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Induction of delayed type hypersensitivity against ultrasonicated *Mycobacterium lepraemurium* bacilli without simultaneous local reactivity against live bacilli or protective immunity

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SUMMARY

Delayed type hypersensitivity (DTH) was induced in C3H mice by subcutaneous immunization with *Mycobacterium lepraemurium* (MLM) antigens in Freund's complete (FCA) or Freund's incomplete (FIA) adjuvant. The total ultrasonicate (MLMSon-P) of MLM bacilli as well as the water soluble fraction (MLMSon-S) of this ultrasonicate was found effective. MLMSon-S was used as the test antigen. Specific DTH also developed after immunization with heat-killed MLM bacilli in FIA, but not with heat-killed bacilli in saline. Some mice were pre-treated with cyclophosphamide (CY) or splenectomized to augment the effect of immunization. In no instance was DTH to MLMSon-S accompanied by detectable local reactivity to live MLM bacilli measured as swelling of the infected footpad or by reduced multiplication or dissemination of the bacilli during the first 11 weeks after inoculation. As determined by testing in the infected footpad 8 weeks after inoculation, MLM infection did not induce DTH to MLMSon-S in non-immunized mice, and MLM infection was found to neither augment nor suppress established DTH to MLMSon-S. The experiments thus demonstrated a clear dissociation between DTH to MLMSon-S and local reactivity to live MLM bacilli, as well as between DTH to MLMSon-S and protective immunity to MLM infection.

Keywords *Mycobacterium lepraemurium* leprosy hypersensitivity delayed immunization C3H mice

INTRODUCTION

Resistance against the intracellular bacterium *Mycobacterium lepraemurium* (MLM) is thought to depend on cell-mediated immunity (Closs & Haugen, 1975a, 1975b; Alexander, 1979). Although the nature of the relationship between DTH and acquired protective cell-mediated immunity is a matter of controversy (Lefford, 1975; Salvin & Neta, 1975; Youmans, 1975), DTH often occurs together with protective immunity and has been considered an important component of cell-mediated resistance against intracellular parasites (Mackaness, 1964). DTH against water soluble MLM antigens can be induced by immunization with ultrasonicated bacilli in FIA (Løvik & Closs, 1980). Inbred C3H mice are highly susceptible to infection with MLM (Closs, 1975). We therefore did experiments to determine whether the induction of DTH to MLMSon-S in C3H mice was

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accompanied by the induction of local reactivity against live MLM bacilli or by a manifest reduction in susceptibility to MLM infection. Furthermore, we wanted to see if MLM infection would induce DTH to MLMSon-S or suppress already established DTH to this antigen.

MATERIALS AND METHODS

Animals. Five to six week old female mice of the inbred strain C3H/Bom (C3H) were obtained as specific pathogen free animals directly from the breeder, Gl. Bomholtgård Ltd., Ry, Denmark (Løvik, Collins & Closs, 1982).

MLM bacilli. The Douglas strain of MLM was maintained by repeated passage in C3H mice. The bacilli were harvested, prepared and counted after acid-fast staining with auramin as previously described (Løvik & Closs, 1981). The bacilli were stored overnight at 4°C before use, or for some experiments the bacilli were stored on liquid nitrogen. Because the viability of MLM bacilli cannot be determined by conventional bacteriological methods, acid-fast bacilli prepared and stored as described are referred to as live MLM bacilli.

Heat-killed MLM bacilli. Purified MLM bacilli that had been stored on liquid nitrogen were washed and kept for 1 h in a water bath at 70°C. The bacilli were stored at −20°C. They were thawed and used after appropriate dilution without further washing.

Preparation of MLMSon-P and MLMSon-S. MLM bacilli were ultrasonicated as previously described (Closs, Harboe & Wassum, 1975). Insoluble material was removed from the sonicate by centrifugation at 20,000 *g* for 20 min. The protein concentration of the supernatant was 0·8 mg/ml as determined by the modified Folin–Ciocalteu method with bovine serum albumin as a standard (Lowry *et al.*, 1951). The sonicate was stored undiluted and in dilutions of 1 : 10 and 1 : 100 with 0·9% NaCl at −20°C. The same batch of sonicate was used for all footpad injections to determine DTH throughout the experiments. In crossed immunoelectrophoresis MLM sonicate prepared in this manner has been shown to contain more than 40 antigenic components (Closs *et al.*, 1975). Separate batches of sonicate (protein concentration 0·7–0·9 mg/ml) to be used for immunization only were prepared in the same manner. In some experiments the sonicate to be used for immunization was reconstituted with the amount of insoluble material removed by centrifugation, and such reconstituted sonicate is referred to as MLMSon-P. The insoluble material removed by centrifugation of the sonicate has been found to consist for a large part of cell wall fragments (Closs, unpublished results). Sonicate containing only the water soluble material is referred to as MLMSon-S.

Splenectomy. Hair on the abdomen was removed by Surgex hair remover cream (Cooper Laboratories, Inc., Wayne, New Jersey, USA). Anaesthesia was accomplished by the intraperitoneal injection of 1·2 mg pentobarbital sodium dissolved in 0·2 ml sterile water (Nembutal, Abbott Laboratories, North Chicago, Illinois, USA). After cleaning the skin with 70% ethyl alcohol, a 5–10 mm long incision was made through the abdominal wall just above the spleen. The spleen was moved out through the opening, and the pedicle tied off with a 3/0 polyester suture (Flexafil, J. Pfrimmer & Co., Erlangen, W. Germany). The pedicle was cut distally, and the abdominal wound was closed with metal clips and covered with an acrylic spray (Nobecutan, AB Bofors, Nobel-Pharma, Mølndal, Sweden). No antibiotics were used.

Cyclophosphamide (CY) treatment. CY (Sendoxan, Pharmacia AB, Uppsala, Sweden) was dissolved in sterile water and injected intraperitoneally in a single dose of 100 mg/kg. The interval from CY treatment to immunization or infection is given in the Results section for each experiment. The effect of CY has been reported to differ in various mouse strains (Mitsuoka *et al.* 1979; Hurme, Bång & Sihvola, 1980). In preliminary experiments we immunized C3H mice with 1×10^8 sheep red blood cells in Freund's complete adjuvant (FCA) with and without CY pre-treatment. CY was found to enhance DTH to sheep red blood cells in C3H mice (unpublished results).

Immunization with MLMSon preparations and heat-killed MLM bacilli. Equal volumes of MLMSon-P or MLMSon-S and adjuvant were thoroughly mixed and exposed to repeated brief pulses of ultrasound until a thick, stable emulsion was obtained. A single injection of 50 μl of emulsion was given subcutaneously in the upper right side of the thorax after removal of the hairs

with Surgex hair remover cream. An emulsion with heat-killed bacilli suspended in saline was prepared and used in a similar manner. Freund's incomplete adjuvant (FIA) and FCA were obtained from Behringwerke AG, Marburg/Lahn, W. Germany. FCA contained 1 mg/ml of heat-killed and dried *M. tuberculosis*.

Footpad injection of MLMSon-S and experimental infection. For all footpad injections a volume of 10 μl of the appropriate material was given through a 30 gauge needle from a 100 μl syringe (Hamilton Bonaduz, Bonaduz, Switzerland). The needle path was sealed immediately afterwards with Nobecutan.

At appropriate intervals after the inoculation with bacilli, groups of five or six mice were killed, and the infected footpad, the draining popliteal lymph node and in some experiments the spleen or the liver were removed. Preparations for counting the bacilli after acid-fast staining with auramine were made from the removed organs as previously described (Closs, 1975).

DTH against MLMSon-S and local reactivity against MLM bacilli. The footpad swelling assay was used (Crowle, 1959). The thickness of the hind feet was measured with a modified dial gauge caliper (Closs & Løvik, 1980). The instrument causes a slight compression of a swollen footpad, especially if there is little induration.

Statistical methods. Wilcoxon's rank sum test for unpaired samples was used to test differences between groups (White, 1952). Differences with $P > 0{\cdot}05$ were regarded as not statistically significant (n.s.).

RESULTS

Dissociation between reactivity against MLMSon-S and live MLM bacilli

Normal C3H mice and mice that had been given CY 2 days before were immunized with MLMSon-P in FIA. Emulsions were made from three different dilutions of MLMSon-P: undiluted, 1:10 and 1:100. In addition, two groups of CY pre-treated mice were immunized with MLMSon-S (dilution 1:10): one with FIA, the other with FCA as an adjuvant. Finally, one group of CY pre-treated mice was injected subcutaneously in the thorax with 1×10^8 heat-killed MLM bacilli suspended in saline, and one group of mice was given CY not followed by any immunization. Six weeks later the immunized mice and the control groups were injected with $2{\cdot}5 \times 10^7$ live MLM bacilli in one hind footpad and a corresponding amount of MLMSon-S in the other footpad (10 μl, dilution 1:20). This bilateral testing procedure has been found not to alter the local reaction to

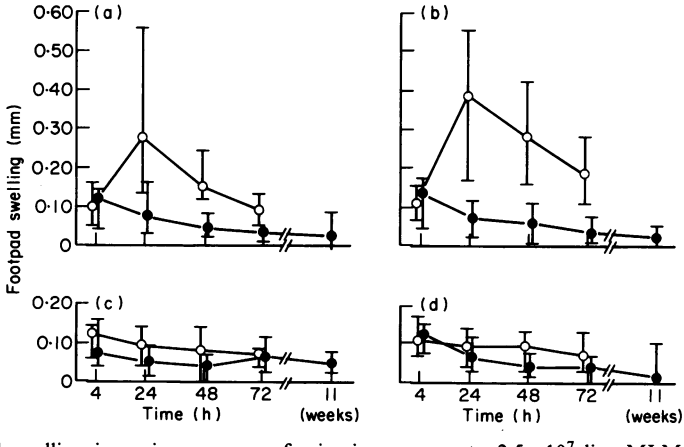

Fig. 1. Footpad swelling in various groups of mice in response to $2{\cdot}5 \times 10^7$ live MLM bacilli (●) or a corresponding amount of MLMSon-S (10 μl, dilution 1:20) (○). (a) Mice immunized with undiluted MLMSon-P in FIA; (b) mice immunized with MLMSon-S (dilution 1:10) in FCA after CY pre-treatment; (c) CY pre-treated mice immunized with 1×10^8 heat-killed MLM bacilli in saline and (d) normal control animals. Median and range for groups of six mice tested simultaneously with live MLM bacilli and MLMSon-S.

MLMSon-S or live MLM bacilli. The thickness of the footpads was measured before and 4, 24, 48 and 72 h after the injection. All the different groups immunized with MLMSon-S or MLMSon-P developed a measurable footpad swelling with the kinetics of a DTH reaction in response to MLMSon-S. Two representative groups are shown in Fig. 1a & b (see figure legend). In contrast to the response to MLMSon-S, no significant local reactivity in response to live MLM bacilli was seen in any of the immunized groups. Not even the mice immunized with MLMSon-S in FCA that tended to have the strongest DTH to MLMSon-S (n.s.) were different from the normal controls in their local reaction to live MLM bacilli (Fig. 1b). CY treated non-immunized mice were not different from the normal controls (not shown). In mice immunized with undiluted MLMSon-S in FIA, increasing the dose of live bacilli from $2{\cdot}5 \times 10^7$ to $1{\cdot}25 \times 10^8$ bacilli did not bring about a statistically significant increase in local reactivity the first 72 h after the injection (data not shown). Thus, as determined by local reactivity up to 72 h after footpad testing, the MLMSon preparations had induced DTH to MLMSon-S but failed to sensitize against live bacilli regardless of CY pretreatment, antigen dose, presence of MLM cell wall fragments, or the use of FCA instead of FIA as an adjuvant. Heat-killed MLM bacilli in saline had no detectable immunizing effect (Fig. 1c).

The thickness of the foot inoculated with live MLM bacilli was measured weekly for 11 weeks. As in preliminary experiments (Løvik & Closs, 1980), no statistically significant footpad swelling developed in any of the immunized groups as compared to the normal controls (Fig. 1). The viability of the bacilli used for challenge was verified by counting the bacilli in the infected footpad and the draining popliteal lymph node (data not given).

In a second experiment, mice were splenectomized or given CY. Five weeks or 5 days later, respectively, the mice were immunized subcutaneously in the thorax with MLMSon-S in FIA or with 3×10^8 heat-killed bacilli in FIA. Seven weeks after immunization the immunized mice and the appropriate controls (see legend to Fig. 2) were injected with MLMSon-S diluted 1:100 in the left hind footpad and $4{\cdot}2 \times 10^6$ MLM bacilli in the right hind footpad. A statistically significant footpad swelling with the kinetics of a DTH reaction developed in CY pre-treated (Fig. 2a) as well as in splenectomized (Fig. 2b) mice immunized with MLMSon-S, but the swelling was enhanced in the CY pretreated mice, especially at 48 h after injection of the test antigen. In CY pre-treated mice there was also a significant swelling in mice immunized with heat-killed MLM bacilli in FIA (Fig. 2a). Thus, DTH to MLMSon-S had also been induced by heat-killed MLM bacilli in FIA. The footpad inoculated with live MLM bacilli was measured initially at daily and later at weekly intervals for 8 weeks. As in previous experiments, none of the groups developed a significant swelling in response to live MLM bacilli as compared with the normal controls (data not given).

Effect of immunization on multiplication of MLM bacilli

The mice from the last experiment described above were sacrificed 9 weeks after being inoculated with bacilli. In the footpad there were no differences in bacillary numbers between the various

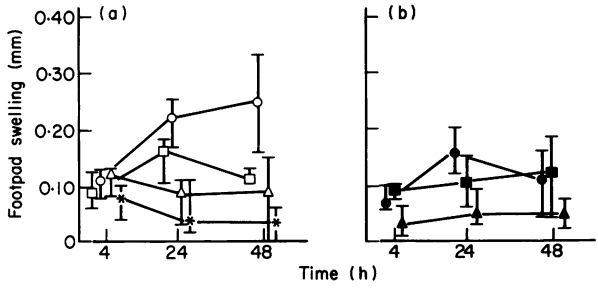

Fig. 2. Footpad swelling in response to MLMSon-S (10 μl, 1:100) in various groups of mice. (a) CY pre-treated mice immunized with MLMSon-S in FIA (○) or heat-killed MLM bacilli in FIA (□); CY treated non-immunized mice (△) and normal controls (*). (b) Splenectomized mice immunized with MLMSon-S in FIA (●) or with heat-killed MLM bacilli in FIA (■) and splenectomized non-immunized mice (▲). Median and range for groups of six mice.

immunized or non-immunized groups (Table 1). In the draining popliteal lymph node, there was a tendency toward lower bacillary numbers in the two groups immunized with MLMSon-S, but the differences were not statistically significant and all the groups had high numbers of bacilli (Table 1). Thus, immunization with MLMSon-S or heat-killed MLM bacilli in FIA after CY pre-treatment or splenectomy had not reduced the susceptibility of C3H mice to MLM infection, as determined by bacillary multiplication and dissemination 9 weeks after the inoculation. Neither had splenectomy or CY treatment without immunization reduced bacillary multiplication and dissemination. Later experiments in C3H mice immunized with MLMon-S in FIA with or without CY pre-treatment showed a lack of an effect of immunization on bacillary numbers in the spleen also 9 weeks after the inoculation with bacilli (data not given).

The adjuvant as well as the insoluble material removed from the MLM ultrasonicate by centrifugation could be of crucial importance for the induction or suppression of protective immunity. Groups of C3H mice were therefore immunized with emulsions of MLMSon-S in FCA, saline in FCA, MLMSon-S in FIA, MLMSon-P in FIA and insoluble material removed from the MLM ultrasonicate (suspended in saline) in FIA. Six weeks later the immunized mice and control animals were inoculated in one hind footpad with 1×10^6 live MLM bacilli. Eleven weeks after the inoculation the mice were sacrificed. None of the immunized groups had a statistically significant reduction of bacillary multiplication and dissemination as determined by bacillary numbers in the footpad and the draining popliteal lymph node (data not given). Thus, all the combinations of antigens and adjuvants failed to induce a manifest reduction of susceptibility to MLM infection as determined 11 weeks after the inoculation with bacilli.

Effect of MLM infection on DTH against MLMSon-S

The lack of local reactivity to MLM bacilli and the lack of a significant effect of immunization on bacillary multiplication up to 11 weeks after the inoculation with bacilli could be due to general or local suppression of immunity by the bacilli.

We therefore did an experiment in which MLMSon-S immunized mice not pre-treated with CY and control animals were tested with MLMSon-S (1:20) before inoculation in the footpad with a high ($1{\cdot}25 \times 10^8$) and a low (1×10^6) dose of live MLM bacilli. Eight weeks after the inoculation with bacilli groups of mice were again tested with MLMSon-S. Mice that had been inoculated with bacilli were tested in the infected footpad. The experiment was done with parallel groups of mice so that all groups were tested only once. Fig. 3a shows that DTH against MLMSon-S had been induced by the immunization and was present after 5 weeks, just before the inoculation with bacilli. Fig. 3b shows that 8 weeks after infection, 14 weeks after immunization, the size of the footpad swelling in response to MLMSon-S was not significantly different in infected and non-infected immunized

Table 1. Bacillary numbers in the footpad and draining popliteal lymph node in various groups of mice 9 weeks after footpad inoculation with $4{\cdot}2 \times 10^6$ live MLM bacilli. Median and range for groups of six mice

		Number of acid fast bacilli $\times 10^{-6}$	
Immunization	Pre-treatment	Footpad	P. lymph node
None	None	34 (20–42)	6·4 (2·7–41)
None	CY	28 (25–42)*	6·8 (2·3–10)*
None	Splenectomy	41 (26–62)*	5·8 (4·3–10)*
MLM heat-killed	CY	26 (19–40)*	8·4 (1·0–33)*
MLM heat-killed	Splenectomy	27 (17–43)*	4·3 (1·6–6·8)*
MLMSon-S	CY	30 (27–46)*	3·1 (2·0–5·8)*
MLMSon-S	Splenectomy	35 (20–38)*	2·7 (1·7–5·4)*

* Difference from normal controls not statistically significant (Wilcoxon's rank sum test).

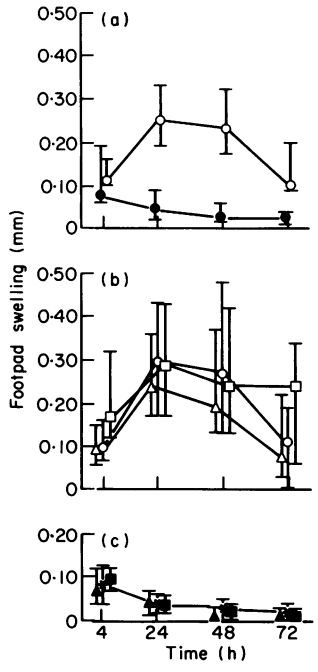

Fig. 3. Footpad swelling in response to MLMSon-S (10 μl, 1:20) in various groups of mice. (a) Five weeks after immunization with MLMSon-S in FIA, 1 week before inoculation with bacilli: immunized mice (○); normal controls (●). (b) Fourteen weeks after immunization, 8 weeks after inoculation with live MLM bacilli: Immunized mice inoculated with 1×10^6 MLM bacilli (△); immunized mice inoculated with $1{\cdot}25 \times 10^8$ MLM bacilli (□); immunized mice not inoculated with bacilli (○). (c) The response to the same dose of MLMSon-S in non-immunized mice 8 weeks after inoculation with 1×10^6 (▲) or $1{\cdot}25 \times 10^8$ (■) bacilli and in normal mice (*). Range for groups of six mice is given, and lines are drawn between medians. In (c) lines are drawn only for normal mice.

mice. Further, the footpad swelling in response to MLMSon-S was nearly identical in size and kinetics 5 and 14 weeks after the immunization in non-infected, immunized mice (Fig. 3a & b). Fig. 3c shows that 8 weeks after the inoculation with bacilli, no detectable DTH to MLMSon-S was present in non-immunized mice infected with $1{\cdot}25 \times 10^8$ or 1×10^6 live MLM bacilli. Thus, the DTH induced by immunization with MLMSon-S in FIA was long lasting and was neither significantly suppressed nor enhanced by MLM infection. Similar results have been found in CY pre-treated mice immunized with MLMSon-P (data not given).

DISCUSSION

The present experiments showed a clear dissociation between DTH to soluble MLM antigens and protective immunity, and between DTH to soluble MLM antigens and local reactivity against intact, live MLM bacilli. Regardless of adjuvants used, CY pre-treatment or splenectomy, MLM bacilli for several weeks multiplied at a normal rate without evoking a detectable local reaction in the tissue of mice that showed strong DTH to an ultrasonicate of the same bacillus. Tests for DTH, for local reactivity to live bacilli and for protective immunity (reduced bacillary multiplication) were all performed in the footpad. In addition, protective immunity was determined in the draining popliteal lymph node and the spleen. Therefore, it is not likely that the observed dissociation may be explained by a compartmentalization of various manifestations of the immune response (Salvin & Neta, 1975).

A dissociation between DTH and protective immunity could be expected if the DTH were

Jones–Mote type and not tuberculin type, while this is less certain concerning the CY modified Jones–Mote hypersensitivity (Alexander, 1978; Alexander & Curtis, 1979; Lagrange, 1979). However, even if the DTH were Jones–Mote type, this would not readily explain the dissociation between DTH to MLMSon-S and local reactivity against live MLM bacilli (Curtis, Adu, & Turk, 1981). Jones–Mote DTH is typically induced by protein antigens in FIA, i.e. adjuvant without mycobacterial components, while the use of FCA, i.e. adjuvant with mycobacterial components, strongly favours the development of tuberculin type DTH (Askenase, 1980). The cell walls of MLM have been found to have potent adjuvant properties (Azuma *et al.*, 1972). The use of FCA instead of FIA did not significantly increase the protective effect of the immunization, and even after immunization with MLMSon-S in FIA, the kinetics of the reaction elicited by MLMSon-S was tuberculin type rather than Jones–Mote type (Fig. 3) (Turk, Polak & Parker, 1976). Also, the long lasting reactivity (14 weeks or more) is a feature typical of tuberculin type DTH and unusual in Jones–Mote hypersensitivity (Turk *et al.*, 1976).

The various antigenic components in MLMSon-S are present in different amounts. The amount of antigen used for immunization can be critical for both the type and the intensity of the immunity induced (Benacerraf & Unanue, 1979), and low doses of antigen have been reported to preferentially induce cell-mediated immunity (Salvin, 1958). The amount of undiluted MLMSon used for immunization corresponds to 1×10^9 MLM bacilli, but lowering the dose by using MLMSon diluted 1:10 and 1:100 did not increase the effect of immunization with regard to local reactivity against live bacilli. The adjuvants or some fraction of MLMSon might have a suppressive effect on components of the immune response (Reinisch, Gleiner & Schlossman, 1976; Neta & Salvin, 1979). In the present experiments neither removal of the insoluble fraction nor immunization with this fraction alone had any significant effect on the protective quality of the immune response. However, a possible suppressive effect of FIA and FCA on certain components of the immune response after immunization with the MLMSon preparations cannot be determined by the protocols used. MLMSon-S with *C. parvum* as an adjuvant has been found to induce very weak DTH against MLMSon-S after subcutaneous administration in C3H mice, and repeated injections of MLMSon-S without adjuvant into the footpad of normal C3H mice apparently had no immunizing effect (unpublished data).

Heat-killed MLM bacilli in saline were not found to be immunogenic. This is consistent with earlier reports (Rook, 1980). Heat-killed MLM in FIA induced an immune response that apparently had the same qualities as the immune response to the MLMSon preparations, i.e. DTH to MLMSon-S but no local reactivity to live MLM bacilli and no protective immunity.

CY is cytotoxic for certain suppressor cells and their precursors (Kaufmann, Hahn & Diamantstein, 1980). Genetically low responder mice can be made to exhibit an antibody or DTH response provided they are pretreated with CY before sensitization (Debre *et al.*, 1976; Miller *et al.*, 1976), and CY abolishes the inhibitory mechanisms of DTH that are activated by a large dose of antigen (Lagrange, Mackaness & Miller, 1974b). CY sensitive suppressor cells are also involved in antigenic competition (Dwyer, Parker & Turk, 1981; Turk & Parker, 1982). However, we found no effect of CY pretreatment on local reactivity against live bacilli or on protective immunity. Thus, it is not likely that the lack of a protective effect of immunization with MLMSon preparations or heat-killed MLM was due to an induction of CY sensitive suppressor cells during immunization. Neither was there any effect from the use of FCA that has been found to enable mice to overcome genetically determined low responsiveness (Newton & Warner, 1977).

The spleen may be important in suppressor-cell mediated unresponsiveness (Bullock, Carlson & Gershon, 1978; Sy *et al.*, 1977). Splenectomy has been shown to potentiate certain immune responses (Lagrange *et al.*, 1974a; Hudson *et al.* 1979). An effect on parameters related to specific cellular immunity has been found 2–3 months (Schrier, Allen & Moore, 1980) and up to 600 days after splenectomy (Slavin, Zan-Bar & Strober, 1980). However, no statistically significant increase in local reactivity against live MLM bacilli or in protective immunity was found in the splenectomized mice. This result argues against a significant role for spleen-dependent suppressor cells in the apparent lack of a cell-mediated immune response against MLM in C3H mice, provided that an intact spleen is not necessary to generate such a response (Skamene & Chayasirisobhon, 1977).

Detectable non-specific suppressor cell activity inhibiting the expression of established DTH was not induced by the MLM infection. If MLM bacilli suppressed the reactivity against some crucial antigens in MLMSon-S, reactivity against these antigens must have represented a small part of the total reactivity since DTH to MLMSon-S was not altered by the MLM infection (Fig. 3b). However, a suppression of components of the immune response not expressed as DTH by MLM cannot be excluded.

The dissociation between DTH to MLMSon-S and protective immunity as well as reactivity against live bacilli could be caused by a hole in the antigen repertoire of C3H mice, affecting some crucial antigens. Or, the defect may have been with the antigen used for immunization so that the crucial antigen(s) was missing in the MLMSon preparations or present in an amount too small for the effector cells to be induced. The crucial antigen could be some surface antigen that is easily lost or inactivated during ultrasonication or heat-killing of the bacilli. In C57BL mice immunized by a previous infection, we have observed that the footpad swelling after injection with live MLM bacilli develops rather slowly and often is small for the first 48–72 h (Closs & Løvik, 1980). This indicates that some antigen is lost even during the preparation of live bacilli and has to be re-expressed before a local reaction will develop. The absence of the crucial antigen activity from the MLMSon preparations also fits well with the finding of a dissociation between DTH to MLMSon-S and local reactivity against live bacilli as well as protective immunity in the resistant C57BL mice (Closs & Løvik, 1980; Løvik & Closs, 1980).

The present paper describes the effects of immunization with MLMSon preparations on the early course of MLM infection, up to 11 weeks after the inoculation with bacilli. In a subsequent paper, we show that the immunization of C3H mice with MLMSon-S and MLMSon-P conditions for a certain reduced susceptibility to MLM during the later phase of the infection (Løvik & Closs, 1983).

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