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Properties of human thymic B cells

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SUMMARY

B cells, distinct from those seen in myasthenia gravis, are present in normal human thymic medulla, concentrated around the Hassall's corpuscles. We have shown that they constitute $33 \pm 4{\cdot}8\%$ of the total cells in the thymic medulla. In tissue sections they were often seen to have rosettes of thymocytes around them, a relationship which was maintained when the cells were isolated from the thymus. Thymic B cells expressed cytoplasmic immunoglobulins IgD, IgM and IgG but only rarely IgA. Unlike murine thymic B cells, human thymic B cells were $CD5^-$. Freshly isolated thymic B cells were activated cells, but they rapidly became quiescent and died in culture over a 10-day period unless stimulated with mitogens. Thymic B cells responded to polyclonal B-cell activators SAC and TPA and when stimulated, maintained their relationship with thymocytes. Electron microscopic studies showed that two morphologically different thymocyte populations associated with the B cells. The plasma membranes of larger thymocytes were juxtaposed to the B-cell membrane, but smaller thymocytes with darker cytoplasm were associated with the B cells via cytoplasmic strands. Studies in mice have suggested that B cells are involved in thymic negative selection. The close association between activated B cells and thymocytes observed in this study supports this hypothesis.

INTRODUCTION

The presence of a unique B-cell population in the human thymus was described by Isaacson *et al.* in 1987.[1] Using immunocytochemistry, they showed that B cells in normal thymus are diffusely distributed in thymic medulla, being most concentrated around the Hassall's corpuscles. This distribution is distinct from that seen in the thymus in myasthenia gravis where B cells are present in the extrathymic compartment as organized lymphoid follicles similar to those in other lymphoid tissues.[2] Normal thymic B cells are activated cells, but unlike activated B cells in normal follicle centres they are not associated with a follicular dendritic cell network. Their phenotype is consistently that of activated B cells; $CD19^+$, $CD20^+$, $CD22^+$, $CD35^-$, $CDw32^-$. Thymic B cells are a morphologically heterogeneous population which includes cells with round and 'asteroid' morphology.[2,3] They are present in foetal, postnatal and adult thymus suggesting that they are a fundamentally important population, and that their stimulus to proliferate is endogenous.[1] Thymic B cells have been described in the mouse where the majority are $CD5^+$, and, unlike most murine B cells, they have been shown to respond poorly to lipopolysaccharides (LPS) suggesting distinct functional characteristics.[4] Ontogenetically, they are the first immunoglobulin-secreting cells in the mouse.[5,6] The significance of B cells in the mouse thymus has recently been highlighted by experiments showing that B cells can influence the T-cell repertoire.[7,8]

Despite growing evidence that murine thymic B cells are a functionally distinct population, little is known of their properties in man. The aim of this study, therefore, was to further characterize the fundamental properties of human thymic B cells.

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MATERIALS AND METHODS

Tissue

Normal thymic tissue from 40 children (aged 2 weeks to 12 years) was obtained during open heart surgery. Tissue was either snap frozen in liquid nitrogen and stored at -70° or fixed in 10% formal saline and routinely embedded in wax for paraffin sections or teased apart in culture medium [RPMI-1640 containing 10% foetal calf serum (FCS) was used throughout] to prepare cell suspensions for *in vitro* analysis or cytocentrifuge preparations. Likewise, tonsil tissue was either snap frozen and stored at -70° or teased apart to prepare cell suspensions. All cell suspensions were centrifuged on Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) before use.

Preparation of suspensions of thymocytes for analysis in vitro *and in cytospins*

B cells were not rigorously purified from the thymocyte suspension to minimize any selection of those thymic B-cell populations which fail to associate with T cells. The suspension was depleted of $CD2^+$ cells however by rosetting with

2-aminoethylisothiuronium bromide (AET) treated sheep erythrocytes. The resulting suspensions of thymocytes contained approximately 5–10% B cells.

Immunocytochemistry

Single immunoperoxidase staining was carried out on frozen sections, rehydrated 3 μm paraffin sections and cytospin preparations using the indirect immunoperoxidase technique with the monoclonal anti-B-cell antibody L26 (CD20),[9-11] and peroxidase anti-peroxidase technique with polyclonal antibodies to IgD, IgM, IgG, IgA and IgE.[9] All primary and secondary antibodies were purchased from Dako Ltd, High Wycombe, Bucks, U.K.

Double immunoenzymatic staining of cytospin preparations was used to quantitate B-cell activation. Indirect immunoperoxidase with the diaminobenzidene reagent (brown reaction product) using monoclonal antibody Ki67 (Dako Ltd)[12] was used to pinpoint the nuclei of activated cells. Preparations were then stained using the alkaline phosphatase anti-alkaline phosphatase technique (Dako Ltd) with a fast blue substrate, using monoclonal anti-CD20 to identify B cells.

Double immunofluorescence was carried out by incubating acetone fixed frozen sections of thymus and tonsil (as controls) overnight in fluorescein isothiocyanate (FITC)-conjugated CD5 and biotinylated CD20. Conjugated antibodies were purchased from Dako Ltd. Sections were washed and incubated with extravidin tetramethylrhodamine isothiocyanate (TRITC) conjugate (Sigma Ltd, Poole, Dorset, U.K.). Double exposure photomicrography at the appropriate wavelengths was used to study the distribution of CD20 and CD5.

Electron microscopy

Suspensions of thymocytes to be used for electron microscopy were enriched for B cells as described and samples of the preparations were used to make cytospins to be stained for L26 to confirm the presence and to determine the percentage of B cells present. The rest of the cells were fixed in phosphate-buffered glutaraldehyde and pelleted in 2% agarose (Sigma Ltd) in 0·1 M phosphate buffer, post-fixed in osmium tetroxide, dehydrated and embedded in Araldite. Thin sections were contrasted with uranyl acetate and lead citrate prior to examination in a Joel 100S at 80 kV.

Studies of responses to mitogens

B-cell-enriched thymocytes or tonsil cells were cultured at 5×10^4 cells per well with either medium alone or with dilutions of phorbol 12-myristate 13-acetate (TPA) (Sigma), *Staphylococcus aureus* Cowan I (SAC) (Sigma), pokeweed mitogen (PWM) (Gibco, Paisley, Renfrewshire, U.K.) or LPS from *Escherichia coli* (Sigma). The activation index after 3 days in culture was ascertained by double staining with Ki67 and CD20 as described above and determining the percentage of B cells expressing Ki67 in triplicate wells.

Changes in B-cell number in culture

The survival of B cells in culture over a 10-day period with and without optimal doses of mitogen was established by counting the changes in cell number in the culture wells and the corresponding percentage of $CD20^+$ cells in cytospins of cells taken from triplicate wells.

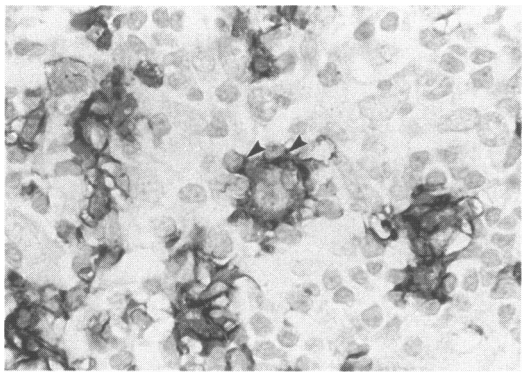

Figure 1. B cells in paraffin section of thymic medulla stained with CD20. Thymocytes which form rosettes around the B cells in thymic medulla are often surrounded by the dendritic processes of the B cells. The CD20 staining is thickened at the point of contact (arrow heads). Immunoperoxidase, original magnification × 200.

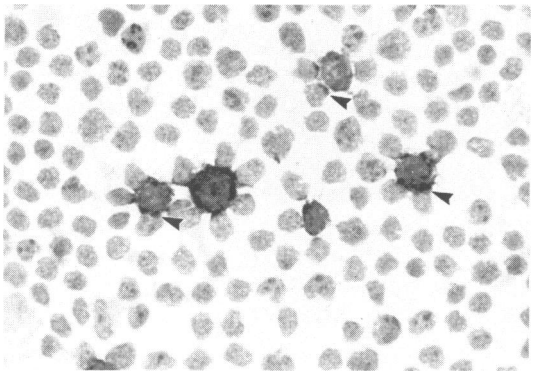

Figure 2. Cytospin preparation of B-cell-enriched thymocytes stained with CD20. Thymocytes associate very closely with the B-cell membranes. The CD20 staining is thickened at the area of contact (arrow heads). Immunoperoxidase, original magnification × 200.

RESULTS

Quantitative studies of thymic B cells in frozen sections and cytospin preparations

Medullary $CD20^+$ cells were enumerated in frozen sections of thymuses from 20 children. All cells in three medullas were counted in each specimen. We observed that B cells constitute approximately $33 \pm 4{\cdot}8\%$ of the total number of cells in the medulla. B cells with rosettes of thymocytes were observed in paraffin sections (Fig. 1).

In cytocentrifuge preparations of B-cell-enriched populations of thymocytes from five thymuses (200 B cells counted in each thymus preparation), we observed a great deal of heterogeneity in the size and morphology of the $CD20^+$ cells. Approximately $54 \pm 8\%$ of the $CD20^+$ B cells were morphologically dendritic, having cytoplasmic processes, the rest being round cells. Large and small cells were observed with each of these appearances. Approximately $86 \pm 13\%$ were surrounded by rosettes and thymocytes (Fig. 2). Cells of all sizes and with both round and dendritic morphology formed rosettes.

Immunoglobulin isotype expression by thymic B cells was highly variable. In all five preparations studied, however, cells

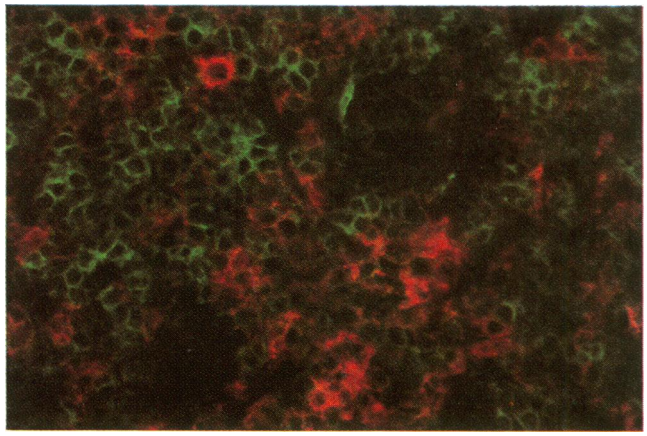

Figure 3. Frozen section of thymic medulla stained for CD5 (FITC, green) and CD20 (TRITC, red). We did not observe any evidence of colour mixing in our studies suggesting that the B cells are $CD5^-$. Immunofluorescence, original magnification × 128.

expressing cytoplasmic IgM (43·5 ± 19·4%), IgD (30·9 ± 6·2%) and IgG (44·7 ± 25·8%) were observed. Few expressed IgA (3 ± 0·6%) and none expressed IgE. There was no difference between the round and dendritic thymic B cells in the immunoglobulin isotypes expressed.

By double staining freshly isolated thymic B cells with Ki67 and CD20, we observed that approximately 10% of the $CD20^+$ cells co-expressed Ki67, an indicator of cellular division.[12,13] In parallel experiments we observed that approximately 20% of tonsillar B cells were $Ki67^+$ when freshly isolated from the tonsil.

Double immunofluorescence

We were unable to demonstrate the presence of CD5 on $CD20^+$ human thymic B cells using double immunofluorescence (Fig. 3). Such cells could be identified as a small population of B cells in the mantle zone of tonsils studied as controls.

Electron microscopy

Following identification and quantitation of B cells in cytospin preparations of cells from the samples to be used for electron microscopy, the cells that we considered to be analogous were identified in semi-thin and then thin sections. Thymic B cells were identified as large (8–9 μm) non-phagocytic cells with prominent filopodia between which thymocytes could be seen forming rosettes (Fig. 4). Many thymocytes were medium-sized cells (6–7 μm), while others were smaller (4–5 μm) and denser than the more abundant medium-sized population. In the nuclei of some of the small darker cells the chromatin was condensed along the nuclear membrane. Some nuclei also showed apparent segregation of the nuclear fragments which were completely filled with condensed chromatin and surrounded by a double membrane. The cytoplasm of these cells also displayed condensation of the cytosol (Fig. 4). Many of the thymocytes forming rosettes appeared to make contact with the B cells where the plasma membranes of the medium-sized thymocytes and the B cells were closely apposed. Small thymocytes made additional contact with the B cells in the form of narrow antenna-like structures which projected from the thymocytes to the plasma membranes of the B cells.

Effect of B-cell mitogens on human thymic B cells

By double staining cytocentrifuged preparations of thymic cells we found that although approximately 10% of the freshly isolated $CD20^+$ cells were $Ki67^+$, only 0–3% of thymic B cells were $CD20^+$, $Ki67^+$ after 3 days ($n = 5$). Thymic B cells gradually died over a 10-day culture period unless treated with polyclonal B-cell activators (Figs 5 and 6). Thymic B cells were activated by a broad range of concentrations of TPA and high doses of SAC (Fig. 6). Only cultures with TPA resulted in both activation and a temporary increase in B-cell numbers (Fig 5). Although activation with SAC was observed it was not clear whether the failure to detect an increase in cell numbers was due to proliferation balanced by cell death or activation but no proliferation. Similar results were obtained in parallel experiments with tonsil B cells (data not shown). Although PWM stimulation resulted in a 15-fold increase in the percentage of tonsillar B cells expressing Ki67 on Day 3 when compared to the control with medium alone, thymic B cells consistently responded relatively poorly to PWM. Experiments were repeated five times with similar results. In contrast to other B-cell activators studied, LPS used at dilutions between 0·1 and 100 ng/ml, was not a potent stimulator of tonsil B cells. In three experiments, the percentage of tonsillar B cells expressing Ki67 on Day 3, was raised from mean values of 1·7% with medium alone to 4·2% in the presence of 1 ng/ml LPS. A similar small increase from 2·5% in medium alone to 4·5% in the presence of 1 ng/ml LPS was observed in thymic B-cell cultures (mean value from three experiments). Stimulated thymic B cells expressed IgG, IgM, IgD and IgA. The mitogen-stimulated cells were large cells with dendritic morphology and retained their rosettes of thymocytes (Fig. 7).

DISCUSSION

We have confirmed that B cells constitute a substantial population in the thymic medulla, comprising approximately 33% of the total medullary population. When extracted from the thymus and studied in cytospin preparations approximately half of the B cells can be seen to have dendritic processes. Most contain cytoplasmic immunoglobulin, including some with cytoplasmic IgD. This is in marked contrast to B cells from other organs. B cells extracted from tonsils are mostly small cells derived from the mantle zone expressing surface immunoglobulin, mostly IgM and IgD. Although cells with cytoplasmic Ig can be extracted from tonsil, these are rarely IgD^+. Some larger cells, mostly derived from the follicle centres, are present in freshly isolated suspensions of tonsil cells but dendritic B-cell processes are rarely observed (ref. 14 and J. Spencer, unpublished results).

The majority of both round and dendritic thymic B cells form distinctive rosettes with thymocytes. Electron microscopic study of the association between B cells and thymocytes showed the presence of cytoplasmic protrusions from small, dense thymocytes to the B cells. Although segregation of the nucleolar components, the classic hallmark of apoptotic thymocyte death was not observed in the thymocytes associated with the B

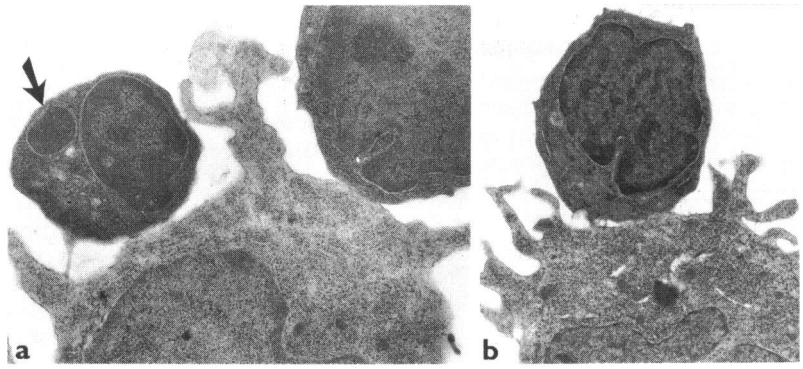

Figure 4. Ultrastructural features of thymocytes forming rosettes with B cells. The plasma membrane of the medium-sized thymocytes abuts onto that of the B cell. The smaller, more dense thymocytes also extend antenna-like processes to contact the B cell. One of these thymocytes shows a separate nuclear fragment (arrow). Original magnification: (a) × 10, 350; (b) × 9, 100.

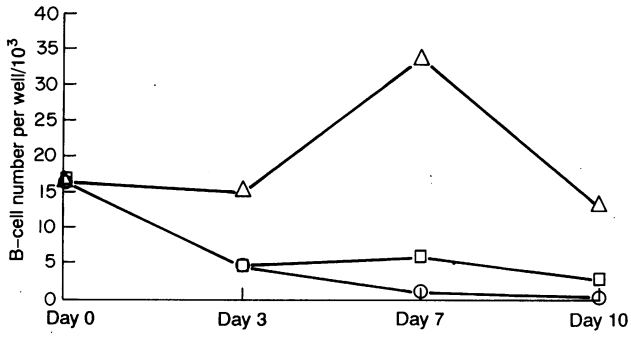

Figure 5. Graph showing the survival of thymic B cells over a 10-day culture period with and without optimal mitogenic stimulation. B-cell number per well was determined by counting the total number of lymphocytes per well and the percentage of B cells in CD20-stained cytospin preparations of the cells from that well. Values are the means of triplicate counts in a single typical experiment (a) TPA (ng/ml) (△); (b) SAC (0·01 v/v) (□); (c) no mitogen (○).

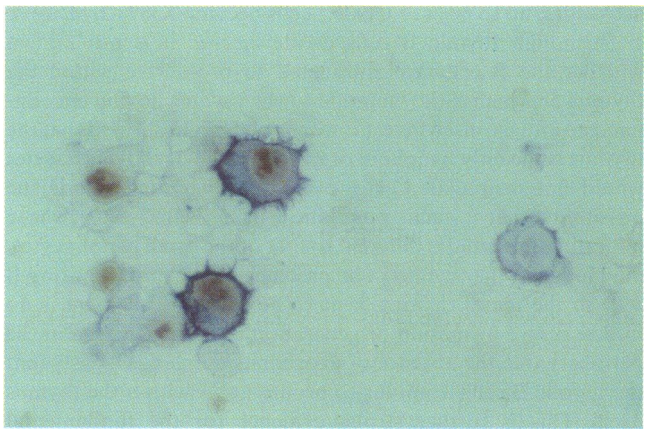

Figure 7. Cytospin preparation of B-cell-enriched thymocytes, double stained for B cells using CD20 (blue) and the nuclear marker of activation Ki67 (brown), 3 days after stimulation with 1 ng/ml TPA. Two activated B cells are indicated with arrows. Stimulated thymic B cells maintain their relationship with thymocytes which are just visible between the B-cell processes despite the lack of nuclear counterstain. Immunoperoxidase and immunoalkaline phosphatase, original magnification × 200.

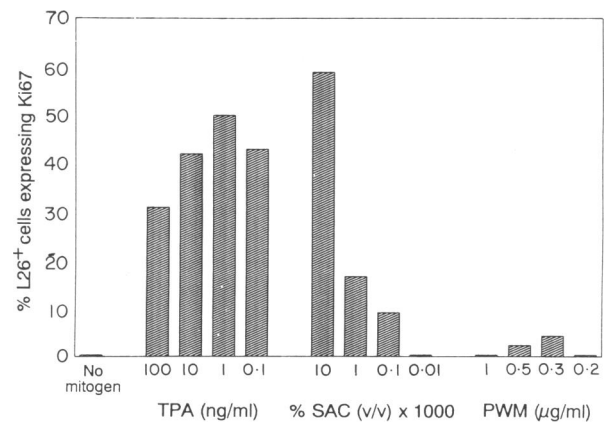

Figure 6. B-cell activation (percentage of B cells expressing Ki67 in cytospin preparations) after 3 days in culture either alone or with a range of doses of polyclonal B-cell activators TPA, SAC or PWM. The values are the means of triplicate counts in a single typical experiment.

cells,[15,16] there was condensation of nuclear chromatin and apparent segregation of nuclear fragments, changes which have been associated with cell death.[17] B cells together with dendritic cells have been shown to influence directly the T-cell repertoire.[7,8] The contact between cytoplasmic processes from T cells showing signs of cell death and the plasma membranes of thymic B cells suggests that B cells may be directly involved in delivering the signal to the T cells to undergo apoptosis.

Thymic B cells differ in man and the mouse. Miyama-Inaba *et al.* showed that thymic B cells in mice respond poorly to mitogenic stimulation by LPS and that the major population contributing to this unresponsiveness was the CD5⁺ B-cell subpopulation.[4] We have shown that the human thymic B cells do not express CD5. They respond well to stimulation by TPA and SAC at a range of concentrations effective in experiments with tonsillar B cells. PWM is a T-cell-dependent B-cell mitogen and the failure of thymic B cells to respond to PWM is probably due to the lack of T-cell help due to the immaturity of the thymocyte population. Although poor responses of thymic B cells to LPS were observed, poor responses were also observed in

the tonsil cell preparations. Failure of human B cells to respond well to LPS has been reported previously and we do not consider the poor response of thymic B cells to LPS in our experiments to be unusual.[18] The expression of CD5 and the associated unresponsiveness to mitogens of murine thymic B cells are not therefore characteristic of the human thymic B-cell population.

Thymic B cells have the phenotype of activated B cells.[1] Consistent with this, approximately 10% of freshly isolated thymic B cells are Ki67$^+$. Ki67 reactivity has been shown to be a reliable marker of cellular activation, giving results which are directly proportional to those obtained in assays using the incorporation of radiolabelled nucleotides.[13] When cultured in the absence of mitogens thymic B cells rapidly become quiescent and have reduced nuclear Ki67 positivity after 3 days. They gradually die over a period of 10 days and are therefore dependent on factors from the thymic environment to support their proliferation. When cultured with optimal doses of B-cell activators, up to 60% of CD20$^+$ cells become Ki67$^+$ by Day 3.

Although thymic B cells divide *in situ*, it is not known whether the B cells are stimulated to proliferate within the thymus or whether they migrate to the thymus having encountered an antigen elsewhere. Gowans and Knight showed that the thymus is the only lymphoid organ through which lymphocytes would not recirculate.[19] However, this does not exclude the possibility that a small population of activated cells, whose migratory potential is different to that of the small lymphocytes, may lodge in the thymus. The presence of such proliferating B cells in the foetal thymus *in utero* however[1] when there is no follicle centre formation or generation of immunoblasts in the periphery due the absence of exogenous antigen, suggests that the thymic B cell's stimulus to proliferate is within the thymus itself. This is further strong evidence for the fundamental importance of B cells in the thymus. B cells are known to be potent antigen presenters.[8,20] It is possible that B cells present autoantigens to thymocytes as part of the negative selection process.

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