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Distribution of Lyt antigens on the surface of thymocytes associated with thymic macrophages and dendritic cells

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Summary. Thymocyte subpopulations that are associated with macrophages and dendritic cells of the thymus in vivo were isolated from the thymuses of C57Bl/6 mice, and their Lyt phenotypes were analyzed. Electron-microscopic examination of immunogold-labeled cells revealed that the thymic complexes formed by macrophages mainly contained Lyt-2-positive thymocytes, while Lyt-1-positive thymocytes were more frequently associated with dendritic cells. The characteristic distributions of Lyt antigens on the surface of thymocytes in regions of reciprocal contact with macrophages (Lyt-2-positive cells) and dendritic cells (Lyt-1-positive cells) suggest that these antigens play a role in specific interactions between thymocytes and stroma cells.

Introduction

The individual stages of thymocyte maturation, which are revealed by stage-specific phenotypic patterns in these cells, are associated with specific localizations in the thymus. This maturation process is controlled by nonlymphoid cells present in particular thymic regions, i.e., epithelial cells and macrophages (Ms) in the cortex and epithelial cells and dendritic cells (DCs) in the thymic medulla (Beller et al. 1976; Rouse and Weissman 1981; Van Ewijk 1984). Thymocytes of the cortex, whose maturation seems to be induced by direct contact with epithelial cells (apart from Ms) in the region (so-called thymic nurse cells; Kyewski and Kaplan 1982) are hydrocortisone sensitive and immature with respect to function and surface phenotype (Lyt 123; high-density Thy 1; low-density H-2 K,D; Van Ewijk et al. 1980, 1981; Scollay and Shortman 1983). On the other hand, a significant proportion of medullar thymocytes are immunocompetent and cortisone resistant, express low amounts of Thy 1 and high-density H-2 K,D, and can be classified as belonging to the Lyt-1 or Lyt-123 subsets (Mathieson et al. 1979; Ledbetter et al. 1980; Ceredig et al. 1982). Mouse thymuses have also been found to contain a subpopulation of functionally mature cells that express an immature surface phenotype (Kyewski et al. 1982; Fink et al. 1984). These cells have been isolated from the thymus taking advantage of their association with Ms and DCs in the form of rosettes (Ros). It has been reported that Ros-associated thymocytes exhibit a homogeneous phenotype corresponding to that of cortical thymocytes, and it is thought that these cells represent an intermediate stage in T-cell differentiation induced by contact with Ms and DCs (Kyewski et al. 1982). As DCs and Ms have distinct and separate localizations in the thymus (Duijvestijn and Hoefsmit 1981; Brelińska et al. 1985), differ from one another with respect to numerous biological features (Duijvestijn et al. 1983; Papiernik and Homo-Delarche 1983; Voorhis et al. 1983), it might be expected that these two cell types would have an affinity to different thymocyte subsets. Moreover, in previous studies, we have demonstrated that the fraction of Ros-associated thymocytes is not uniform, since these thymocytes bind both to mesenchymal nonlymphoid cell surfaces and to epithelial cells of the cortex and medulla (Brelińska et al. 1986).

Therefore, in the present study, we used the immunogold technique to characterize thymocytes in Ros-Ms and Ros-DCs with respect to their Lyt-antigen expression. The central cells of the complexes, i.e., Ms and DCs were identified from their ultrastructural features (Brelińska et al. 1985, 1986).

Materials and methods

Antisera and reagents. A rat monoclonal antibody against mouse Lyt 1 was obtained from culture supernatants of the hybridoma clone, 53.6.7, while that against mouse Lyt 2 was derived from supernatants of the hybridoma clone, 57.7.3. Goat anti-rat Ig-colloidal gold complexes with a mean colloidal-gold-particle diameter of 20000 Å (Gar 20) and a mean colloidal-gold-particle diameter of 5000 Å (Gar 5) were purchased from Janssen Pharmaceuticals (Beerse, Belgium). The goat anti-rabbit Ig serum-gold complex was prepared in our own laboratory. The enzymes used in this study were obtained from Boehringer (Mannheim, FRG) while fetal calf serum (FCS) was obtained from Gibco (Gibco Europe Limited, Scotland).

Isolation of cellular complexes from thymuses. The isolation method used has been described in detail elsewhere (Brelińska et al. 1986). In brief, M- and DC-thymocyte complexes were isolated from the thymuses of 2-month-old C57Bl/6 mice of both sexes by digesting thymic fragments several times with 2% collagenase (from Clostridium histolyticum) in phosphate-buffered saline (PBS). Sequential 30-min incubations with this enzyme were conducted at 0° C, until complete dissolution of the thymic structure had taken place. Supernatants that contained cell complexes (collected after each diges-

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tion period) were pooled and freed of unbound thymocytes by repeated sedimentation at 1 g through layers of FCS.

Immunogold labeling. Samples of the suspension of thymic complexes were incubated with the monoclonal antibody against Lyt 1 (diluted 1:10) or the monoclonal antibody against Lyt 2 (diluted 1:25) for 30 min at 0° C. The suspension samples were then washed three times with PBS at 0° C and incubated for 30 min at 0° C with Gar-20 or Gar-5 particles.

The control reactions included incubation with normal rat serum followed by incubation with goat anti-rat Ig gold complexes and substitution of the goat anti-rat Ig gold complexes with goat anti-rabbit Ig gold complexes.

Electron microscopy. After three washes in PBS the complexes were fixed in 2.5% glutaraldehyde in phosphate buffer (0.1 *M*, pH 7.4) and postfixed in phosphate-buffered 1% osmium tetroxide (pH 7.4) at 4° C. The multicellular complexes were dehydrated (ethanol/acetone) and embedded in Epon 812. Ultrathin sections were cut using a Reichert Ultramicrotome (OmU3) and examined using a JEM 100-C electron microscope after counterstaining with uranyl acetate and lead citrate (Reynolds 1963). Thymocytes in complexes with Ms or DCs were examined in six to eight cross-sections cut at their respective levels.

Differences between the Ros-M and Ros-DC complexes with respect to their content of Lyt-1- and/or Lyt-2-positive thymocytes were statistically analyzed using the Chi^2 test.

Results

Using the immunogold technique, we examined Lyt-antigen expression on thymocytes bound to the surface of Ms (in Ros-M complexes) and on those bound to the surface of DCs (in Ros-DC complexes). The numbers of Lyt-1- and Lyt-2-positive thymocytes present in these two types of complexes are shown in Table 1. In Ros-M complexes, 71% of the thymocytes were labeled by the antibodies against Lyt 1, while 95% of the thymocytes reacted with antibodies against Lyt 2. In Ros-DC complexes, 93% of the thymocytes were Lyt 1 positive, while 55% carried the Lyt-2 antigen. The Chi^2 value calculated for Ros-M and Ros-DC complexes with respect to their content of Lyt-1- and Lyt-2-positive thymocytes was 20.061, this being higher than the expected value and having a significance level of $P=0.001$. This indicates a clear difference between the two studied complexes.

The distribution of the Lyt-1 and Lyt-2 antigens on the surface of thymocytes was analyzed in the two complexes. In the reaction for the Lyt-1 antigen, colloidal-gold labeling was uniformly distributed over the cell surfaces (Fig. 1a). Moreover, the gold particles did not directly adhere to the cell membrane but were located at a certain distance away from it, with the gap being filled with electron-dense material (Fig. 1b and c). This gap between the Lyt-1 labeling zone and the thymocyte cell membrane was characteristic and was particularly evident when GAR-5 particles were used for labeling. On the surface of Lyt-1-positive thymocytes both in Ros-M and Ros-DC complexes, the gold labeling usually covered small fragments of the cell circumference. However, in contrast, the thymocytes with an irregular outline and numerous villous projections, which were occasionally encountered in Ros-DC complexes, exhibited labeling on large segments of their circumference. In approximately 10% of the Lyt-1-positive thymocytes in Ros-DC complexes, labeling was observed close to sites of reciprocal contact between the thymocyte and DCs (Fig. 2). In contrast, such a distribution of the Lyt-1 antigen was never seen in Lyt-1-positive thymocytes in Ros-M complexes.

Table 1. Total number of Ros-M or Ros-DC thymocytes, and the total number and proportion of cells carrying the Lyt-1 and Lyt-2 antigens

	Lyt 1			Lyt 2		
	Total number of cells	Number of positive cells	[%]	Total number of cells	Number of positive cells	[%]
Macrophage-associated thymocytes	248	176	71	194	184	95
Dendritic-cell-associated thymocytes	310	288	93	318	176	55

The colloidal-gold labeling for the Lyt-2 antigen tended to accumulate over small segments of the cell circumference. It should be noted that the Lyt-2-positive thymocytes in Ros-M complexes were more intensely labeled than those in Ros-DC complexes. In Ros-M complexes, a significant proportion of Lyt-2-positive thymocytes (approximately 30%) exhibited colloidal-gold labeling at sites of direct contact, i.e., in the space between the thymocyte and the M (Fig. 3). A similar distribution of the Lyt-2 antigen was observed close to the site where some thymocytes contacted together (Fig. 4). No Lyt-2 antigens were visible in regions of contact between thymocytes and DCs.

No labeling was detected in any of the control specimens. The observed differences with respect to the Lyt-1 and Lyt-2 labeling of thymocytes in contact with Ms and those in contact with DCs might be considered to represent further evidence for the specificity of the reactions.

Discussion

We attempted to characterize the expression of Lyt antigens on the surface of thymocyte subpopulations selected on the basis of their association with Ms and DCs in vivo (Kyewski et al. 1982; Wu and Thomas 1983; Brelińska et al. 1985, 1986). Our observations indicated that M- and DC-associated thymocytes are heterogenous with respect to their expression of Lyt-1 and Lyt-2 antigens. We also found that the Ros-M and Ros-DC complexes differ in the proportions of Lyt-1- and Lyt-2-positive thymocytes that they contain, as well as in distribution of these antigens on the cell surface. Among thymocytes bound to the surface of Ms, cells with high levels of Lyt 2 are predominant, while among DC-associated thymocytes, most cells carry the Lyt-1 antigen. In Ros-M complexes, the Lyt-2 antigen is frequently present in regions of thymocyte-M contact, while the Lyt-1 antigen is often present close to sites of contact between thymocytes and DCs. Thus, distinct types of thymic nonlymphoid cells seem to bind preferentially to different subsets of thymocytes. This conclusion was further strengthened by statistical analysis, which demonstrated significant differences ($P=0.001$) between Ros-M and Ros-DC complexes with respect to their content of Lyt-1- and Lyt-2-positive thymocytes.

The present observations are in agreement with our earlier results demonstrating the nonrandom distribution of

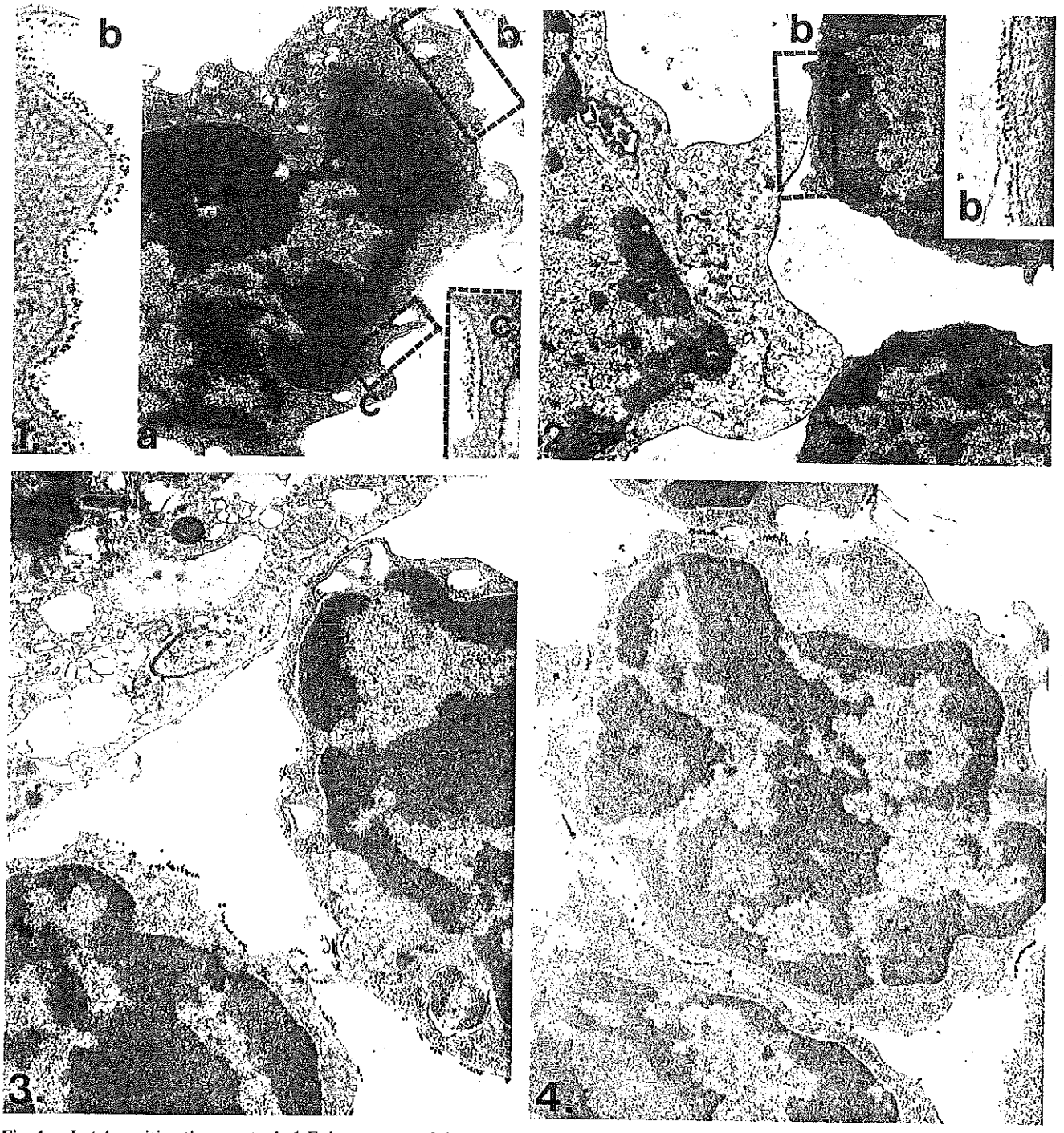

Fig. 1. **a** Lyt-1-positive thymocyte. **b**, **c** Enlargements of the areas indicated in **a**. **a** × 14500; **b**, **c** × 50750

Fig. 2. **a** Lyt-1-positive thymocytes in a Ros-DC complex; labeling is present in the area of cell-to-cell contact. **b** Enlargement of the area shown in **a**. **a** × 14000; **b** × 49000

Fig. 3. Lyt-2-positive thymocytes in a Ros-M complex. The labeling is distributed in an area of thymocyte-M contact. × 23500

Fig. 4. Thymocytes associated with each other. Note the concentration of gold granules in the area of thymocyte contact. × 23500

thymocytes in contact with various types of nonlymphoid cells in the thymus (Brelińska et al. 1985). Other studies of the distribution of thymocytes with distinct surface phenotypes (Van Ewijk et al. 1981; Scollay and Shortman 1983) and of the localization of Ms and DCs have suggested that different Ros-thymocyte complexes might exhibit differences in their antigen expression. Comparison of the labeling of thymocyte subsets by monoclonal antibodies as well as of the intensity of the gold labeling indicated that about 50%–55% of DC-associated thymocytes are positive for Lyt 1 and Lyt 2, while 40%–45% are positive for Lyt 1 but negative for Lyt 2. The remaining 5% of these thymocytes may express other varieties of the Lyt antigen. The nonrandom contacts between DCs and thymocytes suggest a possible role for DCs in thymocyte differentiation. These cells may induce the transition of Lyt-$1^+/2^-$-L3T4$^-$ thymo-

cytes to the pool of helper cells (Lyt-$1^+/2^-$-L3T4$^+$ phenotype) or to the pool of cytotoxic cells (Lyt-$1^+/2^+$-L3T4$^-$ phenotype; Chen et al. 1983; Scollay and Shortman 1985). The results of the present study cast no light on this problem, as we only studied Lyt-antigen expression. However, previous investigations have demonstrated that DC cells are Ia positive (Barclay and Mayrhofer 1981; Duijvestijn et al. 1983), produce interleukin 1 (Papiernik and Homo-Delarche 1983), and are capable of trapping blood-borne antigens and presenting them to thymocytes (Kyewski et al. 1984, 1986); these data suggest that DC cells promote the differentiation of a helper line of thymocytes (Kruisbeek et al. 1985).

Our results concerning the phenotype of M-contacting thymocytes are difficult to interpret in the context of current notions of thymocyte differentiation in the thymus. In contrast to DCs, Ms are present throughout the whole thymus structure, although they are more numerous in the cortex. Therefore, the present results for Ros-M complexes cannot be taken to indicate a role for particular thymic zone (i.e., in contrast to the situation for DCs), so that numerous plausible hypotheses could be devised concerning the roles of thymic Ms.

However, bearing in mind that Ms are primarily in contact with Lyt-$1^-/2^+$ thymocytes, it can be assumed that, apart from eliminating certain thymocytes, Ms probably play a role in inducing the transformation of Lyt-$1^+/2^+$ thymocytes into Lyt-$1^-/2^+$ thymocytes, as noted by Vatteroni and Papiernik (1984) in vitro. More precise definitions of the roles of DCs and Ms will require further study.

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