Acanthocheilonema viteae (Dipetalonema viteae) in mice: differences in the relative binding of microfilarial surface-specific antibody may explain the contrasting response phenotypes of BALB/c and C57BL/10

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ABSTRACT

Experiments were carried out to obtain additional data concerning the role of IgM antibodies, specific for the cuticular surface of the microfilariae (mf) of *A. viteae*, in clearing microfilaraemia from high- and low-responder mice infected by transplanted adult worms. Although BALB/c mice, which sustain a chronic microfilaraemia, produced IgM mf surface-specific antibodies, the binding to target mf was weak when compared to that of antibodies from the serum of the resistant C57BL/10 mice. Furthermore, antibodies from BALB/c mice were not as efficient as those from C57BL/10 mice in promoting the adherence of immune or control leukocytes to mf *in vitro*. Evidence is provided to show that mf shed surface bound antibody. Although the results do not establish conclusively the mechanism underlying the contrasting response phenotypes of C57BL/10 and BALB/c mice, they provide support for the involvement of antibody in controlling microfilaraemia and suggest that quantitative and qualitative differences in the amount and affinity of IgM antibody specific for the mf surface, together with the natural tendency of the mf to shed surface bound antibody at 37°C, may combine to allow the former strain to clear microfilaraemia efficiently whilst the latter sustains a chronic infection.

KEY WORDS: *Acanthocheilonema viteae*, mouse, immunity, microfilariae, IgM

INTRODUCTION

Acanthocheilonema viteae is a natural filarial parasite of jirds, in which the adult worms are located subcutaneously and give rise to a microfilaraemia with reservoirs of microfilariae (mf) in the lungs, liver and muscles (WORMS *et al.*, 1961). In experimental infections in hamsters the parasite produces a heavier parasitaemia than in its natural host (WEISS, 1970), but the resultant microfilaraemia is generally cleared within 120 days of infection, despite the continued persistence of live adult worms. Mice, in contrast, are resistant to infection and inoculated infective larvae do not reach maturity. However, if gravid female worms are implanted surgically into mice they survive long enough to produce a microfilaraemia (HAQUE *et al.*, 1980) which varies greatly in intensity and duration among different strains (STOREY *et al.*, 1985). This variation in degree of infection is reminiscent of that seen (reflects the different clinical and immunological states arising) in humans with filariasis although, as in the human disease, the factors responsible are still not understood.

Initial studies on the immune mechanisms that result in the clearance of microfilaraemia in mice provided evidence for an IgM mediated immune attack (THOMPSON *et al.*, 1979; PHILIPP *et al.*, 1984). Thus, post-infection IgM that bound

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to the surface of mf was found in CBA/H (resistant) mice, but not in CBA/N (susceptible) mice which have defective antibody responses (particularly IgM) to certain T-independent antigens (SCHER et al., 1975). BALB/c mice were also found to be incapable of controlling microfilaraemia and hence sustained chronic infections lasting some 5–6 months (HAQUE et al., 1980; STOREY et al., 1985). In contrast many other strains, including C57BL/10 mice, were found to clear mf within 2 months of transplantation. Initially this strain-dependent difference in response was believed to be related to the ability to generate mf-specific IgM antibodies (STOREY et al., 1987), however, ALMOND et al. (1987), using more sensitive assays than those employed by previous workers, were able to demonstrate IgM mf-specific antibodies in BALB/c mice. The earlier interpretation of the mechanism underlying the responder/non-responder phenotypes of different mouse strains therefore requires some revision.

A variety of approaches have been used in attempts to define the differences in response between BALB/c and C57BL/10 mice which might account for the differential susceptibility to microfilaraemia. It is clear that antibody is involved in some way; passive transfer of post-microfilaraemia (immune) serum from C57BL/10 mice into microfilaraemic BALB/c mice resulted in a substantial but transient reduction in microfilaraemia (STOREY et al., 1989). Numbers of circulating mf dropped by over 80% but then returned to pre-transfer levels within 6 hours. Radiation chimaera experiments, in which bone marrow stem cells from resistant B10/D2n (H-2 matched with BALB/c) were injected into lethally irradiated BALB/c recipients, established that resistance was mediated through the progeny or the products of cells developing from bone marrow precursors (STOREY et al., 1985). It thus appears that, as both strains can produce anti-mf IgM, the difference in response phenotype reflects either some cellular component necessary for the anti-mf function of that antibody and/or some qualitative difference in the antibody itself.

In the present paper we report that IgM antibodies reactive with the surface of the mf of A. viteae are indeed detectable by the indirect fluorescent antibody assay (IFAT) in BALB/c mice infected with this parasite, but that there is a significant difference in the stability of the binding between mf-specific IgM antibody from the two mouse strains and target mf, which could explain the difference in their ability to control microfilaraemia. We also present evidence from both IFAT assays and leukocyte adherence assays for the shedding of antigen bound to the mf surface.

MATERIALS AND METHODS

Maintenance of parasite life cycle

A. viteae was cyclically maintained in jirds (Meriones unguiculatus) and ticks (Orinthodoros moubata) as described (WORMS et al., 1961; STOREY et al., 1985). Hamsters were also infected to provide additional parasite material.

Isolation of parasites

Microfilariae were isolated from the blood of infected jirds using a DEAE-cellulose column, as described previously (STOREY et al., 1987). Adult worms were removed from the pelt and carcass of hamsters killed 96 days after infection.

Adult A. viteae were implanted subcutaneously into six- to ten-week-old female BALB/c Ola (BALB/c) and C57BL/10/ScSn/Ola as described by STOREY et al. (1985). Mice were killed 27 days post implantation to provide sera and cells for use in the experiments.

Indirect fluorescent antibody test (IFAT)

Microfilariae used in this assay were either freshly isolated from the blood of infected jirds or were used following one cycle of freezing and thawing in liquid nitrogen (cryopreserved). Cryopreservation has previously been shown to preserve mf in a viable condition, although not all recover from such treatment (HAM & BIANCO, 1981). Serum was collected not more than one day prior to use, to avoid the need for freezing and thawing.

Microfilariae (1×10^4) were resuspended in 100 µl test serum and incubated for one hour at either 37°C or at 4°C. The mf were then washed three times in PBS and resuspended in 100 µl of a 1/10 dilution of rabbit anti-mouse IgM (Nordic) and incubated for 30 min at 4°C. They were then washed another three times and resuspended in 100 µl of a 1/20 dilution of a FITC conjugated sheep anti-rabbit IgGAM (H and L) (Serotec), and incubated for a further 30 min at 4°C. After a further three washes the mf were resuspended in PBS with glycerol (1:1), mounted on a microscope slide under a cover slip and examined for fluorescence under a UV light source. Two different methods of washing were used: mf were either centrifuged in an MSE Chilspin centrifuge in 15 ml plastic conical centrifuge tubes, in which case the pellet was gently resuspended using a Pasteur pipette ('gentle washing'), or in a microcentrifuge (Sarstedt MH2) in 1·5 ml plastic tubes, with the pellet resuspended more vigorously on a vortex mixer ('vigorous washing').

Some of these assays were carried out in the presence of sodium azide as a metabolic inhibitor. This was added to the test sera at a final concentration of 20 mM, and also included in the PBS for washing at the same concentration, as used by VETTER & KLAVER-WESSELING (1978).

Leukocyte adherence assays

The culture medium used was RPMI 1640 medium buffered with $NaHCO_3$ and 20 mM Hepes to pH 7·2, supplemented with penicillin (100 u/ml) and streptomycin (100 µg/ml). Murine peritoneal exudate cells were collected by lavage using 5 ml of heparinized medium. Leukocyte adherence assays were carried out in flat-bottomed microtitre plates using a method adapted from MACKENZIE *et al.* (1981). Cultures were set up in duplicate, with each well containing: 50 µl of microfilarial suspension in medium (200 mf); 50 µl cells in medium (2×10^5 cells); 50 µl of serum. Serum was collected fresh on the day of use and handled on ice to preserve complement activity. Control cultures were set up with the missing component replaced with 50 µl of medium to maintain the same volume. Cultures were incubated at 37°C in 5% CO_2 and observed after 20 h incubation. A scoring system was devised that took account of three parameters of cellular attack: killing of mf, as judged by motility; the total number of parasites within a culture with adherent cells; the extent of the covering of the surface of those mf with adherent cells. Each parameter was scored 0 to 3 and by totalling the scores for the three categories an overall adherence score of 0 to 9 was obtained. The maximum score of 9 therefore indicates positive adherence with killing of mf, a score of 0 indicates no effect.

RESULTS

IgM reactive to the surface of cryopreserved mf was found in the serum taken from both C57BL/10 and BALB/c mice, while normal mouse serum (NMS) was completely negative. However, the degree and intensity of staining of individual mf varied from negative, through weak and patchy positive to intense confluent positive. The results are recorded as % of mf staining − to +++ in each test.

A variable found to affect staining in the IFAT was the washing procedure. A more vigorous washing method (see Methods) resulted in a marked reduction in the number of mf staining with BALB/c serum (to 7%) but had less effect on the binding by C57BL/10 serum, some 54% of larvae remaining positive (Table I). Subsequent experiments were carried out using the gentle washing procedure.

A second variable to affect the IFAT result was the temperature of incubation. Those carried out at 37°C resulted in greatly reduced staining by C57BL/10 sera compared to those tests carried out at 4°C (Table II). In addition, the use of freshly isolated mf (as opposed to those that had been cryopreserved prior to use) reduced the amount of positive staining at 4°C (Table II) and produced a negative result at 37°C. Both observations suggest the shedding of label by live mf.

To determine whether larvae were shedding antigen or bound antibody from their surfaces by a metabolically active process, IFATs on freshly-isolated mf were also carried out in the presence of sodium azide as a metabolic inhibitor. This enhanced the positive staining seen with C57BL/10 serum and reduced, but did not totally prevent a gradual loss of positivity when the stained larvae were maintained at 20°C for 24 h (Table II). Significantly, a weak positive result with BALB/c serum and freshly isolated mf (as opposed to cryopreserved) was seen only in the presence of sodium azide: this serum was otherwise negative in the assay.

TABLE I. Immunofluorescent staining of cryopreserved microfilariae of *A. viteae*, using sera from C57BL/10 and BALB/c mice. Effect of washing procedure on degree of staining at 4°C.

Washing (1) procedure	Source of (2) serum	IFAT Score (3)			
		−	+	+ +	+ + +
Gentle	C57BL/10 (infected)	13	13	31	43
	BALB/c (infected)	27	43	25	5
	NMS	100	0	0	0
Vigorous	C57BL/10 (infected)	46	10	31	13
	BALB/c (infected)	93	7	0	0
	NMS	100	0	0	0

(1) Assays carried out at 4°C on cryopreserved, live mf.
(2) Infected serum obtained 27 days after incubation with transplanted adult *A. viteae*.
(3) Percentage of Mf staining − to + + +.
NMS = normal serum pooled from control C57BL/10 and BALB/c mice.

TABLE II. Immunofluorescent staining of microfilariae of *A. viteae*. Effects of treatment of microfilariae and different incubation temperatures on the binding of surface-reactive IgM.

Treatment of microfilariae	Source of serum (1)	Temperature of 1° incubations	Surface staining reaction (2)			
			−	+	+ +	+ + +
Cryopreserved	C57BL/10	4°C	13	13	31	43
	C57BL/10	37°C	91	9	0	0
Fresh	C57BL/10	4°C	29	40	24	7
	C57BL/10	37°C	100	0	0	0
	BALB/c	37°C	100	0	0	0
Fresh +	C57BL/10	37°C	8 (35)	30 (45)	50 (14)	12 (6)
Sodium azide	BALB/c	37°C	89	11	0	0

(1) Pooled sera taken 27 days after infection with transplanted adult *A. viteae*. (Normal mouse sera were negative in all assays).
(2) Percentage of mf staining − to + + +. Figures in brackets are scores made after 24 h at 20°C.

TABLE III. Adherence of mouse peritoneal exudate cells (PECs) to *A. viteae* mf.

Source of serum	Source of cells Adherence score (1)				Motility of mf in serum alone
	C57BL/10 infected (2)	BALB/c infected (2)	C57BL/10 control (3)	BALB/c control (3)	
C57BL/10 infected (2)	7	6	6	6	+++
BALB/c infected (2)	2	4	1	1	+++
C57BL/10 control (3)	4	4	4	5	+++
BALB/c control (3)	3	2	4	4	+++

(1) Results expressed as adherence score (0–9) after 24 h of incubation. Mean of two experiments.
(2) Pooled sera/cells taken 27 days after infection with transplanted adult *A. viteae*.
(3) Pooled sera/cells from uninfected mice.

TABLE IV. Evidence that antibodies mediating adherence of mouse peritoneal exudate cells (PECs) to *A. viteae* microfilariae, are shed during preincubation in post infection serum.

Preincubation (1) Serum	Incubation (2) Serum	Adherence (3) Score	Motility of mf in serum alone
Infection serum (4)	Infection serum	5·5	+++
Infection serum	NMS	3·0	+++
NMS	Infection serum	5·0	+++
NMS	NMS	2·0	+++

(1) Preincubation serum was added to mf 2 h prior to the addition of cells.
(2) Incubation serum was added immediately (less than 5 min) before addition of cells.
(3) Results are expressed as adherence score (0–9) after 24 h of incubation. Mean of two duplicate experiments. Cells from infected C57BL/10 mice were used.
(4) Infection sera were taken 27 days after transplantation of adult *A. viteae* to C57BL/10 mice.

Sera from implanted mice were then examined in a leukocyte adherence assay to see if antigen/antibody shedding could also be detected in this system. In initial experiments (Table III) adherence of PECs to mf in the presence of B10 serum was observed. The highest adherence score was seen when the cells were also from C57BL/10 mice. There was also a background adherence score in serum from uninfected C57BL/10 mice with all cell types used. Lower adherence scores were seen in the presence of sera from BALB/c mice (Table III), both infected and uninfected. In the case of C57BL/10 serum, adherence was reduced if a period of time was allowed to elapse after the addition of immune serum to mf, but prior to the addition of cells (Table IV).

DISCUSSION

The results presented here provide an explanation for previous conflicting reports regarding microfilarial surface-reactive IgM in the *A. viteae*/mouse model, and also show a difference between the C57BL/10 and BALB/c mouse strains in the stability of binding of the IgM. It is also demonstrated that mf actively shed surface antigen and/or bound antibody under *in vitro* conditions. Furthermore, it is clear from these results that cryopreservation of mf, although preserving larvae in a viable condition, alters their surface properties to a greater or less degree, so that care must be taken in interpreting previously reported findings where this technique was used.

In the current study, IgM reactive with the surface of cryopreserved mf was detected by IFAT in both C57BL/10 and BALB/c sera, but the staining seen with the latter was unstable and was easily reduced if a vigorous washing method was used. In experiments carried out by STOREY et al. (1987) such a process had been employed and surface bound IgM was not detected. This might indicate that BALB/c mice produce less IgM antibody or antibody of weaker affinity than that of C57BL/10 mice, or, alternatively, it might be that the target antigens recognized by the antibodies of the two strains are different and that those recognized by BALB/c mice are more easily lost from the surface of the worm. Unfortunately, comparison of affinities between the IgM produced by these two strains require purification of the antigens concerned and, as the target antigens of filariae are undefined at present this theory cannot yet be tested. Studies by YEN et al. (1986) have suggested that low affinity receptor interaction exists in a natural chronic filarial infection, that of quokkas infected with Breinlia macropi. The data obtained with C57BL/10 serum when the IFAT assay was carried out on larvae at 4° or 37°, and when a metabolic inhibitor was employed, strongly suggest that the degree of staining observed was influenced by the extent to which antigen, or antigen/antibody complexes were shed from the cuticle. However, even under conditions when shedding should have been minimal (+ azide) few mf bound BALB/c antibody. Under the conditions previously used in detection of anti-mf antibody (STOREY et al., 1987) such shedding coupled with the reduced and lower affinity binding of BALB/c IgM would also have contributed to the negative results obtained with sera from this strain.

In the leukocyte adherence assays a maximum score of 9 was never achieved. Even in positive adherence wells some individual larvae were seen free from any cells and vigorously motile. Similar observations have been reported in other systems, notably with regard to the mf of Dirofilaria immitis. Some mf did not show fluorescence in an IFAT assay using occult infection serum (RZEPCZYK & BISHOP, 1984) and some remained free of cells in an in vitro cytotoxicity assay (RZEPCZYK et al., 1986). These variations between mf in susceptibility to immune effectors were concluded to be related to intrinsic differences in antigenicity. However, HAMMERBERG et al. (1984) found that the mf of D. immitis shed surface-bound normal immunoglobulin within 5 min or so, although immunoglobulins from immune dogs persisted longer. Hence variable reactivity of mf may simply reflect a range of microfilarial viability, the more viable mf being able to evade host effector mechanisms more effectively through a more active shedding of surface antigens (EGWANG & KAZURA, 1987) and cuticular turnover (CARLOW, et al., 1987; SCOTT et al., 1988; DEVANEY, 1988).

At the other end of the scale a totally negative score was rarely seen, one or two cells adhered to a small minority of mf even in controls. For this reason, scores of 3 or less were considered negative. Thus, an attempt to quantify the adherence assay and take account of all parameters of adherence still gave only relative positive and negative results. The assays did reveal, however, that C57BL/10 serum promoted adherence of C57BL/10 PECs to mf and, to a lesser degree, PECs from C57BL/10 control mice and from infected and control BALB/c mice: In studies with Brugia pahangi, OXENHAM et al. (1984) also reported that cells from infected mice performed better in the adherence assay than control cells, and this is probably due to the increase in receptors for the Fc portion of antibody seen on the surface of activated macrophages and indicates that cell activation states are altered during infection. BALB/c serum did not mediate marked adherence in any of the assays.

The final experiments were carried out to test whether antigen shedding would

influence the degree of cell adherence. If shedding of bound antibody or antigen was taking place, then it could be expected that if mf were incubated at 37°C in positive adherence serum prior to the addition of cells, available antibody would be bound to the surface antigen, and then shed, rather than remaining on the microfilarial surface, resulting in a lower adherence score. This prediction was found to be correct when larvae were incubated in C57BL/10 serum for 2 h before the addition of cells.

Antigen shedding by mf would be a useful immune evasion mechanism for the parasite as has been demonstrated in other nematodes, e.g. in infective larvae of the hookworm *Ancylostoma caninum* (VETTER & KLAVER-WESSELING, 1978) and of the ascarid *Toxocara canis* (SMITH *et al.*, 1981). It has also been indicated in mf of *Brugia malayi* (EGWANG & KAZURA, 1987) and suggested, but not confirmed, in the *B. macropi*/quokka system (YEN *et al.*, 1986). Furthermore, the transient nature of the drop in microfilaraemia seen in implanted BALB/c mice following passive transfer of C57BL/10 serum (STOREY *et al.*, 1989) might be explained by this process. Larvae might become sequestered out of circulation following reaction with transferred antibody, but could escape if bound antigens and/or antibody were subsequently shed from the surface. The same reasoning might explain why some individual mf remain free of cells in a leukocyte adherence assay with C57BL/10 serum: bound antigen would be most rapidly shed from the healthiest mf until all the available antibody is complexed. Presumably permanent clearance of mf, as occurs in C57BL/10 mice, must involve the participation of a further as yet unidentified component. One possibility may be that temporary sequestration enables cytotoxic effectors to destroy mf through such mechanisms as the release of damaging oxygen radicals (RZEPCZYK & BISHOP, 1984).

The principal difference in response to *A. viteae* mf, between resistant and susceptible mouse strains, highlighted by this study was the relative stability of binding of IgM antibodies to the cuticular surface of target larvae. The mf surface-reactive IgM response of BALB/c mice was characterized by transient attachment, relatively easy dislodgement and ineffective promotion of leukocyte adherence when compared to the response of antibody from infected C57BL/10 mice. Whether this difference is in itself sufficient to explain the contrasting response phenotype of the two mouse strains, or whether other genetically-determined characteristics such as complement levels are involved, is not certain, but these results identify an important direction for future studies. Recent papers on antigen shedding from both *A. viteae* (APFEL & MEYER, 1990) and *Onchocerca* spp. (EDWARDS *et al.*, 1990) support this view.

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