Depletion of Thymic Macrophages in the Rat by Liposome-Encapsulated Dichloromethylene Diphosphonate*

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Summary. Liposome-entrapped dichloromethylene diphosphonate (Cl_2MDP) was injected locally into the thymus of adult rats. This treatment, which has been found to deplete resident macrophages in other organs, also reduced the number of thymic macrophages. The depletion was evident in the cortex and the cortico-medullary zone at 5, 13 and 23 days after Cl_2MDP treatment, while the decrease in medullary macrophages only became significant 13 and 23 days after injection. However, the maximal reduction of macrophages was evident 13 days after this treatment. Thirty-five days after Cl_2MDP treatment, macrophages repopulated the thymus. Our results suggest that the cortical and cortico-medullary macrophages have a high phagocytic activity consistent with their predominant role as scavengers of the large numbers of thymocytes that die under physiological conditions. It is concluded that liposome-mediated depletion of thymic macrophages can serve as an experimental model to study the roles of these cells.

Macrophages are an important component of the thymic microenvironment (KENDALL, 1991; BOYD et al., 1993). It is believed that monocytes enter the gland with other circulating stem cells and then differentiate into the conventional macrophages which are found in the cortex, cortico-medullary junction and medulla (HAMBLIN and EDGEWORTH, 1988). Thymic macrophages are multipotential cells and may develop a variety of characteristics depending on the nature of the signals they receive (FEDORKO and HIRSCH, 1970; BELLER and UNANUE, 1978; UNANUE and ALLEN, 1987; PAPADIMITROU and ASHMAN, 1989; COLIC et al., 1990).

It has recently been shown macrophages can be eliminated by local (THEPEN et al., 1989; BERGH et al., 1993) or systemic injection of liposome-entrapped dichloro-methylene diphosphonate (Cl_2MDP) (VAN

ROOIJEN and VAN NIEWMEGEN, 1984; VAN ROOIJEN et al., 1985; VAN ROOIJEN, 1989; DELEMARRE et al., 1990; RYMAN and TYNEL, 1980). This substance is ingested by macrophages when encapsulated in liposomes, but not when given free in solution. Once phagocytosed, the lysosomal membranes are digested by phospholipases and the drug released into the cytoplasm of these cells (SUNG et al., 1983; VAN ROOIJEN and VAN NIEWMEGEN, 1983). The exact mechanism of macrophage elimination by the intracellular liposome-delivered drug is not known, but one of the main characteristics of diphosphonates in general is their strong affinity for Ca^{2+} .

Therefore the depletion of intracellular Ca^{2+} from the cytoplasm of cells by diphosphonates and the formation of Ca-diphosphonate complexes could be a part of the mechanism which destroys the macrophage cells. As reported by VAN ROOIJEN et al. (1990), since the intravenous route for administering Cl_2MDP -liposomes did not eliminate macrophage populations in the thymus or the other organs observed (e.g. popliteal-paratracheal, and mesenteric lymph nodes), we injected Cl_2MDP -containing liposomes locally into the thymus to investigate macrophage response in this organ. For this reason we used the cytochemical method for acid phosphatase and immunocytochemical method using TRPM1 and TRPM2 monoclonal antibodies (TAKEYA et al., 1989) that recognize macrophage cells. The TRPM1 monoclonal antibody recognizes also interdigitating cells (IDCs).

MATERIALS AND METHODS

Cl_2MDP -containing liposomes were prepared as described by VAN ROOIJEN and VAN NIEWMEGEN (1984).

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Thirty-two adult (12-week old) male Wistar rats (250–300 g) were studied: 16 animals were injected with liposomes containing dichloromethylene diphosphonate (the kind gift of Boehringer Mannheim) intrathymically; the remaining 16 served as controls and received liposomes containing phosphate saline (PBS), again intrathymically. The other 5 animals were used as controls and received neither Cl_2MDP -liposomes nor PBS-liposomes (untreated animals). Each animal was anaesthetised with xilazine/ketamine (xilazine: 10 mg/kg; ketamine: 50 mg/kg) and the thorax opened by cutting the sternal manubrium and pectoral muscles. The clearly visible thymus gland was injected with 200 μl (4–6 injections within the thymic lobules) of Cl_2MDP -liposome suspension (prepared from 100 mg Cl_2MDP in 0.5 ml PBS) using a Hamilton syringe (treated animals) or 200 μl of PBS-liposome suspension (untreated animals). The thorax was closed with silk sutures and the animals given standard laboratory chow and tap water *ad libitum*. They were sacrificed 5, 13, 23 and 35 days after treatment (4 rats for each day) to determine its maximum effect.

Immuno- and enzyme histochemistry

Cryostat sections (10 μm) from the thymi of treated (with Cl_2MDP) and control (with PBS-liposomes

injection) and untreated rats (without injection) were cut and assayed for the presence of macrophages using TRPM1 and TRPM2 monoclonal antibodies (TAKEYA et al., 1989), which recognize respectively macrophages, IDCs and macrophages (A), and acid phosphatase, the enzyme marker for macrophages (B).

A) The sections were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde for 10 min, washed in a phosphate buffer of 0.1 M pH 7.4 and incubated with TRPM1 and TRPM2 (Technogenetics-Recordati-Italy) using the method described by HSIAO et al. (1991) (with some modifications noted below). TRPM1 recognizes a membrane bound antigen located in cytoplasmic vacuoles and the cell surface of rat IDCs and macrophages. TRPM2 recognizes an intracytoplasmic component on the nuclear envelope and on vacuoles of rat macrophages. A rabbit antimouse secondary antibody (Labometrics s.p.a.—Italy) and a PAP mouse complex (Labometrics s.p.a.—Italy) were also used. Peroxidase activity was visualized by 3-amino-9-ethylcarbazole (AEC) solution (Sigma Chemical Co., U.S.A.). This solution was prepared by adding 5 ml of AEC solution (0.4 g/100 ml dimethylformamide) and 40 μl of H_2O_2 to 100 ml of sodium acetate buffer (0.1 M, pH 5.2).

Table 1. Acid phosphatase, TRPM1 and TRPM2 positive macrophages in the cortex, cortico-medullary zone and medulla of thymi of control (C), untreated (U) and rats 5, 13, 23 and 35 days after liposome-encapsulated Cl_2MDP treatment. Data shown are means (X) and standard deviations (SD) of positive cells as determined by ANOVA (* $P < 0.01$, $N = 1200$)

	C	U	Days after treatment			
			5	13	23	35
Acid phosphatase						
Cortex	14.0 $\pm$ 2.5	13.6 $\pm$ 3.1	9.8 $\pm$ 3.2*	3.6 $\pm$ 1.0*	7.1 $\pm$ 1.7*	12.3 $\pm$ 3.2
Cortico-medullary junction	11.7 $\pm$ 3.2	12.4 $\pm$ 2.7	7.9 $\pm$ 3.5*	3.2 $\pm$ 1.3*	6.7 $\pm$ 1.4*	10.5 $\pm$ 2.8
Medulla	10.8 $\pm$ 3.0	11.0 $\pm$ 2.0	10.2 $\pm$ 2.7	3.5 $\pm$ 1.2*	6.3 $\pm$ 1.5*	8.2 $\pm$ 2.5
TRPM1						
Cortex	8.0 $\pm$ 2.1	8.5 $\pm$ 1.8	6.2 $\pm$ 2.0*	1.6 $\pm$ 1.0*	4.0 $\pm$ 1.4*	6.9 $\pm$ 1.0
Cortico-medullary junction	9.3 $\pm$ 3.2	9.0 $\pm$ 2.5	7.4 $\pm$ 1.4*	1.8 $\pm$ 1.0*	3.2 $\pm$ 1.1*	8.5 $\pm$ 2.5
Medulla	9.2 $\pm$ 1.6	9.4 $\pm$ 2.7	9.6 $\pm$ 1.8	3.6 $\pm$ 1.4*	5.3 $\pm$ 2.0*	8.0 $\pm$ 2.3
TRPM2						
Cortex	10.7 $\pm$ 1.8	10.2 $\pm$ 1.4	7.8 $\pm$ 2.1*	2.6 $\pm$ 1.0*	5.0 $\pm$ 1.3*	8.1 $\pm$ 2.1
Cortico-medullary junction	8.3 $\pm$ 2.2	8.5 $\pm$ 2.1	6.2 $\pm$ 2.1*	2.7 $\pm$ 1.3*	4.8 $\pm$ 1.0*	7.6 $\pm$ 2.1
Medulla	7.9 $\pm$ 1.3	7.6 $\pm$ 2.0	7.5 $\pm$ 1.6	2.4 $\pm$ 1.0*	4.6 $\pm$ 1.2*	7.0 $\pm$ 1.6

Fig. 1. Photomicrographs showing AcP positive cells (macrophages: *arrows*) in the medulla (*M*) and cortex (*C*) of rat thymus respectively in controls (*a*) and on the 13th day after Cl_2MDP treatment (*b*). $\times 230$

B) The sections were fixed with cold acetone for 30 sec. Acid phosphatase was demonstrated using a staining solution (Sigma Chemical Co., U. S. A.), containing naphthol-AS-BI phosphate as substrate and Fast Red TR salt as a capture agent (BURSTONE, 1958). The sections were then counterstained with Mayer hemalum.

Statistical analysis

Quantitative analysis was performed on macrophages found in the cortex, cortico-medullary junction and medulla in $10\ \mu\text{m}$ cryostat sections stained with acid phosphatase, TRPM1 and TRPM2 from treated, control and untreated rats. Under the light microscope ($\times 250$), 100 randomly selected fields of the thymic cortex and medulla ($0.10\ \text{mm}^2$) were analysed in each section (three sections per animal). The cortico-medullary junction was evaluated taking 100 lengths ($0.35\ \text{mm}$) in each section (three sections per animal). Means and standard deviations (SD) in treated control and untreated animals were calculated and

the results studied by the analysis of variance (ANOVA) and Bonferroni test.

RESULTS

Distribution of macrophages in the thymus of rats injected with liposomes containing saline suspension (controls) and in the thymus of rats not injected (untreated)

The macrophages of control and untreated rats in the cortex, cortico-medullary zone and medulla showed strong, weak or moderate acid phosphatase activity (presenting cytoplasmic granules) (Figs. 1a, 4a). Furthermore, a large number of IDCs were weakly positive to acid phosphatase and clearly identified by the TRPM1 monoclonal antibody (Figs. 2a, 4d). The same distribution of macrophages was found with TRPM1 and TRPM2 monoclonal antibodies (Figs. 2a, 3a, 4b, c). Macrophages identified by these methods were rounded, sometimes dendritic and appeared isolated

Fig. 2. Photomicrographs showing TRPM1 positive cells (arrows: interdigitating cells, arrowheads: macrophages in the cortex (C) and medulla (M) of the rat thymus respectively in controls (a) and on the 13th day after Cl_2MDP treatment (b). $\times 230$

or in clusters. The distribution pattern of these cells varied in different parts of thymus as shown in Table 1. No difference was observed between the number and distribution of macrophages in control and untreated rats.

Effect of Cl_2MDP -liposome treatment on macrophages in rat thymus

Five days after administration of Cl_2MDP -liposomes, the thymic morphology had not changed greatly and the cytochemical and immunocytochemical pattern was similar to that observed in control rats. Specifically, macrophage positivity was of variable intensity by acid phosphatase, TRPM1 and TRPM2. However, the average number of macrophages in the cortex and cortico-medullary zone appeared reduced. This was confirmed by statistical analysis which showed a significant difference among macrophage numbers in treated and control or untreated rats (Table 1). In the medulla, no significant difference in macrophage

numbers was found.

Thirteen days after the injection of Cl_2MDP -liposome, the thymic morphology still appeared grossly unaffected by liposome injection: the cortex, cortico-medullary zone and medulla were clearly evident. However, as revealed by acid phosphatase, TRPM1 and TRPM2 histochemistry, macrophages were much reduced in the cortex, cortico-medullary zone and medulla. Statistical analysis revealed that the reductions were significant compared to control or untreated rats ($p < 0.01$) (Table 1). A small number of acid phosphatase, TRPM1 and TRPM2 positive macrophages (Figs. 1b, 2b, 3b) was still evident in the cortex (mainly outer cortex), cortico-medullary zone and medulla; these cells appeared morphologically normal.

Twenty-three days after Cl_2MDP , the number of acid phosphatase, TRPM1 and TRPM2 positive macrophages was noticeably increased as shown in Table 1. The number of TRPM1, TRPM2 and acid phosphatase positive macrophages was similar to normal

Fig. 3. Photomicrographs showing TRPM2 positive cells (*arrows*: macrophages) in the medulla (*M*) and cortex (*C*) of the rat thymus respectively in controls (**a**) and on the 13th day after Cl_2MDP treatment (**b**). $\times 230$

values by Day 35. Cl_2MDP had no apparent effect on the interdigitating cells revealed by enzyme histochemistry and TRPM1 monoclonal antibody, and distinguished by their dendritic morphology with many processes and interdigitations between the thymocytes. This finding was consistent with our ultrastructural data (unpublished observations) showing the IDCs in the thymic medulla.

DISCUSSION

The present study shows that it is possible to reduce the number of thymic macrophages by a single intrathymic administration of Cl_2MDP -containing liposomes. It is interesting to note that the administration of PBS-liposome does not cause morphological alterations in the thymus. Maximal reduction of the macrophages by liposomes containing Cl_2MDP was observed 13 days after Cl_2MDP treatment (23 days after this treatment macrophages were notice-

ably increased and were similar to controls by Day 35).

That acid phosphatase positive macrophages disappear from the cortex, cortico-medullary zone and medulla does not prove that these cells were eliminated, since they could simply have lost their enzymatic activity. For this reason, we also studied their immunological properties, targeting the antigen recognized by TRPM1 and TRPM2 antibodies. Thymic gross morphology compared to controls or untreated animals did not change following the treatment. Interdigitating cells, TRPM1 positive and weakly acid phosphatase positive, were apparently not affected by Cl_2MDP , consistent with the fact that are more differentiated and show little phagocytic activity. It is interesting that 5 days after treatment, statistical analysis revealed a significant decrease in cortex and cortico-medullary zone macrophages, but not among those in the medulla (Table 1).

Thirteen days after treatment, marked macrophage reductions were evident in all zones of the

Fig. 4. Photomicrographs showing respectively a macrophage positive to phosphatase acid (a), a macrophage positive to TRPM2 monoclonal antibody (b), a macrophage (c) and an interdigitating cell (d) positive to the TRPM1 monoclonal antibody. $\times 1,200$

thymus. The reduction in cortex and cortico-medullary zone macrophages 5 days after Cl_2MDP treatment could be explained in two ways: 1) the thymic microenvironment impedes early phagocytosis of the liposomes by medullary macrophages, which are therefore first ingested by cortex macrophages; 2) cortical and cortico-medullary macrophages are more actively phagocytic, since one of their main roles is probably to scavenge the large numbers of thymocytes that die under normal conditions. The latter may therefore ingest more liposomes, resulting in higher doses of Cl_2MDP per cell. This second hypothesis is

supported by works (DELEMARRE et al., 1990) indicating that macrophage phagocytic activity in the popliteal lymph node of mice also differs from zone to zone.

Thirteen days after intrathymic injection of liposome-entrapped Cl_2MDP , macrophage depletion was not complete. The macrophages observed could have arrived from the circulation, as in other organs (VAN ROOIJEN et al., 1990; CLASSEN et al., 1990) or could be more differentiated and thus have reduced their phagocytic function. We lean towards the second suggestion, because in previous (unpublished) studies we

demonstrated that a group of thymic macrophages (present principally in the outer cortex) shares an antigen with interdigitating cells, and for this reason probably constitutes a distinct macrophage subpopulation. We have argued that these cells may give rise to interdigitating cells.

Our data show that this method of reducing phagocytic cells may serve for investigation of the functional aspects of thymic macrophages. It may also be useful in studies on drug targeting, where macrophages represent an undesirable trapping mechanism for the drug, either encapsulated in liposomes or conjugated to antibodies (CLASSEN and VAN ROOIJEN, 1984).

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