Immunology 1994 **82** 275–286

Studies on the thymus of non-obese diabetic (NOD) mice: effect of transgene expression

L. A. O'REILLY, D. HEALEY, E. SIMPSON,* P. CHANDLER,* T. LUND,† M. A. RITTER‡ & A. COOKE *University of Cambridge, Department of Pathology, Division of Immunology, Cambridge, *Transplantation Biology Section, Clinical Research Centre, Harrow, Middlesex, †Department of Immunology and Molecular Biology Unit, The Windyer Building, University College London Medical School, London and ‡Department of Immunology, Royal Postgraduate Medical School, Hammersmith Hospital, London*

SUMMARY

The non-obese diabetic (NOD) mouse is a good model of insulin-dependent diabetes mellitus. Autoreactive T cells may play a fundamental role in disease initiation in this model, while disregulation of such cells may result from an abnormal thymic microenvironment. Diabetes is prevented in NOD mice by direct introduction of an Eα^d transgene (NOD-E) or a modified I-Aβ chain of NOD origin (NOD-PRO or NOD-ASP). To investigate if disease pathology in NOD mice, protection from disease in transgenic NOD-E and NOD-PRO and partial protection from disease in NOD-ASP can be attributed to alterations in the thymic microenviroment, immunohistochemical and flow cytometric analysis of the thymi of these mouse strains was studied. Thymi from NOD and NOD-E mice showed a progressive increase in thymic B-cell percentage from 12 weeks of age. This was accompanied by a concomitant loss in thymic epithelial cells with the appearance of large epithelial-free areas mainly at the corticomedullary junction, which increased in size and number with age and contained the B-cell clusters. Such thymic B cells did not express CD5 and were absent in CBA, NOD-ASP and NOD-PRO mice as were the epithelial cell-free spaces, even at 5 months of age. Therefore the mechanism of disease protection in the transgenic NOD-E and NOD-ASP/NOD-PRO mice may differ if these thymic abnormalities are related to disease.

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is a disease with an autoimmune aetiology resulting in the selective destruction of pancreatic β cells.[1] The precise immunological events involved in both the initiation and destruction of the insulin-containing β cells remain to be resolved and animal models of IDDM may provide some insight. One such animal model is the non-obese diabetic (NOD) inbred mouse strain,[2] which displays many of the characteristics of the human disease. Lymphocytic infiltration of the pancreas is observed from 5 to 6 weeks of age in both male and female NOD mice. Concomitant β-cell destruction ensues, resulting in diabetes in 70–80% of female mice by 250 days of age.

The NOD mouse has an unusual major histocompatibility complex (MHC) as it does not express I-E because of a deletion in the promoter region of the I-Eα chain gene.[3,4] The direct introduction of an Eα^d transgene[5,6] or I-E^k [7] into NOD mice, and hence expression of I-E, results in prevention of IDDM. Additionally, the first external domain of NOD I-A is unique.[8] Our recent studies show that a transgene encoding a modified Aβ chain of NOD (NOD-PRO), that contains proline at position 56 instead of the histidine found in NOD is capable of preventing disease.[5] However, introduction of an Aβ transgene with an aspartic acid at position 57 instead of serine (NOD-ASP) does not completely prevent disease. Such mice have a low incidence of IDDM [~15% by 6 months of age (J. Picard, personal communication)] with extensive peri- and intra-islet infiltration (L. A. O'Reilly, unpublished observations).

NOD mice develop lymphocytic infiltration not only in the pancreas but also in other organs including the submandibular glands, lacrimal glands, thyroid, adrenal glands, ovaries and testes.[9] It is therefore conceivable that the NOD mouse develops polyendocrine autoimmune disease as a consequence

Received 6 September 1993; revised 4 February 1994; accepted 12 February 1994.

Abbreviations: BSA, bovine serum albumin; mAb, monoclonal antibody; MHC, major histocompatibility complex; NOD, non-obese diabetic; NOD-ASP, non-obese diabetic mouse expressing transgenic I-A molecule; NOD-E, non-obese diabetic mouse expressing transgenic I-E molecule; NOD-PRO, non-obese diabetic mouse expressing transgenic I-A molecule; PBS, phosphate-buffered saline.

Correspondence: Dr L. A. O'Reilly, University of Cambridge, Dept. of Pathology, Division of Immunology, Tennis Court Road, Cambridge, CB2 1QP, U.K.

of disregulation of autoreactive cells. This may result from an abnormal thymic microenvironment as T cells play a major role in disease development.

The thymus plays a crucial role in the development of the vertebrate immune system. Tolerance is achieved in the thymus by clonal deletion (negative selection)[10] or inactivation of T cells rendering them anergic.[11] Failure to delete or control anti-self responses in the thymus may lead to T-cell-mediated autoimmunity.

Various accessory cell types may be implicated in the successive steps of T-cell selection in the thymus. Positive selection is thought to be effected by interaction of the T-cell receptor on developing thymocytes with MHC molecules on radioresistant thymic epithelial cells,[12,13] whereas interaction with bone marrow-derived dendritic cells in the thymic medulla is thought to be responsible for negative selection by clonal deletion.[14] However regulatory elements in the periphery may also have a role to play.

Cytokeratin expression characterizes the cells of epithelial origin, while monoclonal antibody (mAb) analysis has revealed considerable heterogeneity within the epithelial component of the thymic microenviroment.[15,16] Thymic epithelial cell subsets might correspond to specific microenvironments which could be related to distinct functions affecting T-cell maturation within the thymus. Disruption of thymic epithelial architecture could alter the processes of positive and negative selection and thus play a role in the propensity to develop autoimmunity. Therefore it seemed logical to investigate whether there were any structural or cellular abnormalities present in NOD or transgenic NOD mouse thymi which could be correlated with the development of, or protection from, autoimmune diabetes. Immunohistology of thymi from NOD and transgenic NOD mice enabled the study of cells such as thymic epithelial cells that do not readily form suspensions. This, together with a detailed flow cytometric analysis, allowed us to determine the relationship between disease development in NOD mice and abnormalities in thymic architecture.

MATERIALS AND METHODS

Mice

A breeding nucleus of NOD mice was established at the CRC (Northwick Park, U.K.) from mice provided by Dr E. Leiter (Jackson Laboratory, Bar Harbor, ME). NOD mice expressing the transgenes encoding a $E\alpha^d$ (NOD-E)[5] or a modified $A\beta$ chain (NOD-PRO)[5] were derived and maintained at the CRC. The transgenic NOD-ASP mouse was derived by the same methods as that for the NOD-PRO mouse,[5] except that the single amino acid substitution in the NOD I-$A\beta$ chain was from serine to aspartic acid at position 57 instead of from histidine to proline at position 56 in the NOD-PRO transgenic. The incidence of diabetes in our colony is 70% for NOD females, $<10\%$ for male NOD mice, 0% for NOD-E or NOD-PRO mice of either sex, and 15% for NOD-ASP female mice at 6 months of age (J. Picard, personal communication), when kept under conventional conditions. CBA mice were obtained from a breeding colony at the CRC (Northwick Park). Male and female mice from all strains (five to 10 mice of each sex) were

Table 1. Panel of antibodies used for immunohistology

Name	Molecular specificity	Ig class species	Cells recognized	Reference
Anti-keratin	Predominantly MW 56,000 and 64,000	Rabbit anti-human polyclonal	Whole thymic epithelial cell network	17
4F1E4	CTES III	Rat IgM	Cortical, subset of medullary epithelial cells	15
IVC4	CTES II	Rat IgM	Subcapsular and medullary epithelial cells	15
B220 (clone RA3–6B2)	B-cell-restricted determinant of Ly-5	Rat IgG2a	Mouse B cells	18
Anti-mouse Ig	Mouse Ig	Rabbit polyclonal	All Ig bearing cell (B cells)	Dako
MRC-OX6	Class II MHC I-$A^{k,s,nod}$	Mouse IgG1	All MHC class II-bearing cells	19
Mac-1/70	C3bi receptor	Rat IgG2b	Mouse neutrophils, macrophages and NK cells	20
F4/80	Monoclonal, F4/80 antigen	Rat IgG2b	Mature mouse macrophages	21
SER-4	Sheep erythrocyte receptor	Rat IgG2a	Stromal tissue macrophages in lymph node, liver and spleen	22
YTS 169.4.2.1	$CD8^+$ T cells	Rat IgG2b	Tc/Ts cells	23
Mac-261*	I-$A\alpha^d$, I-$A\beta^{g7}$	Mouse IgG2a	NOD MHC class II-bearing cells	*
TIB120 (M5/114.15.2)	I-$A^{b,d,q}$, I-$E^{d,k}$	Rat IgG2b	MHC class II-bearing cells	24
YTS 191.1.1.2	$CD4^+$ T cells	Rat IgG2b	Th/Ti cells	23
CD5 (clone 53-7·3)	Ly-1 antigen	Rat IgG2a	Mouse T cells + subpopulation B cells	25
Anti-laminin	Connective tissue glycoprotein	Rabbit anti-human	Blood vessels, basement membranes	Sigma

CTES clusters of thymic epithelial staining patterns: II, mAb stains subcapsular and perivascular TEC (only one cell layer), in addition medullary TEC and Hassall's corpusles (HC) are stained; III, mAb stains virtually all cortical thymic epithelial cells. *Kind gift G. Butcher (AFRC, Cambridge, U.K.).

analysed for thymic abnormalities at 1, 3 and 5 months of age.

Immunohistochemistry of NOD and transgenic NOD thymus
Thymi were excised from mice, and one lobe was snap frozen in isopentane. Five-micrometre cryostat sections were air dried and fixed in 100% acetone at room temperature for 10 min. Air-dried fixed sections were stored at −70° before staining with the panel of rabbit polyclonal and rodent mAb in Table 1. Thymi were assessed at three different levels at least 100 μm apart and staining for the different cellular markers was carried out on sequential sections. The remaining thymic lobe was prepared for flow cytometric analysis.

Indirect immunofluorescent staining of sections was used to detect thymic epithelial cells. Sections were first preblocked with 20% normal mouse serum for 30 min, incubated with polyclonal rabbit anti-keratin antibody for 60 min[17] (Dako, Glostrup, Denmark) and, after washing, incubated for 30 min with swine anti-rabbit Ig conjugated to tetramethylrhodamine isothiocyanate (TRITC) (Serotec, Bicester, U.K.), washed and mounted. Rabbit anti-laminin antibody (Sigma, Poole, U.K.) was used to detect basement membranes of blood vessels and perivascular spaces using the same staining protocol. The medullary and cortical epithelial cell populations were defined by indirect immunofluorescence using the rat mAb IVC4[15] and 4F1E4[15] respectively. Following preblocking with normal mouse serum, sections were incubated in primary antibody for 60 min followed by a 30-min incubation with TRITC-labelled goat anti-rat IgM antibody (Southern Biotechnology, Birmingham, AL). B cells were identified by direct immunofluorescence by first preblocking with normal mouse serum then either incubated for 30 min using B220 directly conjugated to fluorescein isothiocyanate (FITC)[18] (Dako) or rabbit anti-mouse Ig directly conjugated to FITC (Dako), washed and mounted. NOD and CBA MHC class II was similarly detected by MRC-OX6 FITC[19] (Serotec). Thymic macrophage cell-surface antigens were detected by the above indirect protocol with the following rat anti-mouse mAb used as tissue supernatants (kind gifts from Drs S. Gordon and P. Crocker, Oxford, U.K.): Mac-1,[20] F4/80[21] and SER-4.[22] The macrophage-specific antibodies were detected by FITC-conjugated goat anti-rat Ig (Jackson Immunoresearch, West Grove, PA). All antibodies and conjugates were titrated before use. Omission of the primary antibody or replacing it with an irrelevant antibody during staining gave a negative result. All immunostaining was assessed by two independent observers.

Flow cytometric analysis of thymic cell suspensions
Fresh thymus was digested in 0·5 mg/ml collagenase (Sigma) and 0·02 mg/ml deoxyribonuclease I (DNase I) (Boehringer Mannheim, Mannheim, Germany) for several minutes, washed in Hanks' balanced salt solution (HBSS) and frozen in 90% fetal calf serum (FCS)/10% DMSO and stored at −70°. For analysis vials were rapidly thawed, washed, aliquoted at 10^5–10^6 cells/sample into a U-bottomed 96-well plate and resuspended in 50 μl of biotinylated mAb in 1% normal mouse serum. Cells were incubated for 30 min on ice and washed three times in 100-μl changes of PBS/1% bovine serum albumin (BSA). Biotinylated mAb OX6 (1 μg/ml), anti-CD8[23] (Coulter Cytometry, Luton, U.K.), Mac-261,[22] or TIB120 (1 μg/ml[24]) were used in the first incubation step. The biotinylated antibodies were detected by a 30-min incubation with either streptavidin R613 (Gibco BRL, Paisley, U.K.) or avidin phycoerythrin (PE; Biogenesis, Bournemouth, U.K.). For double and triple staining we included in this cocktail one or two of the following mAb. conjugates: anti-CD8–FITC[23] (Coulter Cytometry), B220–FITC, anti-CD4–PE[23] (Coulter Clone, Luton, U.K.). Cells were then washed and fixed in 1% formaldehyde. B cells expressing CD5 from NOD (I-A^{g7}) and C57BL/10 (I-A^b) mouse peritoneal lavage and spleen were detected by the above protocol using CD5–PE[25] (Pharmingen, San Diego, CA). All samples were analysed by the FACScan LYSIS 2 program (Becton Dickinson, Mountain View, CA), with fresh NOD spleen cell suspensions stained as above to define the FACScan gate settings. All reagents were titrated before use and either replacement of the primary antibody with an irrelevant antibody or its omission served as negative controls.

RESULTS

Immunohistological analysis of NOD and transgenic NOD thymus

Thymic MHC class II expression. Sections of 1-, 3-, and 5-month-old thymus of CBA mice were stained with OX6 (detects class II MHC), anti-keratin which detects the whole thymic epithelium and IVC4 and 4F1E4 which detect medullary and cortical epithelia respectively. The pattern of expression of each of the anti-epithelial antibodies on thymus sections from both male and female CBA mice and all mouse strains used in this study did not differ. The normal expression of class II MHC, observed as a typically dense staining pattern in the medulla (Fig. 1A) with a surrounding fine spidery network of epithelial cells throughout the cortex was seen for CBA mice at all ages analysed. Thymi from NOD-ASP (Fig. 1B) and NOD-PRO (Fig. 1C) mice at 1, 3 and 5 months of age had an identical staining pattern to that of CBA mice as shown by the dense epithelial staining in the medulla with both MHC class II and keratin and the fine epithelial array in the surrounding cortex which was positive for both MHC class II and keratin. In both CBA (Fig. 1A) and NOD-PRO (Fig. 1C) thymic cortical expression of MHC class II was confined to the cortical epithelial network (as defined by staining with the mAb 4F1E4) with thin lines of positive staining separating small collections of lymphocytes. In contrast, thymi of NOD (Fig. 1D) and NOD-E (Fig. 1E) mice demonstrated a more intense and confluent staining pattern with anti-class II MHC (OX6) both in the cortex and the medulla at 1, 3 and 5 months of age than that seen in age-matched CBA mice (Fig. 1D,E at 5 months).

Thymic epithelial cell architecture. The normal distribution of thymic epithelial cells can be demonstrated by the polyclonal anti-keratin antibody. When CBA thymi are stained at 1 and 3 months a keratin network (Fig. 2A) is revealed throughout the thymus; this is more dense in the medulla (as detected by double staining with IVC4) than in the cortex (double staining with 4F1E4). Thymi from NOD-ASP (Fig. 2B) and NOD-PRO (data not shown) mice at 1 and 3 months of age had an identical staining pattern. However, in all NOD-ASP and NOD-PRO thymi at 5 months of age small gaps in the confluent keratin-staining pattern were apparent in the medulla and at the corticomedullary junction. Such epithelial-free areas although

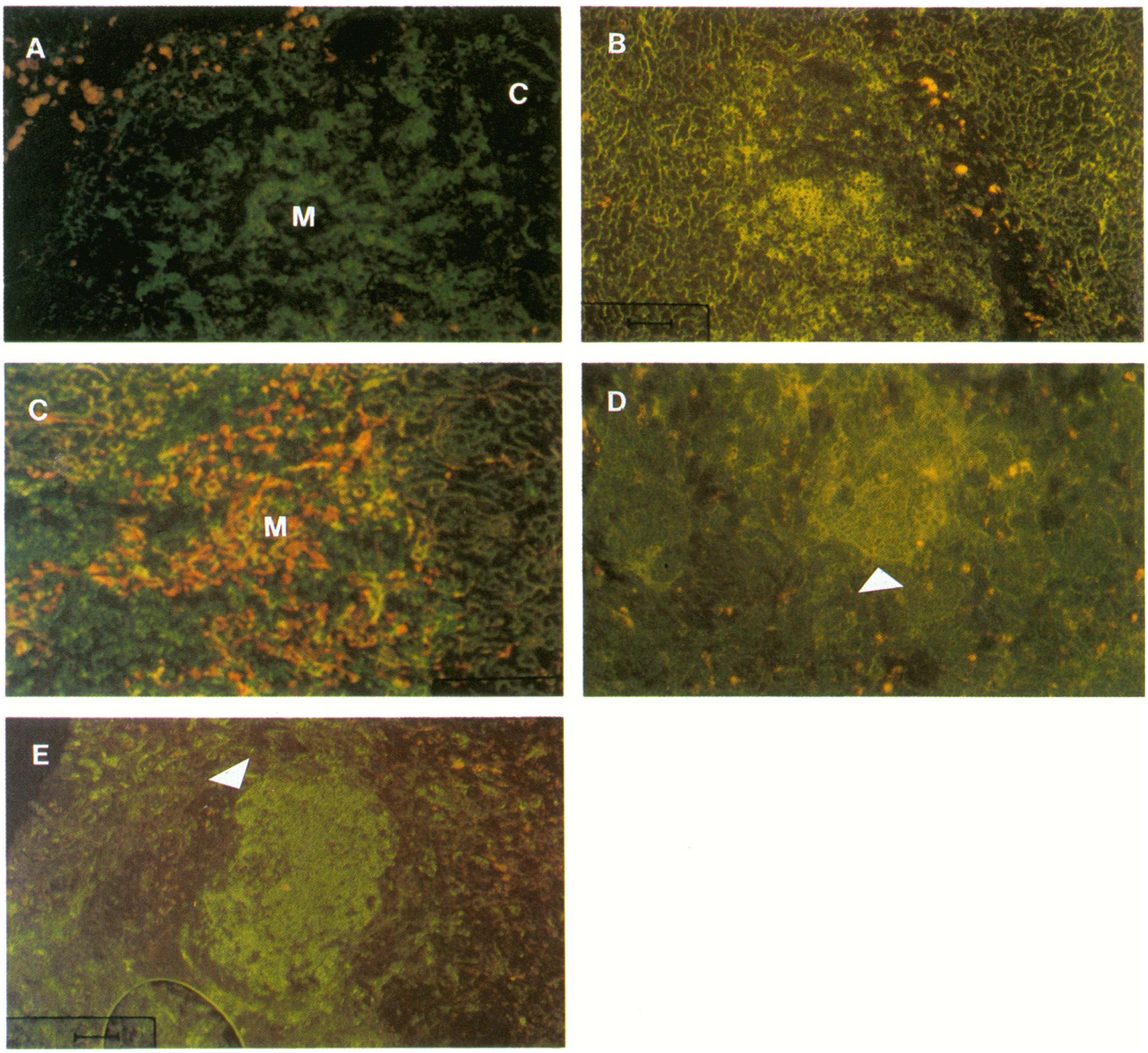

Figure 1. Sections of thymi stained for MHC class II with OX6: (A) CBA, 5 months; (B) NOD-ASP, 5 months; (C) NOD-PRO, 5 months, also stained with anti-keratin (orange) which detects epithelial cells; (D) NOD, 5 months; (E) NOD-E, 5 months. Photomicrographs (A–C) show MHC class II expression is densely packed in the medulla (M) and is also expressed on the fine epithelial array in the cortex. (D, E) Increased expression of MHC class II in both the cortex and medulla and the presence of MHC class II-positive collections of lymphoid cells. C, cortex; M, medulla; arrows show class II expression on small collections of lymphocytes. Magnification ×200, (C) ×400.

present, were not as frequent in CBA mice, occurring in only 40% of thymi examined at this age; an observation that is typical of the initial stages of thymic atrophy. These small spaces in the epithelial layer did not contain any focal accumulations of either MHC class II positive cells (Fig. 2C,D stained with OX6) or B cells (stained with B220) unlike those seen in NOD and NOD-E mice (Fig. 2G,H).

In addition to the small epithelial-free spaces, large perivascular areas in the thymic epithelium, particularly surrounding vascular structures at the corticomedullary junction, were detected when the thymi of 3–5-month-old NOD and NOD-E were stained with anti-keratin (Fig. 2E,F) and anti-laminin antibodies (Fig. 3E,F). The frequency of these spaces and area of thymus occupied by them increased in size with age. Their appearance was associated with an apparent concomitant disappearance of medullary epithelium; this may result from the epithelium being pushed aside by the enlarged perivascular spaces (Fig. 2G,H).

Thymic B-cell expression. The high MHC class II expression in the NOD and NOD-E thymi could be the result of increased macrophage presence in this tissue. However this was not the case as there was no increase in macrophage number detected with the macrophage markers Mac-1, F4/80 and SER-4.

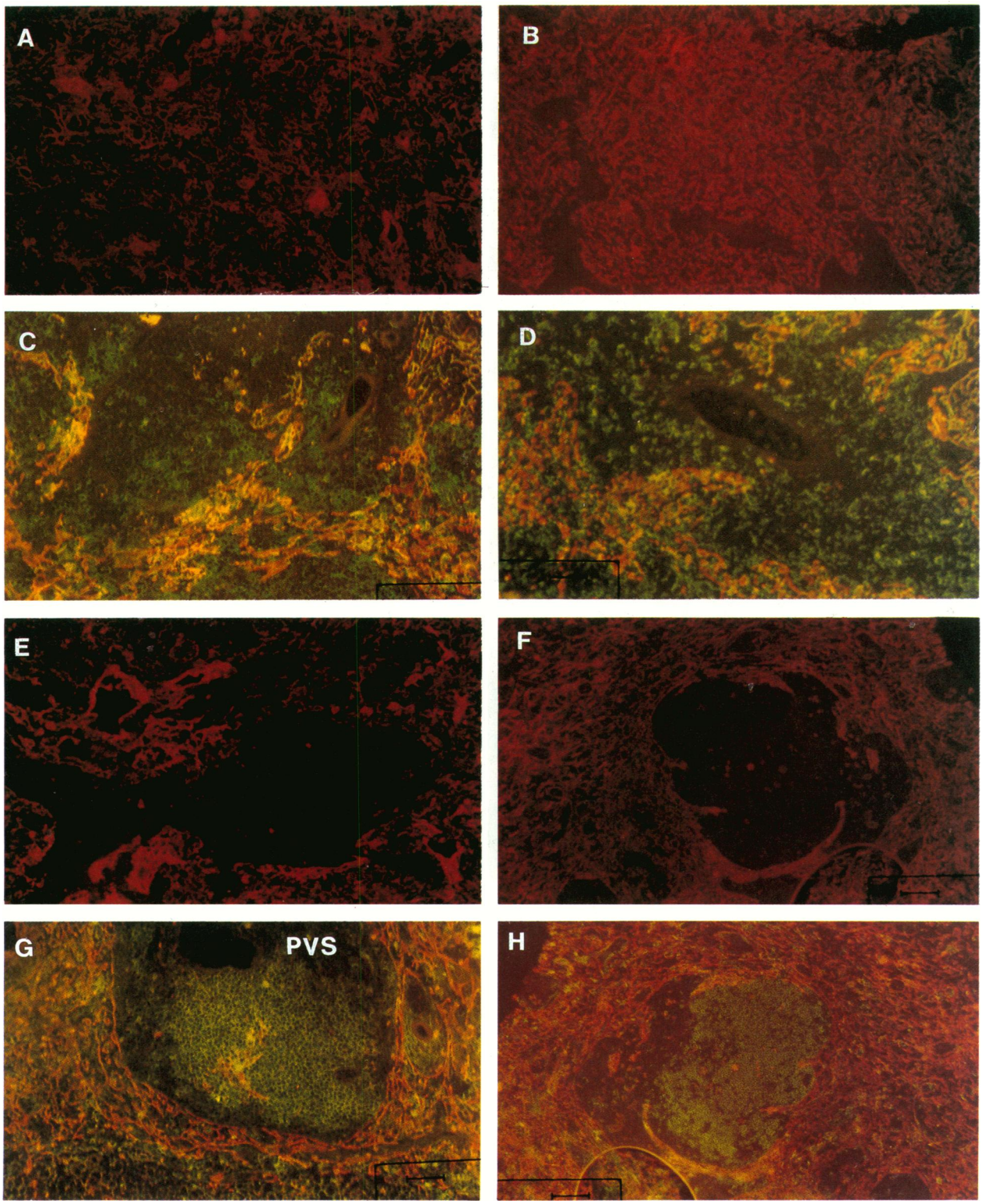

Figure 2. Epithelial cell staining [anti-keratin (red)] of thymi sections: (A) CBA, 5 months; (B) NOD-ASP, 5 months; (C) NOD-ASP, 5 months, also stained for MHC class II with OX6 (green); (D) NOD-PRO, 5 months, also stained for MHC class II with OX6 (green); (E) NOD, 5 months; (F) NOD-E, 5 months; (G) NOD, 5 months, also stained for MHC class II with OX6 (green); (H) NOD-E, 5 months, also stained for MHC class II with OX6 (green). Sections show the absence of both perivascular spaces (A, B) and large clusters of class II^+ cells (C, D) in the few perivascular spaces that were observed in CBA and NOD-ASP thymi. (E and F) show the presence of large perivascular spaces containing MHC class II^+ cells (G, H) in NOD and NOD-E thymi respectively. PVS, perivascular space. Magnification: $\times 200$.

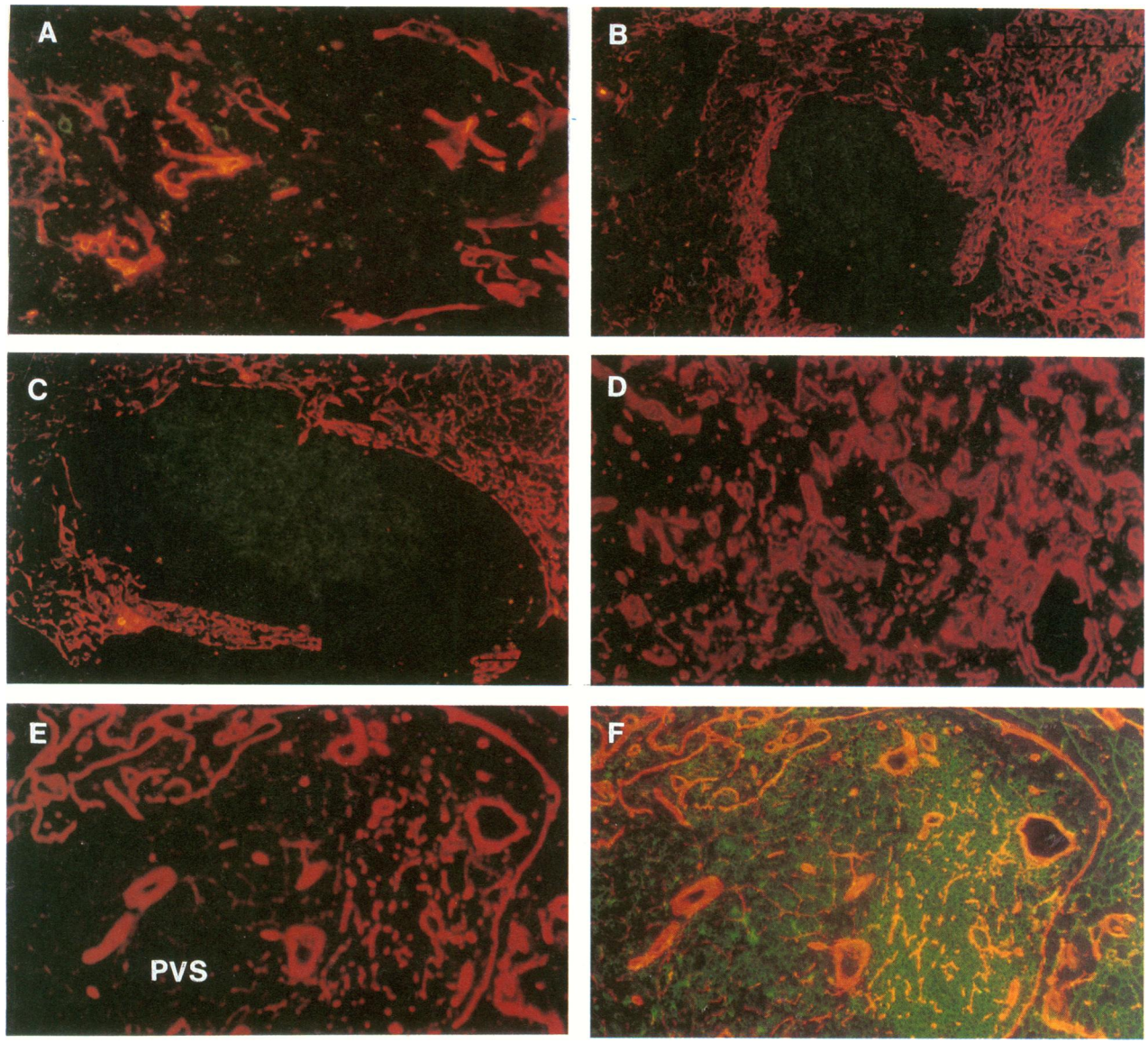

Figure 3. Sections of thymi stained for epithelial cells [anti-keratin(red)] and B cells [B220 (green)] or anti-laminin (red): (A) NOD-ASP, 5 months, stained for anti-keratin and B220; (B) NOD, 5 months, stained for anti-keratin and B220; (C) NOD-E, 5 months, stained for anti-keratin and B220; (D) CBA, 5 months, stained with anti-laminin; (E) NOD, 5 months, stained with anti-laminin; (F) NOD, 5 months, stained with anti-laminin and OX6 (green). Photomicrographs show a few scattered B cells in CBA thymi (A) and the presence of B cells within the PVS in NOD and NOD-E (B, C). A dense anti-laminin pattern is observed in CBA thymi (D). The perivascular origin of the epithelial spaces in NOD thymi is shown in (E) which contains class II^+ cells (F). Magnification: ×200; (A) ×400. PVS, perivascular space.

Furthermore, macrophages showed a normal distribution in perivascular spaces and interlobular septa. Therefore these non-epithelial thymic class II positive cells may be B cells. Normally B cells ($B220^+$, Ig^+, class II^+) are rare and scattered throughout the cortex and the medulla. Such a distribution of B cells was observed in CBA, NOD-ASP (Fig. 3A) and NOD-PRO thymi at 1, 3 and 5 months and even in NOD-ASP thymi at 8 months of age (data not shown). Accumulations of $B220^+$ cells were rare in these strains and few in number when observed. In contrast lymphoid-like clusters of class II MHC positive cells were observed throughout the medulla and corticomedullary junction in NOD (Fig. 2G) and NOD-E mice (Fig. 2H). The clusters of class II-expressing cells in both NOD and NOD-E thymus at 3–5 months of age appeared to be B cells as they expressed both B220 and Ig (Fig. 3B,C). Staining with the anti-laminin antibody indicated that such B-cell clusters were surrounded by a continuous layer of basement membrane and were thus perivascular in location (Fig. 3D–F). Such $B220^+$ cells were also seen at the corticomedullary junction. However both the distribution and numbers of B cells did not fully account for the increased expression of class II MHC.

Table 2. Percentage of $OX6^+/B220^+$ cells in thymus of NOD, NOD-E, NOD-ASP, NOD-PRO and CBA mice

Mouse strain	Age (weeks)	No. analysed (sex)	$OX6^+/B220^+$	Range
CBA	4	6 M, 6 F	0·01 ± 0·01	0·01–0·02
NOD	4	5 M, 5 F	0·03 ± 0·01	0·00–0·06
NOD-E	4	5 M, 5 F	0·03 ± 0·03	0·00–0·08
NOD-ASP	5	6 M, 5 F	0·05 ± 0·02	0·01–0·08
NOD-PRO	6	6 M, 6 F	0·03 ± 0·16	0·01–0·07
NOD*	6	3 M, 5 F	0·08 ± 0·05	0·03–0·14
CBA	12	8 M, 10 F	0·07 ± 0·07	0·01–0·32
NOD	12	8 M, 10 F	1·44 ± 1·57	0·03–4·88
NOD-E	12	10 M, 10 F	0·20 ± 0·13	0·03–0·57
NOD-ASP	12	5 M, 5 F	0·06 ± 0·02	0·03–0·10
CBA	20	5 M, 5 F	0·09 ± 0·05	0·03–0·19
NOD	17–22	15 M, 14 F	0·58 ± 0·66	0·08–2·50
NOD-E	16–23	10 M, 10 F	0·62 ± 0·52	0·07–2·32
NOD-ASP	20	6 M, 6 F	0·09 ± 0·05	0·04–0·21
NOD-PRO	20	5 M, 6 F	0·05 ± 0·02	0·03–0·09
NOD*	20	6 M, 6 F	0·38 ± 0·39	0·15–1·59

Fresh thymus was digested in 0·5 mg/ml collagenase and 0·02 mg/ml DNase for several minutes then washed in BSS and frozen in 90% FCS/10% DMSO at −70°. For analysis vials were rapidly thawed, washed, resuspended and stained with biotinylated OX6 in 1% normal mouse serum for 30 min followed by B220–FITC and avidin–PE (1/80) pooled as the second layer. All samples were then analysed by FACScan (Becton Dickinson). The FACScan gates were set on the $OX6^+$–$B220^+$ fresh NOD spleen cell population and these same parameters used to analyse thymus cells for expression of class II MHC (OX6) and B cells (B220). The percentage of the total thymocyte population from 20,000 events that was positive was recorded. The values indicated are ± SD M, male; F, female. *NOD-PRO, transgene negative littermates.

Flow cytometric analysis of thymus cell populations

Thymus cell suspensions from 4-, 12- and ~20-week-old male and female CBA, NOD, NOD-E, NOD-ASP and NOD-PRO mice were stained for FACScan analysis. Table 2 shows the frequency of B cells ($OX6^+/B220^+$) expressed as a percentage of the total thymus cell population. The $OX6^+/B220^+$ thymic cell population showed no increase in number in CBA mice from 0·01 ± 0·01% at 4 weeks to 0·09 ± 0·05 at 16–23 weeks with little variation in range. This population showed a greater increase in both male and female NOD mice from 0·03 ± 0·01% at 4 weeks to 1·44 ± 1·57 at 12 weeks and 0·58 ± 0·66% by 17–22 weeks of age. Thymi of NOD-E mice also showed an increase in the $OX6^+/B220^+$ thymic population from 0·03 ± 0·03% at 4 weeks to 0·62 ± 0·52% at 16–23 weeks, although there was a range in values. However such an increase in thymic $B220^+/OX6^+$ cells with age was not apparent in NOD-ASP mice 0·09 ± 0·05% or NOD-PRO mice 0·05 ± 0·02% at 20 weeks compared to 0·38 ± 0·39% for transgene negative littermates (Table 2). As the sex of mice did not alter the percentage of $B220^+/OX6^+$ cells present in thymi at all ages and in each strain examined, the results were combined in Table 2. It must be noted that the increase in thymic B cells in NOD and NOD-E thymi appeared to increase in a stochastic manner. At 5 months of age only 72% of NOD and 85% of NOD-E mice had elevated thymic B cells. Therefore at a particular time-point a range of thymic B cells in NOD and NOD-E thymi is anticipated whereas CBA, NOD-ASP and NOD-PRO thymic B cells remained stable. Staining of thymic cell suspensions with Mac-261, an antibody specific for $I\text{-}A^{g7}$, was compared to the staining with OX6 which recognizes a polymorphic mouse I-A determinant common to many mouse strains as an additional specificity control. Thymi from 5-month-old NOD, NOD-E, NOD-ASP, NOD-PRO and CBA mice were stained either with OX6 or the $I\text{-}A^{g7}$-specific mAb and B220. Quantitative expression of class II MHC/B220 in the thymus of NOD and NOD-E mice was similar with the NOD $I\text{-}A^{g7}$-specific mAb and OX6 (data not shown). Thus the large numbers of thymic B cells detected in individual NOD/NOD-E mice with OX6 and low numbers in NOD-ASP and NOD-PRO mice, were also reflected in the specific $I\text{-}A^{g7}$ staining. The $I\text{-}A^{g7}$ mAb also acted as an excellent negative control as effectively no B cells ($Mac\text{-}261^+/B220^+$) were detected in CBA thymus.

No significant differences were observed in either the percentage of $CD4^+$ $CD8^+$ single-positive T cells or the percentage $CD4^+$ $CD8^+$ double-positive T cells and thus no difference in the CD4/CD8 ratio in the NOD, NOD-E, NOD-ASP and NOD-PRO strains compared to CBA (Table 3). The immature $CD4^+$ $CD8^+$ thymocytes decreased in percentage in all strains with a corresponding increase in the mature single-positive CD4 and CD8 T cells as a function of thymic age.

It is clear that there is an age-related increase in the $OX6^+/B220^+$ double-positive population in the thymus of NOD and NOD-E mice compared to NOD-ASP, NOD-PRO and CBA mice. Such B-cell populations are likely to correspond to the $OX6^+/B220^+/Ig^+$ clusters observed by immunohistochemical analysis of thymus tissue sections of these mice. This population does not become apparent until 12 weeks of age and by 20 weeks of age there were six times as many $OX6^+/B220^+$ cells in NOD or NOD-E thymi compared to CBA mice of the same age. The NOD-ASP and NOD-PRO thymi on the other hand did not contain such cells.

Flow cytometric analysis of thymic B cells for CD5

In order to attribute some kind of function to the thymic B cells it was decided to assess their expression of CD5 (Ly-1). The Ly-1 differentiation antigen is found at high densities on all murine T cells and also is expressed at low levels on a small population of B cells which has been linked to production of autoantibodies.[25]

$CD5^+$ B cells frequently comprise 20% of peritoneal exudate cells and more than half of those B cells recoverable from the peritoneum. This population is present to a lesser extent in the spleen comprising 2% of the total splenocytes at 6 weeks of age. Female NOD peritoneal exudate cells were used to characterize the FACS staining profile of the $CD5^+$ B-cell population. FACScan gate settings used to analyse NOD peritoneal exudate cells (Fig. 4A) were then used to determine the $OX6^+/B220^+$, $CD5^+$ population in the spleen and subsequently to define the CD5-expressing B cells in the thymus of male and female mice of each strain at 5 months of age. At least four to five mice of each sex and strain were analysed.

Table 3. Percentage of thymocytes $CD4^+$ and $CD8^+$ in NOD, NOD-E, NOD-ASP, NOD-PRO and CBA mice

Mouse strain	Age (months)	Sex	No. of mice	$CD4^+$	$CD8^+$	$CD4^+$ $CD8^+$
CBA	1	M	6	1·90 ± 0·31	7·70 ± 0·93	88·14 ± 1·77
CBA	1	F	6	2·18 ± 0·39	8·62 ± 1·40	86·52 ± 1·53
NOD	1	M	5	1·68 ± 0·46	7·19 ± 0·53	89·08 ± 1·46
NOD	1	F	5	1·27 ± 0·95	6·16 ± 2·51	90·89 ± 3·98
NOD-E	1	M	5	1·77 ± 0·30	8·75 ± 0·73	86·76 ± 1·68
NOD-E	1	F	5	2·02 ± 0·60	9·66 ± 1·85	84·52 ± 2·15
CBA	3	M	4	2·02 ± 0·64	9·19 ± 2·31	86·08 ± 2·13
CBA	3	F	4	1·84 ± 0·28	8·30 ± 1·13	86·72 ± 1·37
NOD	3	M	5	1·52 ± 0·28	6·08 ± 0·99	89·23 ± 1·72
NOD	3	F	4	3·08 ± 1·11	11·23 ± 3·26	82·37 ± 4·47
NOD-E	3	M	5	1·86 ± 0·51	9·41 ± 1·84	85·65 ± 2·35
NOD-E	3	F	5	1·62 ± 0·36	9·15 ± 1·88	85·37 ± 2·47
CBA	5	M	5	14·18 ± 1·32	3·53 ± 0·24	76·35 ± 0·78
CBA	5	F	5	18·88 ± 4·20	4·66 ± 1·28	70·19 ± 6·40
NOD	5	M	10	13·77 ± 3·20	4·34 ± 1·68	78·90 ± 6·41
NOD	5	F	11	16·42 ± 7·83	3·86 ± 1·99	73·51 ± 11·17
NOD-E	5	M	10	16·20 ± 6·11	3·57 ± 0·82	74·66 ± 3·44
NOD-E	5	F	10	19·06 ± 7·06	4·11 ± 2·22	65·04 ± 2·33
NOD-PRO	5	M	6	11·0 ± 1·40	4·34 ± 1·00	81·79 ± 2·65
NOD-PRO	5	F	6	14·40 ± 2·15	4·58 ± 1·14	75·84 ± 4·84
NOD-ASP	6	F	6	12·6 ± 1·86	4·03 ± 0·67	80·6 ± 2·68
NOD-ASP	8	F	6	18·35 ± 5·56	6·03 ± 0·91	70·83 ± 2·68
Mouse strain	Age (months)	Sex	No. of mice	00.00 ± 0.00	00.00 ± 00.00	00.00 ± 0.00

Fresh thymus was digested in 0·5 mg/ml collagenase and 0·02 mg/ml DNase for several minutes then washed in BSS and frozen in 90% FCS/10% DMSO at −70°. For analysis vials were rapidly thawed, washed, resuspended and stained with biotinylated OX6 (1 μg/ml) in 1% normal mouse serum followed by the triple label cocktail anti-CD4–PE (1/40), anti-CD8–FITC (1/40) and streptavidin R613 (1/100) which binds to the biotynbylated OX6. All samples were then analysed by flow cytometry. The FACScan gates were set on either the CD4 single-positive or CD8 single-positive thymocytes (as confirmed by gating on splenic lymphocytes) or on the $CD4^+$ $CD8^+$ double-positive thymocyte population. The percentage of each population was based on 20,000 events recorded. M, male; F, female.

Analysis of thymi from NOD, NOD-E and CBA mice at 5 months of age showed that thymic B cells in these mice do not express CD5 (30 000 total events were recorded, Fig. 4B,C,E). Expressed as a percentage of the total number of thymocytes they are negligible. CBA thymi effectively had no $CD5^+$ B cells, as expected (Fig. 4E). Insufficient $B220^+/OX6^+$ or Mac-$261^+/B220^+$ B cells were obtained from NOD-PRO mice to give an accurate figure of $CD5^+$ B cells, although they do not appear to bear this marker (Fig. 4D). Similarly because B cells were so infrequent in the NOD-ASP transgenic thymi analysis of their CD5 expression was not performed.

The thymic populations analysed above were also labelled with anti-$I\text{-}A^{g7}$/B220/CD5 confirming the result with the OX6/B220/CD5 staining and thus the specifcity of the OX6 label. The few $CD5^+$ thymic B cells detectable in NOD and CBA mice did not stain with the negative control mAb TIB120 (data not shown).

DISCUSSION

Generation of immunocompetent T cells requires the influence of an intact thymic microenvironment. T cells are selected with T-cell receptors biased toward MHC-associated recognition of foreign peptides (positive selection)[26] and away from reactivity against self-peptides associated with MHC molecules (negative selection).[27] In order to influence such a selection process, MHC antigens would have to be expressed on thymic stromal cells of the thymic microenvironment. It is thought that the cortical epithelial network influences positive selection[28] whereas bone marrow-derived dendritic cells in the thymic medulla regulate clonal deletion.[29]

There is considerable evidence from human and animal studies that T cells play a fundamental role in autoimmune IDDM development. Disease can be prevented in the NOD mouse model by cyclosporin A treatment,[30] or by antibodies to CD4,[31–33] CD8,[34] and I-A,[35] or by neonatal thymectomy.[36] Thymectomy at 3 weeks increases the disease incidence in females,[37] suggesting the existence of a regulatory role of late thymic-derived cells. It therefore seemed appropriate to undertake a morphological inspection of the thymic architecture of NOD and transgenic NOD-E, NOD-ASP and NOD-PRO mice in view of the influence on diabetes susceptibility of the two transgenes.

Previously, it has been shown that class II MHC antigens are strongly expressed on the reticular network of cortical epithelial cells.[38,39] The pattern in the medulla is more confluent because of strong cytoplasmic staining of stromal cells. Such a pattern of class II expression was observed in CBA, NOD-ASP and NOD-PRO mice at all ages analysed with MRC-OX6. NOD and NOD-E thymi, however, exhibited a peculiar expression pattern of I-A molecules in which the staining in the medullary region was confluent and in the cortical region the epithelia seemed to form a much tighter network, although invariant chain expression was normal and similar to that of

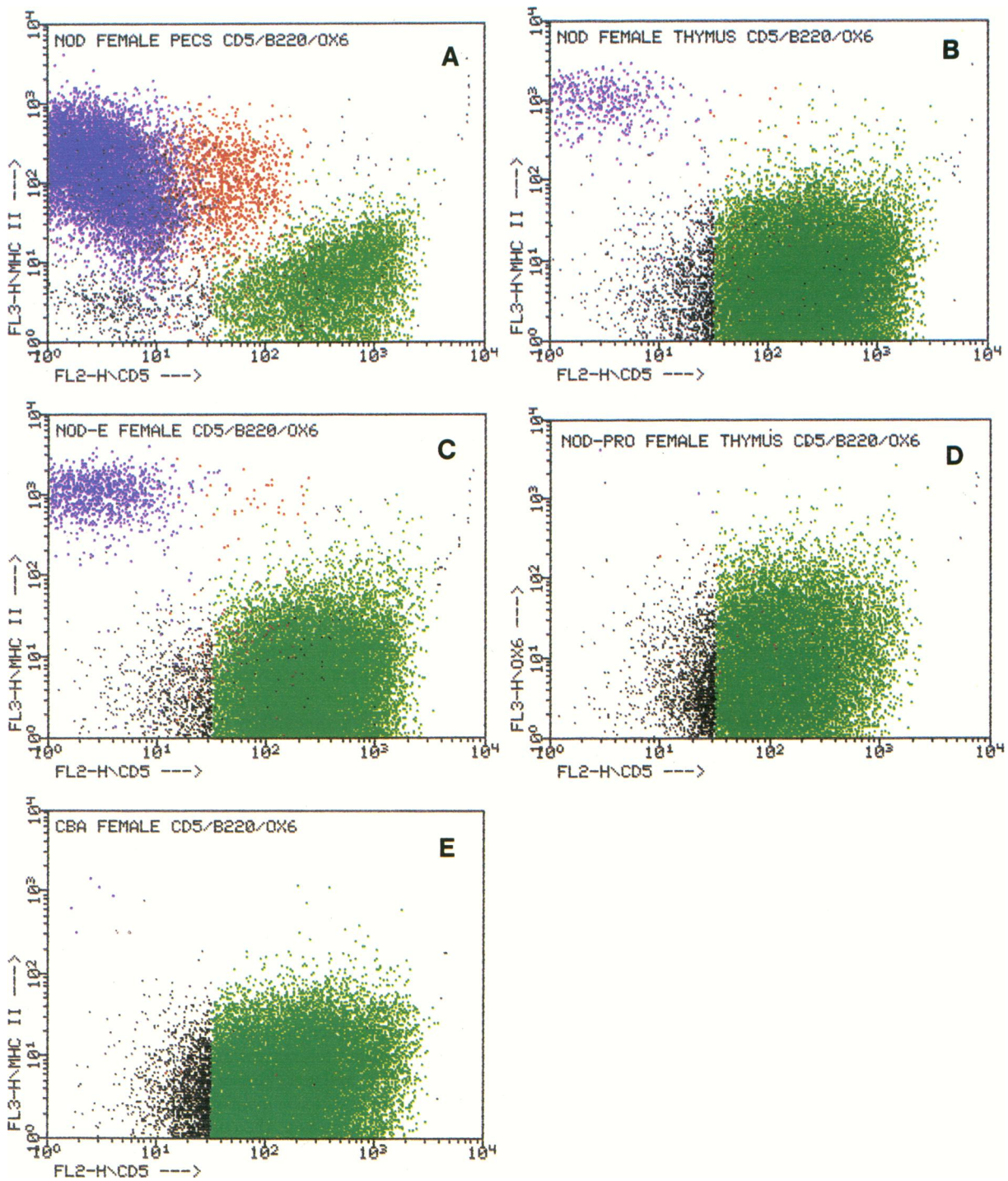

Figure 4. $CD5^+$ B cells are more numerous in the peritoneal cavity, therefore this population from female NOD mice was used to define $CD5^+$ ($OX6^+/B220^+$) B cells. NOD PEC were stained with OX6 and B220 (to define the B-cell population) and CD5 for flow cytometric analysis. Gates were set on the $OX6^+/B220^+$ population [(A) purple dots] and the percentage of this population expressing CD5 from 30,000 events recorded [(A) red dots]. These same parameters were then applied to NOD, NOD-E, NOD-PRO and CBA 1-, 3- and 5-month-old thymic cell suspensions. B cells were present ($OX6^+/B220^+$) in the thymi of 5-month-old NOD and NOD-E mice (purple dots), [(B,C) typical examples], but not in 5-month-old NOD-PRO or CBA thymi [(D,E) typical examples]. CD5 expression appeared to be present almost wholly on the class $II^-/B220^-$ population (green dots representing T cells) and not on the $OX6^+/B220^+$ thymic B cells in all mouse strains examined.

CBA mice (L. A. O'Reilly, unpublished observations). Some of the increased expression of MHC class II could be accounted for by the presence of $OX6^+/B220^+$ 'B cells' in the medulla. In addition MHC proteins secreted by stromal cells may be picked up by surrounding thymocytes in the cortex.[40]

Increased expression of class II MHC in the NOD and NOD-E thymi may influence both positive and negative selection as MHC-bearing thymic epithelial cells and dendritic cells have been implicated in these processes.[41,42] The significance of increased expression of MHC class II and its relationship to disease, if any, awaits further study. In this respect, however, the presence of insulitis in allophenic chimeras of the NOD↔C57BL/6 strain combinations correlated with the presence of NOD MHC molecules on thymic cortical regions; thus positive selection of T cells could play a crucial role in the development of IDDM.[43]

Probably of greater significance in thymic selection were the signs of premature ageing commencing around 12 weeks of age in both NOD and NOD-E mice. In particular, there were changes in the thymic epithelial architecture, with epithelium pushed aside by enlarged perivascular spaces in the medulla and at the corticomedullary junction in these mice. Such abnormalities were not detectable in the thymus of CBA mice, even at 5 months of age, and were very rarely seen in NOD-ASP and NOD-PRO mice. Similar structures, but on a smaller scale, have been described in ageing human and rodent thymi.[44,45]

Alterations in the ultrastructural morphology of the thymus in mice with genetic autoimmune diseases such as lupus (NZB, BXSB and MRL mice)[46] and in other strains of mice with diabetes such as the db/db mouse[47] and autoimmune diabetes such as the NOD mouse have been reported.[48] Thymi from such mice had lesions consisting of a patchwork of involuted and non-involuted zones and disorganization of the thymic structure. Such alterations appeared early in life (from 5 weeks) in autoimmune-prone mice compared to 10 months in normal mice, giving the thymus an aspect of premature and permanent ageing.[48]

Analysis of distinct cytokeratin-defined thymic epithelial cell subsets has shown that the numbers of keratin $3/10^+$ and keratin 19^+ (complex stratified medullary epithelia[49]) decrease in ageing normal mice.[50,51] This phenomenon however is more pronounced, and seems to occur earlier, in autoimmune mice such as (NZB × NZW)F_1 and MRL/1 as well as both male and female NOD mice.[46,50] A reduction in epithelial subsets could result in the loss of specific thymic microenvironments and lead to impaired T-cell selection. Savino[51] also noticed abnormally enlarged perivascular spaces in NOD thymi; these increased in size with age, and contained T, and to a lesser extent, B cells. Such structures were not observed in NZB mice.

Detailed histological and flow cytometric analysis presented in this study would suggest that the interruptions in the thymic epithelial network in NOD and NOD-E but not CBA, NOD-ASP or NOD-PRO thymi consisted of collections of B cells ($B220^+/sIg^+$/class II MHC^+). The accumulation of lymphocytes in these spaces could be associated with a defect in intrathymic lymphocyte traffic, or to abnormal regulatory or effector T cells. It remains to be elucidated whether the B cells enter the thymus from the blood or if the B cells increase in number by *in situ* expansion in pathological situations. Progressive accumulation of recirculating B cells in the thymus of $MLR^{+/+}$ and MRL/Mp-*lpr/lpr* mice (*Mrl/lpr*)[52] and in the thymi of myasthenia gravis patients[53] however, has been suggested.

$CD5^+$ B cells are a minor subpopulation (1–2%) in the spleen, and below detectability in lymph nodes or Peyer's patches. Increased numbers of $CD5^+$ thymic B cells may occur in situations where thymic hypertrophy occurs, as in patients with the autoimmune diseases myasthenia gravis and systemic lupus erythematosis.[53,54] Similar findings have been reported in autoimmune-prone mice such as (NZB × NZW)F_1 and MRL/Mp-*lpr/lpr* (*Mrl/lpr*)[55] in which 60–70% of these thymic B lymphocytes expressed CD5.[56] These thymic B cells produced autoantibodies *in vitro* with or without stimulants.[57] Organ-specific antibodies have been reported in myasthenia gravis[58] and in Rasedow's disease,[59] a condition which is also characterized by increased numbers of thymic B cells. Thymic B cells are a minor subset in normal and germ-free mouse strains;[45] 0·2–1% of thymic lymphoid cells are B cells, being present from the perinatal period onwards. Of these, a high proportion are Ig secreting and show no differences in V_H repertoire expression when compared to their peripheral counterparts.[52]

Given the current interest in $CD5^+$ B cells, in particular those that reside in the thymus of autoimmune diseased patients and animals, it seemed logical to assess the CD5 phenotype of B cells in the thymus of NOD, NOD-E, NOD-ASP and NOD-PRO relative to control CBA mice. Thymic B cells from these strains did not express CD5 to any appreciable level. This finding correlates with that of Watanabe *et al.*[60] who also demonstrated that thymi of 12–16-week-old NOD were hyperplastic with follicular B-lymphocyte aggregation in the medulla and corticomedullary junction; these B cells expressed sIgM and/or sIgD but not sIgG2a. Southern blot analysis of NOD thymus showed a distinct rearranged band at 3·8 kb which was not apparent in I-E-expressing NOD thymus when hybridized with a J_H probe, suggesting that a monoclonal expansion of thymic B cells occurred in NOD mice.[60]

There are two major roles that the B cells within the thymi of NOD and NOD-E mice could perform: (1) they may act as antigen-presenting cells for T-cell repertoire selection or (2) they may produce antibody. There is some evidence that thymic B cells are capable of peptide antigen presentation in addition to the class II-expressing thymic stromal cells.[61] Furthermore, with respect to the presentation of endogenous superantigens, thymic but not splenic B cells deleted Mls^a reactive $V\beta6^+$ T cells and induced tolerance when injected into neonatal mice across an Mls^a antigenic barrier.[62] Thymic B lymphocytes are the best candidates to be involved in these phenomena as they form germinal centres at the corticomedullary junction which subdivides unselected/selected populations of thymocytes, express Mls antigens which T cells do not[63] and are able to process and present antigen.[64] Another possible role for the thymic B lymphocyte is the production of autoantibody to islet cell-surface antigen, islet cell antigen and insulin (ICSA, ICA and IAA respectively[1]), all of which are predictive markers of IDDM.

Although NOD-E mice are protected from disease the thymi from these mice still display many abnormalities characteristic of NOD mice. The bearing of these defects on disease pathology must therefore come into question, particularly as the CD4 : CD8 ratio within the thymus is not altered in NOD or NOD-E mice when compared to control strain CBA .

Recent published data suggest that there is no evidence for Vβ deletion or anergy either in NOD or NOD-E mice.[65] Furthermore, no clear candidate for a major Vβ T-cell family deletion has emerged in either NOD-ASP or NOD-PRO mice in comparison to control strains (N. Parish, personal communication). This does not rule out the possibility of antigen-specific T-cell deletion. T-cell selection in the thymus however may not be the only mechanism that prevents disease and events in the periphery may also be important. Serreze and Leiter have shown that development of diabetogenic cells from NOD/Lt marrow is blocked when an allo-H-2 haplotype is expressed on cells of hematopoietic origin but not thymic epithelium.[66] Parish has shown that chimeric NOD mice given a NOD-E thymus and NOD bone marrow can still develop disease,[67] suggesting that some kind of regulatory cell exists in the periphery of NOD-E mice, and that in NOD-E mice a peripheral mechanism of tolerance maintenance may pertain. This would be in line with the suggestion of Bohme and Mathis that the mechanism of protection may not be via deletion in the thymus but involve a peripheral presentation step.[14] The finding that NOD-ASP and NOD-PRO thymus at 5 months of age is similar to that of CBA mice would suggest that the mechanism of protection from diabetes in this case may differ from that of NOD-E mice. Therefore, protection from diabetes in the NOD-ASP and NOD-PRO transgenic mice that have a normal thymic architecture may be the result of competition for peptide binding with endogeneous I-A, while in NOD-E mice a peripheral mechanism for tolerance maintainance supervenes.

ACKNOWLEDGMENTS

We would like to thank A. Baxter (Dept. of Pathology, University of Cambridge, U.K.) for his comments and critical reading of this manuscript, N. Parish (Dept. of Pathology, University of Cambridge, U.K.) and J. Picard (CRC, Harrow, London, U.K.) for access to unpublished data. This study was supported by grants from the Wellcome Trust and the British Diabetic Association.

REFERENCES

1. Castano L. & Eisenbarth G.S. (1990) Type-1 diabetes: a chronic autoimmune disease of human mouse and rat. *Annu. Rev. Immunol.* **8,** 647.
2. Makino S., Kurimoto T., Mizushima Y., Katagiri K. & Tochino Y. (1980) Breeding of a non-obese, diabetic strain of mice. *Exp. Anim.* **29,** 1.
3. Hattori M., Buse J.B., Jackson R.A., Glimcher L., Makino S., Moriwaki K. *et al.* (1986) The NOD mouse: recessive diabetogenic gene within the major histocompatibility complex. *Science,* **231,** 733.
4. Lund T., Simpson E. & Cooke A. (1990) Restriction fragment length polymorphisms in the major histocompatibility complex of the non-obese diabetic mouse. *J. Autoimmun.* **3,** 289.
5. Lund T., O'Reilly L., Hutchings P., Kanagawa O., Simpson E., Gravely R., Chandler P. *et al.* (1990) Prevention of insulin-dependent diabetes mellitus in non-obese diabetic mice by transgenes encoding modified I-Aβ chain or normal I-E α-chain. *Nature,* **345,** 727.
6. Uehira M., Uho M., Kurner T., Kikatani H., Mori K., Inomoto T. *et al.* (1989) Development of autoimmune insulitis is prevented in $E\alpha^d$ but not $A\beta^k$ NOD transgenic mice. *Int. Immunol.* **1,** 209.
7. Nishimoto H., Kikutani H., Yamamura K. & Kishimoto T. (1987) Prevention of autoimmune insulitis by expression of I- E molecules in NOD mice. *Nature,* **328,** 432.
8. Acha-Orbea H. & McDevitt H.O. (1987) The first external domain of the non-obese diabetic mouse class II I-Aβ chain is unique. *Pro. natl. Acad. Sci. U.S.A.* **84,** 2435.
9. Hanafusa T., Miyazaki A., Yamada K., Miyagawa J., Fujino-Kurihara H., Nakajima H., Kono N., Nonaka K. & Tarui S. (1981) Autoantibodies to islet-cells and multiple organs in the NOD mouse. *Diab. Nutr. Metab.* **1,** 273.
10. Kappler J.W., Roehm N. & Marrack P.C. (1987) T cell tolerance by clonal elimination in the thymus. *Cell,* **49,** 273.
11. Ramsdell F. & Fowlks B.J. (1990) Clonal deletion versus clonal anergy: the role of the thymus in inducing self tolerance. *Science,* **248,** 1342.
12. Schwartz R.H. (1989) Acquisition of immunologic self tolerance. *Cell,* **57,** 1073.
13. Lo D. & Sprent J. (1986) Identity of cells that imprint H-2 restricted T cell specificity in the thymus. *Nature,* **319,** 672.
14. Bohme J., Schuhhaur B., Kanagawa O., Benoise C. & Mathis D. (1990) MHC-linked protection from diabetes dissociated from clonal deletion of T cells. *Science,* **249,** 293.
15. Kanariou M., Ruby R., Ladyman H., Colic M., Sivolapenko G., Lampert I. & Ritter M. (1989) Immunosupppression with cyclosporin A alters the thymic microenviroment. *Clin. exp. Immunol.* **78,** 263.
16. De Maagd R.A., Mackenzie W.A., Schuurman H.-J., Ritter M.A., Price K.M., Broekhuizen A. & Kater L. (1985) The human thymus microenviroment: heterogeneity detected by monoclonal anti-epithelial cell antibodies. *Immunology,* **54,** 745.
17. Schlegel R., Banks-Schlegel S. & Pinkus G.S. (1980) Immuno-histochemical localization of keratin in normal human tissues. *Lab. Invest.* **42,** 91.
18. Holmes K.L. & Morse H.C. (1988) Murine hematopoietic cell surface antigen expression. *Immunol. Today,* **9,** 344.
19. McMaster W.R. & Williams A.F. (1979) Identification of Ia gycoproteins in rat thymus and purification from rat spleen. *Eur. J. Immunol.* **9,** 426.
20. Beller D.I., Springer T.A. & Schreiber R.D. (1982) Anti-Mac-1 selectively inhibits the mouse and human complement receptor. *J. exp. Med.* **159,** 1000.
21. Austyn J.M. & Gordon S. (1981) F4/80 a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* **11,** 805.
22. Crocker P.R. & Gordon S. (1989) Mouse macrophage hemagglutinin (sheep erythrocyte receptor) with specificity for sialyated glyconjugates characterized by a monoclonal. *J. exp. Med.* **169,** 1333.
23. Cobbold S.P., Jayasuriya A., Nash A., Prospero T.D. & Waldmann H. (1984) Therapy with monoclonal antibodies by elimination of T cell subsets *in vivo*. *Nature,* **312,** 548.
24. Bhattacharya A., Dorf M.E. & Springer T.A. (1981) A shared determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol.* **127,** 2488.
25. Hardy R. & Kayakawa K. (1986) Development and physiology of Ly-1 B cell and its human homologue, leu-1 B. *Immunol. Rev.* **3,** 53.
26. Moller G. (1988) The T cell repertoire. *Immunol. Rev.* **101,** 1.
27. MacDonald H.R. & Lees R.K. (1990) Programmed death of autoreactive thymocytes. *Nature,* **343,** 642.
28. Marrack P., McCormack J. & Kappler J. (1989) Presentation of antigen, foreign major histocompatibility complex protein and self by thymus cortical epithelium. *Nature,* **388,** 503.
29. van Ewijk W. (1991) T-cell differentiation is influenced by thymic microenviroments. *Annu. Rev. Immunol.* **9,** 591.
30. Mori Y., Suko H., Okudaira I., Matsuba A., Tsuruoka A., Asaki H. *et al.* (1986) Preventative effects of cyclosporin on diabetes in NOD mice. *Diabetologia,* **29,** 244.
31. Hutchings P., O'Reilly L., Parish N.M., Waldmann H. & Cooke A. (1992) The use of a non-depleting anti-CD4 monoclonal antibody to re-establish tolerance to β cells in NOD mice. *Eur. J. Immunol.* **22,** 1913.

32. Koike T., Itoh Y., Ishii T., Ito I., Takabayashi K., Mauryama N., Tomioka H. & Yoshida S. (1987) Preventative effect of monoclonal anti-L3T4 antibody on the development of diabetes on NOD mice. *Diabetes*, **36,** 539.
33. Shizuru J.A., Taylor-Edwards C., Banks B.A., Gregory A.K. & Fathman C.G. (1988) Immunotherapy of the non-obese diabetic mouse: treatment with an antibody to T-helper lymphocytes. *Science*, **240,** 659.
34. Hutchings P.R., Simpson E., O'Reilly L. A., Lund T., Waldmann H. & Cooke A. (1990) The involvement of $Lyt2^+$ cells in beta cell destruction. *J. Autoimmun.* **3,** 101.
35. Boitard C., Bendelac A., Richard M.F., Carnaud C. & Bach J.F. (1988) Prevention of diabetes in nonobese diabetic mice by I-A monoclonal antibodies: transfer of protection by splenic T cells. *Proc. natl. Acad. Sci. U.S.A.* **85,** 9719.
36. Ogawa M., Marauyama T., Hasegawa T., Kanaya T., Kobayashi F., Tochino Y. & Uda H. (1985) The inhibitory effect of neonatal thymectomy on the incidence of insulitis in non-obese-diabetic (NOD) mice. *Biomed. Res.* **6,** 103.
37. Bendelac A., Carnaud C., Boitard C. & Bach J.-F. (1987) Syngeneic transfer of autoimmune diabetes from diabetic mice to healthy neonates, requirement for both L3T4- and Lyt-2-T cells. *J. exp. Med.* **166,** 823.
38. Rouse R.V., van Ewijk W., Jones P.P. & Weisssman I.L. (1979) Expression of MHC antigens by mouse thymic dendritic cells. *J. Immunol.* **122,** 2508.
39. Farr A.G. & Nakane P.K. (1983) Cells bearing Ia antigens in the murine thymus: an ultrastructural study. *Am. J. Pathol.* **111,** 88.
40. Sharrow S.O., Mathieson B.J. & Singer A. (1981) Cell surface appearance of unexpected host MHC determinants on thymocytes from radiation bone marrow chimeras. *J. Immunol.* **126,** 1327.
41. van Ewijk W., Ron Y., Monaco J., Kappler J., Le Meur M., Gerlinger P., Durand B., Benoist C. & Mathis D. (1988) Compartmentalization of MHC class II gene expression in transgenic mice. *Cell*, **53,** 357.
42. Jenkinson E.J., Anderson G. & Owen J.J.T. (1992) Studies on T cell maturation on defined thymic stromal cell populations *in vitro*. *J. exp. Med.* **176,** 845.
43. Forsgren S., Dahl U., Soderstrom A., Holmberg D. & Matsunaga T. (1991) The phenotype of lymphoid cells and thymic epithelium correlates with development of autoimmune insulitis in NOD-C57BL/6 allophenic chimeras. *Proc. natl. Acad. Sci. U.S.A.* **88,** 9335.
44. Steinman G.G., Klaus B. & Muller-Harmelink H.K. (1985) The evolution of the aging human thymus is independent of puberty. *J. Immunol.* **22,** 563.
45. Benner R., Rijubeck A.M., Bernabe R.R., Alonos-Martininz C. & Coutinho A. (1981) Frequencies of background immunoglobulin-secreting cells in mice as a function of organ, age and immune status. *Immunobiology*, **158,** 225.
46. Nabarra B., Dardenne M. & Bach J.-F. (1990) Thymic reticulum of autoimmune mice: II ultrastructural studies of mice with lupus-like syndrome (NZB, BXSB, MRL/1). *J. Autoimmun.* **3,** 25.
47. Nabarra B. & Andrianarison I. (1991) Thymic reticulum of autoimmune mice: I ultrastructural studies of the diabetic (db/db) mouse thymus. *Exp. Pathol.* **29,** 45.
48. Nabarra B. & Andrianarison I. (1991) Thymus reticulum of autoimmune mice: III ultrastructural study of NOD mouse thymus. *Int. J. Pathol.* **72,** 275.
49. Brekelmans P. & van Ewijk W. (1990) Phenotypic characterization of murine thymic microenviroments. *Sem. Immunol.* **2,** 13.
50. Savino W. & Dardenne M. (1988) Developmental studies on expression of monoclonal antibody defined cytokeratins by thymic epithelial cells from normal and autoimmune mice. *J. Histochem. Cytochem.* **36,** 1123.
51. Savino W., Boitard C., Bach J.-F. & Dardenne M. (1991) Studies on the thymus in nonobese diabetic mouse. I. Changes in the microenviromental compartments. *Lab. Invest.* **36,** 405.
52. Andreu-Sanchez J.L., Faro J., Alonso J.M., Paige C.J., Martinez-A C. & Marcos J.R. (1990) Ontogenic characterization of thymic B lymphocytes. Analysis in different mouse strains. *Eur. J. Immunol.* **20,** 1767.
53. MacKay I.R. (1963) Thymic germinal centres' and plasma cells in systemic lupus erythematosis. *Lancet*, **2,** 667.
54. Fujii N., Itoyama Y., Tebira T. & Kuroiwa T. (1983) Subsets of lymphoid cells in the blood and thymus in myasthenia gravis. Monoclonal antibody analysis. *J. Immunol.* **4,** 151.
55. Ikehara S., Tanka H., Nakamura T., Furukawa F., Inoue S., Sekita K., Shimizi J., Hamashima Y. & Good R.A. (1985) The influence of thymic abnormalities on the development of autoimmune diseases. *Thymus*, **7,** 25.
56. Inaba M., Kuma S.-I., Inaba K., Ogata H., Iwai H., Yasimizu R., Muramatsu S., Steinman R.M. & Ikehara S. (1988) Unusual phenotype of B cells in the thymus of normal mice. *J. exp. Med.* **163,** 811.
57. Scadding G.K., Vincent A., Newson-Davies J. & Henry K. (1981) Acetylcholine receptor antibody synthesis by thymic lymphocytes correlation with thymic histology. *Neurology*, **31,** 935.
58. Patrick J. & Listrom J. (1973) Autoimmune response to acetylcholine receptor. *Science*, **180,** 871.
59. Manley S.W., Burke J.W. & Hawker R.W. (1974) The thyrotrophine receptor in guinea-pig thyroid homogenate interaction with the long-acting thyroid stimulator. *J. Endocrinol.* **61,** 437.
60. Watanabe K., Tanaka R., Nishimura T., Kumagai Y., Miyazaki J.-I., Yamamura K.-I. & Habu S. (1991) I-E restricted monoclonal expansion of B lymphocytes in the thymus of NOD mice. *Int. Immunol.* **3,** 839.
61. Zoller M. (1991) Intrathymic presentation by dendritic cells and macrophages: their role in selecting T cells with specificity for internal and external nominal antigen. *J. Immunol.* **74,** 407.
62. Inaba M., Inaba K., Hosono M., Kumamoto T., Ishida T., Muramatsu S., Masuda T. & Ikehara S. (1991) Distinct mechanisms of neonatal tolerance induced by dendritic cells and thymic B cells. *J. exp. Med.* **173,** 549.
63. Molina I.J., Thyman N.A.R. & Huber B.T. (1989) Macrophages and T cells do not express Mls^a determinants. *J. Immunol.* **143,** 39.
64. Weiss S. & Bogen B. (1989) B-lymphoma cells process and present their endogenous immunoglobulin to major histocompatibility complex-restricted T cells. *Proc. natl. Acad. Sci. U.S.A.* **86,** 282.
65. Parish N.M, Acha-Orbea H., Simpson E., Qin S.-X., Lund T. & Cooke A. (1993) A comparative study of T-cell receptor $V\beta$ usage in non-obese diabetic (NOD) and I-E transgenic NOD mice. *Immunology*, **78,** 606.
66. Serreze D.V. & Leiter E.H. (1991) Development of diabetogenic T cells from NOD/Lt marrow is blocked when allo-H-2 haplotype is expressed on cells of hemapoietic origin, but not on thymic epithelium. *J. Immunol.* **147,** 1222.
67. Parish N.M., Chandler P., Quartey-Papafio R., Simpson E. & Cooke A. (1993) The effect of bone marrow and thymus chimerism between non-obese diabetic (NOD) and NOD-E transgenic mice, on the expression and prevention of diabetes. *Eur. J. Immunol.* **23,** 2667.