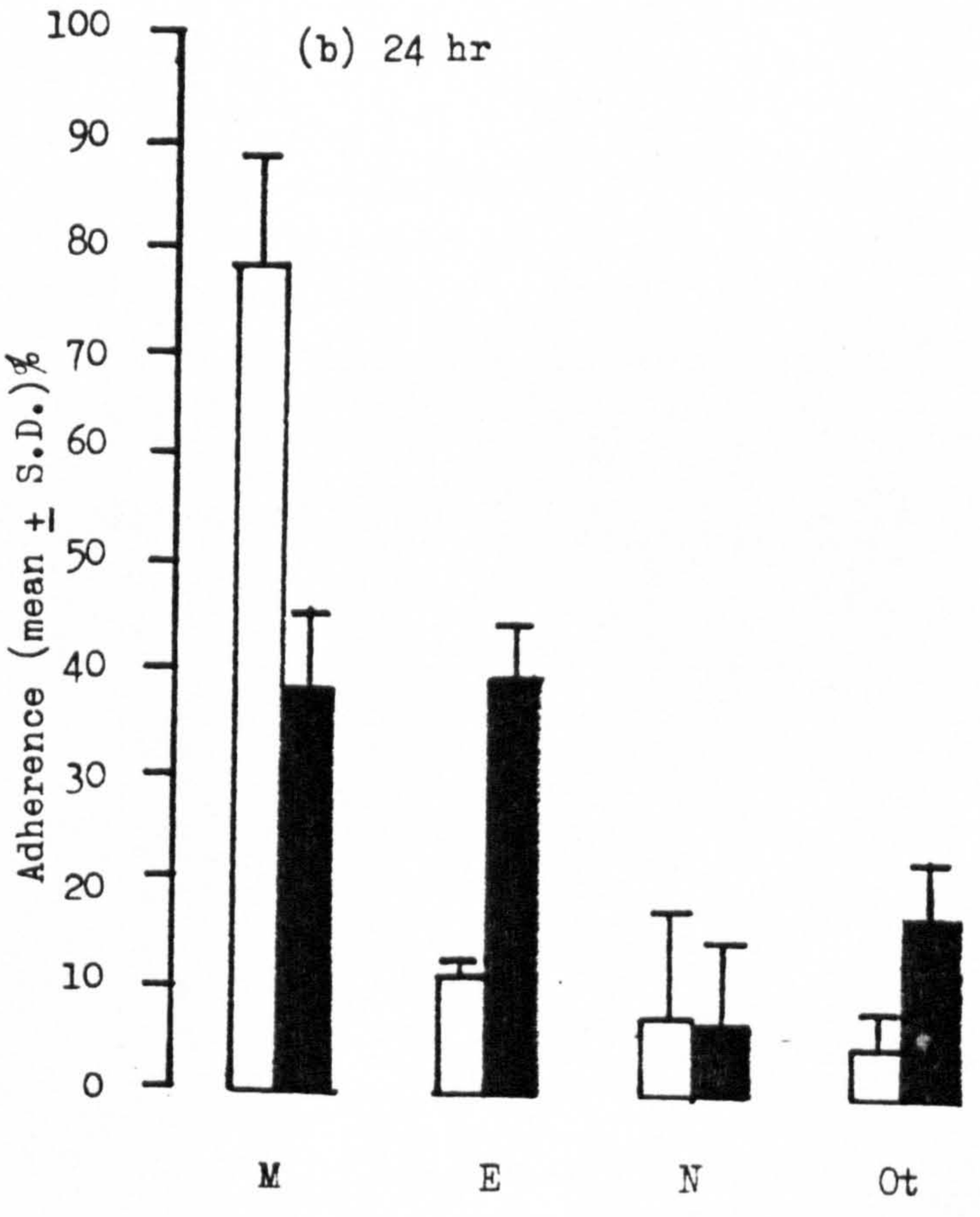

(b) 24 hr
100
90
80
70
60
50
40
30
20
10
0
Adherence (mean ± S.D.)%
M
E
N
Ot

Plate 7.1.

Photomicrograph of a schistosomulum with its attached cells (exhibiting R+++ cellular adherence; stained cytocentrifuged preparation).

Plate 7.2.

Photomicrograph of a schistosomulum with its attached cells (exhibiting R+ cellular adherence; stained cytocentrifuged preparation).

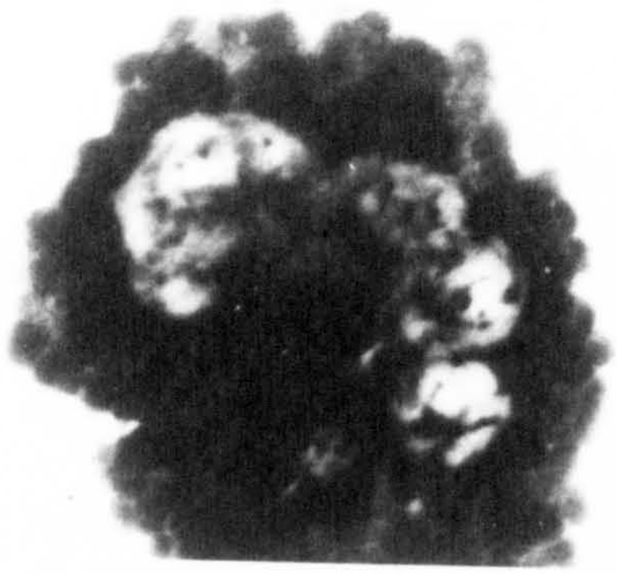


Plate 7.1.

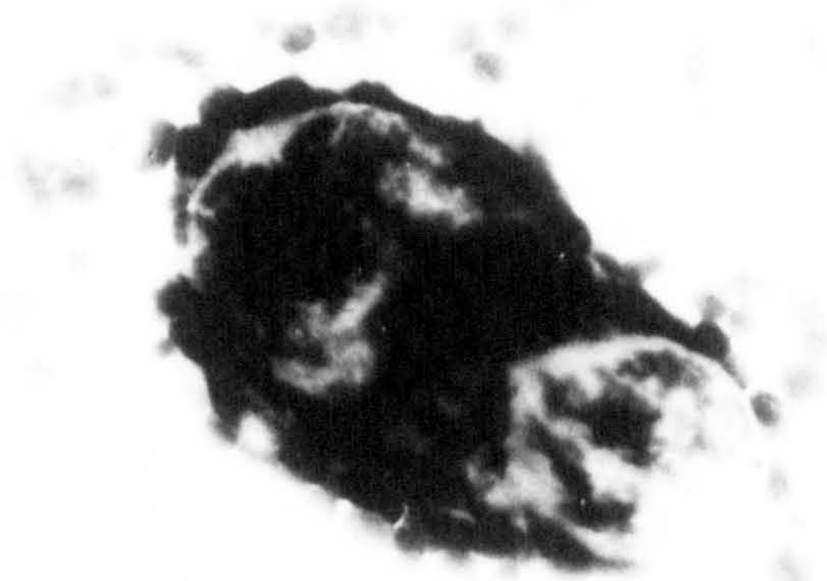


Plate 7.2.

Plate 7.3.

Photomicrograph of a schistosomulum with its attached cells together with small clusters (arrows) nearby. These were collected 2 hr after the intraperitoneal inoculation of schistosomula into normal mice (stained cytocentrifuged preparation).

Plate 7.4.

Photomicrograph of a small cell cluster consisting mostly of mononuclear cells with a few granulocytes. This was taken 2 hr after the intraperitoneal inoculation of schistosomula into 12 week-infected mice (stained cytocentrifuged preparation).

Plate 7.5.

Photomicrograph of released mast cell granules (arrows) within the phagocytic inclusion of a macrophage. This was taken at 2 hr after the intraperitoneal inoculation with schistosomula into normal mice (stained cytocentrifuged preparation).

Plate 7.6.

Representative photomicrograph of cells dislodged enzymatically from schistosomula that were recovered 30 min after the inoculation of 13 week-infected mice with schistosomula)(stained cytocentrifuged preparation).

Plate 7.3.

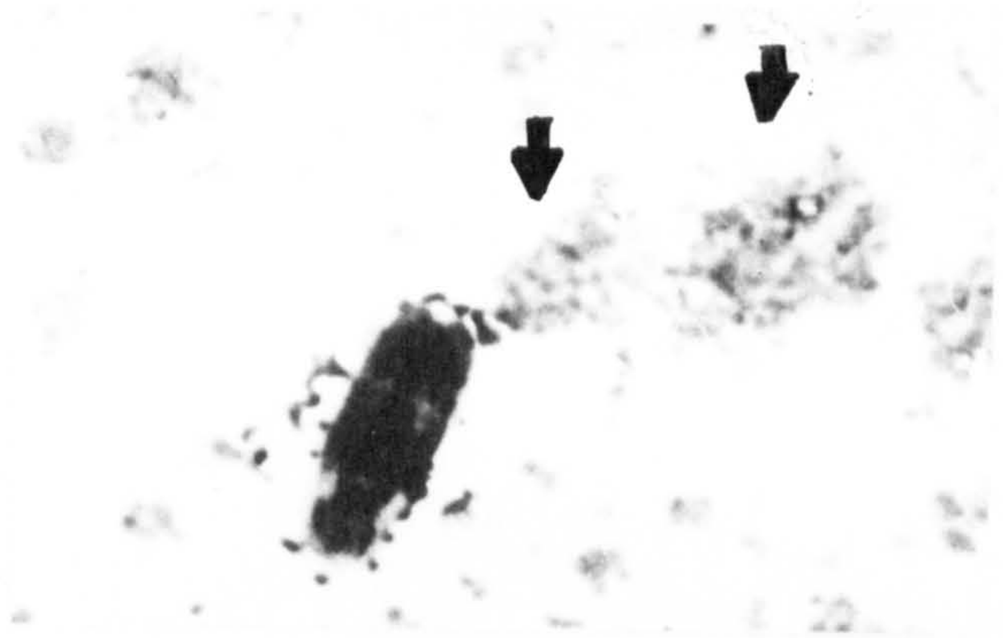


Plate 7.4.

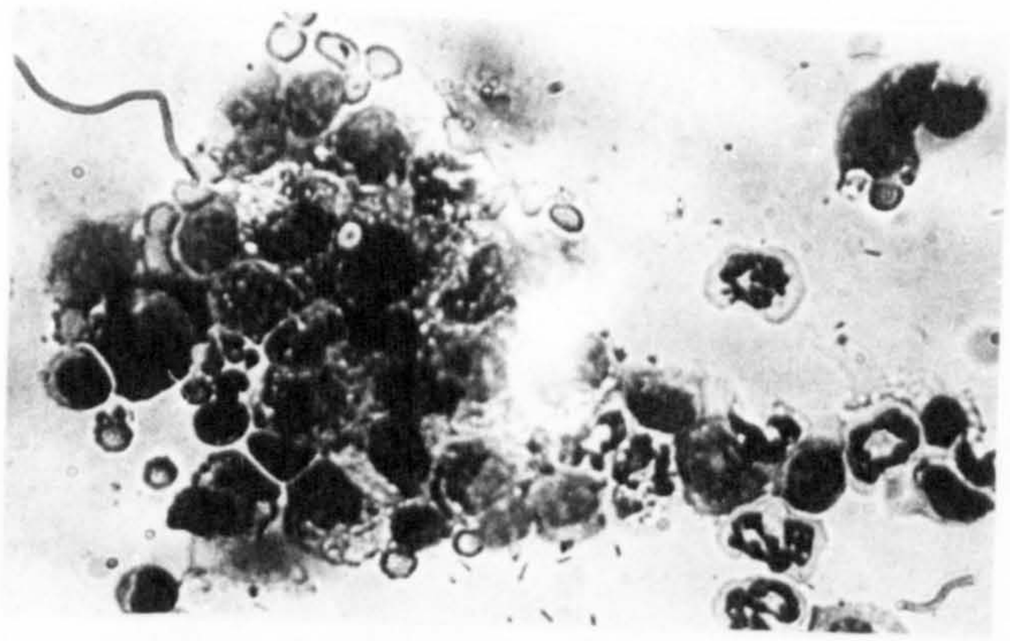


Plate 7.5.

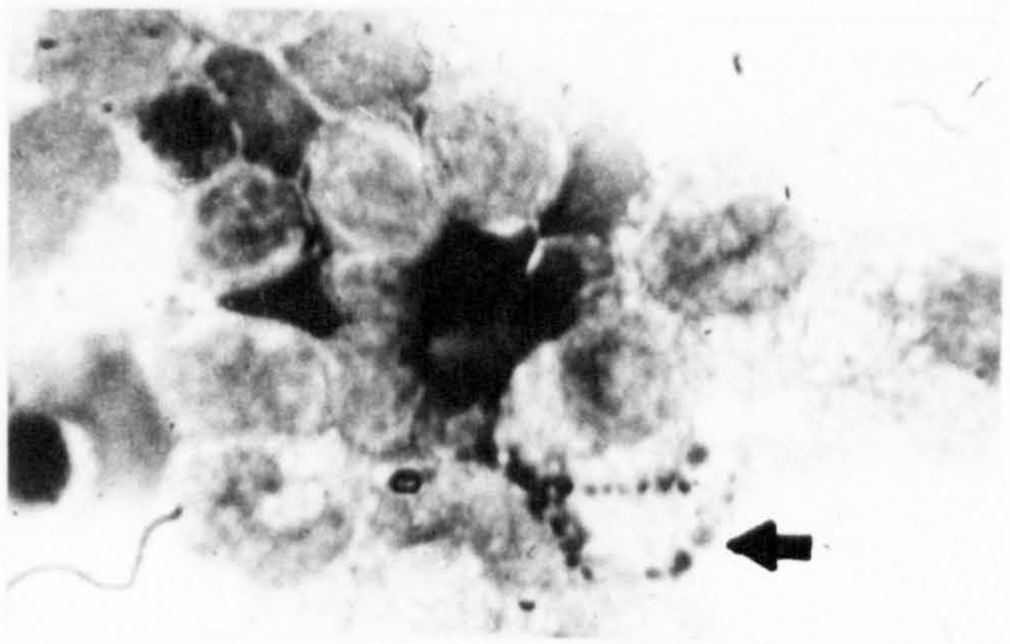


Plate 7.6.

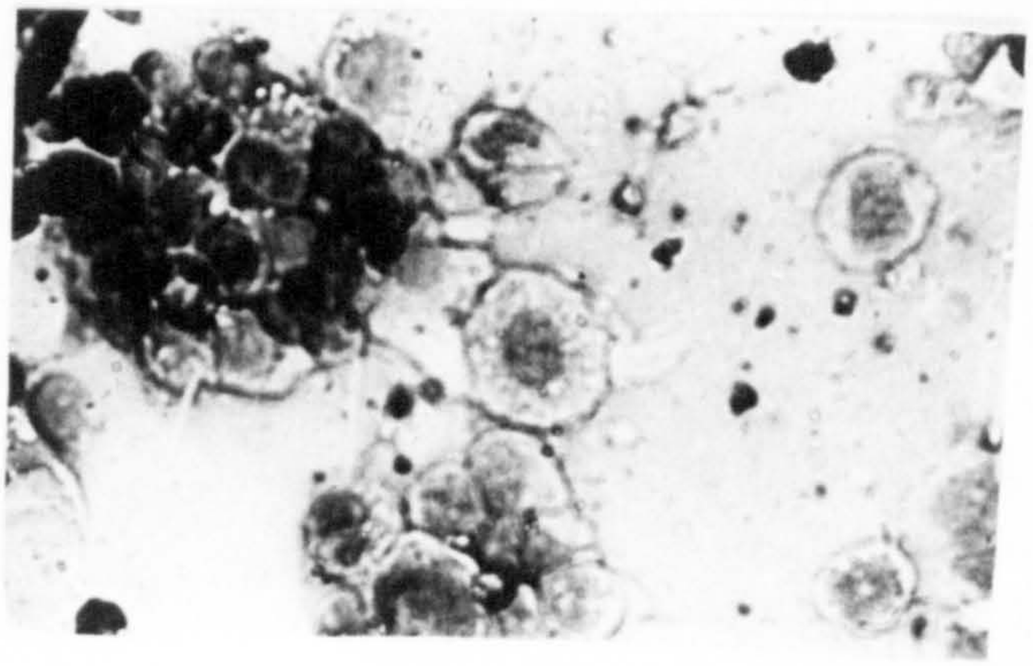


Plate 7.7.

Paraffin section (4 micron thickness) of parasite-cell focus stained with haematoxylin and eosin. It was harvested from a 10 week-infected mouse 24 hr after the inoculation with schistosomula. Some cells are within vacuoles (arrows) around the schistosomulum.

Plate 7.8.

Paraffin section (4 micron thickness) of parasite- cell focus stained with haematoxylin and eosin. It was harvested from a normal mouse 24 hr after the inoculation with schistosomula.

Plate 7.9.

Paraffin section (4 micron thickness) of parasite-cell focus stained with haematoxylin and eosin. It was harvested from an 11 week-infected mouse 24 hr after the inoculation with schistosomula.

Plate 7.7.

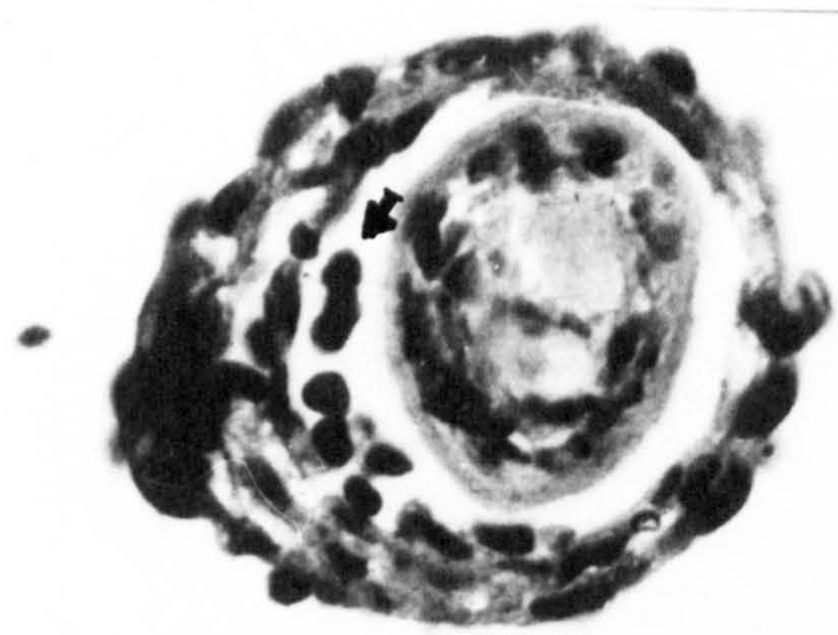


Plate 7.8.

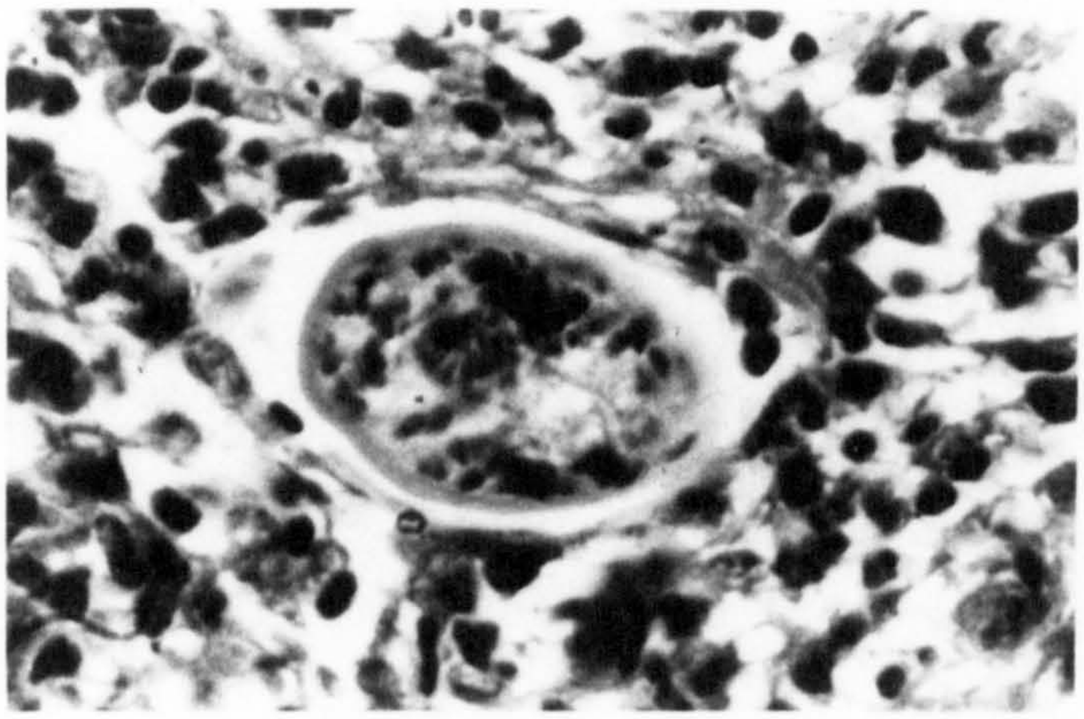


Plate 7.9.

Plate 7.10.

Photomicrograph of representative aggregates dominated by cells that have taken up carbon particles. These were harvested from 11 week-infected mice 2 hr after the inoculation with schistosomula and carbon particles.

Plate 7.11.

Photomicrograph of a schistosomulum with its attached carbon-uptake cells. It was harvested from a normal mouse 2 hr after the inoculation with schistosomula and carbon particles.

Plate 7.12.

Photomicrograph of neutrophil-dominated cell clusters. These were harvested from normal mice 2 hr after the inoculation with schistosomula and carbon particles.

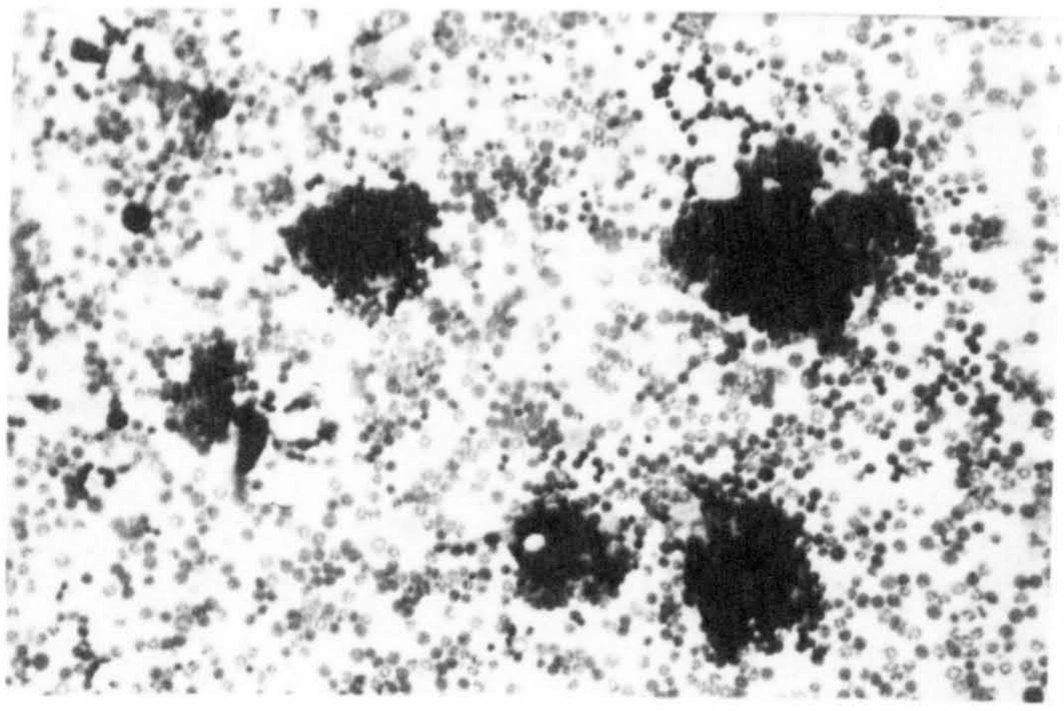


Plate 7.10

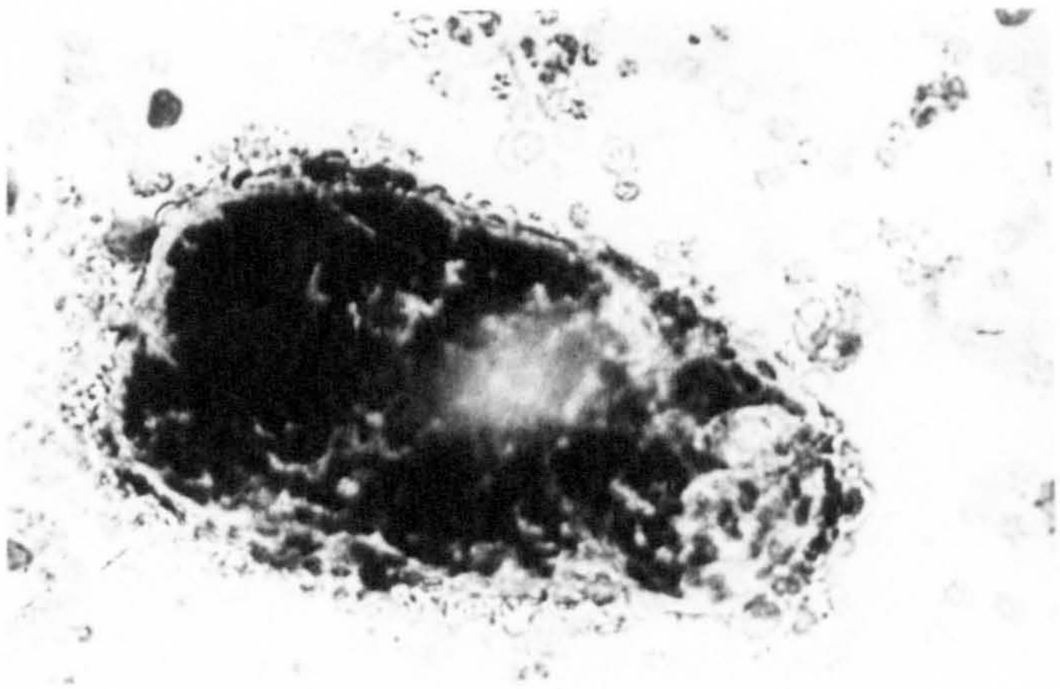


Plate 7.11.

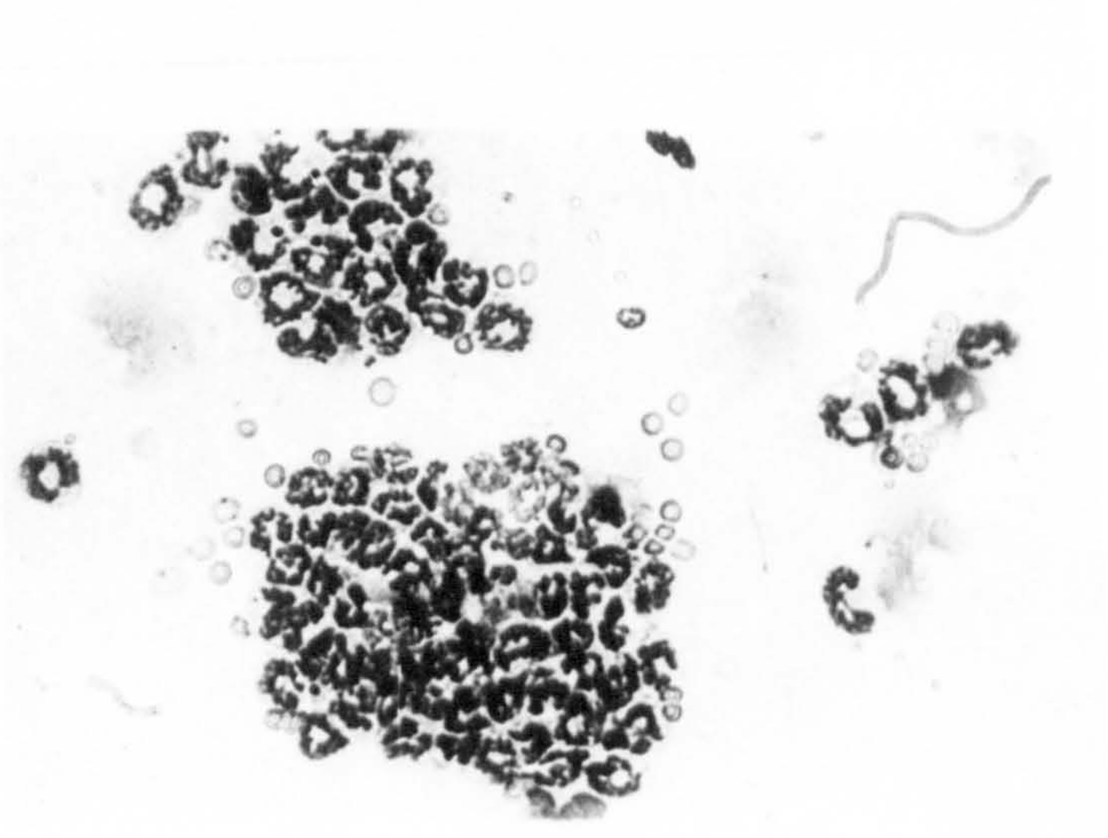


Plate 7.12.

Plate 7.13.

Photomicrograph of schistosomula stained with fluorescein conjugated to wheat germ agglutinin (wet mount preparation).

Plate 7.14.

Photomicrograph of dead (arrows) and living schistosomula labelled with fluorescein conjugated to wheat germ agglutinin (wet mount preparation).

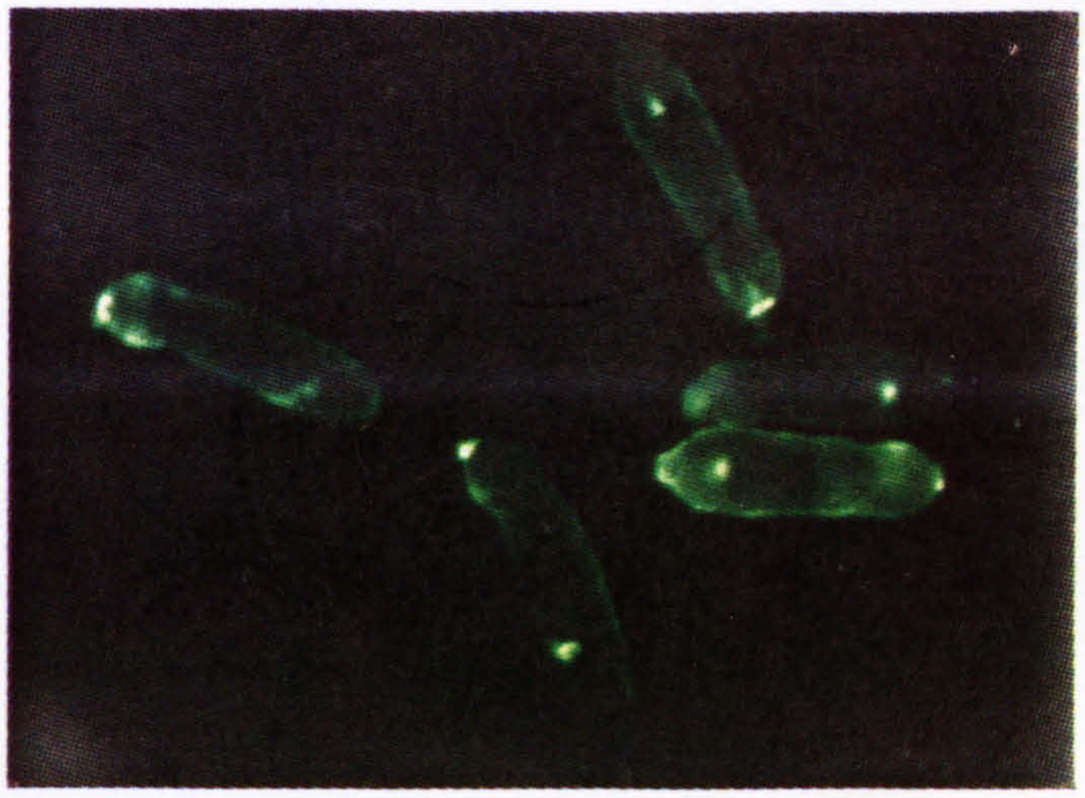


Plate 7.13.

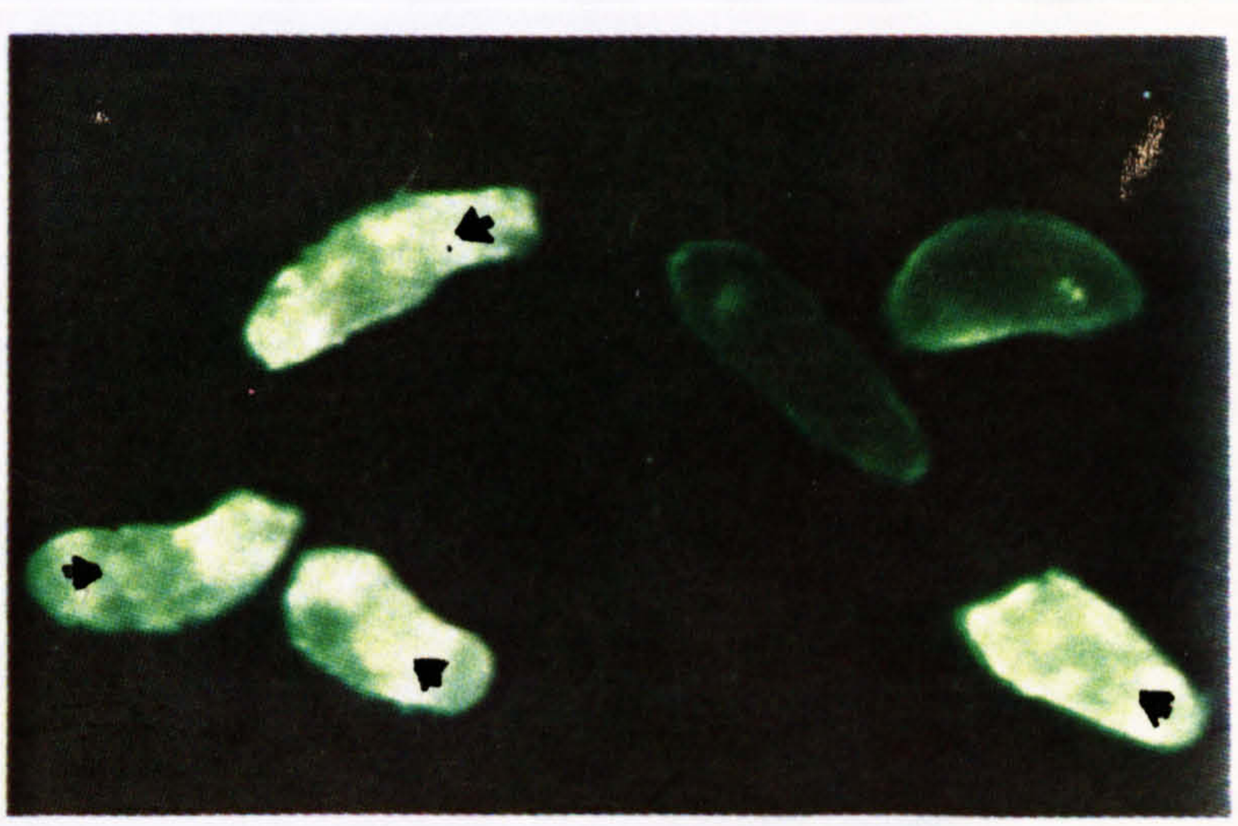


Plate 7.14.

Plate 7.15.

Photomicrograph of a schistosomulum with attached cells that had taken up fluorescent worm materials (wet mount).

(a) white light illumination

(b) U.V. light illumination

Plate 7.16.

Photomicrograph of a schistosomulum with attached cells that had taken up fluorescent worm materials. There are also large globule-like objects exhibiting fluorescence (arrows) near by (cytocentrifuged preparation).

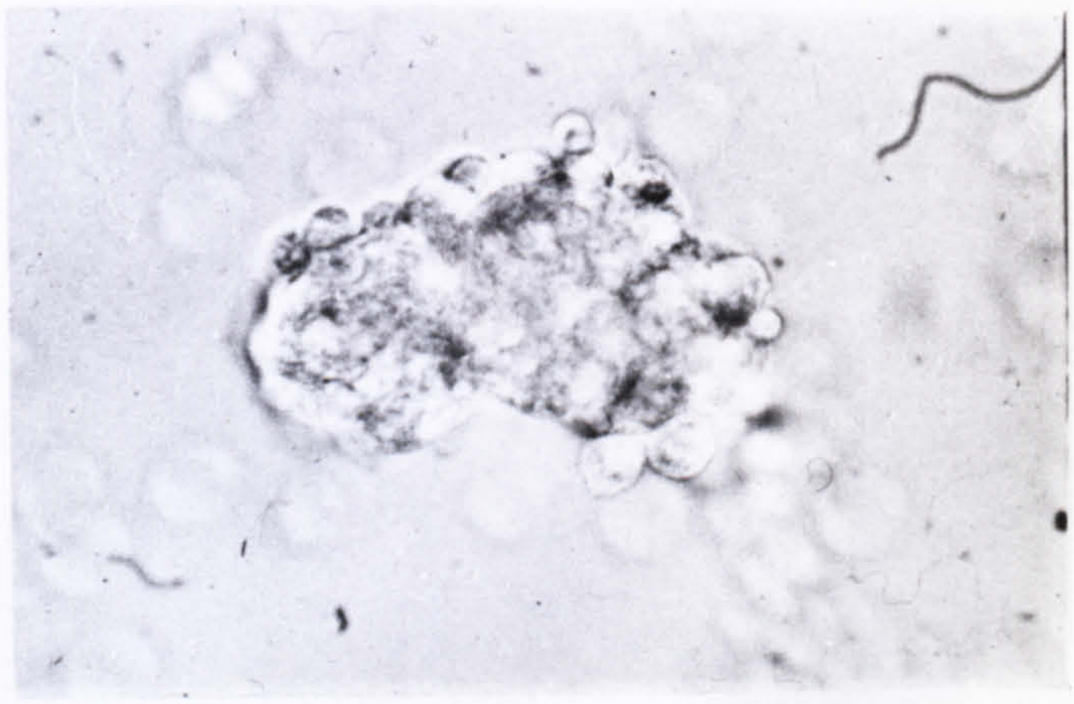


Plate 7.15a.

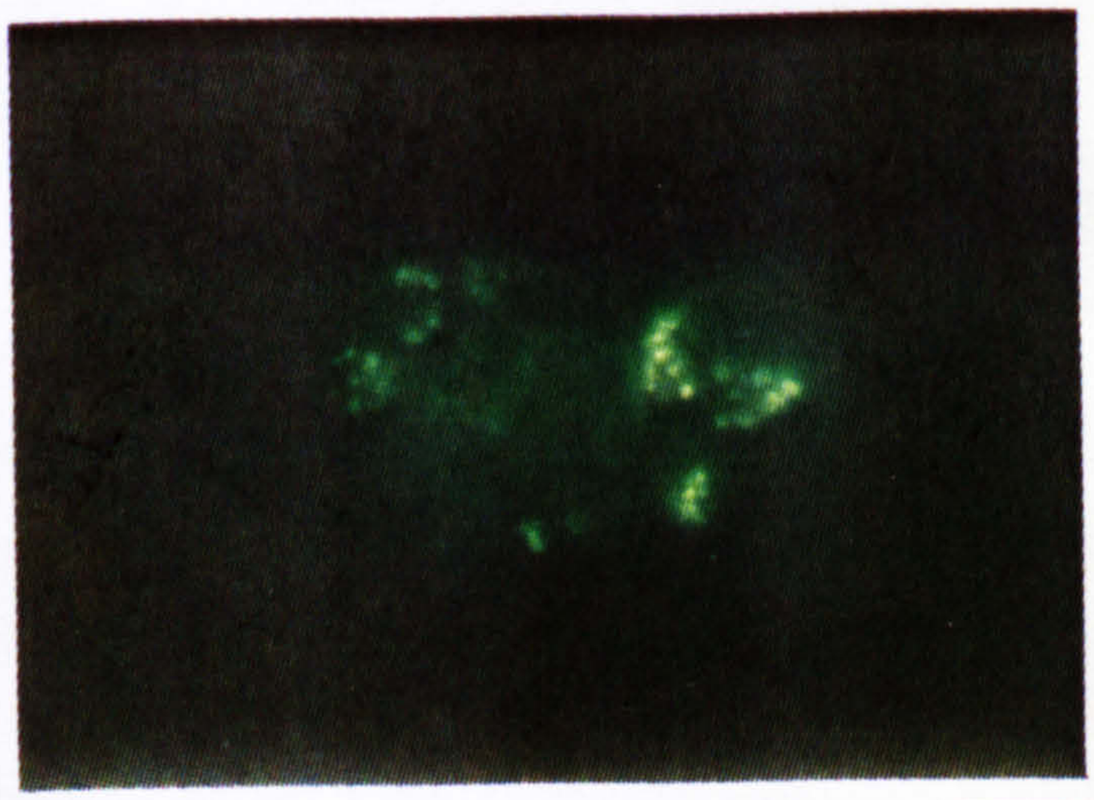


Plate 7.15b.

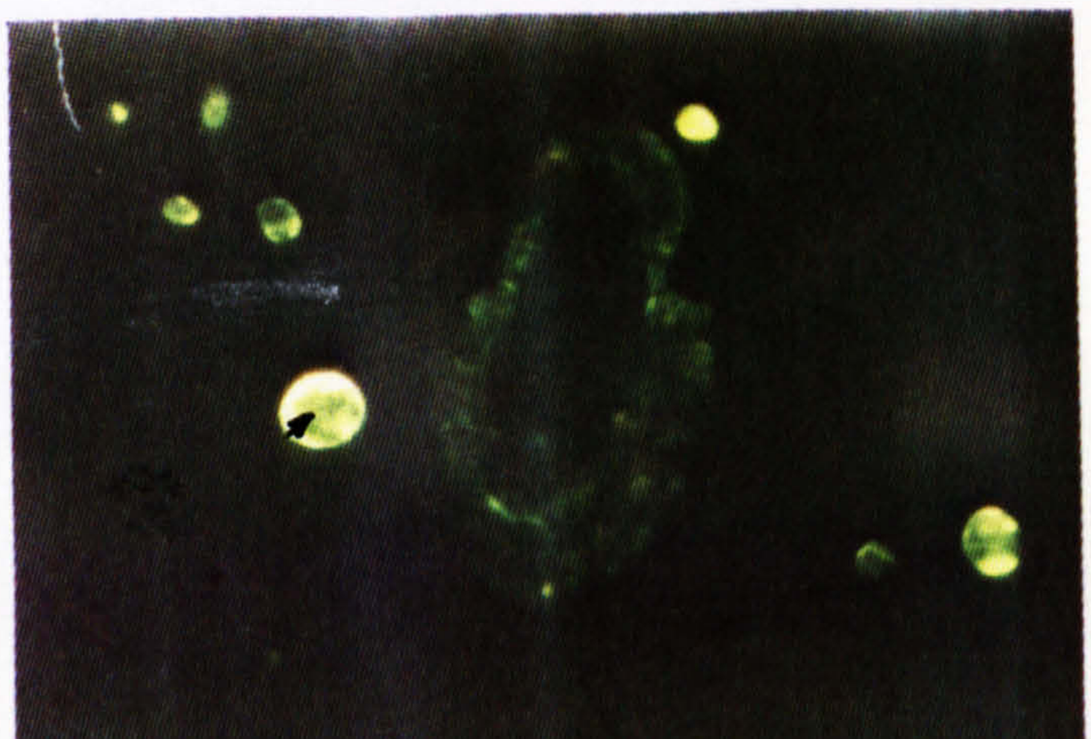


Plate 7.16.

Plate 7.17.

Photomicrograph of a large parasite-cell focus containing fluorescent worm debris and cells (wet mount preparation).

(a) white light illumination

(b) U.V. light illumination

Plate 7.17a.

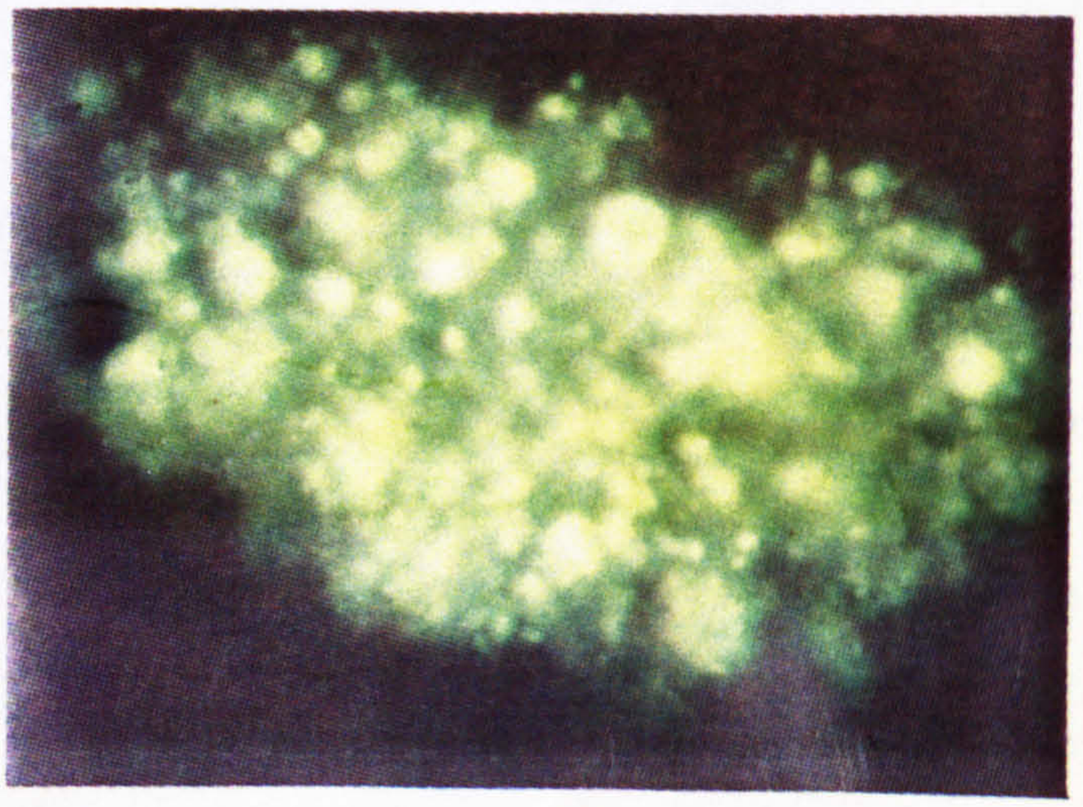


Plate 7.17b.

8. Fractionation of adult worm homogenate to isolate the macromolecule responsible for inducing peritoneal eosinophilia

In a pilot test, it was shown that, like the schistosomula (see Section 4.2.), the homogenate of adult worms also elicited the secondary increase in the number of eosinophils in the peritoneal cavity of infected mice. Thus, a series of fractionation experiments with extracts of adult worms was carried out in an attempt to isolate the antigenic determinants which were responsible for inducing this cellular reactivity using the present intraperitoneal challenge system.

8.1. Experimental design

8.1.1. Preparation of crude extracts of adult worms

The soluble extract was prepared from adult worms collected from experimentally infected hamsters. The worms were supplied in the freeze-dried form by the World Health Organization. Worms weighing 132 mg were homogenized in 15 ml PBS (see Section 2.1.2.) briefly by hand to form a thick brownish slurry. To complete the disintegration, the homogenate was sonicated in a MSE sonicator for 10 second / burst with 2 min rest period on ice between each sonication. The break up of worms was examined microscopically and found to be completed after a total 70 seconds sonication. Insoluble debris was removed by a slow centrifugation

at 2K r.p.m. for 15 min in a bench centrifuge followed by a high speed centrifugation at 30K r.p.m. for 1 hr at 4°C in a Beckman L5-50B Ultracentrifuge (USA) using a swing-out rotor. The soluble phase of the adult worm homogenate thus prepared was designated as 'crude PBS extract'. The total protein content of the crude PBS extract was approximately 37 mg. A portion of the extract was concentrated using Aquacide II and extensively dialyzed against 0.01 M Tris-HCl (pH 8.6) for the subsequent ion exchange chromatography.

8.1.2. Fractionation of crude PBS extract by linear salt gradient and pH gradient

Since isoelectric focusing in thin layer polyacrylamide gel showed that 16 components of the crude extract of adult worms were focused between pH 3.9 and 8.2 (El-Adhami, 1980), most of the components were negatively charged at pH 8.6. Thus, the anion exchange chromatography and elution buffer of pH 8.6 was employed in fractionation to obtain maximum adsorption of the worm proteins.

DEAE- Bio-gel A (BIO-RAD Labs. Richmond, U.S.A.) was packed into a 0.9 x 11 cm column (Pharmacia Fine Chemicals, Uppsala, Sweden) and transferred to the cold room (4°C). The gel was equilibrated by using 0.01M Tris-HCl (pH 8.6) buffer. The flow rate was set at 3 ml/hr. Approximately 10.5 mg of worm proteins in 0.8 ml buffer was deposited onto the column. The elution was performed initially with

a linear salt gradient made of 45 ml of 0.01M Tris-HCl (pH 8.6) buffer and 45 ml of the same buffer containing 0.05M NaCl to elute the minor basic proteins.(El-Adhami, 1981). A second linear salt gradient was made of 40 ml of 0.05 M NaCl in 0.01M Tris-HCl (pH 8.6) buffer and 40 ml of 0.5 M NaCl in the same buffer. The residual proteins in the column were then eluted by a linear pH gradient made of 40 ml of 0.01M Tris-HCl (pH 8.6) in 0.5M NaCl and 40 ml of 0.01M Tris-HCl (pH 6.2) in the same NaCl solution. The eluted fractions were collected in 1 ml aliquots by an LKB UltraRac fraction collector. The presence of protein in fractions was monitored at 280 nm spectrophotometrically.

8.1.3. SDS polyacrylamide gel electrophoresis

Electrophoresis was performed on a slab gel of 10% polyacrylamide at pH 8.8 and stained with Coomassie brilliant blue (R 250)(see Section 2.1.3.).

8.1.4. Determination of the fraction responsible for the elicitation of peritoneal eosinophilia

Two groups of mice infected with approximately 50 cercariae for 9 -13 weeks were used (four experiments) Volumes of 0.2 ml of PBS containing a fixed quantity of worm materials, either crude extract or fraction, was intraperitoneally injected into each infected mouse. Cellular reactions were quantified 24 hr after injection

(see Section 1.1.4.). The cellular reactivity was assayed by varying the quantity of protein given and comparing the dose-response relationship with that of the other fractions. The worm materials used in the test were dialysed against PBS at 4° C overnight prior to intraperitoneal challenge experiments. The controls were mice intraperitoneally injected with column washings (0.01M Tris-HCl, pH 8.6) collected just before the application of crude extract. The washing was dialysed against PBS and stored at -20° C until use.

8.2. Results

8.2.1. Ion exchange chromatography of crude PBS extract of adult worms

The proteins of the crude worm extract were eluted into fractions according to the increase of molar concentration of NaCl in a concave gradient by chromatography on DEAE-BIO-gel. The representative elution profile obtained by the monitoring of absorption at 280 nm is shown in Fig. 8.1. The constituents of the crude extract were separated by the second linear salt gradient (0.05-0.5M NaCl gradient). Individual fractions under each peak were pooled and designated as S2, S3 and S4 according to the order of elution. The elutions from the first salt gradient (0.01-0.05M NaCl gradient) with no detectable protein when measured spectrophotometrically at 280 nm were also pooled and designated

as S1. A portion of each pooled fraction. was removed for protein estimation using the method described by Lowry et al (1951). The remainder was concentrated using Acquacide II and desalted by extensive dialysis against PBS at 4°C.

The recovered total protein was approximately 40% of the original sample applied to the column. Protein estimation showed that the percentage distributions by weight of protein were 65%, 32% and 3% for S2, S3 and S4, respectively. Some of the residual proteins in the column were eluted at pH 8.4 by 0.5M NaCl (Fig. 8.2.). The pooled fraction was designated as S5. Due to the limited material, the protein concentrations of S1 and S5 were not estimated. In the subsequent study, the fraction pools, S2, S3, S4 and S5 were subjected to SDS gel electrophoresis, and S1, S2 and S3 were used in the intraperitoneal challenge experiments.

8.2.2. <u>Comparative analysis of electrophoretic patterns of crude extracts of adult worms and fractions eluted from anion exchange chromatography</u>

Electrophoresis of the crude PBS extract of adult worms in 10% polyacrylamide gel containing 1% SDS distinguished at least 28 major components. For the comparative studies, fourteen bands were selected and designated arbitrarily as 1-14. The intensity of staining of each band was scored at levels ± to +++ (Fig. 8.3). This 14 band-electrophoretic profile served as a basis for the

comparison of homogeneity in the fractions separated by the anion exchange chromatography. On the basis of band positions, it appeared that S2, S3 and S4 shared at least 8, 5 and 2 components, respectively, with that of crude extract (Fig. 8.3.). The differences in the number of bands and their relative staining intensity suggest selective elution by the salt gradients. The fraction S5 eluted by pH gradient revealed a more complex electrophoretic profile than those of S2, S3 and S4.

8.2.3. Comparative analysis of cellular responses to the intraperitoneal injection of crude extract and fractions

It was shown that intraperitoneal injection of crude extract induced the infiltration of eosinophils in infected mice and neutrophils in normal mice (Fig. 8.4.). The minimum dose required to stimulate the eosinophilic response was 50 μg when the mice were tested at doses ranging from 10 μg to 500 μg per mouse. The injection of crude extract up to 250 μg did not stimulate eosinophilic response in the normal mice (Fig. 8.4.). However, the injection of 250 μg triggered a neutrophilic reaction in the normal mice when compared with those injected with column washing (Fig. 8.4.). Not enough material was available for finding the minimum dose, so the minimum dose for the induction of neutrophilia was not investigated .

The results of the test of the three pooled fractions collected by anion exchange chromatography of crude extract, S1, S2 and S3, at various doses are shown in Fig. 8.5.; 8.6.; and 8.7.. Both S1 and S2 were active in the stimulating the eosinophilic response

in the infected mice when tested at the minimum dose of 5 μg per mouse (Fig. 8.5.; 8.6.). S1 gave more response than S2 when compared by the ratio of mean of activity induced at 5 μg to controls. In contrast, S3 tested at three times the minimum dose mentioned above (16 ug per mouse) failed to produce an eosinophilic response (Fig. 8.7.). However, this fraction induced a significant increase in the number of neutrophils in normal mice when tested at doses of 4 μg per mouse (Fig. 8.7.). Both eosinophil-reactive fractions, S1 and S2, failed to induce neutrophilia at their respective maximum doses tested (10 μg for S1 and 40 μg for S2) (Fig. 8.5.; 8.6.). Mice with a hyperactivity to the respective minimum dose did not demonstrate increase in the magnitude of the infiltration of eosinophils and neutrophils when tested at higher dose (Fig. 8.5.; 8.6.; 8.7.). There was no correlation between the magnitude of hyperreactivity and the injection dose.

8.3. Conclusion

The intraperitoneal injection of crude PBS extract of adult worms elicited infiltration of eosinophils in schistosome infected mice and of neutrophils in normal mice (Section 8.2.3.). Some of that crude extract was separated by anion exchange chromatography into four fractions (S1, S2, S3 and S4) using two salt gradients and then a fifth (S5) eluted using a pH gradient (Section 8.2.1.). The electrophoretic analysis of the eluted fractions showed variations in the number of bands and in their staining intensity (Section 8.2.2.). The intraperitoneal injection of S1 and S2 induced the

infiltration of eosinophils in infected mice, whereas S3 elicited infiltration of neutrophils in normal mice (Section 8.2.3.). The minimum dose required to induce eosinophilia and neutrophilia were 5 μg and 4 μg per mouse, respectively (Section 8.2.3.). There was no correlation between the magnitude of hyperreactivity and the injection dose (Section 8.2.3.).

Fig. 8 .1.

Anion exchange chromatography of crude PBS extract of adult worms (step 1)

The crude extract of adult worms was adsorbed to a column (0.9 x 11 cm) of DEAE-Bio. gel equilibrated in 0.01 M Tris-HCl buffer (pH 8.6). The elution was accomplished with two linear gradients of NaCl,e.g. 0-0.05 M and 0.05-0.5 M NaCl in 0.01 M Tris-HCl at rate of 3 ml/hr at 4° C. Aliquots of approximately 1 ml fractions were collected and their absorbance at 280 nm and conductivity in milliSiemens (mS) were measured. The bars (S1, S2, S3 and S4) represent fractions which were pooled, concentrated and characterized.

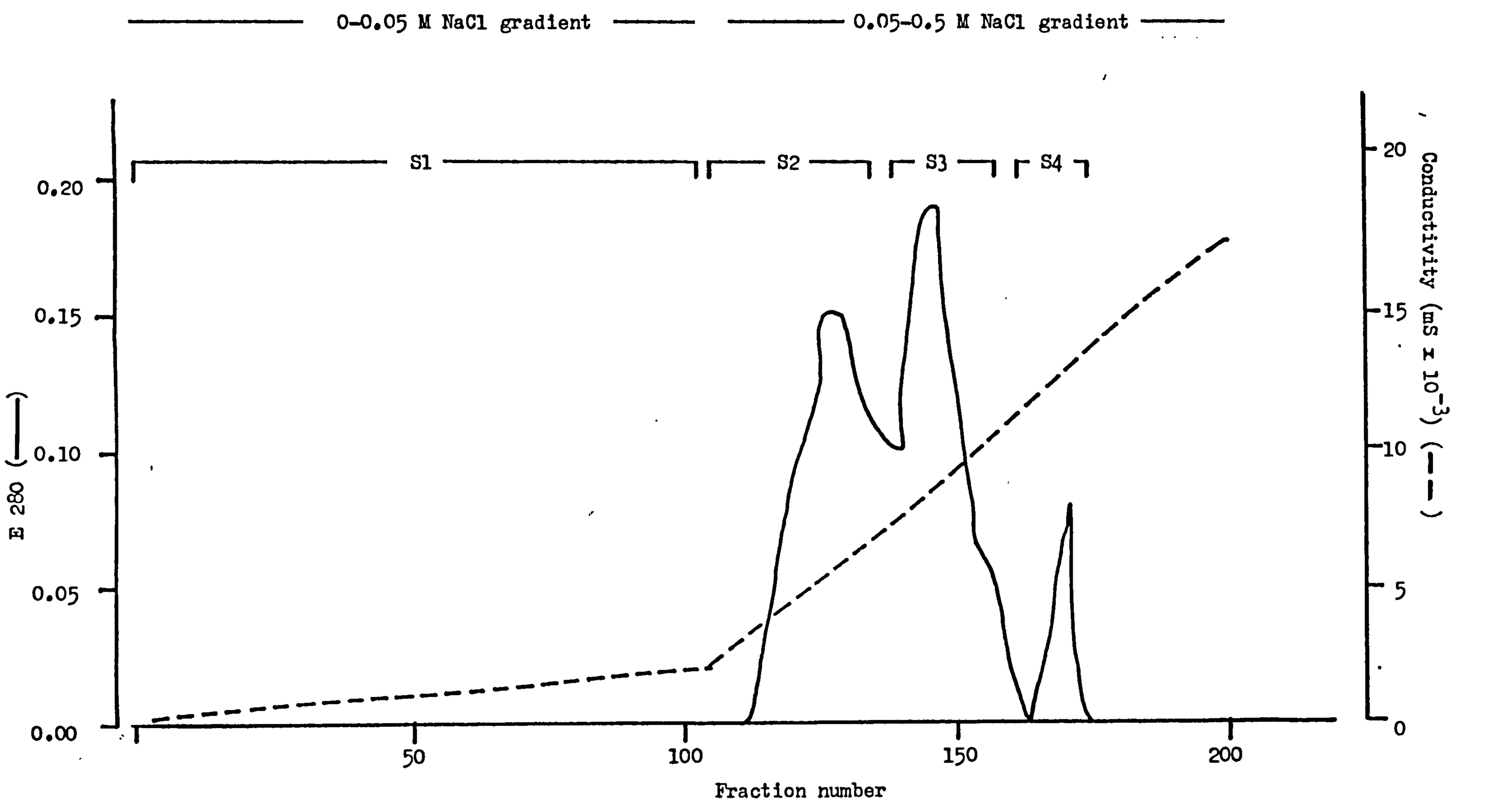

0-0.05 M NaCl gradient
0.05-0.5 M NaCl gradient
S1
S2
S3
S4
0.20
0.15
0.10
0.05
0.00
E 280 (——)
20
15
10
5
0
Conductivity (mS x 10-3) (– – –)
50
100
150
200
Fraction number

Fig. 8.2.

Anion exchange chromatography of crude PBS extract of adult worms (step 2)

The residual worm material after the treatment of two linear gradients of NaCl (Fig. 8.1.) was further eluted by one linear gradient of pH, e.g. 0.01 M Tris-HCl (pH 8.6- 6.2) in 0.5 M NaCl solution at a rate of 3 ml/hr at 4°C. Aliquots of approximately 1 ml fractions were collected and their absorbance at 280 nm and pH were measured. The bars (S5) represent fractions which were pooled, concentrated and characterized.

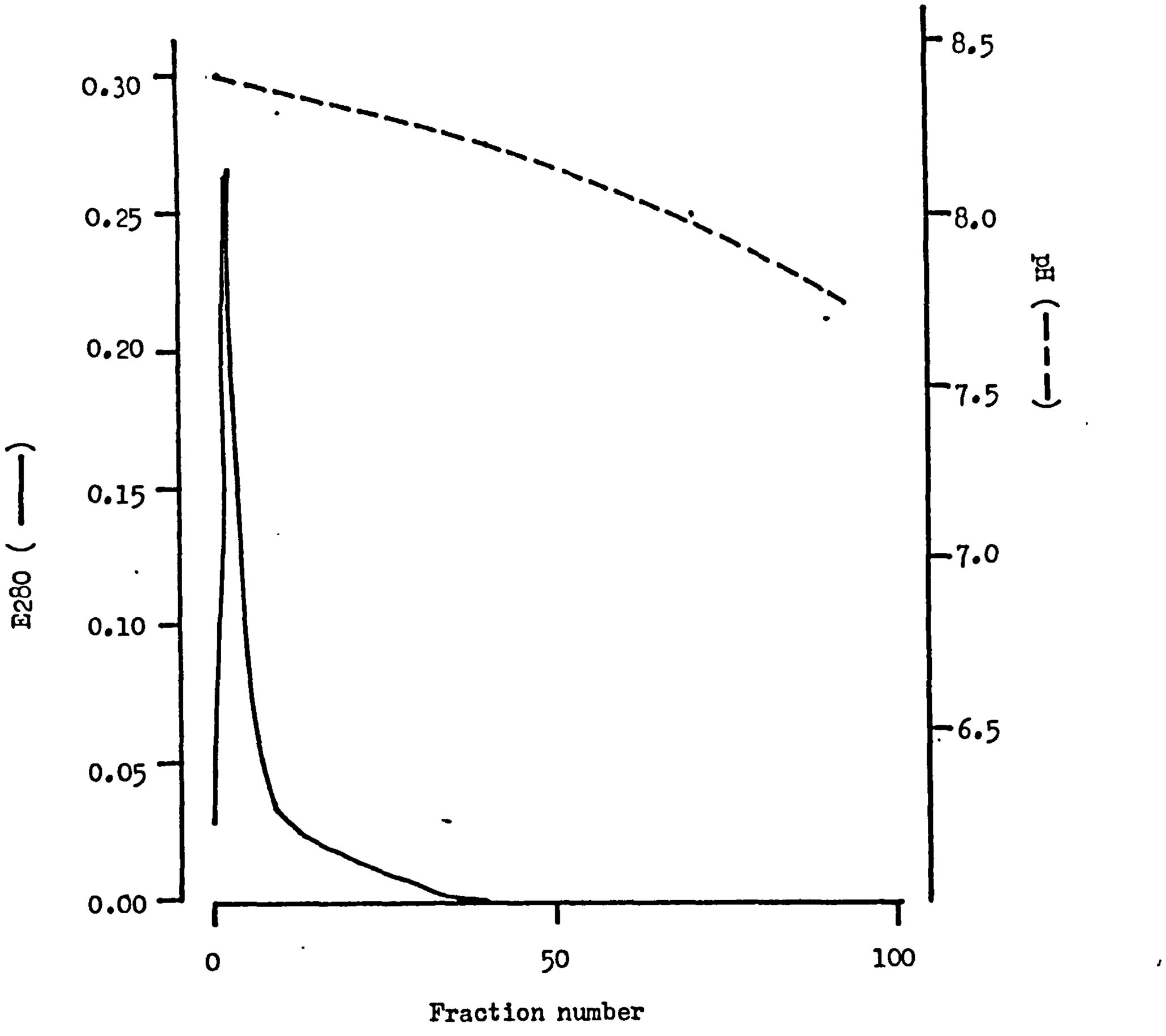

E280 (—)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
Fraction number
8.5
8.0
7.5
7.0
6.5
pH (- - -)

Fig. 8.3.

Representative electrophoretogram of crude extract of adult worms and fractions eluted by anion exchange chromatography

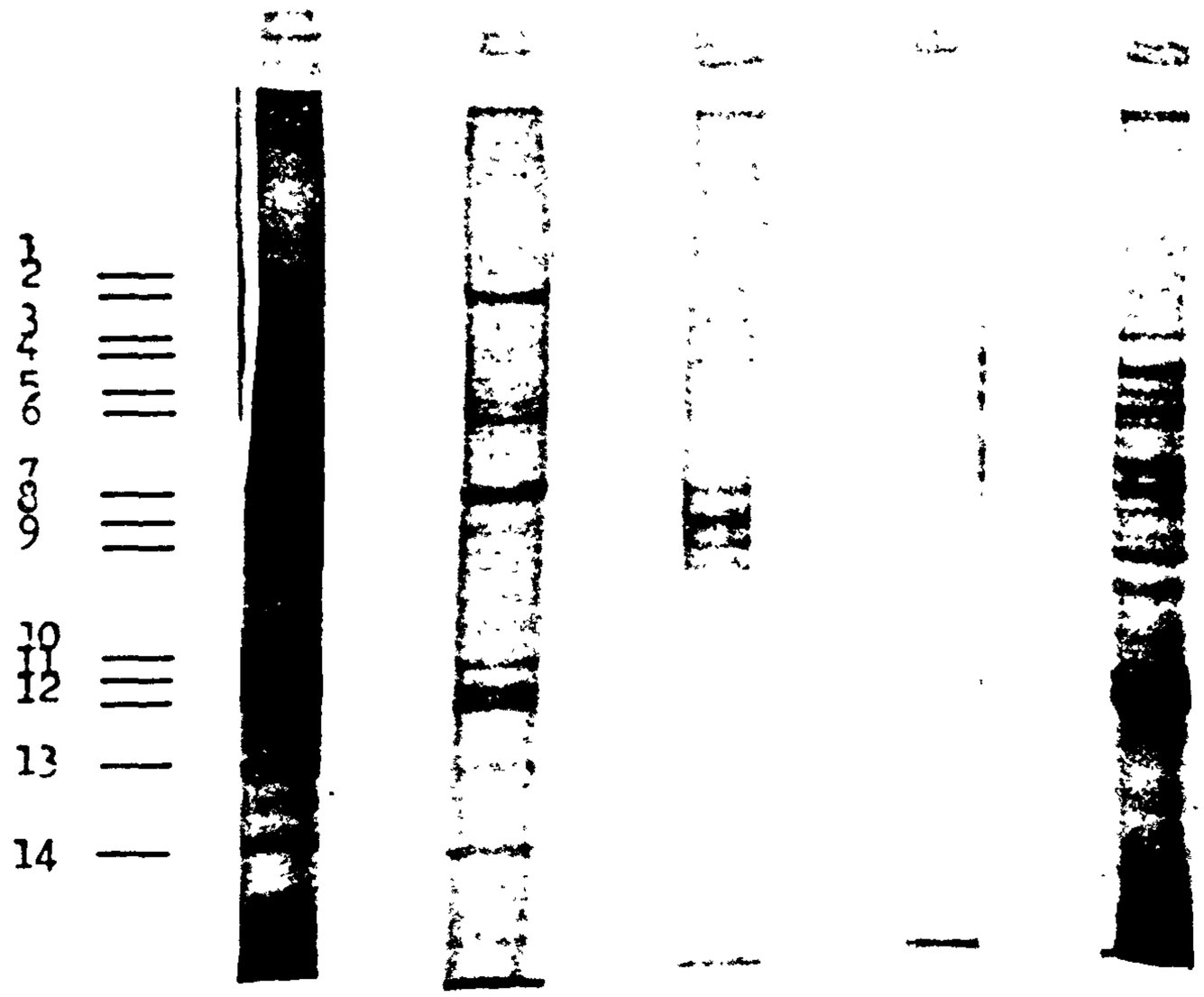
The electrophoresis was carried out in the Tris-buffered slab gel of 10% polyacrylamide at pH 8.8. Anode is at the bottom of the gel. Protein bands were stained with Coomassie brilliant blue R 250.

Samples were (1) crude PBS extract of adult worms

(2) S2
(3) S3 | Pooled fractions eluted by salt gradients
(4) S4

(5) S5 | Pooled fractions eluted by the pH gradient

Fourteen bands exhibited by the crude extract were selected and used as a basis for the comparison of homogeneity in the fractions. The intensity of staining of each band in each fraction was scored at levels ± to +++.



Band number	Crude homogenate	Chromatography: Salt gradient S2	Salt gradient S3	Salt gradient S4	pH gradient S5
1	++	++	±	?	±
2	++	±	±	?	±
3	+	±	-	?	+
4	++	±	-	?	++
5	+	-	-	+++	++
6	++	+	±	+++	+
7	+++	+++	+++	?	++
8	+++	±	+++	?	++
9	++	±	+++	?	+
10	++	++	-	?	+
11	++	++	++	?	+++
12	++	++	-	?	+++
13	++	+	-	?	++
14	++	++	+	?	++

Fig. 8.3.

Representative electrophoretogram of crude extract of adult worms and fractions eluted by anion exchange chromatography

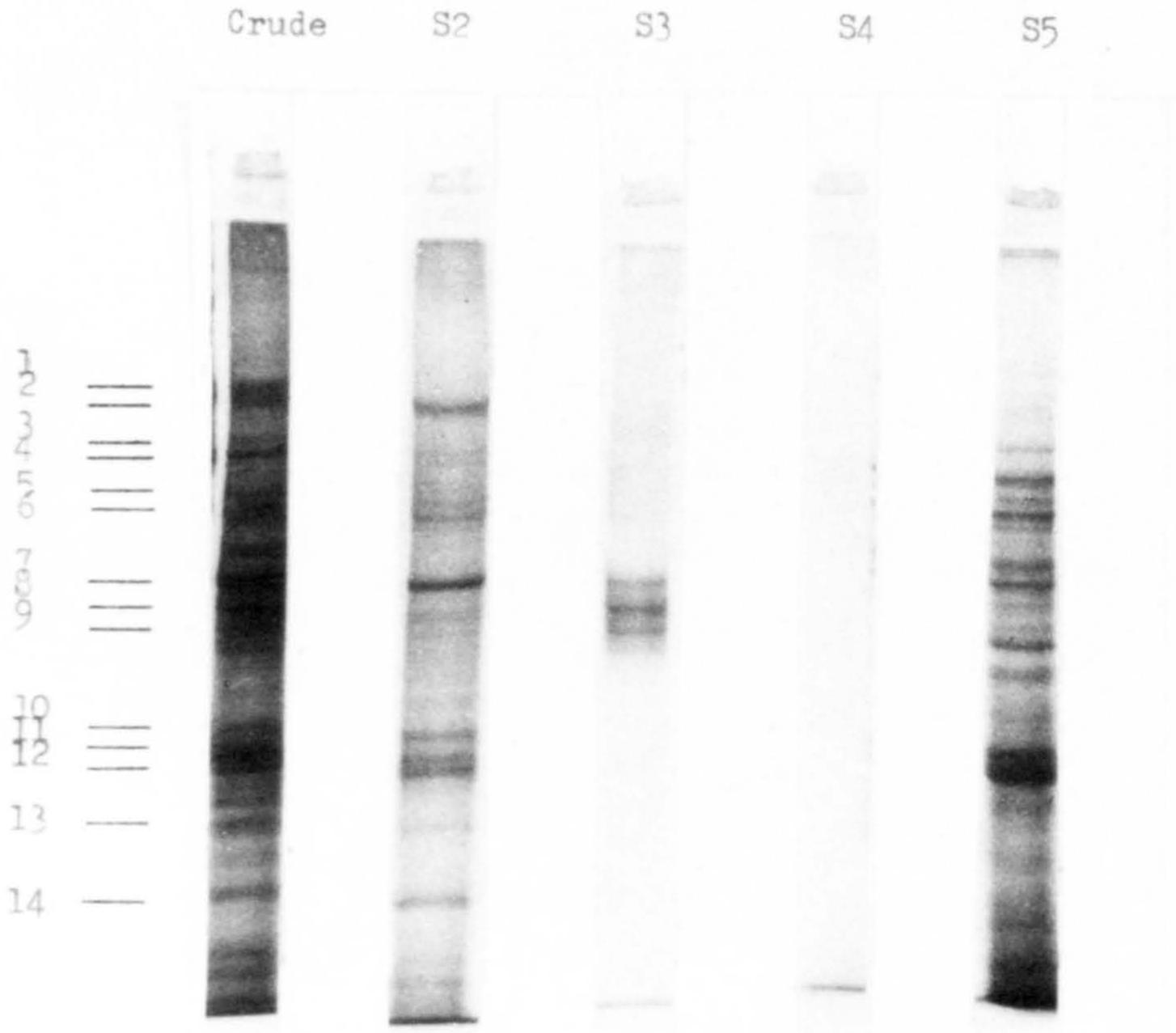
The electrophoresis was carried out in the Tris-buffered slab gel of 10% polyacrylamide at pH 8.8. Anode is at the bottom of the gel. Protein bands were stained with Coomassie brilliant blue R 250.

Samples were (1) crude PBS extract of adult worms

(2) S2 | (3) S3 | (4) S4 | Pooled fractions eluted by salt gradients

(5) S5 | Pooled fractions eluted by the pH gradient

Fourteen bands exhibited by the crude extract were selected and used as a basis for the comparison of homogeneity in the fractions. The intensity of staining of each band in each fraction was scored at levels ± to +++.



Band number	Crude homogenate	Chromatography: Salt gradient S2	Salt gradient S3	Salt gradient S4	pH gradient S5
1	++	++	±	?	±
2	++	±	±	?	±
3	+	±	-	?	+
4	++	±	-	?	++
5	+	-	-	+++	++
6	++	+	±	+++	+
7	+++	+++	+++	?	++
8	+++	±	+++	?	++
9	++	±	+++	?	+
10	++	++	-	?	+
11	++	++	++	?	+++
12	++	++	-	?	+++
13	++	+	-	?	++
14	++	++	+	?	++

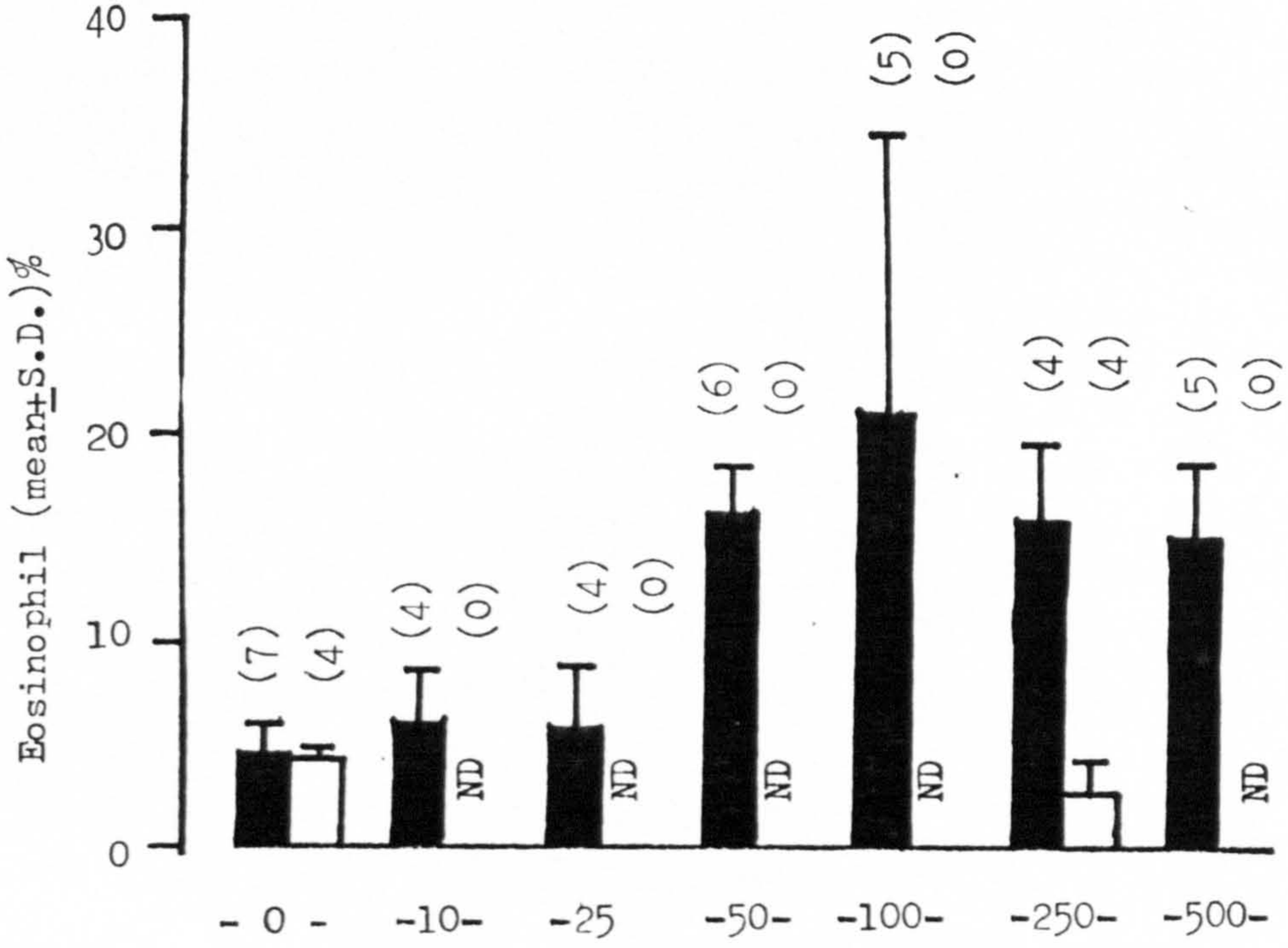
Fig. 8.4

Relationship between injection doses of crude PBS extract and eosinophilic and neutrophilic responses in the peritoneal cavity of mice

Volumes of 0.2 ml of PBS containing 10, 25, 50, 100, 250 and 500 µg protein of the crude PBS extract were intraperitoneally injected into mice previously infected with 50 cercariae for 13 weeks. One group of normal mice were also injected with 250 µg protein of the extract. Controls were mice, either infected or normal, injected with 0.2 ml of column washings. At 24 hr after injection, mice were killed and peritoneal cells were harvested. The proportions of eosinophils and neutrophils were subsequently quantified (see Section 1.1.4.2.).

Each column represents the mean of percentages of (a) eosinophils (b) neutrophils obtained from each group and the vertical bars their respective standard deviations. The closed columns indicate the values obtained from infected mice and the open from normal mice. Numbers in the parenthesis refer to the number of mice used in each group.

(a) Eosinophil



(b) Neutrophil

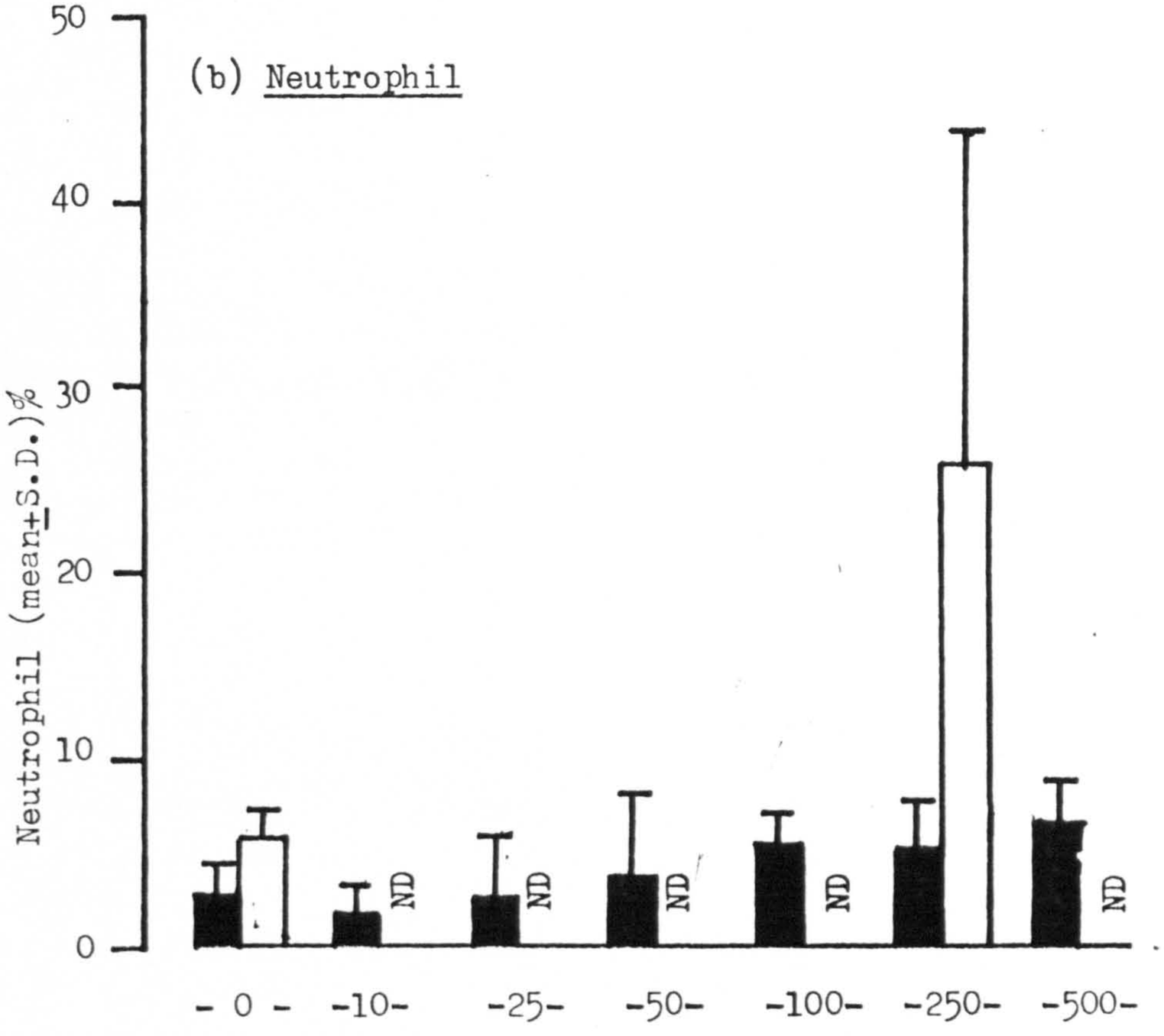
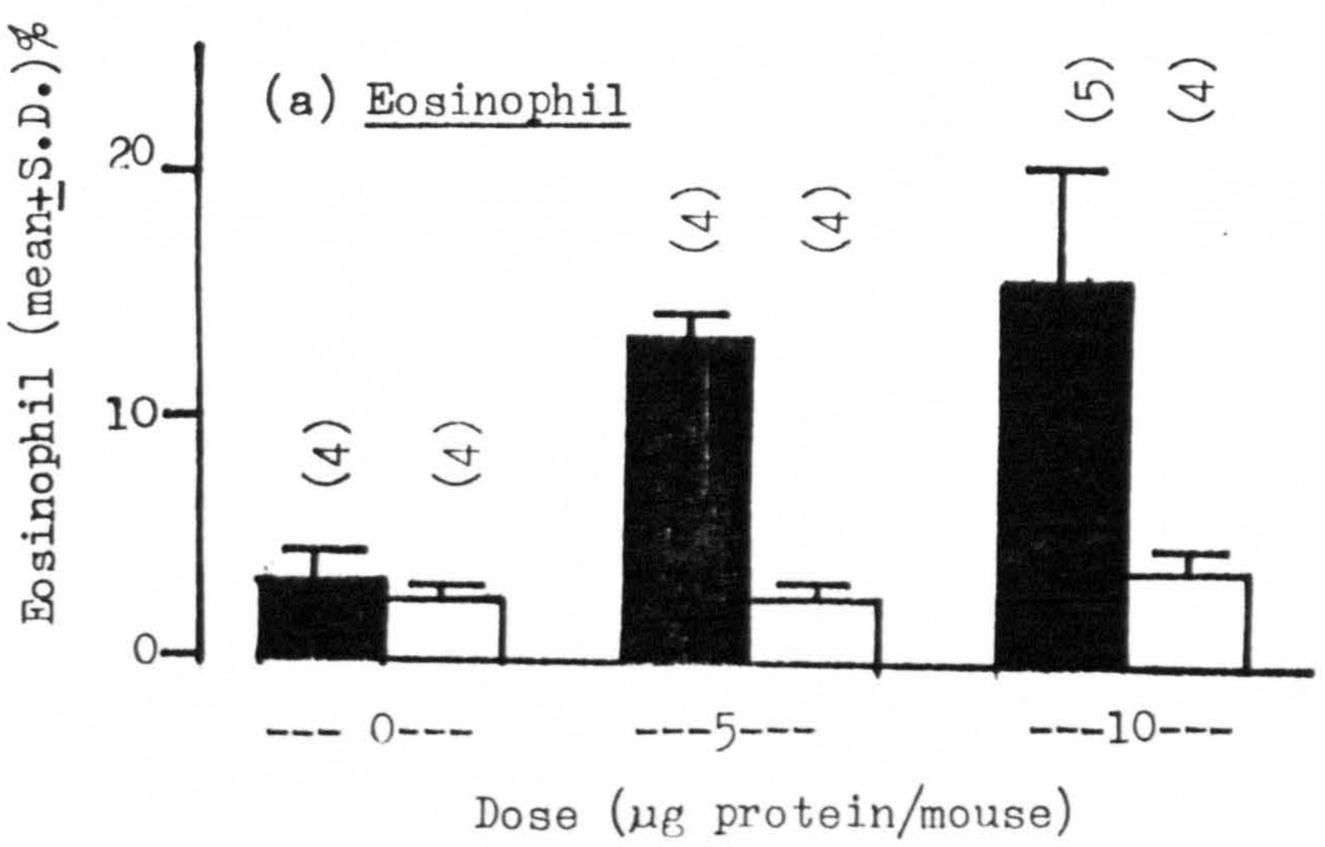


Fig. 8.5.

Relationship between injection doses of fraction pool S1 and eosinophilic and neutrophilic responses in the peritoneal cavity of mice

Volumes of 0.2 ml of PBS containing 5 or 10 µg protein of fraction pool S1 were intraperitoneally injected into normal mice or mice previously infected with 50 cercariae for 9 weeks. Controls were mice, either infected or normal, injected with 0.2 ml of column washings. At 24 hr after injection, mice were killed and peritoneal cells were harvested. The proportions of eosinophils and neutrophils were subsequently quantified (see Section 1.1.4.2.).

Each column represents the mean of percentages of (a) eosinophils (b) neutrophils obtained from each group and the vertical bars their respective standard deviations. The closed columns indicate the values obtained from infected mice and the open from normal mice. Numbers in the parenthesis refer to the number of mice used in each group.


(a) Eosinophil
Eosinophil (mean±S.D.)%
20
10
0
(4)
(4)
(4)
(4)
(5)
(4)
--- 0---
---5---
---10---
Dose (μg protein/mouse)

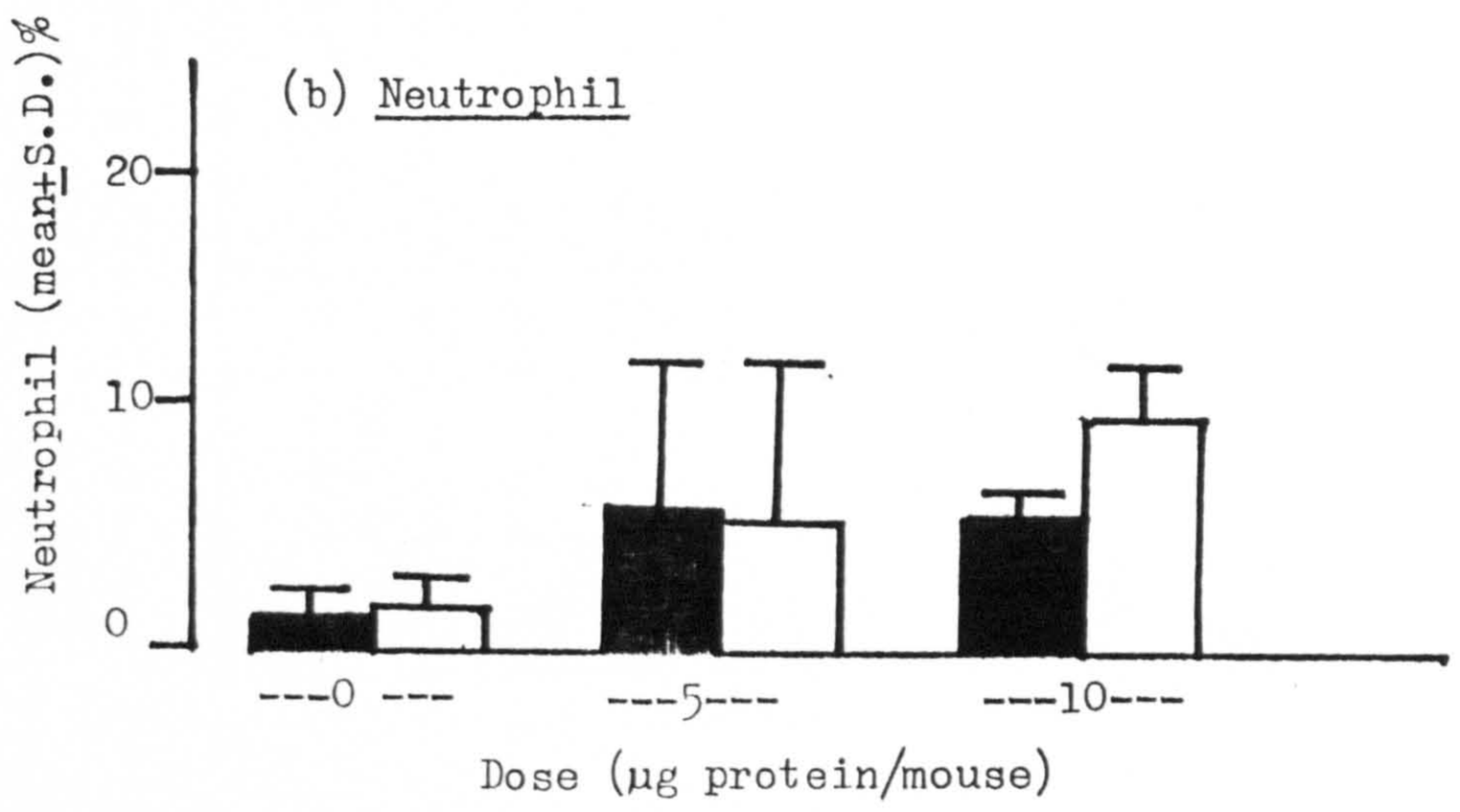

(b) Neutrophil
Neutrophil (mean±S.D.)%
20
10
0
---0 ---
---5---
---10---
Dose (μg protein/mouse)

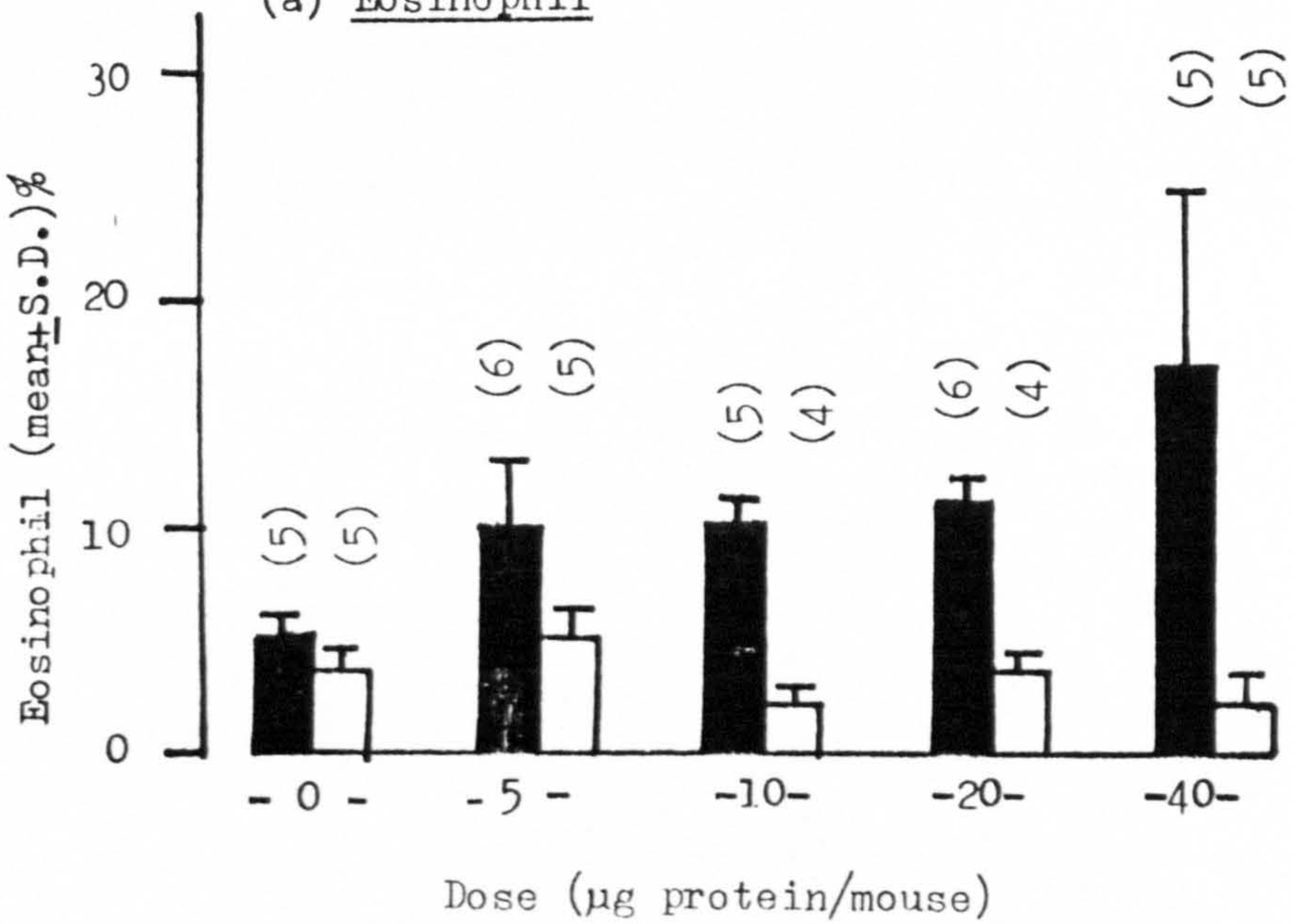
Fig. 8.6.

Relationship between injection doses of fraction pool S2 and eosinophilic and neutrophilic responses in the peritoneal cavity of mice

Volumes of 0.2 ml of PBS containing 5, 10, 20 or 40 µg protein of fraction pool S2 were intraperitoneally injected into normal mice or mice previously infected with 50 cercariae for 11 weeks. Controls were mice, either infected or normal, injected with 0.2 ml of column washings. At 24 hr after injection, mice were killed and peritoneal cells were harvested. The proportions of eosinophils and neutrophils were subsequently quantified (see Section 1.1.4.2.).

Each column represents the mean of percentages of (a) eosinophils (b) neutrophils obtained from each group and the vertical bars their respective standard deviations. The closed columns indicate the values obtained from infected mice and the open from normal mice. Numbers in the parenthesis refer to the number of mice used in each group.

(a) Eosinophil



(b) Neutrophil

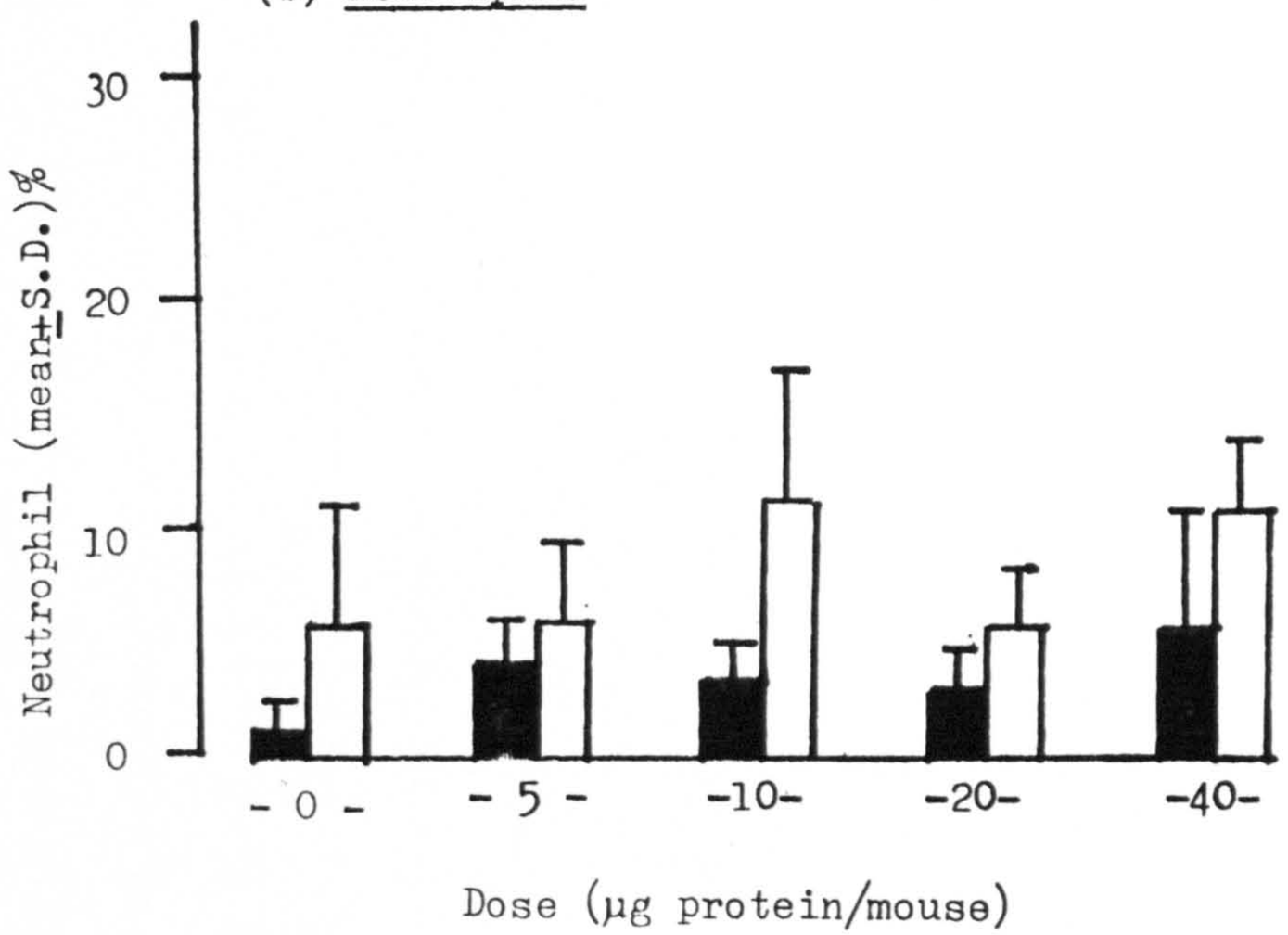
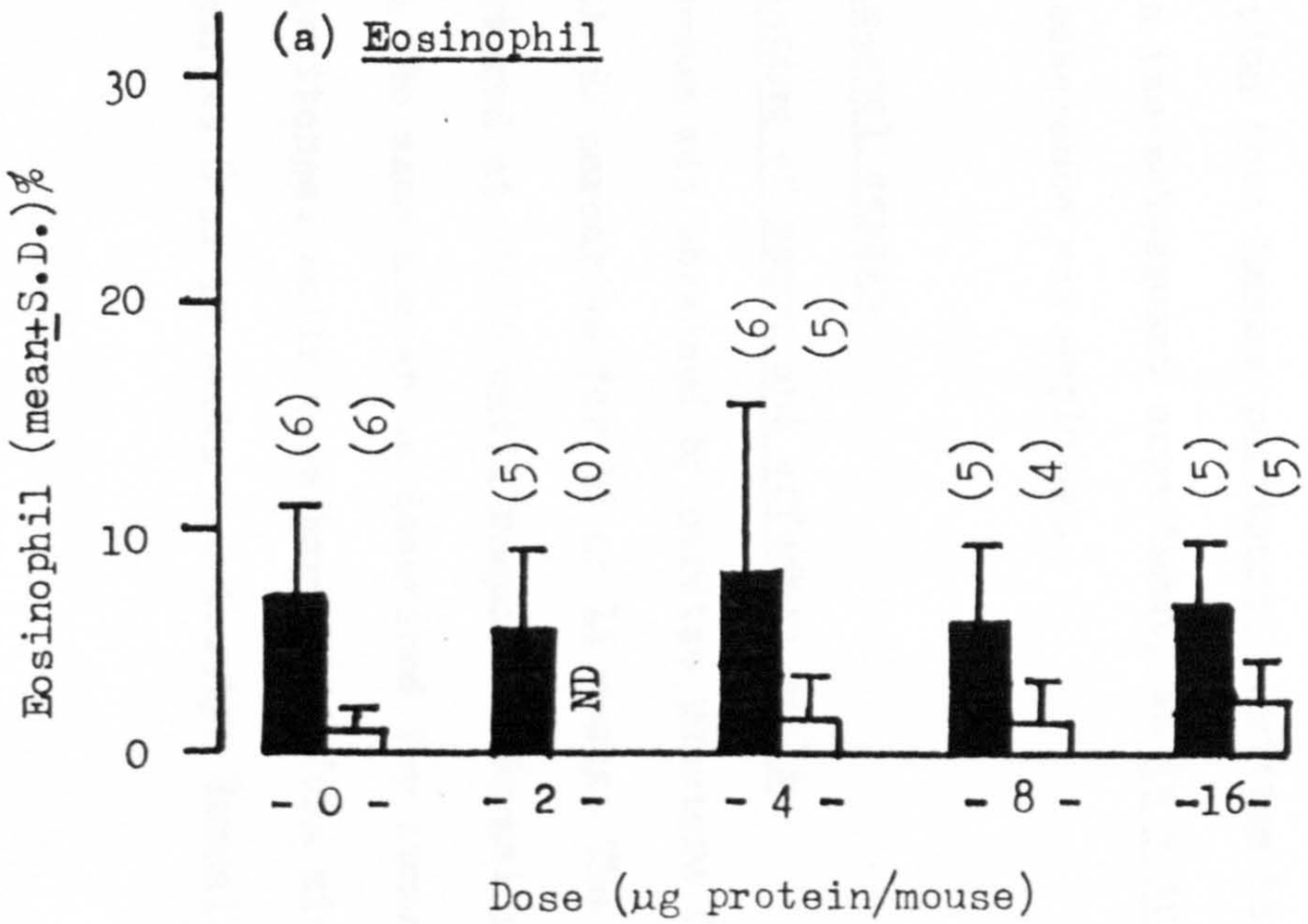


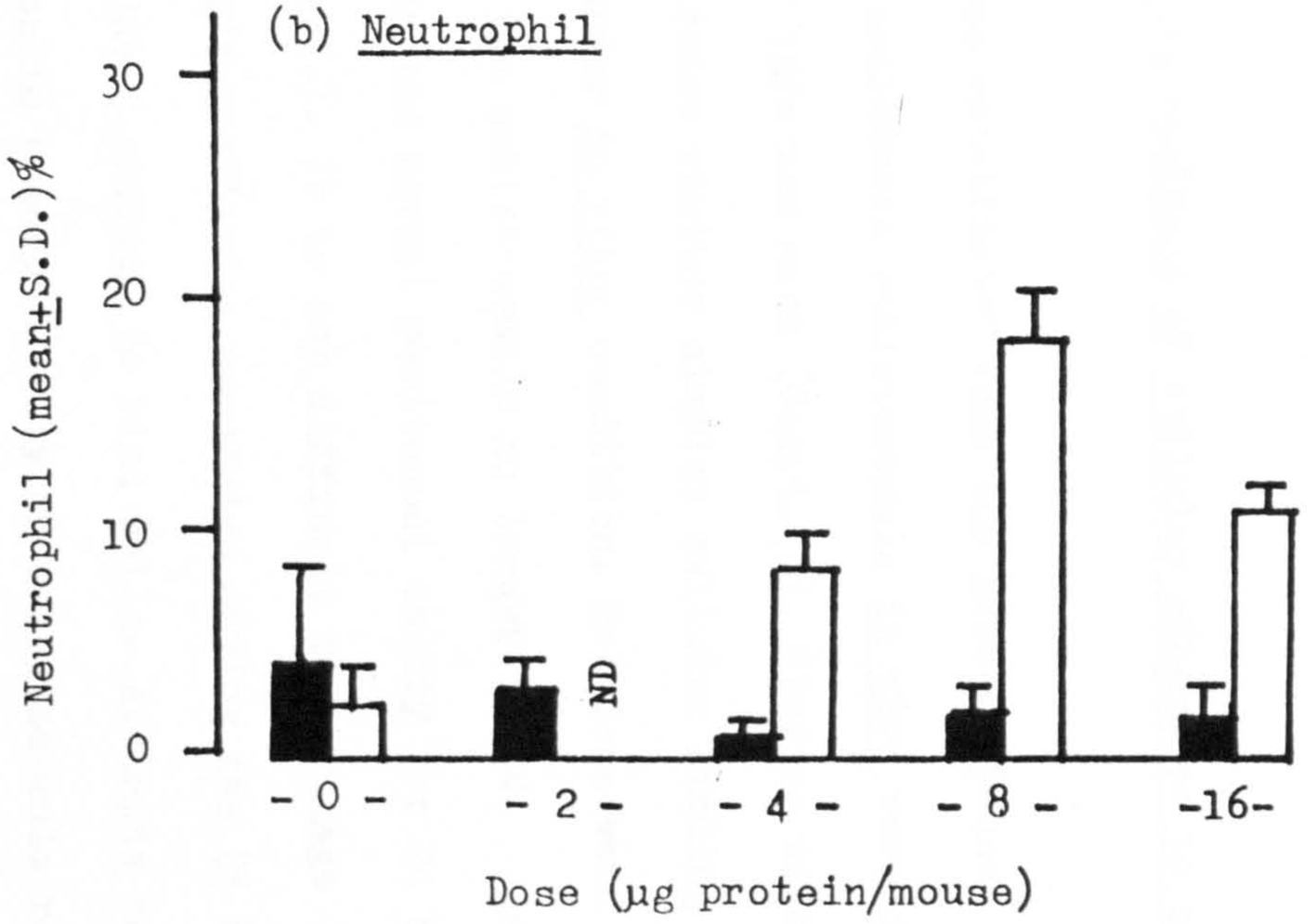
Fig.8.7.

Relationship between injection doses of fraction pool S3 and eosinophilic and neutrophilic responses in the peritoneal cavity of mice

Volumes of 0.2 ml of PBS containing 2, 4, 8 and 16 µg protein of fraction pool S3 were intraperitoneally injected into normal mice or mice previously infected with 50 cercariae for 10 weeks. Controls were mice, either infected or normal, injected with 0.2 ml of column washings. At 24 hr after injection, mice were killed and peritoneal cells were harvested. The proportions of eosinophils and neutrophils were subsequently quantified (see Section 1.1.4.2.).

Each column represents the mean of percentages of (a) eosinophils (b) neutrophils obtained from each group and the vertical bars their respective standard deviations. The closed columns indicate the values obtained from infected mice and the open from normal mice. Numbers in the parenthesis refer to the number of mice used in each group.


(a) Eosinophil
Eosinophil (mean±S.D.)%
30
20
10
0
(6)
(6)
(5)
(0)
(6)
(5)
(5)
(4)
(5)
(5)
ND
- 0 -
- 2 -
- 4 -
- 8 -
-16-
Dose (μg protein/mouse)


(b) Neutrophil
Neutrophil (mean±S.D.)%
30
20
10
0
ND
- 0 -
- 2 -
- 4 -
- 8 -
-16-
Dose (μg protein/mouse)

A. Quantitative analysis of cellular adherence to schistosomula in vitro

It has been established that the host responses to intraperitoneally introduced schistosomula *in vivo* were different in normal and infected mice (Chapt. 6). Studies were next undertaken to determine whether similar cellular binding patterns would occur under *in vitro* condition. Previous work also demonstrated that the schistosomula no longer bound cells *in vivo* after being in the normal peritoneal cavity for 24 hr (see Section 6.2.1.2.). It is not difficult to envisage that the tegumental differentiation occurring during the 24 hr accounts for their reduced ability to bind cells. Attempts to investigate the alteration in susceptibility of schistosomula recovered from normal peritoneal cavities to cellular adherence by direct injection into immune peritoneal cavities have failed. Therefore, in the subsequent experiment, an *in vitro* assay of cellular adherence was employed.

A.1. Experimental design

A.1.1 Harvesting of serum and effector cells

Immune serum was obtained by cardiac puncture from mice infected with 50 cercariae for 10 or 14 weeks. The serum was pooled and stored at -20° C until required. Normal serum was also collected in the same manner as described for immune serum.

Immune peritoneal cells were harvested from mice infected with 50 cercariae 8 or 10 weeks previously. Normal peritoneal

cells were also collected in the same manner. The cells were collected in Eagle's essential medium supplemented with 100 i.u. penicillin and 100 ug/ml streptomycin (Esp medium). Heparin was added at a final concentration of 10 unit /ml. The cells were washed in fresh Esp medium twice and adjusted to 2.2×10^4 cell/ml.

A.1.2. Preparation of fresh mechanically transformed schistosomula

Schistosomula were transformed from cercariae by mechanical separation of tails from the bodies according to the method described by Ramalho-Pinto et al (1974) (see Section 4.1.2.) The resultant mechanically transformed schistosomula were washed three times in fresh Esp medium and adjusted to 100 schistosomula/ml. The schistosomula were 1-2 hr old at the begining of the experiments.

A.1.3. Preparation of peritoneal schistosomula

Volumes of 0.2 ml suspension containing 2,000 mechanically transformed schistosomula were intraperitoneally injected into normal mice. Peritoneal washings were collected at 24 hr after inoculation (see Section 6.1.3.). The washing were pooled and the schistosomula concentrated at 1 x G. The sedimented schistosomula were resuspended in 10 ml of Esp medium supplemented with heat-inactivated (56°C/30 min) normal mouse serum in a size 9 cm petri dish (Becton Dickinson & Co.,U.S.A.) They were depleted of larger worm-cell foci by incubation

at 37°C for 30-45 min in an atmosphere 5% CO_2 and 95% air. The schistosomula with R++ to R++++ bound cells generally adhered to the surface of the petri dish through macrophages present in the foci (see Section 7.2). The schistosomula free of or with few bound cells ($R\pm$ to R+) were recovered and washed twice with Esp medium. The resultant peritoneal schistosomula were adjusted to 100 schistosomula/ml and analysed for their ability to bind cells in the presence of antibodies.

A.1.4. In vitro assay of cellular adherence to schistosomula

The in vitro assay was performed as follows: each petri dish contained 9 ml of the effector cell suspension and 1 ml of the suspension of schistosomula , either freshly prepared or harvested from normal peritoneal cavities. The schistosomula: cell ratio was set at 1: 2000, The serum was then added at a final concentration of 10% for the assay. The dish was incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Cellular adherence was assessed after 1/2, 2, 12 and 24 hr. The cellular binding to schistosomula was scored on a scale of $R\pm$ to R++++ (see Section 6.1.4).

A.2. Results

A.2.1. Comparative study of cellular adherence to fresh mechanically transformed schistosomula by immune and normal cells in vitro

Fig. A.1. compares the time course of the magnitude of cellular adherence to schistosomula in the presence of immune

serum and normal serum. At 1/2 and 2 hr, in contrast to the early responses occurring in the peritoneal cavities where the majority of injected schistosomula had attracted numerous cells, the responses to schistosomula in vitro in the presence of normal cells and serum was minimal. Most of the schistosomula induced only R+ and R++ cellular adherence. Incubation up to 24 hr did not improve the performance of normal cells. Well over 80% of the schistosomula were motile and free of bound cells.

Incubation with immune cells in the presence of immune serum resulted in marginally more foci of R++ and R+++ reactivities. However, the cellular response was limited to an early time up to 2 hr. Nearly 50% of the schistosomula were free or had a few cells attached after incubation for 24 hr.

A.2.2. Comparative study of cellular adherence to fresh and peritoneal schistosomula by immune cells in the presence of immune serum in vitro

The majority of freshly prepared schistosomula attracted cellular binding of R++ and R+++ reactivities (Fig. A.2.). The spectrum in cellular adherence observed at 2 hr did not alter appreciably when the same samples were examined at 12 hr. In contrast, peritoneal schistosomula exhibited a significant increase in resistance to cellular binding at 2 hr in the tested system described (Fig. A.2.). Prolonged incubation for 12 hr did not improve the cellular activity. The suspension of

peritoneal schistosomula invariably contained a small number of individuals with already bound cells. Since there was no apparent difference in the pattern of cellular adherence to peritoneal schistosomula before and after inoculation (Fig. A.2.), it is likely that the cellular binding observed *in vitro* had already occurred in the schistosomula preparation before injection. *In vitro* culture with immune cells and serum probably does not confer additional cellular binding to peritoneal schistosomula.

A.3. Conclusion

The present studies have demonstrated that the presence of antibodies is a prerequisite for cellular adherence to schistosomula *in vitro* (Section A.2.1.). Culture with immune serum gave superior binding compared to that with normal serum. The intensive cellular binding observed in normal peritoneal cavities during the early hours after challenge *in vivo* was not observed *in vitro* when schistosomula were incubated in the presence of normal cells and serum (Section 6.2.1.1.). Additionally, the schistosomula recovered from normal peritoneal cavities were no longer able to attract cell binding in the presence of immune serum *in vitro* (Section A.2.2.).

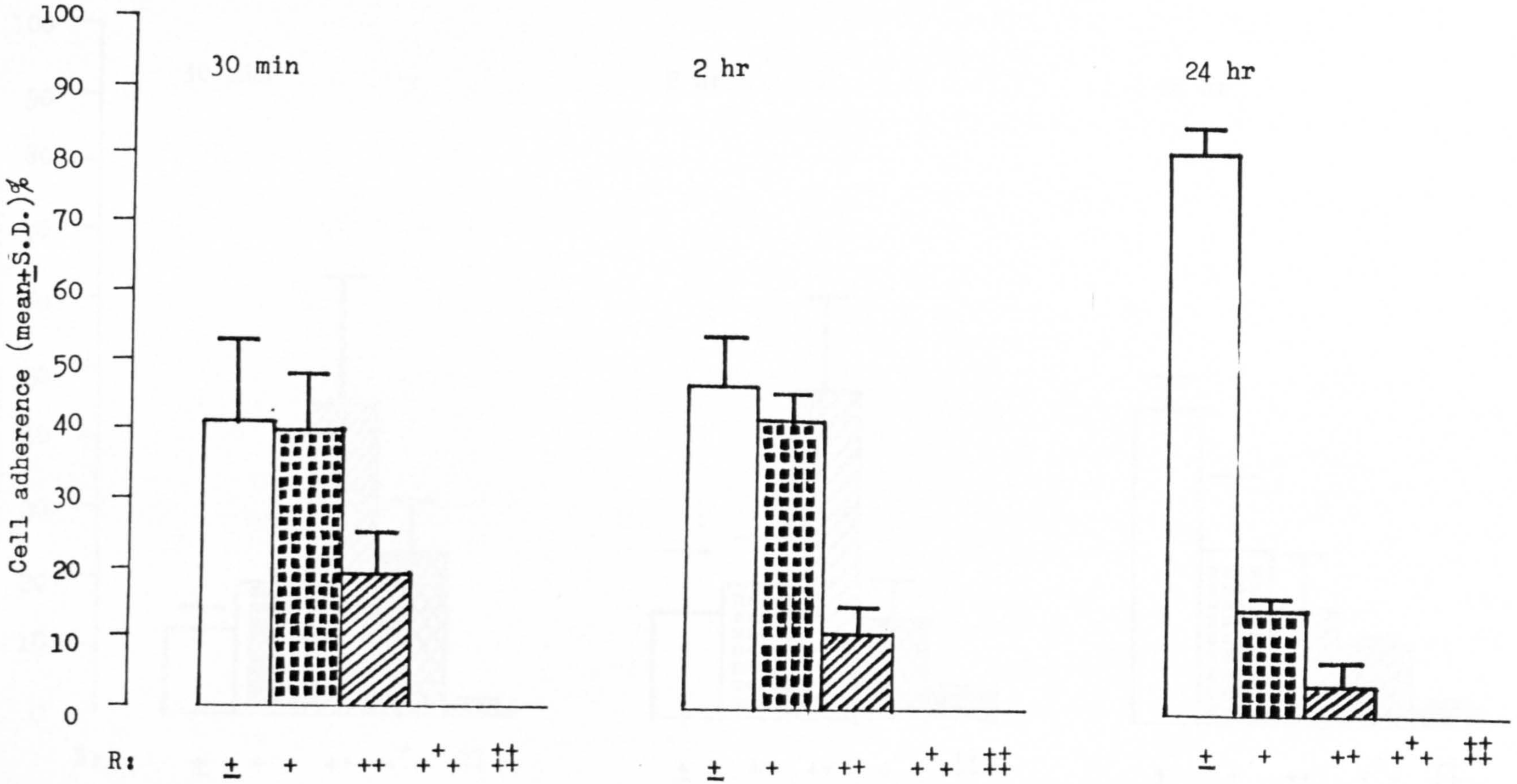
Fig. A.1.

Time course of magnitude of cellular adherence to schistosomula of S. mansoni in vitro

In the in vitro cellular adherence assay, the schistosomulum : cell ratio was set at 1:2000. The donors of cells were either (a) normal mice or (b) mice infected for 8 or 10 weeks. The donors of serum were either (a) normal mice or (b) mice infected for 10 or 14 weeks. At 1/2, 2 and 24 hrs, the cellular adherence to schistosomula was scored.

Each column represents mean value of cellular adherence to schistosomula obtained from two experiments on a scale of R± to R++++, and the vertical bars their respective standard deviations. See Fig. 6.2. for column symbols.

(a) Normal serum and cells



(b) Immune serum and cells

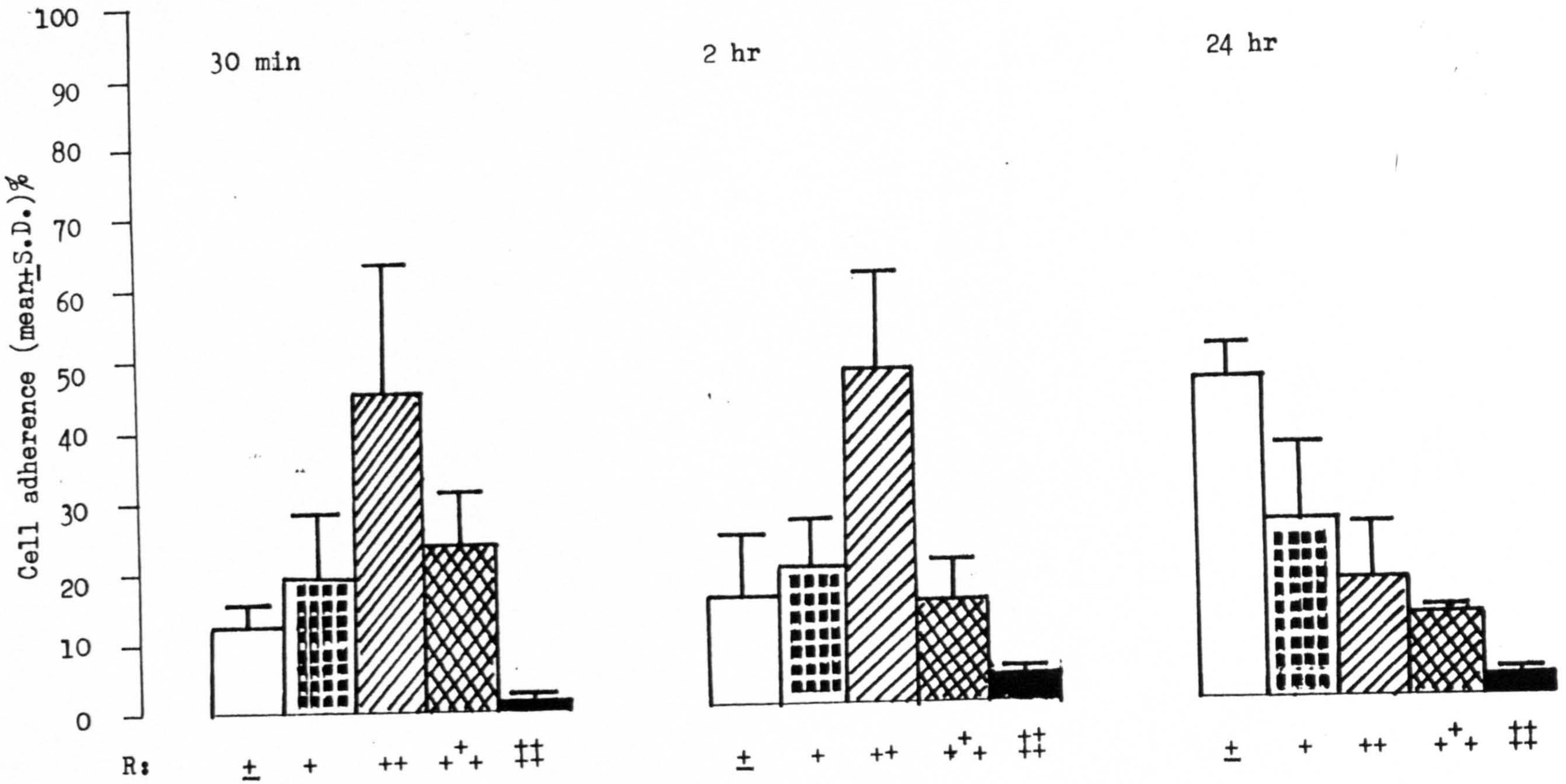


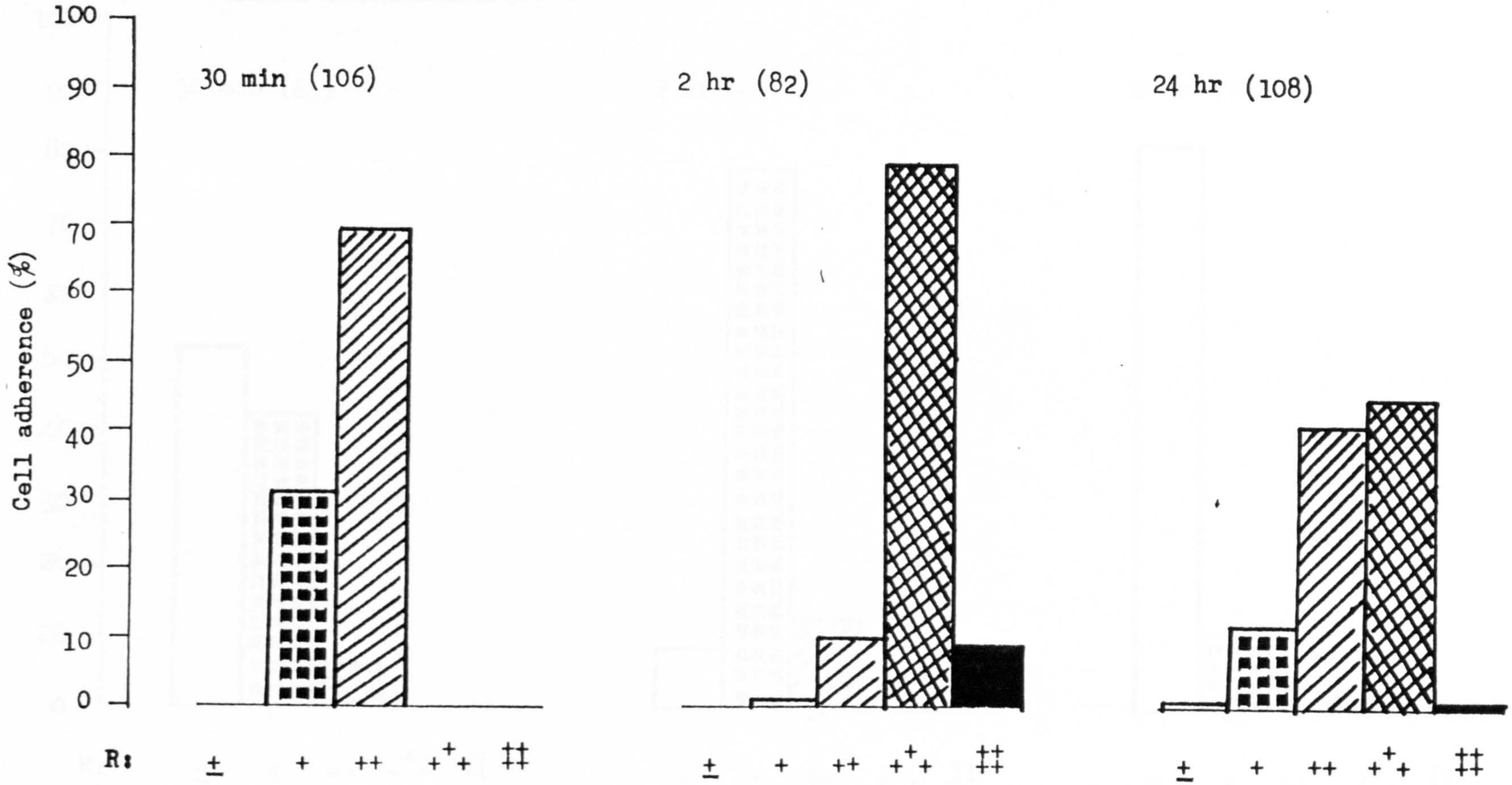
Fig. A.2.

Time course of magnitude of cellular adherence to fresh or peritoneal schistosomula in vitro

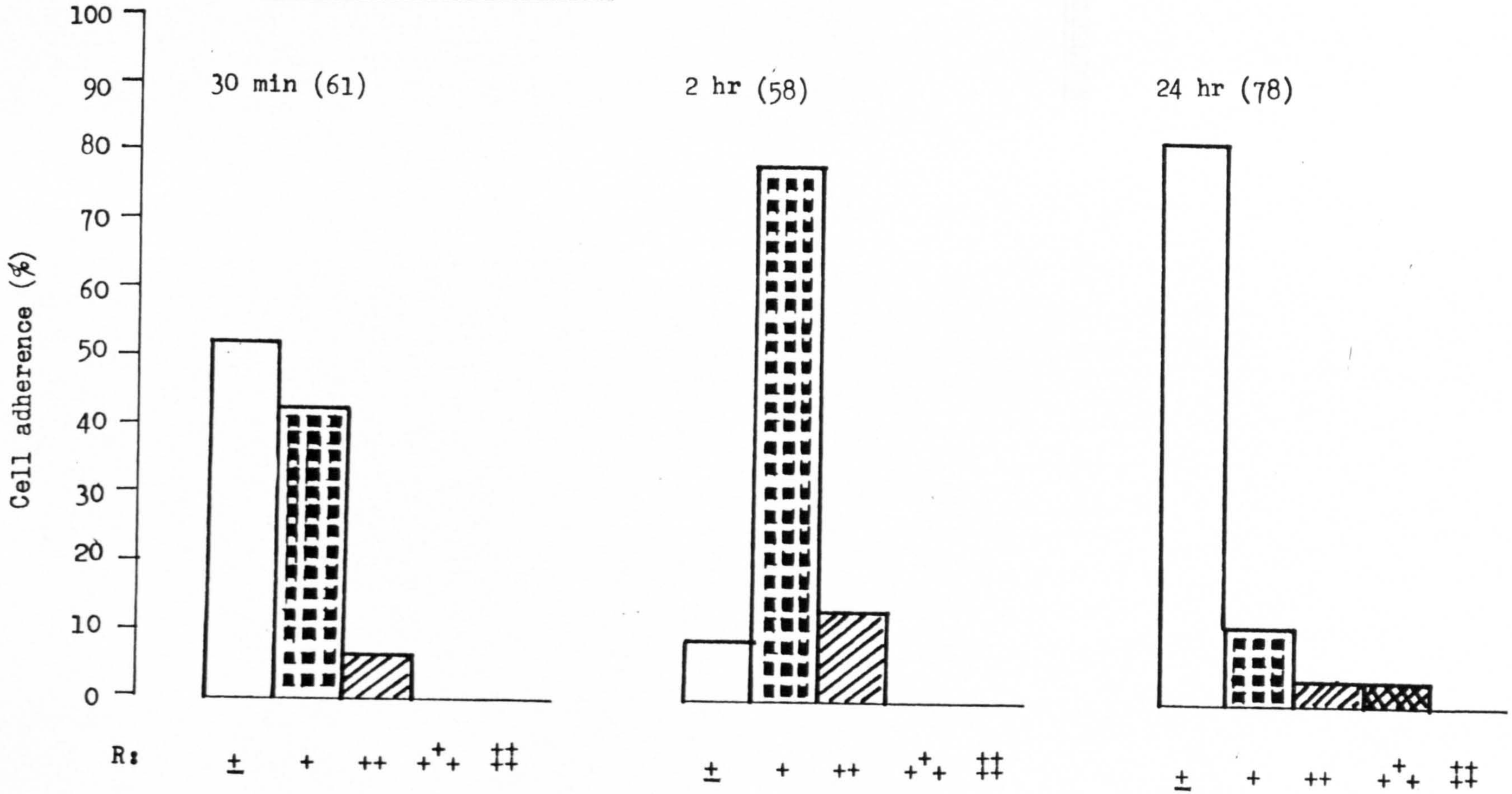
In the in vitro cellular adherence assay, the schistosomulum: cell ratio was set at 1:2000. The donors of cells and serum were mice infected 8 weeks previously. At 1/2, 2 and 24 hr, the cellular adherence to schistosomula was scored.

Each column represents the value of cellular adherence to schistosomula obtained from one experiment on a scale of R± to R++++, and numbers in the parenthesis refer to the number of schistosomula examined. See Fig. 6.2. for column symbols.

(a) Fresh schistosomula



(b) Peritoneal schistosomula



DISCUSSION

Fig. D.1.

Diagrammatic representation of the events which follow the intraperitoneal injection of schistosomula into (a) normal and (b) infected mice

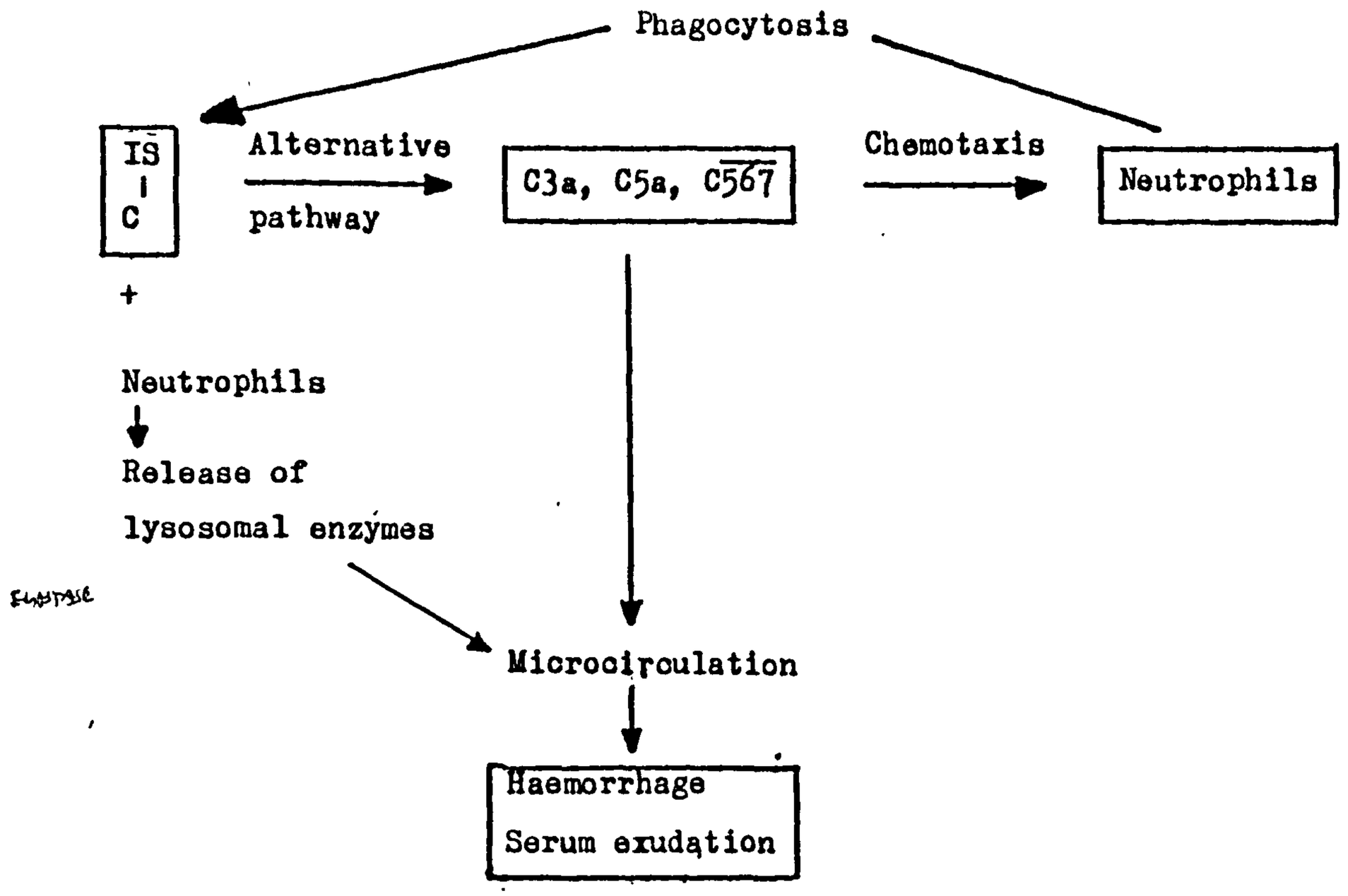
Abbreviations: PS= Permeated antigens from adult worms and eggs of the primary infection via circulation.

IS= Intraperitoneally injected schistosomula and their released antigens.

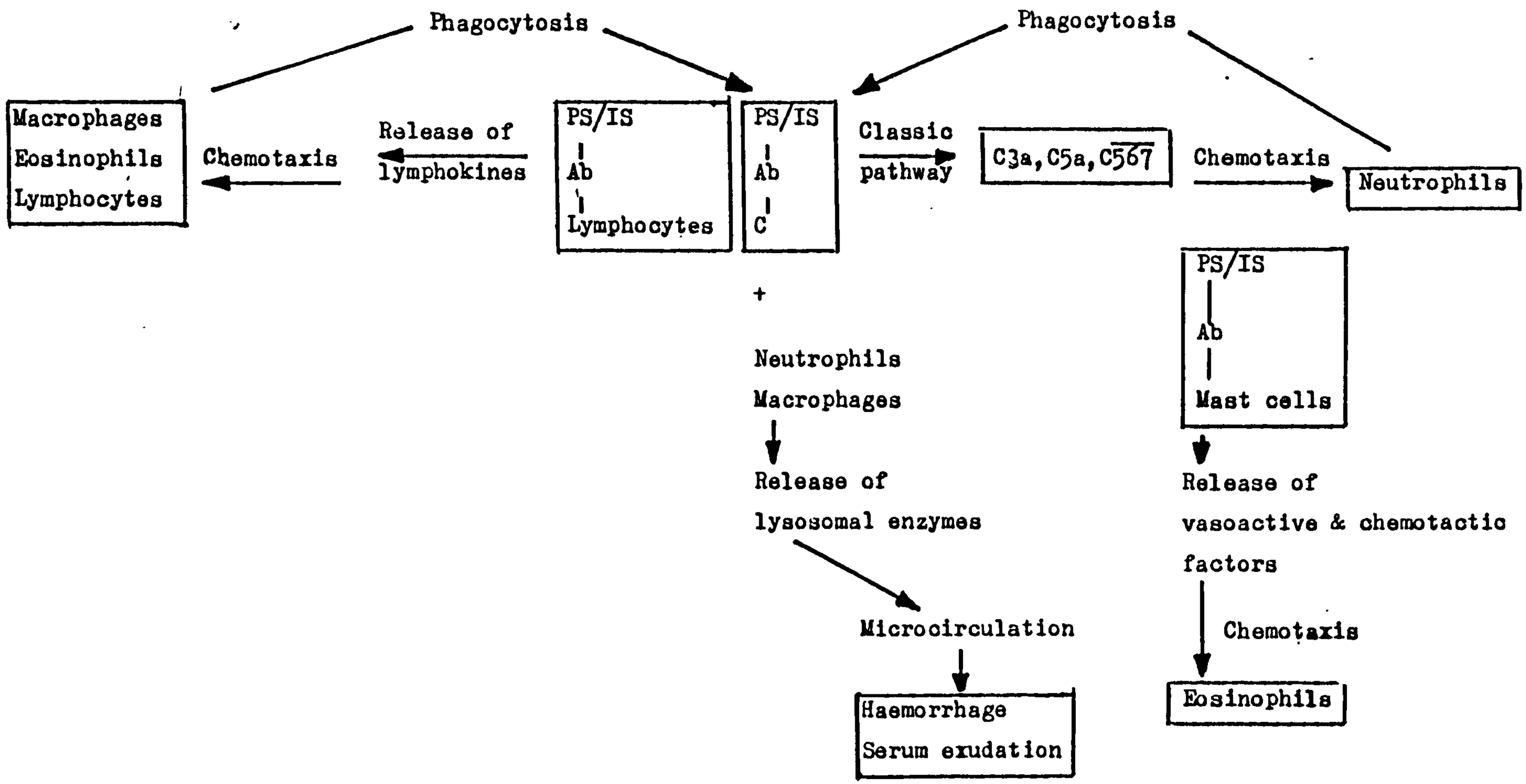
Ab= anti-*S. mansoni* antibodies

C= complement system

(a) Normal peritoneal cavity



(b) Infected peritoneal cavity



The prerequisite for an in vivo model system for cell immunity to any kind of infective organism is that the tested site must give a specific response to a specific stimulus. The response should correspond well with the reactions obtained from a natural infection. These are, in the infection with S. mansoni, chemotaxis induced by invading parasites, the parasitic attrition by leukocytes and evasion of host immunity by the parasites. Ideally, the observations obtained from the model should provide insights into the mechanisms underlying the immunity to S. mansoni in an immune animal.

The peritoneal cavity has been previously used to study the host immunity to tumours (Hopper and Nelson, 1979) and to bacteria (Fishel et al 1976). This thesis describes the cellular reactivity induced by the intraperitoneally injected schistosomula in normal and infected mice; the sequential development of cellular infiltration, the specificity of the infiltration; the quantitative analysis of the cellular adherence to schistosomula, and identities of cells participating in the infiltration and adherence. The fact that the peritoneal cavity is not a natural environment of the schistosome necessitates a careful evaluation to determine the limitations of the intraperitoneal challenge model. Here, the feasibility of using the peritoneal cavity as the site for the investigation of cellular activity is discussed in three major aspects: (1) chemotaxis in S. mansoni infection, (2) parasitic attrition by host leukocytes, and (3) parasitic evasion from host cellular reactivity.

1. Value of the intraperitoneal challenge model in the study of chemotaxis in S. mansoni infection

1.1. The sequence of cellular infiltration into the peritoneal cavity after intraperitoneal injection of schistosomula

The presence of schistosomula in the peritoneal cavity evoked a sequence of alterations in the cellular constitution in normal and schistosome infected mice (Section 4.2.1.). In infected mice, the reaction was characterized by an early (2-6 hr after challenge) infiltration predominantly of neutrophils, followed by the significant accumulation of eosinophils and macrophages (24 hr after challenge). In normal mice, the peritoneal reaction featured only the early, transient infiltration of neutrophils. A scheme of the possible mechanisms underlying the primary and secondary peritoneal chemotaxis and serum exudation is depicted in Fig. D1.

In the peritoneal challenge model, the question whether the schistosomula themselves are chemotactic for leukocytes is unknown. When tested in a chemotaxis chamber in vitro, the direct migration of neutrophils and eosinophils is not stimulated by whole schistosomula, by their excretion/secretion products, nor by the supernatant fluid of schistosomula after freeze-thaw disruption (James and Sher, 1980). Although both the injection of suspending medium and puncturing the peritoneal wall with a needle induced an infiltration of neutrophils (Section 4.2.2.), the greater infiltration of neutrophils induced by schistosomula

implies that the neutrophilic response was not solely due to the needle trauma. The abdominal puncture or injection of medium did not significantly alter the number of either eosinophils or macrophages compared with their respective unchallenged controls (Section 4.2.2.).

Several in vitro studies point to some contribution of complement components to chemotaxis. The residual glycocalyx on the newly transformed schistosomula is capable of activating the complement cascade via either the alternative (Machado et al., 1975; Santoro et al., 1979; Ramalho-Pinto et al., 1978) or the classical (Capron et al., 1974; Tavares et al., 1978) pathways. The resulting products, anaphylatoxin (C5a) and trimolecular complex ($C\overline{567}$), may be responsible for the early neutrophilic infiltration on challenge in normal and infected mice (James and Sher, 1980). This concept has also been applied to explain the neutrophil accumulation in the mouse skin (Lichtenberg et al., 1976) and in the hamster lung (Smith et al., 1975) after an initial infection or a reinfection. It was occasionally found that the acute neutrophilic infiltration was accompanied by mild to severe haemorrhage in the peritoneal cavity. In the present intraperitoneal challenge system, the visceral congestion was more often seen in challenged infected than in challenged normal mice. Lack of marked erythrophagocytosis indicates that the peritoneal bleeding did not take place during the primary infection but occurred as a consequence of the intraperitoneal challenge. Although a direct modulating effect on the blood vessel wall permeability

by the anaphylatoxin (Lepow, 1971) is a possibility, the microvascular injuries by the lysosomal proteases released by phagocytosing neutrophils and, possibly, macrophages (Section 7.2.2.1.; Movat et al., 1971; Allison, 1968; Weissmann et al., 1971; Hawkins,1971) may be responsible for the extravasation of red blood cells in the peritoneal cavity. The mice showing severe haemorrhage were not used in the present study. The haemorrhage occuring as a consequence of Arthus reaction has been previously observed in the lungs of hamsters reexposed to cercariae (Smith et al., 1975).

It has been demonstrated that the complement cleavage products are chemotactic for both neutrophils and eosinophils (Kay et al., 1973; Ward and Newman, 1969; James and Sher, 1980; Lachman et al., 1970) and capable of releasing eosinophil chemotactic histamine from mast cells (Lepow,1971). The failure of the peritoneal response to schistosomula to contain similar activity to eosinophils and macrophages in normal mice is unexplained. The lack of eosinophilic response in normal mice could not be attributed to a low level of recruitable peripheral cells, since mice with a peripheral eosinophilia induced by a previous infection with T. spiralis could not mount a peritoneal reaction with an augmented eosinophil content when intraperitoneally challenged with schistosomula (data is not shown) Perhaps this was because that there was too little chemotactic anaphylatoxin generated in the normal mice. It has been shown that the interaction between schistosomula and complement-fixing

antibodies was required to generate sufficient mouse complement activity to stimulate the migration of eosinophils *in vitro* (James and Sher, 1980; Mahmoud, 1982). The neutrophilic response occurring without accompanying infiltration of eosinophils excludes the possible role of eosinophil chemotactic factors released by phagocytosing neutrophils (Konig *et al.*, 1978). In this context, critical experiments to determine the role of the complement system in the early neutrophilic or the late eosinophilic responses should include studies of the effect on peritoneal reactivity of decomplementation, either by treating mice with cobra venom factor to ihduce *in vivo* complement depletion (Cochrane *et al.*, 1970; Pepys, 1975) or using mice congenitally deficient in complement components. Additionally, low levels of complement components has been taken as evidence of consumption secondary to complement-mediated neutrophilic infiltration. By measuring several components of either the classical or alternative pathway, the pathway can be identified which is likely to be involved in the neutrophilic infiltration. One should keep in mind, however, that the static measurement of complement level may not detect significant changes in complement degradation and compensatory production.

Unlike the chemotaxis of neutrophils, the recruitment of eosinophils in the peritoneal cavity requires a previous infection with *S. mansoni* and a homologous challenge (Sections 4.2.1.2. and 4.2.7.). The ability to mount an eosinophilic response could be transferred by an intravenous injection of immune serum shortly

before intraperitoneal challenge to normal recipients (Section 5.2.). At present, the identity of the factors in the donor serum which are active in the transferrence of eosinophilic response can not be deduced with certainty. The intravenous injection of immune serum resulted in an increase in peritoneal antibodies in the recipients (Section 5.2.). It seems reasonable to suppose that humoral antibodies are involved. That these antibodies against worm antigens themselves are sufficient to transfer the eosinophilic reactivity is likely, since such a response was observed in newly infected mice before the egg laying by the worms of the primary infection had started and anti-egg antibodies were detected (Sections 4.2.4.;3.2.2.4.). However, antibodies against egg antigens may have an enhancing effect on the peritoneal eosinophilia since greater infiltration of eosinophils, both primary and secondary, took place in mice with detectable peritoneal egg-specific antibodies (Sections 4.2.4.; 3.2.2.4.).

At present, it is not known what class of antibodies is responsible for the primary and secondary eosinophilic responses. The depletion of mast cells from the peritoneal exudates that occurred synchronously with the eosinophilic infiltration in a primary infection (Section 1.2.2.)suggests the activity of homocytotropic antibodies. Both IgG1 and IgE can function as homocytotropic antibody in the mouse species (Schwartz and Levine, 1973). The drastic decrease in-mast cells suggests IgE activity since mast cells have been shown to undergo an explosive type of degranulation and release all of their granules upon exposure

to the specific antigen (Barnett and Justus, 1975). Increase in serum IgE can be stimulated by an infection with S. mansoni (Rousseaux-Prevost et al., 1977). The relevant antigen may be the antigens of relatively small molecular weight secreted or excreted by worms and eggs and which passed through the vascular wall into the peritoneal cavity. The mild eosinophilic response without the accompanying decrease of mast cells observed in newly infected mice upon challenge (Section 4.2.4.) may suggest a phenomenon akin to the piece-meal mast cell degranulation noted in cutaneous reaction to cercarial reexposure (Lichtenberg et al., 1976; Askenase et al., 1976). Although the anaphylactic release of chemotactic factors may initiate the peritoneal eosinophilia in a primary infection, the extent to which the chronic eosinophilia in a primary infection (Section 1.2.2.3.) and the secondary eosinophilic infiltration induced by an intraperitoneal challenge (Section 4.2.1.2.) is a result of mast cell degranulation is unknown. The anaphylaxis may be a transient reaction since the content of mast cells remains low after the initial reduction and was not seen to return to the pre-infection level (Section 1.2.2.5.) and since the eosinophilic histamine is broken down rapidly in the tissue (Miles, 1956). The peripheral basophils are rare, and hence there may be no ready supply of recruitable basophils for the manifestation of mast cell hyperplasia and the subsequent basophil anaphylaxis (Askenase et al., 1978) following challenge. In this context, it is important to include an investigation of effects on primary and secondary peritoneal eosinophilia of cromolyn (Evans et al., 1975) in a

future study of the role of mast cells in the peritoneal reactivity.

It is likely that lymphocytes contribute heavily to the maintenance of chronic peritoneal eosinophilia in a primary infection and the secondary eosinophilic infiltration after challenge with schistosomula. Exposure of sensitized lymphocytes from schistosome infected mice to soluble schistosome antigens, in vitro, results in the generation of a lymphokine, eosinophil stimulation promoter factor, which exerts a chemotactic effect on eosinophils (Pelley et al., 1976; Mahmoud et al., 1979b; Phillips et al., 1977; Fine et al., 1973; Greene and Colley, 1976). It is intriguing to note that newly infected mice lacking antibodies showed eosinophilic reaction on challenge though this was not as marked as that occurring in mice with detectable antibodies (Section 4.2.3.; 4.2.4.). This may suggest that the ELISA used is not sensitive enough to detect trace antibodies or that antibody level does not necessarily reflect the degree of systemic sensitization. Several studies have indicated the involvement of T lymphocytes in the more fundamental initial supply process of eosinophilopoiesis (Mahmoud et al., 1975a,b; Miller et al., 1976). The sustained eosinophilopoietic response in the bone marrow of mice starts between 6-8 weeks after a schistosome infection. The onset of eosinophilopoietic activity apparently corresponds to that of peritoneal eosinophilia in a primary infection (Section 1.2.2.3.). This suggests that the eosinophilopoiesis may be causally related to the primary

peritoneal eosinophilia.

In contrast to the success in the induction of peritoneal eosinophilia by passive transfer of immune serum (Section 5.2.2.), the same procedure failed to confer a macrophage response in normal recipients (Section 5.2.2.). It may be relevant to mention a recent work which showed that immune serum did not significantly affect the diameter of macrophage-enriched egg granulomata in a mouse serum recipient and that the capacity of T lymphocyte-deprived mice to form normal size granulomata could not be restored by transferance of immune serum into these mice (Doenhoff et al., 1981). Therefore, it is unlikely that the macrophage accumulation is solely dependent on antibodies. The following reported works give support to the putative role of lymphokines in the macrophage response. In the cutaneous response to cercarial exposure or to intradermal injection of cercarial antigenic preparation in the mouse, a component of mononuclear hypersensitivity was transferable with immune spleen cells (Katz and Colley, 1976). The magnitude of cutaneous reactions to cercarial reexposure and of egg granulomata in the lungs and livers were found to be greatly diminished in animals deprived of functioning T lymphocytes (Buchanan et al., 1973, Domingo and Warren, 1967; Doenhoff et al., 1979; Byram et al., 1977). In vitro correlations of delayed-type hypersensitivity in schistosome infection have also been demonstrated (Lewis et al.,1977; James and Sher, 1980). Substances which influence the migration of macrophages, namely the macrophage migration inhibition

factor and macrophage/monocyte chemotactic factor, have been detected in the supernatant fluid of cultured sensitized spleen cells from schistosome infected mice in the presence of specific antigens. Since the onset of increase in peritoneal macrophages (Section 1.2.2.1.) corresponds with the reported time at which egg granulomata develop in the liver in a primary infection (Byram et al., 1979), and the increase in macrophages, both primary and secondary, only takes place in mice in which antibodies against egg antigens can be detected in serum and peritoneal fluids (Section 3.2.2.4.), it is possible that sensitization of mice with egg antigens is a prerequisite for the macrophage response in the peritoneal cavity. The cells, presumably T lymphocytes, in the egg granulomata are likely to be responsible for the peritoneal reactivity. The absence of macrophage infiltration in mice previously infected with a unisexual population of cercariae (Section 4.2.5.) when intraperitoneally challenged supports this concept. Mice are apparently sensitized to egg antigens by 8 weeks after infection as judged by their capacity to mount the primary (Section 1.2.2.1.) and the secondary (Section 4.2.4.) macrophage responses. Lymphokines are also known to stimulate the chemotaxis of lymphocytes (Cohen et al., 1973; Ward et al., 1977). Thus, the peritoneal lymphocytes, either residential or recruited, may be constantly stimulated by permeated schistosome antigens and to produce cascades of lymphokine activity. This may be responsible for the prolonged peritoneal leukocytosis in a primary infection (Section 1.2.1.).

The presence of schistosomula means much more pronounced antigenic stimulation of peritoneal lymphocytes that results in the secondary infiltration of eosinophils and macrophages in the infected mice (Section 4.2.1.2.; 4.2.4.; 4.2.6.).

1.2. Development of the protein concentration and specific antibodies in the peritoneal fluid

In addition to changes in the total number of and the composition of peritoneal exudates, the present study demonstrates that infection with S. mansoni for 7 weeks or longer also induces accumulation of protein within the peritoneal cavity (Section 2.2.1.).

From the electrophoretic patterns (Section 2.2.3.), it is clear that the peritoneal fluid from mice contains a wide variety of protein molecules. Since the focus of attention of this study is on the inflammatory and immune systems and since also the other proteins present in the peritoneal fluid are little known, the production of immunoglobulins will be therefore the subject for discussion.

The presence of IgG (IgG1 and IgG3), IgA and IgM were detected in the lavaged peritoneal fluids from normal and infected mice by the immunodiffusion method (Section 2.2.3.). The increase in the concentration of specific IgG to worm and egg antigens of S. mansoni was demonstrated by ELISA (Section 3.2.2.4.). Subsequent experiments revealed that the initiation of the increase in IgG in mice previously infected for 7 weeks depends on the

size of cercarial dose used for the infection (Section 3.2.2.6.). Two sources of peritoneal immunoglobulins warrant consideration: production by cells within the peritoneal cavity and transudation from serum. Several studies have demonstrated that peritoneal cells isolated from sensitized animals are capable of differentiating into specific antibody-producing plasma cells both in vivo and in vitro (Shelton et al., 1976; Gisler et al., 1974; Pages et al., 1974). At present, the relative quantity of activated lymphocytes that synthesize anti-schistosome IgG is unknown. While the local production should not be disregarded, the direct exudation of serum into the peritoneal cavity is likely to be the major source for the peritoneal immunoglobulins. This is supported by the similarities of the major proteins in SDS-electrophoretic analysis and the precipitin lines in immunoelectrophoretic patterns (Section 2.2.3.) of serum and lavaged peritoneal fluids. The increase in the concentration of MW 96K and MW 50K electrophoretic bands in the serum also occurred simultaneously in their respective peritoneal fluids (Section 2.2.3.). Furthermore, the rise in antibody titre in the peritoneal fluids generally coincides with that of serum (Section 3.2.2.5.). The existence of positive correlation in antibody levels between serum and their corresponding peritoneal fluids was also established (Section 3.2.2.5.).

Intravenous injection of immune serum resulted in an increase in the peritoneal IgG (Section 3.2.2.). This finding indicates that the vascular-peritoneal barrier is permeable to molecular size of IgG under the described experimental condition. IgM was

detected in the normal and infected peritoneal fluids (Section 3.2.1.) and an increase in serum IgM due to a. schistosome infection has been previously reported (Bout et al., 1980; Sher et al., 1977b). It is not yet known whether its increase in serum is also reflected in their corresponding peritoneal fluids. This is an interesting question for two reasons: firstly, the transport of larger molecules may give insight into changes in the permeability of the blood vascular wall during a schistosome infection. Secondly , the polymeric IgM may contribute to the pathological symptoms (Tada et al., 1975;Hillyer and Lewert, 1974; Natali and Cioli, 1976;Sogandares-Bernal and Brandt, 1976) and immunity against S. mansoni (Smith et al., 1982).

Speculations regarding the mechanism underlying the serum exudation for the microvessels may include the release of vaso-active mediators from mast cells following interaction of anaphylactic antibodies on the cell surface with antigens. Such a sequence of events induced by schistosomula leading to tissue oedema was previously demonstrated in the skin of guinea pig at the site where basophil anaphylaxis has taken place (Askenase et al., 1978). It would be interesting to investigate the effect of reserpine on the accumulation of proteins in the peritoneal fluid, since it depletes the serotonin in mouse mast cells which are responsible for the change of vascular permeability (Askenase et al., 1978).

The factor causing the prolonged exudation in the chronic infection (Section 2.2.I.) is unknown. The number of mast cells did not return to pre-infection level after the initial

decrease at 7 weeks after infection (Section 1.2.2.5.). Therefore, the anaphylactic mediator release cannot be the sole cause of the chronic serum exudation. Anaphylatoxins generated from activation of complement cascade have been previously shown to enhance vascular permeability (Osler et al., 1959). Additionally the portal hypertension caused by egg granulomata is an established clinical and experimental manifestation of schistosomiasis (WHO Memorandum, 1974; Warren, 1973a; Lichtenberg, 1970). The marked elevation of concentration of protein (Section 2.2.1.) and specific IgG (Section 3.2.2.4.) started at the time at which egg granulomata are known to appear in the liver (Byram et al., 1979). The portal hypertension is the consequence of the constriction of veins which in turn leads to increased pressure in the venules. This is followed by formation of gaps between adjacent venular endothelial cells which allow the exudation of serum. The resulting exudation may contribute to the increase of protein contents in peritoneal fluids of mice which have been previously infected for 7 weeks when egg laying has been commenced, and to the chronic state of peritoneal oedema.

1.3. Use in the study of inflammatory responses to schistosomula

1.3.1. As the source of chemotactic factors involved in cellular infiltration induced by schistosomula

The present study was designed to establish the feasibility of the intraperitoneal challenge model in studies of chemotaxis

in S. mansoni infection. By definition, an in vivo model should recapitulate in vivo events of cellular responses to S. mansoni during natural infection. The cellular response to schistosomula in the skin of normal mice has been shown to be primarily neutrophilic, whereas the reaction to challenge larvae in immune mice is enriched with eosinophils or macrophages depending on the site of challenge (Colley et al., 1972; Lichtenberg et al., 1976). All these types of cells demonstrate schistosomacidal activity in vitro (Review see Introduction, Phillips and Colley, 1978; McLaren, 1980). The ingress of peritoneal blood leukocytes to the locus of parasitic invasion is likely to be of central importance in the immunity against S. mansoni. The preferential accumulation of neutrophils, eosinophils and macrophages has been attributed to various chemotactic factors generated by immediate-type hypersensitivity, by Arthus reaction and by delayed-type hypersensitivity that selectively attract these classes of leukocytes in vitro (Review see Introduction, Phillips and Colley, 1978). However, there has been no direct study of the biologic activity of extracts collected from skin reaction sites to show that the cutaneous reaction is so mediated.

The present study has established that the development of cellular responses to schistosomula in the peritoneal cavity (Section 4.2.1.) correlates well with previous histological observations made in the skin exposed or reexposed to cercariae (Colley et al., 1972; Lichtenberg et al., 1976). The intraperitoneal challenge model has an advantage in that the cellular

infiltration can be accurately quantitated. The peritoneal responses proved to be immunologically specific as intraperitoneal challenge with T. spiralis, T. canis and E. coli failed to produce cellular responses similar to those induced by the schistosomula (Section 4.2.7.). The intraperitoneally introduced E. coli elicited an acute accumulation of neutrophils (Section 4.2.7.). A neutrophilic response could be induced in skin when the bacteria were injected intradermally (Kopaniak et al., 1980). Using non-schistosome systems, several studies have demonstrated that intraperitoneal injection of extracts from skin sites exhibiting hypersensitivity reactions could produce peritoneal hypersensitivity similar to the cutaneous reaction of the donor (Cohen et al., 1973) Substances with migration enhancing activities, such as histamine, a lipid chemotactic factor for neutrophils, macrophage migration inhibition factor and a macrophage disappearance factor have been isolated from the peritoneal fluids lavaged from immunologically challenged animals(Postethwaite and Snyderman, 1975; Valone and Goetzl, 1978; Maarsseveen, 1977). Therefore, isolation and characterization of chemotactic factors in the peritoneal fluids of schistosome infected mice before and after the intraperitoneal challenge with schistosomula should determine the onset and involvement of immunologic reactions of immediate and delayed-type hypersensitivity as well as of activation of complement pathways in the peritoneal cavity. The understanding of mechanisms underlying the peritoneal hypersensitivity should provide insights into those involved in the skin immunity.

The intraperitoneal challenge model permitted an analysis of immunologic events culminating in the cellular infiltration. The eosinophilic reaction can be transferred by the intravenous injection of immune serum to normal recipients (Section 5.2.2.). Such systemic passive sensitization was previously shown to elicit an eosinophilic infiltration in the skin of normal recipients when subsequently exposed to cercariae (Hsu and Hsu, 1976; Katz and Colley, 1976). Several workers, using non-schistosome systems, have also demonstrated that systemic or local introduction of or depletion of one or more participating humoral (antibody, complement) or cellular (mast cell, lymphocyte) elements could alter the cellular reaction to a specific challenge in the peritoneal cavity of the treated animals (Cohen et al., 1974; Hopper and Nelson, 1979). Hence, the intraperitoneal challenge model described here provides three advantages in the studies of chemotaxis in S. mansoni infection: (1) providing a qualitative and quantitative way for monitoring the cellular infiltration, (2) permitting the observation of interactions between each factor (humoral or cellular) leading to the infiltration of cells into the challenge site, and (3) permitting the isolation and characterization of chemotactic factors responsible for the cellular reactions.

1.3.2. As the source of anti-S. mansoni antibodies involved in immune response

The increase in peritoneal IgG (Section 3.2.2.4.) could be used as an indicator of the initiation of antibody production

elicited by an infection with S. mansoni. In fact, the IgG against adult worm antigens could be detected in peritoneal fluids earlier than in their corresponding sera (Section 3.2.2.4.). However, it does not allow periodic assessment of antibody level in peritoneal fluids in the same animal throughout the infection course as one may do with serum, nor might it be used for diagnosis of schistosomiasis in man.

The S. mansoni infection has been shown to result in dramatic elevation in serum IgG level in mice (Section 3.2.2.4.) (Bout et al., 1980). Passive sensitization experiments have implicated IgG1 in immunity in vivo (Sher et al., 1975). Several in vitro studies indicate that antibodies promote adherence and killing of schistosomula by various types of leukocytes (Review see Introduction; Phillips and Colley, 1978; McLaren, 1980). Sensitization of mediator-producer cells by cytophilic antibodies or interaction of complement-fixing antibodies with complement may lead to further attraction of effector cells (Colley et al., 1972; Katz and Colley, 1976). There have been no investigations of the activity of antibodies in skin extracts prepared from sites exposed and reexposed to cercariae. In any case, preparation of sufficient amount of skin extract has proved difficult (Kopaniak et al., 1980).

The present study showed that serum exudation could be induced by a chronic schistosome infection (Section 2.2.1.), and that IgG could cross a vascular wall of normal immune serum-recipients (Section 5.2.2.). It is conceivable that serum IgG could pass

not only into the peritoneal cavity but also into the skin tissue. The immunological dependence of accumulation of eosinophils and macrophages around the invading schistosomula in the skin (Colley et al., 1972; Lichtenberg et al., 1976) gives some support to this hypothesis. The coincidence in the onset of the increase in peritoneal IgG and in serum IgG encourages the further exploration of using peritoneal IgG or other protein as a standard of serum exudation induced by a schistosome infection into a tissue space. The isolation and characterization of antibodies in the peritoneal fluids may, therefore, lead to identification of relevant antibodies involved in the acquired skin immunity.

1.3.3. As a site for determination of schistosome macromolecules which are responsible for the cellular infiltration

Much of the interest in schistosome antigens has centered on their actual or potential use as reliable skin-test material for the diagnosis of schistosomiasis in the field. Progress with the isolation of skin test material has been based largely on empirical attempts to induce specific cutaneous reaction in laboratory animals with various antigenic preparations. Several methods, such as homologous passive cutaneous anaphylaxis, systemic sensitization with local challenge and Prausnitz-Kustner type reaction have been devised for the assay of schistosome antigen fractions as skin test materials (Williams et al., 1965; Sato et al., 1969; Fife, 1971; Harris, 1973).

The present study showed that the intraperitoneal injection of crude PBS extract of adult worms elicited a significant infiltration of eosinophils (Section 9.2.3.). Of the three fractions (S1, S2 and S3) isolated from anion exchange chromatography of crude extract, both S1 and S2 could induce eosinophilic infiltration in infected mice. Previous studies have shown that a continuous local antigenic stimulus is the prerequisite for the maintenance of peritoneal eosinophilia (Berg et al., 1980). The surface membrane and gut epithelium of adult worms and eggs constantly secrete or excrete antigenic substances (Kusel et al., 1975; Kusel and Mackenzie, 1975; Lichtenberg et al., 1974; Houba et al., 1976). Some of the released antigens were found in the circulation (Nash et al., 1974; 1977; Gold et al., 1969; Lichtenberg et al., 1974). It is conceivable that the portal hypertension causes some of the circulating smaller macromolecules, possibly including those in S1 and S2, to pass through the vascular wall into the peritoneal cavity. The permeated antigens may then interact with sensitized cells and induce them to release chemotactic and vasoactive substances locally, resulting in the eosinophil-enriched leukocytosis and serum exudation observed in the chronic primary infection (Section 2.2.1.).

The failure of homogenates of schistosomula/adult worms to stimulate infiltration of macrophages in infected mice is an interesting observation (Section 9.2.3.). This may be for one or more of the following reasons: (1) homogenization of schistosomes results in conformational changes in some of the

antigenic determinants essential for the macrophage responses, (2) the antigenic determinants responsible for the macrophage reactivity reside in the insoluble fraction of the homogenate, and (3) macrophage reaction is not manifested at the tested site because of the paucity of antigen retention. The assumption that the manifestation of delayed-type hypersensitivity may depend on the local retention of antigens at the tested site has been previously put forward to explain the negative footpad reaction to soluble proteins in sensitized mice (Katsura et al., 1977). Interestingly, the crude extract of adult worms induced more infiltration of neutrophils into the normal peritoneal cavity than did PBS alone (Section 9.2.3.). This non-specific peritoneal reactivity was detected in the S3 fraction isolated by ion exchange chromatography (Section 9.2.3.). The mechanism underlying the neutrophilic activity is not known. The macromolecules in S3 may activate complement via the alternative pathway. The significance of this neutrophilic activity in the host-parasite relationship is unclear. The demonstration of non-specific neutrophilia-inducing factor in the crude worm extract may help to explain the false positives obtained when crude extract was used as material for skin tests previously reported (Kloetzel and Da Silva, 1967; Sato et al., 1969).

The present study shows that the injection of as little as 5 µg protein (Section 9.2.3.) could induce peritoneal reactivity, and that by examining the nature of infiltrating cells, the specific and non-specific active factors in an antigenic prepa-

ration could be identified. Therefore, the possibility of using the intraperitoneal challenge model, in the same manner as the conventional skin test in experimental animals, to assess the chemotactic activity of antigenic preparations should be further explored. Further study also should be made of S1 and S2 in an attempt to purify and characterize the eosinophilia-inducing factor, and to investigate whether the S1 and S2 or purified proteins derived from S1 and S2 could be used as reliable skin test materials in the field or used as a vaccine to enhance the host effector mechanisms against the percutaneous infection with S. mansoni in man.

2. Value of the intraperitoneal challenge model in the study of cellular adherence and parasitic attrition

The development of *in vitro* assay systems for the study of immunity to *S. mansoni* has added considerably to our knowledge of its mechanisms (Review see Introduction, Phillips and Colley, 1978; McLaren, 1980). However, some of the data obtained from *in vitro* experiments can not be reconciled with histological observations made from a percutaneous infection. For example, an intense cellular adherence to invading schistosomula takes place in the normal mouse skin (Lichtenberg *et al.*, 1976). The innate skin immunity accounts 65% of the deaths of the infective population in mice (Smithers and Gammage, 1980), whereas only negligible adherence and killing activities by normal cells are obtained *in vitro* (Section A.2.1.) (Kassis *et al.*, 1979 ; James *et al.*, 1982). The present intraperitoneal challenge model demonstrates results parallel to those in the skin in adherence patterns over 24 hr (Section 6.2.1.), thus the peritoneal cavity may reflect host skin reactivity more closely than do *in vitro* systems.

The investigation of the identity of cells participating in the early adherence to schistosomula in the peritoneal cavity revealed a discrepancy with the histological observation of cutaneous reactions. In a percutaneous infection, the neutrophils appear to be of the major responding cell type in both innate and acquired skin immunity (Lichtenberg *et al.*, 1976). In contrast,

macrophages represent at least 80% of the population of adherent cells even during the peak of infiltration of neutrophils (2 hr after challenge) in the peritoneal cavity regardless of the immune status of mice (Sections 7.2.2.; 7.2.3.; 7.2.4; 7.2.5.). This finding suggests that the type of cells which are active may depend on the site of challenge, and that the cells infiltrating to the challenge site do not necessarily participate in adherence or phagocytosis, or that the adherence by neutrophils is a transient event lasting for less than 30 min (the time for the first sample collection for the enzymatic treatment). The relative ineffectiveness of mouse neutrophils was previously demonstrated *in vitro* (Kassis *et al.*, 1979).

The macrophage adherence may be a non-specific response to foreign bodies administered intraperitoneally, since similar observations have also been made in animals injected with non-schistosome organisms (Jeska, 1969; Greenberg and Wertheim, 1973). Although macrophages are the basic constituent of young worm-cell foci harvested from both normal and infected mice at 2 hr after intraperitoneal challenge (Section 7.2.), enzymatic treatment to dissociate adhering cells has conclusively shown that macrophages in the immune foci are as tightly apposed to each other as to schistosomula to which they adhere (Section 7.2.2.). This is indicated by the difficulty encountered in dispersing them enzymatically as compared with the easy dissociation of normal cells from schistosomula. Some schistosomula

enzymatically freed from normal cells were motile. These findings as well as the self-induced dissociation of normal macrophages from schistosomula after 24 hr (Section 6.2.1.2.) indicate that, at least functionally, macrophages present in immune foci may differ from those in foci recovered from normal mice. Previous studies have shown that macrophages from chronically infected mice are in an activated state (James et al., 1982). The activation may be generated as a consequence of phagocytosing immune complexes (Capron et al., 1975) or of exposure to lymphokines (Bout et al., 1981). The activated macrophages demonstrated greater affinity to and killing of schistosomula than normal macrophages (James et al., 1982). The presence of antibodies enhances the innate ability of macrophages, both normal and activated, to adhere to and kill schistosomula in vitro (Kassis et al., 1979; James et al., 1982). The enhanced effect was found to be mediated by IgG1 (Kassis et al., 1979). These findings may have relevance to the persistent adherence to and phagocytosis of surface materials of schistosomula by macrophages in the immune peritoneal cavity (Section 7.2.5.) in which anti-schistosome IgG was detected (Section 3.2.2.4.). The intensive mononuclear cell infiltration at the site of cercarial exposure in immune mice (Colley et al., 1972) suggests a possible function for macrophages in the acquired skin immunity. The decreased resistance to reinfection in mice congenitally deficient in macrophage function (Blum et al., 1978) gives some support to this hypothesis. It was also interesting

to note that some of the macrophages in the peritoneal cavity, regardless of the immune status of the tested mice, had mast cell granules in their vacuoles (Section 7.2.5.1.). Such exocytosis of granules by mast cells and the subsequent phagocytosis of granules by macrophages have been previously noted in mouse reactions to T. spiralis (Justus and Morakote, 1981) and to keyhole limpet haemocyanin(Askenase et al., 1978). The significance of this feature is unknown.

By 24 hr, the number of eosinophils active in adherence increased and the adhering cell population obtained from infected mice contained almost equivalent percentages (approximately 45%) of eosinophils and macrophages (Section 7.2.5.). Homing of eosinophils toward schistosomula in the skin of immune mice has been demonstrated in several histological studies (Lichtenberg et al., 1976, Savage and Colley, 1980). The capability of eosinophils in killing of schistosomula has been established in vitro and their role in the acquired skin immunity has been suggested (Review see Introduction, Phillips and Colley, 1978). The collaboration between eosinophils and macrophages in killing of schistosomula has been demonstrated in vitro (Kassis et al., 1979). The relationship between eosinophil and macrophage activity on the surface of schistosomula is presently unknown. In conclusion, the adherence of peritoneal cells in the immune peritoneal cavity to schistosomula may be a multiphasic process involving macrophages in an early stage

and eosinophils at a later stage. In normal mice, in contrast, the foci collected at a later time were smaller and the percentage of free schistosomula increased (Section6.2.1.2.) when compared to the earlier (2 hr) (Section 6.2.1.1.) cellular adherence pattern. The failure of adherent macrophages from normal mice to achieve significant killing of schistosomula *in vitro* was previously reported (James *et al.*, 1982). The conclusion is therefore inescapable that schistosomula become progressively less susceptible to attack by host leukocytes. The possible mechanisms underlying the immunoevasion will be discussed later.

Systemic transfer of immune serum conferred a significant increase in R++ to R++++ peritoneal cellular adherence activities to normal mouse recipients (Section 6.2.3.). The intravenous injection of immune serum induced an increase in specific IgG in the peritoneal fluid of the recipient (Section 5.2.2.). This result suggests that the augmented adherence rates attained by immune mice over the 24 hr is not fully accounted for by the greater initial number of leukocytes (Section 1.2.1.) alone, Rather it suggests that the leukocytes adhere more efficiently in the presence of IgG. This finding appears to be relevant to a previous report showing that significant protection against a percutaneous infection can be transferred by serum or the IgG fraction of serum from immune mice to normal mouse recipients (Sher *et al.*, 1975; 1977). A significant increase in the cellular adherence to intraperitoneally injected schistosomula was induced in mice previously infected with

T. spiralis compared to those in normal mice (Section 6.2.2.). Since serum from T. spiralis -infected mice was previously shown to be ineffective in inducing cellular adherence to schistosomula (Kassis et al., 1979), the non-specific cellular adherence is therefore probably due to macrophages activated by a previous trichinella infection (Wing et al., 1979).

The destruction of schistosomula in the peritoneal cavity was not quantitatively analysed in the present work. The schistosomula completely covered with cells were generally immotile and had morphological signs of death (relative opacity and flattened appearance) when the lavaged sample was collected and examined 24 hr after challenge. Thus, the percentages of schistosomula with R+++ to R++++ adhering cells at 24 hr may reflect the mortality of injected schistosomula in the peritoneal cavity. An additional criterion of mortality, such as a dye exclusion method, would increase the reliability of the quantitative data and should be included in a future study employing the intraperitoneal challenge model.

The schistosomula preparation used for challenge experiments invariably included a small percentage of dead individuals ranging from 1% to 3% of the population. Since these dead individuals could not be separated from the 1,000 schistosomula intraperitoneally injected, it is difficult to distinguish the schistosomula which had been killed by host effector mechanisms from those which had been killed during the mechanical transformation procedure. In addition, not all schistosomula were recovered.

In general, only 2-8% of the intraperitoneally inoculated schistosomula could be recovered by the peritoneal washing procedure described. The fate of the rest of the challenge schistosomula is unknown. The possibility that the low recovery is a consequence of adhesion of foci to the peritoneal surface could not be excluded (Melo et al., 1980). A lymphokine causing macrophages to adhere to the peritoneal surface has been described (Sonozake and Cohen, 1971; Nelson and Boyden, 1963). This adherence may lead to the removal of macrophage-bound schistosomula from the fluid phase of the infected peritoneal cavity. Some portal worms derived from intraperitoneally injected schistosomula could be recovered 8 weeks later. The number of adult worms surviving the peritoneal immunity may demonstrate the immune status of tested animals. It is therefore suggested that the intraperitoneal challenge model can be a useful in vivo method for studies of preferential adherence of various cell types to schistosomula in the presence or absence of antibodies, as well as for quantitative analysis of cellular adherence to and killing of schistosomula. This model may also provide information analogous to portal worm counts (Smithers and Terry, 1965) which measure the long term survival resulting from challenges.

3. Value of the intraperitoneal challenge model in the study of immunoevasion by S. mansoni

In mice, the schistosomula surviving the innate and acquired skin immunity evoke little cellular reactivity when they arrive in the lungs 3-5 days later (Lichtenberg et al., 1976). The worms recovered from the lungs are resistant to immune attack in vitro (McLaren et al., 1975; Ramalho-Pinto et al., 1978; Incani and McLaren, 1981). The critical stage of differentiation related to the acquisition of resistance to host immunity, therefore, occurs within 48 hr after penetration of the skin. However, entrapment of schistosomula in cutaneous tissue hinders the recovery of skin schistosomula for the analysis of their immunological features and the investigation of the conditions required for the development of the resistance to immunity. Several in vitro techniques have been devised to overcome this sampling difficulty and obtain late-stage schistosomula by preparing schistosomula using either mechanical transformation method (Ramalho-Pinto et al., 1974) or allowing cercariae to penetrate an isolated skin in vitro (Clegg and Smithers, 1972), followed by culture in the presence of host macromolecules for 2 or more days. Such schistosomula exhibit a significant increase in resistance to immune attack in vivo (Lichtenberg et al., 1977) and in vitro (Tavares et al.,1978b; 1980; Dessein et al., 1981; Samuelson et al., 1980; McLaren and Incani, 1982). The present study shows that within 24 hr in the normal and immune peritoneal

cavity, some of the injected schistosomula are capable of avoiding recognition by host cells *in vivo* (Section 6.2.1.) and *in vitro* (Section A.2.2.). It is not difficult to envisage that the schistosomula at this time have a reduced number of antibody or C3b receptor sites on their surface. These results are in good agreement with studies in mice showing that passive transfer of immunity with immune sera is effective if the transfer is performed shortly before, as opposed to administered several days after the challenge infection (Sher *et al.*,1977c). The cell-free living schistosomula recovered from the peritoneal cavity, referred to as 'peritoneal schistosomula', therefore, provide an interesting candidate for a study of evasion of non-specific and specific recognition by schistosomula.

It was intriguing to note that the peritoneal schistosomula did attract cellular adherence during the early hours after their inoculation into normal peritoneal cavities. There might be several reasons why the schistosomula could survive the adherence of macrophages. The adhering macrophages may not discharge their lysosomes onto the parasite surface due to the lack of signal that may be given by bound antibodies (Sajnani *et al.*, 1974) as in an infected animal. On the other hand, the macrophages may have discharged their enzymes, but schistosomula, after an appropriate environmental stimulation encountered in the peritoneal cavity, starts membrane turnover at such a rate that the damage by adhering macrophages does not take place before the replacement of the target membrane together

with its adhering cells by a new membrane. The metabolic surface changes by peritoneal schistosomula were demonstrated by two observations made in our laboratory (manuscript in preparation); (1) the cercarienhüllen reaction (Kemp et al., 1973) did not take place on the surface of peritoneal schistosomula when incubated with immune serum, which indicates the loss of glycocalyx, and (2) the antisera against antigens that were shed from mechanically transformed schistosomula during the three days in culture was raised in rabbits. The fluorescein conjugated antisera detected small quantities of the early tegumental antigens on the surface of schistosomula recovered from skin 24 hr after percutaneous infection, and on the surface of peritoneal schistosomula. In contrast, the same fluorescent antisera bound very strongly to the antigens in 3 hr- and 48-old mechanically transformed schistosomula and 3 hr-old in vitro skin penetrated schistosomula. This evidence suggests that the schistosomulum in the peritoneal cavity has a rate of membrane turnover more similar to that of the skin schistosomulum than to those prepared in vitro. The loss of fluorescein conjugated wheat germ agglutinin label from the surface of living schistosomula could be demonstrated within 24 hr after intraperitoneal inoculation (Section 7.2.5.1.), whereas schistosomula incubated in culture enriched with heat-inactivated normal mouse serum for the same length of time still retain their label. Therefore, it is likely that there are factors present in the peritoneal fluid which are able to stimulate in mechanically transformed schistosomula the initial changes promoted by skin penetration.

The stimuli which potentiate the tegumental change by schistosomula in the perittoneal cavity remains unclear. Since the schistosomula used for challenge were prepared by the mechanical transformation method and were subsequently directly injected intraperitoneally (Section 4.1.), the surface abrasion that may happen to those migrating through the skin could not, therefore, account for the glycocalyx loss by peritoneal schistosomula. It appears that the schistosomulum undergoes an intrinsic differentiation step that results in the shedding of glycocalyx soon after inoculation. Several in vitro studies have shown that mechanically transformed schistosomula exhibit a significant level of protectiom against cellular attack after being cultured in the presence of serum for 2 days (McLaren and Incani, 1982; Tavares et al.,1978b) The serum factor responsible for the enhanced resistance is a macromolecule with a molecular weight between 7S and 19S, but that is neither IgG nor a component of complement (Tavares et al., 1978b;1980). The serum protection factor may be active in the peritoneal cavity since sera and peritoneal fluid demonstrate similar immunoelectrophoretic profiles (Section 2.2.3.).

It is also likely that, from the observations above, the membrane turnover by schistosomula is more rapid in vivo than in vitro.

In the immunologic sense, the loss of complement-activating glycocalyx suggests a reduction in the number of C3b molecules generated on their surface. Therefore, it is conceivable that the loss of receptors essential for the cellular binding to the later stage schistosomula is responsible for their escape

from cellular binding in the normal peritoneal cavity.

The mechanism underlying the immunoevasion by schistosomula in the immune peritoneal cavity is still undefined. The survival of parasites from the secondary percutaneous infection in immune mice, though small compared to that in challenged normal controls, has been reported (Smithers and Gammage, 1980). Several hypotheses, such as antigen mimicry and antigen masking, have been proposed as evasion mechanisms (reviewed by Smithers and Terry, 1976; McLaren, 1980). However, none of the papers have addressed the question of how the young schistosomula survive the initial immune attack and develop into immune resistant schistosomula. The observations made from <u>in vitro</u> experiments performed in our laboratory (manuscript in preparation) may shed some light on the mechanism for this immunoevasion in immune animals. The fresh mechanically transformed schistosomula labelled with fluorescein conjugated wheat germ agglutinin showed an intense, smooth staining pattern. When they were incubated with immune serum at 37°C, a cercarienhüllen reaction (Kemp, 1970) took place and a large fluorescent envelope was seen around each schistosomulum by 2 hr. A few parasites showed localization of fluorescence as a cap over the posterior end. The shedding of the bulk of fluorescent complexes that resulted in living schistosomula with unlabeled membrane was seen in a few after 24 hr in culture. Interestingly, when peritoneal cells from immune mice were subsequently added to the 2 hr-old cultures containing schistosomula exhibiting fluorescent caps, the incubation mixture contained a few schistosomula with

highly localized cellular adherence over part of the body. On each of these schistosomula there was a sharp line of separation between negative and positive areas. Positive reactions were generally associated with fluorescent regions (fluorescent caps), while the unlabelled area of the same individual was free of bound cells. The schistosomula, presumably dead or damaged, that were totally covered by cells invariably retained their whole fluorescent label. This suggests that evasion of schistosomula from immune response is closely tied to metabolic process during the first hours in culture and that the tegumental change results in the loss of binding sites for cells. This observation appears to be compatible with a recent study using human neutrophils. It was shown that surface areas on the schistosomulum cleared of fluorescent wheat germ agglutinin label and bound cells no longer attract cellular binding in the presence of antibodies in vitro (Caulfield et al., 1982). Accordingly, the role of glycocalyx may be considered not only in terms of acting to waterproof the free swimming cercariae (Stirewalt, 1963) and to act as an adhesive to stick cercariae together to facilitate their migration in the skin (Kruidenier, 1953), but also functions to protect schistosomula during their skin penetration by concentrating host leukocytes via receptors for complement and antibodies onto the thick fibrillar surface coat. While the cell-covered glycocalyx is subsequently stripped off, the schistosomulum may simultaneously develop an immune rssistant membrane

by acquiring host macromolecules (Review see Smithers and Terry, 1976; McLaren et al., 1980; Samuelson, 1980), such that it no longer has the capacity to bind C3 or antibodies in a manner that is sufficiently stable to permit subsequent attack by host leukocytes.

In conclusion, an experimental model has been put forward whereby the hypersensitivity induced by migrating schistosomula and also their elimination in the skin can be reproduced within the peritoneal cavity by an intraperitoneal inoculation of schistosomula. The problems of identifying the immunological and biochemical pathways involved in the skin immunity, both innate and acquired, should be more easily approached by characterizing the responsible factors released in the peritoneal fluids. This model also provides an in vivo system to study these changes in the tegument of the schistosomula which permits their survival in spite of the intensive cellular activity which lets them establish an infection.

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