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Strain-dependent protective effect of adult thymectomy on murine infection by *Mycobacterium lepraemurium*

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SUMMARY

C57BL/6, DBA/2, BALB/c and CBA mice were thymectomized as adults, or sham-thymectomized, and infected subcutaneously with 10^6 MLM. The number of MLM in the spleen and in the inoculated footpad was measured after 1 year of infection as well as the DTH reactions and the IgM and IgG antibody levels to MLM. Non-thymectomized mice exhibited a broad spectrum of resistance to MLM infection and of T cell mediated immunity grading from the highly resistant C57BL/6 strain to the highly susceptible CBA strain. In between, DBA/2 was found more resistant than BALB/c mice. Adult thymectomy reduced by 100 times the MLM number in the spleen of infected DBA/2 mice, without affecting that measured in the inoculated footpad, and significantly decreased DTH reaction in the same strain. No effect of adult thymectomy was observed in any other strain, except for an increase of anti MLM antibodies in BALB/c mice. These results may suggest that the medium-resistant DBA/2 strain develops after MLM infection suppressor T cells which favour MLM dissemination and are sensitive to adult

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INTRODUCTION

Experimental infection by *Mycobacterium lepraemurium* (MLM) in mice represents a model of chronic mycobacterial infection, a 'murine leprosy', which resembles the human disease in some aspects, with large strain-dependent variations in the resistance to the infection, mimicking the leprosy spectrum (Kawaguchi, 1959; Closs, 1975a). In this model, some studies (Closs, 1975b; Bach *et al.*, 1983) have shown in MLM-infected mice suppressor T-cells capable of decreasing the resistance of naive recipients to the infection in transfer experiments.

In the present study, we have used adult thymectomy as another tool to approach the problem of the role of suppressor T cells in the development of tolerance to MLM. Indeed adult thymectomy (ATx) in contrast to neonatal thymectomy, does not dramatically impair cell-mediated immunity, but leads to specific alteration of some functions of short-lived T cells, the most commonly and profoundly affected being the suppressor function (Kappler *et al.* 1974; Borel, 1980). In four mouse strains, chosen to cover the spectrum of murine resistance to MLM infection, we have followed ATx and sham-thymectomized mice, subcutaneously infected with MLM, for bacillary multiplication and dissemination, as well as for MLM-specific humoral and cellular immune responses. Effects of ATx on the outcome of MLM infection and on its immunological consequences markedly differed according to the strain, the most affected being the DBA/2 strain where ATx mice exhibited a pronounced reduction of bacillary dissemination, suggesting that thymus dependent suppressor cells develop in the course of infection and regulate protective T cell immunity.

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MATERIALS AND METHODS

Mice. Female C57BL/6, DBA/2, BALB/c and CBA inbred strains of mice were obtained from Pasteur Institute (Paris, France).

Surgical procedures. Groups of nine to 10 mice were thymectomized at 10–12 weeks of age by a suction method under pentobarbital anaesthesia. Control groups of nine to 10 mice were sham-thymectomized with the same procedure except for thymus suction.

MLM infection. The MLM strain, kindly donated by P. Lagrange (Pasteur Institute, Paris, France), was maintained as previously described (Hoffenbach, Lagrange & Bach, 1985). ATx or control mice were inoculated subcutaneously 3 weeks after the operation with 10^6 MLM into the left hind footpad, in 50 microlitres of saline. One year after the inoculation, all surviving mice were killed. The number of acid fast bacilli (AFB) in the inoculated footpads and in the spleens was counted using the auramin-staining method as already reported (Hoffenbach *et al.*, 1985).

Assessment of local granuloma size. Both the inoculated and the contralateral footpad were measured at intervals of 2 weeks during 46 weeks, using a dial gauge caliper (Schnelltaster, Hessen, West Germany). Results were expressed as the size difference between the infected footpad and the contralateral footpad.

Delayed-type hypersensitivity (DTH) to MLM. Forty-six weeks after inoculation, all mice were injected subcutaneously in the right hind footpad with 10^7 MLM, previously killed by heating for 1 h at 60°C. The size of the injected footpad was measured as described above before injection, and 24 h, 48 h and 72 h later. Footpad swelling resulting from the DTH reaction was measured as the difference between the footpad size before and after the injection of the heat-killed MLM suspension, expressed in $\text{mm} \times 10^{-1}$. Control non-infected mice were also injected into the right hind footpad with the same MLM suspension: the mean increase of the sensitized footpad was of 1·33 (SD:0·78), 0·77 (SD:1·01) and 0·58 (SD:0·9) at 24, 48 and 72 h respectively.

IgM and IgG antibodies to MLM. Mice were bled 46 weeks after inoculation, just before DTH assessment by retro-orbital puncture. Sera were stored at −20°C until tested. For each serum, IgM and IgG antibodies to MLM were concurrently measured by an ELISA assay as already described (Hoffenbach & Bach, 1986). In brief, a solution of heat-killed, sonicated and ultracentrifuged (100,000 *g* for 1 h) MLM was adjusted to a concentration of 5 μg protein/ml, coated into microtitre plates (Immulon 2, Dynatech laboratories, Virginia, USA), and dried at 37°C overnight. Plates were then saturated by phosphate buffered saline containing 5% bovine serum albumin. Two-fold serial dilutions of individual sera were introduced into the plates and incubated for 2 h at 37°C. Plates with no MLM antigen coating were also set up and served as controls for background measurement. After washing, plates were revealed by incubation with peroxidase conjugated anti mu or anti gamma mouse chain antibodies (Biosys, Compiègne, France) (2 h at 37°C), followed after extensive washing, by the addition of orthophenyl ethylene diamine (Sigma, St Louis, MO, USA) (0·4 mg/ml in citrate phosphate buffer 0·15 M, pH 5, containing 0·04% H_2O_2). After 30 min, the reaction was stopped by the addition of H_2SO_4 (2·5 N) and optical densities (OD) were read at 492 nm.

For each serum dilution, background OD without antigen was substracted from that obtained with antigen (Δ OD). Δ OD were plotted against log of reciprocal dilutions. Best fitting straight lines were calculated and compared to that obtained with a standard reference serum which was arbitrarily considered as containing 10,000 units of antibody activity.

Statistics. Groups of mice were compared by using the two-tailed Student's *t*-test or the non parametric Mann-Whitney test.

RESULTS

Influence of adult thymectomy on the control of MLM infection and on the size of the local granuloma. The ability of non thymectomized mice to control the local growth of bacilli and their dissemination, as measured by the AFB number in the spleen, varied from one strain to another as

Table 1. Effect of adult thymectomy on control of MLM growth and dissemination (mean ± s.d.)

Strain	Number of surviving mice/number inoculated	ATx	Log AFB number 1 year post-inoculation	
			In the inoculated footpad	In the spleen
C57BL/6	9/9	−	7·69 ± 0·46	5·73 ± 1·04
	8/10	+	7·59 ± 1·02	5·97 ± 0·98
DBA/2	10/10	−	7·50 ± 0·49	6·76 ± 0·61
	9/9	+	7·35 ± 0·75	4·52 ± 1·02*
BALB/c	7/9	−	8·34 ± 0·80	7·56 ± 0·68
	10/10	+	7·65 ± 0·85	7·66 ± 1·48
CBA	4/9	−	9·60 ± 0·44	9·11 ± 0·21
	3/10	+	9·34 ± 0·31	8·74 ± 1·23

* Significantly different from sham Tx control ($P < 0.001$).

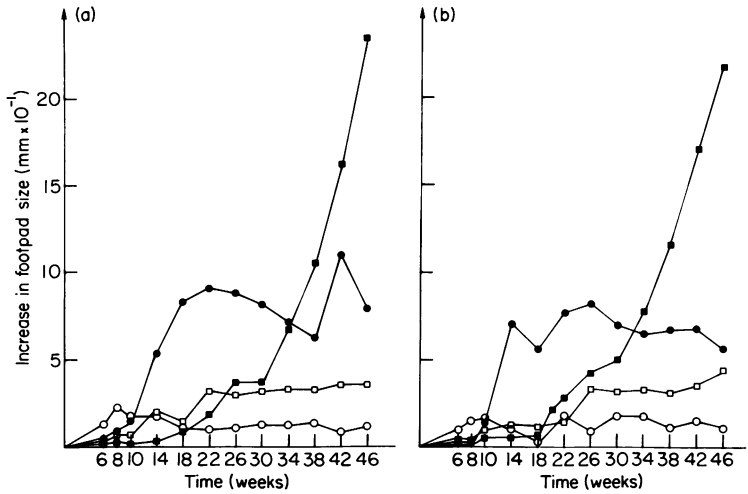

Fig. 1. Progression of the local granuloma size during the course of MLM infection in different mouse strains. (a) in control mice; (b) in ATx mice. Mice were infected subcutaneously into the left hind footpad with 10^6 MLM. Both footpads were measured at 2 weeks intervals and results are expressed as the difference between the inoculated and the contralateral footpad. For clarity, only data recorded every 4 weeks are shown. C57BL/6 (○—○); DBA/2 (□—□); BALB/c (●—●); CBA (■—■).

shown in Table 1, where strains have been classified by order of increasing susceptibility to the infection, and in Fig. 1a. Thus, C57BL/6 and DBA/2 similarly exhibited relatively low numbers of AFB both in the inoculated footpad and the spleen, and developed early and small granulomas. On the other hand, CBA mice infected with the same 10^6 MLM inoculum displayed 100 times more AFB in the footpad and 1000 times more in the spleen, than C57BL/6 mice, with late-appearing and exponentially swelling granulomas, while BALB/c mice showed intermediate susceptibility. The difference observed in the control of MLM multiplication was associated to similar differences in the health status of the mice. Among C57BL/6, DBA/2 and BALB/c mice, only a few died before killing: two ATx C57BL/6 and one control BALB/c died from bleeding; one control BALB/C mouse died 9 months after infection with hind leg paralysis but could not be examined post mortem.

Table 2. Strain influence on anti MLM antibody and DTH responses

Strain	ATx	anti MLM antibodies* IgM	anti MLM antibodies* IgG	DTH† 24 h
C57BL/6	–	37±41‡ (9)	19±20 (9)	3·44±1·13 (9)
	+	36±38 (9)	10±10 (9)	3·70±2·05 (10)
DBA/2	–	3±2 (10)	3±5 (10)	4·30±2·21 (10)
	+	2±3 (9)	1±1 (9)	2·44±1·23§ (9)
BALB/c	–	536±602 (8)	169±112 (8)	4·71±4·02 (7)
	+	222±167 (10)	639±614§ (10)	3·20±2·42 (10)
CBA	–	12±9 (7)	109±184 (7)	1·00±0·92 (8)
	+	10±11 (6)	239±382 (6)	0·85±0·89 (7)

* IgG and IgM activity to MLM sonicate was measured by an ELISA assay and expressed in arbitrary activity units by reference to a standard serum.

† Difference expressed in $mm \times 10^{-1}$ between the footpad size measured before, and 24 h after injection of 10^7 heat-killed MLM, that is at the time of maximal footpad enlargement.

‡ Mean±s.d. Number of mice are indicated in parenthesis.

§ Significantly different from sham Tx controls, $P<0·05$.

Conversely as many as seven out of 10 ATx CBA mice and six out of nine CBA controls died between 10 months and 12 months after inoculation, with seven and four respectively suffering hind leg paralysis. Four of these mice that could be examined post mortem exhibited widespread infection.

Adult thymectomy did not affect the number of AFB recovered from the infected footpad in any strain tested (Table 1). Neither did it affect health status nor granuloma size (Fig. 1b). However dissemination of bacilli as measured by the number of AFB recovered from the spleen was reduced by 100 times ($P<0·001$) in thymectomized DBA/2 mice as compared to controls (Table 1). No such effect of adult thymectomy on the number of AFB in the spleen could be observed in CBA, BALB/c or C57BL/6 strains.

Influence of adult thymectomy on antibody production and on DTH to MLM. DTH and antibody production to MLM were measured 10 months after inoculation. DTH was assessed 24, 48 and 72 h after challenge in the non-infected footpad. Footpad swelling was found to be greater at 24 h than at 48 h and 72 h in all mice ('Jones-Mote' type DTH), but was still significant at 72 h in C57BL/6 and DBA/2 mice, (Tuberculin-type DTH) (Rook & Stanford, 1979; Pelletier *et al.*, 1984). In BALB/c mice, a significant DTH reaction could be seen at 24 h only. CBA/mice, highly susceptible to the infection, were found unable to mount any DTH reaction to MLM. Adult thymectomy significantly reduced DTH reactions to MLM in DBA/2 mice ($P<0·05$) but not in other strains.

Marked contrasts in levels of anti-MLM antibodies were also seen among the various strains investigated, irrespective of their ability to control MLM infection. Thus the BALB/c strain was the best antibody producer and DBA/2 the weakest, with intermediate values for C57BL/6 and CBA strains. Levels of IgM antibodies were similar in thymectomized versus control mice of all strains tested, whereas IgG antibody levels were increased in thymectomized BALB/c mice as compared to their sham-thymectomized controls.

DISCUSSION

Several authors have underlined the role of genetic factors on the mouse resistance to mycobacterial infection (Kawaguchi, 1959; Closs, 1975a), but the level at which such a genetic control would operate remains poorly elucidated. Resistance to intravenous inoculation of MLM is partly governed by the *bcg* gene (Gros, Skaméné & Forget, 1981; Hoffenbach, Lagrange & Bach, 1985), which controls the natural ability of macrophages to eliminate mycobacteria independently of T cell activation (Stach *et al.*, 1984). The *bcg* gene however was shown to play little role if any in the control of subcutaneous MLM infection (Curtis & Turk, 1984; Hoffenbach & Bach, 1986). The possibility remains that strain differences in the control of subcutaneous MLM infection reflect strain variations in the T cell repertoire to mycobacterial antigens via immune response genes (Benacerraf & Germain, 1978), or genes leading to immune suppression, as primarily described (Kapp *et al.*, 1974) for copolymers of amino acids.

In the present work, we have followed for 1 year the course of a subcutaneous MLM infection in mice from four different strains. The effect of adult thymectomy was studied on the resistance to the infection and on the development of antibody production and DTH reactions, since adult thymectomy had been shown to abrogate the T cell mediated suppression which develops in various types of cellular and humoral immune responses (Kerbel & Eidinger, 1972; Kappler *et al.*, 1974; Asherson *et al.*, 1976; Sy *et al.*, 1979; Borel *et al.*, 1980).

The four strains studied cover a broad spectrum of susceptibility to *M. lepraemurium* as already noted (Alexander, 1978; Turcotte, 1980; Patel, 1981; Saito & Hirooka, 1983; Curtiss & Turk, 1984; Hoffenbach & Bach, 1986). C57BL/6, the most resistant strain, controlled MLM local growth and dissemination well, developed an early and small granuloma and displayed both Jones-Motes and tuberculin type DTH. DBA/2 was significantly more susceptible, with higher numbers of AFB collected from the inoculated footpad and from the spleen and with a larger local granuloma, but a similar pattern of DTH reaction. BALB/c mice were still more susceptible with poor control of MLM growth and dissemination, large local granulomas and a DTH reaction of the Jones-Mote-type exclusively. CBA mice were found the most susceptible with exponential local MLM growth and extensive bacilli dissemination, and no DTH reaction.

Adult thymectomy showed long-lasting protective effect on MLM dissemination in the DBA/2 strain, suggesting that thymus-dependent suppressor mechanisms favour MLM dissemination in intact DBA/2 mice. Adult thymectomy did not modify the course of MLM infection in the most MLM-resistant strain C57BL/6, a result in agreement with the previously reported lack of effect of cyclophosphamide (Alexander, 1978; Saito & Hirooka, 1983) suggesting that the low MLM dissemination in this strain might result from a reduced generation of suppressor T cells by subcutaneous infection. Adult thymectomy also did not affect the progress of MLM infection in the susceptible BALB/c and CBA strains, at variance with cyclophosphamide treatment, which reduced growth of bacilli in these strains (Alexander, 1978; Saito & Hirooka, 1983). Such an effect was however transient (Alexander, 1978). It must be stressed in addition that the small number of surviving CBA mice in our study may not allow us to ascertain a lack of effect of thymectomy in this strain. On the other hand the increase of anti-MLM antibody level observed in ATx BALB/c mice also suggests that MLM-triggered suppressor T cells do exist in this strain. Taken together, these results may indicate that suppressor T cells are generated by MLM infection in BALB/c and CBA mice but are not required to maintain a long term T cell tolerance to MLM, as already observed in other models of tolerance (Borel, 1980; Parks, Doyle & Weigle, 1978).

It is interesting to note that in DBA/2 mice the local bacillary growth at the site of inoculation

was not affected by adult thymectomy which may indicate that different regulatory mechanisms are involved in controlling local growth and systemic dissemination. It is also noteworthy that Atx DBA/2 mice, protected from MLM dissemination, exhibited lower DTH reaction to MLM than control mice. Other authors (Anderson & Crowle, 1981; Lovik & Closs, 1982) have already underlined the absence of a direct relationship between DTH reactions and T cell mediated protective immunity. The mechanism of the decrease of DTH reaction by adult thymectomy is not clear. DTH has been shown to involve the cooperation of different T cell subsets (Wright & Ramshaw, 1983), one of which could be sensitive to adult thymectomy. Actually the effect of both adult thymectomy or cyclophosphamide treatment on DTH were found to vary according to the conditions of immunization: DTH was depressed in optimally sensitized mice, when stimulation of suppressor T cells was minimized, and was increased in supraoptimally immunized or in tolerized mice, when the generation of suppressor T cells was favoured (Kappler *et al.*, 1974; Asherson *et al.*, 1976; Schwartz, Askenase & Gershon, 1978; Erard *et al.*, 1979).

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