INTRATHYMIC PRESENTATION OF CIRCULATING NON-MHC ANTIGENS BY MEDULLARY DENDRITIC CELLS

An Antigen-dependent Microenvironment for T Cell Differentiation

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The generation of the T cell repertoire, the site and mechanism of T cell subset bifurcation, and the selective constraints exerted on the developing repertoire to ensure self-tolerance are still poorly understood. The assignment of these events to either the pre-, intra-, or postthymic stages of T cell maturation has been aided by the recent development of mAb to the antigen-specific T cell receptor(s) (1), and the cloning of genes encoding this receptor(s) (2). The available data strongly suggest that the differentiation events mentioned above take place during intrathymic T cell differentiation. T cell receptor gene rearrangement and subsequent T cell receptor surface expression seem to occur early during thymic differentiation. It is likely that ~50% of cortical thymocytes already express the receptor heterodimer and the T3 complex on the cell surface (3, 4). Given the low frequency of T cell precursors among cortical thymocytes and the low exit rate of thymocytes (5–8), T cell receptor expression is obviously a necessary but not a sufficient criterion for positive T cell selection. Since early migrants display a T cell repertoire similar to that of peripheral T cells (9), the development of the T cell repertoire can now be more precisely placed between the intrathymic stage where T cells first express their recognition molecules, and their exit. Assuming the initial generation of a random T cell repertoire, it follows that important selection processes must operate intrathymically on the developing T cell population. Thymocyte–stromal cell interactions are thought to be, at least in part, involved in these differentiation and selection events (10, 11). Three different types of such intercellular interactions have been recently characterized in more detail (12, 13), namely associations between thymocytes and (*a*) cortical epithelial cells (thymic nurse cells, TNC),[1] (*b*) cortical macrophages, and (*c*) medullary dendritic cells (DC); the in vitro isolates of the latter two interactions are referred to as thymocyte rosettes, T-ROS. Evidence has been obtained that T cells specifically and nonrandomly associate with these distinct stromal cells in vivo, and that TNC and T-ROS represent the correlate

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[1] *Abbreviations used in this paper:* DC, dendritic cell; Mgb, myoglobin; ROS, rosettes; TNC, thymic nurse cells.

in vitro of these interactions in vivo. Thus, when T-ROS and TNC were isolated from mixtures in vitro of homozygous Thy-1.1 and Thy-1.2 thymuses, T cells of either type segregated into individual complexes. Also, T cells clustered nonrandomly in individual lymphostromal cell complexes isolated from thymuses of Thy-1.1/1.2 chimeras (13–15). To understand the specificity and physiological function of these recognition processes, we studied the accessibility of thymic stromal cells to circulating protein antigens in vivo, and their capacity to present these antigens to T cells. Two questions are relevant in this context. (*a*), Where do developing T cells first recognize class I and II MHC antigens? (*b*), Where do they first encounter non-MHC antigens in the context of self-MHC determinants during their development? The latter question is particularly relevant to the yet unresolved problem of where and how self-tolerance occurs. Recent observations (16–18) indicating that self-tolerance is MHC-restricted imply the involvement of cell-cell recognition in the induction of self-tolerance.

We previously reported (19) that thymic DC are accessible to small circulating protein antigens, and are efficient in presenting these antigens in vitro. Antigen pulsing in situ was dose- and time-dependent. Here, we report experiments extending this approach to proteins of higher molecular weight, on the turnover of thymic APC, and on the ontogeny of DC-thymocyte interactions.

Materials and Methods

Mice. C57BL/Ka(B/Ka), C3H/J, (C57BL/Ka × BALB/c)F_1, (C57BL/Ka × C3H/J)F_1, (C57BL/Ka [Thy 1.1] × C3H)F_1 mice were all bred in the animal facilities at Stanford University. Female mice 3–4 wk of age were used, if not otherwise stated. Radiation chimeras were constructed as described previously (8), by reconstituting lethally irradiated (900 rad, 250 kVp; Philips Medical Systems, Shelton, CT) recipients with 10^7 bone marrow cells pretreated with anti-Thy-1 plus C′). Non–radiation chimeras were constructed by injection of newborn recipients with 2×10^7 untreated bone marrow cells per day. Recipients were injected into the preocular vein on days 0 and 1, and if possible on day 2, and intraperitoneally on days 3 and 4. 3 wk after the last injection, the mice were killed. The gestation stage of pregnant mice was determined by the appearance of vaginal plugs; this was denoted as day zero.

Reagents. Ammonium sulfate precipitate of mAb PB 107 was used for antibody plus C′ treatment, with low-tox-M rabbit serum (1:15 final dilution; Cedarlane Laboratories, Hornby, Ontario, Canada) as a source of C′. mAb 33D1, which specifically recognizes splenic DC (20) has been kindly provided by Dr. R. Steinman, The Rockefeller University, NY, and was used as culture supernatant (1:10 dilution). Biotin-conjugated anti-I-A^k (clone 10-2.16) (13), biotin–anti-Thy-1.1 (clone 19 E12) (14) FITC-conjugated anti-I-A^b (clone PB107) (14) FITC–anti-Thy-1.2 (clone 30 H12; Becton Dickinson Monoclonal Center, Inc., Mountain View, CA) and tetramethyl-rhodamine isothiocyanate–avidin (Vector Laboratories, Burlingame, CA) were used for labeling of cells in vitro.

Sperm whale myoglobin, Mgb, 17,000 mol wt (Sigma Chemical Co., St. Louis, MO), KLH, 3×10^6 mol wt (CalBiochem-Behring Corp., San Diego, CA), GAT, 60,000–100,000 mol wt (Miles-Yeda, Rehovot, Israel) were used as antigens in vitro in the following final concentrations: Mgb, 40 μg/ml; GAT, 10 μg/ml; and KLH, 80 μg/ml. Doses injected in vivo are indicated for each experiment. Newborn mice were injected with antigen in the preocular vein.

T cell clones. T cell clones of the following antigen and MHC restriction specificity were used: GAT/I-A^k (16 F); GAT/I-A^b (14.14); KLH/I-A^k (14); KLH/I-A^b (16.3); KLH/I-$A^{k,b}$ (NA 4.3); Mgb/I-A^b (28.1); allo-I-A^b (OT-4); the derivation and characterization of these T cell clones has been published elsewhere (21–23). T cells were maintained in vitro by restimulation of 10^6 cloned T cells with 6×10^8 irradiated spleen cells (2,000

rad, Cs source), specific antigen, and 0.1% (vol/vol) supernatant of PMA-stimulated EL-4 cells every 10 d.

Isolation of Thymic and Splenic Lymphostromal Cell Complexes. The isolation and purification of T-ROS and TNC has been described elsewhere in detail (8, 12, 13). Briefly, pooled thymuses were trimmed free of adjacent connective tissue, minced, and agitated in medium (RMPI 1640, 20 mM Hepes; 0.5 ml/thymus) for 10 min at 25°C. Tissue fragments were separated and the suspended cells were designated unselected thymocytes. The tissue fragments were then digested successively four times (10 min each) with collagenase type IV (0.5 mg/ml, 0.5 ml/thymus; Millipore/Continental Water Systems, Bedford, MA) at 25–30°C (T-ROS fractions), followed by three to five digestions with dispase (Boehringer-Mannheim, NY) used at 0.3 mg/ml, 0.25 ml/thymus plus 4 μg/ml DNase I (Sigma Chemical Co.) at 37°C until the thymic tissue was completely digested (TNC fractions). TNC and T-ROS were purified by sedimentation at unit gravity through gradients of FCS at 4°C to obtain lymphostromal cell aggregates in which <5% of the total lymphocytes consist of free thymocytes. Splenic lymphostromal cell clusters were isolated as for thymic T-ROS, e.g. the first four collagenase fractions (each 10 min at 25–30°C) were pooled, and the splenic rosettes were purified to the same degree as T-ROS.

Nonadherent T-ROS and spleen-ROS were obtained after incubation of the mixed ROS fractions on glass coverslips in 24-well plates (Costar, Cambridge, MA) at 5×10^5 T ROS/well for 1.5 h. The nonadherent cells were recovered by gently swirling the plates and collecting the supernatant.

Proliferation Assay. T cells were cocultured at 1 or 2×10^4 cells/well with graded numbers of thymic or splenic cell suspensions or lymphostromal cell complexes, respectively, for 72 h in round-bottom, 96-well microtiter plates (Costar). If not otherwise stated, purified lymphostromal cell clusters were used as stimulator cells in the proliferation assay without depleting the associated thymocytes. Stimulator cells were irradiated with 2,000 rad. Cells were cultured in RPMI 1640 supplemented with 10% FCS (Gibco). 5×10^{-5} M 2-ME, 10 mM Hepes, and penicillin (100 U/ml)/streptomycin (100 μg/ml). Proliferation was measured by the extent of [^{3}H]thymidine uptake (0.5 μCi/well; New England Nuclear, Boston, MA) during a 6-h pulse. Cells were harvested and processed for counting by standard procedures.

Immunoelectron Microscopy. Suspensions of T-ROS and TNC from C3H mice were incubated first with biotin–anti-I-A^k, followed by peroxidase-avidin (Vector Laboratories), each incubation step being 20 min at 4°C. The stained cells were pelleted and fixed in 1% glutaraldehyde in 0.067% cacodylate buffer for 45 min. The pellet was then incubated with 0.1% diaminobenzidine (Sigma Chemical Co.) in 0.3% H_2O_2 in PBS for 20 min, washed, and incubated with 0.5 M $CuSO_4$ in 0.9% NaCl for 20 min. After washing, the pellet was fixed again in 1.25% glutaraldehyde, stained with 2% OsO_4 for 30 min, embedded in epon, and processed for routine electronmicroscopy.

Results

Class II MHC Antigen Expression by Thymic Lymphostromal Cell Clusters. Among T-ROS, surface I-A expression was consistently found on cells resembling the DC (interdigitating) type (24). These cells had a rather irregular shape with long cytoplasmic protrusions extending between the attached thymocytes, with a paucity of lysosomes or other cytoplasmic organelles, and irregular nuclei with dense chromatin lining the inner nuclear membrane (Fig. 1*B*). The second major stromal cell type forming T-ROS (50–60%) were typical macrophages, which were distinguishable from the former cells by their more regular circumference, their larger diameter, and the abundance of lysosomes and phagolysosomes. Cells of such morphology did not express I-A antigens (Fig. 1*A*). These results are consistent with double-labeling fluorescence studies using zymosan and anti-I-A mAb, in which highly phagocytic readily adherent stromal cells from purified

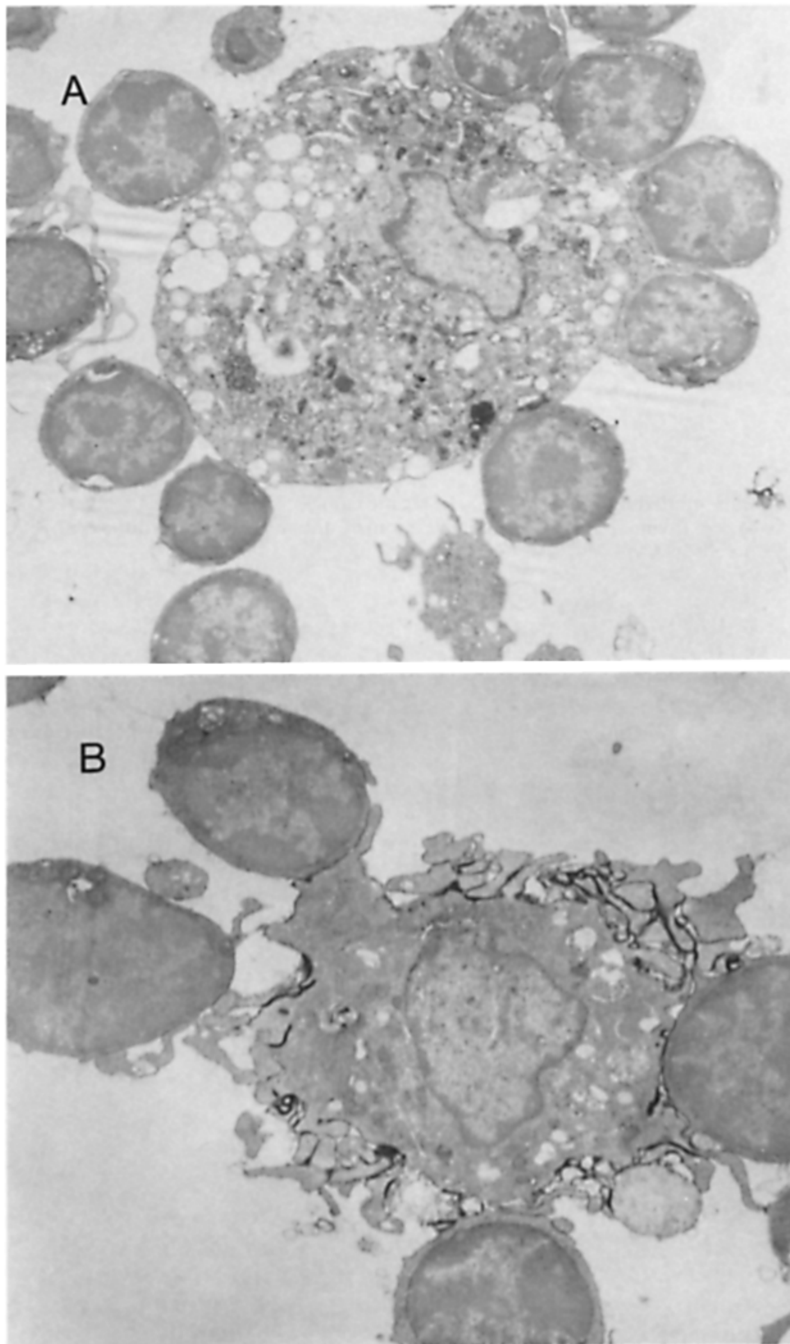

FIGURE 1. I-A antigen expression by T-ROS. T-ROS were isolated from C3H/J mice and stained with anti-I-A^k–biotin/avidin–peroxidase. Typical macrophage-ROS (*A*, × 3,600) and DC-ROS (*B*, × 6,000) are shown. Note the expression of I-A antigens on the surface of DC, and their absence on the macrophage membrane.

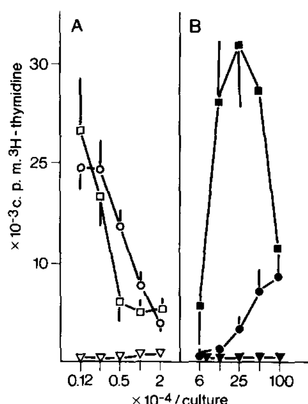

FIGURE 2. Enrichment of APC in thymic and splenic lymphostromal cell clusters. Purified T-ROS (*A*, ○) and spleen-ROS (*A*, □) were compared with nonaggregated thymocytes (*B*, ●) and splenocytes (*B*, ■) from C57BL/Ka mice for their frequency of APC. Stimulator cells were cocultured in graded numbers with GAT-specific cloned T helper cells and antigen. Control: T-ROS (▽) or thymocytes (▼) without addition of antigen. Note the different titration of stimulator cells in *A* and *B*.

T-ROS did not express class II MHC antigens, whereas dendritic-like cells were nonphagocytic and strongly I-A^+ (data not shown). The outer epithelial membrane of TNC was found to express I-A antigens, as has been previously reported (12).

Thymocyte and Spleen Rosettes Are Highly Enriched in APC. When purified T-ROS were compared with unselected thymocytes for APC function, T-ROS were >100-fold enriched in cells inducing antigen-specific proliferation of cloned T helper cells (Fig. 2, *A* and *B*). By the same method, lymphostromal cell complexes can be isolated from spleen (termed spleen-ROS herein), which are composed of central nonlymphoid cells and rosetting T and B cells (our unpublished data). These structures bear resemblance to the DC-lymphocyte clusters observed in vitro (25). Spleen-ROS purified to the same degree as T-ROS are also significantly enriched in APC over nonaggregated splenocytes (Fig. 2). In both, cases, optimal proliferation is induced with ~1,000 ROS/well or less. Antigen presentation by T-ROS or spleen-ROS was not affected by pretreatment of these populations with anti-Thy-1.2 mAb and C′, and could be inhibited by >90% in the presence of appropriate anti-I-A mAb (data not shown). These results illustrate the efficient purification of APC from both organs by taking advantage of their tight interactions in vivo with lymphocytes.

Thymic APC Are Accessible to Circulating Antigens of Different Molecular Size. C57BL/Ka mice were injected with Mgb i.v. (1.0 mg Mgb/g body weight), and 2 h later, T-ROS were isolated. Coculture of these T-ROS with Mgb-specific cloned T helper cells resulted in a specific proliferation in vitro (Fig. 3, *A* and *B*) (19). A similar dose-dependent proliferation of cocultured T cells was elicited by using unpurified splenocytes as stimulator cells from the same animals. To assess the molecular weight range of antigens accessible to thymic APC, we further tested GAT (M_r 60,000–100,000) and KLH (M_r 3 × 10^6) using the same experimental design. After i.v. injection of 1.6 mg GAT/g body weight or 1 mg

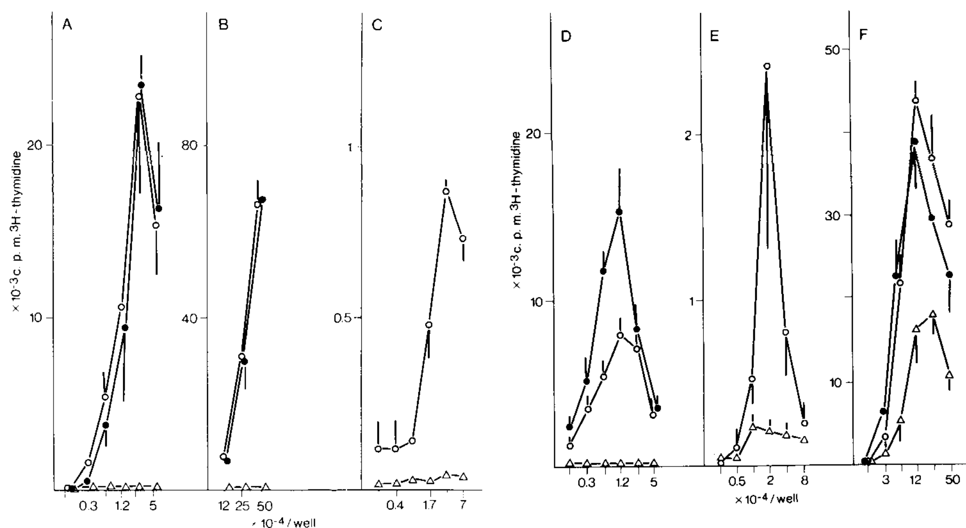

FIGURE 3. Pulsing in vivo with different protein antigens. *A* and *B*: 10 C57BL/Ka were injected with 1.0 mg Mgb/g body weight i.v. 2 h before isolation of APC. Nonadherent T-ROS (*A*) or splenocytes (*B*) were titered as stimulator cells with (●) or without (○) addition of Mgb in vitro. Control: same stimulator cell populations from 10 uninjected mice (△). *C* and *D*: nine C57BL/Ka mice were injected with 1.6 mg GAT/g body weight i.v. 2 h before isolation of APC. T-ROS (*C*) and spleen-ROS (*D*) were titered with (●) and without (○) addition of GAT in vitro. Control: the same stimulator cell populations were isolated from nine uninjected mice (△). *E* and *F*: 14 C57BL/Ka mice were injected with 1 mg KLH/g body weight i.v. and 2 h later APC were isolated. Nonadherent T-ROS (*E*) and splenocytes (*F*) were titered as stimulator cells with (●) and without (○) addition of KLH in vitro. Control: the same stimulator populations isolated from 14 uninjected mice (△).

KLH/g body weight, highly enriched thymic APC led to significant stimulation of GAT-specific or KLH-specific T helper cells, respectively (Fig. 3, *C–F*). A quantitative difference between high and low molecular weight antigens should be pointed out. Whereas a dose of 1 mg Mgb/g body weight leads to saturation of thymic and splenic APC in vivo (further addition of antigen in vitro does not increase the T cell response (Fig. 3, *A* and *B*), in the case of the high molecular weight antigen GAT, the response by splenic APC could be further elevated by addition of GAT in vitro (Fig. 3*D*). Moreover, the response elicited by thymic APC isolated from mice injected with GAT and KLH was suboptimal (compare Fig. 2*A* with Fig. 3*C*). These results confirm the previous observations that molecules of a wide molecular weight range can permeate the vessels of the thymic medulla (26). Whether the quantitative differences between thymus and spleen in the extravasation of high molecular weight proteins are due to specific properties of the molecules (e.g. charge) remains open.

Effect of mAb on Thymic Antigen Presentation. When T-ROS-forming stromal cells were split into adherent and nonadherent stromal cells, antigen presentation function copurified with the nonadherent cell fraction (19). Adherent cells are to >90% I-A⁻ macrophages, whereas the nonadherent ROS are highly enriched in DC-ROS (as assessed by morphology, I-A expression vs. phagocytosis, and Fc receptor expression). Pretreatment of nonadherent T-ROS with the DC-specific mAb 33D1 (20) plus C′ reduced their antigen presentation capacity by ~50%

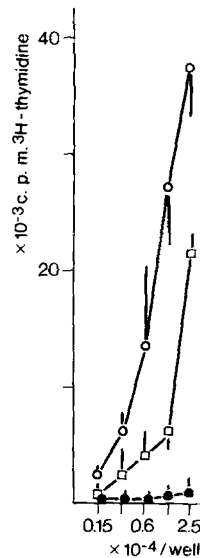

FIGURE 4. Effect of mAb 33D1 and PB 107 on APC function of nonadherent T-ROS. 15 C57BL/Ka mice were injected with 0.5 mg Mgb/g body weight and 2 h later nonadherent T-ROS were purified and titered as stimulator cells after pretreatment with C′ alone (○), mAb 33 D1 plus C′ (□), and mAb Pb 107 plus C′ (●). The numbers of cells seeded refer to counts before treatment.

after pulsing in vivo. Pretreatment with an anti-I-A mAb resulted in abrogation of T cell proliferation by ~90% (Fig. 4). While this result further supports the role of DC in thymic antigen presentation, further detailed studies using lineage-specific mAb and APC of different organs will be necessary to delineate the functional contributions of different cell lineage(s).

Turnover of Thymic APC. To assess whether thymic DC are long-lived in situ or continously replaced by extrathymic DC, as is the case for splenic DC (27), we chose a non-radiation chimera model. Substantial chimerism of the hematopoietic system can be achieved in adult mice without prior ablation of host bone marrow (28). Similarly, chimerism can be induced in newborn mice by multiple injections of congenic or semiallogeneic bone marrow cells. In both models, the degree of stem cell chimerism is strictly dependent on the dose of donor cells injected and the route of injection (28 and our unpublished data). Groups of newborn C3H mice were injected five times with either (C3H × C57BL/Ka)F_1 or (C3H × BALB/c)F_1 BM cells. 3 wk after the last injection, purified nonadherent T-ROS from these chimeras were cocultured with anti-I-A^b alloreactive T helper cells. As shown in Fig. 5, *A* and *B*, thymic and splenic APC of b × k → k, but not c × k → k chimeras induced specific T cell proliferation, which corresponded to ~10–20% of the response induced by age-matched untreated (b × k)F_1 mice. Additionally, in a second group of chimeras, T cell chimerism was determined by using Thy-1.1/1.2 congenic mice (Fig. 5, *C* and *D*). Among unselected thymocytes, 5 ± 1.4% (n = 4) were of donor type, as determined by double immunofluorescence (500 thymocytes were counted per sample). The data suggest that these mice established a stem cell chimerism that was reflected both in the T cell and DC lineage.

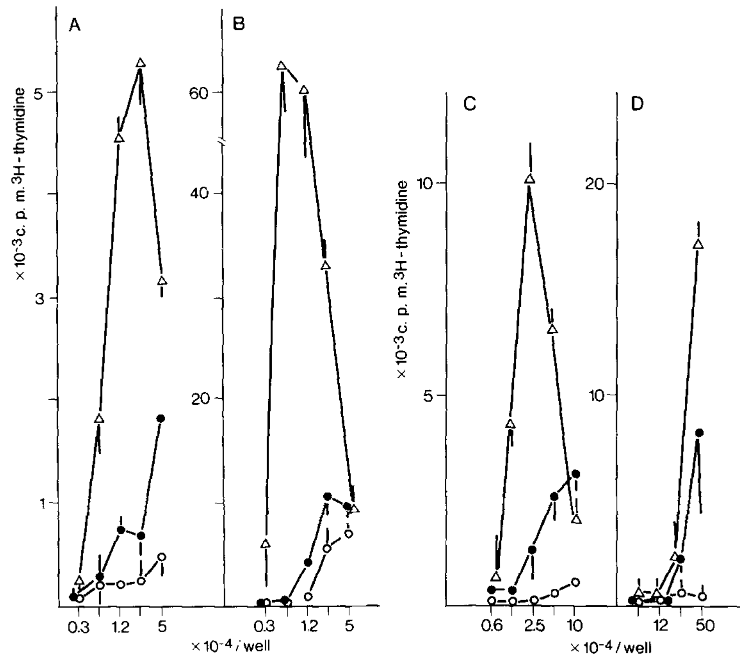

FIGURE 5. Turnover of thymic and splenic APC in non-radiation chimeras. *A* and *B*: nonadherent T-ROS (*A*) and nonadherent spleen-ROS (*B*) were isolated from seven (C3H × C57BL/Ka)F_1 → newborn C3H (●), eight (C3H × BALB/c)F_1 → newborn C3H non-radiation chimeras (○), and eight (C3H × C57BL/Ka)F_1 mice (△). All thymuses and four spleens of each group were pooled for isolation of T-ROS and spleen-ROS, respectively. Spleen-ROS were pretreated with anti-Thy-1.2 mAb and C'. *C* and *D*: nonadherent T-ROS (*C*) and unseparated splenocytes (*D*) were isolated from 12 (C3H × C57BL/Ka [Thy-1.1])F_1 → newborn C3H non-radiation chimeras (●), 13 (C3H × C57BL/Ka [Thy-1.1])F_1 mice (△), and 12 C3H mice (○). In both experiments, stimulator cells were cocultured in graded numbers with an allo-I-A^b-specific T cell clone. For details of chimeras see Materials and Methods.

Second, lethally whole body-irradiated (900 rad) C3H mice were reconstituted with (C3H × C57BL/Ka)F_1 BM cells. 16 or 33 d after reconstitution, purified T-ROS isolated from these chimeras were compared with those from untreated F_1 mice for their capacity to present KLH to I-$A^{b,k}$ hybrid determinant-restricted T cells. As shown in Fig. 6, *A* and *B* 16 or 33 d postirradiation, sufficient thymic APC within T-ROS were derived from donor cells to completely restore the antigen presentation activity. The same degree of reconstitution was observed when spleen cells of these mice were used as a source of APC (data not shown). These results combined with those of the previous section suggest a rapid and complete replacement of thymic DC in radiation chimeras within the first 5 wk.

Ontogeny of Thymic Antigen Presentation In Vitro and In Vivo. Both T-ROS and TNC can be isolated from prenatal thymuses, and their recovery parallels the ontogeny of thymic T cell differentiation (Table I). A direct comparison of antigen presentation by T-ROS and spleen-ROS of fetal (day 18–19 of gestation), newborn, 7-d, and 5-wk-old mice is illustrated in Fig. 7, *A* and *B*. Whereas T-ROS of all four age groups elicit similar T cell proliferation, spleen-ROS of newborn and 1-wk-old mice still contain significantly fewer APC then of adult mice. The antigen presentation capacity of T-ROS and spleen-ROS directly

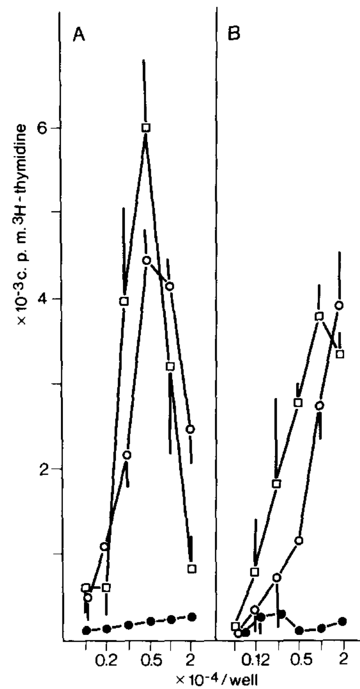

FIGURE 6. Turnover of thymic APC in radiation chimeras. *A*: T-ROS were isolated from 14 (C57BL/Ka × C3H)F_1 → C57BL/Ka radiation chimeras (900 rad) 16 d after reconstitution (□), from 10 F_1 mice (○) and 16 C57BL/Ka mice (●). *B*: T-ROS were isolated from nine (C57BL/Ka × C3H/J)F_1 → C57BL/Ka radiation chimeras 33 d after reconstitution (□), six F_1 mice (○), and eight C57BL/Ka mice (●). In both experiments, T-ROS were cocultured with KLH/I-$A^{b,k}$–specific T helper cells and antigen.

TABLE I
Ontogeny of Lymphostromal Cell Complexes

Age	Cells found in thymuses (× 10^{-3} cells/thymus)	
	T-ROS	TNC
Days 14–16	+	–
Day 17	+	+
Newborn	0.42*	NC
Day 3	4.4 (9.5)	1.2
Day 6	19 (9.2)	3.2 (2.5)
4–5 wk	100–200	20–40
5 mo	20	4

* T-ROS including those containing a central I-A^+ cell and 4–5 surrounding lymphocytes can be isolated. Values refer to C57BL/Ka mice and represent the mean of at least seven pooled thymuses. The last two age groups include only female mice. Replicate values are given in parentheses. NC, Not counted.

correlated with the frequency of I-A^+ stromal cells within these complexes. T-ROS isolated from newborn mice contained the same relative frequency of DC-ROS (40–50%) as those from adult mice (13), whereas the frequency of I-A^+ stromal cells within spleen-ROS increased from 2% in neonatal to ~50% in adult

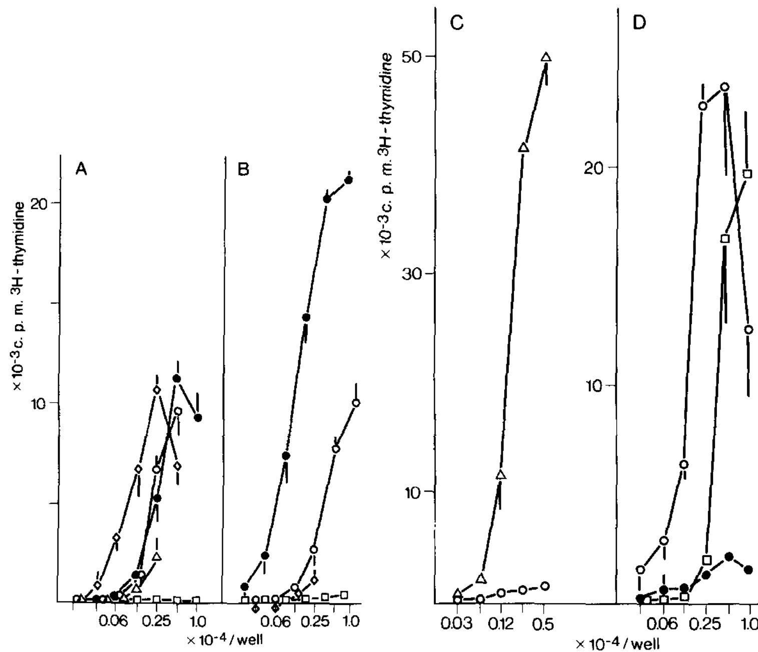

FIGURE 7. Ontogeny of thymic and splenic APC. *A* and *B*: T-ROS (*A*) and spleen-ROS (*B*) were isolated from 18 pooled prenatal (2 litters on days 18 and 19) (△), 12 newborn (◇), nine 1-wk-old (○), and nine 5-wk-old C57BL/Ka mice (●). Stimulator cells were cocultured with GAT/I-A^b–specific T helper cells and antigen. Control: the same stimulator populations from 5-wk-old mice without antigen (□). *C*: three pregnant mice (days 18–19) were injected with 0.5 mg Mgb/g body weight and 2 h later, T-ROS were enriched from the three maternal thymuses (△) and 23 pooled fetal thymuses (○). *D*: 7 newborn (●) and 10 4-wk-old (□) C57BL/Ka mice were injected with 0.5 mg Mgb/g body weight i.v., and 2 h later, T-ROS were isolated and cultured in graded numbers with Mgb/A^b–specific T helper cells. Control: T-ROS isolated from seven uninjected newborns (●).

mice. These results confirm previous findings (29–31), and in addition demonstrate that T cell recognition of thymic DC correlates with T cell ontogeny, and thus precedes similar recognition events in peripheral lymphoid organs.

Antigen traffic to the thymus during ontogeny was further studied in fetal and newborn mice. Three pregnant C57BL/Ka mice (day 18–19 of gestation) were injected with 0.5 mg Mgb/g body weight, and 2 h later, T-ROS were isolated from 23 pooled fetal and the maternal thymuses and assessed for antigen presentation in vitro. Only maternally derived thymic APC induced T cell proliferation of the Mgb-specific T cell clone (Fig. 7*C*). The same result was obtained when pregnant mice were injected with the same dose of Mgb and T-ROS were isolated 16 h after injection, or when pregnant mice were injected with 2 mg Mgb/g body weight (a dose saturating thymic and splenic APC in vivo [Fig. 3, *A* and *B*]) (data not shown). Given the presence and functional detectability of APC in fetal thymuses of this age (Fig. 7*A*) (30) this result speaks for a tight seclusion of fetal thymuses from Mgb in the maternal circulation. Next, newborn animals were directly compared with young adult mice (4–5 wk of age) for accessibility of thymic APC to blood-borne antigens. T-ROS were isolated

from both age groups 2 h after injection of the same relative amount of Mgb i.v. (0.5 mg Mgb/g body weight). Both APC populations induced a similar T cell proliferation (Fig. 7*D*). The thymuses of newborn and adult mice are thus equally exposed to circulating antigens, and the relative frequency of thymic DC in neonates has already reached adult levels.

Discussion

The experiments described in this report address the ability of distinct thymic stromal cells participating in lymphostromal cell-cell interactions in vivo to present antigen intrathymically. The accessibility of thymic stromal cells in vivo to circulating antigens and their capacity to present these antigens was assessed independently. Antigen presentation copurified with thymic DC after pulsing in vivo and in vitro. Thymic DC were equally exposed as splenic APC to blood-borne antigens of different molecular size (M_r 17,000–3 × 10^6). After in situ pulsing, antigen persisted longer in association with thymic than with splenic APC, when APC from both organs were purified to the same degree (19) (data not shown). In contrast to DC, macrophages contained within T-ROS did not present antigen under the same experimental conditions. This I-A^- macrophage subset may thus not be identical with the adherent I-A^+ stromal cells described by Beller and Unanue (32) and Papiernik et al. (33). Enriched TNC isolated from P → F_1 radiation chimeras did not present antigen to F_1 hybrid I-A determinant–restricted T cell clones after pulsing with antigen in situ (19) or in vitro (data not shown), in spite of their strong expression of I-A antigens. Again, TNC may represent only a subset of epithelial cells, and may not be fully representative of all thymic epithelial cells. Further studies using subset-specific markers (31) and positive and negative selection methods will be necessary to precisely assess the accessory function of epithelial cells in vivo. With these qualifications in mind, however, the data indicate a functional compartmentalization of thymic stromal cells. Thymic DC have been shown to be strictly confined to the medulla and corticomedullary junction in man (34), rats (35, 36), mice (37), and chickens (38). TNC have been localized to the outer cortical region (14) and rosetting macrophages to the cortex (in traffic studies using Thy-1 congenic mice the association of donor thymocytes with macrophages coincides in corresponding tissue sections with their appearance in the cortex (our unpublished data). Interestingly, the vessels in the medulla allow the extravasation of blood-borne macromolecules, whereas cortical lymphocytes seem to be protected from circulating antigens by a specialized vascular architecture (26). This apparent confinement of functional APC to the compartment that is open to the circulation probably points to an important functional difference between cortex and medulla.

Our studies confirm and extend previous findings with regard to the ontogeny of thymic APC (29–31). T-ROS can be isolated first at day 14/15 of gestation, at the same time as the first MLR stimulator cells can be detected (Table I). The expression of class II antigens on medullary DC at day 14 in mice (31) and at 7 wk in humans (39) represents the first expression site of these genes in vivo.The relative frequency of APC among T-ROS are similar when day 18–19 fetal, newborn, 1-wk-old, and 4–5-wk-old animals are compared. In contrast, in 1-wk-

old mice, the frequency of splenic APC still lags behind that of adult mice. Furthermore, thymic APC from newborn mice were as accessible to blood-borne antigens as those of young adult mice. Thymic APC of fetuses, however, seem to be secluded from antigen (here shown for Mgb) in the maternal circulation. The results show (*a*) that the frequency of thymocyte-DC interactions in situ strictly correlates with the ontogeny of T cell differentiation (the frequency of T-ROS remained unchanged after immunization with soluble antigens i.v. and cellular antigens i.p. and i.v., data not shown), and (*b*) that the accessibility of this microenvironment is not altered during postnatal development. We presume that thymic DC are exposed to circulating self antigens from day 14 of gestation onwards.

Thymic DC in the medulla are bone marrow–derived (35, 37, 38, 40), however, there has been controversy as to the rate of turnover and function of thymic APC in radiation bone marrow chimeras (40, 41). The functional analysis of splenic and thymic APC (as isolated by purification of lymphostromal cell complexes) in $P \rightarrow F_1$ and $F_1 \rightarrow P$ radiation chimeras (900 rad) shows that APC in both organs are functionally replaced within the first 2–5 wk after reconstitution. These results are consistent with phenotypic studies (13, 35, 37), and suggest a higher turnover of these cells than previously reported (40). This conclusion is further supported by results from non–radiation chimeras. Multiple injections of F_1 bone marrow cells into newborn parental mice leads to stem cell chimerism without ablation of host bone marrow. The extent of this chimerism is dependent on the dose of donor cells injected and the route of injection (28 and our unpublished data).

After five injections of 2×10^7 F_1-type bone marrow cells, APC of donor origin were present among nonadherent T-ROS, as assessed by proliferation of T cells specific for donor-type I-A antigens. In addition to the DC-lineage, ~5% of thymocytes in these chimeras expressed the Thy-1.1 phenotype of donor origin. A similar degree of chimerism (5–15%) in two distinct hemopoietic cell lineages most likely reflects stem cell chimerism in these animals. The equilibration of DC between the stem cell compartment and the thymus within 3 wk indicates a continuous physiological turnover of these cells. Thus, thymic DC do not differ significantly from splenic DC (27) or Langerhans cells in skin (42) with respect to the kinetics of their turnover in vivo. In addition to the direct entry of blood-borne antigens into the thymus, circulating antigen-laden DC may contribute to the spectrum of intrathymically presented antigens.

What physiological role do DC-thymocyte interactions play in vivo? DC are highly efficient APC in the peripheral lymphoid organs. They interact with T and B cells to initiate the immune response (25, 43). Are thymocytes likewise primed to foreign antigens by recognition of antigen-bearing thymic DC? Priming of thymocytes in situ to non-self antigens (minor histocompatibility antigens) has been reported (44). Certain observations, however, cannot easily be reconciled with the notion that DC-thymocyte interactions merely represent the thymic equivalent of DC-lymphocyte interactions in the periphery. The frequency of DC-ROS is strictly correlated with T cell differentiation, and not with the immune status of the animal: thymic DC-ROS clearly precede analogous multicellular structures in spleen and lymph nodes during ontogeny. Second, DC-

associated thymocytes bear an immature surface antigen phenotype, and thus are distinct from functionally mature medullary thymocytes and peripheral T cells (13 and our unpublished data).

A correlation between the presence of I-A-bearing bone marrow–derived APC in the thymus and the MHC restriction and differentiation of T helper cells has been demonstrated in radiation chimeras (40) and anti-I-A–treated mice (45), suggesting a specific role of thymic APC in the development of this T cell lineage. While our results support the notion that DC-thymocyte interactions participate in T cell development, it is not known yet whether only T helper cells or their precursors interact with DC.

Thymic DC may be involved in T cell tolerance. Numerous experimental observations suggest that induction of T cell-tolerance is an intrathymic event that necessitates the continuous intrathymic presence of the tolerogen (for review see 46). Recent observations may provide new clues to the definition of the site of tolerance imposition. Tolerance induction seems to be MHC-restricted, i.e. T cells are only tolerant to self antigens in the context of their own MHC, but not in the context of allo-MHC (16–18), implying that self antigens need to be presented to T cells before tolerance induction. Second, when thymuses were transplanted, T cells residing in the graft did not become tolerant to class I and II MHC antigens expressed by the grafted thymic epithelial cells (47, 48). By exclusion, only DC fulfill the requirements imposed by these experiments on cellular inducers of T cell tolerance. MHC-restricted recognition of circulating (self) antigens by thymocytes at this stage of their differentiation may lead to their functional deletion or the induction of antigen-specific suppressor cells.

Summary

We present evidence for intrathymic presentation of soluble circulating antigens in vivo. Our results show that proteins of different molecular weight enter the mouse thymus rapidly after i.v. injection. The intrathymic presence of antigen was assayed by proliferation of cloned antigen-specific T helper cells, which were cocultured with purified thymic stromal cells; stromal cells were isolated and purified as lymphostromal cell complexes, which preexist in vivo. Antigen presentation copurified with non–adherent medullary dendritic cells (DC) (interdigitating cells). I-A$^-$ cortical macrophages forming thymocyte rosettes in vivo and I-A$^+$ cortical epithelial cells forming thymic nurse cells (TNC) in vivo did not act as antigen presenting cells (APC) after antigen pulsing in vivo or in vitro. Thymic APC turn over physiologically and are rapidly replaced (within 2–5 wk) after lethal irradiation by donor bone marrow–derived cells. The frequency of thymocyte-DC interactions in vivo strictly correlates with thymic T cell differentiation, and is independent of the immune status of the animal. Fetal thymic APC seem to be secluded from antigen in the maternal circulation. Thymic DC-ROS probably represent the microenvironment where maturing T cells first encounter non-MHC antigens in the context of self-MHC antigens.

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