THE INDUCTION OF PROTECTIVE IMMUNITY TO *SCHISTOSOMA MANSONI* IN MICE: *IN VIVO* LYMPHOCYTE RESPONSES IN THE DRAINING LYMPH NODES

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CONTENTS

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DECLARATION

All the work presented in this thesis is my own, with the exception of the following:

Chapter 2: Autoradiographic tracking of parasites, carried out by Dr A.P. Mountford.

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ABSTRACT

The *in vivo* cellular responses associated with the induction of protective immunity to *Schistosoma mansoni* in mice were investigated, with particular emphasis on events in the draining lymph nodes (LN).

Exposure to optimally-irradiated cercariae provoked changes in total cell numbers in the skin-draining (axillary) and lung-draining (mediastinal) LN only, the temporal pattern of responses being coincident with the kinetics of parasite migration. Phenotypic analysis of the cellular changes revealed an increase both in T lymphocytes and, to a greater extent, in B lymphocytes. When protected mice were compared with animals exposed to non-immunizing parasites, smaller and more transient cellular changes were observed in the latter, suggesting that the induction of high levels of protection requires a prolonged priming of lymphocytes within draining LN.

The changes in cell proliferation in the axillary and mediastinal LN of vaccinated animals was measured, using *in vivo* 5-bromo-2′-deoxyuridine incorporation. A marked increase in the number of dividing cells was detected in both groups of LN. When the proliferation of T and B lymphocytes was determined separately, there was a striking preferential increase in the proportion of dividing T, relative to B, cells. The data highlight the importance of T cell proliferation within draining LN for successful vaccination.

Several dynamic components of lymphocyte traffic were examined to assess their relative contribution to the overall changes in the LN of immunized mice. A significant part of the observed accumulation of cells in the axillary LN was due to the effect of hyperaemia, although the ratio of T:B lymphocytes recruited from the blood into the draining nodes was not altered. However vaccination led to a significant change in the retention pattern of T and B cells in the draining LN, such that B lymphocytes failed to exit from that site.

The expression of cytokine mRNA was used as a marker for the *in vivo* activation of T helper subsets in the axillary LN. The ^{32}P-labelled probes generated by *in vitro* transcription proved highly specific and detected the relevant mRNA in cytokine-producing cell lines. However, the probes failed to hybridize to RNA isolated from the LN cells of vaccinated mice, suggesting the frequency of cytokine-secreting lymphocytes after immunization is extremely small.

CHAPTER 1

INTRODUCTION

PART I: IMMUNITY TO SCHISTOSOMES

1.1 Background

Schistosomiasis is considered to be one of the most widespread parasitic diseases of man with an estimated 200 million people currently infected (Mott 1987). The disease is caused by digenean trematodes of the genus Schistosoma and is distributed in tropical and sub-tropical regions. Human schistosomiasis is caused primarily by three species, S. mansoni (Africa, Central and South America), S. japonicum (Asia), and S. haematobium (Africa and Middle East).

Schistosomes have a complex life cycle, involving a molluscan intermediate host. Free-swimming cercariae are released from the freshwater snails and infect mammalian hosts by direct skin penetration. Within hours of entering the skin, the cercaria completes metamorphosis into the schistosomulum stage. Further maturation takes place as the schistosomula migrate by an intravascular route to their final destination: the hepatic portal system (S. mansoni, S. japonicum) or the bladder (S. haematobium). Adult worms pair and, following sexual reproduction, large numbers of eggs are released by the female. Many of the eggs cross the tissues to the lumen of the intestinal or urinary tract and are excreted by the host. The eggs will hatch in fresh water, releasing miracidia capable of locating and infecting the appropriate intermediate host.

Worm pairs can persist within their host for many years, causing only trivial direct pathology. In fact, most of the pathology associated with infection is caused, not by activities of the parasite, but by the immunological responses of the host to the eggs released by the female schistosome. More than half the eggs laid become trapped in local tissue sites or get swept away by the blood (von Lichtenberg 1987). In the case of S. mansoni, the eggs enter the hepatic portal vein, pass into the liver and then lodge in pre-sinusoidal venules. Long-term exposure to the

potent immunogens released by the miracidia within the eggs elicits intense localised inflammatory reactions and each egg becomes the focus for the production of a granuloma. The fibrous scar tissue which develops around the egg progressively impedes liver circulation, causing increased blood pressure in the hepatic portal vein (portal hypertension). Further complications include hepatomegaly, splenomegaly and the development of oesophageal varices (Warren 1972). Thus, the disease is regarded as chronic and debilitating, but the extent of pathology is directly dependent on the egg burden in the host. The abundance of eggs is related to the number of worm pairs, which in turn is determined by the host's frequency of exposure to contaminated water and susceptibility to reinfection.

Several approaches have been used in an attempt to control the disease. These include the introduction of health education programmes, upgrading sanitation facilities, improving environmental management and the eradication of snails with molluscicides. On the whole, these have had limited success (reviewed by Mott 1987). Research into a chemotherapeutic approach has resulted in the development of several effective drugs eg. praziquantel for S. haematobium and S. japonicum, and oxamniquine for S. mansoni infections (Mott 1987). However, chemotherapy can only be regarded as a temporary solution to the problem, since reinfection is almost certain in endemic regions. Furthermore, the sheer cost of this approach makes it an unfeasible long-term option for the affected Third World countries (Prescott 1987). Efforts must be channelled into finding a more definite solution, in the form of an effective vaccine.

Much work is currently centred on studying the immunology of the disease, in the hope of identifying potential protective antigens. There is evidence of acquired resistance to reinfection by schistosomes in humans (reviewed by Butterworth & Hagan 1987; Hagan et al. 1991), but most of these studies require many years to yield useful information. In order to gain more knowledge of how immunity develops against the parasites in humans,

the bulk of research is now performed on experimental animal models.

1.2 Experimental models of schistosomiasis

Several animal models are currently in use for the study of acquired immunity to schistosomes, including rats, mice, guinea-pigs and non-human primates. Each animal has its own advantages and disadvantages since host-parasite relationships vary considerably according to the combination of host and schistosome. However, the mouse is particularly well-suited as a model for the human situation because it is a permissive host (unlike the rat) and shows all the clinical symptoms associated with egg-induced pathology. Furthermore, the availability of a wide range of inbred strains of mice with specific immunological traits enables dissection of any anti-parasite response.

Homologous reinfections have been carried out in mice (reviewed by Dean 1983), with moderate to high levels of resistance being obtained with a number of schistosome species. However, much of the research on schistosomes has focused on S. mansoni, mainly because it is the easiest to maintain under laboratory conditions. Since the experimental work presented in this thesis is concerned with a murine model of immunity to S. mansoni, the remainder of the studies described will deal with that particular host-parasite combination.

In the following sections various aspects of an infection with normal or attenuated parasites will be discussed in order to present the background which led to the work for this thesis.

1.3 Chronic infection model

1.3.1 Development and migration of normal parasites in naive mice

Immediately following penetration of host skin the cercaria begins its substantial transformation into the

schistosomulum stage, a process which takes less than one hour to complete in vivo (Cousin, Stirewalt & Dorsey 1981). During this period significant changes occur both in the structure of the tegument (Hockley & McLaren 1973) and the biochemical composition of the surface molecules (reviewed by Wilson 1987). Changes in surface morphology are also apparent, the most obvious being the gradual loss of all mid-body spines (Crabtree & Wilson 1980). By 72 hours post-infection, the schistosomulum is ready to leave the skin.

Of the infecting cercariae, only 30-50% will eventually mature (Wilson 1987). It was originally suggested that the skin was a major site of parasite attrition in naive mice, with the elimination of approximately 65% of penetrating cercariae (Smithers & Gammage 1980). However, histological studies have failed to detect dead or dying parasites in the skin, despite evidence of dermal inflammatory responses (Mastin, Bickle & Wilson 1983). The notion is further refuted by compressed organ autoradiography which showed that the majority of applied parasites had left the site of exposure by 7 days post-infection (Mangold & Dean 1983; Wilson, Coulson & Dixon 1986). The data from both studies strongly indicate that most of the attrition to schistosomula in naive mice occurs after their migration from skin to lungs.

Estimates for $t^1/_2$ of migration out of the skin range from 3.8 days (Georgi 1982) to 5.3 days (Wilson et al. 1986). The route of exit from the skin is mainly intravascular (Miller & Wilson 1978) although at least 10% of applied parasites migrate via the lymphatics (Wheater & Wilson 1979; Mountford, Coulson & Wilson 1988). Schistosomula can first be detected in the lungs on day 3 post-infection (Miller & Wilson 1980), rising to a peak number between day 6 (Miller & Wilson 1980; Dean et al. 1984) and day 8 (Georgi, Dean & Mangold 1983; Wilson et al. 1986). Following their arrival in the lungs, the parasites undergo a sequence of developmental changes, including significant elongation (Wilson et al. 1978; Crabtree & Wilson 1980). This enables the lung schistosomula to

negotiate the narrow pulmonary capillaries and eventually leave via the pulmonary vein.

Autoradiographic tracking experiments have shown that, of the schistosomula which travel to the lungs, only a minority actually reach the hepatic portal system (Wilson et al. 1986). Since the level of maturation achieved is the same following either a conventional percutaneous infection or the intravenous injection of day 7 lung-stage schistosomula (ie. administration of parasites directly to the pulmonary vasculature), the lungs have been implicated as a dominant site of parasite attrition in naive mice (Mangold et al. 1986). However, the attrition is not achieved by direct interaction with the parasites themselves. Following their arrival in the lungs, an increasing number of the schistosomula enter the alveoli (Crabtree & Wilson 1986a), probably accidentally, whilst attempting to traverse pulmonary blood vessels. Inflammatory foci are associated with the alveoli (von Lichtenberg & Byram 1980; Crabtree & Wilson 1986a) but these do not appear to damage the parasites in any way (Coulson & Wilson 1988). Instead, once in the alveoli the schistosomula are incapable of re-entering the circulation. These retained parasites account for a large proportion of the non-maturing larvae in naive animals.

For the remaining larvae, the majority of evidence supports an intravascular route of migration from lungs to the hepatic portal system (reviewed by Wilson 1987). Schistosomula are distributed to all systemic organs in proportion to cardiac output (Wilson et al. 1986). A study of the dynamics of parasite migration through the vasculature revealed the probability of a schistosomulum being trapped in the hepatic portal distributaries within the liver as 0.72-0.86 (Wilson & Coulson 1986). Therefore, the entire hepatic portal population of schistosomes is accumulated with 2-3 circuits of parasites around the pulmonary-systemic vasculature (Wilson & Coulson 1986).

Schistosomula can first be detected in the liver on day 6 post-infection (Miller & Wilson 1980) and accumulation is complete by day 21 (Wilson et al. 1986).

Once in the liver the parasites are unable to negotiate the sinusoids and instead transform into blood-feeding worms. These adult schistosomes ultimately pair and migrate upstream to the mesenteric venules for oviposition.

1.3.2 Migration and elimination of challenge parasites in infected mice

The pattern of skin- and lung-phase migration of challenge parasites in previously infected mice is essentially identical to that in naive animals. Using autoradiographic tracking, Dean & Mangold (1984) detected equal numbers of challenge schistosomula in the lungs of both naive and 6-week infected mice on day 6 post-challenge. Inflammatory responses in the lungs were minor following a secondary infection, failing to initiate any schistosomulum attrition by direct cell contact (von Lichtenberg & Byram 1980).

However, the number of schistosomula in the livers of previously infected mice 21 days after challenge was reduced by 59% in comparison with control animals (Dean & Mangold 1984). This points to a significant level of parasite elimination after arrival in the liver, suggesting the animals have acquired some immunity to reinfection. Whether this represents a genuine acquisition of protection has been a controversial subject, as will now be discussed.

1.3.3. Concomitant immunity vs. egg-induced pathology

High levels of resistance (up to 100%) have been recorded in mice harbouring a chronic infection (reviewed by Dean 1983). This elimination appears to be directed against the migrating larvae of the challenge infection, whilst the adult worms from the primary exposure remain unharmed. The term 'concomitant immunity' was used to describe this situation (Smithers & Terry 1969). However, it was noted that the level of resistance elicited in the infected animals was clearly correlated with the presence of eggs in the tissues of the liver (Dean et al. 1978a;

Harrison, Bickle & Doenhoff 1982). Furthermore, infections using single-sex parasites usually resulted in low levels of protection (Dean et al. 1978b; Bickle 1982).

One explanation is that the eggs are a source of antigens shared with the newly penetrated parasites, thus stimulating immunity to the latter. However, immunization with isolated eggs or egg extracts has, in general, failed to demonstrate any protective effect (Ritchie, Garson & Erickson 1962; von Lichtenberg, Sadun & Bruce 1963; Bickle, Ford & Andrews 1983).

An alternative, and now widely accepted, hypothesis is that of the 'leaky liver', originally proposed by Wilson (1980). This was based on studies of the alterations in hepatic vasculature observed during a chronic infection. The portal hypertension which results from egg-induced granulomas is followed by the appearance of porta-systemic anastamoses which direct blood flow around the liver (Warren 1972). In order to chart the progressive changes in the liver's integrity and hence its 'leakiness', Wilson, Coulson & McHugh (1983) injected microspheres into the portal vein of chronically infected mice and examined the distribution to the lungs and liver. They reported significant shunting of blood in the hepatic vasculature, with leakiness developing exponentially 6-12 weeks after infection. It was proposed that the porta-systemic connections enable challenge schistosomula to escape from the liver into the blood and ultimately lodge in locations unfavourable to parasite development (reviewed by Wilson 1990). In other words, the concomitant immunity which develops in chronically infected mice is not immunological in nature but largely due to an artefact of egg-induced hepatic pathology (McHugh, Coulson & Wilson 1987). In support of this notion, it has not proved possible to transfer the resistance observed in chronically-infected mice via a parabiotic union (Dean, Bukowski & Clark 1981a) or by serum (Dean 1983). Furthermore, the resistance induced is not species-specific (Dean 1983).

Clearly, a chronic infection model is of no value in the study of acquired immunity to schistosomes, because of the complications associated with egg-induced pathology. Instead, investigators have turned to models of infection which use attenuated parasites. One of the main advantages with these models is that the schistosomula die before reaching maturity, so there is no egg deposition. In addition, attenuated parasites can induce very high levels of protection in mice.

1.4 Attenuated parasite infection model

1.4.1 Parasite attenuation

Several approaches have been used for the attenuation of schistosome larvae, including chemicals (Bickle & Andrews 1985), ultraviolet light (Dean et al. 1983) and X-rays (Hsu, Hsu & Burmeister 1981). However, the most popular method is that of γ-irradiation from a ^{60}Co source. The subsequent level of protection induced by the attenuated larvae is significantly influenced by the dose of radiation, with resistance becoming progressively greater as the dose is increased (Minard et al. 1978a). In the USA, the highest levels of resistance are obtained with cercariae irradiated with 50 krad. (Minard et al. 1978a; Mangold & Dean 1984). However, in the UK the optimum attenuation dose is 20 krad. (Bickle, Dobinson & James 1979a), a result confirmed in our own laboratory. This discrepancy is thought to reflect differences in parasite maintenance and/or irradiation conditions (James & Dobinson 1985). Therefore, in order to avoid confusion, the term 'optimum irradiation' will be used in the remainder of this thesis. It should be noted that cercariae irradiated with doses above the optimum level have a reduced capacity to induce protection (Minard et al. 1978a).

Following a single vaccination with optimally-attenuated larvae, resistance peaks between days 17 and 24 (Ratcliffe & Wilson 1991) and persists for at least 15 weeks (Minard et al. 1978a). The level of protection

induced ranges from 30-80% depending on the strain of mouse (James, Labine & Sher 1981). High responder strains, such as C57BL/6 mice, routinely develop around 70% resistance to a challenge infection, as assessed by reduced worm burdens. The standard immunization protocol employed in our laboratory involves a single percutaneous exposure of C57BL/6 mice to 500 optimally-irradiated cercariae. A percutaneous challenge with 200 normal cercariae is performed 5 weeks later to determine the efficacy of the vaccination. Variations in methods used at different laboratories have been reviewed by Dean (1983).

1.4.2 Effect of optimal irradiation on parasite development and migration

An explanation to account for the irradiated schistosome's success in eliciting high levels of protection has been the subject of much debate. One obvious possibility is that radiation modifies the parasites in some way, thus exposing usually unseen characteristics eg. morphological or biochemical features. Mastin, Bickle & Wilson (1985) performed an ultrastructural study on radiation-attenuated parasites in the lungs in order to determine whether the larvae were morphologically different from normal schistosomula. They concluded that this was not the case as attenuated larvae had undergone the normal developmental changes associated with the lung stage of migration. The biochemical changes which might be associated with irradiation have also been investigated. Using immunoprecipitation, Simpson et al. (1985) examined the expression of surface antigens on normal and attenuated schistosomula. The same antigens were detected on both groups of parasites, suggesting that δ-radiation does not significantly alter larval antigenicity.

The most noticeable influence of irradiation is on the schistosomulum's migration and survival (as previously mentioned, optimally-irradiated parasites do not reach maturity). Using quantitative histology, Mastin et al. (1983) found evidence for a slight retardation in the exit

of irradiated, relative to normal, larvae from the skin. Death at this site was negligible and most of the attenuated schistosomula eventually reached the lungs. However, at 21 days post-vaccination the majority of irradiated parasites were still in the lungs, in contrast to only 2% of normal schistosomula (Mastin et al. 1983). Furthermore, at least 81% of the immunizing parasites observed in the lungs were dead. The same conclusions can be drawn from autoradiographic tracking data (Mangold & Dean 1984; Mountford et al. 1988).

As there is no apparent damage to the schistosomula following irradiation, the reason for their curtailed migration and death is difficult to determine. It has been suggested that the high dose of radiation impairs DNA synthesis in the parasites, in turn leading to a significant reduction in protein synthesis. The subsequent exhaustion of energy reserves thus renders the larvae incapable of migrating beyond the pulmonary capillary beds. Although the hypothesis is a plausible one, there is as yet no direct evidence to support it.

However, there is still the question of how the irradiation procedure results in larvae capable of generating high levels of protection in mice. One of the major differences between vaccinating and normal parasites is their migration kinetics through particular lymph nodes (LN). Following skin penetration, a proportion of both normal and attenuated schistosomula migrate to the skin-draining LN (Mountford et al. 1988). Whereas normal parasites spend only a very short period of time in the nodes, a significant number of irradiated larvae remain there for up to 2 weeks. Given that LN provide a potent environment for the processing and presentation of antigen to immunological cells (Austyn 1989), the persistence of attenuated larvae in the skin-draining LN could be a key feature in the success of the irradiated vaccine. If this is the case, the role played by the optimal-irradiation procedure is to retard the schistosomulum's migration sufficiently such that a prolonged release of parasite

antigens can take place in a site relevant for host sensitization. In support of this hypothesis, a proportionally greater amount of parasite-released material can be detected in the nodes of vaccinated mice, relative to mice infected with normal cercariae (Mountford et al. 1988). The full importance of the draining LN in the induction of protective immunity will be discussed in detail later (see 1.6.3).

1.4.3 Immunological responses associated with vaccination by optimally-irradiated parasites

Unlike a chronic infection, the resistance elicited to challenge parasites in mice protected by exposure to irradiated larvae has an immunological basis. For example, the immunity generated in vaccinated mice can be transferred to naive animals across a parabiotic union (Dean et al. 1981a). Furthermore, the mechanism of resistance is dependent on functional T and B lymphocytes (Sher et al. 1982) and is species-specific (Bickle et al. 1985; Aitken, Coulson & Wilson 1988). The role of different immunological components in relation to the resistance developed by vaccinated mice will be discussed in the next few sections.

(a) Humoral responses

Exposure to optimally-irradiated cercariae elicits specific anti-schistosome antibody responses. Titres are first detectable in the serum 2 weeks post-vaccination, peak at 5-6 weeks, and persist at least until week 15 (James et al. 1981). The isotypes in vaccinated mouse serum are predominantly IgM, IgA and IgG (James et al. 1981), although after challenge all of the anamnestic circulating antibodies belong to the IgG class (Correa-Oliveira, Sher & James 1984). Despite these observations, the actual titres of antibody do not correlate well with the immune status of the host eg. highly resistant animals often develop the lowest levels of antibody (James et al. 1981; Bickle et al. 1985; Roberts, Boot & Wilson 1988).

An alternative approach in the study of humoral responses has been to examine the effect on protection of treating mice with anti-μ-chain antisera in order to make them deficient in B lymphocytes (Sher et al. 1982). Resistance to a challenge infection was very significantly reduced in the μ-suppressed animals implying a role for B cells in vaccine-induced immunity. However, since this study, the validity of anti-μ treatment as a means of ablating solely humoral responses has been brought into question (Kim et al. 1984; Sher et al. 1987). Kim et al. (1984) examined the effect of μ-suppression on T lymphocyte responses both in vivo and in vitro. They concluded that the immunological, specific as well as non-specific, function of T cells was significantly impaired in μ-treated mice. Therefore, the evidence presented by Sher et al. (1982) that B cell responses are a requisite for vaccine-induced immunity is inconclusive.

The passive transfer of either serum from vaccinated mice or monoclonal antibodies raised against schistosome antigens, confers variable levels of protection to the naive recipients. Attempts to transfer immunity with serum from once-vaccinated resistant donors have generally failed (Bickle et al. 1985; Mangold & Dean 1986). However, serum from mice hyperimmunized by multiple exposure to optimally-irradiated cercariae confers partial protection (Mangold & Dean 1986; McLaren & Smithers 1988). Similarly, the in vivo administration of anti-schistosome monoclonal antibodies to naive mice elicits little resistance in the recipients unless used at very high titres (Smith et al. 1982; Hadzai et al. 1985; Bickle, Andrews & Taylor 1986). It may be that the titre of antibody, rather than its affinity, is a more critical factor in the development of optimal immunity (Vignali, Bickle & Taylor 1989a; Vignali et al. 1990). Alternatively, as suggested by Kelly & Colley (1988), the immune responses which are elicited in mice exposed to repeated immunization differ from those operating in the once-vaccinated animal.

On the whole, firm evidence to support an important role for antibody in immunity induced by a single exposure to irradiated parasites is still lacking.

(b) T cell-mediated responses

Compared with humoral responses, the evidence for the requirement of T cells in the development of resistance is more compelling (reviewed by James & Sher 1990).

In particular, the P/N (P) strain of mouse has proved a valuable tool in the study of protective immunity to S. mansoni. These mice are classified as 'low responders' to vaccination as they routinely develop only 2-25% resistance to a challenge infection (James & Sher 1983). The immunological abnormality of the P strain is an inability to activate macrophages and hence induce responses such as DTH (James & Sher 1983; James, Correa-Oliveira & Leonard 1984a). This inability has been pin-pointed to a selective defect in T cell function for production of macrophage-activating lymphokines such as interferon-gamma (James et al. 1986). Thus, the P mouse model provides evidence that resistance to reinfection requires, not only T lymphocytes, but the release of specific cytokines.

The role of different T lymphocyte subsets in vaccine-induced immunity has also been examined. Several groups have investigated the effect of in vivo depletion of particular T cell subsets (Phillips et al. 1987; Kelly & Colley 1988; Vignali et al. 1989b). In all three studies, the administration of a monoclonal antibody specific for $CD4^+$ T cells (ie. T helper) almost completely ablated resistance in the once-vaccinated mouse, provided it was administered prior to or shortly after the time of challenge infection. In contrast, depletion of $CD8^+$ T cells either had no effect on (Vignali et al. 1989b) or actually enhanced (Phillips et al. 1987) the level of resistance.

Schistosome-reactive T cells are generated following exposure to irradiated larvae, as demonstrated by in vitro studies of lymphocyte blastogenesis (James et al. 1981;

Lewis & Wilson 1982; James et al. 1984a; Pemberton et al. 1991). Cells isolated from the LN of vaccinated mice show significant responses to both live schistosomula (James et al. 1981; James et al. 1984a) and schistosome-derived antigens (Lewis & Wilson 1982; James et al. 1984a; Pemberton et al. 1991). In two of these studies the blastogenic responses after vaccination have been compared with those following exposure to normal cercariae (Lewis & Wilson 1982; Pemberton et al. 1991). The levels of proliferation were similar at early time points (the period thought to be most relevant to the induction of protection, see section 1.6.3), suggesting that the extent of T cell blastogenesis is not a good correlate of immunity. However, there were significant differences in the patterns of cytokines produced by the LN cells from the two groups of mice (Pemberton et al. 1991), once again implying an important role for T cell derived factors (see 1.5.3 (b)).

(c) Other factors

The requirement for various other components of the immune system in vaccine-induced resistance has been investigated, mostly with the use of mouse strains deficient in specific immune responses. Sher et al. (1983) have shown that neither mast cells nor IgE antibodies are important, as both WBB6F-W/W^{V} (lacking mast cells) and SJL/J (mounting very weak IgE responses) mice display the same levels of resistance to challenge infection as animals developing potent immediate hypersensitivity responses. Similarly, mice genetically defective in the C5 component of complement (Sher et al. 1982) or depleted of C3 by cobra venom factor (Sher et al. 1982; Vignali et al. 1988b) are unaffected in their ability to develop immunity following vaccination. The presence of natural killer cells or granulocytes does not seem important either, since normal protective responses are observed in the beige strain of mouse (James & Sher 1983).

Having presented the range of humoral and cellular responses generated as a result of immunization with

attenuated schistosomes, the events will now be discussed in the context of the effector and induction phases of immunity. The reason for describing the effector phase first is in order to establish the immunological interactions which make up a successful challenge elimination. These interactions then provide the criteria which must be fulfilled during the induction period.

1.5 The effector mechanism in protective immunity

1.5.1 Site of challenge parasite elimination

The issue of the site of challenge parasite elimination has been the subject of much debate, as essentially identical experiments performed in different laboratories have generated conflicting data (Wilson & Coulson 1989; McLaren 1989). A major phase of immune attrition can occur in either the skin or the lungs of vaccinated mice, depending on the strain of mouse and / or parasite isolate used in the various laboratories. Therefore, the evidence for both arguments will be presented as though each were true.

(a) Evidence for skin-phase immunity

Results from mincing and incubation of skin and lung tissue in order to recover challenge parasites suggest that the major attrition of larvae occurs within the first 4 days after challenge (Miller & Smithers 1980). When the cutaneous tissues of vaccinated mice were examined by histopathology, challenge larvae were found trapped in subdermal focal reactions (Ward & McLaren 1988). Interestingly, the trapped larvae still accomplished morphological transition from skin-stage to lung-stage worms, suggesting that the foci block the migration potential of the parasites rather than damage them.

Evidence that the cutaneous inflammatory response is a crucial element of protective immunity comes from experiments in which a monoclonal antibody directed against neutrophils was administered to immune mice at the time of

challenge (McLaren, Strath & Smithers 1987). This treatment significantly depleted the cutaneous reaction around the challenge parasites and decreased expected levels of resistance by an average of 67%. The specific ablation of $CD4^+$ T cells during the skin phase of challenge worm migration also reduced the expression of vaccine immunity (Kelly & Colley 1988).

If the skin is by-passed by direct injection of challenge schistosomula into the lungs, the mice are only marginally resistant (Miller, Smithers & Sher 1981; McLaren, Pearce & Smithers 1985). Furthermore, attrition of the injected lung worms is minor, suggesting a lack of pulmonary responses to the larvae (McLaren et al. 1985). Tracking the challenge parasites by compressed organ autoradiography revealed that, of those applied, 58.5% fail to reach the lungs of vaccinated mice (Kamiya, Smithers & McLaren 1987), once again supporting a major role for skin-phase immunity.

(b) Evidence for lung-phase immunity

Using a mincing and incubation technique, similar numbers of challenge parasites have been recovered from the lungs of vaccinated and normal mice (Minard et al. 1978b; Stek, Dean & Clark 1981). Peak recoveries were obtained from immunized mice around 8 days post-challenge, with at least 80% of the worm elimination occurring 8 or more days after the challenge infection (Stek et al. 1981).

Data from histological studies have revealed that the number of challenge parasites which eventually leave the skin of vaccinated mice is not significantly different from that in normal mice (Mastin et al. 1983; von Lichtenberg, Correa-Oliveira & Sher 1985). Furthermore, comparable numbers of larvae can be detected in the lungs of naive and immunized animals on day 4 post-challenge (Mastin et al. 1983). Schistosomula arriving in the lungs of vaccinated mice provoke marked inflammatory responses in the pulmonary tissues, resulting in localized foci forming around the parasites (von Lichtenberg et al. 1985). As with the schistosomula observed in the skin-phase model of immunity

(Ward & McLaren 1988), the challenge parasites in the lungs appear unharmed by the inflammatory foci.

Experiments in which the challenge schistosomula are administered intravenously, such that they by-pass the skin, have shown little or no reduction in the level of resistance expressed (Dean, Cioli & Bukowski 1981b; Mangold et al. 1986). Dean et al. (1981b) recorded the elimination of 32-44% of day 6 lung schistosomula injected into the lungs of previously vaccinated mice. In a similar study, 50.6% of the injected lung parasites were eliminated (Mangold et al. 1986). As a result of these studies, Mangold et al. concluded that immune attrition does not begin until at least 7 days post-challenge and is still detectable at day 35.

The most convincing evidence in support of lung-phase immunity is provided by autoradiographic studies (Dean et al. 1984; Wilson et al. 1986). These showed that, although the migration of challenge parasites from the skin to the lungs is delayed for several days in vaccinated mice, equal numbers of schistosomula eventually reach the lungs of control and immunized animals. Results from autoradiographs in which all organs were sampled (in order to obtain an estimate of the total numbers of parasites in the mouse) revealed that the challenge schistosomula fail to progress further than the lungs in vaccinated animals (Wilson et al. 1986). By day 21 post-challenge (when migration to the liver should be complete), autoradiographic foci were distributed in the ratio 3.7 : 1.3 : 1.0, lungs : systemic organs : tail skin infection site, suggesting that the majority of challenge parasite elimination takes place in the lungs (Wilson et al. 1986).

Despite the consensus of opinion that the lung-stage schistosomula is the target of immune elimination, the controversy associated with identifying the site of challenge attrition is still ongoing. However, several studies have addressed the problem directly in an attempt to clarify the differences observed between laboratories. For example, Elsaghier & McLaren (1989) examined the effect

of varying the sites selected for immunization and challenge of the mice, since each laboratory has its own routine combination. Despite the variations, the authors found that the skin was consistently the principal site of challenge elimination in vaccinated mice. They concluded that the strain variability of schistosomes used in their laboratory and elsewhere must be responsible for the observed discrepancy. In response to this, Dean et al. recently reported at the "VIIth International Congress of Parasitology" meeting in Paris (August 1990) that they had compared the parasite strain from their own and D.J. McLaren's laboratory and found that, regardless of the strain, most or all immune elimination occurs during the lung phase of migration. Instead, the variations between groups may involve differences in combinations of mouse and parasite strains or other unrecognized factors.

The results obtained in our laboratory have consistently pointed to a lung phase of immunity, so our research into events following a challenge infection has focused primarily on pulmonary responses. Furthermore, judging by recent publications and meetings, the current weight of opinion from the majority of schistosome research groups now appears to favour the lungs as the site of immune elimination. Therefore, the mechanism of parasite attrition described in the following sections will pertain to the lungs only.

1.5.2 Mechanism of challenge parasite elimination

As previously discussed (section 1.4.3), the majority of evidence supports a role for T cell mediated resistance in the attenuated parasite model of immunity. However, the nature of the protective immune response will be dependent on the type of T cell involved, since different T lymphocyte classes generate very different effector mechanisms.

The *in vivo* ablation of either $CD4^+$ or $CD8^+$ T cells (*eg.* Vignali *et al.* 1989b) has shown that the presence of the T helper subset is required for the successful elicitation of protective immunity. Furthermore, a flow cytometric analysis of lung cell populations recovered at the time of challenge infection revealed 89% T cells, of which 72% were $CD4^+$ (Aitken *et al.* 1988). An immunocytochemical study of the focal aggregates in the lung tissues of mice vaccinated and challenged has further highlighted the predominance of $CD4^+$ over $CD8^+$ T lymphocytes (Kambara & Wilson 1990). T helper cells are capable of playing a variety of roles in immunological responses. In the context of this attenuated parasite model of immunity, the function of most interest is the ability of the $CD4^+$ T cell to initiate delayed-type hypersensitivity (DTH) reactions. Several lines of evidence point to a DTH effector mechanism operating against challenge schistosomula.

For example, a histological and ultrastructural examination of challenge parasites in the lungs of vaccinated mice has shown that the schistosomula attract foci of inflammatory leukocytes (Crabtree & Wilson 1986b). The leukocytes were always 85% or more mononuclear, containing both lymphocytes and macrophages; the authors concluded that the observed inflammation had many of the features of a DTH reaction.

Further evidence for a DTH effector mechanism is obtained from the many studies carried out in the P strain of mouse (James & Sher 1983). As described in section 1.4.3 (b), this strain is deficient in macrophage function, leading to an inability to mount DTH responses. This deficiency is thought to be the reason for the failure of P mice to become resistant following exposure to irradiated cercariae (James *et al.* 1984a). These observations have led to several examinations of macrophage populations obtained from high responder mice, in order to assess directly the macrophage's ability to kill schistosomula *in vitro* (reviewed by James 1986). For example, peritoneal macrophages recovered from C57BL/6 vaccinated mice after

intraperitoneal challenge with soluble schistosome antigens, demonstrated larvacidal activity towards skin-stage larvae (James et al. 1984b). Similar results have been obtained using post-lung stage schistosomula as the target (Pearce & James 1986). Furthermore, genetic studies have revealed that macrophage larvacidal activity and the level of resistance to challenge infection are highly correlated (James et al. 1987).

Despite in vitro evidence for schistosome killing by activated macrophages, this is not thought to be the mechanism of immune elimination which operates in vivo. As previously discussed (section 1.5.1 (b)) the target for the immune response is the lung-stage parasite, yet larvacidal assays have shown that 7 and 10 day old schistosomula are refractory to the macrophage's effects in vitro (Pearce & James 1986). Also, in their ultrastructural study, Crabtree & Wilson (1986b) observed inflammatory foci around the challenge parasites in the lungs of vaccinated mice, but noted an absence of damaged or dead schistosomula. The migratory potential and viability of challenge schistosomula recovered after residence in the lungs of vaccinated mice has been assessed by their introduction into the vasculature of naive recipients (Coulson & Wilson 1988). The results indicate that significant numbers of schistosomula are capable of maturation, reiterating the suggestion that the parasites do not suffer cytotoxic injury as a result of DTH reactions.

Instead, the mechanism of parasite elimination is thought to be indirect (reviewed by Wilson & Coulson 1989). Larvae newly arrived in the lungs of immunized mice are all intravascular in location but with time an increasing proportion can be detected in the alveoli (Crabtree & Wilson 1986b). The reason is that the infiltration of mononuclear cells into the lungs damages the host capillary and alveolar epithelium, effectively destroying the vascular path of parasite migration (Crabtree & Wilson 1986b). As a result, the schistosomula are deflected into alveoli and are incapable of re-entering blood vessels because of the marked pulmonary inflammation around them.

Hence, immunologically specific DTH responses (presumably triggered by the presence of the challenge parasites in the lungs) result in non-specific larval elimination.

In order to ascertain that pulmonary DTH operates *in vivo* during the effector phase of this model of immunity, Menson & Wilson (1989) carried out a study to examine the activation state of alveolar macrophages following a challenge infection. They measured the ability of the macrophage population recovered from the lungs of mice vaccinated and challenged, to produce an oxidative burst. Highly activated cells were recorded at 7 and 14 days post-challenge, the times associated with parasite arrival and retention in the lungs.

1.5.3 *DTH-inducing cells and cytokines*

The field of cellular immunology has recently been revolutionized by the observation that cloned $CD4^+$ T cells can be separated into at least two phenotypes, on the basis of differential cytokine production (Mosmann & Coffman 1987; Bottomly 1988). According to this definition, TH1 cells preferentially produce interferon-gamma (IFN-γ), interleukin 2 (IL-2) and lymphotoxin. In contrast, TH2 cells release IL-4 and IL-5. The two clones have different functions, attributable to the cytokines they produce. Thus, TH1 cells are unique in mediating DTH responses (Cher & Mosmann 1987). Furthermore, the T lymphocyte subsets exhibit different helper cell capabilities for stimulating the production of particular immunoglobulin isotypes (*eg.* IgG2a for TH1, IgE for TH2; reviewed by Coffman *et al.* 1988).

Although most of the early studies concerned with the heterogeneity of $CD4^+$ T lymphocytes have been carried out using cloned cells, there is now substantial evidence that the dichotomy *does* have biological relevance, particularly in parasitic infections (reviewed by Scott *et al.* 1989). The best characterized example of this is in leishmaniasis, a chronic protozoal disease.

(a) TH1/TH2 cells in leishmaniasis

While most inbred strains of mice develop self-healing infections, those which develop in BALB/c mice are non-healing and fatal. A series of experiments has indicated that $CD4^+$ cells are responsible for modulating both types of infection (Liew 1989; Scott 1989). However, the cells isolated from the draining LN of BALB/c mice produce a TH2-biased response, characterized by the presence of IL-4 messenger RNA (mRNA) in the cells (Heinzel et al. 1989) and the generation of IL-4 and IL-5 after antigen stimulation in vitro (Locksley & Scott 1991). LN cells sampled from resistant mice contain high levels of IFN-δ mRNA (Sadick et al. 1987; Heinzel et al. 1989), but little IL-4 message (Heinzel et al. 1989), typical of a TH1 subset.

The protective immune response of resistant mice can be ablated by the administration of anti-IFN-δ monoclonal antibody within the initial 10 days of infection (Belosevic et al. 1988). Conversely, anti-IL-4 treatment of infected BALB/c mice results in their cure (Sadick et al. 1990). Furthermore, the adoptive transfer of TH1 clones, derived from the spleens of chronically infected mice, is capable of conferring significant levels of protection to naive BALB/c mice (Holaday, Sadick & Pearson 1988). Thus, the generation of susceptibility or resistance in a leishmaniasis infection seems to be related directly to the differential development of TH1 and TH2 cells.

(b) TH1/TH2 cells in schistosomiasis

On the whole, helminth-induced infections are associated with the production of TH2-type cytokines (reviewed by Finkelman et al. 1991). Acute S. mansoni infections result in high levels of total serum IgE and circulating eosinophils (James & Sher 1990), responses usually under the regulation of TH2-produced lymphokines. The administration of anti-IL-5 monoclonal antibody results in significantly fewer eosinophils in the circulation and egg granulomas of infected mice (Sher et al. 1990).

In contrast, mice vaccinated with irradiated cercariae display responses characteristic of a TH1 subset. As described in section 1.4.3 (a), circulating antibodies following a challenge infection of vaccinated mice belong to the IgG2a sub-class (Correa-Oliveira et al. 1984), the isotype associated with stimulation by TH1 cells. However, peak levels of IgG antibodies produced by immunized P strain mice are equivalent to those in high responder strains (Correa-Oliveira et al. 1984), suggesting once again that antibodies play only a minor role in protective immunity.

Spleen cells taken from 3-10 week vaccinated mice produce significant levels of IFN-δ and IL-2 in response to larval antigens (Pearce et al. 1991; Caulada-Benedetti et al. 1991). Interestingly, T lymphocytes from 1x immunized mice are at least as efficient as those from multiply immunized animals at IFN-δ production (James & Sher 1990), which may explain why little, if any, increase in resistance is achieved by multiple exposure to irradiated cercariae. However, hyperimmunization also leads to increased IL-5 and IL-4 production, associated with an enhanced TH2 response (Caulada-Benedetti et al. 1991). The resistance following a multiple vaccination may represent a mixture of TH1 and TH2-associated effector mechanisms.

The cytokines released both by draining LN (Pemberton et al. 1991) and lung (Smythies et al., unpublished data) lymphocytes following either a single vaccinating or normal infection, have been compared. The production of IFN-δ from skin-draining LN cells was 24-fold greater in immunized than infected animals at day 22 post-exposure (Pemberton et al. 1991). Similarly, an 8-fold greater release of IFN-δ from lung cells was recorded in vaccinated, relative to normally-infected, mice at day 21 (Smythies et al., unpublished data). An estimation of the IFN-δ production from the lung cells of vaccinated animals following a challenge infection revealed a faster and greater response than after vaccination alone. In the context of lung-phase immunity mediated by DTH, the

presence of TH1-type activity in the lungs certainly provides supporting evidence. Of course, whilst it is assumed that IFN-ɣ producing TH1 cells are responsible for the resistance observed in vaccinated mice, this remains to be definitely proven in transfer studies using T cell clones.

The definition of antigen characteristics and immunization techniques which stimulate preferentially one T cell subset over another would be of applied interest. For example, the co-administration of cytokines and antigen may alter TH1/TH2 ratios. The type of antigen-presenting cell involved will also play a critical role (Bottomly 1988; Liew 1989). In the case of the attenuated S. mansoni vaccine model, events which take place during the induction phase of immunity are poorly understood.

Despite this, certain features have already been identified as critical for obtaining successful immunization, as will now be discussed.

1.6 The induction phase of protective immunity

1.6.1 Developmental stage of parasite used

Attempts have been made to protect mice by vaccinating them with parasites of different ages. The authors of such studies concluded that, as schistosome larvae mature from cercarial to liver stage, their ability to induce resistance diminishes (Dean et al. 1981b). However, a closer examination of the work reveals that in the majority of studies, the route of schistosome administration as well as parasite age was varied (eg. Bickle et al. 1979b; Sher & Benno 1982), making a clear-cut interpretation of the data difficult.

In order to clarify the situation, Coulson & Mountford (1989) addressed the question directly and compared the immunogenicity of intradermally (ID) administered 3 hour and day-8 schistosomula . In contrast to Dean et al. (1981b) and Sher & Benno (1982), similar

levels of immunity were generated with both larval stages. Furthermore the resistance was comparable to that induced by percutaneously (PC) administered irradiated cercariae. The reason for the greater immunogenicity of lung schistosomula observed by Coulson & Mountford may be related to the larvae having been attenuated at the cercarial stage rather than after recovery from normally-infected donors. The authors argue that normal schistosomula irradiated at the lung stage, and lung schistosomula derived from attenuated cercariae, are likely to differ with respect to their migratory potential.

1.6.2 Site of parasite administration

Relative to parasite age, the route of administration has a significantly greater effect on the level of resistance induced by attenuated larvae. When Bickle et al. (1979b) compared the protection elicited by PC applied irradiated cercariae with that by intramuscularly (IM) injected schistosomula, the PC route was significantly more effective (60% cf. 40%). Similar results have been reported by other groups (Sher & Benno 1982; James & Dobinson 1985). A comparison of parasites injected via IM, ID and intravenous (IV) routes, resulted in a high level of resistance following the ID administration, but lower levels with the other two routes (Bickle et al. 1979b).

The efficacy of vaccination protocols using non-living antigens has also shown that the method of administration is critically important for the development of high levels of resistance (reviewed by James & Sher 1986). Immunization by IV route using schistosomula killed by freezing and thawing failed to induce protective immunity (James 1985). However ID administration of the same immunogen + bacterial adjuvant (BCG), resulted in strong protection (51%) against challenge parasites. Interestingly, administration by the IV route elicited significantly higher levels of antibody production against larval surface antigens than ID immunization (James 1985), suggesting an inverse correlation between antibody titres

and the induction of protection (see 1.4.3(a)). The same results were obtained using SM97/paramyosin antigen + BCG (James 1987).

It has been shown that the distribution of radiolabelled compounds in host tissues varies according to the route of injection (Humphrey 1982). In particular, IV presentation favours delivery to the spleen and liver, whereas ID or subcutaneous administration results in sequestration in the skin-draining LN. As a corollary, antigens sequestered in the spleen are thought to stimulate suppressive immunological mechanisms, whereas antigen accumulation in the LN triggers a state of responsiveness, preferentially via cell-mediated immunity (Greene & Benacerraf 1980). The PC method of parasite administration (used in routine vaccination) probably most resembles an ID immunization. If this is the case, then high levels of protection following exposure to irradiated parasites should correlate with the localization of antigens in particular LN.

In order to test the role of parasite localization in the LN, Coulson & Mountford (1989) have monitored the distribution of attenuated lung schistosomula after injection via various routes, and related their fate to the extent of resistance. Following IV, intratracheal (IT) and intraperitoneal (IP) immunization, few parasites were detected in organs other than the injection site. In contrast, 40% of the parasites injected via the ID route were located in other sites on day 9 post-vaccination. Furthermore, after ID administration, 9% of injected schistosomula were recorded in the draining LN on day 5, and 6% on day 9. Once again, the ID immunization gave the highest level of protection, compared to the IT and IP routes which were significantly lower. The IV administration of schistosomula failed to elicit any resistance.

In conclusion, it appears that some parasite migration, particularly to the draining LN, is obligatory for the induction of high levels of protection. The reason why the non-living vaccines described by James (1985; 1987)

were successful is probably related to the adjuvant, acting as a delivery system to the relevant skin-draining LN.

1.6.3 The importance of draining lymph nodes

Indirect evidence for the requirement of parasites and/or parasite antigens to enter the draining LN for the induction of significant protection, has been obtained from early studies on variations of cercarial irradiation dosage. For example, parasites attenuated with twice the optimal dose of radiation were rarely detected in the lungs or liver, and induced only 20-25% resistance (Bickle *et al.* 1979a+b). Very high irradiation doses can result in negligible protection (Minard *et al.* 1978a), because the majority of attenuated parasites fail to leave the skin (Mangold & Dean 1984). The removal of the skin exposure site within the first 4 days of optimal-dose immunization, such that parasite material is prevented from reaching the draining LN, completely blocks the induction of resistance (Bickle 1982; Mangold & Dean 1984). Excision of the site between days 4 and 6 allows progressively greater levels of protection to develop, with no effect on induction from day 8 onwards (Mangold & Dean 1984).

Optimally attenuated parasites can also be prevented from reaching the skin-draining LN using schistosomulicidal drugs at times soon after vaccination. Bickle & Andrews (1985) have used the drug Ro 11-3128 to study the time required for parasites to survive before a protective immune response is elicited. Attenuated cercariae induced significantly less resistance when the drug treatment was carried out 24, 48 or 96 hours post-vaccination. The level of resistance was comparable to that elicited by untreated irradiated parasites only when the drug was administered 8 days after vaccination (59% *cf.* 65%). Thus, a minimum of one week appears necessary for attenuated parasites to induce protective immunity. Furthermore, the presence of parasites primarily in the skin, even over an extended period, is not sufficient to permit the development of very high levels of resistance.

As already described (1.6.2), when schistosomula are given IV, such that they by-pass draining LN, they fail to elicit protection (Coulson & Mountford 1989). Lymphadenectomy studies have shown that the removal of the skin-draining LN from mice either before, or shortly after, PC vaccination leads to a significant decrease in the resistance which develops (Mountford & Wilson 1990). Excision of the nodes 5 days before vaccination reduces the level of immunity by two-thirds. Removal of nodes at intervals after immunization was progressively less effective, with ablation of resistance being greatest when the excision is performed on days 5 and 10 post-vaccination.

The autoradiographic tracking of irradiated parasites has shown that a significant proportion migrate to the LN draining the exposure site and persist there for several days (Mountford et al. 1988). In contrast, normal schistosomula spend only a very transient period in that site. The persistence of attenuated larvae and the prolonged release of parasite material in the draining LN is likely to generate a relevant population of sensitized lymphocytes, required for the eventual recognition and elimination of a challenge infection. Therefore, the brief residence of normal schistosomula in the draining LN means that these parasites will be incapable of inducing host protection because an insufficient number of primed lymphocytes is generated (this forms the basis for the experimental work described in Chapter 2).

In vitro studies of lymphocyte blastogenesis have shown that cells from the skin-draining LN of immunized mice can proliferate in response to larval antigens (Pemberton et al. 1991). Peak responses occurred around day 5 post-vaccination, and then declined rapidly. As previously described (1.5.3(b)), these cells also produce several cytokines, including high levels of IFN-γ (24-fold more than LN cells taken from normally-infected mice). Thus, we can speculate that the DTH-inducing effector cells which can be detected in the lungs of vaccinated mice

following a challenge infection have originated from the draining LN.

1.6.4 Arming of the lungs

Following percutaneous vaccination, there is a significant expansion of the lymphocyte pool in the circulation (Menson, Coulson & Wilson 1989), presumably the result of intense lymphocyte proliferation in the draining LN in response to attenuated parasites (see Chapter 3). In order to test whether these circulating cells are schistosome-reactive, their ability to mediate DTH responses to either soluble worm antigen or live lung schistosomula, has been assessed using footpad assays (Ratcliffe & Wilson 1991). The authors observed a strong DTH reaction to the antigen preparation; the responses peaked at day 17, and declined to background levels by day 35. The reactivity to lung parasites was weaker and peaked earlier at day 10. Also, both responses could be completely abrogated by prior in vivo administration of anti-$CD4^+$ monoclonal antibody (Ratcliffe & Wilson 1991).

Bronchoalveolar lavage (BAL) studies of the lungs of vaccinated mice have shown a marked and persistent mononuclear infiltration into the pulmonary airways, peaking at day 21 after vaccination (Aitken et al. 1988; Menson et al. 1989). The infiltration represented a 6-fold and 58-fold increase in the number of macrophages and lymphocytes, respectively, with elevated levels of both for at least 10 weeks (Menson et al. 1989). Flow cytometric analysis of the lymphocytes present in the lungs of vaccinated animals revealed that the majority were T cells, of the $CD4^+$ phenotype (Aitken et al. 1988). In addition, the BAL cells are capable of producing high levels of IFN-γ when restimulated in vitro with schistosome antigens (Smythies et al., unpublished data). Therefore, the lungs of vaccinated mice accumulate a large number of schistosome-reactive IFN-γ-releasing T cells. Furthermore, the accumulation of peak numbers of lymphocytes into the pulmonary airways occurs over the

period of time during which footpad DTH is diminishing, suggesting that the DTH-mediating T lymphocytes are being recruited from the circulation.

The presence of dying vaccinating parasites in the alveoli must provide a powerful inflammatory stimulus for circulating sensitized T cells. In addition, the newly-recruited lymphocytes are likely to trigger their own inflammatory responses, due to their contact with lung schistosomula. The release of a variety of cytokines from these cells will result in further recruitment from the circulation, albeit non-specific. The kinetics of this non-specific recruitment have been investigated using ^{51}Cr-labelled mononuclear cells obtained from naive donor spleens and injected into vaccinated mice (Ratcliffe & Wilson, unpublished data). Infiltration of ^{51}Cr-labelled cells into the lungs commenced shortly after parasite arrival in that site and was at its peak 14-16 days after vaccination. Throughout the study, the results correlated closely with the observed changes in BAL total cell numbers (Aitken et al. 1988; Menson et al. 1989).

The 'arming' of the lungs during vaccination is thought to have a significant role in the induction phase of protective immunity, notably in preparing the lungs for the arrival of challenge parasites, such that the schistosomula can be eliminated rapidly. The importance of a secondary infiltration of cells into the lungs (ie. following challenge) is unclear, as one study has reported an anamnestic increase in T lymphocytes (Aitken et al. 1988), but another claims that no additional recruitment is evident (Menson et al. 1989). Studies of whole-body irradiation suggest that the conditions required for pulmonary responses to challenge schistosomula are fully established during the vaccinating infection (Aitken et al. 1987; Vignali, Bickle & Taylor 1988a). The whole-body irradiation of vaccinated animals a few days prior to challenge depleted their circulating leukocyte population but leukocyte numbers in the lungs were undiminished. The observation that the treatment failed to abrogate subsequent levels of immunity, suggests that no further

recruitment of cells to the lungs is necessary after the 5-week period of immunization, in order to obtain maximal resistance.

That arming of the lungs is required at all for the development of optimal protection has been demonstrated by parabiotic studies in which a union between a vaccinated and a naive partner was established after vaccination (Coulson & Wilson, unpublished data). Immune reactivity to schistosomes was transferred from the vaccinated to the naive partner, as measured by the in vitro proliferative responses of spleen cells to larval antigens. However, unlike the immunized partner, there was no detectable recruitment of cells to the lungs of the naive animals. When the separated partners were challenged, the naive mice showed around 70% of the immunity of the vaccinated animals. Therefore, the presence of cells in the lungs at the time of challenge accounts for approximately 30% of the total resistance. Coulson & Wilson suggest that the observed results are related to the speed with which the challenge schistosomula are able to reach the lungs of vaccinated mice. If sensitized cells are not already on site (as in the naive parabiotic partner), then the fastest migrating parasites will traverse the lungs before the relevant cells can be recruited to the lungs and the pulmonary effector mechanism activated. This inference was supported by the observation that the vaccinated, but not the naive, partner was able to resist an IV challenge. In conclusion, pre-arming of the lungs represents a bonus to the immunity attained, but is essential to achieve maximal protection.

1.7 Summary: the hypothesis

The experimental work described in this thesis has been derived from a hypothesis (reviewed by Wilson & Coulson 1989), incorporating a specific sequence of events required for the induction and expression of protective immunity to S. mansoni. This hypothesis has been

formulated from the evidence discussed in the previous sections, and will now be summarized.

The percutaneously applied attenuated parasites have two potential roles during the induction phase. First, they act as a natural adjuvant, delivering themselves to skin-draining LN where they initiate antigen presentation and generate a population of schistosome-reactive lymphocytes. Secondly, following their migration to the lungs, the parasites act as a recruitment signal for schistosome-specific $CD4^+$ T lymphocytes, resulting in the arming of that organ.

The subsequent arrival of challenge schistosomula triggers a pulmonary DTH response. The presentation of antigen to memory T lymphocytes stimulates the release of cytokines, particularly IFN-γ , which in turn activate the surrounding macrophages. The resulting inflammatory foci block the migration of the challenge parasites, causing their trapping and eventual death in the lungs.

PART II : CELL TRAFFIC AND THE LYMPH NODE

This thesis is concerned with investigating the cellular events associated with the induction of protective immunity to S. mansoni, with particular emphasis on the role played by draining LN. Since the experimental work described in Chapters 2 and 3 has been presented as papers, this second part of the Introduction is designed to provide a more complete background of previous studies on cell traffic through draining nodes, and the way in which primary immunological responses affect that traffic.

1.8 Lymph node structure

Lymph nodes are members of the 'secondary lymphoid' group of organs, which also includes the spleen, Peyer's patches and appendix. The distribution of mouse LN has been described in detail by Dunn (1954). In all cases, they are located either between muscles or adjacent to viscera. The structure varies with different groups of nodes and is somewhat dependent on the strain and health status of the animal. As LN are sites of immune response initiation, they have an elaborate internal architecture that maximizes the interaction of various cell types within them. A typical LN consists of an outer layer, the cortex, and an inner core, the medulla. The bulk of the lymphoid tissue is found in the cortex and one of the striking features about its organization is that different lymphocyte classes are mostly separated into specific anatomical compartments.

Discrete aggregations of B cells, termed 'primary follicles', can be seen in the outer cortex. In the unstimulated node, these domains consist of a spherical collection of small lymphocytes (Gutman & Weissman 1972). After antigenic challenge they form 'secondary follicles', consisting of a mantle of packed resting small B lymphocytes, surrounding a 'germinal centre' of large,

often proliferating, B cell blasts. Secondary follicles can also contain a variety of antigen-presenting cells, as well as a few T and natural killer cells (reviewed by Heinen, Cormann & Kinet-Denoel 1988). T lymphocytes are largely confined to a region of the node referred to as the 'paracortex', 'diffuse cortex' or 'thymus-dependent areas' (Waksman, Arnason & Jankovic 1962; Parrott, de Sousa & East 1966). When a T-cell-mediated response is elicited, there is a marked proliferation of cells in this area, and typical lymphoblasts are evident. Many interdigitating cells are also present. A network of fibrous tissue, called the 'reticulum' extends throughout the LN, particularly into the medullar region. The connective fibres are separated by large sinuses in which the majority of plasma cells in the node reside. In addition, scavenger phagocytic cells, including macrophages, interdigitating cells and dendritic reticular cells, are organized along the fibrous cords.

The secondary lymphoid organs are interconnected by a dual circulatory system composed of the bloodstream and lymphatics. Each node has its own arterial and venous supply. However, whilst lymphocytes can enter the LN from the blood, they can only leave in efferent lymph. The lymphatics are an extensively branched and widely dispersed network of thin-walled vessels which originate in the interstitial spaces of tissues. Fluid (lymph) is pulled into and pumped through the lymphatics by osmotic pressure and muscular contraction of the surrounding tissues. The lymph enters LN through afferent lymphatic vessels, percolates through the tissues of the LN, before exiting via the efferent lymphatics. During the passage of lymph across the node, the reticulum and its adherent cells trap particulate antigen (Nossal et al. 1968), resulting in the generation of antigen-charged accessory cells for interaction with the incoming circulating lymphocytes. Antigen can also enter LN via the blood or within certain mononuclear cells eg. Langerhans and dendritic cells (Streilein, Stein-Streilein & Head 1986). Thus, the design of the LN is such that a wide variety of antigens can be

presented to a range of antigen-specific lymphocytes in a relatively short period of time.

1.9 Lymphocyte recirculation : an introduction

1.9.1 Background:

Recirculation is now regarded as normal lymphocyte behaviour in most mammals, including man (reviewed by Anderson, Anderson & White 1982). The process is a crucial aspect in the efficiency of the immune system because it allows the full repertoire of clonal lymphocyte specificities to be available throughout the body. The phenomenon was first investigated after several authors noted that lymphocytes entered the vasculature from major lymphatic ducts, in numbers sufficient to replace the total blood content several times daily. Initially it was postulated that the lymphocyte turnover rate must be extremely high, since the level of lymphocytes in the blood remains almost constant. This was shown to be unlikely, as only 2-3% of lymphocytes in efferent lymph are newly-formed (Hall & Morris 1965a). The work of Gowans provided the first indications that those cells leaving the lymphatics eventually return to those same sites. He demonstrated that chronic thoracic duct drainage in rats produces a severe decrease in the number of lymphocytes later emerging in efferent lymph (Gowans 1957). However, the depletion could be prevented by re-infusion of thoracic duct lymphocytes (TDL) intravenously into the cannulated animal. Furthermore, radiolabelled TDL injected intravenously reappeared several hours later in the lymph and lymphoid tissue of recipient animals (Gowans & Knight 1964), firmly establishing the principal of recirculation.

Of the lymphocytes entering a LN, approximately 10% will be derived from afferent ducts, while 85-90% originate from the blood (Hall & Morris 1965a). Both T and B lymphocytes recirculate from blood to efferent lymph, although B cells are thought to migrate less frequently and more slowly (Sprent 1973; Ford 1975). The mean transit

time of T lymphocytes across LN is 18-20 hours, compared with 28-30 hours for B cells (Ford 1975). Upon entering LN, B cells will migrate specifically to the primary follicles, whilst T cells remain in the diffuse cortex. After slowly traversing their specific cortical domains, B and T lymphocytes enter the medulla which serves as a collection point for the sinusoids that lead to the efferent lymphatic vessels. From the node, the lymphocytes are carried via main lymphatic ducts back to the bloodstream to begin the cycle again. In the rat, this amounts to 4 X 10^7 lymphocytes per hour being returned to the blood (Yednock & Rosen 1989).

1.9.2 Lymphocyte - endothelial recognition

In order to enter the various secondary lymphoid tissues involved in recirculation (except the spleen; see Pabst 1988), blood lymphocytes have to cross the endothelial vascular lining. This process of extravasation occurs at specialized post-capillary vascular sites called 'high endothelial venules', or HEV (reviewed by Jalkanen *et al*. 1986; Woodruff, Clarke & Chin 1987; Pabst & Binns 1989). High endothelial cells are, in contrast to other vascular endothelial cells, typical in their plump, cuboidal morphology (Anderson, Anderson & Wyllie 1976). Although HEV are a permanent feature in lymphoid tissues, their number and length varies, under the control of local immune activity. For example, antigenic stimulation of a LN results in a rapid increase in its high endothelial vasculature. In contrast, blockade of antigen supply from afferent lymph leads to an impaired function of HEV followed by flattening of the cells (Duijvestijn & Hamann 1989).

Within LN, HEV are situated between B cell follicles, in the T cell domains of the outer and inner cortex (Anderson *et al*. 1982). Lymphocyte-HEV interactions have been studied extensively using both short-term *in vivo* circulation experiments, and *in vitro* adhesion assays. In the latter, lymphocytes overlaid on frozen tissue sections

of lymphoid organs were found to bind specifically to HEV (Stamper & Woodruff 1976). A microscopic and ultrastructural examination of rat HEV revealed that the endothelial cells are linked together by discontinuous macular junctional complexes (Anderson et al. 1976). Blood lymphocytes adhere to HEV surfaces through microvilli which attach to shallow pits on the luminal surfaces of high endothelial cells (Anderson & Anderson 1976). The lymphocytes then emigrate from HEV by passing between adjacent endothelial cells, a phenomenon which has been calculated to take an average 5-10 minutes (Smith & Ford 1983) and been observed in real time using fluoromicroscopy (Bjerknes et al. 1986).

The process of lymphocyte-endothelial interaction depends on the existence of membrane receptors for recognition and attachment of the two cell components (reviewed by Hamann & Thiele 1989; Berg et al. 1989; Yednock & Rosen 1989). The endothelial receptors are known as 'vascular addressins' (Streeter et al. 1988), whereas the corresponding molecules on lymphocytes are 'homing receptors' (Gallatin, Weissman & Butcher 1983). Several homing receptors on murine lymphocytes have been described (Berg et al. 1989), based on the ability of specific antibodies to inhibit lymphocyte-HEV cell binding eg. MEL-14, LFA-1, etc... There is a strong genetic component involved, in that adhesion is impaired in allogeneic combinations of lymphocytes and HEV (Heslop & Hardy 1971). Furthermore, the capacity of lymphocytes to bind HEV and the specificity they display for HEV in particular tissues appears to be determined by a variety of factors, including class, state of differentiation and the site at which the cells initially responded to antigen. These factors all contribute to lymphocytes showing preferential homing properties.

1.9.3 Selective lymphocyte homing

There are at least three distinct lymphocyte-HEV recognition systems. One modulates lymphocyte traffic to

peripheral LN (PLN), another directs traffic to mucosal lymphoid organs such as Peyer's patches (PP) or the appendix (Streeter et al. 1988), and a third controls traffic to the synovium of inflamed joints (Jalkanen et al. 1986). The existence of these tissue-specific lymphocyte-HEV recognition systems was initially demonstrated with the aid of certain murine and human lymphomas that bind selectively to either PLN or mucosal HEV (Butcher, Scollay & Weissman 1980). In this way, MEL-14 antibody is capable of blocking adhesion to the HEV of LN, but not PP (Gallatin et al. 1983). There is substantial evidence that PLN and PP HEV exhibit distinct recognition ligands (reviewed by Yednock & Rosen 1989). Interestingly, mesenteric LN are thought to express both PLN and PP-like HEV ligands (Jalkanen et al. 1986), perhaps reflecting the fact that these nodes receive lymphatic input from both the intestine and peritoneal cavity.

In general, most normal naive lymphocytes are capable of binding to several types of HEV. However, there is a tendency for different lymphocyte classes to display particular homing characteristics, independent of their organ of origin (Kraal & Twisk 1984). Using a combination of short-term in vivo homing studies and in vitro adherence assays, mouse T cells were found to home 5 times more efficiently than B cells to PLN (Stevens, Weissman & Butcher 1982). In contrast, B lymphocytes were more likely to localize in PP and the spleen. Similarly, the migratory properties of T cell subsets have been investigated (Butcher et al. 1982; Kraal , Weissman & Butcher 1983). Mouse Lyt-2^- (probably = $CD4^+$) T cells were 1.5 times more likely than Lyt-2^+ (=$CD8^+$) to localize in PP, whereas both cell types migrated equivalently to PLN (Kraal et al. 1983). In fact, the preference of lymphocyte populations to adhere to the different types of HEV is a direct reflection of the in situ distribution of these populations in the organs examined (Kraal & Twisk 1984). Thus, for the majority of small, unstimulated lymphocytes in secondary lymphoid organs, migratory specificities seem to be developmentally preprogrammed as a function of cell type.

One can speculate that the relative preference of B (versus T) cells for mucosal PP over PLN, reflects a greater evolutionary pressure for precursors of humoral immunity in mucosal sites.

A variety of studies has shown that blasts and effector / memory cells, compared with resting cells, have an altered expression of certain homing receptors. For example, blast cells resulting from the stimulation of a mixed lymphocyte culture were predominantly MEL-14$^-$ (Dailey et al. 1983). In parallel with the loss of MEL-14 antigen, the blasts lost their HEV binding ability. This phenomenon has also been demonstrated in vivo. As previously described, B lymphoblasts accumulate in the germinal centres of LN, where they proliferate and differentiate in response to antigen deposits in those sites. All germinal centre B cells were shown to be MEL-14$^-$ (Kraal, Weissman & Butcher 1988). Furthermore, dividing B cells isolated from these areas did not adhere to HEV in either PP or PLN sections (Reichart et al. 1983). MEL-14$^-$ populations of L3T4$^+$ T cells have also been identified in mouse germinal centres (Rouse, Ledbetter & Weissman 1982). These findings suggest that, in mice, most antigen-reactive lymphocytes undergo a period of migratory incompetence during which they lack functioning homing capability. Therefore, lymphocytes are retained in a relevant site of antigenic stimulation, allowing them time to respond with the appropriate clonal expansion and differentiation.

After several days retention, activated lymphocytes leave their lymphoid site of sensitization, having regained their migratory phenotype (Kraal et al. 1988). However, these blast populations display homing properties that are strikingly different from those of virgin T and B lymphocytes (reviewed by Hamann & Thiele 1989). In particular, their multiple specificity seems to be lost. For example, blasts in peripheral lymphatics will migrate selectively back to PLN, whereas dividing T and B cells in intestinal lymph traffic preferentially through mucosal sites (Hall, Parry & Smith 1972). In other words, once lymphocytes have been stimulated by antigen, the resulting

memory cells are restricted to circulate among tissues where the same antigen is most likely to be re-encountered. Following secondary stimulation with the same antigen, the circulatory properties of effector and memory lymphocyte populations are further narrowed in that there is an enrichment for cells expressing unique HEV recognition and homing characteristics (Butcher 1986).

In summary, the expression of different homing receptors by lymphocytes becomes increasingly restricted upon antigen-dependent differentiation. Virgin lymphocytes express several homing receptor classes, allowing promiscuous recirculation through many lymphoid organs. Upon exposure to cognate antigen in the context of a particular lymphoid organ, memory cells are generated that exhibit preferential recirculation to the same and related lymphoid organs. Finally, terminally differentiated immunoblasts, such as plasma cell precursors, express only one class of homing receptor, directing them to localize selectively within the organ where antigen was initially encountered.

1.10 Effects of antigen on lymphocyte traffic

In non-stimulated animals, lymphocyte recirculation is characterized by a balanced flux of cellular traffic across afferent and efferent terminals of lymphatic tissues. This kinetic equilibrium is rapidly distorted in the blood (Westermann & Pabst 1990) and regional nodes draining sites of inflammation, infection or antigenic challenge.

1.10.1 Changes in cell influx to draining lymph nodes

Within three hours of antigenic stimulation, there is a significant increase in the number of recirculating lymphocytes entering the LN (Cahill, Frost & Trnka 1976). However, an examination of the specificities of these lymphocytes shows them to be largely unrelated to the

antigen which initiated the response. In fact, a similar influx of lymphocytes into the LN can be induced simply by injecting adjuvants such as B. pertussis (Dresser, Taub & Krantz 1970). The observed influx of circulating cells into stimulated LN has been extensively investigated and is now thought to be the result of several interacting components.

(a) Increased blood flow

The increase in lymphocyte traffic from blood to lymph during an immune response was first described by Hall & Morris (1965b). By continuously infusing ^{3}H-thymidine into a LN which was responding to antigen, they proved that the increased output of small lymphocytes observed in the first 48 hours following stimulation, was not due to an enhanced proliferation of lymphocytes within the node. Also, experiments involving the chronic drainage of lymphatic vessels leading from the site of subcutaneous antigen administration, demonstrated that the increased numbers of lymphocytes entering the LN were not derived from afferent lymph (Cahill et al. 1976). The authors concluded that the observed influx of lymphocytes into antigen-stimulated LN was the result of an augmented cell recruitment from the bloodstream.

A number of studies have shown that the total blood flow to a LN increases significantly following antigenic stimulation (Ottaway & Parrott 1979; Hay et al. 1980; Drayson, Smith & Ford 1981), with rates of flow approaching 30 times that in unstimulated nodes (Hay & Hobbs 1977). Ottaway & Parrott (1979) have tested the hypothesis that changes in lymphocyte traffic to LN draining an immune reaction are directly related to alterations in blood flow to that tissue. They examined the distribution of ^{86}RbCl (to monitor regional blood flow) and ^{51}Cr-labelled lymphocytes in mice, following sensitization with oxazolone. The two components were highly correlated in peripheral LN ($r=0.85$), a phenomenon which has also been reported in the rabbit (Herman et al. 1976) and rat (Drayson et al. 1981).

A close examination of the circulation changes in stimulated LN revealed that the increase in blood flow following antigen inoculation is separated into two distinct phases. The first increase in flow is evident within 1.5 hours post-stimulation, reaching a maximum by 14 hours (Hay & Hobbs 1977). This phase is thought to be associated with the effects of 'hyperaemia' ie. the local dilatation of blood vessels, which includes the opening of arteriovenous shunts within the node (Herman, Utsunomiya & Hessel 1979). It is still not understood how the presence of antigen in lymphoid tissue brings about this vasodilatation. However, three common vasoactive agents (histamine, bradykinin and prostaglandin E_1) have been investigated in their ability to induce hyperaemic effects in the skin of rabbits (Hay et al. 1975). An examination of the agents' effects over a dose range of 4-400 ng, revealed a clear dose-response relationship with changes in blood flow to the skin, prostaglandin giving the most potent response. Vasoactive mediators may be released by incoming cells themselves. For example, afferent lymph cells draining a granulomatous area were shown to release a material into culture medium which, when injected into recipient skin, produced a visible hyperaemia (Vadas et al. 1979). Interestingly, a role for vasoactive amines has been implicated in the infiltration of mononuclear cells in DTH lesions in mice (Gershon, Askenase & Gershon 1975).

The effects of hyperaemia gradually decrease until 24 hours after the immune stimulation, when a second phase of increased blood flow becomes evident (Hay & Hobbs 1977). This second phase is thought to be mostly due to the process of 'angiogenesis' ie. the growth of new blood vessels, which results in a prolonged influx of blood, and hence circulating lymphocytes, into the LN. However, as well as an augmented arterial blood supply, part of the second prominent influx of lymphocytes into a stimulated LN has been attributed to the enhanced capability of HEV to capture lymphocytes from the blood.

(b) Alterations in HEV

Following stimulation by a skin allograft, HEV in LN have been shown to undergo significant structural changes (Anderson, Anderson & Wyllie 1975). These included an increase in the concentration of polyribosomes, endothelial reticulum and Golgi saccules, suggesting that HEV cells were undergoing division. Furthermore, autoradiography following infusion of ^{3}H-thymidine revealed that the endothelial cells of HEV located nearest to primary follicles in the LN became labelled between 3 and 6 days after stimulation (Anderson et al. 1975). ^{35}S-sulphate has been used to study the temporal responses of rat LN HEV cells to antigenic stimulation, as HEV selectively incorporate this compound as a sulphated glycolipid into their Golgi apparatus (Drayson et al. 1981). Peak incorporation (2.5x) was recorded on day 4 post antigen administration, with subsequent relative increases being almost identical to those for lymphocyte influx. Thus, the extended branching of the network of HEV has the effect of enlarging the area of endothelium available for transvascular lymphocyte migration (Herman, Yamamoto & Mellius 1972).

It has been suggested that certain cytokines induce a special phenotype of lymphoid HEV, capable of enhancing the adherence of recirculating lymphocytes to these tissues. For example, the in vitro incubation of mouse lung or bone marrow-derived endothelial cells with IFN-γ (but not IFN-β or IL-1) stimulated the expression of MECA-325, an endothelial differentiation antigen (Duijvestijn, Schreiber & Butcher 1986). When mice are given a systemic inoculation with an IFN inducer, there is a marked increase in both the size of the draining LN and the number of small lymphocytes in the HEV of the paracortex of these nodes (Levy et al. 1980). The effects of IFN-γ on lymphocytes with respect to their interaction with HEV endothelium in LN has been studied further, using the frozen section assay (Hendriks et al. 1989). Lymphocytes showed an increase in binding to HEV of up to 35% compared to control lymphocytes, after in vivo administration of IFN-γ.

Increased adherence was also found by pre-incubation of the lymphocytes with IFN-ɣ *in vitro*. When homing receptors on the lymphocytes displaying an enhanced binding capacity were examined, neither MEL-14 nor LFA-1 expression was augmented (Hendriks *et al*. 1989), suggesting that IFN-ɣ induces an additional adhesion mechanism on lymphocytes, independent of the MEL-14 / LFA-1 system. In addition, the effect was non-specific in terms of lymphocyte classes, as no change in the T:B cell ratio of the adherent cells was observed, a feature which has also been reported following LN stimulation with various antigens (Kraal & Twisk 1984).

In conclusion, a part of the observed changes in lymphocyte influx to stimulated LN can be explained by alterations in HEV, both in terms of their expanded structure and increased binding capacity for lymphocytes. The lymphocytes themselves are also affected by the immunological environment, further enhancing their adherence to HEV. Interestingly, secretory factors and lysosomal enzymes from activated macrophages have also been shown to alter lymphocyte surface adhesiveness and transit time within a LN (Unanue *et al*. 1976), which may be a contributing factor in certain immune responses.

1.10.2 *Proliferative responses within lymph nodes*

. As the early phase of recirculating lymphocyte sequestration within a stimulated node subsides, blast cell transformation and mitotic activity appear in the T and B cell zones of the cortex (Anderson *et al*. 1982), reflecting antigen-dependent cellular proliferation. In general, there is little data available on the qualitative, let alone quantitative, aspects of *in vivo* lymphocyte proliferation in lymphoid organs, mainly because of the difficulty in measuring these responses. Most research in this field has centred on the study of abnormal cellular replication, such as that found in tumours and certain cell lines (Darzynkiewickz, Traganos & Kimmel 1986).

Of the *in vivo* studies on lymphoid organs, the earlier approaches relied on the injection of radioactive

precursors of DNA, such as ^{14}C- or ^{3}H-thymidine, followed by scintillation counting or autoradiography. The precursors can be used to label cells either within a specific LN (Hall & Morris 1965b; Pabst & Trepel 1979) or on a systemic level (Rieke, Caffrey & Everett 1963). Using these methods, some useful information concerning the kinetics of lymphocyte proliferation has been obtained in both unstimulated and stimulated individual LN. The primary immune responses in rat popliteal LN to Salmonella adelaide flagella has been studied, using the intravenous administration of ^{3}H-thymidine followed by autoradiography (Mitchell, McDonald & Nossal 1963; Nossal, Mitchell & McDonald 1963). Within one day of antigenic stimulation, there was a significant increase in the number of labelled blasts, with a peak 10% to total LN cells labelled by day 3. Similarly, a 2-fold increase in cell proliferation was recorded in the popliteal LN of rats within 24 hours of administering sheep red blood cells in the hind footpads (Drayson et al. 1981). The peak response was on day 5, with a 10-fold greater incorporation than control LN.

Despite the popularity of the radioactive DNA precursor method, there is a major drawback with its use in that it provides no information on the cell types within a proliferating population. An alternative approach has been the administration of hydroxyurea, a compound which kills replicating, but not resting, cells (Sinclair 1965). An indication of the cell renewal rate within a particular organ is obtained by examining the decrease in cell numbers in that organ at different times after hydroxyurea treatment. The technique has the advantage that it can be combined with immunofluorescence, enabling the identification of different cell subsets. The population dynamics of both B (Freitas et al. 1982) and T (Rocha, Freitas & Coutrinho 1983) lymphocytes in mouse LN have been studied using this approach. The results obtained suggest that the renewal rate of the majority of peripheral B and T cells is 2-3 days. However, this approach has been severely criticised by several authors who claim that hydroxyurea has serious side-effects on by-stander cells,

leading to an overestimation of cycling populations.

The newest, and arguably most accurate, method of measuring lymphocyte turnover is that using 5-bromo-2'-deoxyuridine (BrdUrd) incorporation, first described by Gratzner (1982). BrdUrd is a thymidine analogue which is incorporated into DNA-synthesizing cells. Those cells which contain the analogue are then recognized by incubation with fluorescent-labelled anti-BrdUrd antibody, which can be combined with staining for lymphocyte phenotypes. The proportion of dividing cells is then observed either by fluorescence microscopy (enabling visualization of the distribution of cells within a particular organ) or flow cytometry (giving a highly accurate estimate at the single cell level). This method has been used successfully for the detection of very low levels of lymphocyte proliferation within the lymphoid organs of rats (Westermann et al. 1989). An examination of B, T and T lymphocyte subsets in unstimulated LN revealed that no more than 2% of the cells were dividing in any peripheral LN. As for stimulated nodes, the injection of phytohaemagglutinin into the skin of pigs results in a significant increase in the number of $BrdUrd^+$ lymphocytes in the draining LN (Fritz, Pabst & Binns 1990). By 48 hours post-antigen administration, approximately 8% of the LN cells were in a state of proliferation. No studies on the proliferation of stimulated rodent LN cells using BrdUrd incorporation have been reported to date. Thus, the experimental work described in Chapter 3 of this thesis provides new information on the pattern and kinetics of lymphocyte proliferation in mice.

1.10.3 Changes in cell output from draining lymph nodes

Following antigenic stimulation there is a considerable increase in the output of cells from a draining LN, reaching 10-fold that from resting nodes (Trnka & Cahill 1980). As with influx, the increased egress of lymphocytes usually occurs in two peaks, with a time delay between maximal input and output accounted for

by the cells' transit time through the node (Hay & Hobbs 1977). The first peak, detectable at around 48 hours post antigenic stimulation, is entirely composed of small lymphocytes. However, in the second (4-5 days after antigen) as much as 30% of the output may be blast cells.

Numerous studies have shown that, prior to an increased efflux of cells from stimulated LN, there is a transient dramatic fall in lymphocyte output, a phenomenon known as the 'shutdown effect' or 'lymphocyte trapping' (Zatz & Lance 1971). The effect was first noticed following the administration of human albumin to cannulated sheep LN (Hall & Morris 1962), but has since been described in response to a wide range of antigens (Cahill et al. 1976; Trnka & Cahill 1980). An investigation of the cell types being trapped in the LN suggests an entirely non-specific mechanism, as both blast and small lymphocytes are depleted from efferent lymph (Cahill et al. 1976). However, it should be noted that the phenomenon is extremely unpredictable and varies considerably with each antigen (eg. it is most severe with viral antigen). Indeed, in some cases it does not occur at all (Trnka & Cahill 1980).

Despite its unpredictability, the mechanism controlling the shutdown effect has received much attention and several biological agents have now been implicated in a regulatory capacity. For example, Gresser et al. (1981) reported that the injection of partially- or highly-purified IFN into mice caused a 2-3 fold decrease in the number of lymphocytes in the thoracic duct lymph, with a concurrent reduction in the peripheral blood. Similarly, the output of recirculating lymphocytes from the cannulated popliteal LN of sheep following the administration of IFN-alpha-2a, fell to below 1% of the pre-treatment level (Hein & Supersaxo 1988). Numbers remained depressed for nearly 35 hours, but a seemingly compensatory surge of cell traffic occurred for 2-3 days after recovery. Furthermore, the endogenous production of IFN in mice, using either artificial inducers such as poly I:poly C or vaccinia virus has been shown to suppress almost completely the egress of

cells into thoracic duct lymph (Korngold, Blank & Murasko 1983). The number of TDL were restored to normal levels by 48-64 hours. Other potential effectors of cell trapping include prostaglandin E_2 (Hopkins, McConnell & Pearson 1981) and certain corticosteroids (Hall 1986). There is also some evidence that complement activation within LN initiates decreased cell exit (McConnell & Hopkins 1981).

The actual mechanism which leads to LN shutdown is still unknown, although it has been suggested that agents such as interferons have a direct effect on the lymphocytes, rather than the LN itself (Kimber et al. 1987). Rat TDL incubated in vitro with IFN prior to intravenous injection into recipients displayed a reduced ability to migrate out of the LN. As described in section 1.9.3, antigenic stimulation leads to a significant reduction in the expression of MEL-14 receptor molecules on the surface of circulating cells, which results in their failure to exit the node. Perhaps the shutdown effect and altered homing receptor expression are connected in some way? If there is an association between the two observations, it is unlikely to be through the action of IFN, as the cytokine has no direct effect on the density of MEL-14 expression (Hendriks et al. 1989). Of course this does not preclude a correlation with an alternative adhesion molecule on the lymphocyte surface. However, it should be remembered that lymphocyte trapping affects all cells, not just the stimulated population.

In conclusion, the increase in cell input to a stimulated LN, and the reduction in output from that node, may be regulated by the same factors. From the evidence presented, the interferons seem to be particularly potent candidates, although no other cytokines have been investigated in this respect. Specific patterns of cytokine release are generated, according to the immune response in question, so it is highly unlikely that the interferons alone are responsible for the control of lymphocyte recirculation through the LN. It would be of interest to study those cases where antigenic stimulation

leads to an increased influx of cells without an observed transient decrease in output. Also, models of immunity dominated by humoral responses are likely to be under the influence of alternative regulatory mechanisms. Although it is easy to draw analogies between the effects of antigen- and cytokine-induced alterations in lymphocyte migration, care should be taken when interpreting the relevance of the data, as differences do exist between the two systems. For instance, unlike antigenic stimulation, the cellular responses which are triggered by the administration of IFN do not include a component of proliferation. Does this mean that cytokines involved in the regulation of lymphocyte stimulation and / or proliferation can be ruled out as participants in the monitoring of LN cell circulation?

Whatever the mechanism involved, the combined effect of altered cell influx and efflux plays a crucial role in the initiation of immune responses, by allowing the requisite time for antigen-specific cells to expand their pool for recirculation.

1.10.4 Summary

Antigenic stimulation influences the localization of lymphocytes to draining LN in a variety of ways, resulting in a specific sequence of changes in the kinetics of cell input and output. Using the data of Drayson *et al*. (1981), these changes will now be summarized in the order of their appearance in the sequence, so as to get some idea of the temporal relationship between each event.

1) Within 1 hour of sheep red blood cell administration, blood flow to the popliteal LN increases by a factor of 3. The flow reaches a plateau at 36 hours and is maintained up to day 4, before gradually falling. This change in circulation to the LN results from the effects of hyperaemia and angiogenesis. 2) An increase in lymphocyte influx is first detectable around 12 hours post antigen, reaching a peak of 2.5-fold greater influx than control LN between days 3 and 4. Thus, the increased influx of

lymphocytes is mainly a consequence of augmented delivery to the LN in its arterial blood supply, although HEV in the stimulated LN are also thought to become more effective at capturing and transmitting lymphocytes from the blood. 3) Within 24 hours of antigen administration, cell proliferation has doubled and peaks on day 5, with a 10-fold increase in ^{3}H-thymidine incorporation. 4) Structural changes in HEV (as measured by ^{35}S-sulphate incorporation) are not apparent in the first 24 hours of the response. However, by day 4, there is a maximal 2.5-fold increase in incorporation, which persists until at least day 8 post-stimulation. 5) The study of Drayson et al. did not measure the changes in cell output from the stimulated node. However, using other data discussed in he earlier sections, the majority of lymphocyte efflux will take place 4-5 days after antigen administration (Hay & Hobbs 1977).

Thus, an observed increase in LN weight following antigenic stimulation is the result of several contributing factors. The close inter-relationship between these factors ensures the maximal probability that the appropriate lymphocyte will come into contact with a particular antigen and generate the required immune response.

PART III : AIMS OF THIS STUDY

In general, little is known about the immunological events associated with the induction of protective immunity to *S. mansoni*. One important feature in the success of the irradiated-vaccine model is the requirement of schistosomula to enter and persist in LN draining the area of parasite exposure. Thus, the skin-draining LN have been implicated as the site of host sensitization.

The aim of this study was to investigate the *in vivo* LN cellular responses following immunization with attenuated larvae, in order to establish the immunological sequence of events during the induction of protection. Chapter 2 (presented as a published paper) describes initial work carried out to confirm a role for the draining LN in the elicitation of maximal levels of immunity. The temporal pattern of cellular changes in various nodes following different parasite-exposure regimes is described, and includes a phenotypic analysis of the observed responses. The work in Chapter 3 (presented in the format submitted for publication) is an in-depth characterization of the dynamic components of cell traffic which contribute to the changes observed in Chapter 2. This includes an *in vivo* measure of T and B lymphocyte proliferation, cell cycle kinetics, hyperaemia, and lymphocyte recruitment / efflux patterns, within the draining nodes of vaccinated mice. Finally, Chapter 4 details the attempts made to identify the subset (TH1 / TH2) of $CD4^+$ T cells generated in the skin-draining LN during the induction phase of immunity, using the expression of cytokine mRNA as a marker. This was carried out using RNA:RNA Northern hybridization analysis.

CHAPTER 2

Phenotypic analysis of the cellular responses in regional lymphoid organs of mice vaccinated against *Schistosoma mansoni*.

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Phenotypic analysis of the cellular responses in regional lymphoid organs of mice vaccinated against *Schistosoma mansoni*

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SUMMARY

The cellular responses in regional lymphoid organs of C57Bl/6 mice were examined, following protective immunization with 20 krad.-irradiated cercariae of *S. mansoni*. Marked changes in total cell number were observed, with peak increases of 13·5-fold in the skin-draining (axillary) lymph nodes and 6·9-fold in the lung-draining (mediastinal) lymph nodes. In contrast, cellular responses were small in the spleen and undetectable in the brachial lymph nodes. The temporal pattern of responses was coincident with the kinetics of parasite migration, events in the mediastinal lymph node being apparent only after day 7. Phenotypic analysis of the cellular changes revealed an increase both in T lymphocytes and, to a greater extent, in B lymphocytes. The changes in Thy1$^+$ cells comprised an increase in both L3T4$^+$ and Lyt2$^+$ populations. A comparison of mice exposed to non-immunizing parasites (normal or 80 krad.-irradiated cercariae) with protected animals, revealed smaller and more transient cellular changes in the axillary lymph nodes of the former. We suggest that the successful immunization of mice with attenuated parasites depends upon the prolonged priming of lymphocytes within the lymph nodes draining the skin-exposure site and that the persistence of 20 krad.-irradiated parasites within these nodes provides the requisite stimulus.

Key words: *Schistosoma mansoni*, vaccination, lymphoid organs, lymphocyte phenotypes.

INTRODUCTION

The mechanism wherby optimally irradiated cercariae of *Schistosoma mansoni* elicit specific acquired immunity in mice is still unclear. One possibility is that, following exposure of parasites to radiation, expression of their antigens is modified, either quantitatively or qualitatively, relative to normal. However, neither biochemical (Simpson *et al.* 1985) nor ultrastructural (Mastin, Bickle & Wilson, 1985) studies have revealed evidence for altered antigenicity. Alternatively, the explanation may lie in differences between the migration kinetics of normal and attenuated parasites, as demonstrated by histology (Mastin, Bickle & Wilson, 1983) and autoradiographic tracking (Mangold & Dean, 1984; Mountford, Coulson & Wilson, 1988).

We have observed that, following skin penetration, both normal and attenuated schistosomula migrate to the lymph nodes draining the exposure site (Mountford *et al.* 1988). Whereas normal parasites spend only a very transient period in the nodes, a significant number of irradiated parasites persist there for several days. Coincidentally, we noted that skin- and lung-draining lymph nodes were more enlarged after vaccination than after exposure to normal parasites. Since lymph nodes provide a potent environment for the processing and presentation of antigen to immunological cells (Austyn, 1989), the extended residence of attenuated parasites within specific nodes may be the crucial factor leading to priming of the host.

Further evidence for the importance of local rather than systemic antigen presentation has been provided by vaccination experiments with attenuated day 8 schistosomula (Coulson & Mountford, 1989). When these parasites are administered intradermally, with subsequent migration to the draining lymph nodes, high levels of protection ensue. When given intravenously, bypassing the skin-draining nodes, they fail to elicit protection. Furthermore, if the relevant skin-draining lymph nodes are excised before or shortly after percutaneous immunization, resistance to challenge parasites is significantly reduced (A. P. Mountford & R. A. Wilson, unpublished observations). *In vitro* studies of lymphocyte blastogenesis have also highlighted local rather than systemic events. Cells extracted over the period 1–6 weeks post-vaccination from lymph nodes draining the skin (James, Labine & Sher, 1981; Lewis & Wilson, 1982) and the lungs (Lewis & Wilson, 1982) proliferated in response to live schistosomula, cercarial antigen, and adult antigen preparations. In contrast, spleen cell responses were not overwhelming (Lewis & Wilson, 1982).

Given the supposed involvement of T lymphocytes in immune elimination of challenge schistosomula from vaccinated mice (Crabtree & Wilson, 1986; James *et al.* 1986; Aitken, Coulson & Wilson, 1988), we might anticipate that successful immuniza-

tion would preferentially stimulate the proliferation of T-cells in particular regional lymph nodes. In the present study, we examine the relationship between parasite persistence, cellular events in lymphoid tissues, and the induction of protective immunity. We have selected three different protective and non-protective exposure regimes in which parasites fail to reach (80 krad.-irradiated cercariae), are sequestered in (20 krad.-irradiated cercariae), or pass through (normal cercariae) the skin-draining lymph nodes. As an index of responses we measured changes in the numbers of T and B lymphocytes and T-cell subsets in lymphoid organs, following exposure to parasites. Cells were phenotyped using fluorescent-labelled antibodies and analysed by flow cytometry. We show that responses are local in character and relate to the extent of protection induced.

MATERIALS AND METHODS

Host and parasite

Female C57Bl/6 mice, weighing 18–20 g, were used throughout the study. A Puerto Rican strain of *Schistosoma mansoni* was maintained in albino *Biomphalaria glabrata*. For autoradiographic tracking experiments, patent snails were incubated in pond water containing [^{75}Se]methionine (SC 12 Amersham; 25 μCi/snail/ml) for 5 h on two successive days (modification of Wilson & Coulson, 1986). Radio-isotope labelled cercariae were shed 6 days later.

Attenuation of parasites and exposure regimes

Parasites were attenuated either with a 20 krad. or 80 krad. radiation dose from a ^{60}Co source (Department of Radiobiology, Cookridge Hospital, Leeds). Mice were anaesthetized with 10% Sagatal (May & Baker) in 10% ethanol (0·01 ml/g body weight) and exposed to 500 cercariae via the shaved abdomen (Smithers & Terry, 1965).

Three exposure regimes were examined: V20 – 20 krad.-irradiated cercariae, inducing a high level of immunity; V80 – 80 krad.-irradiated cercariae, inducing negligible resistance; NI – normal cercariae, representing a standard against which to compare the cellular changes stimulated by attenuated parasites. A major component of the resistance developing in this last group after bisexual infection is due to non-specific factors, related to egg-induced hepatic pathology, and not specific immunity (Wilson, Coulson & McHugh, 1983). In the absence of pathology, following exposure to large numbers of single-sex cercariae, insignificant levels of protection are observed (Bickle, 1982).

For each experiment, normal and attenuated cercariae were obtained from the same pool.

Resistance measurements

Thirty-five days post-vaccination, groups ($n = 5$) of naive (C), V20 or V80 mice (T) were challenged via the tail with 200 normal cercariae (Olivier & Stirewalt, 1952). Five weeks later, adult worms were recovered by portal perfusion (Smithers & Terry, 1965) and the level of resistance (R) calculated by comparing the mean worm burdens in C and T mice, using the formula: $R = (C-T)/C \times 100$.

Autopsy of mice

For autoradiographic tracking, groups of V20 and V80 mice ($n = 5$) were autopsied on days 2, 4, 5, 7, 10 and 14 post-vaccination. The skin, lungs, liver, plus the inguinal, axillary, brachial and mediastinal lymph nodes (LN) were prepared for autoradiography as described by Wilson & Coulson (1986) and Mountford *et al.* (1988); intensifying screens were used for tissues prepared on days 7, 10 and 14.

To investigate the cellular events following parasite exposure, groups of mice ($n = 5$) were sacrificed on days 0, 7, 14, 21, 28 and 35 post-exposure. The axillary LN (draining the abdominal exposure site) and mediastinal LN (draining the lungs and peritoneal cavity) were examined for localized cellular changes associated with parasite migration. The brachial LN (draining the forelimbs) were used to monitor responses in sites distant from the migration route. The spleen was examined for cellular changes stimulated by parasite antigens released into the systemic circulation.

Preparation of cell suspensions

The left and right axillary and brachial LN, the single mediastinal LN, and the spleen were excised from each mouse and cleaned of adipose tissue. The LN capsules were then gently teased apart using curved forceps, whereas the spleens were crushed with a silicone bung. The LN and spleen cells were washed out of the capsules usng 1 ml of ice-cold phosphate-buffered saline (PBS)-medium (PBS pH 7·2 containing 0·01% calcium chloride, 0·01% magnesium chloride and 0·1% bovine serum albumin). After filtration through a 400-gauge stainless steel mesh, all lymphoid cells were kept on ice. Spleen cells were treated with Tris-buffered ammonium chloride to lyse red blood cells (Hunt, 1987).

An estimate of the cell number in each sample was obtained using a Neubauer haemocytometer; an aliquot of the cell suspension was diluted in an appropriate volume of 0·5% saturated Malachite Green solution in 1·5% acetic acid.

Phenotyping of lymphocytes

T- and B-cells were identified using a one-step immunophenotyping procedure. T lymphocytes were stained with fluorescein isothiocyanate (FITC)-conjugated anti-Thy 1.2 antibody (Becton Dickinson) at a final dilution of 1/80, and B lymphocytes with phycoerythrin (PE)-conjugated anti-Ig antibody (Southern Biotechnology Assocs. Inc.) at a final dilution of 1/40. L3T4$^+$ and Lyt2$^+$ cells were stained with FITC-conjugated anti-L3T4 or anti-Lyt2 antibody (Sera Lab) at a 1/100 dilution in 10% heat inactivated normal rabbit serum. Aliquots of 5×10^5 cells in a 0·1 ml volume, were incubated with the appropriate antibody for 30 min on ice. Unbound antibody was then removed by 2 washes in PBS-medium.

A population of 'null' cells (Thy1$^-$,sIg$^-$) was tested for natural killer phenotype using a two-step procedure. Anti-LGL-1 antibody (a marker for murine natural killer cells) was obtained from L. Mason, National Cancer Institute, Frederick, MD, USA (Mason *et al.* 1988) and used at a 1/1000 dilution. LGL-1$^+$ cells were then detected with FITC-goat anti-rat IgG (Sigma) diluted 1/50 in 50% heat-inactivated normal mouse serum.

Flow cytometric analysis

Stained cell suspensions were analysed on an EPICS C flow cytometer (Coulter Electronics Ltd) equipped with a 488 nm emission argon-ion laser. Fluorescence emitted by FITC and PE was passed through a short-pass 560 nm interference and long-pass 570 nm absorbance filter, respectively.

The lymphocyte population in the samples was identified on the basis of its forward and 90° light scatter properties. A gated area was created around the live lymphocyte population (as previously determined by propidium iodide exclusion (Yeh, Hsi & Faulk, 1981)), and the proportion of fluorescence-positive cells was then calculated within this area.

Statistical analysis

Differences in mean worm burdens and cell numbers were tested for significance using Student's *t*-test.

RESULTS

Autoradiographic tracking of 80 krad.-irradiated parasites

We first showed that, whilst 80 krad.-irradiated parasites penetrated the skin successfully, they neither migrated to the skin-draining LN in significant numbers, nor generated resistance. The migration of 80 krad.- and 20 krad.-irradiated parasites from the same pool is illustrated in Fig. 1. During the first 7 days post-vaccination, greater

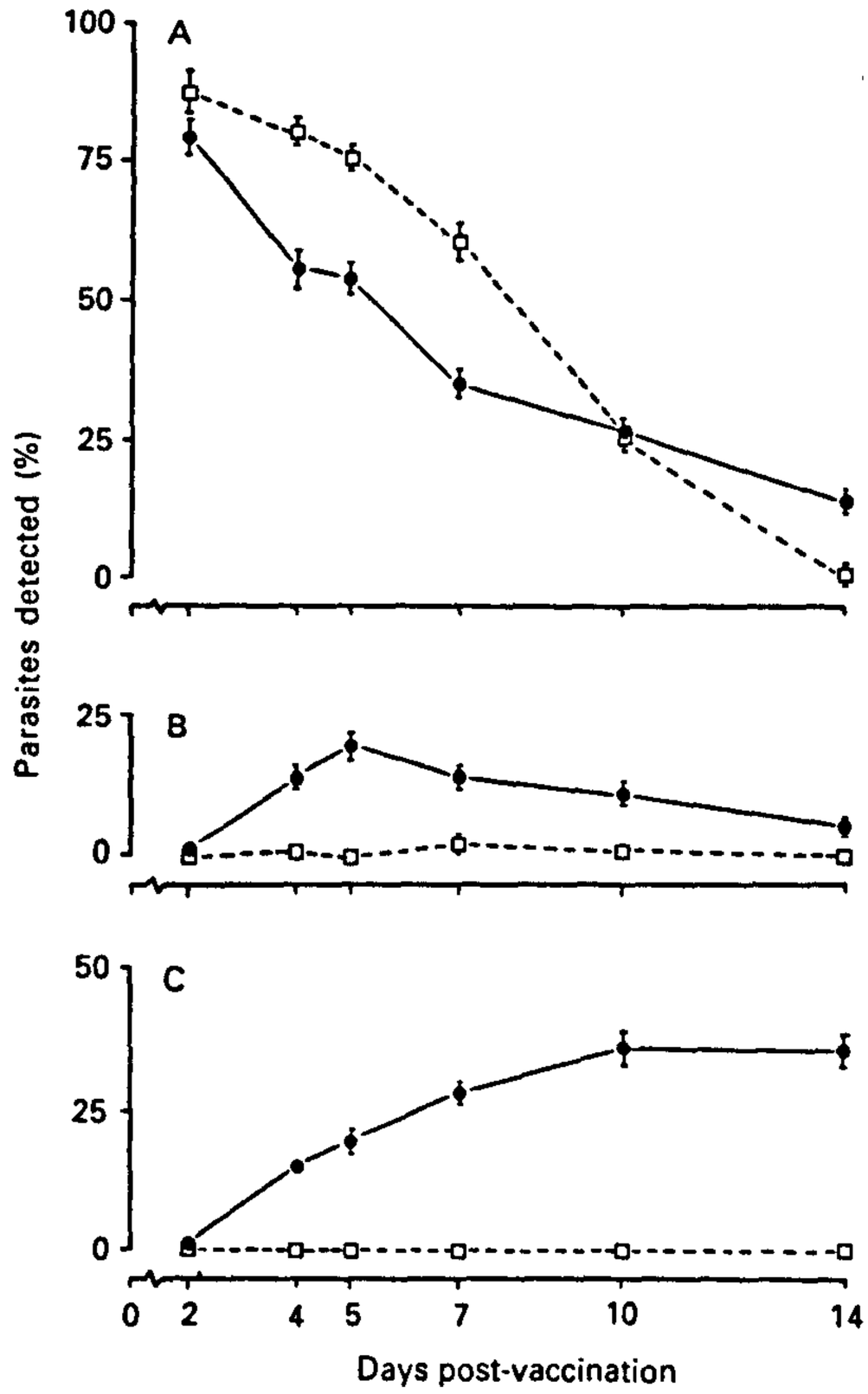

Fig. 1. The number of parasites at times post-vaccination in the (A) skin-exposure site, (B) axillary and inguinal LN and (C) lungs, expressed as a percentage of the number of applied cercariae. Values for V20 (●——●), and V80 (□– – –□) mice are the mean from 7 mice (±S.E.).

numbers of parasites were detected in the skins of V80 than V20 mice (Fig. 1A); the values on day 7 were 61% and 34%, respectively. Thereafter, the number in the skins of V80 mice declined rapidly to only 0·8% on day 14, compared to 13% in V20 mice. A maximum of only 2% of parasites was detected on day 7 in the axillary and inguinal LN of V80 mice (Fig. 1B). However, in the same nodes of V20 animals a peak of 19% of applied parasites was present on day 5. In the lungs of V20 mice, up to 37% of applied parasites were detected on days 10–14 (Fig. 1C). In contrast, only an occasional parasite reached the lungs of V80 mice. No parasites were present in the livers or the brachial and mediastinal LN of either V20 or V80 mice (data not shown).

In this experiment, the mean worm burden in challenge control mice was 50·0 (S.E. ±4·1) compared with 22·6 (S.E. ±2·0) in V20 mice ($P < 0·001$) and 53·0 (S.E. ±6·1) in V80 mice ($P > 0·05$). The degree of resistance induced was 54·8% in V20 mice and −6·0% in V80 mice.

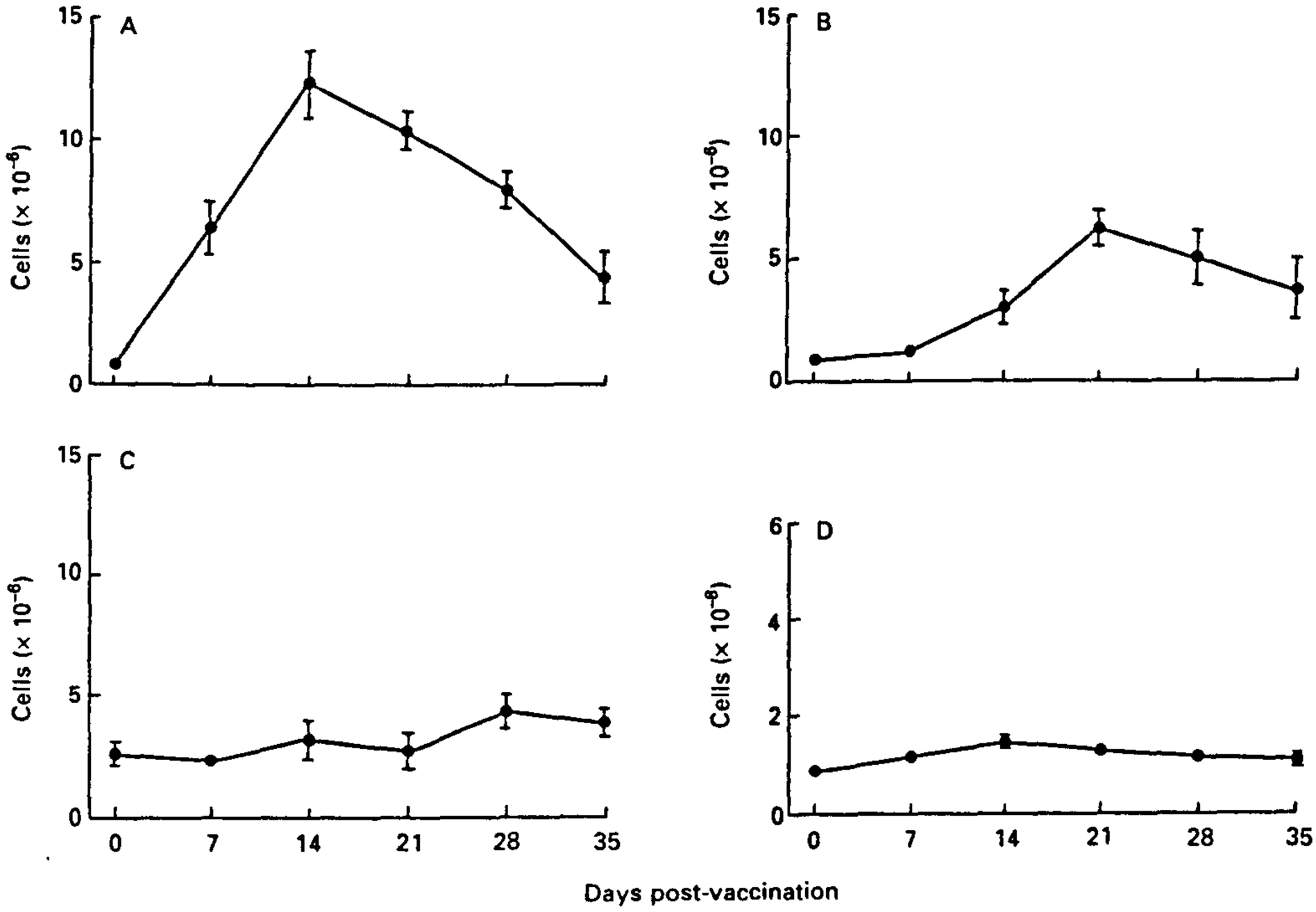

Fig. 2. Changes in total cell number in the LN and spleen of mice vaccinated with 20 krad.-irradiated cercariae. (A) Axillary LN; (B) mediastinal LN; (C) brachial LN; (D) spleen. Values are the mean from 5 mice (±S.E.).

Cellular events following exposure to parasites inducing maximum immunity

We next examined the events occurring in both draining and distant lymphoid tissues of mice exposed to 20 krad.-irradiated parasites in 2 separate experiments. In one we investigated the T- and B-cell composition, in the other, T-cell ($L3T4^+/Lyt2^+$) subsets.

Changes in total cell number. A rapid increase in cell number (7·1 × day 0) was detected in the axillary LN by 7 days post-vaccination, reaching a peak 13·5-fold increase at day 14 (Fig. 2A). Thereafter, a gradual decrease in cell number was observed, although levels were still elevated at day 35, compared to day 0 ($P < 0·05$). A large increase in cell number was also observed in the mediastinal LN (Fig. 2B) but in contrast, the initial rise was delayed to day 14 and peak number was reached at day 21 post-vaccination; this peak represents a 6·9-fold increase over the day 0 value. No changes in cell number were observed in the brachial LN at any time (Fig. 1C). A significant increase was detected in the spleen (Fig. 1D) only at days 14 and 21 ($P < 0·05$), the maximum at day 14 representing a 1·7-fold increase over day 0. In two replicate experiments, no significant changes in spleen cell numbers were observed at any time-point after vaccination (data not shown). Consequently, the remainder of this report focuses on events in the axillary and mediastinal LN.

Lymphocyte phenotypes. The absolute number of T and B lymphocytes, together with the ratio of T:B-cells is plotted in Fig. 3. In the axillary LN (Fig. 3A), a significant change in T- and B-cell numbers was evident by day 7 post-vaccination. T- and B-cells increased and decreased in unison, the main difference between the two populations being a 7·8-fold increase in T-cells versus a 28·3-fold increase in B-cells at the day 14 peak. The effect this had was to alter the T:B-cell ratio from 4:1 to 1:1, although the total number of T-cells exceeded B-cells almost throughout. Significant changes in T- and B-cell numbers were also observed in the mediastinal LN (Fig. 3B), although these were not detected until day 14 post-vaccination. There were peak increases in T-cells at day 21 (4·9-fold) and in B-cells at day 28 (11-fold), resulting in a decrease of the T:B-cell ratio from 3:1 to 1:1. Changes in the proportions of T and B lymphocytes also coincided with the appearance of a population of 'null' lymphocytes in the responding nodes, comprising up to 15% of the total. Only 1–2% of these lymphocytes were $LGL\text{-}1^+$ indicating that the null cell population was not composed of natural killer cells (data not shown). No changes in the abundance or proportions of T and B lymphocytes in the brachial LN and spleen were observed over the course of the experiment (data not shown).

In both the axillary and mediastinal LN, the changes in $L3T4^+$ and $Lyt2^+$ cell number mirrored

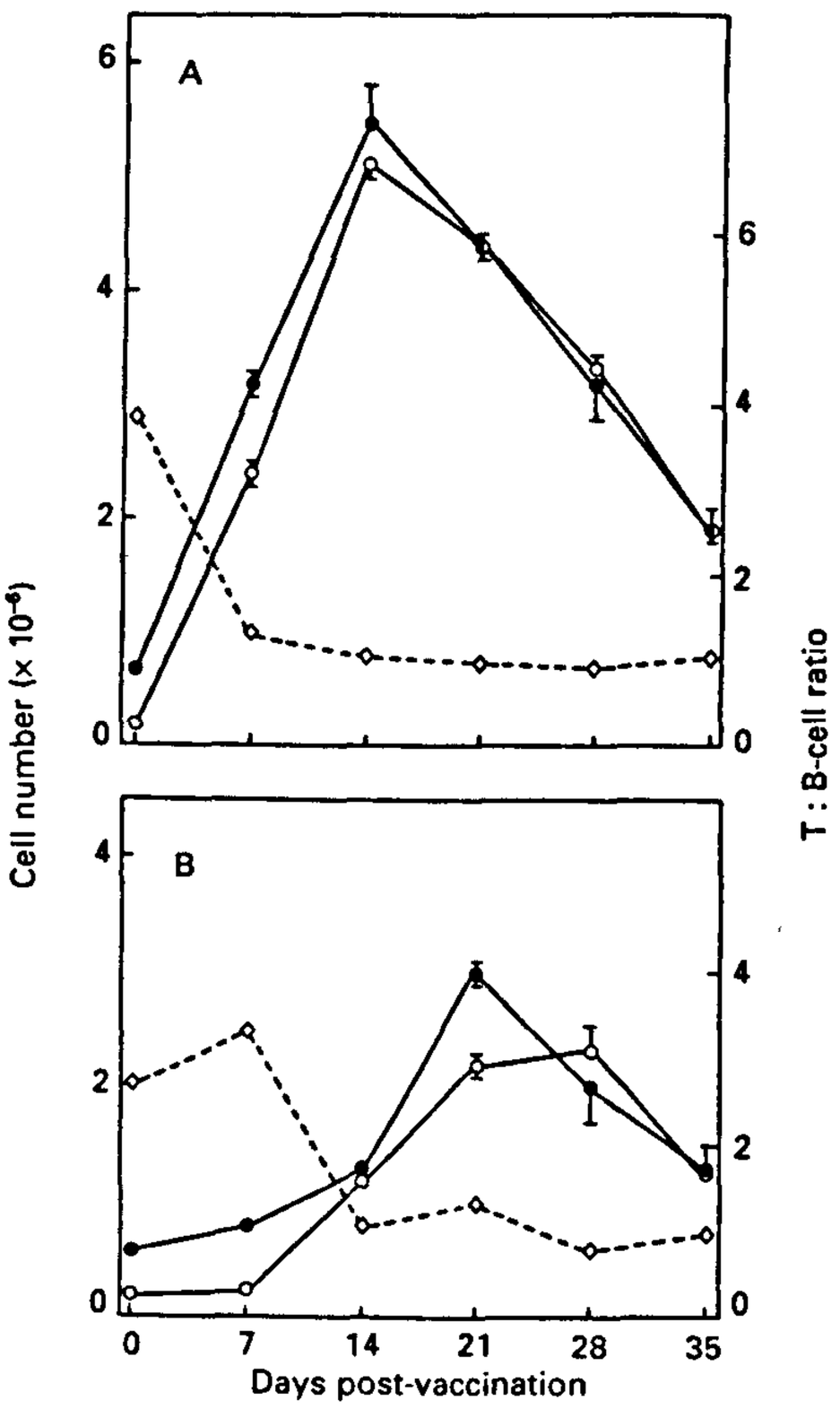

Fig. 3. Changes in the number of T lymphocytes (●——●) and B lymphocytes (○——○), and changes in the T:B-cell ratio (◇– – –◇) in the LN of mice vaccinated with 20 krad.-irradiated cercariae.
(A) Axillary LN; (B) mediastinal LN. Values are the mean from 5 mice (±S.E.).

the pattern in Thy1$^+$ cells (data not shown). The axillary ratio was 1:1 at the start of the experiment, changed to 1·3:1 by day 7, and remained at that value thereafter. The ratio in the mediastinal LN also started at 1:1 and fluctuated between 1·2:1 and 1·4:1 from day 14 onwards.

Resistance measurements. For the T/B experiment, the mean worm burden of the challenge control mice was 59·6 (S.E. ±5·9) compared with 15·2 (S.E. ±4·3) in the V20 mice ($P < 0·001$), resulting in resistance of 74·5%. For the L3T4$^+$/Lyt2$^+$ experiment the mean worm burden of the challenge control mice was 81·0 (S.E. ±3·9) compared with 34·0 (S.E. ±4·0) in the V20 mice ($P < 0·001$), resulting in resistance of 58·0%.

A comparison of LN cell phenotypes in mice exposed to immunizing versus non-immunizing parasites

The relevance of events in the LN of V20 mice to the induction of protective immunity was assessed in a separate experiment, by comparison with responses in V80 and NI animals.

Within each experimental group, T- and B-cell numbers changed in unison, the T lymphocytes outnumbering the B lymphocytes at almost every sampling time. At day 7 post-exposure in all 3 groups, the axillary T- and B-cell numbers had increased approximately 2-fold and 4·5-fold, respectively (Fig. 4A and B). However, by day 14 marked differences had emerged, with a decrease in T and B lymphocytes in the NI group, no further change in the V80 mice, and a continued increase in the V20 animals. At day 21, the cell numbers in the V80 and NI groups had returned virtually to baseline values, unlike the V20 cell numbers which persisted at elevated levels to day 35. The changes in T:B-cell ratio observed in the axillary LN were short-lived in the V80 mice and less marked in the NI mice, relative to V20 animals (Fig. 4C).

In the mediastinal LN, no significant changes in cell numbers were observed in V80 mice ($P > 0·05$) throughout the experiment. In contrast, marked increases in T- and B-cells were recorded in the V20 and NI animals after day 14. However, neither the T-cell nor B-cell numbers differed significantly between V20 and NI groups at any time after vaccination (Fig. 4D and E). Peak responses were seen at day 28, with numbers remaining elevated levels at day 35. The T:B-cell ratio of both V20 and NI mice showed a downward trend, resulting in a 2-fold decrease by day 35 (Fig. 4F). There was some fluctuation of the ratio in V80 animals, but no overall pattern of change.

Resistance measurements. The mean worm burden in the challenge control mice in this experiment was 68·0 (S.E. ±2·9) compared with 23·0 (S.E. ±4·9) in V20 mice ($P < 0·001$) and 66·2 (S.E. ±9·6) in V80 mice ($P > 0·05$). This resulted in resistances of 66·1% and 2·6% in the V20 and V80 groups, respectively.

DISCUSSION

Mountford *et al.* (1988) suggested that successful vaccination of mice with irradiated cercariae of *S. mansoni* depended on the persistence of the parasites, and prolonged release of antigen, in LN draining the exposure site. This led us to examine events in the draining LN of mice exposed to different infection or vaccination regimes. Throughout the study, 20 krad.-irradiated cercariae were used to elicit high levels of protection, ranging from 58% to 74%. This protocol was compared with exposure to normal parasites which fail to induce resistance independent of pathology (Bickle, 1982), and 80 krad.-irradiated parasites which induced negligible levels (−6·0 to 2·6%) of protection.

Examination of the changes in cell number in

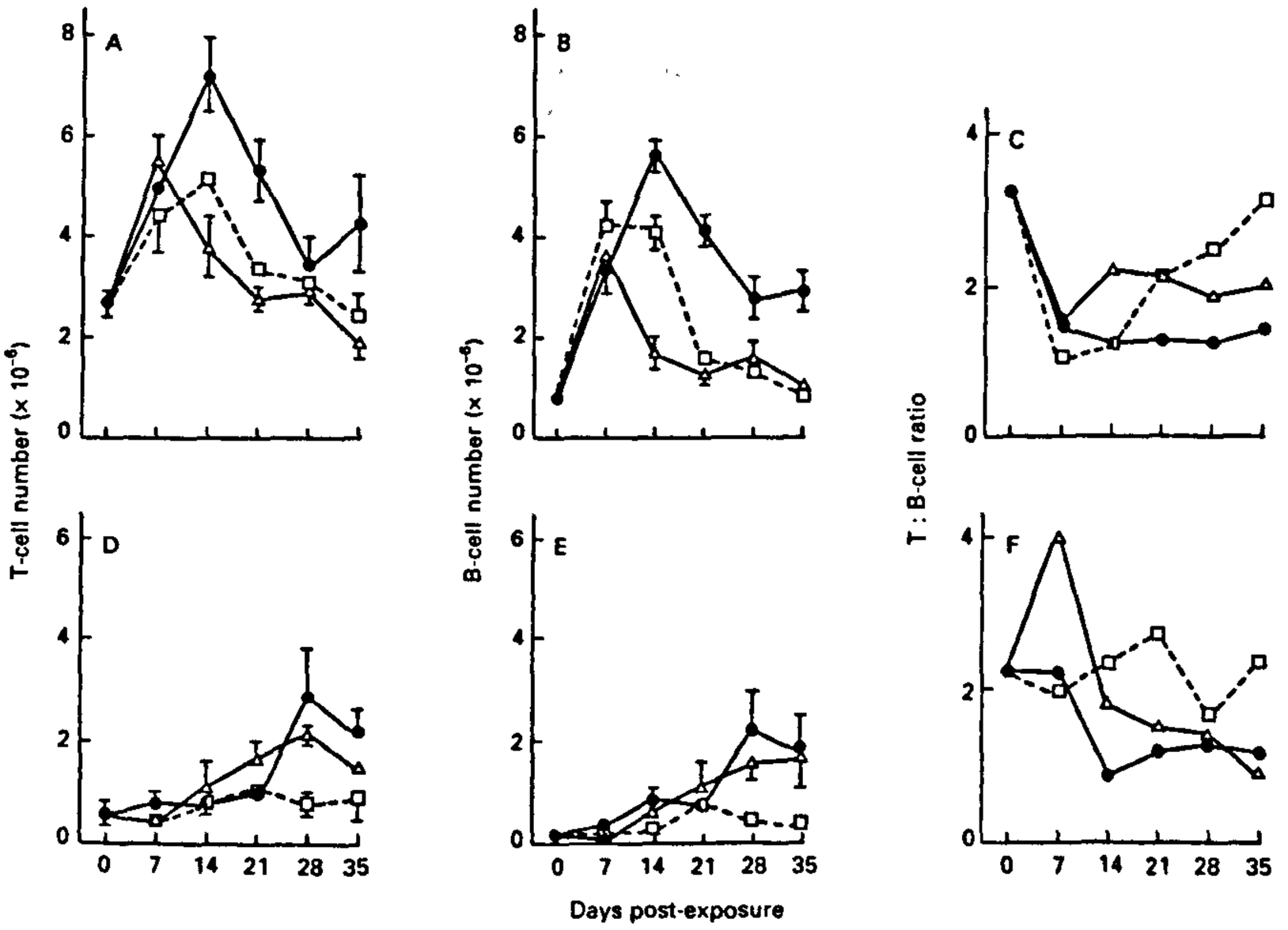

Fig. 4. Changes in cell phenotypes in the LN of mice exposed to either 20 krad.-irradiated (●——●), 80 krad.-irradiated (□– – –□), or normal cercariae (△——△). Axillary LN: (A) T lymphocytes, (B) B lymphocytes, (C) T:B-cell ratio. Mediastinal LN: (D) T lymphocytes, (E) B lymphocytes, (F) T:B-cell ratio. Values are the mean from 5 mice (±S.E.).

various lymphoid organs of V20 mice revealed marked localized, rather than systemic responses. There was a large-fold increase in cell number in the axillary and mediastinal LN; changes in the brachial LN and spleen were undetectable or slight in comparison. A prominent feature of the responses was their close correlation with the kinetics of attenuated-parasite migration (Mountford *et al.* 1988), events in the axillary LN preceding those in the mediastinal LN by 7 or more days. Studies by other workers, employing *in vitro* assays of antigen-stimulated proliferation, have revealed a similar pattern of response with cells from the skin- and lung-draining LN of vaccinated mice (Lewis & Wilson, 1982). Although blastogenic responses have been reported with spleen cells, they were less pronounced than in the LN (Lewis & Wilson, 1982; James, Correa-Oliveira & Leonard, 1984); Pemberton & Wilson, unpublished observations), and occurred later (Lewis & Wilson, 1982; Pemberton & Wilson, unpublished observations). The limited splenic response is not surprising as no parasites and little of their released antigens were detected in the spleen after vaccination (Mountford *et al.* 1988). Since the spleen is the most important organ in lymphocyte recirculation (Pabst, 1988), lymphocytes primed in the draining LN will reach it in normal cell traffic. This would provide an explanation for the presence of schistosome-reactive cells in the spleen in the absence of a marked increase in overall cell number. A comparison of the axillary and mediastinal LN of V20, V80 and NI mice, revealed significant differences, both in magnitude and duration of responses, which can be related to variations in the pattern of parasite migration. We showed in the tracking experiment that most 80 krad.-irradiated parasites failed to migrate from the skin. In contrast, few normal schistosomula, and intermediate numbers of 20 krad.-irradiated parasites remained in the skin of NI and V20 animals, respectively (Mastin *et al.* 1983; Mountford *et al.* 1988). We might expect that the magnitude of response in the axillary LN would reflect the level of parasite death and antigen release in the skin; it clearly does not, the V20 response being greatest. This substantiates the idea that persistence of parasites in skin-draining LN, with prolonged release of antigen, is vital to vaccination success (Mountford *et al.* 1988). In contrast, the rapid passage of normal parasites through these nodes is obviously insufficient to provoke more than a transient response.

The truncated migration of 80 krad.-irradiated parasites also accounts for the total lack of response in the mediastinal LN of V80 mice. The change in cell number in this node in the V20 and NI groups was almost identical, both in magnitude and duration. Since neither attenuated nor normal parasites have been detected in the mediastinal LN (Mountford *et al.* 1988), the changes observed must be stimulated by parasite antigens draining from the lungs. The elimination of approximately equal proportions of 20 krad.-irradiated and normal parasites in mouse lungs (Wilson, 1987) would explain the similarity of response. The magnitude of the response in the mediastinal LN, judged by the fold

increase in cell number, is greater than that of the axillary nodes. However, the maximum cell number attained is only equal to that of the unstimulated axillary nodes. Taking into account both fold increase, and total cell number attained, events in the axillary LN, rather than in the mediastinal node, correlate best with the level of immunity induced. This point is reinforced by the failure of attenuated lung-stage parasites administered intravenously to the lungs of naive mice, to induce immunity (Coulson & Mountford, 1989).

A further dimension to the success of immunization is provided by the observation that significant numbers of lymphocytes and other leucocytes infiltrate the lungs after vaccination with 20 krad.-irradiated parasites (Aitken *et al.* 1988; Menson, Coulson & Wilson, 1989). It has been suggested that the stimulus for recruitment is provided by the presence of attenuated parasites in the lungs and serves to 'arm' the organ against a subsequent challenge (Aitken *et al.* 1988). Failure of 80 krad.-irradiated parasites to reach the lungs could explain the absence of leucocyte recruitment and arming of the organ, even though responses were stimulated in the axillary LN (Mountford, unpublished data). Conversely, the death of normal parasites in the lungs would provide a recruitment signal but arming would fail, due to the low level of response in axillary nodes. We are currently investigating the interrelationships between lymphocyte proliferation and recruitment.

The changes in cell number in the axillary and mediastinal LN of V20 mice reflected increases in both T and B lymphocytes, the latter being more marked. When the T-lymphocyte subsets were examined in these nodes both L3T4$^+$ and Lyt2$^+$ cells increased, the ratio also changing from 1:1 to 1·3:1. Although this change is relatively small, it might contribute to the predominant L3T4$^+$ composition of the pulmonary infiltrates described by Aitken *et al.* (1988). Compared to the responses in V20 mice, the changes in T/B proportions were less marked in the axillary LN of the NI group and transient in the V80 group. Again these patterns are presumably the product of parasite migration kinetics. The proportionally greater increase in B-cells compared to T-cells in both the axillary and mediastinal LN is difficult to equate with an effector mechanism mediated by DTH (Crabtree & Wilson, 1986; James *et al.* 1986; Aitken *et al.* 1988). Furthermore, anti-parasite antibody titres in this model are a poor correlate of the immune status of the host (James *et al.* 1981; Correa-Oliveira, Sher & James, 1984; Roberts, Boot & Wilson, 1988). A predominant increase in the absolute number of B-cells within regional LN has been reported in other models of immunity where T-cells are known to be the key *in vivo* effector. For example, Lynch, Doherty & Ceredig (1989) found that the T:B-cell ratio in the LN of mice exposed to lymphocytic choriomeningitis virus decreased from 2:1 to 0·9:1 at peak cell numbers; they offered no explanation for this phenomenon.

A possible role for the B lymphocytes could be the priming of the host via their capacity as antigen-presenting cells for T-helper (CD4$^+$) lymphocytes (Lanzavecchia, 1985). It has been suggested that B-cells are the main antigen-presenting cell in peripheral LN, compared to other types of accessory cell in the spleen (Janeway, Ron & Katz, 1987). Recently, Mosmann & Coffman (1987) have divided the murine T-helper subset into Th1 and Th2 cells on the basis of the profile of cytokines produced by cloned lines. Bottomly & Janeway (1989) have proposed that the two subsets require distinct accessory cells, with B cells preferentially presenting antigen to Th1 cells. Since this subset mediates DTH responses (Cher & Mosmann, 1987), we speculate that an equivalent Th1/B-cell interaction may operate in our model of protective immunity and we are currently investigating the potential role of T-helper subsets *in vivo*. A differential role for the two subsets has already been established in murine leishmaniasis (Heinzel *et al.* 1989).

Throughout this study we have described changes in the LN in terms of an increase or decrease in cell number. These changes are likely to comprise several interacting components, as observed in the stimulated LN of rats (Drayson, Smith & Ford, 1981). As well as the active proliferation of specific anti-parasite lymphocytes, the non-specific recruitment of circulating lymphocytes, and variations in the efflux of cells will be important factors determining the cell number and composition of the draining LN. In a subsequent paper (Constant & Wilson, manuscript in preparation) we will describe experiments to dissect out the contribution of the different components to the changes described here. Of course, the mere presence, as well as changes in dimensions, of particular lymphocyte subsets within the responding LN does not establish the nature of the immune responses involved. In order to gain further insight into the mechanism of resistance, it will be necessary to investigate the functional roles of the various subsets, both *in vitro* and *in vivo*.

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CHAPTER 3

In vivo lymphocyte responses in the draining lymph nodes of mice exposed to Schistosoma mansoni: preferential proliferation of T cells is central to the induction of protective immunity.

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In Vivo Lymphocyte Responses in the Draining Lymph Nodes of Mice Exposed to *Schistosoma mansoni*: Preferential Proliferation of T Cells is Central to the Induction of Protective Immunity

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Summary: We have investigated the *in vivo* cellular responses associated with the induction of specific immunity by attenuated larvae of *Schistosoma mansoni* in mice. Using *in vivo* 5-bromo-2′-deoxyuridine incorporation, we measured the changes in cell proliferation in the skin- and lung- draining lymph nodes (LN) of vaccinated animals. A marked increase in the number of dividing cells was detected in both groups of LN, with a preferential increase in the proportion of proliferating T, relative to B, lymphocytes. Several dynamic components of cell migration have been examined to assess their relative contribution to the overall changes in the LN of immunized mice. We determined that a significant part of the observed accumulation of cells is due to the effect of hyperaemia. There was no alteration in the affinity of the LN for T and B lymphocytes, but we concluded that the majority of recruited B cells failed to exit the LN. Our results have highlighted the importance of T cell proliferation within the draining LN for the successful immunization of mice with attenuated parasites.

INTRODUCTION

Following a single exposure to radiation-attenuated cercariae of *Schistosoma mansoni*, C57BL/6 mice develop high levels of specific immunity. We believe that the successful induction of resistance to reinfection takes place in lymph nodes (LN) draining the skin-exposure site (1, 2). Evidence to support our hypothesis is provided by vaccination experiments in which irradiated parasites are administered such that they bypass the relevant skin-draining LN. For example, if attenuated day 8 schistosomula are administered intravenously, they fail to elicit protection (3). Furthermore, excision of the skin-draining LN before or shortly after percutaneous

vaccination significantly reduces resistance to a challenge infection (4). When we examined the migration kinetics of normal versus irradiated parasites, we found significant differences (1). Following skin penetration, a proportion of both normal and attenuated schistosomula migrated to the LN draining the exposure site. However, in contrast to the normal infection, attenuated parasites persisted in the LN for several days. Thus, we suggested that the prolonged release and presentation of antigen within specific draining LN provides the requisite stimulus for priming of the host.

In order to gain further insight into the sequence of events leading to the induction of protection, we examined the cellular responses within regional nodes following exposure of mice to either immunizing or non-immunizing parasites (2). We observed a marked increase in cell numbers in the skin-draining LN of both groups of mice, however, the magnitude and duration of the responses in protected animals were significantly greater. Phenotypic analysis of the cellular changes revealed an increase both in T and B lymphocytes, but the T:B cell ratio was altered from 4:1 to 1:1. These results are difficult to equate with the substantial evidence that the effector mechanism operating in this model of immunity is T cell-mediated (5-7). However, it is unlikely that the observed alterations in absolute cell numbers within the draining LN are due solely to the proliferation of cells in response to antigen. Studies on the LN of rats stimulated with a model antigen have shown that, as well as the active proliferation of specific lymphocytes, factors such as non-specific recruitment from the circulation, and variations in efflux will contribute to changes in cell number (8).

To probe more deeply the cellular events reported in our previous studies, we have investigated the dynamic components which determine the cell number and composition of the draining LN of mice vaccinated with attenuated larvae of S. mansoni. We first examined the in vivo proliferation of cells within the draining LN in order to establish the extent to which changes in cell number can be

accounted for by cell division. For this, we used the technique of *in vivo* bromodeoxyuridine (BrdUrd) incorporation, originally developed by Gratzner (9). BrdUrd, a thymidine analogue, is incorporated into DNA-synthesizing cells and can be detected by monoclonal antibodies. Combined with flow cytometric analysis, the technique is an extremely sensitive and rapid means of quantifying small numbers of cells undergoing division. We have examined the proliferation of LN cells following exposure to immunizing parasites and then compared the responses with those following two non-protective regimes. In a further experiment the individual contribution of proliferating T and B lymphocytes to the overall pattern of response in vaccinated animals has been investigated. An additional factor likely to influence the composition of cells in stimulated LN is a localized hyperaemia. As reported by Drayson et al. (8), variations in blood flow to responding nodes are closely associated with changes in cell influx. We have measured the extent of hyperaemia in mice after vaccination to assess its potential contribution to the cellular changes previously described (2). We also sought evidence for the preferential recruitment and retention of specific cells in the draining LN since antigenic stimulation changes the localization patterns of T and B cells within nodes (10). As a result of this study we have defined more precisely the cellular events required for the successful induction of protective immunity to *S. mansoni* in mice.

METHODS

Mice and parasites

Adult, female C57BL/6 mice were bred in the Department of Biology, at the University of York. A Puerto Rican strain of *S. mansoni* was routinely maintained by passage through LACA mice and albino *Biomphalaria glabrata* snails. For vaccination experiments, cercariae were attenuated with 20,

or 80 krad γ-radiation from a ^{60}Co source at the Department of Radiobiology, Cookridge Hospital, Leeds, UK). Where the effect of different exposures was compared (20 krad, 80 krad or untreated) the parasites were all taken from a single pool.

Exposure of mice to parasites and assay of acquired resistance

Mice were anaesthetised with 10% Sagatal (May & Baker, Dagenham, UK) in 10% ethanol (0.01 ml/g body weight) and 500 cercariae applied to the shaved abdomen (11). Thirty-five days post-exposure, groups (n=5) of mice vaccinated with 20 krad-irradiated (V20) or 80 krad-irradiated (V80) parasites were challenged via the tail with 200 normal cercariae (12). Five weeks after challenge, adult worms were recovered by portal perfusion (11) and the level of resistance (R) calculated by comparing the mean worm burden of challenge control mice (C) with that of vaccinated mice (V), using the formula: %R = (C-V)/C X100.

Preparation and phenotyping of LN cell suspensions

At various times post-exposure to parasites, the axillary and mediastinal LN (draining sites associated with parasite migration) and the brachial LN (draining sites distant from the migration route) were excised; the cells were then isolated, filtered and enumerated as described previously (2). Phenotyping of T and B lymphocytes was carried out using fluorescein isothiocyanate (FITC)-conjugated anti-Thy 1.2 antibody (Becton Dickinson, Oxford, UK) and phycoerythrin (PE)-conjugated anti-Ig (Southern Biotechnology Associates, Birmingham, AL) respectively. For cell sorting experiments, two-colour immunophenotyping was used, lymphocytes being stained for the B cell marker first, washed, then stained for the T cell marker.

In vivo cell proliferation assay

At various intervals post-exposure to parasites, 5-bromo-2'-deoxyuridine (Sigma, Poole, UK) in phosphate buffered saline (PBS) pH 7.2 was administered (50 mg/kg body weight) to 5 mice of the appropriate experimental group, by lateral tail vein injection. The mice were sacrificed 2 hours post-administration and the axillary, mediastinal and brachial LN excised. Harvested LN cells were fixed in ice-cold 70% ethanol, and stored at $4^{o}C$ until use. The cell nuclei were isolated and partially denatured by simultaneous proteolytic enzyme digestion / acid denaturation, and stained for BrdUrd and DNA content, based on the method of Van Erp et al. (13). Briefly, fixed cells were washed once in PBS-medium (PBS pH 7.2, containing 0.1% bovine serum albumin, 0.01% $CaCl_2$ and 0.01% $MgCl_2$) and resuspended in 2M HCl containing 0.2 mg/ml pepsin (Sigma) for 30 min at room temperature. The resulting nuclei were then washed once in Borax buffer (0.1M di-sodium tetraborate pH 8.5), followed by two washes in PBS-medium. Incorporated BrdUrd was detected by incubating the washed nuclei in a 1/100 dilution of rat anti-BrdUrd (Sera-Lab, Crawley Down, UK) followed by a 1/50 dilution of FITC-conjugated goat anti-rat IgG (Sigma). Control samples received the FITC-antibody only. All antibody binding was carried out at $4^{o}C$ for 30 min, with 2 washes in PBS-medium after each incubation. The stained nuclei were then resuspended in propidium iodide (Sigma) at a final concentration of 5 µg/ml of suspension, and syringed through a 27-gauge needle to dissociate aggregates. The samples were maintained at room temperature for at least 15 min before flow cytometric analysis.

In a further experiment we examined the in vivo proliferation of T and B cells in order to establish their separate contribution to the total LN cell response. To obtain sufficient lymphocytes for analysis, the relevant LN from 5 V20 mice were pooled for each suspension of cells; triplicate samples were prepared at each time point. The cells were phenotyped for T and B surface markers using

two-colour immunofluorescence and fixed in 70% ethanol. An aliquot of the fixed cells was then sorted into T and B lymphocytes classes by flow cytometry and the BrdUrd / DNA content of each, plus that of the original unsorted cells, assayed as described above.

Estimation of cell cycle kinetics

DNA synthesis-time (Ts) was calculated using data from a single sample of cells taken several hours after labelling with BrdUrd, as described by Begg et al. (14). BrdUrd was administered to V20 mice 14 days post-vaccination and the axillary LN harvested 3 hours later. Cell suspensions (n=3) were each made up from the pooled LN of 7 mice. The pooled suspensions were phenotyped for T and B lymphocytes, fixed, and an aliquot sorted by flow cytometry. The BrdUrd / DNA content was determined as described earlier. The movement of S-phase cells relative to the positions of G1 and G2 (RM, relative movement) was calculated using the formula RM = (FL-FG1)/(FGM-FG1), where FL is the mean red fluorescence of the green-labelled cells and FG1 and FGM are the mean red fluorescence values of G1 and G2+M cells, respectively. Ts was then calculated as follows: Ts = 0.5/(RM-0.5) X t, where t is the sampling time in hours.

Measurement of hyperaemia

The radio-labelled microsphere method (15) was used to measure the longitudinal changes in blood flow to different organs after protective vaccination. Microspheres labelled with ^{57}Co (10.36mCi/g) of 16.1 $\pm$ 0.1 μm diameter (DuPont NEN, Wilmington, DE) were sonicated and diluted 1:8 in 0.9% NaCl containing 0.05% Tween 80 and 0.9% benzoyl alcohol. At various intervals post-vaccination, approximately 1.5 X 10^4 microspheres (1 X 10^6 counts per minute (cpm)) in a 0.1 ml volume were administered to anaesthetised V20 and naive mice (n=5) by injection into the left ventricle of the heart, as described by Wilson & Coulson (16). Twenty minutes later, the skin vaccination site, the skin-draining

LN (pooled axillary and inguinal LN), and the mediastinal LN were excised and weighed. The total amount of labelled material in each sample was determined by gamma-counting for 1 min (Packard Autogamma). In order to check that the distribution of microspheres was consistent between mice, the kidneys were also sampled and used as a reference organ. The accuracy of the aliquots delivered to each group of animals was monitored by gamma-counting 0.1 ml samples of the microsphere suspension throughout each sequence of injections.

In vivo cell tracking

The migration pattern of T and B lymphocytes was compared between groups (n=5) of naive and day 14 V20 mice. Spleen cell suspensions from naive donors were labelled using the PKH26-GL fluorescent cell linker kit (Zynaxis Cell Science Inc., Malvern, PA; ref. 17). Samples of 5 X 10^7 spleen cells were suspended in 2 ml of PKH26-GL diluent and 5 µl of the dye added. The reaction mixture was incubated at room temperature for exactly 90 seconds, with periodic gentle pipetting to assure uniform labelling of the cells; the staining reaction was quenched by 2 washes in excess PBS-medium. The labelled cells were then resuspended in 0.2 ml PBS-medium and administered to the appropriate (naive or V20) recipient mouse by lateral tail vein injection. In order to monitor the distribution of donor cells within the circulation, 20 µl of blood were sampled from the tail at 0, 15 and 120 min post-administration. The red blood cells were lysed in Tris-buffered NH_4Cl and the proportion of $PKH26^+$ lymphocytes determined by flow cytometry. Following the 120 min sampling time, the recipient was anaesthetised and 1 ml of blood extracted (by cardiac puncture) and treated with Tris-NH_4Cl for phenotyping purposes. The axillary LN and spleen were excised and the cells isolated. Blood, LN and spleen lymphocytes were stained either with FITC-conjugated anti-Thy 1.2 or -Ig in order to determine the phenotype of resident (non-PKH^+) and recruited (PKH^+) cells. Dual

fluorescence analysis was carried out by flow cytometry. The data were displayed as a bivariate distribution of PKH^+ versus $FITC^+$ events to calculate the proportions of single and double stained populations.

Isolation and phenotyping of efferent lymph and blood cells

Because the lymphatic drainage from the axillary LN is inaccessible for sampling purposes, mice were immunized to stimulate the LN draining into the cysterna chyla (thoracic duct). In preliminary studies, we identified the nodes which drain directly into the duct: the caudal LN (draining the tail), the lumbar, renal and popliteal LN (draining the hind feet) and the mesenteric LN (draining the intestinal tract). A group of 6 mice was exposed to 250 irradiated cercariae on the tail and 125 cercariae on each hind foot. After 14 days, vaccinated and naive animals received a subcutaneous injection of 0.2 ml Evans' Blue dye into each footpad and 0.2 ml olive oil into the stomach by gavage to permit distinction between output from the LN draining the feet and intestine, respectively. Thirty minutes later, the mice were anaesthetised and the peritoneal cavity opened to expose the cysterna chyla, as described by Korngold & Bennick (18). In order to collect cells only from LN stimulated by vaccination, the lymphatic channels connecting the mesenteric LN to the main duct were ligatured using Mersilk W529 thread (Ethicon Ltd., Edinburgh, UK). Once the cysterna chyla was clear of milky white fluid and the lymph was well stained with Evans' Blue, a small incision was made in the wall of the duct. PBS (0.1 ml) was injected into each footpad and the feet gently massaged to induce flow of lymph; approximately 10 µl of thoracic duct lymph were collected from each mouse with a fine glass capillary and kept on ice until required. Whilst the mice were still under anaesthesia, 200 µl of blood were sampled by cardiac puncture and immediately transferred into 1 ml Tris-buffered NH_4Cl to lyse the red blood cells. The mice were then sacrificed and the caudal, lumbar, renal and popliteal LN excised. Lymph, blood and LN

lymphocytes were diluted in an appropriate volume of PBS-medium and phenotyped for T and B cell markers as described earlier.

To assess the level of resistance induced by the tail / footpad route of immunization, 5 vaccinated and 5 naive mice were challenged with 200 normal cercariae on the abdomen five weeks post-vaccination; the two groups of animals were then perfused five weeks later and R calculated.

Flow cytometry

Stained cell / nuclei suspensions were analysed and sorted on an EPICS C flow cytometer (Coulter Electronics Ltd., Luton, UK) equipped with a 488 nm emission argon-ion laser. Fluorescence emitted by FITC and propidium iodide (PI) was passed through a short-pass 560 nm interference and long-pass 610 nm absorbance filter, respectively. A 570 nm long-pass absorbance filter was used for both PE and PKH26. Each fluorescence signal was amplified logarithmically except for that of PI which was amplified on a linear scale. In all experiments where two-colour analysis was required, electronic correction for spectral overlap of FITC and PE/PKH/PI fluorescence was calibrated by using the appropriate single-stained control samples. For phenotyping experiments, a gated area was created around the live lymphocyte population and the proportion of fluorescence-positive cells was then calculated within this area. Sort gates were designed to include FITC-stained T cells or PE-stained B cells only; an overall purity of >95% was obtained, with <1% contamination from the complimentary sort gate. For cell proliferation studies forward light scatter (FLS), 90^{o} light scatter (90^{o} LS) and FITC fluorescence scatter were gated onto the PI signal to ensure that only particles of nuclear material were analysed.

Statistical analysis

Differences in mean worm burden, cell numbers and cpm were tested for significance using Student's t-test.

RESULTS

In vivo lymphocyte proliferation following exposure to 20 krad-irradiated parasites

Mice were exposed to optimally-irradiated cercariae and at a series of time points thereafter the in vivo proliferative response of the axillary, mediastinal and brachial LN cells was determined. Figure 1 shows bivariate distributions (expressed as contour plots) of DNA content versus BrdUrd content for the axillary (Fig.1a), mediastinal (Fig.1b) and brachial (Fig.1c) LN of V20 mice at days 0 and 14 post-vaccination. In all three LN, the distributions were virtually identical at day 0; by day 14 however, a very marked rise in BrdUrd content (as denoted by the increased green fluorescence signal) was evident in axillary and mediastinal LN cells but not in the brachial LN. A quantitative measure of these changes over the course of immunization is illustrated in Figure 2. A rapid increase in the percentage of $BrdUrd^+$ cells was detected in the axillary LN by 7 days post-vaccination, reaching a peak 3.4-fold increase at day 14 (Fig.2a). By taking into account the cell number in the axillary LN at the time of sampling a 22.2-fold increase in $BrdUrd^+$ cells was observed at the peak (Fig.2b). A large increase in the percentage (Fig.2c) and absolute number (Fig.2d) of cells was also detected in the mediastinal LN but in contrast to the axillary LN, the initial rise was delayed by 7 days. Responses were maximal on days 14 (2.1-fold) and 21 (10.3-fold) post-vaccination in the percentage and absolute number of positive cells, respectively. In both the axillary and mediastinal LN, the proportion and number of $BrdUrd^+$ cells were still elevated at day 31, compared to

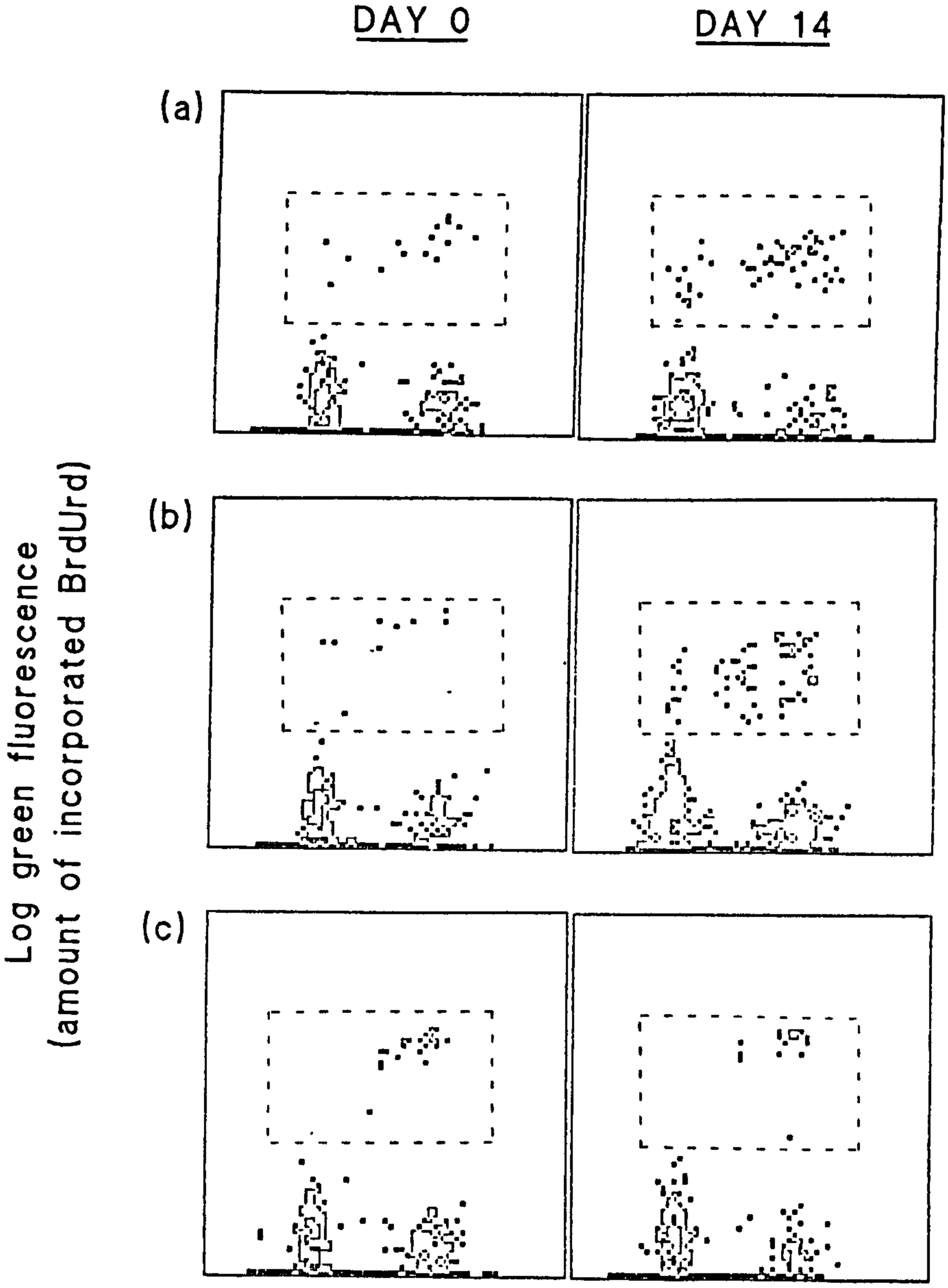

Figure 1. Representative bivariate DNA / BrdUrd distributions of LN cells at day 0 and 14 post-exposure to 20 krad-irradiated cercariae. The events in the rectangle represent the cells in S-phase. (a) Axillary LN; (b) Mediastinal LN; (c) Brachial LN.

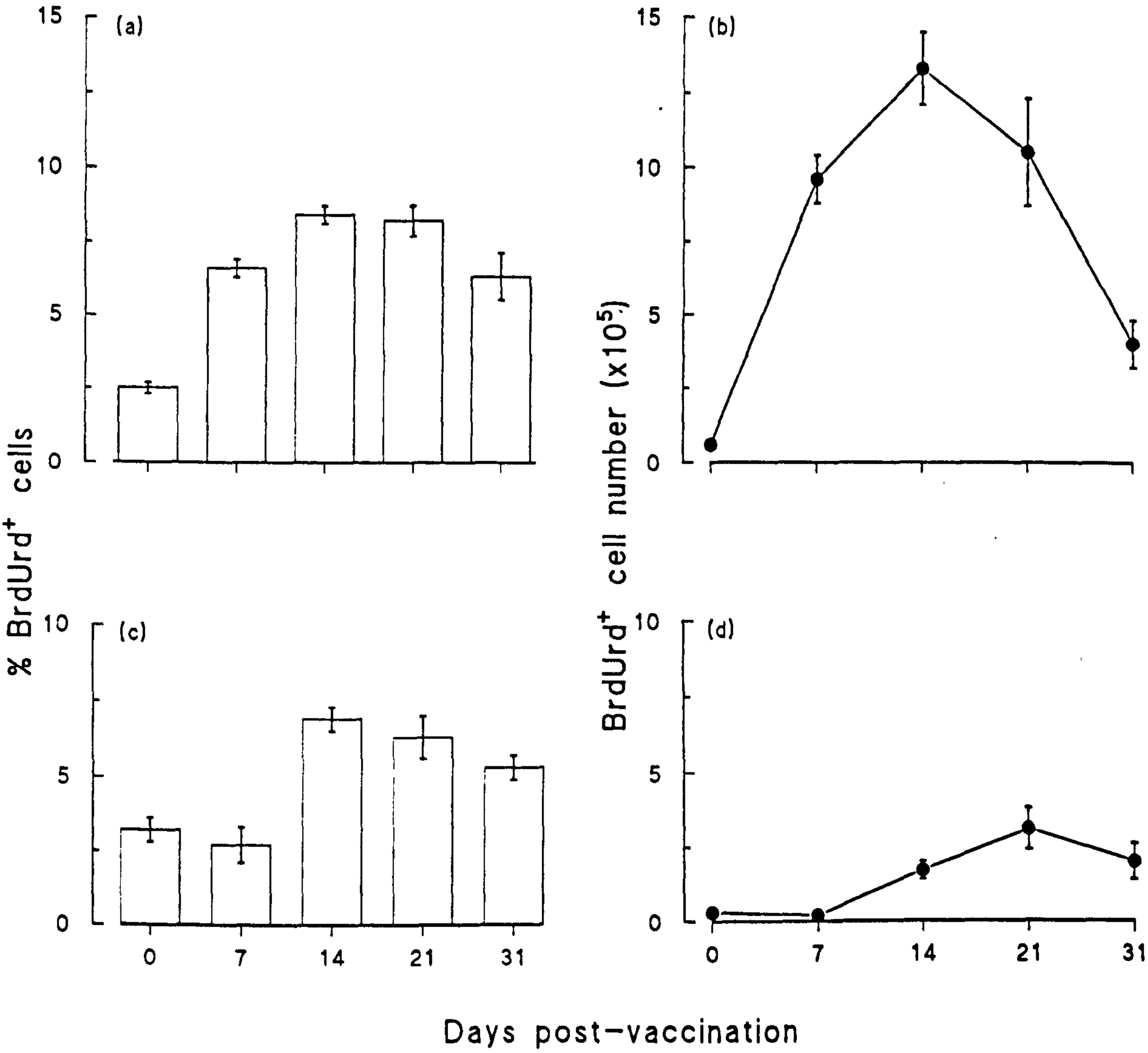

Figure 2. Changes in the proportion and absolute number of $BrdUrd^+$ cells in the LN of mice vaccinated with 20 krad-irradiated cercariae. Axillary LN (a + b); Mediastinal LN (c + d). Values are the mean from 5 mice (± S.E.).

day 0. The proportion of $BrdUrd^+$ cells in the brachial LN remained at baseline levels (1.5%) throughout the experiment (data not shown).

Cross-sectional comparison of lymphocyte proliferation in mice exposed to immunizing versus non-immunizing parasites

To relate LN cell proliferation to the induction of protective immunity, a cross-sectional experiment was performed, comparing V20 responses with those following non-immunizing regimes (V80 and normal infection, NI), with sampling of axillary LN at day 14 and mediastinal LN at day 21 post-exposure. Although a marked increase in $BrdUrd^+$ cell number was observed in the axillary LN of all three groups (Fig.3a), the change in V80 and NI mice was significantly smaller relative to that in V20 animals ($P<0.05$ and $P<0.001$, respectively). In the mediastinal LN (Fig.3b) an increase in $BrdUrd^+$ cells was recorded in V20 and NI mice only, with no statistical difference between the two ($P>0.05$).

The mean worm burden in the challenge control mice in this experiment was 68 ± 2.9 compared with 23 ± 4.9 in V20 mice ($P<0.001$) and 66.2 ± 9.6 in V80 mice ($P>0.05$). This resulted in resistance of 66.1% and 2.6% in the V20 and V80 groups, respectively.

Phenotype of proliferating cells

In order to characterize further the proliferative responses observed in V20 mice, axillary and mediastinal LN cells were phenotyped for T and B lymphocyte markers and the BrdUrd content of the two subsets determined separately. Consistently, we observed that the proportion of B cells proliferating in unstimulated LN was significantly greater ($P<0.01$) than that of T cells (2.6% versus 1.2% in the axillary LN; 3.7% versus 2.0% in the mediastinal LN). Therefore, to compare directly the changes in T and B cell proliferation, the data obtained at each time point post-vaccination has been displayed as a

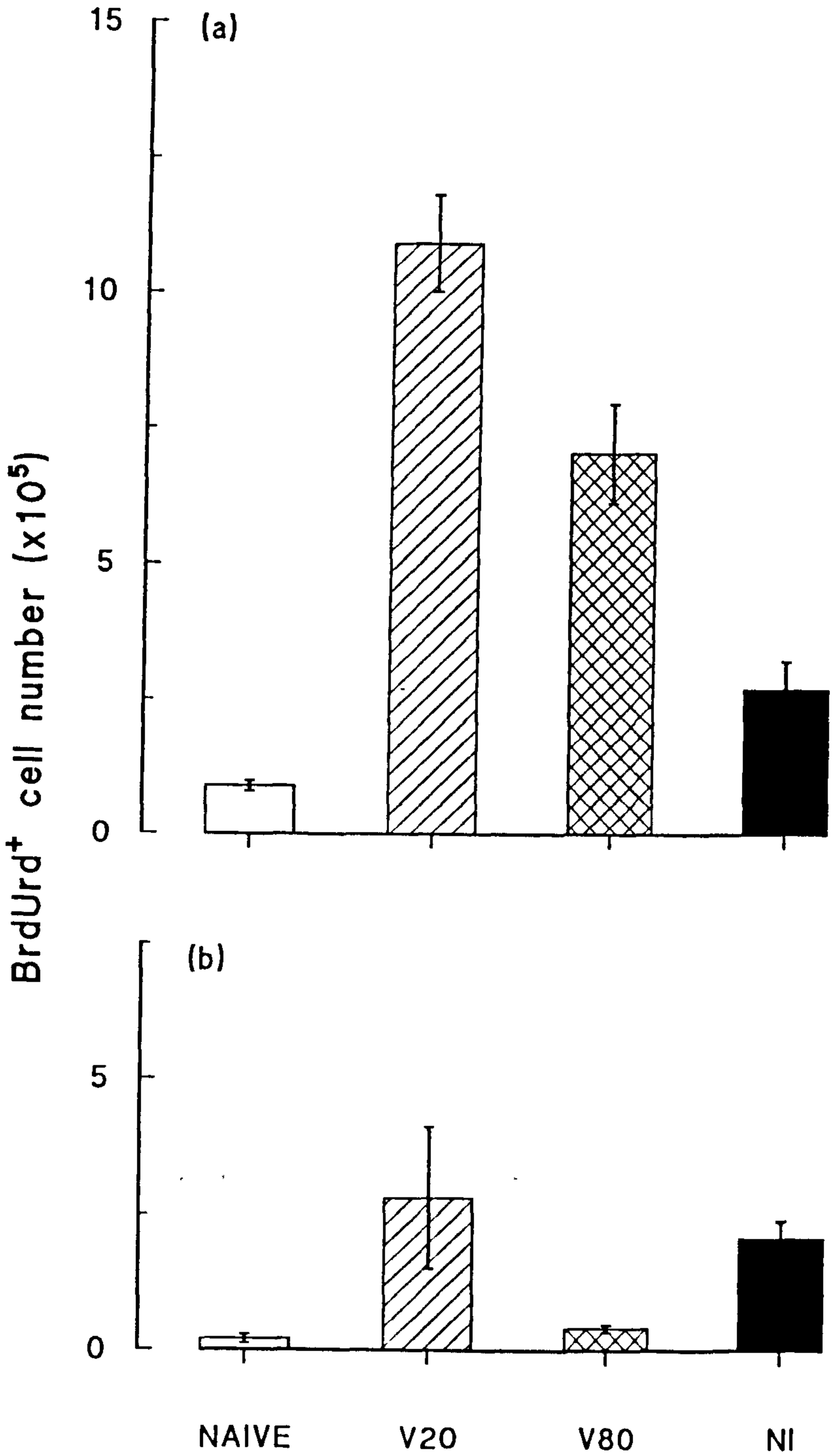

Figure 3. Comparison of BrdUrd$^+$ cell number in the LN of naive, protected (V20) and non-protected (V80 and NI) mice. (a) Axillary LN at day 14 post-exposure; (b) Mediastinal LN at day 21 post-exposure. Values are the mean of 5 mice (± S.E.).

fold-increase over respective baseline values (Fig.4). The salient feature observed in both the axillary (Fig.4a) and mediastinal (Fig.4b) LN was the preferential increase in the proportion of proliferating T lymphocytes. In the axillary LN the peak increase in $BrdUrd^+$ T cells was 4.2-fold whereas that of $BrdUrd^+$ B cells was only 1.6-fold. By day 21 the proportion of proliferating B cells had returned virtually to baseline values but $BrdUrd^+$ T cells persisted at elevated levels to day 35. Changes in $BrdUrd^+$ B cells in the mediastinal LN were negligible throughout the experiment. In contrast, there was a marked increase in the proportion of proliferating T cells after day 7, with a peak 3.0-fold change at day 35.

Duration of DNA synthesis in T and B lymphocytes

Axillary LN T and B cells showed a Ts of 3.6 and 4.0 hours, respectively (Table 1), but these values were not significantly different from each other ($P>0.05$).

Hyperaemia stimulated by immunizing parasites

The administration of microspheres to the systemic circulation was highly reproducible throughout the experiment, as found by the consistent localisation of approximately 15% of the label in the kidneys (Fig.5a). Following exposure to irradiated parasites there was a sharp increase in the proportion of microspheres lodging in the skin-vaccination site (Fig.5b) with 2.8% total cpm / g tissue present at day 4. From then on, the level of label recorded in that site decreased gradually, returning to the baseline by day 20. Changes in blood flow to the LN mirrored closely the changes in organ weight ($r=0.70$ and 0.84 for axillary and mediastinal LN, respectively). A 2.9-fold increase in cpm was observed in the skin-draining LN by day 4 (Fig.5c), reaching a peak 3.9-fold increase (0.6% total cpm) at day 16. A high level of hyperaemia persisted in these LN until day 24. The initial rise in cpm in the mediastinal LN (Fig.5d) was delayed to day 16

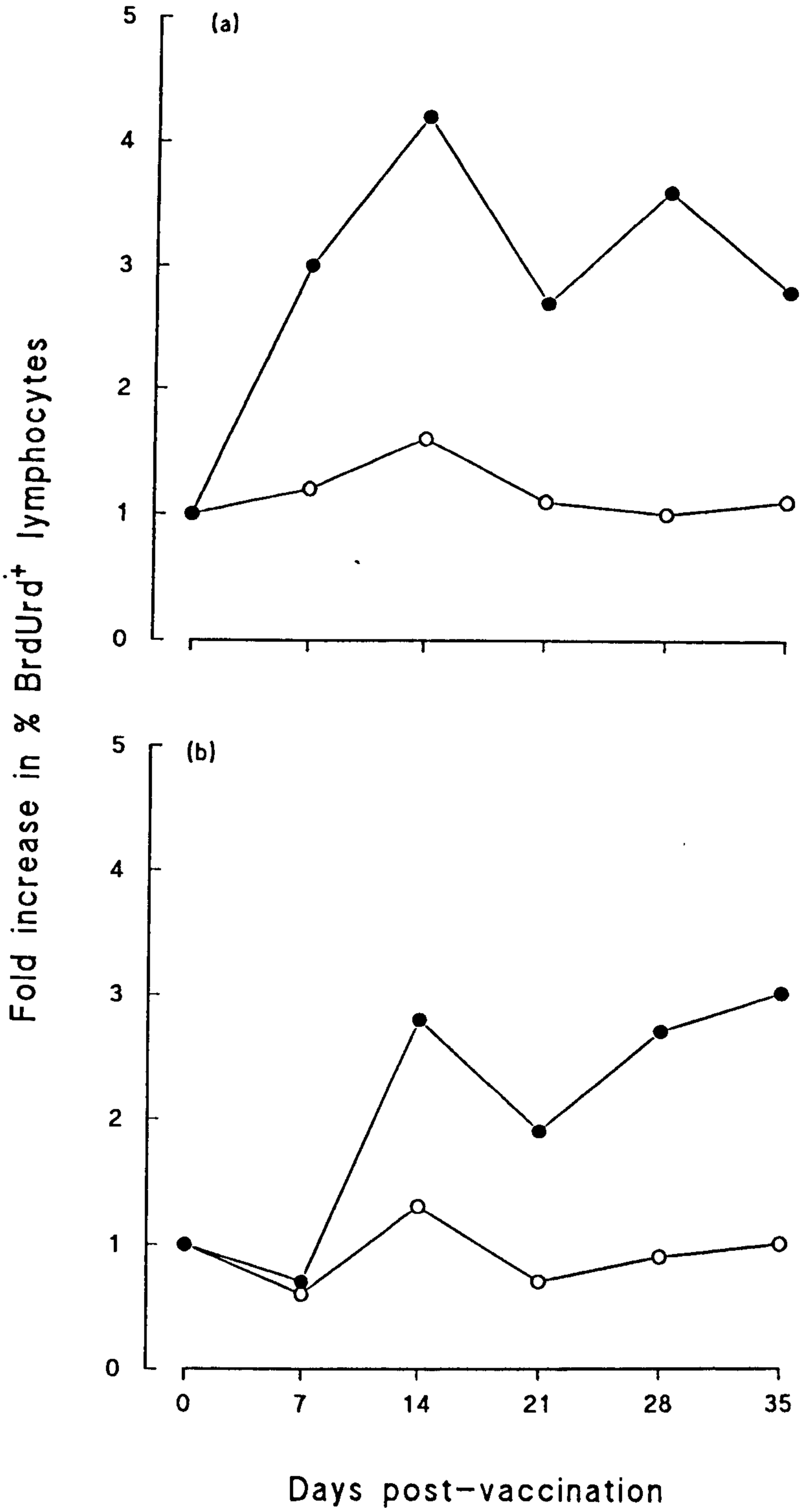

Figure 4: Changes in the proportion of BrdUrd$^+$ T lymphocytes (●——●) and B lymphocytes (○——○) in the LN of mice vaccinated with 20 krad-irradiated cercariae. The data is displayed as a fold-increase over respective baseline values. (a) Axillary LN; (b) Mediastinal LN.

Table 1. Measurement of lymphocyte cycle kinetics

Cells	RM [a]	Ts (h) [b]
Axillary LN total	0.89 + 0.03	3.8
Axillary LN T cells	0.92 ± 0.06	3.6
Axillary LN B cells	0.87 ± 0.01	4.0

(a) Relative movement. Values are the mean from 3 samples (± S.E.)

(b) DNA synthesis time

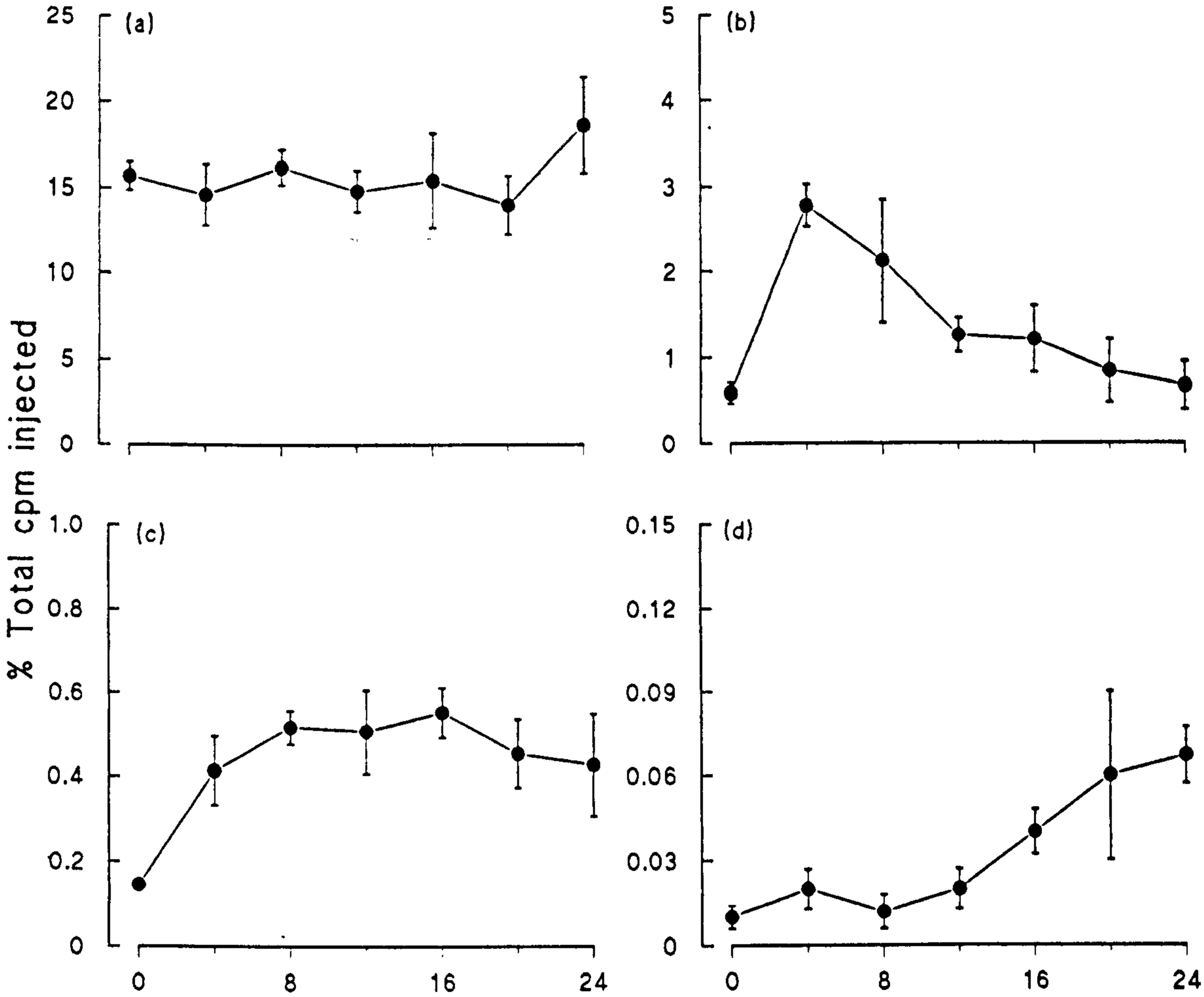

Figure 5: Changes in the proportion of ^{57}Co-labelled microspheres lodging in the organs of mice vaccinated with 20 krad-irradiated cercariae. (a) kidneys; (b) skin (per gram of tissue); (c) skin-draining LN (axillary + inguinal); (d) mediastinal LN. Values are the mean of 5 mice (± S.E.).

(4.0-fold), but levels continued increasing to day 24 (6.7-fold). No significant changes in cpm were observed in the organs of naive animals throughout the experiment (data not shown).

Differential recruitment of T and B cells

The sampling of blood at 0 and 15 min post-administration of labelled cells revealed a sharp decrease in the proportion of circulating PKH^+ cells in both naive and V20 recipients (data not shown). After 120 min, donor cells composed 3.5 - 4.5% of the total lymphocyte population in the blood and had a T:B cell ratio of 0.7:1. A greater influx of B versus T cells was recorded in the spleen of both naive and V20 mice, with phenotypes being recruited in a ratio almost identical to that of the resident population (data not shown). The results from the axillary LN are plotted in Figure 6 and are expressed as absolute numbers of cells. Both resident PKH^- (Fig.6a) and recruited PKH^+ (Fig.6b) T and B cells were present in significantly higher numbers following vaccination. The number of resident B cells had expanded such that they outnumbered the T cells (0.9:1, T:B cells). However, this was not matched by the nodes recruiting proportionally more B cells. No significant change ($P>0.05$) in the T:B cell ratio of lymphocytes recruited to the nodes was observed, despite an overall increase in the total number of cells. Thus there was still a preferential influx of T lymphocytes into the LN.

Phenotypic composition of blood, LN and efferent lymphocytes after vaccination

Using the change in LN weight as an index of stimulation, only the popliteal nodes showed a significant increase ($P<0.001$) after vaccination via the tail and hind feet (data not shown); therefore cellular events in the popliteal LN were taken to represent responses analogous to those observed in the axillary LN following a conventional

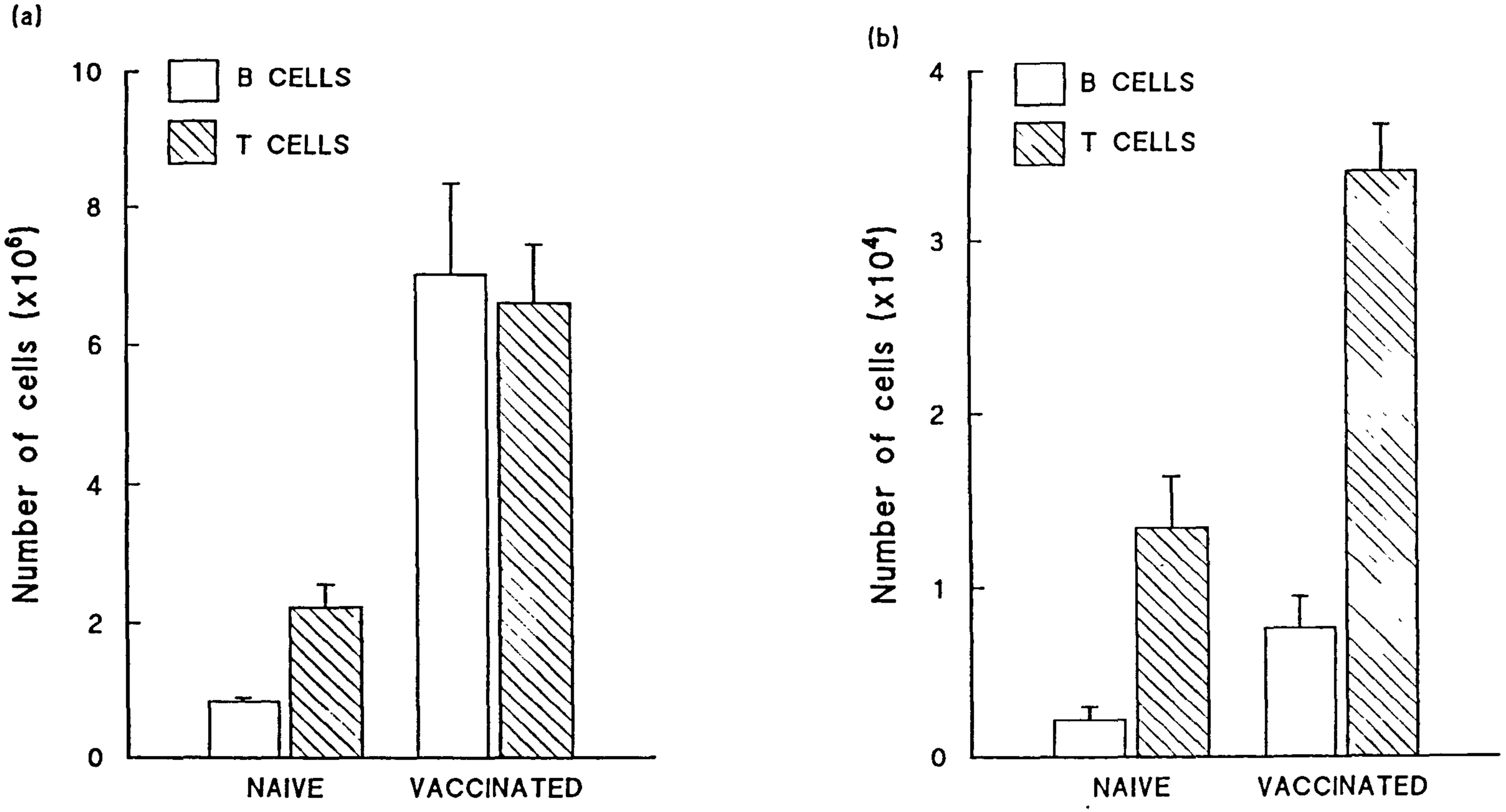

Figure 6: Comparison of the number of (a) resident (PKH^-) versus (b) recruited (PKH^+) T and B lymphocytes in the LN of naive and immunized mice. Values are the mean of 5 mice ($\pm$ S.E.).

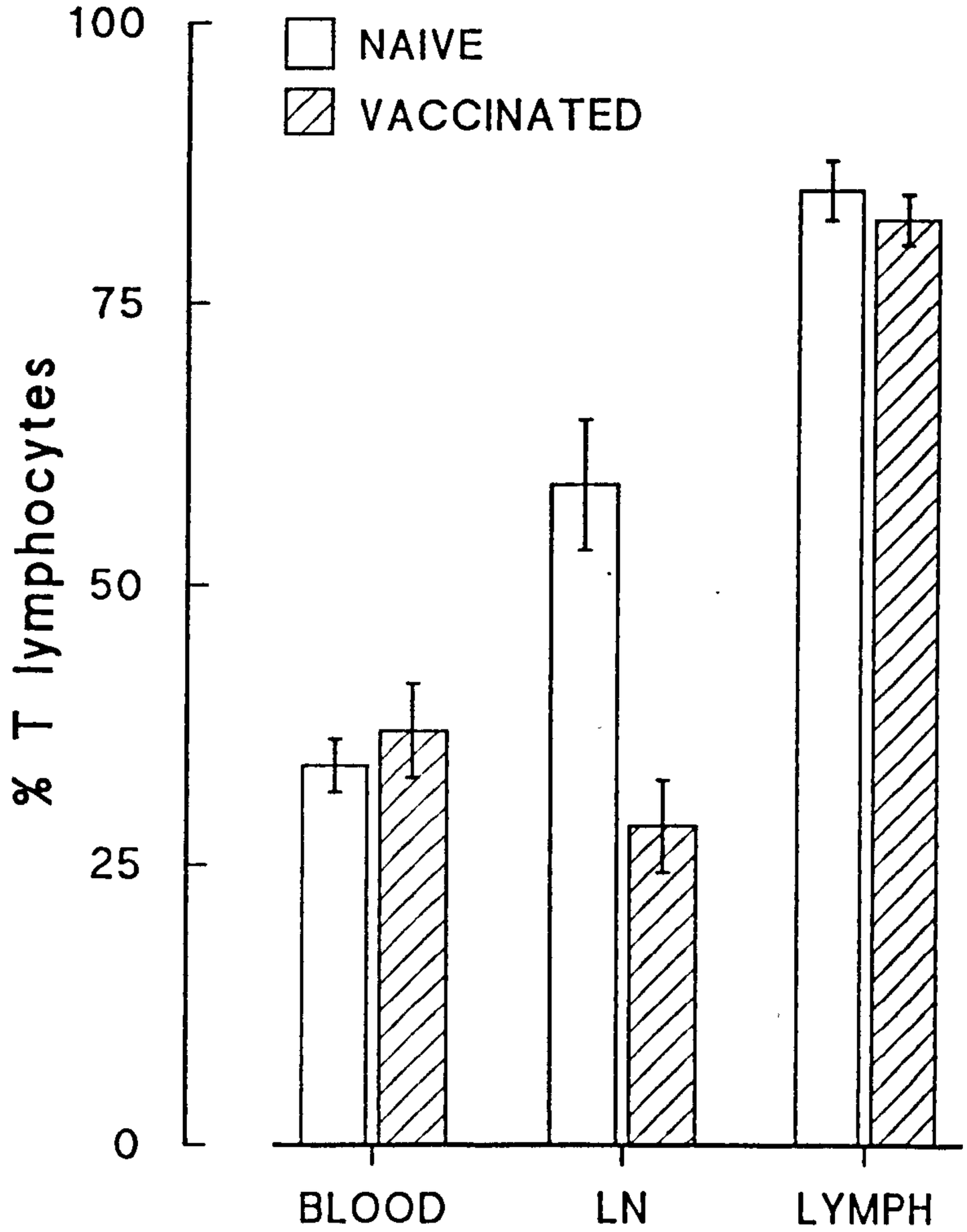

Figure 7: Comparison of the proportion of T lymphocytes in the blood, popliteal LN and thoracic duct lymph in naive and vaccinated mice. Values are the mean of 5 mice (± S.E.).

abdominal immunization. In Figure 7, the proportion of T lymphocytes in the blood, popliteal LN and thoracic duct lymph of naive and 14-day vaccinated animals have been compared. No significant differences between control and V20 animals were recorded in either the blood (Fig.7a) or lymph (Fig.7c). The percentage of T cells in the LN (Fig.7b) however, decreased from 58.8% to 28.4% ($P<0.01$) post-vaccination.

Resistance measurements

R Values for all experiments (excluding the cross-sectional comparison) ranged from 48.5 - 78.9% (n=6), giving a mean resistance of 63.7%.

DISCUSSION

In previous studies we reported a significant correlation between changes in the total lymphocyte number in draining LN and the induction of protective immunity to *S. mansoni* in mice (2). In an attempt to define more precisely the cellular events required for successful vaccination, we have investigated several interacting components in the draining LN. We first examined the *in vivo* proliferative responses of LN cells, using the BrdUrd incorporation technique. This has been used successfully by other workers to label very small numbers of cells in S-phase, particularly in lymphoid organs (19, 20). It allowed us to detect proliferation levels as low as 1.5% positive cells. Both the axillary and mediastinal LN of V20 mice showed a significant increase in the proportion and absolute number of proliferating cells. As in our previous studies (2) the responses were closely correlated with the kinetics of parasite migration, events in the mediastinal LN being delayed by 7 days. A prominent feature of the data was the sustained pattern of proliferation; $BrdUrd^+$ cells were still above baseline levels at day 35 post-vaccination. This presumably reflects the persistence of parasites and

the prolonged release of parasite antigen into the regional LN (1). When we compared the proliferative responses in V20 mice with those generated by non-protective regimes (ie. in V80 and NI mice), we found significant differences, particularly in the skin-draining LN. We know from autoradiographic tracking data that normal parasites spend only a very transient period in the axillary LN (1) which would account for the small number of $BrdUrd^+$ cells present at day 14 post-exposure. In the case of V80 mice, most parasites fail to migrate from the skin (2) and this was reflected by a smaller proliferative response than that observed in V20 animals. This truncated migration also explains the negligible change in $BrdUrd^+$ cells in the mediastinal LN of the V80 group. An almost identical number of proliferating cells in the lung-draining LN of V20 and NI mice reinforces our earlier suggestion that events in the axillary LN, rather than in the mediastinal node, correlate best with the level of immunity induced (2). Furthermore the failure of 80 krad-irradiated cercariae to elicit significant levels of immunity substantiates the idea that persistence of the parasites in the skin-draining LN is vital to vaccination success; release of antigen in the skin-exposure site alone is obviously not sufficient to generate intense and prolonged proliferation of the relevant cells.

In our previous work we reported a proportionally greater increase in B cells compared to T cells in both the axillary and mediastinal LN of V20 mice (2). This is somewhat paradoxical in the light of the proposed delayed-type hypersensivity (DTH) effector mechanism operating in this model of immunity (5-7, 21). We therefore wanted to establish the reason for the change in T:B cell ratio and the prominent accumulation of B cells in the draining LN of V20 mice. Using <u>in vivo</u> BrdUrd incorporation we examined the proliferation patterns of T and B lymphocytes separately, to look for differences between the two subsets. The most striking feature of the results was the preferential increase in the proportion of proliferating T, relative to B, cells. Moreover the T lymphocytes showed a

slightly shorter Ts than the B lymphocytes, although there was no statistical difference between the two values. These results lead us to conclude that the significant increase in B cell numbers in the draining LN cannot be accounted for by the preferential stimulation of that subset. However, the prominent in vivo stimulation of the T cell class does support the hypothesis that events in the draining LN are crucial for the generation of schistosome-specific T lymphocytes. Confirmation for the accumulation of specific T cells in the draining LN is obtained from in vitro assays of antigen-stimulated blastogenesis. Schistosome-reactive cells are present in both the skin- and lung-draining LN of V20 mice (22, 23). However, if these cells are treated with anti-Thy1 antibody prior to exposure to antigen, the observed blastogenic responses are almost completely ablated (23). Furthermore, in vivo depletion studies support the idea that it is the $CD4^+$ subset of schistosome-specific T cells which must be generated in order for the DTH effector mechanism to operate successfully against the challenge parasites (24, 25). Ideally, we would have liked to compare the in vivo proliferative responses of $CD4^+$ versus $CD8^+$ lymphocytes in the LN. However, this was not feasible using our cell sorting approach. Recently, Bayer et al (26) have developed a new method for the simultaneous detection of BrdUrd and lymphocyte markers in murine bone marrow cells, which may provide us with a means of examining the proliferation of LN T cell subsets in future studies.

Since the prominent change in T:B cell ratio in the LN of V20 mice could not be explained in terms of a preferential stimulation of B cells, we examined several components of lymphocyte migration. In particular, we wanted to assess the relative contributions of cellular influx and efflux to the overall changes in cell numbers observed in the draining LN. These components have been extensively studied in the sheep (27), rabbit (28, 29) and rat (8), yet very little is known of the events in the nodes of mice. We first investigated the phenomenon of hyperaemia in the draining LN, as this is closely associated with a non-

specific influx of cells from the peripheral circulation (8, 30). In our measurements of fractional distribution of cardiac output we found that approximately 15% of total radio-labelled microspheres was distributed to the kidneys (used as our reference organ) which agrees well with the figure obtained by Ottaway & Parrott (30). Following exposure to attenuated parasites an increase in blood flow was detected at the skin-vaccination site. This is not surprising since Mastin et al. (31) have shown that penetration of murine skin by cercariae leads to the development of marked inflammatory lesions. The close association between the kinetics of dermal inflammation and changes in blood flow has already been established in several models of skin sensitization (15, 32). Significant levels of hyperaemia were also detected in the skin- and lung-draining LN of V20 animals but, in contrast to the skin-vaccination site, responses in the nodes were prolonged. The kinetics of these responses correlate very well with the pattern of changes in cell numbers observed in the axillary and mediastinal LN (2). Although we were unable to compare directly the changes in hyperaemia with those in cell number, we used LN weight as an index of the latter. We have previously found (unpublished observations) that variations in V20 LN weight mirror very closely the changes in cellularity ($r=0.82$ and 0.95 for skin- and lung-draining LN, respectively). Thus, the marked correlation between LN weight and hyperaemia leads us to conclude that a significant part of the observed accumulation of cells in the draining LN must be due to enhanced recruitment.

Hall & Morris (33) calculated that approximately 90% of the lymphocytes emerging from LN are originally recruited from the peripheral circulation. However, lymphocyte migration into specific organs is not random. Entry into lymphoid organs such as LN and Peyer's patches is directly controlled by the interaction of lymphocytes with high endothelial venules, HEV (34). Furthermore, the binding affinity of LN HEV for recirculating lymphocytes can be significantly enhanced by antigenic stimulation (10) or in

vivo administration of particular cytokines (35). We examined the influence of exposure to 20 krad-irradiated cercariae on the influx of T and B lymphocytes into the draining LN. We tracked lymphocytes in vivo using the fluorescent lipophilic dye, PKH26; this yielded highly reproducible data. In naive mice, T and B lymphocytes were recruited to the spleen and draining LN in the same proportions as those already present in the organs. We observed a preferential infiltration of B cells in the spleen and T cells in the nodes, confirming that the recirculation potential of neither subset was harmed by the labelling procedure. Such organ-selective localization has been reported by other workers (10, 36). Following vaccination, the ratio of T:B cells recruited into the axillary LN remained unchanged, despite a significant increase in the proportion of resident B lymphocytes. This suggests that HEV affinity, but not specificity, is altered by the immunising parasites. Thus, the marked increase in the number of B lymphocytes in the draining LN cannot be due to a preferential influx of that subset.

We also compared the proportions of T and B cells in the peripheral blood and draining LN with those in the efferent lymph of naive and V20 mice. An unconventional vaccination protocol had to be used for this particular experiment, but the level of protection induced was not significantly different from that of a conventional V20 protocol carried out in parallel. We found no difference in the percentage of T cells in the efferent lymph of vaccinated compared to naive mice. However, the proportion of LN T cells showed a decrease of 50% in V20, relative to naive, animals. We might expect a change in the ratio of T:B cells in the LN to be reflected by a change in the efferent lymph. This is clearly not the case. It could be argued that only the popliteal LN responded after vaccination, therefore minor changes in the ratio of T:B cells in the thoracic duct would be difficult to detect amongst the substantial output of cells from other LN. However, using LN weight as an index of the potential output of cells from each node, the percentage contribution of the popliteal LN to the thoracic

lymph pool increased from 24.9% to 46.7% after vaccination, so any changes in lymph T cells should be well within our detection range. As reported in our earlier studies (2), there is a proportionally greater increase in the number of B cells compared to T cells in the LN of vaccinated mice. On the basis of the results obtained here, this phenomenon is not due to the preferential influx or proliferation of the B lymphocyte subset. However, the lack of any noticeable increase in B cell numbers in the efferent lymph suggests that vaccination leads to a significant change in the retention patterns of T and B cells in the draining LN. The majority of B cells, originally recruited during hyperaemia, are failing to exit the LN. A similar conclusion was reached by Kraal & Twisk (10) when they compared unstimulated with oxazolone-primed peripheral LN; B cells were retained specifically in the stimulated nodes.

The present study has enabled us to gain insight into some of the dynamic components contributing to changes in the stimulated draining LN of mice. How do these components relate to the sequence of events leading to the induction of protective immunity to S. mansoni? We suggest that the persistence of attenuated parasites and sustained release of antigen in the skin-draining LN of V20 mice results in a marked hyperaemia, which in turn leads to a prolonged non-specific influx of cells into the node. Once in the node, schistosome-reactive cells will proliferate in response to the parasite antigen; as judged by the present data, most of these cells are T lymphocytes. After stimulation, a significant proportion of these T cells will leave the node either as memory or effector cells. In contrast to T lymphocytes, there is no enhanced proliferation of B cells in the LN. Furthermore, a significant proportion of the B cells fail to exit the LN, leading to their prominent accumulation. Why they are retained at that site remains to be explained. A predominant increase in the absolute number of B cells within regional LN has been reported in other models of immunity where T cells are known to be the key in vivo effector (37, 38). Of course, we cannot exclude the

possibility that some of these B cells play a part in the priming of the host, for example via their capacity as antigen-presenting cells for T-helper ($CD4^+$) lymphocytes (39). In fact, Janeway et al. (40) have suggested that B lymphocytes are the main type of antigen-presenting cell in peripheral LN. Furthermore, Bottomly & Janeway (41) have proposed that B cells preferentially present antigen to DTH-inducing cells. This is of particular interest to us as we are currently investigating the functional role of the $CD4^+$ population sensitised in the draining LN of V20 mice. By examining the profile of cytokines produced during the induction (23) and effector (Smythies et al., submitted for publication) phases of immunity, we have found a strong correlation between the levels of δ-IFN and the elicitation of protection. A similar association has recently been reported by other workers (42). However, the essential question remains how does processing and presentation of schistosome-released antigen in the nodes give rise to a preferential priming of δ-IFN producing T cells? Factors such as the type of antigen-presenting cell involved and the cytokine environment that is present during the primary response are likely to be important influences on the type of T cell that will be generated. We are currently investigating some of these components in order to characterize and eventually recreate the appropriate immunological environment for successful vaccination.

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CHAPTER 4

The detection of cytokine mRNA in the skin-draining lymph nodes of mice vaccinated with attenuated *Schistosoma mansoni*.

1.0 Introduction

In the previous two chapters, it was established that vaccination of mice with attenuated larvae of S. mansoni results in significant changes in cellularity in the LN draining the exposure site. In particular, there is a preferential proliferation of T lymphocytes at that site, which is believed to generate the anti-schistosome DTH-effector cells needed for the elimination of a subsequent challenge infection. The importance of $CD4^+$ T cells for the induction of protection in this model of immunity has already been discussed in detail in Chapter 1. However, with the division of $CD4^+$ lymphocytes into TH1 and TH2 classes (Mosmann & Coffman 1987) an extra dimension has been added to the study of immune responses in general. The original classification was based on the profile of cytokine production by long-term murine T helper cell clones. Using various bioassays, it was revealed that TH1 cells produce IFN-δ , lymphotoxin and IL-2, and are capable of transferring a DTH response (Cher & Mosmann 1987). In contrast, the TH2 class secretes IL-4 and IL-5, and is thought to be particularly important for B cell growth and development (Killar et al. 1987).

It has since been shown that in vivo derived $CD4^+$ T lymphocytes also demonstrate differential expression of cytokines (reviewed by Janeway et al. 1988). However, the subdivision of these cells may not be as clear-cut as that observed with clones (Kelso & Gough 1988; Erb et al. 1991). Furthermore, there is little data available concerning the pattern of cytokine production during in vivo immune stimulation, especially in response to infectious agents. The study of a T cell functional dichotomy is of paramount importance for the understanding of host / disease interactions, as the ability to direct a particular immune response towards humoral or cell-mediated immunity is likely to be a critical aspect for the development of vaccines.

Cells isolated from the draining LN of S. mansoni-vaccinated mice release significant levels of both IFN-γ and IL-2 when restimulated in vitro with schistosome antigens (Pemberton et al. 1991). On this basis, it was suggested that the lymphocytes generated during the induction phase of immunity can be categorized as belonging to the TH1 subset. However, having to use an in vitro system to study the supposed cellular reactions taking place within the host is not ideal and may not be a true representation of the in vivo situation. The in vitro restimulation of cells can often lead to the introduction of immunological artefacts, particularly if the antigens used are not analogous to the full range presented to the cells in vivo.

Therefore, in order to study cytokine production in vivo, it was decided to use the expression of mRNA in the cells of attenuated S. mansoni-stimulated axillary LN, as a marker for synthesis of the proteins during immunization. Of course the presence of mRNA within a cell does not prove that the corresponding protein is synthesized and secreted, but work on cytokine production from other laboratories supports the validity of the RNA procedure (Cherwinski et al. 1987). A similar study has already been carried out in Leishmania-infected mice (Heinzel et al. 1989), using the expression of IFN-γ and IL-4 mRNA as markers for TH1 and TH2 cells, respectively. Whereas the LN of resistant C57BL/6 mice contained high levels of mRNA coding for IFN-γ and none for IL-4, the inverse was true for the susceptible BALB/c strain, suggesting a TH1 / TH2 heterogeneity was in operation. With the understanding of the immunological dichotomy between the two strains, the investigators have since been able to confer protection to usually susceptible animals (Sadick et al. 1990).

The experimental work in this chapter has been divided into two parts. The first describes the preliminary work carried out in order to establish the optimal conditions for the detection of murine cytokine mRNA by Northern hybridization, since no molecular biology

techniques were available in the laboratory at the time. The second part focuses on the attempts made to detect cytokine message for IFN-ϒ , IL-2, IL-4 and IL-5 in the axillary LN of vaccinated mice.

2.0 Materials & methods

2.1 Cell lines and culture conditions

Three variants of the X63Ag8-653 (X6310) myeloma cell line transfected with cDNA encoding murine IL-2, 4, or 5 (Karasuyama & Melchers 1988) were obtained from Dr F. Melchers, Basel Institute for Immunology, Basel, Switzerland. The cells were cultured in RPMI-1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% foetal calf serum (FCS; Gibco BRL, Paisley, Scotland), 2mM L-glutamine, 200 U/ml penicillin, 100 µg/ml streptomycin and 5 X 10^{-5}M 2-mercaptoethanol. To ensure that the transfectant phenotype was maintained, 1 mg/ml of the selection drug G418 (Geneticin; Gibco BRL) was occasionally added to the medium.

The CHO-211A cell line (a Chinese Hamster Ovary cell line transfected with cDNA encoding murine IFN-ϒ ; Morris & Ward 1987) was obtained from Dr A. Morris, Warwick University, Warwick, UK. The cells were cultured in GMEM medium (Flow Laboratories) containing 10% FCS, 200 U/ml penicillin and 100 µg/ml streptomycin. Since this line grows as a monolayer, the cells were harvested from culture flasks using 0.25% trypsin (Sigma, Poole, UK).

Cells harvested from the different cultures were washed twice in diethylpyrocarbonate (DEPC)-treated phosphate-buffered saline (PBS) pH 7.2 and either used immediately or frozen in liquid nitrogen and stored at -80^{o}C until needed.

2.2 Parasites and mice

A Puerto Rican strain of S. mansoni was maintained by passage through LACA mice and Biomphalaria glabrata. Cercariae were attenuated by exposure to 20 krad. of gamma radiation from a ^{60}Co source (Department of Radiobiology, Cookridge Hospital, Leeds). Male and female C57Bl/6 mice, weighing 18-25g, were used throughout the study. The mice were anaesthetised with 10% Sagatal (May & Baker, Dagenham, UK) in 10% ethanol (0.01 ml/g body weight) and exposed to 500 cercariae via the shaved abdomen (Smithers & Terry 1965).

Groups of mice were sacrificed on days 0, 7, 14 and 21 post-vaccination. The left and right axillary LN (draining the abdominal exposure site) were excised from each mouse, using DEPC-treated forceps, and immediately transferred into guanidium isothyocyanate homogenization buffer (see 2.3b).

N.B. All procedures involving RNA were carried out using DEPC-treated reagents and glassware in order to avoid RNAse activity.

2.3 Isolation of total RNA

RNA from cultured cells and LN cells was isolated using different methods (see 3.1).

a) Cell lines - A solution of freshly prepared 3M LiCl, 6M Urea, 10mM NaAc pH 5.0 was added to a pellet of approximately 1 X 10^7 cells. The cell suspension was homogenized on ice using an Ystral shearer (Fryma Ltd., Hemel Hempstead, UK) at full speed for 2 mins, and the RNA allowed to precipitate from the homogenate by overnight incubation at 4^oC. The homogenate was then centrifuged at 20 000g for 30 mins at 4^oC and the resulting RNA pellet redissolved in SE (0.5% SDS, 10mM EDTA pH 8.0) containing 200 µg/ml Proteinase K (Gibco BRL). The mixture was incubated for 1 hour at 37^oC, followed by a

phenol:chloroform: isoamylalcohol extraction step to remove the enzyme. The RNA was finally recovered by overnight NaAc:ethanol precipitation at 0°C and resuspended in 400 µl DEPC-treated water. The yield and quality of the RNA extracted from each batch of cells was determined by E_{260} spectrophotometry and gel electrophoresis, respectively. Samples were stored at -20°C until required.

b) LN cells - Freshly isolated LN were homogenized in guanidium isothyocyanate homogenization buffer (4.0M guanidium isothyocyanate, 0.1M Tris.Cl pH 7.5, 1% 2-mercaptoethanol) using an Ystral shearer. Sodium lauryl sarcosinate was then added to a final concentration of 0.5% and the resulting suspension spun at 5000g for 10 mins at room temperature to precipitate LN capsule debris. The supernatant from the spin was layered onto a cushion of 5.7M CsCl, 0.01M EDTA pH 7.5 and centrifuged in an SW41 swing-out rotor at 32 000 rpm for 24 hours, 20°C. Most of the mixture above the resulting RNA pellet was carefully drawn off with a pasteur pipette and the bottom of the centrifuge tube (containing the RNA) cut just above the level of the remaining fluid using a red-hot razor blade. The RNA pellet was washed once in 70% ethanol and then left to dissolve overnight in TES (10mM Tris.Cl pH 7.5, 1mM EDTA pH 8.0, 0.1% SDS) at 4°C on a shaking platform. The resulting solution of RNA was extracted once with phenol: chloroform:isoamylalcohol and twice with chloroform: isoamylacohol, followed by overnight NaAc:ethanol precipitation at 0°C. RNA pellets were resuspended in 200 µl DEPC-treated water and the yield and quality of each estimated as already described.

2.4 Poly A^+ RNA selection

Oligo (dT)-cellulose columns were prepared using sterile 1 ml Gilson tips plugged with DEPC-treated glass wool. Oligo (dT)-cellulose (Pharmacia Ltd, Milton Keynes, UK) was suspended in 0.1M NaOH and poured to produce 200 µl volume columns. These were washed in 1x column-loading

buffer (50mM Sodium citrate pH 7.6, 0.5M NaCl, 1mM EDTA pH 8.0, 0.1% sodium lauryl sarcosinate) until the pH of the effluent was less than 8.0. Samples of total RNA were heated to 65^{o}C for 5 mins, cooled, and diluted with an equal volume of 2x column-loading buffer. Each solution was then applied to a prepared column and the eluate collected, heated to 65^{o} for 5 mins, and reapplied. The columns were then washed through with several volumes of 1x column-loading buffer, to remove non-polyadenylated RNA (as monitored by 260nm absorbance readings on a spectrophotometer). Bound poly A^{+} RNA was eluted from the oligo (dT)-cellulose with 2-3 column volumes of sterile elution buffer (10mM Tris.Cl pH 7.6, 1mM EDTA pH 8.0, 0.05% SDS) and 100 µl fractions collected. The 260nm absorbance of each fraction was measured and those containing RNA pooled. The mRNA was precipitated by overnight incubation in NaAc:ethanol at 0^{o}C and redissolved in 40-100 µl DEPC-treated water. The degree of enrichment for poly A^{+} RNA was checked by comparing an aliquot with samples of total RNA by gel electrophoresis.

2.5 Formaldehyde gel electrophoresis and Northern blotting

Formaldehyde gels (1.2%) were obtained by adding 70 ml dissolved agarose to a mixture of 10 ml 10x MOPS (200mM morpholinopropanesulfonic acid, 50mM NaAc pH 5.0, 1mM EDTA pH 7.0) and 18 ml 37% formaldehyde pH >4.0, at 60^{o}C. RNA samples (0.5-10 µg) were prepared as 25 µl aliquots, containing 50% deionized formamide, 1x MOPS, 2.2M formaldehyde and 1x loading buffer (3% glycerol, 10mM EDTA pH 8.0, 0.1% SDS, 0.025% bromophenol blue). The samples were heated to 65^{o}C for 10 mins, chilled on ice, and then loaded onto the gel. For each gel, a sample of RNA molecular-weight markers (Gibco BRL) was also loaded, in one of the outer lanes. Gels were run submerged in 1x MOPS at 25V, overnight, 4^{o}C. At the end of the run, the gels were washed 3 X 5 mins in DEPC-treated water. The lane containing the markers was cut off, stained with ethidium bromide (0.5 µg/ml in 0.1M ammonium acetate), and

photographed alongside a ruler. The remainder of each gel was partially hydrolyzed by soaking in excess 50mM NaOH, 10mM NaCl for 45 mins at room temperature, and then neutralized by soaking 2 X 25 mins in 0.1M Tris.Cl pH 7.5. Gels were left standing for at least 1 hour in 10x SSC (1.5M NaCl, 150mM sodium citrate, pH 7.0) before the next stage.

The required size of Hybond-C Extra nitrocellulose (Amersham, Aylesbury, UK) was cut, floated on DEPC-treated water at 80°C for 5 mins and then immersed in 10x SSC. Transfer of RNA from gel to membrane was carried out by overnight capillary blotting (Sambrook, Fritsch & Maniatis 1989), using 10x SSC as the transfer buffer. After transfer, the membrane was rinsed several times in 3x SSC, allowed to dry at room temperature and then baked at 80°C for 2 hours. To assess the efficiency of RNA transfer, blotted gels were stained for 1 hour with ethidium bromide and examined by ultraviolet illumination.

2.6 Probe preparation

Plasmids containing cytokine-specific cDNA were gifts from the following individuals:

1) IFN-γ , pBR322-MuIFN-γ , Dr A. Morris (Warwick University, Warwick, UK).
2) IL-4, pSP6K.m.IL-4-374, Dr T. Honjo (Kyoto University, Kyoto, Japan), ref. Noma et al. (1986).
3) IL-5, pEDFM-2/3, Dr C. Sanderson (MRC, London, UK), ref. Campbell et al. (1988).
4) IL-2, pMIL2-45, Dr T. Taniguchi (Cancer Institute, Tokyo, Japan), ref. Kashima et al. (1985).
   A second plasmid, pSP65-mIL-2, supposedly containing the IL-2 gene (Dr P. van de Meide, TNO, Rijswijk, Holland) was also used in initial studies, but later discarded (see 3.3).

The cytokine-coding region was isolated from each plasmid by restriction digest: pBR322-Mu IFN-γ (643-bp PstI fragment), pSP6K.m.IL-4-374 (705-bp BamHI), pEDFM-2/3 (604-bp EcoRI-HindIII), pMIL2-45 (1000-bp PstI) and pSP65-

mIL2 (555-bp EcoRI-PstI). Fragments were separated out on 1% agarose gels, excised, and cloned into the SK polylinker site of the Bluescript M13$^-$ plasmid (Stratagene Ltd, Cambridge, UK) using the method of Zhu et al. (1985). Resulting clones were then expanded and purified on CsCl by standard techniques (Sambrook et al. 1989). The orientation of cDNA inserts in the polylinker site was determined by sequencing the region upstream of the M13 -20 primer, using double-stranded DNA as the template (Pharmacia T7 sequencing kit).

In order to generate labelled probes for hybridization, recombinant plasmids were linearized with the appropriate restriction enzyme (depending on the orientation in the linker site) for 1 hour at 37^{o}C and an aliquot run on a gel to ensure complete digestion before the next step. DNA (1 µg) was transcribed in vitro using 10U of either T3 or T7 RNA polymerase and a ribonucleotide mixture containing 100 µCi ^{32}P-UTP (6000 Ci/mmol; NEN DuPont, Stevenage, UK), according to the manufacturer's recommendations (Stratagene Transcription Kit; Stratagene). Prior to hybridization, an aliquot of each transcription product was subjected to electrophoresis and the dried gel then exposed to autoradiographic film (Hyperfilm-MP; Amersham) for 30 mins to check for any transcript degradation.

2.7 Northern hybridizations

Membranes were pre-incubated for 2 hours at 65^{o}C in hybridization solution (50% formamide, 5x SSC, 50mM Tris.Cl pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% ficoll 400, 5mM EDTA, 0.2% polyvinylpyrolidone, 0.2% bovine serum albumin, 150 µg/ml denatured herring sperm DNA and 150 µg/ml yeast RNA). Hybridizations with the RNA probes were carried out for at least 16 hours. The blots were then washed at 65^{o}C, twice for 15 mins each in 2x SSC, 0.1% SDS, and twice in 0.1x SSC, 0.1% SDS. Where required, membranes were then rinsed in 0.1x SSC and incubated for 1 hour at 37^{o}C with 2 µg/ml pancreatic ribonuclease A (Sigma, Poole,

UK) in 10mM Tris.Cl pH 7.4, 300mM NaCl, to remove non-specifically bound probe (see 3.5). The blots were washed for 30 mins with 0.1x SSC, 0.1% SDS at 65^{o}C and rinsed with 0.1x SSC. All membranes were exposed to autoradiographic film at -80^{o}C in the presence of intensifying screens for up to 4 weeks.

3.0 Results & discussion of experimental problems

3.1 Optimization of total RNA extraction

Precipitation of large RNAs (eg. rRNAs and mRNAs) using LiCl salt, resulted in reproducibly high yields of undegraded total RNA from cultured cells. Table 1 gives typical yields from the different cell lines, with examples of the banding pattern from intact total RNA shown in Fig.1a. The same protocol was initially used for the isolation of total RNA from LN cells. However, this consistently resulted in low yields (Table 2) of markedly degraded (Fig.1b) RNA. Compared to cultured cells, the fragility and high RNAse content of freshly isolated cells is thought to make them particularly difficult to handle for RNA extraction (Dr B. Bennetts, personal communication), so several different methods were tried (data not shown) in an attempt to overcome these problems. A protocol using guanidium isothyocyanate proved the most successful, with intact RNA being extracted in significantly higher quantities than with the LiCl approach (Table 2). Furthermore, the yields were greater than those which have been reported for fresh lymphocytes by other groups (Weinberg, English & Swain 1990).

Less RNA / 10^6 cells was obtained from LN samples after vaccination, compared to those taken at day 0. A measure of the RNA content of a cell is a direct reflection of that cell's activation state (Darzynkiewickz 1988), so an increase in RNA / lymphocyte was expected in the vaccine-stimulated LN samples. This was clearly not the case in the present study. An equivalent number of

Table 1

Yields of total RNA extracted from cultured cells

CELL LINE	TOTAL RNA/10^6 CELLS (μg)
CHO-211A { I	25.0
CHO-211A { II	37.0
X6310-mIL2	36.5
X6310-mIL4	19.7
X6310-mIL5	28.2

Table 2

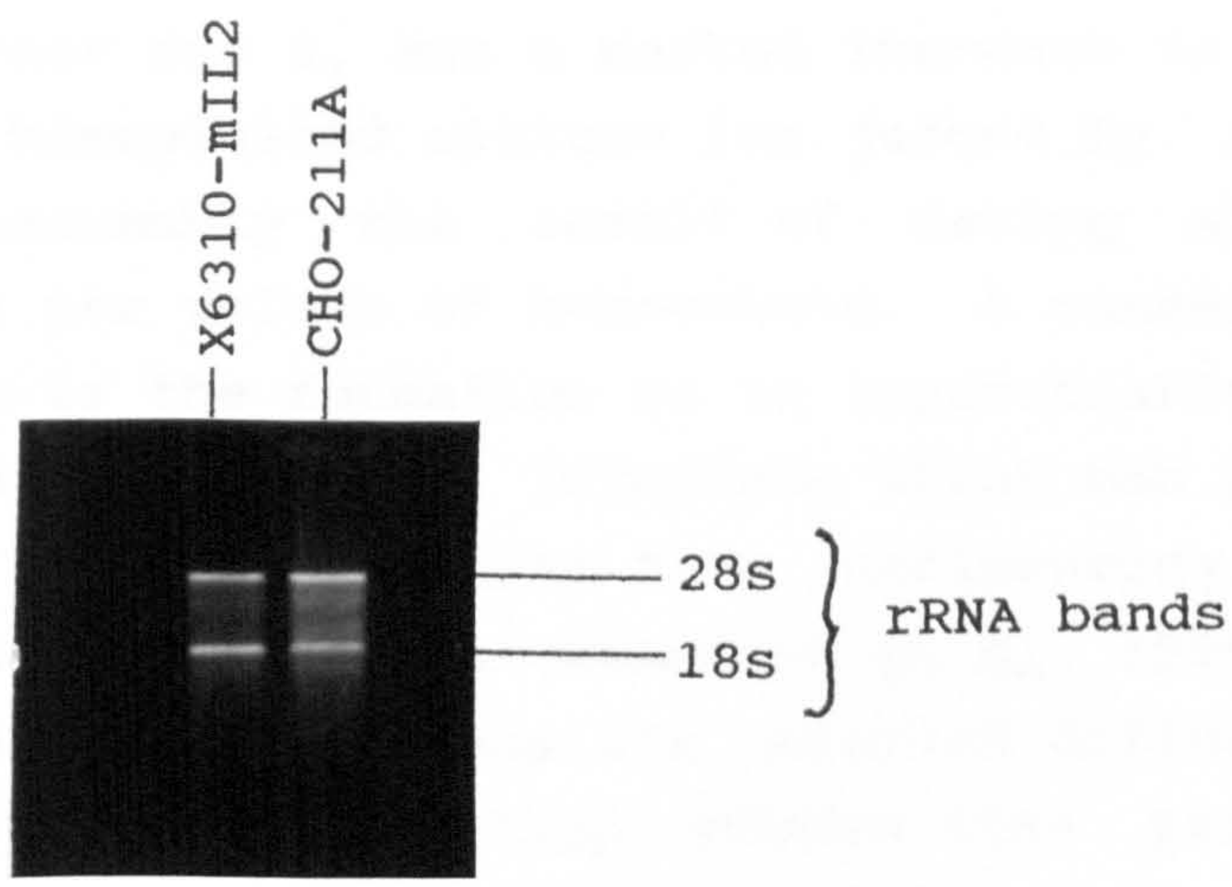
Yields of total RNA extracted from LN cells: lithium chloride versus guanidium isothyocyanate

DAYS POST-VACCINATION	TOTAL RNA/10^6 CELLS (μg) LiCl method	Gua.thi. method
0 { I	0.09	6.2
0 { II	0.05	7.5
7 { I	0.10	1.6
7 { II	0.08	2.1
14 { I	0.06	1.8
14 { II	0.05	2.3
21 { I	N.D.	3.3
21 { II		2.9

N.D. Not Done

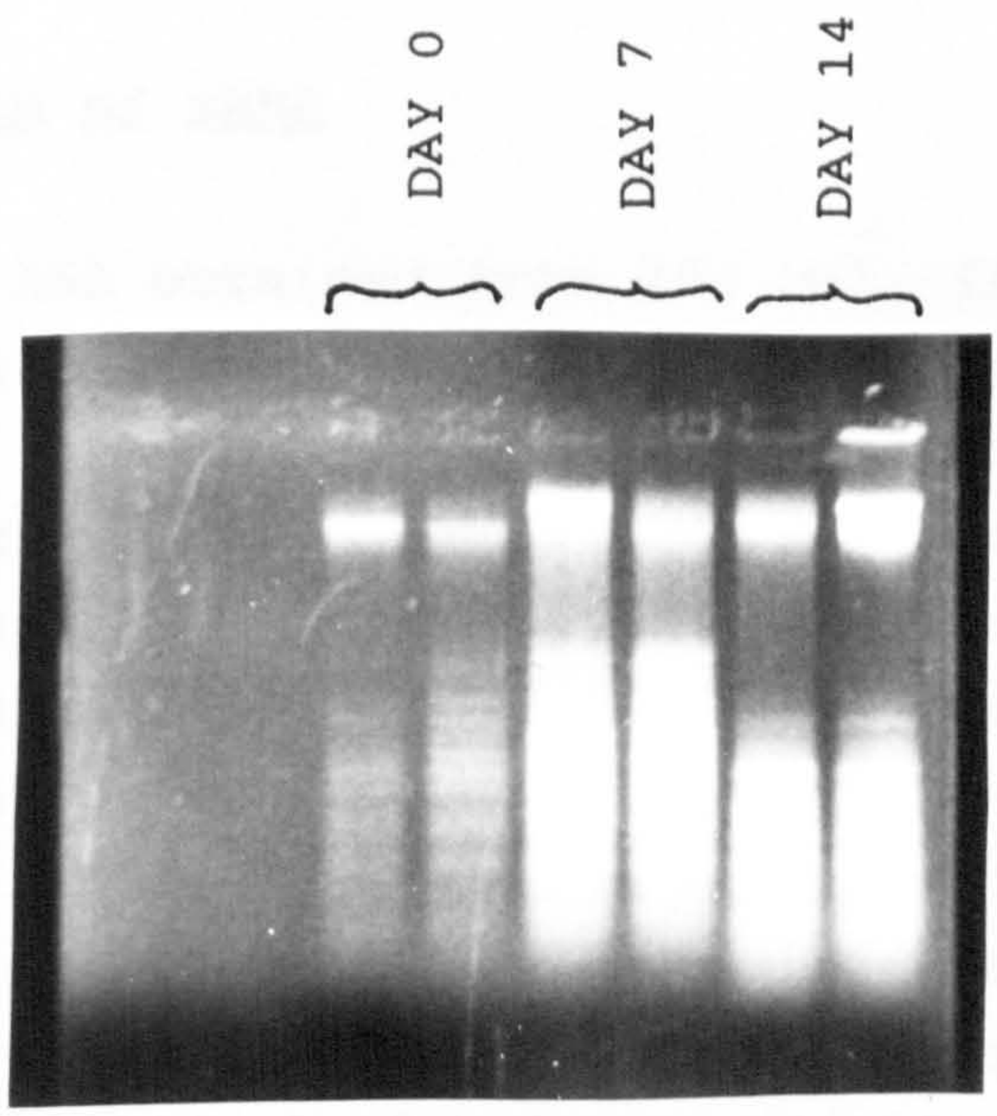
Fig 1: Examples of RNA quality following LiCl extraction

(a) Cell lines



(1 µg RNA / sample; 1% agarose gel)

(b) LN cells, at different days post-vaccination



(1 µg RNA / sample; 1% agarose gel)

axillary LN were homogenized at each sampling time, regardless of the cell content in the nodes. Yet, there is a significant increase (up to 13-fold) in the cellularity of the draining LN following vaccination (see Chapter 2). One problem which was encountered during RNA extractions at time points after day 0, was a marked increase in the DNA content of the homogenized mixture (as judged by augmented viscosity), presumably the result of having a greater number of cells per volume of homogenate. A consequence of this excess DNA is the formation of an impenetrable viscous mat at the supernatant / CsCl interface which can prevent a proportion of the RNA fraction from sedimenting at the bottom of the centrifuge tube (Sambrook et al. 1989). This may provide the explanation for the observed differences in the efficiency of RNA extraction between time points ie. the greater the number of cells per volume of homogenate, the greater the RNA loss per cell. In fact, a calculation of the correlation coefficient between the cell number and RNA yield for this study has revealed an almost direct negative association ($r = -0.992$). In conclusion, it is suggested that in future studies the number of cells, rather than LN, per homogenization should be standardized. Alternatively, it might be possible to reduce the viscosity of the DNA by a more prolonged shearing step.

3.2 Isolation of mRNA

Total RNA obtained from the cell lines was used to optimize poly A^+ RNA selection, due to the greater quantity of material available. Table 3 shows the yield of mRNA from duplicate samples of the different cell lines, following one round of poly A^+ extraction. Approximately 1-2% of the total RNA applied to the column is expected to be recovered as mRNA. Therefore, for each sample, the quantity of poly A^+ RNA obtained has also been calculated as a percentage of the original total (Table 3). Judging by the close duplicate values, the technique was extremely reproducible, particularly as the procedure was carried out on samples I and II from each line on two different days.

Table 3

Yields of cell line mRNA after 1 round of oligo(dT)-cellulose selection

CELL LINE		TOTAL RNA LOADED (μg)	mRNA AFTER 1X OLIGO(dT) (μg)	RECOVERY FROM TOTAL RNA (%)
CHO-211A		544	29.6	5.4
X6310-mIL2	I	800	92.0	11.5
	II	800	85.5	10.7
X6310-mIL4	I	776	80.0	10.3
	II	776	89.6	11.5
X6310-mIL5	I	448	26.8	6.0
	II	448	33.6	7.5

Table 4

Yields of cell line mRNA after 2 rounds of oligo(dT)-cellulose selection

CELL LINE	1X OLIGO(dT) mRNA LOADED (μg)	mRNA AFTER 2X OLIGO(dT) (μg)	RECOVERY FROM TOTAL RNA (%)
CHO-211A	29.6	4.2	0.8
X6310-mIL2	92.0	8.8	1.1
X6310-mIL4	80.0	8.5	1.1
X6310-mIL5	26.8	2.2	0.5

A qualitative example of the enrichment for mRNA is shown in Fig.2, where 1 µg of total and poly A^+ RNA were compared by gel electrophoresis. There is a significant reduction in the rRNA content of the purified sample.

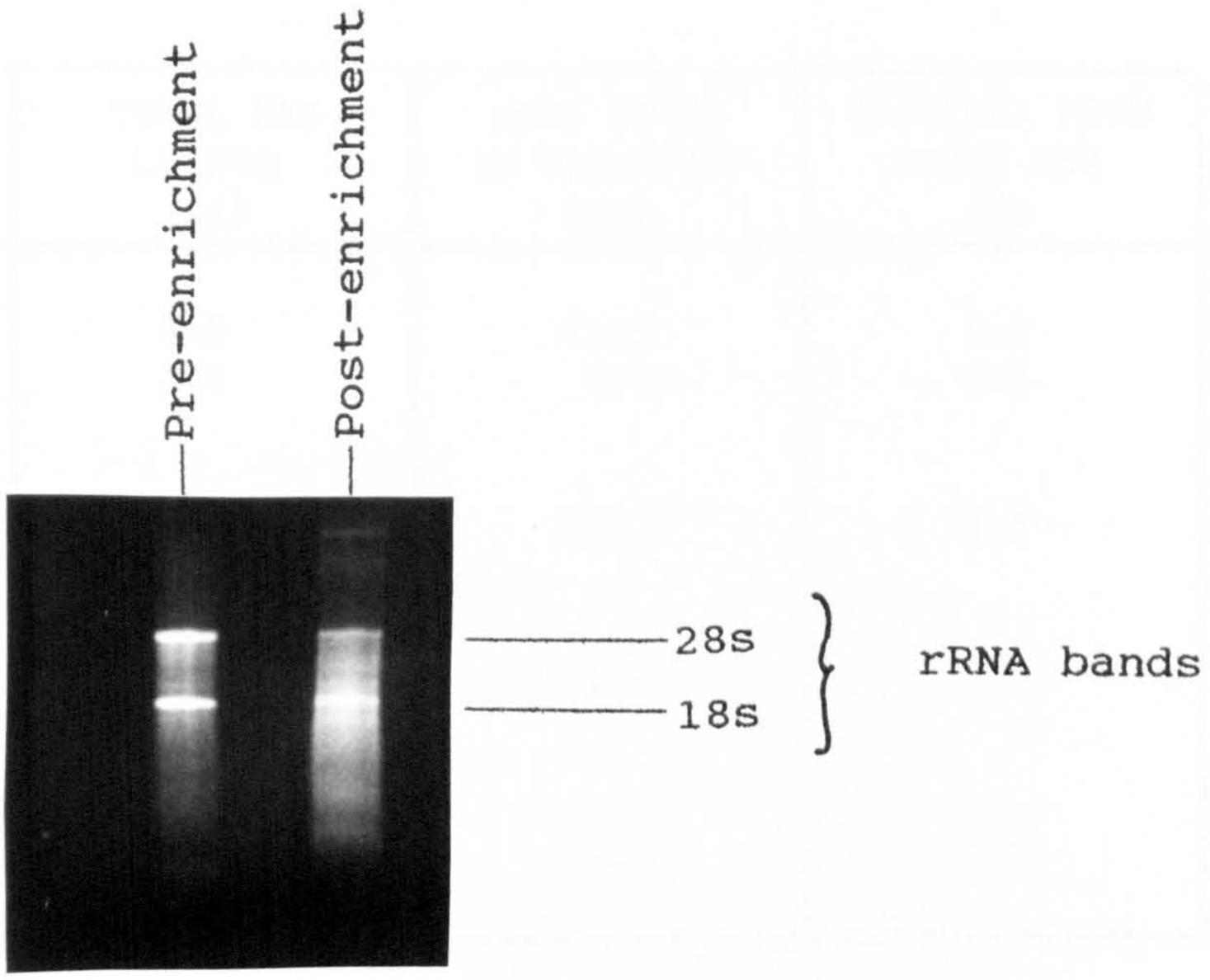
Some protocols recommend that samples be put through a second round of oligo (dT)-cellulose in order to purify the poly A^+ fraction further. This was carried out on mRNA sample I from each cell line. The yields and percentage recovery (as compared to original total RNA) can be seen in Table 4. Although the figures for percentage recovery are somewhat closer to the expected values of 1-2%, the final yields of mRNA after two rounds of selection are extremely small, resulting in little material available for Northern hybridization. It is highly unlikely that the binding of poly A^+ RNA to oligo (dT)-cellulose is 100% efficient, so the significant reduction in RNA yield after a second extraction round will have included a substantial loss in poly A^+, as well as non-polyadenylated, RNA. Also, when samples obtained following either a single or double selection procedure were compared by gel electrophoresis (data not shown), the further enrichment for mRNA after two rounds of purification was almost negligible.

On the basis of these results, it was decided that any increase in the mRNA content of a sample following double purification was not sufficient to compensate for the significant reduction in final yield. This is particularly pertinent to LN cell RNA, where there is little starting material. Therefore, a single poly A^+ selection step was used for all subsequent samples. The results from LN cell RNA purification are shown in Table 5.

3.3 Cloning of cytokine cDNA into Bluescript

No major problems were encountered during the recloning of cytokine cDNA into Bluescript, except that single digests (used for Mu-IFN-δ, mIL-4-374 and mIL2-45) resulted in a high proportion of cDNA fragments re-annealing to themselves rather than to the plasmid DNA, following the addition of T4 ligase. This was solved by

Fig 2: Enrichment for X6310-mIL-2 mRNA after 1 round of oligo (dT)-cellulose selection



(1 µg RNA / sample; 1% agarose gel)

Table 5

Yields of LN cell mRNA after 1 round of oligo(dT)-cellulose selection

DAYS POST-VACCINATION	TOTAL RNA LOADED (µg)	mRNA AFTER 1X OLIGO(dT) (µg)	RECOVERY FROM TOTAL RNA (%)
0 I	648	14.3	2.2
0 II	300	7.6	2.5
7	285	10.3	3.6
14	444	12.5	2.8
21	608	23.5	3.9

treating the linearized DNA with alkaline phosphatase to remove 5′-phosphate groups prior to the ligation step and led to a significant increase in the number of recombinant clones obtained.

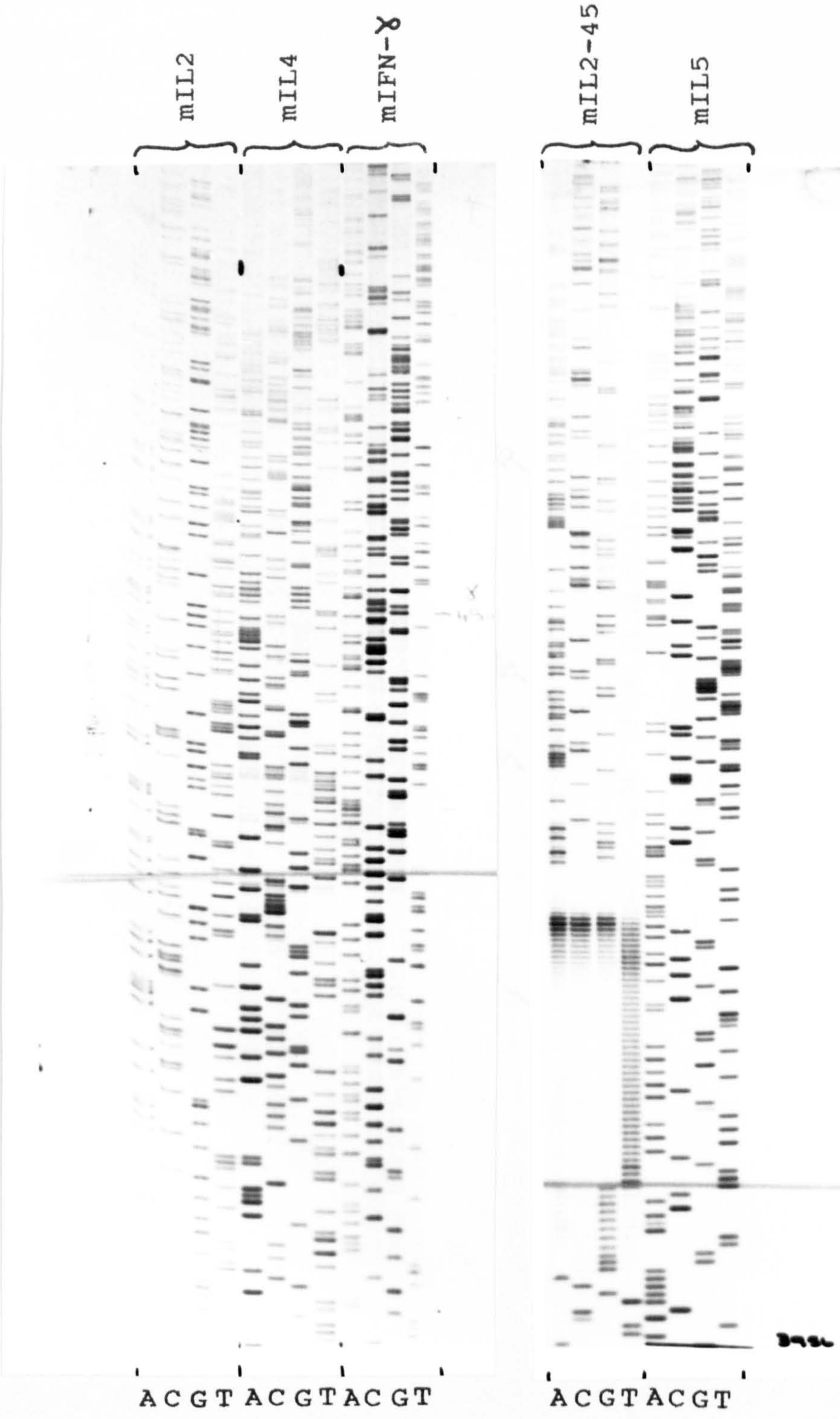
The orientation of each inserted fragment was determined by comparing the resulting DNA sequences (shown in Fig 3) with standard published ones. Thus, IFN-ɣ and IL-4 cDNA inserts both read in the same direction as the 5′ to 3′ published sequence, whereas the IL-5 fragment is in an inverted orientation. However, an examination of the sequence generated from the IL-2 insert (from pSP65-mIL-2) revealed a complete lack of homology with the relevant published data. It is unlikely that a fragment other than the IL-2 gene was excised from the original plasmid, since the EcoRI / HindIII restriction sites are unique in the vector. Similarly there is only one of each site in the Bluescript polylinker, so the fragment could not have been inserted in a different region. The sequence obtained was also compared with that of Bluescript in case plasmid DNA had been sequenced by mistake, but there was no homology between the two. Therefore, it was concluded that the original vector sent contained a fragment other than murine IL-2 cDNA. The plasmid was discarded and a different one (pMIL-2-45) obtained from an alternative source. Sequencing of the new fragment inserted into Bluescript revealed that the IL-2 gene was present this time, and had been cloned in an inverted orientation.

Using the Bluescript SK^- polylinker sequence (Fig.4), the correct promoter (T7 or T3) and restriction digest enzyme required for generating anti-sense RNA probes by <u>in vitro</u> transcription, were determined for each fragment: IFN-ɣ (T3, HindIII); IL-2 (T7, BamHI); IL-4 (T3, HindIII); IL-5 (T7, EcoRI).

3.4 <u>The generation of ^{32}P-labelled RNA probes</u>

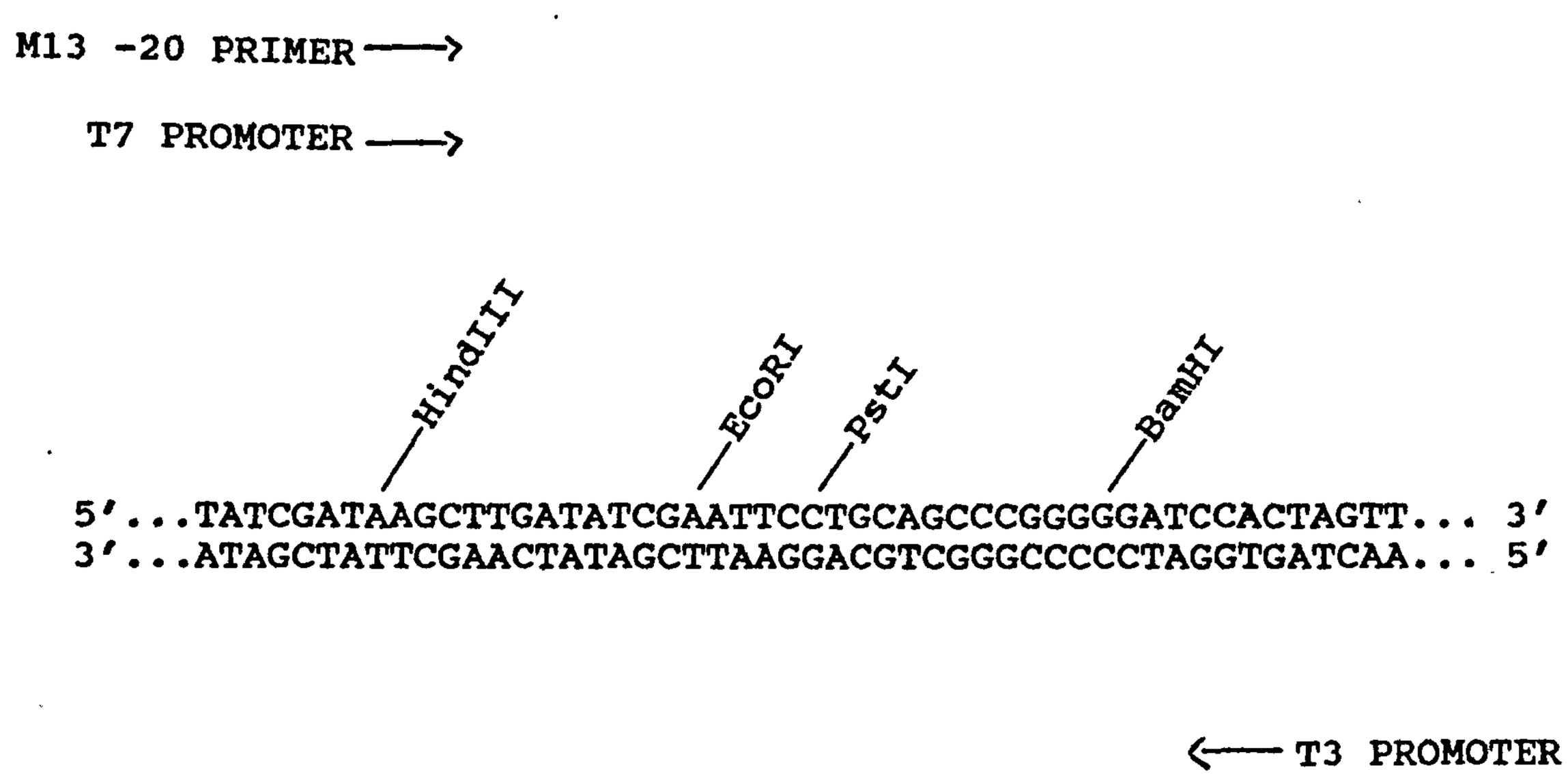
One of the main drawbacks associated with the use of RNA (<u>cf</u>. cDNA) probes, is the extreme care which must be taken to avoid degradation due to the activity of RNAses.

Fig 3: Sequencing of Bluescript SK⁻ polylinker site containing murine cytokine cDNA



(ds pBS-SK⁻ from -20 primer; 6% wedge gel)

Fig 4: Bluescript SK⁻ polylinker sequence, showing relevant restriction sites



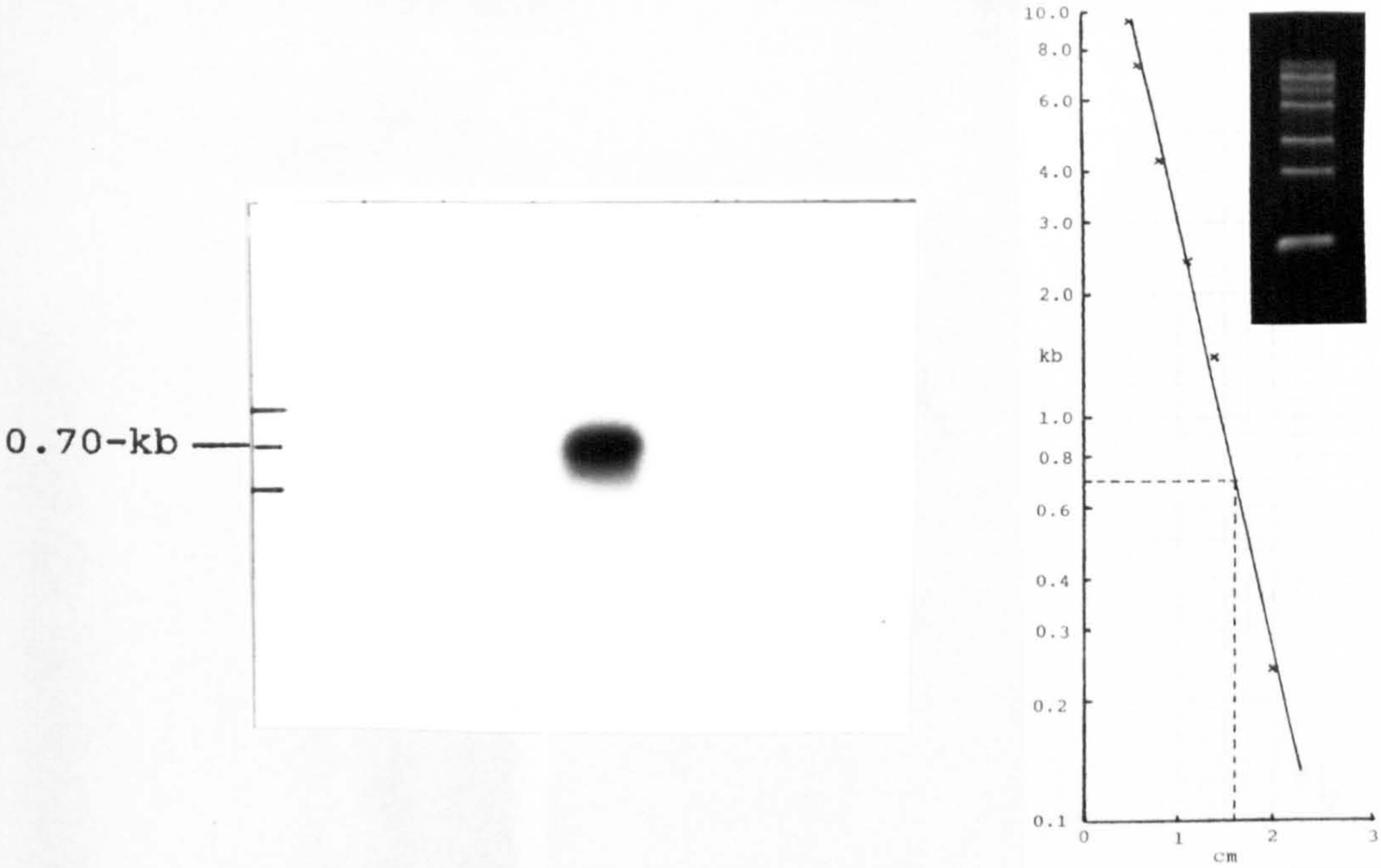
This is particularly so during the transcription procedure. Therefore, transcription products were always checked by gel electrophoresis and autoradiography immediately after synthesis, to ensure the probes were in a sufficiently good condition for hybridization purposes. As an extra check, a sample of RNA molecular-weight markers was sometimes run alongside a transcript to determine band sizes on the gel / autoradiograph. Examples of the results obtained from these checks can be seen in Fig.5. An autoradiograph of intact IFN-δ probe (revealing a single band) is shown in Fig.5a. When the position of the band was plotted against standard RNA molecular weights, a size estimate of 0.7 kb was obtained (cf. IFN-δ cDNA = 643-bp). Fig.5b shows the results from aliquots of IL-2, IL-4 and IL-5 transcripts. In contrast to IFN-δ , there is clearly some degradation in all three products, although to a differing extent. In this case, it was decided to proceed with hybridizations using these probes.

3.5 Detection of cytokine mRNA by hybridization

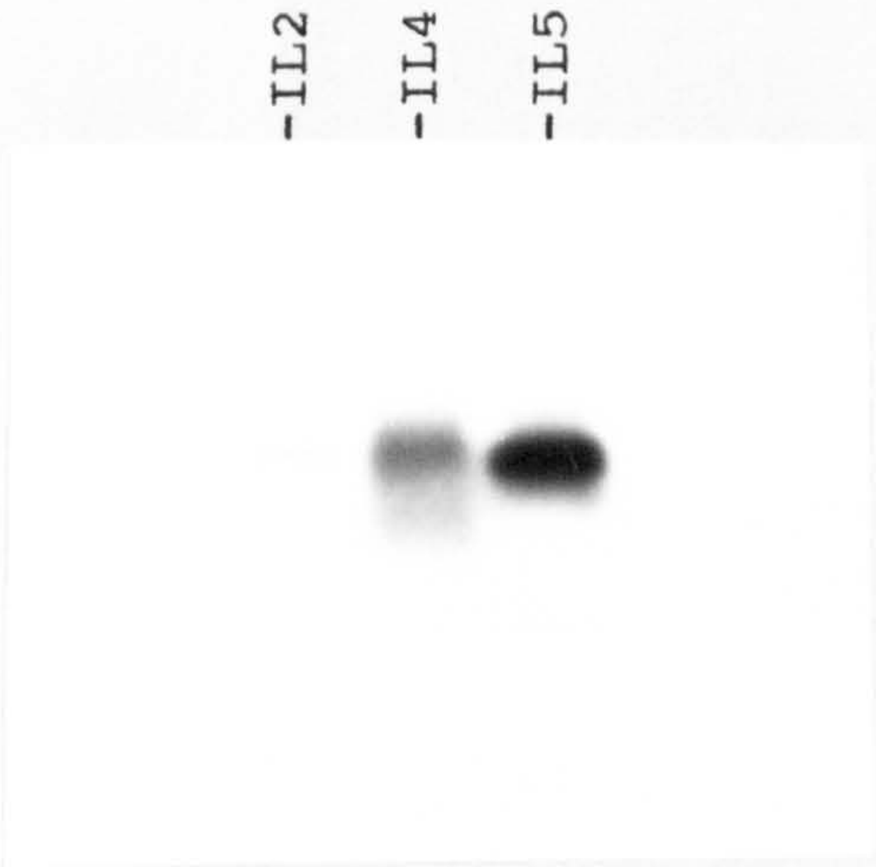
Before attempting to detect mRNA extracted from LN cells, a pilot study was carried out to determine the sensitivity of the probes. A range of mRNA concentrations obtained from cell lines was used for this purpose. Fig.6 shows the results obtained from the hybridization of X6310-mIL2 mRNA (10, 5, 2, 1, 0.5 µg) with an anti-sense IL-2 probe. To check for hybrid specificity, a sample (5 µg) of X6310-mIL4 mRNA was included as a negative control. Most hybridization protocols do not include an RNAse incubation step prior to autoradiographic exposure, so initial studies were carried out without the treatment. However, this led to significant problems. The autoradiograph (24-hour exposure) in Fig.6a illustrates the lack of specific detection which resulted when the RNAse treatment step was omitted, although one feature of interest was noted. The same bands were present in each lane (albeit in varying degrees) except for one, at the 0.94 kb position, which was absent from the negative control, mIL-4 sample (lane 1).

Fig 5: ^{32}P-labelled anti-sense RNA probes generated by in vitro transcription of murine cytokine cDNA

(a) Autoradiograph of intact anti-IFN-ɣ probe: the size of band was determined by plotting distance from well vs log RNA molecular-weight markers

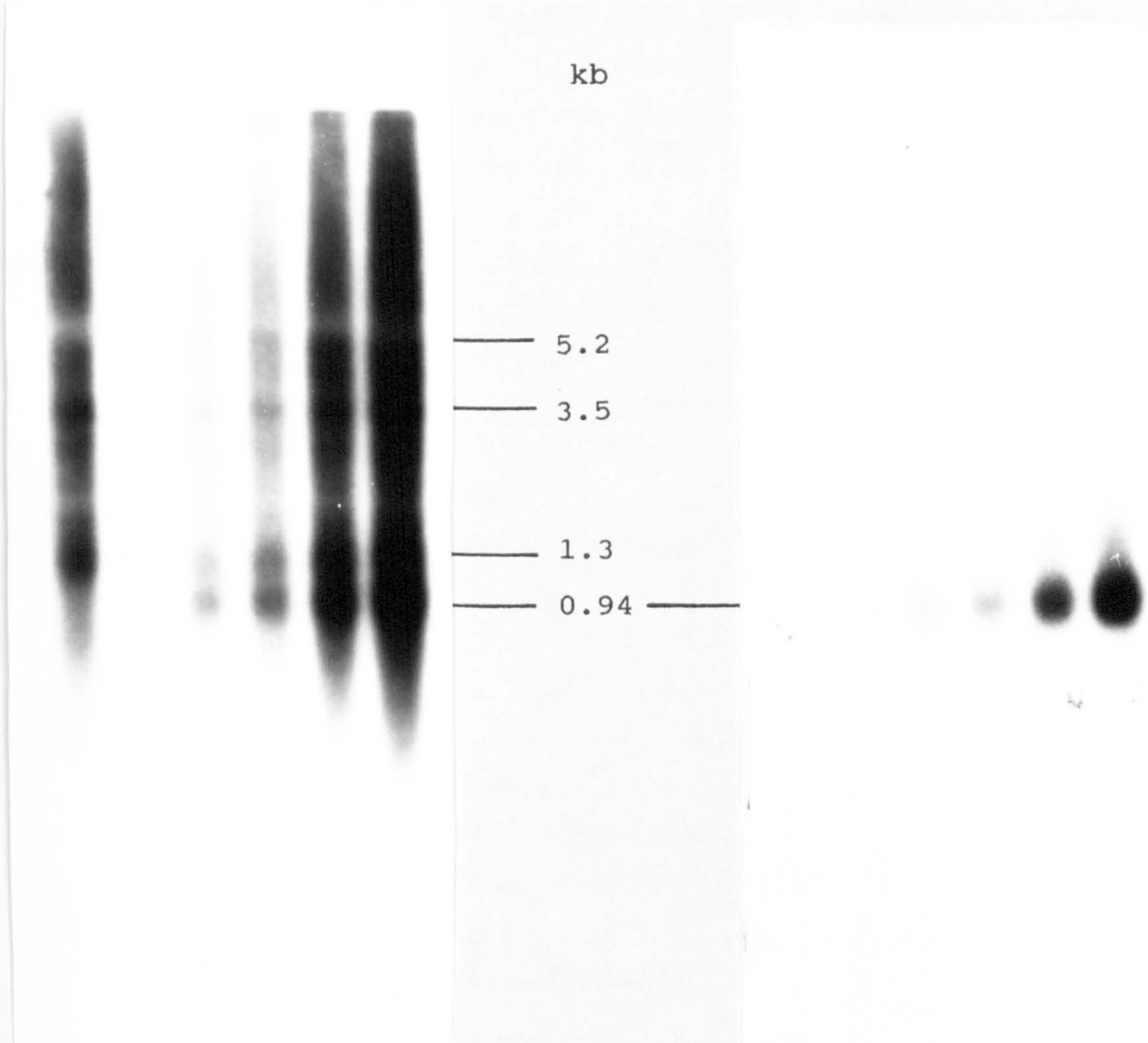


(b) Autoradiograph of anti-IL-2, -IL-4 and -IL-5 probes



(2 µl transcription product / sample; 30 mins exposure)

Fig 6: Hybridization of anti-IL-2 probe to different quantities of X6310-mIL2 mRNA



Lanes:
1) 5 µg X6310-mIL4
2) 0.5 µg
3) 1 µg
4) 2 µg
5) 5 µg
6) 10 µg
} X6310-mIL2

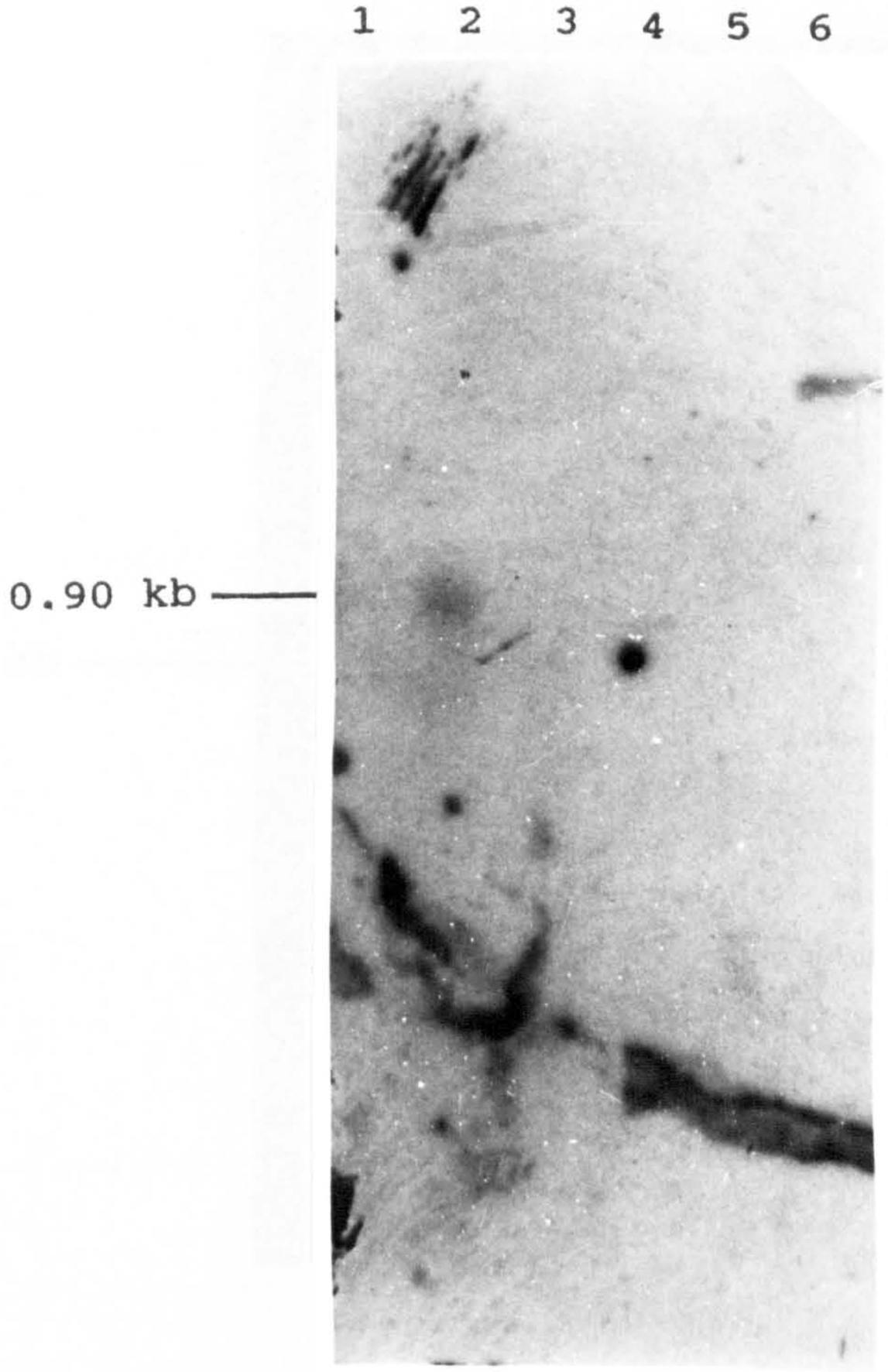
Since a similar sized band (0.90 kb) has been reported for murine IL-2 by other groups (Swain et al. 1988), it was concluded that the 0.94 kb band must be a specific hybrid. In fact, this was confirmed when all non-specific binding was removed by incubating the blot in RNAse A and then exposing it to autoradiographic film for a further 72 hours (Fig.6b). The only band which remained was that at the 0.94 kb position, at various intensities, according to the concentration of mRNA applied. The probe's specificity for IL-2 message was further proven by the complete lack of signal in the mIL-4 lane. On the basis of this result, it was decided that the sensitivity threshold of the hybridization probes is such that at least 1 µg mRNA per sample is required.

Fresh anti-sense IL-2 RNA was synthesized and used to probe different samples (day 0, 7, 14 and 21 post-vaccination) of LN cell mRNA (5 µg / lane). On the same blot was included 5 µg each of 'positive' (from X6310-mIL2) and 'negative' (from X6310-mIL4) mRNA, to check for the specificity of hybridization. The probe did indeed bind specifically to the positive control (Fig.7a), but no IL-2 RNA was detected in any of the LN samples. Possible interpretations of this will be discussed later.

Labelled probes were then synthesized for the detection of IFN-δ , IL-4 and IL-5 message, to determine whether cytokines, other than IL-2, were generated by LN cells after vaccination. Separate blots were used for the different hybridizations, with appropriate positive and negative controls on each. For the IFN-δ blot, as well as 5 µg CHO-211A RNA, an additional 'positive' sample was included in the form of 5 µg mRNA isolated from the T-cell clone, 79C. This is an FCS-specific $CD4^+$ clone, originally derived from the LN of vaccinated mice, which produces significant levels of IFN-δ when restimulated in vitro (R.M. Pemberton, unpublished data). Hence, it was decided that the use of a clone, such as 79C, would represent a good intermediate between CHO-211A and LN cells, since it is derived from a mouse T lymphocyte, but IFN-δ will be produced by all the cells in the population.

Fig 7: Hybridization of probes to LN cell mRNA, at different days post-vaccination

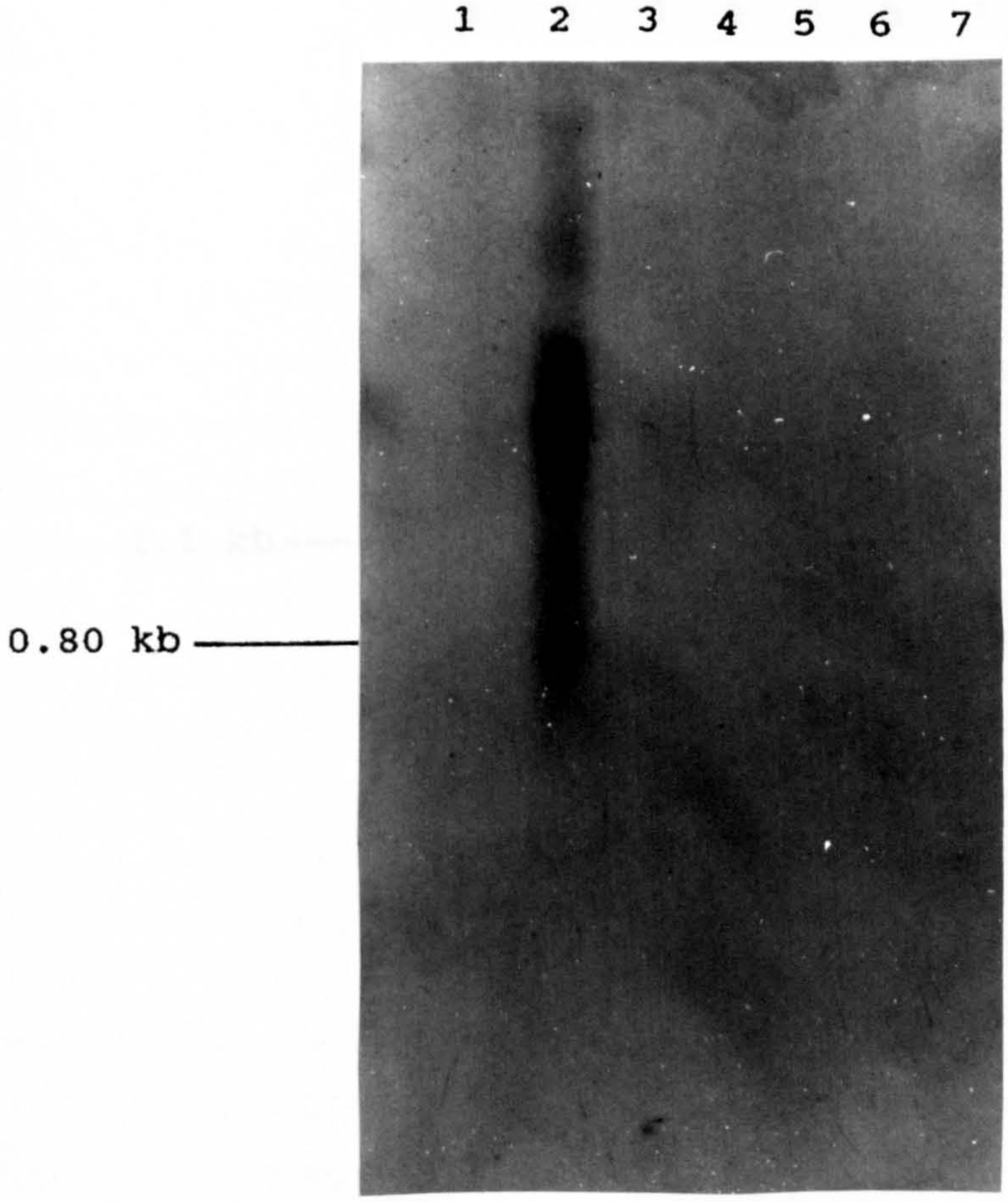
(a) Detection of IL-2 mRNA



Lanes: 1) 5 µg X6310-mIL4
2) 5 µg X6310-mIL2
3) 5 µg Day 0
4) 5 µg Day 7
5) 5 µg Day 14
6) 5 µg Day 21
} LN

Fig 7:

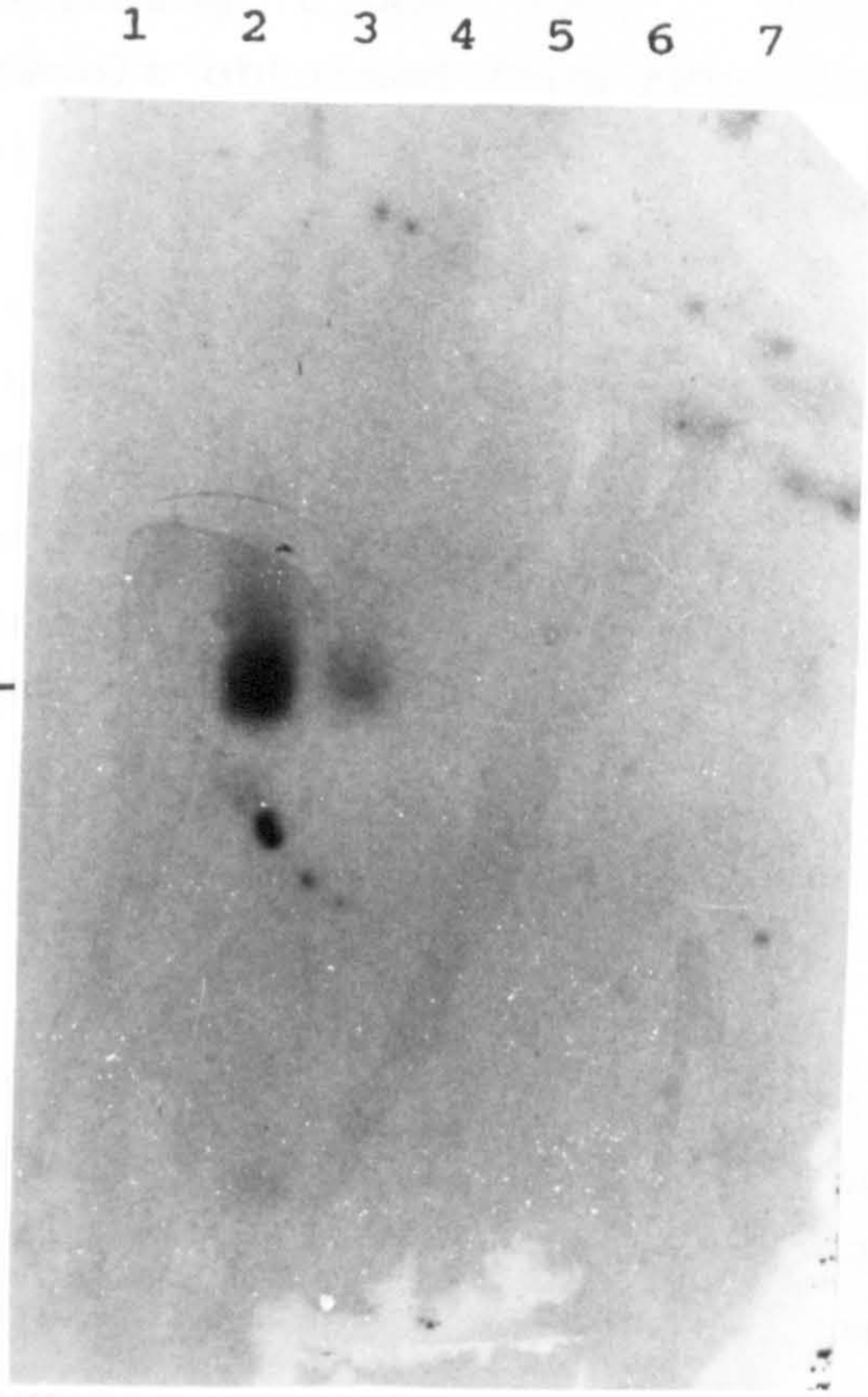
(b) Detection of IFN-δ mRNA



Lanes: 1) 5 µg X6310-mIL4
2) 5 µg CHO-211A
3) 5 µg Clone 79C
4) 5 µg Day 0
5) 5 µg Day 7
6) 5 µg Day 14
7) 5 µg Day 21
} LN

Fig 7:

(c) Detection of IL-5 mRNA



Lanes: 1) 5 µg X6310-mIL2
2) 5 µg } X6310-mIL5
3) 1 µg }
4) 5 µg Day 0 }
5) 5 µg Day 7 } LN
6) 5 µg Day 14 }
7) 5 µg Day 21 }

The results obtained with anti-sense IFN-δ probe, following 2 weeks' autoradiography, is shown in Fig.7b. Some hybridization is evident in the CHO-211A lane, although the RNAse treatment did not remove all non-specific binding. However, a band of approximately the correct size for IFN-δ message (0.8 kb) can still be pin-pointed. There was no binding of the probe to any other sample, including clone 79C. Similarly, an examination of the result obtained from hybridization with the IL-5 specific probe (Fig. 7c) revealed bands of the correct size in the cell line samples only. No hybridization was evident with the IL-4 probe (data no shown), a feature which may be related to the level of degradation in the transcription product (see 3.4). This same problem was encountered in preliminary studies (data not shown) and reinforces the importance of using intact or at least virtually intact probes.

4.0 Discussion

The failure to detect cytokine message in the LN of mice immunized against S. mansoni could be due to a variety of factors. The first, and most straight-forward, explanation would be that the irradiated-vaccine does not stimulate cytokine production from the cells in the draining LN. Unless cytokines other than those examined were generated, this is highly unlikely, from an immunological point of view. It is well established that the proliferation and differentiation of T lymphocytes are under the control of a variety of T cell derived lymphokines, of which IL-2 and IL-4 are among the best defined. IL-2 is released from antigen-stimulated lymphocytes within hours of activation, mediating a switch in T cells from G1 into the proliferative phases of the cell cycle (Smith 1984). The detection of proliferating T cells in the draining LN of vaccinated mice (Chapter 3) provides strong evidence that IL-2 is being produced in vivo.

One of the main reasons for choosing to examine the particular four cytokines (ie. IFN-ɣ , IL-2, IL-4 and IL-5) was in order to place the subsets of T cells generated during the induction of protection to S. mansoni, in the context of the TH1 / TH2 classification described by Mosmann & Coffman (1987). Although the original division was derived from the study of long-term mouse T helper cell clones, there are now several studies which demonstrate the presence of TH1 / TH2 cytokine patterns in normal immune responses (reviewed by Janeway et al. 1988). It was expected that message for both IFN-ɣ and IL-2 would be detected with ease in the freshly isolated LN cells of vaccinated animals, since production of the two cytokines was demonstrated following in vitro restimulation of the cells with schistosome antigens (Pemberton et al. 1991). This was not the case, as shown by the lack of RNA hybridization. A closer examination of the studies described in the review of Janeway et al. revealed that most measurements of cytokine protein / mRNA were in fact carried out on lymphocytes isolated from in vivo primed animals, but then restimulated in vitro with an appropriate antigen / mitogen. Thus, these studies are measuring secondary, rather than primary, responses which provides an explanation for the significant levels of cell product expressed. If LN cells are taken from vaccinated mice and cultured for 3 days in the absence of restimulation, the level of IFN-ɣ generated is below the limit of detection (R.M. Pemberton, pers. comm.), emphasizing the difference between looking at primary and secondary responses.

Therefore, the lack of any RNA detection in the present study is most likely due to insufficient cytokine message in the samples. As described in Chapter 2, there is a marked change in the proportion of lymphocyte subsets in the axillary LN following vaccination. In particular, the increase in B lymphocyte numbers is significantly greater than that of the T cells, resulting in an approximate 50:50 ratio of T:B cells at day 14 post-exposure. Of the T lymphocytes present, around 50% are $CD4^+$ (Chapter 2). So, assuming that the DTH effector

mechanism in this model of immunity is mediated solely by $CD4^+$ lymphocytes, only 25% of the total cell population in the draining LN after vaccination have the potential to respond to schistosome antigens and become DTH-inducing effector cells. The results obtained from the proliferative studies in Chapter 3 suggest that, of the 9% $BrdUrd^+$ cells at the peak of proliferation, only $^2/_3$ are T lymphocytes (as B cells make up approximately 3% of the total $BrdUrd^+$ population). It is not known what proportion of $CD4^+$ and $CD8^+$ cells contribute to the 6% of proliferating T lymphocytes. However, if all were of the T helper subset, then a maximum 0.3 µg of the 5 µg mRNA / sample used for hybridization, would have been derived from $CD4^+$ cells and hence contain one of the cytokines of interest. When this is compared to the cultured cell lines, where 100% of the 5 µg of mRNA is from cytokine-producing cells, the limitations in the detection sensitivity of the assay become very obvious. Furthermore, a limiting dilution analysis to enumerate schistosome-reactive / IFN-γ -secreting T lymphocytes after vaccination, has revealed they may represent as few as 1 in 7000 cells (E.C. Ratcliffe, unpublished data).

Although this might provide an adequate explanation for the lack of results obtained with the LN cell samples, there is still the puzzling failure of the anti-IFN-γ probe to detect message in clone 79C. Being a clone it is expected that all the cells are IFN-γ producers, a situation analogous to the CHO-211A line. However, apart from their source of origin, there is one major difference between the two cultures, concerning the levels of IFN-γ protein each produces. Whereas CHO-211A is capable of generating up to 1000 U/ml, clone 79C secretes a mere 43 U/ml, for the same number of cells (L.E. Smythies & R.M. Pemberton, unpublished data). Assuming mRNA and protein levels are correlated, this 23-fold difference in IFN-γ production must be mirrored by low levels of message in the clone. Reasons for the smaller quantity of IFN-γ secretion from 79C, compared to CHO-211A, cells are unknown. It is interesting that cultures of total axillary

LN cells obtained from vaccinated mice can produce as much as 20 U/ml of IFN-δ following restimulation *in vitro* with schistosome antigens, and yet less than 25% of the lymphocytes are likely to be $CD4^+$ (*cf.* clone 79C). This suggests either that $CD8^+$ T cells in the LN cultures are contributing to the generation of cytokine (unlikely, based on the *in vitro* ablation experiments of Pemberton *et al.* 1991), or that FCS-specific clone 79C is simply a poor producer of IFN-δ .

As already discussed, the majority of studies describing cytokine detection (including those at the RNA level) were examining secondary, rather then primary, cellular responses. However, there are several reports which do describe cytokine production following primary stimulation, albeit mostly *in vitro* stimulation (*eg.* Street *et al.* 1990; Weinberg *et al.* 1990). The work has been particularly useful in highlighting some of the difficulties associated with the study of cytokine detection in once-stimulated cells. When normal $CD4^+$ T cells from murine spleen were activated with Con A immediately after their isolation, high levels of IL-2 protein (Street *et al.* 1990) and mRNA (Weinberg *et al.* 1990), were generated. However, restimulation of the cells was required to obtain significant expression of any other cytokine, such as IL-4 (Weinberg *et al.* 1990). Interestingly, when the frequencies of IL-2 and IL-4-secreting $CD4^+$ lymphocytes in the LN of KLH-primed mice were investigated, using limiting dilution analysis, cells capable of IL-4 production were rare (Powers, Abbas & Miller 1988). In contrast, IL-2 producing cells were abundant.

It has been argued that the reason for the failure to detect IL-4 message in the above studies is because IL-2 and IL-4 are secreted in a sequence, rather than simultaneously, following *in vivo* primary stimulation (Mohler & Butler 1990). The authors found that the sensitization of mice with picryl chloride resulted in a discordant production of the two cytokines in the draining LN, as measured by reverse transcription PCR of the mRNA in

the cells. The majority of IL-2 mRNA expression occurred from 1 to 3 days after antigenic exposure, whereas IL-4 expression was mainly from day 3-5. This 'cascade' effect of cytokine production has also been demonstrated by *in situ* hybridization (Carding *et al*. 1989) in which mitogen-stimulated $CD4^+$ spleen cells expressed maximal levels of IL-2 and IL-4 at 24 and 48 hours post-activation, respectively. Other cytokines, such as IL-5, appear even later in the cascade (Gauchat *et al*. 1989; Cardell & Sander 1990).

Taken together, these studies demonstrate the feasibility of detecting cytokines following primary sensitization. However, in order to obtain sufficient material for analysis, either the stimulant was particularly potent (hence the use of mitogens) or some form of mRNA amplification (*eg*. by PCR) was required. These studies also serve to illustrate the complexity of cytokine generation which is likely to follow primary stimulation, particularly if trying to place the results in the context of other immunological responses. For example, Mohler & Butler (1990) found that the changes in iodo-deoxyuridine (IUdR) incorporation of picryl chloride stimulated LN cells were directly related to the levels of IL-2 and IL-4. Cell proliferation increased in response to IL-2 falling, whilst a rise in IL-4 led to a concurrent decrease in IUdR incorporation. Thus, the cytokines had an antagonistic effect on each other, resulting in the control of the extent of lymphocyte proliferation in the nodes. Although only two out of many possible cytokines were examined, it is easy to foresee the complications which are likely to arise when trying to interpret the interactions and cascades of a large number of cell products. In one study, a sequence of cytokine production following *in vitro* stimulation of spleen cells, was based entirely on positive *in situ* hybridization results obtained from only 2-3% of the total cell population (Cardell & Sander 1990). It is difficult to assess the validity of such data, although these values may in fact be more representative of an *in vivo* situation.

Of course, there is also always a danger in trying to relate results obtained from *in vitro* work, with *in vivo* models of immunity. Due to the difficulty associated with measuring the products of once-stimulated cells, there is little data available for *in vivo* systems. The study of lymphoid organs draining either acute viral infections (Gessner *et al*. 1990; Kasaian & Biron 1990) or lesions caused by parasitic infections such as leishmaniasis (Heinzel *et al*. 1989) has yielded valuable information. In the *Leishmania* study, mRNA for IFN-δ and IL-4 was heavily expressed in the lymphoid organs of infected C57BL/6 and BALB/c mice, respectively. The work was unusual in that the message was easily detectable without the need for prior enrichment of the $CD4^+$ T cell population or some form of genetic amplification. Furthermore, Heinzel *et al*. used almost identical RNA isolation and hybridization conditions as in the present study, yet their results differ markedly from those obtained with attenuated *S. mansoni*-stimulated cells. At first, it was postulated that the overall number of *Leishmania*-sensitized cells (*ie*. $CD4^+$ T cells) in organs such as the draining LN might be particularly abundant, hence total levels of message would be high in those organs. The phenotypic analysis of the responding lymphocytes in the draining LN of susceptible and resistant *Leishmania*-infected mice did indeed reveal a rise in activated $CD4^+$ T cells in both strains (Heinzel, Sadick & Locksley 1988). However, as with an attenuated schistosome infection, the greatest increase observed in these nodes was in the number of B lymphocytes. In fact, the proportions of each lymphocyte class at peak response are almost identical in the two parasite infections.

Instead, the answer may lie in the nature of the disease itself. The LN of *Leishmania*-infected animals are draining lesions in which parasite propagation is ongoing (Mauel & Behin 1982), resulting in the continuous release of significant quantities of antigenic material, over a prolonged period of time. Perhaps the sustained exposure to high burdens of parasite antigen in the nodes, with persistent activation of the relevant $CD4^+$ T cells, is the

explanation for the detectable levels of cytokine message in that particular model of immunity. Up to 8 weeks post-infection, high levels of cytokine message were still observed in the draining LN of both C57BL/6 and BALB/c mice (Heinzel et al. 1989). An additional difference between Schistosoma and Leishmania parasites is that the latter are intracellular organisms, residing and multiplying within host macrophages. Therefore the abundance of parasite antigen which is processed and presented by the infected macrophages is likely to be high and may be a significant factor in T cell activation. If this is the case, we might expect particularly high levels of cytokine mRNA expression in other models of infection where the parasites are intramacrophage organisms eg. Mycobacterium tuberculosis, M. leprae, Legionella pneumophila and Brucella abortus. $CD4^+$ T lymphocytes with reactivity to intracellular parasites have indeed been shown to secrete IFN-γ (reviewed by Kaufmann 1988), although it is not known how this relates to the cells' mRNA content.

The aim of this chapter was to investigate the in vivo sequence of cytokine production in the skin-draining LN of mice exposed to attenuated S. mansoni. However, Northern hybridization failed to detect cytokine mRNA in the lymphocytes isolated from the draining nodes suggesting negligible levels of expression following vaccination. Pilot studies in which the sensitivity of the RNA probes was tested, revealed them to be highly specific and capable of detecting cytokine RNA within 1 μg of cell line mRNA. The biggest problem with trying to detect the same message in LN samples is the dilution effect due to mRNA from non-stimulated cells (particularly B lymphocytes). This could be overcome in part by purifying the $CD4^+$ T cells from the total population, prior to RNA isolation. Alternatively, the cells may have to be restimulated in vitro with schistosome antigens before sufficient message is expressed for detection purposes. Although this would confirm the subsets of anti-schistosome T helper cell generated as a result of immunization, it still would not provide any

detail of the immunological events taking place during the induction phase *per se*. In conclusion, it is unlikely that cytokine mRNA can be detected *in vivo* following primary sensitization with *S. mansoni*, unless some means of amplifying the RNA present is used.

5.0 References

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CHAPTER 5

CONCLUDING DISCUSSION

A crucial role for the draining LN in the induction of protective immunity to S. mansoni in mice was first proposed in the studies of Mountford et al. (1988). More specifically, the authors suggested that the success of a vaccination with optimally-irradiated schistosomula is dependent on the persistence of the parasites, and prolonged release of antigen, in the nodes draining the exposure site. The failure to elicit resistance if the larvae are prevented access to the LN has been demonstrated by a variety of studies (reviewed in Chapter 1.I). However, confirmation that the draining LN are the site of immune initiation for this model of vaccination, can only be obtained from immunological data. Thus, the aim of the work presented in this thesis was to investigate the lymphocytic responses which take place in the draining nodes following exposure to 20 krad.-irradiated parasites, in the hope of establishing some of the cellular criteria for successful immunization.

An initial study was performed to pin-point the lymphoid organs responding to the presence of parasites and / or parasite antigens following vaccination (Chapter 2). Changes in cell number were recorded in the axillary and mediastinal LN only, emphasizing the stimulation of localized rather than systemic events. Furthermore the magnitude and duration of these responses were related to the immune status of the host, although changes in the axillary LN rather than in the mediastinal node, correlated best with the level of immunity induced. The results highlighted the requirement for prolonged schistosomular residence in the skin-draining LN (cf. 80 krad.-irradiated and normal parasites) to obtain high levels of resistance.

A phenotypic analysis of the cellular changes in the LN was also carried out to establish the contribution of different lymphocyte subsets to the observed alterations in cell number. Because of the substantial evidence supporting a cell-mediated effector mechanism (see Chapter 1.I), a preferential increase in T lymphocyte numbers was expected in the nodes. Instead, an expansion of both T and B cell populations was found, with a significantly greater

increase in B, relative to T, lymphocyte numbers. This same phenomenon has been reported in other parasite models of immunity where the key effector cell is the T lymphocyte (Heinzel, Sadick & Locksley 1988; Lynch, Doherty & Ceredig 1989), but no explanations to account for this anomaly were put forward by the authors. The main reason for the detailed dissection of LN cellular responses described in Chapter 3, was in order to provide an answer for the apparent inconsistency between changes in lymphocyte phenotype and the generation of relevant effector cells in the nodes.

Alterations in the cellularity of stimulated draining LN are the result of several interacting dynamic components, most of which are non-specific (reviewed in Chapter 1.II). Therefore, of particular importance was a need to demonstrate that part of the observed increase in lymphocyte numbers in the nodes of vaccinated mice was the result of cell proliferation. This would confirm that the draining LN are indeed the site where new, presumably schistosome-specific, lymphocytes are being generated. The BrdUrd incorporation studies (Chapter 3) revealed a marked proliferation of cells in both the axillary and mediastinal LN following vaccination. This is in agreement with the data of Pemberton _et al_. (1991) in which the presence of schistosome-reactive cells in these LN has been demonstrated by _in vitro_ assays of blastogenesis. However, there is a marked difference between the _in vivo_ and _in vitro_ responses, namely their duration. In the case of the axillary LN, the number of $BrdUrd^+$ cells increased up to 14 days post-vaccination and was still well above baseline levels at day 35. In contrast, the cells' responsiveness to restimulation _in vitro_ had decreased significantly by day 7, returning to background values between days 22 and 35. One of the conclusions formulated from the _in vivo_ proliferation results in Chapter 3 was that high levels of immunity require a prolonged stimulation of lymphocytes in draining LN, in order to generate sufficient numbers of effector cells for a subsequent challenge elimination. This is inconsistent with the _in vitro_ data.

An explanation for the discrepancy is that the two assays are measuring different aspects of the responses to the immunization. An antigenic preparation derived from 18-hour-cultured schistosomula was used for *in vitro* restimulation purposes. This may not provide the entire range of antigens encountered by the lymphocytes *in vivo*. The majority of antigens presented to the lymphocytes in the LN are likely to be newly-synthesized proteins secreted by the live parasites. In contrast, somatic antigens (particularly muscle proteins) will predominate in the larval antigen preparation. A characterization of the antigens released by live schistosomula maintained in culture for up to 2 weeks post-transformation is currently underway (R. Harrop, unpublished data), and should shed more light on the qualitative differences between *in vitro* and *in vivo* schistosome-specific T cell priming. Interestingly, the levels of *in vitro* proliferation of axillary LN cells following either an immunizing or normal (*ie*. non-immunizing) infection are almost identical. In contrast, the *in vivo* responses for the two regimes are significantly different, with a smaller and shorter effect following the normal infection. Thus, the *in vitro*-derived data may be a poor correlate of immunity because the assay is not measuring the full complement of responses relevant to the induction of protection. Of course it is still possible that only the T cells generated during the first few days after vaccination are the important ones, but this remains to be proven.

A phenotypic analysis of proliferative responses observed *in vivo* revealed a striking preferential stimulation of the T lymphocyte class. This is a particularly important feature in the results because it provides *in vivo* evidence for T lymphocyte sensitization to attenuated schistosomes and hence reinforces the hypothesis that this vaccine model of immunity is T cell-mediated. However, the virtual absence of any change in the proliferation pattern of B lymphocytes made the substantial increase in B cell numbers recorded in these nodes even more puzzling. The kinetics of T and B lymphocyte

replication were measured to determine whether the latter subset was dividing at a particularly fast rate, accounting for the build-up of B cell numbers from a small proliferating pool. This was not the case. Instead, it was decided to examine some of the better defined aspects of lymphocyte traffic which follow immune stimulation (reviewed in Chapter 1.II). According to the literature, the effects of hyperaemia have the most potent influence on changes in cellularity within draining LN. In the present study, a significant and prolonged hyperaemia was detected in the LN after vaccination, but the proportions of T and B lymphocytes recruited into the nodes as a result of the increased blood flow, remained unchanged. In fact, the influx of T lymphocytes continued to predominate over that of B cells, a feature which has been shown in the studies of other workers (Stevens et al. 1982; Kraal & Twisk 1984).

Since the B lymphocytes were neither proliferating nor being recruited to a greater extent than the T cell class, it was postulated that they must be failing to exit from, and hence accumulate in, the nodes. The retention of B cells in the LN was indeed demonstrated, as judged by the virtual absence of that subset in the efferent lymphatics draining the vaccine-stimulated nodes. Why B lymphocytes persist in the LN in this way is unknown. Apart from a small proportion of the B lymphocyte subset being triggered to proliferate (presumably accounting for the production of anti-schistosome antibodies circulating after vaccination), the majority of the B cells in the LN probably remain unstimulated. Perhaps this influences the likelihood of their leaving the nodes in order to recirculate (cf. the activated T lymphocytes). However, Kraal & Twisk (1984) have shown that B lymphocytes are retained in stimulated draining LN, regardless of the antigen used so the effect must be the result of something other than the cells' state of activation.

The effector mechanism in this vaccine-model of immunity is thought to be mediated by $CD4^+$ DTH-inducing cells. If this is correct, it would be expected that the majority of the T cell proliferation observed in vivo

(Chapter 3) is in the $CD4^+$ subset. Unfortunately it was not possible to measure this. Instead, alternative markers of cell activation have been sought in order to characterize further the anti-schistosome priming events in the draining LN. With the advent of the TH1 / TH2 classification, the obvious choice was to examine the cytokines generated by the cells. As discussed in Chapter 4, the production of both IFN-γ and IL-2 from axillary node lymphocytes has been demonstrated following vaccination (Pemberton et al. 1991), firmly placing the stimulated lymphocytes in the TH1 category. The fact that TH1 cells are being generated in the draining LN of vaccinated mice emphasizes the important role played by these nodes in the immunization strategy. However, the way in which the various cells and their products interact in the LN to regulate the expression of this TH1 functional phenotype, is far from understood.

TH1 and TH2 lymphocytes are thought to be mutually inhibitory. The presence of IFN-γ significantly downregulates the growth of TH2 cells (Gajewski & Fitch 1988), whereas IL-10 (designated by Moore et al. 1990; reviewed by Mosmann & Moore 1991) produced by the TH2 subset inhibits the synthesis of cytokines by TH1 cells (Fiorentino, Bond & Mosmann 1989). Such crossregulation of T lymphocytes has been demonstrated in cells isolated from mice undergoing a normal S. mansoni infection (Pearce & Sher 1991). These cells produced substantial amounts of IL-10 in response to antigen, but in the presence of anti-IL-10 monoclonal antibody, significant levels of (previously undetected) IFN-γ were synthesized. Lymphocytes isolated from the LN of vaccinated mice show a marked decrease in in vitro blastogenesis after day 7 post-exposure (Pemberton et al. 1991). Perhaps this downregulation in lymphocyte proliferation is due to the presence of an inhibitory factor, such as IL-10, in the cultures. It is interesting that IL-10 can induce a reduction in IL-2 synthesis, leading to a decrease in the proliferation of cells without their responsiveness to the growth factor itself being affected (Magilavy, Fitch &

Gajewski 1989). As a corollary, the in vivo proliferation seen at time points later than day 7 post-vaccination (Chapter 3) may represent TH2-dominated cell division. One can speculate that such a switch in the class of dividing cell is a means whereby the immune system is able to return to a state of balance after a strong TH1 or TH2 lymphocyte response. This is an area of immunology about which little is known.

In Chapter 4 an attempt was made to detect the in vivo changes in cytokine mRNA expression in the draining LN cells of vaccinated mice. One of the conditions laid down at the start of the work was that only the primary responses taking place during the induction phase of vaccination were to be investigated ie. cytokine synthesis should be examined in the absence of any in vitro restimulation of the lymphocytes with either antigen or mitogen. Unfortunately this did not prove possible because of the small amount of specific message present within the total pool of LN cells. It was concluded that in order to obtain data of this kind, the relevant cells (ie. $CD4^+$ T lymphocytes) would need to be purified from the rest of the population prior to RNA hybridization. Apart from the standard methods of lymphocyte separation, several new flow cytometric sorting techniques have recently become available, allowing the recovery of intact RNA from sorted cells (Dunne, Thomas & Lee 1989; Khochbin et al. 1990). However, in situ hybridization is probably the approach most likely to succeed in detecting low levels of mRNA in a population of cells, because the actual lymphocyte expressing the message can be pin-pointed, even if it represents only 1 in a 1000 in the field of view. Furthermore, using a differential staining system, it is possible to detect several specific mRNAs simultaneously within the same sample. For example, this would confirm whether the cytokines produced in a cascade are being generated by the same or different lymphocytes. The FISH (Fluorescent In Situ Hybridization) technique, developed for the detection of RNA by flow cytometry (reviewed by Bauman et al. 1989) would prove very powerful in this

respect. The advantages in being able to analyse tens of thousands of cells in a short space of time are obvious. However, the original studies in which this method was used were examining the expression of extremely abundant species of RNA eg. ribosomal RNA (Bauman & Bentvelzen 1988) and β-globin mRNA in haemopoietic tissues (Bayer & Bauman 1990). If the same approach were to be used for the detection of cytokine mRNA, the fluorescence emitted by the fluorochrome-labelled cDNA/RNA probes would undoubtedly require some form of amplification. Despite these limitations, it is an avenue worth further investigation.

The objective behind dissecting the immunological responses in the draining LN of vaccinated mice is to determine the cellular criteria required for the successful induction of protection. This is of fundamental importance if the same conditions are to be recreated for an effective non-living vaccine. The work presented in this thesis has reinforced the role of the draining LN in the induction phase of vaccination by demonstrating the generation of cellular responses specific to immunizing schistosomula. The parasites in this model of immunity are unusual in that they deliver themselves to the requisite site of lymphocyte sensitization. This is an important feature for obtaining high levels of resistance and one that will be difficult to accomplish with a dead vaccine, unless some mechanism of antigen delivery into the LN can be implemented. In addition, the antigen must be delivered into the appropriate LN, as the microenvironment in different LN (eg. those draining mucosal versus nonmucosal sites) is thought to be partially responsible for altering the potential of resident T lymphocytes to produce distinct T cell growth factors (Daynes et al. 1990). Delivery via adjuvants may be the key in this respect, especially as they also have the potential to activate selectively different arms of the immune system. For example, complete or incomplete Freund's adjuvant triggers a TH1-type response whereas the alum adsorption of antigen results in the priming of TH2 cells (Janeway et al. 1988). The

correct combination of antigen and adjuvant could be highly effective in driving the host's immunity in a particular direction.

The type of antigen-presenting cell (APC) which is activated following vaccination is also likely to have an influence on the outcome of the immune response. Several lines of research suggest that B lymphocytes are required for the priming of TH1 cells to protein antigens (eg. Malynn & Wortis 1984; Chang et al. 1990). In contrast, because they require IL-1 for their autocrine growth (Greenbaum et al. 1988), TH2 cells are most likely primed by interaction with macrophages (Kurt-Jones et al. 1987), the APCs which produce the most IL-1. However, other workers argue that the preferred APC for TH1 and TH2 cells is the macrophage and B lymphocyte, respectively (reviewed by Gajewski et al. 1989). The situation is further complicated by the discovery of APC-derived co-stimulatory factors other than IL-1 (Roska & Lipsky 1985; Jenkins, Ashwell & Schwartz 1988). Work to characterize these factors is still underway and it is not yet known whether their effect on the T cells is mediated via membrane-based or secreted products (Mueller, Jenkins & Schwartz 1989; Mueller et al. 1990). The message from such studies is that the cell / cytokine interactions within stimulated LN are even more complex than originally predicted.

With an understanding of the immunology associated with the elicitation of resistance in the attenuated schistosome model of immunity it is eventually hoped that a suitable vaccine against schistosomiasis can be developed for use in humans. The recent demonstration that TH1/TH2-like lineages do indeed exist, and can be induced preferentially, in humans (Romagnani 1991) certainly confirms the pertinence of the murine work. With the appropriate combination of adjuvants and/or cytokines alongside the antigen of choice it may even be possible to achieve levels of immunity as high as those generated by attenuated larvae in mice. Until then, there is still much knowledge to be gained from studying the irradiated parasite model of schistosomiasis.

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