

SUPPRESSION OF HETEROLOGOUS IMMUNITY BY *NEMATOSPIROIDES DUBIUS* ANTIGENS *IN VITRO*

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(Received 20 July 1987; accepted 15 July 1988)

Abstract—CRAWFORD C., BEHNKE J. M. and PRITCHARD D. I. 1989. Suppression of heterologous immunity by *Nematospiroides dubius* antigens *in vitro*. *International Journal for Parasitology* **19**: 29–34. The direct effect of the soluble antigens in the homogenate of adult *Nematospiroides dubius* (AH) on spleen cells from uninfected NIH mice was investigated using a Mishell–Dutton culture system. Parasite antigens were shown to reduce the plaque-forming cell (PFC) response to sheep red blood cells (SRBC) in a dose-dependent manner *in vitro*. A population of suppressor cells was demonstrated in the spleens of infected mice. Furthermore naive spleen cells cultured in the presence of AH gave rise to cells which depressed the PFC response of naive cells when subsequently cultured together *in vitro*. Treatment of these cell populations with anti-thy 1.2 plus complement did not impair suppressor activity, and it was concluded that cells expressing the T-cell phenotype were not involved.

INDEX KEY WORDS: *Nematospiroides dubius*; plaque forming cells (PFC); sheep red blood cells (SRBC); suppressor cells.

INTRODUCTION

THE trichostrongyle nematode *Nematospiroides dubius* survives in the mouse small intestine for 8 months or more in a primary infection (Ehrenford, 1954; Keymer & Hiorns, 1986), and exerts a depressive effect on the host's ability to respond to heterologous (Chowaniec, Wescott & Congdon, 1972; Shimp, Crandall & Crandall, 1975; Price & Turner, 1986a, b) and homologous antigens (Pritchard & Behnke, 1985; Sitepu, Dobson & Brindley, 1985). In mice concurrently infected with *N. dubius* and *T. spiralis*, the survival time of the latter parasite is prolonged compared to controls (Behnke, Wakelin & Wilson, 1978). A primary infection with *N. dubius* is also known to severely depress the host's immune response to concurrently administered sheep erythrocytes (Shimp *et al.*, 1975; Ali & Behnke, 1983), and it can be demonstrated that the degree of suppression is a function of the number of adult worms present in the small intestine (Ali & Behnke, 1983). Suppression has also been shown to be maximal 14 days after infection, corresponding to the presence of the adult stage in the lumen, rather than the L4 stage which resides in the gut wall (Ali & Behnke, 1983). It has been proposed that *N. dubius* facilitates its own survival in the gastrointestinal tract in the face of an immune response by the production of immunomodulatory factors (Behnke, Hannah & Pritchard, 1983; Behnke, 1987) which may also be implicated in suppression of heterologous immunity. Thus mice given a normally immunogenic infection with irradiated larvae at the same time as an

infection with normal larvae do not become immune to challenge (Behnke *et al.*, 1983), and homologous immunity stimulated by an anthelmintic abbreviated infection is reduced on injection with soluble adult antigen (Pritchard & Behnke, 1985), as is the response to the heterologous antigen, sheep red cells (SRBC) (Pritchard, Ali & Behnke, 1984).

The present study was designed to confirm and expand these *in vivo* observations by utilizing a Mishell–Dutton culture system, in which normal splenocytes are stimulated *in vitro* with SRBC, and their responsiveness assessed in a plaque (PFC) assay. This system is advantageous in that it has proven to be more manipulable than *in vivo* systems, and requires significantly less material to modulate immune responsiveness.

MATERIALS AND METHODS

Animals. Female NIH mice, 6–8 weeks old, bred under conventional animal house conditions, were chosen for this study following the demonstration that this strain was responsive to sheep erythrocytes using an *in vitro* Mishell–Dutton culture system.

Antigens. Nematode antigens were prepared as described previously (Pritchard, Williams, Behnke & Lee, 1983) and filter sterilized prior to use. Sheep erythrocytes were kindly supplied by Mr Walker, School of Agriculture, Nottingham University, Sutton Bonington. *Anti-Thy-1.2* was obtained from Serotec Ltd, Blackthorn, Bicester, England. The optimum dose for complement mediated cytotoxicity of 99% of cells was predetermined using NIH mouse thymocytes, and was subsequently used at a concentration of

1/1000. *Low toxicity rabbit complement* was obtained from Cederlane Laboratories Ltd, Hornby, Ontario, Canada, and used at a dilution of 1 in 12.

In vitro culture. An *in vitro* assay to analyse the immunoregulatory effect of *N. dubius* antigens was developed essentially as described by Mishell & Dutton (1966, 1967). Briefly, spleen cells from female NIH mice were re-suspended in RPMI 1640 medium supplemented with 5% fetal calf serum and antibiotics (100 i.u. per ml penicillin and 100 µg per ml streptomycin) to give a final concentration of 1×10^7 cells per ml. To each well of the tissue culture tray 0.5 ml of this suspension was added, together with 0.5 ml of the parasite antigen at the required concentration, or with 0.5 ml of the second cell population (see results). The cultures were then inoculated with 30 µl of a 1% suspension of sheep erythrocytes, and incubated at 37°C in a humidified CO₂ incubator for 4 days. After this time, the cells were harvested and assayed for the presence of direct (IgM) plaque forming cells against sheep erythrocytes using Cunningham's method (Cunningham, 1965). Results are expressed as the number of PFC/10⁶ lymphocytes ± s.d.

Cell counts. Cell viability was determined by using fluorescein diacetate (Mishell & Mishell, 1980).

RESULTS

The effect of infection with N. dubius on the immune response of spleen cells to SRBC in vitro

Female NIH mice were infected with 250 L3 larvae of *N. dubius* on day 0 of the experiment, and their spleens removed on days 6, 9, 15 and 20 post infection. The responsiveness of single cell suspensions prepared from the spleens to SRBC was then assayed *in vitro*. Unchallenged cultures were tested in parallel.

From the data in Fig. 1 it can be seen that the immune responsiveness of the cell suspensions diminished as the infection progressed through the larval stages to adulthood on day 9. Furthermore, splenocytes from mice harbouring adult parasites (days 9–21) when co-cultured with naive cells from uninfected animals reduced the latter cell population's responsiveness to sheep erythrocytes *in vitro*.

The effect of adding the soluble antigens from the homogenate of adult N. dubius on the in vitro immune response of normal splenocytes to SRBC

Normal splenocytes were inoculated with SRBC in the presence of *N. dubius* AH and their ability to respond was assessed 4 days later in a direct PFC assay. In addition, splenocytes cultured in the presence of AH for 4 days were washed thoroughly to remove any extraneous antigen, transferred to naive cell cultures, and their ability to suppress the SRBC response of the naive cell population was investigated.

Table 1 demonstrates that soluble adult worm antigens could directly reduce the number of PFC in inoculated cultures in a dose-dependent manner. Therefore the higher the dose of *N. dubius* antigen added to the cultures, the greater the suppression of the PFC response observed, compared to cultures

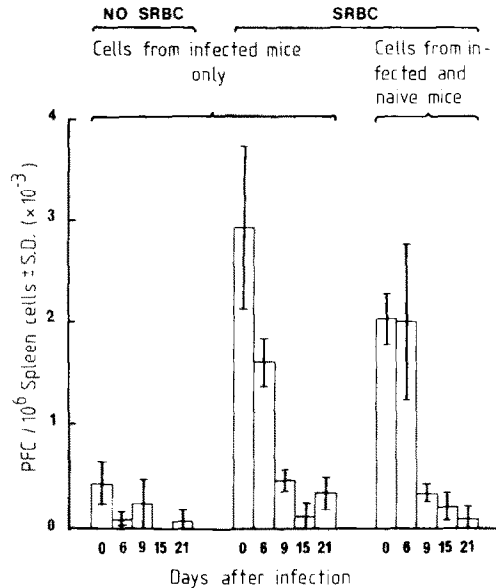


Fig. 1. *In vitro* response to SRBC of spleen cells from mice infected with *N. dubius* for varying periods of time and the effect of these cells on splenocytes from uninfected animals. 5×10^6 cells from mice infected for different periods of time were cultured *in vitro* with 30 µl of 1% SRBC (immunized controls) or without sheep erythrocytes (unstimulated controls). Additionally, cells from infected mice were co-incubated with 5×10^6 cells from naive mice together with SRBC. Four days after initiation of cultures, the cells were harvested and PFC assay carried out.

receiving only sheep erythrocytes. However, the degree of suppression varied between experiments for a defined quantity of adult antigen, possibly because of differences in the batches of fetal calf serum and SRBC. The crude worm homogenate caused suppression in this system, without any noticeable toxic effect on cells (as measured by viability), even when used at high concentrations.

This result was explored further to investigate effector cells in this system, and the data in Table 2 confirm that addition of adult antigen to splenocyte cultures reduced the number of PFC observed to sheep erythrocytes compared to controls. When normal spleen cells were incubated with 10 µg of AH for 4 days and then added to naive cell cultures together with SRBC, the PFC response was reduced. It appears that the greater the number of AH-treated cells added, the more the PFC were suppressed. Supernatants taken from cells incubated with adult antigen did not reduce the ability of normal splenocytes to respond to SRBC.

The effect of adding soluble adult worm antigens to spleen cell cultures at different time intervals after stimulation by SRBC

Normal splenocytes were incubated with SRBC in Mishell-Dutton cultures on day zero (A). Fifty

TABLE 1—THE EFFECT OF SOLUBLE ANTIGENS FROM THE HOMOGENATE OF ADULT *N. dubius* ON THE *in vitro* IMMUNE RESPONSE OF NORMAL SPLENCYTES TO SRBC

Antigen added to tissue culture tray well ($\mu\text{g}/\text{well}$)*	PFC/ 10^6 cells \pm 1 s.d. (% of immunized control)		
	Experiment (1)	Experiment (2)	Experiment (3)
SRBC alone (immunized control)	1625 \pm 683	731 \pm 212	1053 \pm 210
None (unstimulated control)	130 \pm 25 (8)	54 \pm 45 (7)	111 \pm 150 (10)
SRBC + 1 μg adult homogenate	1200 \pm 75 (74)	576 \pm 87 (79)	678 \pm 280 (64)
SRBC + 10 μg adult homogenate	1050 \pm 311 (65)	532 \pm 115 (73)	275 \pm 220 (26)
SRBC + 100 μg adult homogenate	407 \pm 185 (25)	210 \pm 64 (29)	139 \pm 13 (13)

*Each well contained 5×10^6 spleen cells from uninfected female NIH mice.

microgrammes of *N. dubius* adult antigen was then added to separate cultures, either at the same time (C) as the sheep erythrocytes, or 1 (D), 2 (E) or 3 (F) days later. Four days after initiation of the experiment, the cells were harvested and assessed for antibody by the PFC assay.

The results in Fig. 2 demonstrate that the addition of adult antigen concurrently with SRBC suppressed the PFC response of the splenocytes to the latter antigen. Suppression was detected irrespective of whether AH was added on days 0, 1, 2 or 3, although the degree of suppression decreased with the increasing interval between the introduction of SRBC and adult antigen to the cultures.

Effect of incubating AH-treated cells with anti-Thy 1.2 plus complement

Spleen cells from normal NIH mice were incubated for 4 days either in medium alone or with 10 μg AH, after which time they were harvested and washed three times to remove any extraneous antigen. Half of the AH-treated splenocytes were then incubated with anti-Thy 1.2 plus complement to remove any T-cells which were present. The three cell populations were added to naive spleen cell cultures together with SRBC, and the PFC response was assessed.

Figure 3 shows that spleen cells treated with *N. dubius* adult homogenate when added to normal splenocytes reduced the PFC response of the latter cell population to SRBC. However, prior incubation of antigen treated spleen cells with anti-Thy 1.2 plus complement did not restore the PFC response to control levels.

DISCUSSION

The work presented in this paper set out to determine the direct effect of soluble adult *N. dubius* antigen on spleen cell responses to SRBC, in an attempt to understand further the mechanism by which suppression of heterologous immunity operates, and to pave the way for purification of immunomodulatory material from the parasite. Therefore the responsiveness of spleen cells taken from mice at

different times after *N. dubius* infection to a heterologous antigen was investigated *in vitro*. This first experiment (Fig. 1) confirmed the observations that mice harbouring adult worms (days 9–21) were less responsive to SRBC than animals with a larval infection (day 6) (Ali & Behnke, 1983). Moreover, it was

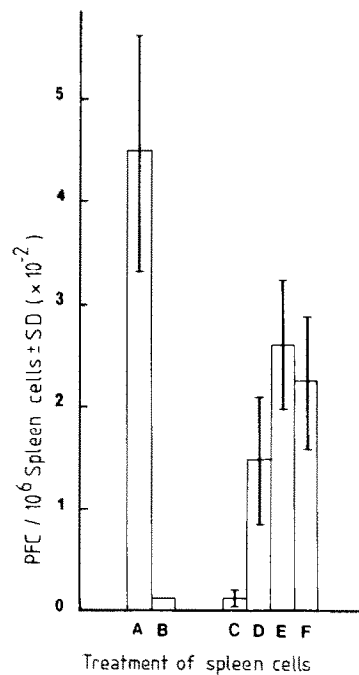


FIG. 2. The effect of adding soluble antigens from the homogenate of adult *N. dubius* (AH) to spleen cells from uninfected mice at different time intervals after stimulation by SRBC *in vitro*. 5×10^6 cells from naive mice were incubated with (A—immunized control) or without (B—unstimulated control) $30 \mu\text{l}$ of 1% SRBC. Splenocytes stimulated by SRBC *in vitro* were also cultured in the presence of 50 μg of AH, the latter being added to culture wells on day 0 (C), day 1 (D), day 2 (E) or day 3 (F) after initiation of incubation. All the cultures were harvested on day 4 for analysis of PFC.

TABLE 2—THE EFFECT OF SPLEEN CELLS CULTURED IN THE PRESENCE OF SOLUBLE ANTIGENS FROM THE HOMOGENATE OF ADULT *N. dubius* (AH) ON THE IMMUNE RESPONSE OF NORMAL SPLENOCYTES TO SRBC *in vitro*

Antigen and/or cells added to tissue culture well*	PFC/10 ⁶ cells ± 1 s.d. (% of immunized well)
SRBC alone (immunized control)	1171 ± 229
None (unstimulated control)	356 ± 96 (30)
SRBC + 0.1 µg AH	1015 ± 63 (87)
SRBC + 1.0 µg AH	950 ± 77 (81)
SRBC + 10.0 µg AH	325 ± 54 (27)
SRBC + supernatant from 0.1 µg AH/well†	1008 ± 118 (93)
SRBC + supernatant from 1.0 µg AH/well	1499 ± 232 (128)
SRBC + supernatant from 10.0 µg AH/well	1960 ± 255 (157)
SRBC + 5 × 10 ⁶ cells from 10.0 µg AH/well‡	0 (0)
SRBC + 1 × 10 ⁶ cells from 10.0 µg AH/well	495 ± 53 (42)
SRBC + 0.5 × 10 ⁶ cells from 10.0 µg AH/well	787 ± 73 (67)

*Each well contained 5 × 10⁶ spleen cells from uninfected female NIH mice.

†Supernatants were obtained from normal splenocytes cultured in the presence of AH at the concentration shown for 4 days.

‡Cells were obtained from cultures of normal splenocytes incubated with 10 µg of AH for 4 days.

demonstrated that splenocytes from mice harbouring adult parasites reduced the response of naive spleen cells to sheep erythrocytes, indicating the presence of a suppressor cell population in these animals. Similarly, soluble adult worm antigens were shown to directly reduce the *in vitro* PFC response of naive splenocytes in a dose dependent manner (Table 1). This also appeared to be due to a suppressor cell population, since the reduction in plaques of normal cells was a function of the transferred AH-treated cells and not the supernatants (Table 2). Furthermore, a reduction in the PFC response of splenocytes was observed even when adult antigen was added 3 days after inoculation of SRBC into Mishell-Dutton cultures (Fig. 2), indicating the rapid generation and influence of the suppressor cell on the production of IgM antibody to sheep erythrocytes. Treating cells incubated with adult *N. dubius* antigen with anti-Thy 1.2 plus complement to remove all T-cells did not eliminate the *in vitro* generated suppressor cell population, therefore T-cells are unlikely to be responsible. This conclusion is supported by the work of Price & Turner (1986a) who investigated *in vivo* responses to ovalbumin administered with adjuvant in *N. dubius* infected mice. Variable suppressor activity was identified in spleen cells taken from infected mice transferred to irradiated recipients, which was not mediated by T-cells. However, a role for T-cells in suppression of heterologous immunity has possibly been implicated by the restorative effect of 2'-deoxyguanosine treatment on the immune response of *N. dubius* infected mice (Pritchard *et al.*, 1984), although macrophage mediated suppression of the proliferative response of thymocytes to concanavalin A in rats can be abolished by 2'-deoxyguanosine, but this has not yet been shown in mice (Bril, van den

Akker, Hussaarts-Odijk & Benner, 1985). However, suppression is not the property of adult *N. dubius* antigen alone, for day 6 larval antigen was also able to reduce the PFC response in culture to SRBC, as were homogenates prepared from *Necator americanus*. Other nematode antigens such as those obtained from *Ascaris suum* gave different results, actually increasing the PFC response *in vitro* (personal observations).

Suppressor cells have been implicated as mediators of non-specific suppression to heterologous antigens in other parasitic infections. For example the response of mice infected with *T. brucei* to SRBC is depressed (Murray, Urquhart, Murray & Jennings, 1973) and in murine malarial infections of *P. yoelii* and *P. berghei* impairment of humoral responses to sheep erythrocytes was observed (Salaman, Wedderburn & Bruce-Chwatt, 1969; Greenwood, Playfair & Torigiani, 1971). Experiments have shown that an excess of normal resident macrophages can profoundly depress lymphocyte proliferation, and if the macrophages are activated, the effect is even more pronounced (Kaufman, Simon & Hahn, 1982). All stages of *N. dubius* can activate complement by the alternative pathway (Prowse, Ey & Jenkin, 1979) so cleaving the complement component C₃ into C_{3a} and C_{3b} fragments, the latter of which is a potent agent for activating macrophages. Additionally the surface of many nematodes is polyanionic (Himmelhoch & Zuckerman, 1983; Murrell & Graham, 1983), a factor which can contribute to the activation of macrophages, and activated macrophages are known to be associated with chronic inflammation, such as occurs during infection with *N. dubius* (see Allison, 1978).

The possibility that a non-T suppressor cell might be involved in the non-specific suppression observed during infection of mice with *T. brucei* was raised by

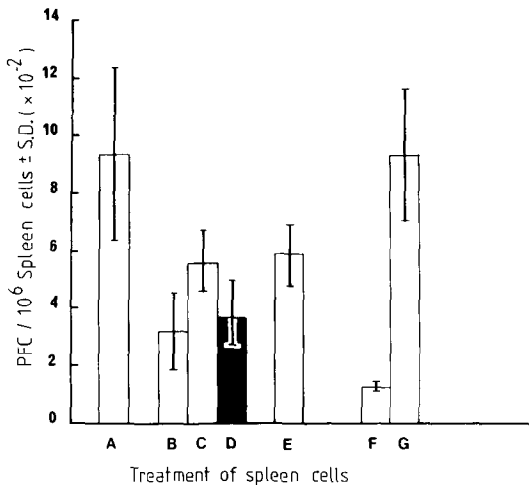


FIG. 3. The effect of anti-Thy 1.2 + complement on the ability of spleen cells cultured in the presence of the soluble antigens from the homogenate of adult *N. dubius* (AH) to suppress the PFC response of naive splenocytes to SRBC. The experiment comprised the following groups: A. 5×10^6 naive splenocytes + SRBC + 5×10^6 naive cells after incubation in medium for 4 days. [Naive splenocytes from uninfected mice were incubated in medium without AH for 4 days but were otherwise treated identically to cells exposed to AH prior to addition to these cultures.] B. 5×10^6 naive splenocytes + SRBC + 5×10^6 AH treated cells. [Naive splenocytes were incubated in the presence of $10 \mu\text{g}$ of AH for 4 days prior to addition to these cultures.] C. 5×10^6 naive splenocytes + SRBC + 2.5×10^6 AH treated cells. [Naive splenocytes were incubated in the presence of $10 \mu\text{g}$ of AH for 4 days prior to addition to these cultures.] D. 5×10^6 naive splenocytes + SRBC + 2.5×10^6 AH treated cells after incubation with anti-Thy 1.2 + complement. [Naive splenocytes were incubated in the presence of $10 \mu\text{g}$ of AH for 4 days and were then treated with anti-Thy 1.2 + complement before addition to these cultures.] E. 5×10^6 naive splenocytes + SRBC + $10 \mu\text{g}$ AH. F. 5×10^6 naive splenocytes [no SRBC; unstimulated controls]. G. 5×10^6 naive splenocytes + SRBC [immunized control].

Murray *et al.* (1973), who found proliferation of mononuclear phagocytes in the lymphoid organs of infected mice. Corsini, Clayton, Askonas & Ogilvie (1977) found that macrophages obtained from mice profoundly reduced the ability of normal mouse spleen cells to proliferate and secrete antibody when cultured with lipopolysaccharide (LPS). Eardley & Jayawardena (1977) also observed the generation of adherent cells, able to depress antibody responses of normal spleen cells, present in spleens of *T. brucei* infected mice. However, other workers (Jayawardena, A. N. 1977. Abstract in *Proceedings of the Vth International Congress on Protozoology*, p. 72) have suggested that thymus dependent lymphocytes are involved in the generation of non-specific adherent suppressor cells during the course of murine malarial infections, and interactions of T-cells and macrophages have been involved in other suppressive

effects, for example of contact sensitivity (Asherson & Zembala, 1974). It has been postulated that the triggering of the release of suppressor factors by macrophages may be antigen specific, while the factors themselves are non-specific (Allinson, 1978).

The significance of the observation that parasitic helminths cause non-specific immunodepression in the host is not clear, but one possibility is that the organisms facilitate their own survival through reducing host immunocompetence (Ogilvie & Wilson, 1976; Behnke, 1987). However, parasite induced immunosuppression does not always prevent the host from responding to parasite antigens. For example, in *Trypanosoma brucei* infections, there was no evidence that the parasite caused any significant reduction of antibody responses in acute infections (MacAskill, Holmes, Jennings & Urquhart, 1981). Furthermore, in the case of *N. dubius*, mice infected with irradiated larvae, which generate good immunity to challenge, have a reduced capacity to respond to SRBC (Ali & Behnke, 1984). Thus the interrelationship of specific and non-specific immunodepression during *N. dubius* infection is still debatable and their relative importance to parasite survival is controversial.

One possibility may be that the role of immunomodulatory factors secreted by *N. dubius* is principally to incapacitate the host's defences in the intestine in the microenvironment created by the parasite (Pritchard & Behnke, 1985), and that the systemic non-specific effects are a consequence of this activity (Behnke, 1987). Further analysis of this system should reveal the precise relationship between parasite-specific and non-specific immunodepression in *N. dubius* infected mice, and the survival value of such strategies to the parasite.

Acknowledgements—We would like to thank Professors D. Wakelin and P. N. R. Usherwood for the facilities provided for the work in the Department of Zoology. We are also indebted to Mrs J. Brown for technical assistance and Mr K. Cosgrove for animal maintenance. The support of the Medical Research Council through grants G8328675/T and G8100159/T is also gratefully appreciated.

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