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Adoptive transfer of resistance to *Nematospiroides dubius* infections of mice and an assay to measure the *in vitro* proliferation of lymphocytes reactive with *N. dubius* antigen

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SUMMARY

Spleen and mesenteric lymph node cells from *Nematospiroides dubius*-infected and normal control mice were cultured *in vitro* with *N. dubius* antigen. Proliferation of these cells in response to antigen was measured by the uptake of [^{3}H]TdR. Cells harvested from mice during a primary infection did not proliferate *in vitro;* however, low levels of specific proliferation could be demonstrated if these mice were treated on Day 5 post-infection with 20 mg/kg of cyclophosphamide i.p. A strong cell proliferative response was measured 6 days following a challenge infection; spleen cells responded more strongly than cells from the mesenteric lymph nodes (MLN), but the addition of lymph node cells to spleen cell cultures did not suppress the latter response. Responsiveness of spleen cells to concanavalin A (Con A) was two-fold higher in infected mice than in normal controls, but the proliferation of MLN cells to Con A was similar in infected and uninfected mice. When *N. dubius*-resistant B10.M (*H-2^f*) mice were compared to the susceptible B10.BR (*H-2^k*) mice, no differences were observed in the spleen cell response to *N. dubius* adult antigen following challenge infections. However, after a tertiary infection, MLN cells from the resistant strain proliferated strongly in comparison to cells from susceptible mice. Spleen or MLN cells from resistant mice transferred immunity to naive recipients provided that the recipients had received a prior injection i.p. with adult *N. dubius* antigen. The injection alone, or cells in the absence of the injection, failed to protect the recipients from *N. dubius* challenge.

INTRODUCTION

Functional immunity against *N. dubius* appears to be induced primarily by the developmental stages of the parasite in the wall of the small intestine, and these larval stages also serve as the targets of such responses when they occur (Barlett & Ball, 1974; Pritchard *et al.*, 1983). Data from several laboratories suggest that adult worms of *N. dubius* living in the lumen of the mouse small intestine are not susceptible to immune attack. In fact, adult worms may actively suppress functional immunity (Barlett & Ball, 1974; Jacobson, Brooks & Cypess, 1982; Behnke, Hannah & Pritchard, 1983).

It is well established that inbred strains of mice differ in their ability to resist challenge infections with *N. dubius* (Cypess *et al.*, 1977; Jacobson *et al.*, 1982; Behnke & Robinson, 1985; F. J. Enriquez, B. O. Brooks, R. H. Cypess, C. S. David and D. L. Wassom, manuscript in preparation), and it has been suggested that susceptible hosts are preferentially immunosuppressed (Behnke & Robinson, 1985; Sitepu, Dobson & Brindley, 1985; F. J. Enriquez, R. H. Cypess and D. L. Wassom, manuscript in preparation). In order to explore the cellular basis for differences in susceptibility to challenge infections, and to assess the possible role of suppressor cells or suppressor phenomena in determining the outcome of such infections, we have established conditions for measuring the *in vitro* proliferation of lymphocytes cultured with *N. dubius*-soluble antigen. In addition, we have defined the conditions necessary for the successful transfer of immunity to *N. dubius* infection using lymph node or spleen cells from *N. dubius*-infected mice.

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Abbreviations: Con A, concanavalin A; i.p., intraperitoneal; i.v., intravenously; L_3, third stage larvae; MLN, mesenteric lymph node; s.c. subcutaneous.

MATERIALS AND METHODS

Mice

Eight- to ten-week-old female mice were used in all experiments. B10.M, B10. BR, and B10.T(6R) mice were obtained originally from the immunogenetics mouse colony at the Mayo clinic

(Rochester, MN), and bred and maintained at Cornell University. DBA/1J mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Antigen

Third stage larvae (L_3) used for preparing antigen were recovered from 7-day cultures (F. J. Enriquez, B. O. Brooks, R. H. Cypess, C. S. David and D. L. Wassom, manuscript in preparation). *N. dubius* adult worms were recovered from the intestines of infected mice 3 weeks post-infection. The worms were placed in a 15-ml centrifuge tube (Corning, NY) containing sterile PBS (pH 7·2) supplemented with 100 IU/ml of streptomycin and 100 IU/ml of penicillin and washed 10 times by allowing the worms to sediment at 1 ***g***. The worms were kept at 4° and ground for 40 min with a teflon-tipped tissue grinder adapted to an homogenizer. The suspension was centrifuged at 40,000 ***g*** for 20 min at 4°, and the supernatant was dialysed against three changes of phosphate-buffered saline using a membrane with a 5,000 MW exclusion limit (Spectrapore, Spectrum Medical Instruments Inc.). The suspension was centrifuged again at 40,000 ***g***, and the supernatant was assayed for protein concentration using the Bio-Rad (Bio Rad Laboratories, Richmond, CA) procedure using a globulin standard. Antigens were stored at $-70°$ until used.

In preliminary experiments, antigens from L_3 and from adult worms were compared using SDS polyacrylamide gel electrophoresis. All protein bands appearing in the L_3 preparation were represented in the preparation made from adult worms. Furthermore, the L_3 and adult worm antigen preparations each supported comparable levels of proliferation in our cell proliferation assay, and serum from infected mice reacted with each of the antigen preparations when in an ELISA assay. Since adult worm antigen was easier to obtain, we used adult worm antigen to stimulate cultured cells in the assay reported below, even when the infected hosts had never been exposed to adult worms. Antigen prepared from encysted L_4 would have been ideal for our experiments but was too difficult to prepare in adequate amounts.

In vitro *cell proliferation assay (CPA)*

The basic protocol for immunization and challenge of mice is outlined in Table 1. Invermectin (Equalan, MSD, Aguet, Barcelona, Puerto Rico) was administered *per os* (8 mg/kg). Four or six days after either the primary, secondary, or tertiary infection, these mice, as well as uninfected controls, were killed by cervical dislocation, and the mesenteric lymph nodes (MLN) and/or the spleens of each mouse were removed aseptically and placed in 17 × 100 mm tubes (no. 2001; Falcon, Baltimore, MD) containing 5 ml of Hanks' balanced salt solution (HBSS), supplemented with 10 mM/ml of HEPES (no. 380–5630; Gibco, Grand Island, NY), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco), 30 mg/l of DNASE (Sigma, St Louis, MO), and 2% horse serum (Gibco). The tissues were gently disrupted with a teflon-tipped tissue grinder (Wheaton, NY), and after a few min, when the debris had settled to the bottom of the tube, the cells in the supernatant were aspirated with a Pasteur pipette, transferred into a 15-ml centrifuge tube (Corning no. 25311) and spun at 400 ***g*** for 5 min at 4°. The supernatant was discarded and the cells were briefly resuspended in 2 ml of ammonium chloride buffer (NH_4Cl 8·19 mg/ml, $KHCO_3$ 1 mg/ml, and EDTA 0·037 mg/ml in distilled water) to lyse erythrocytes, and washed twice as described before. The cells were then resuspended in 2 ml of cell culture media (CCM) [RPMI-1640 (Gibco) containing 2 mM/ml glutamine (Gibco), 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM/ml HEPES buffer, 3×10^{-7} M/ml 2-mercaptoeptanol (Sigma), and 5% horse serum (Gibco)]. Viable cells (trypan blue dye exclusion) at a concentration of 4×10^5/100 μl of CCM were placed into wells of flat-bottomed 96-well sterile microtitre plates (no. 3596; Costar, Cambridge, MA). Cells were cultured with 5 μg of concanavalin A (Con A) (Sigma) in 100 μl of serum-free CCM, or with 100 μl of *N. dubius* adult worm or L_3 antigen (Ag), at concentrations of 25, 50, and 100 μg/ml. Control wells contained culture medium alone. After 2 days (Con A-stimulated cells) or 5 days of culture (*N. dubius* Ag-stimulated cells), 10 μl of [^{3}H]TdR (Research Products International) in HBSS at 160 μCi/ml was added to each well. Twenty-four hours later, the cells were harvested (Microharvester, Otto Hiller Co., Madison, WI) on to filter paper discs (Glass fibre strips nos 7735–10021; Bellco, Vineland, NJ) and dried for at least 24 hr. The discs were punched individually into 13 × 57 mm vials (Wheaton no. 225402) and 3 ml of scintillation fluid (Fisher 50-X-2; Fisher Scientific Co., Louisville, KY) were added to each vial. Disintegrations per min were determined using a Beckman LS 7000 scintillation counter (Beckman Instruments, Fullerton, MA). Results were expressed as the change in c.p.m. (Δ c.p.m.), calculated by subtracting the average count of non-stimulated control cultures from that of stimulated cultures of each sample. Cells from uninfected mice did not proliferate when cultured *in vitro* with either L_3 or adult worm *N. dubius* antigen preparations.

Cyclophosphamide (Cytoxan, Bristol-Meyers, Syracuse, NY) was given at a dose of 20 mg/Kg intraperitoneally (i.p.) in PBS.

Cell transfer experiments

Cells were prepared from infected or uninfected donors as outlined above for the CPA. They were pooled and washed one additional time at 400 ***g*** for 5 min at 4°, prior to resuspension in sterile PBS (pH 7·2) at 5×10^7 cells/ml. Each recipient of cells was injected with at least 1×10^7 cells intravenously (i.v.) in 200 μl. Twenty-four hours after the cell transfer, recipient mice received an i.p. injection of 100 μg of *N. dubius* adult worm extract in Complete Freund's Adjuvant (FCA). Seven days after the antigen boost, mice were challenged with 100 L_3 and the adult worms were counted 21 days later.

Statistics

The non-parametric Mann–Whitney Rank Sum test was used to analyse results of the adult worm counts. A Student's *t*-test was used to analyse the cell proliferation data (Snedecor & Cochran, 1980). *P* values $<0·05$ were considered significant.

RESULTS

Antigen-specific proliferation of spleen and mesenteric lymph node cells

B10.T (6R) mice were divided into four groups of four mice each; two of these groups received a primary *N. dubius* infection and the other two groups remained uninfected. One group of infected mice and their uninfected controls were injected i.p. with cyclophosphamide (20 mg/kg) on Day 5 post-infection; the

Table 1. Experimental design for *N. dubius* infections

	Day						
	0	6	10	16	20	24	26
Uninfected	—	—	—	—	—	CPA	CPA
First infection	—	—	—	Px	100 L_3	CPA	CPA
Second infection	—	Px	50 L_3	Px	100 L_3	CPA	CPA
Third infection	50 L_3	Px	50 L_3	Px	100 L_3	CPA	CPA

Px = ivermectin *per os.*

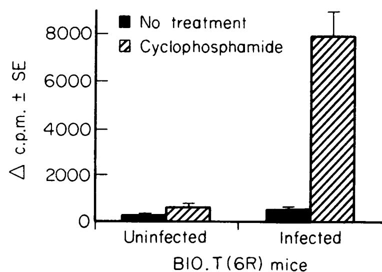

Figure 1. Effect of treatment with low dose (20 mg/kg) cyclophosphamide on the *in vitro* proliferation of MLN cells cultured with 100 μg/ml of adult *N. dubius* Ag 6 days after a *N. dubius* primary infection ($n = 4$ B10.T(6R) mice per group). Cultures were pulsed 5 days post-incubation.

other infected group and their uninfected controls received an i.p. injection of saline solution. Six days post-infection, the MLN cells of all four groups were cultured *in vitro* with adult *N. dubius* antigen. As shown in Fig. 1, only cells from infected mice receiving the cyclophosphamide injection proliferated *in vitro*.

Because we could not demonstrate antigen-specific proliferation following an unmodified (i.e. non-cyclophosphamide treatment) first infection, we compared MLN and spleen cells from B10.T (6R) mice 4, 6, or 8 days following a second *N. dubius* infection. In addition, we compared the response of spleen cells to the response of MLN cells, and pulsed the *in vitro* cultures after either 3, 4 or 5 days of culture (on Day 4 only for 8-day infections). No proliferation was evident on Day 4 post-second infection (Fig. 2a). On Day 6 post-second infection a moderate spleen cell response ($P < 0{\cdot}001$) was observed in cells cultured 4, 5 and 6 days *in vitro* prior to the addition of [^{3}H]TdR (Fig. 2b). By Day 8 post-infection no proliferation was observed for either population of cells (data not shown).

Next, we examined the ability of MLN cells and spleen cells from infected and control mice to proliferate *in vitro* when cultured with Con A. B10.T (6R) mice, four per group, were primed with 50 L_3 on Day 0, treated with ivermectin on Day 14, and challenged with 80 L_3 on Day 45. Six days later MLN cells and spleen cells were harvested from these mice, single cell suspensions prepared, and cells were cultured in the presence of Con A. The strength of the Con A-induced response was similar for MLN cells and spleen cells harvested from uninfected mice (Table 2). In addition, the response of MLN cells from infected mice resembled the response of cells from uninfected controls. However, spleen cells from infected mice responded more strongly to Con A than did cells from controls, suggesting that these cells may be highly activated.

All the experiments reported above were conducted in B10.T (6R) mice, a strain known to be quite susceptible to challenge infection with *N. dubius* (F. J. Enriquez, B. O. Brooks, R. H. Cypess, C. S. David and D. L. Wassom, manuscript in preparation). Therefore, we compared the responses of cells taken from B10.M (*H-2^f*) and B10.BR (*H-2^k*) mice, resistant and susceptible H-2 congenic strains, respectively (F. J. Enriquez, B. O. Brooks, R. H. Cypess, C. S. David and D. L. Wassom, manuscript in preparation), six days following either a primary, secondary, or tertiary infection (Table 1). Consistent with earlier results, spleen cells responded more strongly than MLN cells following a secondary infection in both strains ($P < 0{\cdot}05$) (Fig. 3). However, MLN cells from the resistant B10.M mice responded strongly following the third infection, a response not observed for cells harvested from the susceptible B10.BR mice ($P < 0{\cdot}05$). These results prompted us to explore the possibility that suppressor cells, localized in the gut-associated lymphoid tissue (GALT) were preferentially induced in the susceptible strains. In an attempt to demonstrate the presence of such suppressor cells we cultured spleen cells from secondarily-infected B10.T(6R) mice with increasing numbers (10^2, 10^3, 10^4, and 4×10^4) of their own MLN cells at a total number of 4×10^5 cells/well. As shown in Table 3, the response of spleen cells was unaltered by the addition of MLN cells at all antigen and cell concentrations tested.

Adoptive transfer of immunity

Spleen or MLN cells from multiply infected B10.M mice (Table 1) or from uninfected controls were injected intravenously (1×10^7 cells/mouse) into previously uninfected syngeneic recipients. Because early attempts to transfer immunity to *N. dubius* with cells alone had been unsuccessful in our laboratory, we

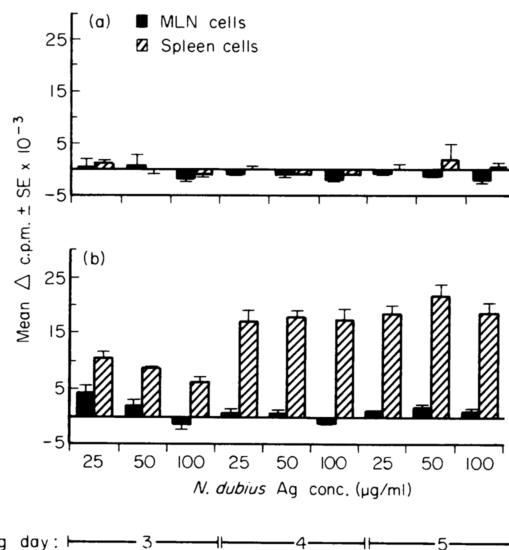

Figure 2. Cell proliferation after a secondary *N. dubius* infection in presence of adult worm antigen. The cells were harvested from mice ($n = 4$ per group) (a) 4 days post-challenge infection, or (b) 6 days post-challenge infection. The cultures were pulsed with [^{3}H]TdR at either 3, 4, or 5 days post-culture.

Table 2. [^{3}H]Thymidine incorporation of cells from uninfected and secondarily infected mice cultured in presence of Con A*

	Mean Δ c.p.m. ± SE × 10^{-3}	
	MLN cells	Spleen cells
Uninfected	84·0 ± 18·5	122·3 ± 52·5
Infected	103·0 ± 16·5	217·7 ± 18·8†

* B10.T(6R) mice ($n = 4$ per treatment). Cultures were pulsed 48 hr post-incubation.
† Significantly different from the other treatments ($P < 0·05$).

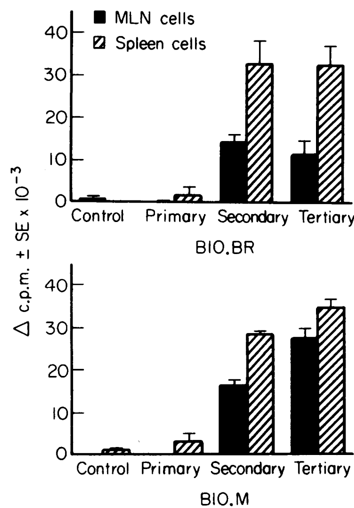

Figure 3. Proliferation of MLN cells and spleen cells of B10.BR and B10.M mice in presence of 100 μg/ml adult worm antigen. C.p.m. shown for uninfected controls or 6 days post-primary, secondary, or tertiary *N. dubius* infection. The cultures were pulsed with [^{3}H]TdR 5 days post-culture.

Table 3. Influence of increasing numbers of MLN cells on spleen cell cultures*

	Mean Δ c.p.m. ± SE × 10^{-3}			
	Number of MLN cells/ml per well			
Ag Conc. μg/ml	10^3	10^4	10^5	4×10^5
25	20·3 ± 4·3	18·8 ± 3·5	20·7 ± 4·1	20·8 ± 3·3
50	32·1 ± 8·0	29·6 ± 7·0	33·7 ± 5·6	32·9 ± 5·8
100	44·0 ± 6·5	47·6 ± 7·1	46·0 ± 7·2	37·8 ± 8·6

* B10.T(6R) mice ($n = 4$ per group).

Table 4. Adoptive cell transfer of resistance to *N. dubius* with Ag boost in B10.M mice

	Boost			Mean % of control ± SE	
Transferred cells*	Ag	FCA	Ag/FCA		
Normal Spleen	–	–	–	118·6	17·1
Normal Spleen	–	–	+	112·4	1·7
Normal MLN	–	–	–	115·5	8·7
Immune MLN	–	–	–	111·4	11·2
Immune MLN	+	–	–	87·2	17·6
Immune MLN	–	–	+	29·4	6·4†
Immune spleen	–	–	+	23·3	6·7†
Immune MLN/spleen	–	–	+	7·8	7·8†
—	+	–	–	96·4	17·7
—	–	+	–	98·8	15·5
—	–	–	+	111·6	8·8

* 1×10^7 cells i.v.
† Significantly different from control ($P < 0·05$)

injected mice with 100 μg of *N. dubius* Ag i.p. in FCA 24 hr following the cell transfer. Cells from uninfected mice were unable to transfer resistance (Table 4). However, either MLN cells or spleen cells from immunized mice, in conjunction with an antigen boost, protected recipients to a significant degree ($P < 0·05$). Immune cells alone, or the antigen boost in the absence of cells, had no effect.

DISCUSSION

Primary infections with *N. dubius* are rejected slowly by mice and it has been suggested that such mice have suppressed immune responses (Pritchard *et al.*, 1983; Ali & Behnke, 1984a). Evidence for parasite-induced immunosuppression comes from studies showing that infected hosts respond poorly to heterologous antigens or mitogens (Shimp, Crandall & Crandall, 1975; Price & Turner, 1983; Ali & Behnke, 1984b; Losson, Lloyd & Soulsby, 1985) and may not respond normally to infection with other parasites (Chowaniec, Wescott & Congdon, 1972; Jenkins, 1975; Jenkins & Behnke, 1977; Hopkins, 1980; Behnke, Wakelin & Wilson, 1978; Bottjer, Hirsh & Slonka, 1978; Hagan & Wakelin, 1982). We found it difficult to demonstrate *N. dubius* antigen-specific proliferation of lymphocytes during primary infection, although some proliferation was evident in mice treated with low doses of cyclophosphamide, a treatment reported to preferentially abrogate suppressor cell activity. We demonstrated *N. dubius*-specific lymphocyte proliferation following challenge infections, but here too the response of cells from the MLN was not augmented when compared to cells from the spleen. If suppressor cells are induced in the GALT and the effects of these cells are localized in the gut, responses of MLN cells may be depressed in comparison to cells from peripheral sites. We were unable, however, to suppress the spleen cell response by adding MLN cells to spleen cell cultures.

Spleen cell-proliferative responses to *N. dubius* Ag were augmented in both resistant and susceptible strains of mice experiencing secondary or tertiary infections (Fig. 3). However, following a tertiary infection, the proliferation of MLN cells from susceptible mice was significantly reduced when compared

to the response of MLN cells from resistant mice. This observation supports the hypothesis that the immune response occurring in the gut may be more easily suppressed in susceptible than in resistant strains of mice. We have shown that susceptible strains of mice will resist *N. dubius* challenge if they are first immunized via infections which are terminated before adult worms emerge into the gut lumen (6-day abbreviated immunizing infection). Thus, adult worms living in the gut lumen may actively suppress an otherwise functional response (F. J. Enriquez, R. H. Cypess and D. L. Wassom, manuscript in preparation). This may have been responsible for the lack of proliferation of both spleen cells and MLN cells on Day 8 following challenge of B10.T(6R) mice. In all the other experiments reported herein, the cell populations studied were harvested from mice which had never been exposed to adult worms (priming infections were always terminated with ivermectin 6 days post-infection). Therefore, if the proliferative response of MLN cells was actively suppressed in susceptible mice, larval worms as well as adult worms may be responsible for this suppression.

Transfer of immunity to *N. dubius* using lymphocytes from immunized hosts has been difficult to accomplish. Cypess (1970) suggested that transfer of some protection occurred using spleen cells from mice immunized with 4000 ex-sheathed larvae. Dobson & Owen (1978) reported transfer of resistance with immune MLN cells but such resistance, although statistically significant, was minimal. Behnke & Parish (1981) were unable to transfer protection using cells alone but could protect mice against challenge with irradiated larvae by injecting recipients with cells plus serum from immune donors. However, because the challenge infection consisted of irradiated larvae, a significant variable was added to the system which does not necessarily allow extrapolation to effector mechanisms associated with a normal larval challenge.

In preliminary studies we found that resistance could be transferred with cells from immune donors if recipients were also injected with worm extract in adjuvant, either FCA or Alum (unpublished observations). The levels of resistance transferred varied from experiment to experiment and in some cases resistance could not be demonstrated in cell recipients. The present studies suffered from some inter-experiment variability. However, the data herein represent an effect seen repeatedly, albeit one which is difficult to reproduce consistently. In these studies, cells alone had no effect. It is known that the transfer of cells to naive recipients may induce suppression such that the effects of the transferred cells are not manifested (Hagan & Wakelin, 1982). MLN from mice concurrently infected with *Trichinella spiralis* and *N. dubius* failed to transfer immunity to *T. spiralis*, in contrast to a successful transfer of resistance from mice infected with *Trichinella* alone; in addition, the blast transformation in the presence of *T. spiralis* Ag of cells obtained from *T. spiralis*-infected mice was depleted when the mice were concurrently infected with *N. dubius* (Hagan & Wakelin, 1982). It is possible that in the *N. dubius* system, the antigen boost serves to prime the system in such a way that preferential stimulation of protective rather than suppressive effectors occurs. At present however, we do not know why the antigen boost is required. However, having characterized an adoptive transfer protocol which allows resistance to be transferred with cells in the absence of serum, it should now be possible to identify and characterize the subpopulations of cells responsible for the observed effect. This can be achieved by selectively eliminating subpopulations of cells prior to the transfer using monoclonal antibodies specific for the cell-surface antigens which correlate with the functional characteristics of these cells. Such studies can be confirmed by Ag-specific *in vitro* proliferation of cells which have been subjected to the same monoclonal antibody treatment. Regarding this, preliminary studies suggest that T cells bearing the Ly 1^+2^- and either the Ly 1^-2^+ or Ly 123^+ phenotypes are required for successful *in vitro* proliferation or adoptive transfer to occur. Further characterization of the cells which participate in these reactions is required before conclusions can be drawn regarding which cells regulate and effect the anti-parasite response.

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