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Influence of cyclophosphamide on delayed hypersensitivity and acquired cellular resistance to *Listeria monocytogenes* in the mouse

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Summary. The effect of a single dose of cyclophosphamide (CY) on delayed type hypersensitivity (DH) and acquired cellular resistance (ACR) to *Listeria monocytogenes* infection in mice was studied. Intraperitoneal or intracutaneous immunization with L forms of *L. monocytogenes* did not result in protection against lethal challenge. A positive DH could be observed when CY-treated mice were intracutaneously immunized with 10^8 or more L forms. Intraperitoneal injection of viable *L. monocytogenes* resulted only in a narrow dose range in survival on immunization and partial protection on challenge. Protection was accompanied by DH. Intracutaneous injection of Listeria in Freund's complete adjuvant permitted the use of even 10^9 viable bacteria for immunization. This figure was reduced to 10^5 or less for CY treated mice. In normal mice protection was afforded on immunization with 10^7 bacteria whereas 10^3 bacteria were sufficient to protect CY treated animals. All protected mice showed a positive DH. These results demonstrate that CY treatment reduces the dose of viable bacteria tolerated for immunization 10^4 times. On the other hand after CY treatment the doses of bacteria effective on immunization for ACR and DH could be reduced in the same order of magnitude. Reduction of the CY dose resulted in a peak DH with 4 mg CY, but the protection was less than that obtained after treatment with 6 mg CY. A dissociation between ACR and DH was observed by varying the interval between immunization and challenge. In normal mice DH was preceded by ACR, with peaks at respectively 10 and 5 days after immunization. CY treatment caused a delay in the onset of the ACR, followed by an enhanced and slightly prolonged response. The effect of CY on DH consisted of enhancement and prolongation.

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INTRODUCTION

L. monocytogenes is a facultative intracellular bacterial parasite. Resistance to Listeria infection in mice is an example of cell-mediated immunity (CMI); it is triggered by specifically sensitized thymus derived (T) lymphocytes (Blanden & Langman, 1972; Lane & Unanue, 1972). Humoral immunity is not essential (Mackaness, 1969). Both delayed type hypersensitivity (DH) and acquired cellular resistance (ACR) are believed to be manifestations of CMI, since they can be passively transferred with viable lymphoid cells (Mackaness, 1969) but not with serum (Mackaness, 1962). It is controversial, however, whether there is a correlation between the two phenomena (Youmans, 1975; Lefford, 1975).

A single injection of cyclophosphamide (CY) selectively depletes non-thymus dependent areas of lymph nodes and spleen in guinea-pig and mouse (Turk & Poulter, 1972). A proportional increase in theta-antigen-carrying lymphocytes in the lymphoid tissue could be observed (Poulter & Turk, 1972). Although the B-cell population is almost completely eliminated the T-cell population is also partly affected by CY treatment (Willers & Sluis, 1975). Whereas the restoration of the B-cell population is slow, the recovery of the T-cell population is faster and considerably accelerated by antigenic stimulation (Willers & Sluis, 1975). Treatment with CY (300 mg/kg) followed by i.c. immunization with sheep red blood cells (SRBC) in FCA or i.p. immunization with low doses of SRBC produced a strongly enhanced DH, whereas the antibody formation was completely suppressed (Kerckhaert, van den Berg & Willers, 1974; Kerckhaert, 1974; Lagrange, Mackaness & Miller, 1974). Tripathy & Mackaness (1969) studied the effects of CY on the primary response to *L. monocytogenes*. The host's immunity was suppressed when CY was injected from 2 days before infection to 4 days after infection Pretreatment with CY 4–7 days before infection resulted in enhanced immunity, which coincided with lymphoid hyperplasia.

In the present investigation the effects of CY pretreatment on DH and ACR is studied simultaneously and extended with variations in route and dose of immunization with viable *L. monocytogenes*, with variations in CY dose and with variations in interval between immunization and challenge.

MATERIALS AND METHODS

Animals

Inbred BALB/c mice were bred and maintained in the Laboratory of Microbiology, State University of Utrecht, The Netherlands. Female mice were used at an age of about 11 weeks (20 g).

Bacteria

L. monocytogenes, strain L347, serotype IVB and the non-virulent penicillin-induced L form (Bartlema & Braunius, 1971) were kindly provided by Dr H. C. Bartlema, RVO-TNO, Rijswijk, The Netherlands. The bacteria were grown on tryptose phosphate broth (Difco Laboratories, Inc., Detroit, Michigan) for 16 h at 37°, centrifuged at 1000 *g* for 15 min and resuspended in saline to the concentration required. L forms were subcultured on 3·7% Brain–Heart Infusion (BHI) broth (Difco) containing 2% NaCl and 0·5% yeast extract (Difco), during 72 h at 37°. Viable cell counts of the bacteria were made after plating on trypticase-soy-agar (Baltimore Biological Laboratories Baltimore, Maryland). For enumeration, L forms were plated on BHI containing 1·1% Noble agar (Difco) and 5% horse serum.

Immunizations

Mice were i.p. immunized with viable bacteria or L forms suspended in 0·5 ml saline. For i.c. immunization the bacteria or L forms were suspended in a volume of 0·05 ml saline and mixed with an equal volume of FCA. Control animals received saline (i.p.) or FCA (i.c.).

Cyclophosphamide treatment

Cyclophosphamide was obtained from Koch-Light Laboratories' (Colnbrook, Buckinghamshire). It was freshly dissolved in 0·5 ml saline and i.p. injected 8 h before immunization. Unless otherwise mentioned a dose of 300 mg CY/kg body weight was used.

Assay for delayed type hypersensitivity

Delayed type hypersensitivity was measured as the increase in footpad thickness (Kerckhaert *et al.*, 1974) 24 h after injecting an eliciting dose of $2{\cdot}5 \times 10^4$ L forms. Reactions are recorded against the day when the test dose of antigen was injected, rather than the day upon which the reaction was measured.

Determination of acquired cellular resistance

The 14-day mean lethal dose (LD50) of viable *L. monocytogenes* in BALB/c mice was found to be 2×10^5 colony forming units after i.p. injection. The LD50 values were calculated by the method of Reed & Muench (1938). Mice were i.p. challenged with 1000 LD50 and the numbers of survivors 2 weeks later were recorded.

RESULTS

DH and ACR after immunization with L forms

Groups of six mice were treated with CY or saline followed by an i.p. injection of graded numbers of L forms. After 10 days the eliciting injection for

Table 1. DH on day 10 after immunization with L forms of *L. monocytogenes*

Immunization dose	Footpad swelling in 1/10 mm ± s.e.			
	i.p. Immunization after:		i.c. Immunization after:	
	Saline	CY	Saline	CY
None	1·2±0·3	1·7±0·3	0·5±0·2	1·4±0·4
3×10^7	n.d.	n.d.	1·7±0·2	2·2±0·7
10^8	1·6±0·5	1·6±0·6	0·2±0·6	5·3±1·0
3×10^8	n.d.	n.d.	1·2±0·3	4·5±1·5

n.d. = Not determined

DH was given, followed 2 days later by the challenge for survival. Similar experiments were done with mice immunized i.c. with L forms emulsified in FCA. Table 1 shows that only following CY treatment and i.c. immunization with 10^8 or 3×10^8 L forms could DH be induced. However none of the immunizations resulted in ACR, as all animals died within 14 days after the challenge injection. As the eliciting injection with L forms did not contribute to the ACR, in further experiments DH and ACR were determined on the same mice with an interval of 2 days between the eliciting and challenge injections.

Survival, DH and ACR after i.p. injections of viable bacteria

The absence of protective immunity after a single injection with L forms, prompted us to use viable bacteria for immunization. Groups of five mice were treated with CY or saline followed by an i.p. injection of graded numbers of viable bacteria. Non-CY treated mice survived injections of 10^6 bacteria or less (Table 2). However only the dose of 10^6 immunized for DH and ACR (two out of four mice survived). CY treatment of mice reduced the dose tolerated for immunization to 10^3. The surviving mice showed DH and some protection.

DH and ACR after i.c. injections of viable bacteria

As protection after i.p. injection with *L. monocytogenes* was only marginal, immunization via the i.c. route was tried. Groups of at least five mice were pretreated with CY or saline and i.c. injected with graded numbers of viable bacteria in FCA. Without pretreatment an immunizing dose of 10^7 bacteria or more resulted in a high percentage of protected mice (Table 3). The optimal immunizing dose for DH was 10^8 bacteria. Pretreatment with CY resulted in increasing mortality following immunization. However with immunizing doses of 10^5 bacteria or less this mortality decreased, while a high degree of protection in the surviving animals was observed.

Table 2. Effect of i.p. immunization with viable *L. monocytogenes* on survival, DH and ACR

Immunization dose	Without CY			With CY		
	No. survivors/ no. immunized	Footpad swelling	No. survivors/ no. challenged	No. survivors/ no. immunized	Footpad swelling	No. survivors/ no. challenged
10^7	0/5			0/5		
10^6	5/5	6·0±0·5	2/4	0/5		
10^5	5/5	2·0±0·9	0/5	0/5		
10^4	5/5	2·0±0·4	0/5	1/5	8·0	1/1
10^3	5/5	1·5±0·3	0/5	3/5	4·0±1·5	1/3

Mice received an eliciting injection with L forms 10 days after immunization. The footpad swelling measured 24 h later, is given in 1/10 mm ± s.e. The challenge injection was given 2 days after the eliciting injection.

Table 3. Effect of i.c. immunization with viable *L. monocytogenes* on survival, DH and ACR

Immunization dose	Without CY			With CY		
	No. survivors/ no. immunized	Footpad swelling	No. survivors/ no. challenged	No. survivors/ no. immunized	Footpad swelling	No. survivors/ no. challenged
10^9	8/10	4·5±1·0	7/8			
10^8	8/10	7·3±0·4	8/8	0/5		
10^7	18/20	5·0±0·9	14/18	0/5		
10^6	14/15	3·8±0·6	4/14	0/20		
10^5	15/15	2·5±0·5	0/15	7/20	10·0±1·4	7/7
10^4	5/5	2·5±0·5	0/5	12/20	7·0±2·8	12/12
10^3	5/5	1·3±0·3	0/5	15/20	5·4±0·9	14/15
10^2	5/5	1·1±0·3	0/5	15/15	5·9±0·8	10/15
0	25/25	1·2±0·3	0/25	10/10	2·0±0·4	0/10

Mice received an eliciting injection with L forms 10 days after immunization. The footpad swelling measured 24 h later, is given in 1/10 mm±s.e. The challenge injection was given 2 days after the eliciting injection.

All surviving animals showed DH, with a maximum after immunization with 10^5 bacteria.

Effect of variation in CY dose on DH and ACR

Mice were immunized i.c. with 10^4 bacteria after pretreatment with saline or graded doses of CY. Treatment with 100, 200 and 300 mg CY/kg resulted in significantly enhanced DH (Fig. 1). The greatest enhancement occurred after 200 mg CY/kg. On the other hand the percentage of survivors after 200 mg CY/kg was only 60%, whereas after 300 mg CY/kg all animals survived the challenge.

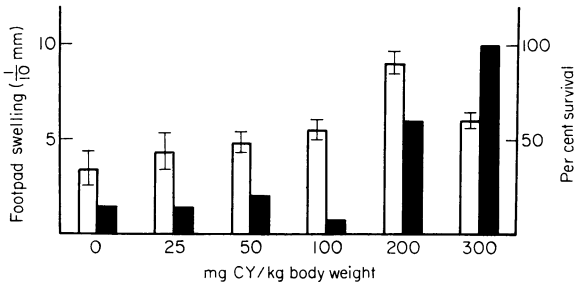

Figure 1. Effect of different doses of CY on DH and ACR. Groups of at least ten mice were treated with saline or graded doses of CY, followed by an i.c. injection of 10^4 viable bacteria in FCA. White columns indicate the footpad swelling (left scale) and black columns the percentage survival (right scale). Vertical bars give s.e.

Influence on DH and ACR of variations in the interval between immunization and challenge

In the preceding experiments the interval between immunizing and eliciting injections was 10 days. As this is possibly not optimal under all conditions, DH and ACR were determined at different intervals after i.c. immunization with 10^4 viable bacteria, with and without CY pretreatment.

Optimal DH was measured 10 days after immunization. A CY induced enhancement of DH was observed between days 10 and 25 (Fig. 2). The protection depended on the interval between immunization and challenge. Without CY treatment, the best protection was found at 5 days after

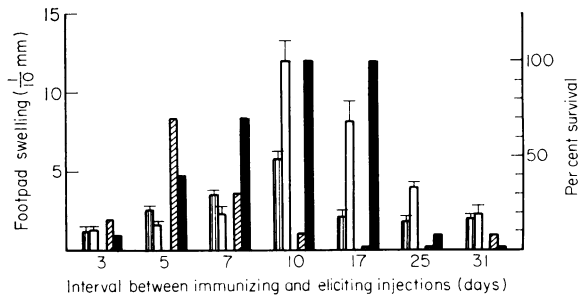

Figure 2. Effect on DH and ACR of variation in the interval between immunization and eliciting and challenge injections. Groups of at least ten mice were treated with saline or 300 mg CY/kg, followed by an i.c. injection of 10^4 viable bacteria. After different intervals the eliciting injection for DH was given (▥ saline-, □ CY-treated; left scale). The challenge injection for ACR was given 2 days later (▨ saline-, ■ CY-treated; right scale). Vertical bars give s.e.

immunization (twenty-one out of thirty survived) but declined rapidly. CY pretreatment seemed to cause an initial suppression of the ACR, followed by an augmented and longer lasting protection. With an interval of 27 days neither treatment gave protection.

DISCUSSION

Attempts to prevent Listeria infection by active immunization with vaccines composed of sublethal numbers of viable *L. monocytogenes* have been succesful (Hasenclever & Karakawa, 1957), but these vaccines sometimes killed experimental animals (Osebold, Njoku-Obi & Abare, 1959). Induction of resistance to listeria infection in mice by the non-viable L forms of Listeria was effective when Bordetella pertussis vaccine was added to the L-form culture (Bartlema & Braunius, 1971). Our attempt to potentiate the active immunization with L forms by pretreatment of the mice with CY, failed. All mice died on challenge, though DH was enhanced after immunization with high numbers of L forms. Intraperitoneal injection of viable bacteria did not result in protection when the dose was 10^5 bacteria or less and killed the mice when the dose was 10^7 or over. Only in the small dose range left, part of the mice were protected. After CY pretreatment the i.p. route of immunization was even less suitable to immunize for protection.

Injection of viable bacteria via the i.c. route reduced the mortality due to immunization considerably. Even 10^9 bacteria were tolerated. The injection route and the emulsification with FCA seems to slow down the release of bacteria. CY pretreatment considerably reduced the sensitivity of mice to the immunizing dose. However with 10^5 or less bacteria the mortality upon immunization was low while the ACR was high. Even after immunization with 100 bacteria, most animals (10/15) were protected. CY pretreatment renders immunizing doses effective, which are not in non-CY-treated mice.

In all instances a positive ACR was accompanied by a positive DH. Kerckhaert, Hofhuis & Willers (1977) using sheep red blood cells showed a pronounced influence on DH of the dose of CY and of the interval between the injections of CY and the eliciting antigen. In the present experiments 4 mg CY caused the greatest enhancement of DH, while the greatest protection was obtained after 6 mg. This suggests a dissociation between DH and ACR. The dissociation was even more pronounced when the interval between immunization and challenge was varied. In the non-CY treated mice, ACR preceded DH, the peak responses were obtained respectively on days 5 and 10. CY pretreatment suppressed DH longer than ACR, but between days 10 and 25 for DH and between 12 and 19 for ACR the responses were greatly enhanced compared with those of the non-CY treated mice. These results suggest that the CY-induced delay in the onset of the reaction followed by an enhancement as described for DH (Kerckhaert *et al.*, 1974) is also found for the ACR after CY treatment and i.c. immunization with viable bacteria. The temporal relationship between the development of ACR and DH corresponds with the results which were described for guinea-pigs (Halliburton & Blazkovec, 1975). Whereas the later appearance of DH might reflect differences in sensitivity between ACR and DH determination this can not explain the longer duration of DH as compared with ACR. The possibility that bacterial antigens mainly involved in the DH differ from those inducing ACR is already suggested by the experiments with L forms. Further experiments with isolated bacterial fractions, for inducing ACR and for eliciting the DH instead of the L forms used in these experiments will be necessary. ACR induced by a single i.c. injection of *L. monocytogenes* was of short duration. McGregor, Koster & Mackaness (1971) found indications that the cells that afford protection against a systemic listeria injection belong to a lymphocyte population that has a rapid turnover and a short-circulating lifespan. Although CY treatment caused an extension of ACR, it disappeared completely at day 25. Considerable prolongation of ACR has been found after multiple injections of non-viable *L. monocytogenes* preparations combined with non-specific stimulation by *Bordetella pertussis* (Bartlema & Braunius, 1971) or lipopolysaccharide (Rodriguez, McClatchy & Campbell, 1974). The effect of CY treatment followed by multiple immunizations with *L. monocytogenes* will be investigated.

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