

Short reports

HHV-8 is not associated with follicular dendritic cell tumours

S J Nayler, L Taylor, K Cooper

Abstract

Follicular dendritic cell tumours are rare malignancies derived from the follicular dendritic cells of lymphoid follicles. These tumours have been associated with Epstein-Barr virus infections and with the hyaline vascular subtype of Castleman's disease. Because many examples of Castleman's disease have been associated with Kaposi's sarcoma associated herpes virus (HHV-8), this study uses polymerase chain reaction technology to examine five cases of follicular dendritic cell tumours for HHV-8. One of these cases had previously been documented to arise from pre-existing Castleman's disease. HHV-8 DNA was not detected in any of the follicular dendritic cell tumours examined, or in the original case of Castleman's disease. These findings suggest that HHV-8 plays no role in the aetiology of follicular dendritic cell tumours and the cause of this tumour remains obscure.

(*J Clin Pathol: Mol Pathol* 1998;51:168-170)

Keywords: follicular dendritic cell tumour; polymerase chain reaction; herpes virus 8; Castleman's disease

Follicular dendritic cell tumours are extremely rare malignant tumours derived from antigen presenting follicular dendritic cells, cells which are integral to the function and support of the germinal centres of lymphoid tissues.¹ The development of these uncommon tumours following pre-existing Castleman's disease has been well documented.² The newly described Kaposi's sarcoma associated herpes virus (HHV-8)³ has been shown to be associated with all subtypes of multicentric Castleman's disease,⁴ and is also implicated in the pathogenesis of a wide number of human neoplasms.⁵⁻⁸ Because follicular dendritic cell tumours have been reported to be associated with other

infective agents, such as Epstein-Barr virus,⁹ our study was undertaken to ascertain whether there is a relation between HHV-8 infection and follicular dendritic cell tumours.

Methods

Five cases of previously diagnosed follicular dendritic cell tumours were retrieved from the archives of this department. Three had been described previously.^{2, 10} The clinical details are summarised in table 1.

Sections of 2-4 µm were cut from the paraffin wax embedded tissue, floated out, and picked up on glass slides. These were then air dried, dewaxed, and hydrated through sequential baths of xylene and alcohol, respectively, before a final rinse in distilled water. The sections were then allowed to dry. Strict anticontamination procedures were followed.

DNA was extracted from the tissue using the QIAmp kit (Qiagen Ltd, Dorking, Surrey, UK). The tissue was digested overnight in proteinase K at 55°C, precipitated with ethanol, and bound in a spin column, before final elution in water.

Two rounds of nested polymerase chain reaction (PCR) were performed on a Perkin Elmer Geneamp PCR system (Perkin Elmer Corp, Norwalk, Connecticut, USA). Initially, 2 µl of DNA was added to a reaction mixture containing 0.2 M each of the primers KS4 (5'-AGCACTCGCAGGGCAGTACG-3') and KS5 (5'-GACTCTTCGCTGATGAACTGG-3'). Each 50 µl contained 10 mM Tris HCl, 50 mM KCl, 2 mM MgCl₂, 1.5 U. Taq polymerase, and 0.1 mM dNTP (Boehringer Mannheim, Randburg, South Africa). A second reaction using internal primers KS1 (5'-AGCCGAAAGGATTCCACCAT1-3') and KS2 (5'-TCCGTGTTGTCTACGTCCAG-3') was performed on 4 µl of the amplicon obtained from the first reaction. The reaction mixture was identical to that used previously.

Thermocycling conditions were as follows: first round, 30 cycles of 45 seconds at 94°C, 30 seconds at 60°C, and 45 seconds at 72°C; the second round was similar, but with an annealing temperature of 55°C.¹¹

Positive controls from a Kaposi's sarcoma, appropriate negative controls, and a blank control, with water substituted for the DNA, were performed. The DNA was electrophoresed on a 3% agarose gel and visualised with ethidium bromide. The size of the DNA fragment (233

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Accepted for publication
11 March 1998

Table 1 Clinical data of patients

Case no.	Sex	Age	Site	Presentation
1	F	18	Tonsil	Bilateral enlarged tonsils
2	M	17	Soft tissue of neck	Two year history of neck swelling
3	F	33	Soft tissue of neck	Neck swelling following Castleman's disease 5.5 years previously
4	F	28	Soft tissue of neck	Rapidly enlarging tumour
5	M	26	Neck lymph node	Cervical lymphadenopathy — possible metastatic nasopharyngeal carcinoma

F, female; M, male.

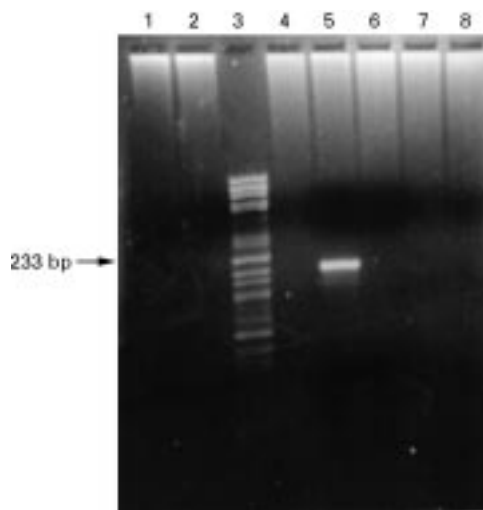


Figure 1 Gel electrophoresis of PCR amplified products corresponding to HHV-8 viral sequences (233 bp). Lane 1, negative control; lane 2, blank; lane 3, molecular weight marker; lane 4, case 1; lane 5, positive control (Kaposi's sarcoma); lane 6, case 2; lane 7, case 3 (lymph node with Castleman's disease); lane 8, case 3 (follicular dendritic cell tumour).

base pairs (bp)) was established by comparison with a standard molecular weight marker (Marker V; Boehringer Mannheim). The sensitivity of this method has been shown to be one copy of HHV-8 DNA/10 000 uninfected cells.¹²

Amplification of the β globin gene, under the same conditions as described above, was performed to demonstrate the integrity of the DNA preparations as described previously.¹³ Extracted DNA (10 μ l) was amplified with primers PCO4 (CAA CTT CAT CgT TCA CC) and GH20 (gAA gAg CCA Agg ACA ggT AC). Each reaction contained 10 mM Tris HCl, 50 mM KCl, 4 mM MgCl₂, 200 μ M each dNTP, 2.5 U Taq polymerase, and 50 μ M each β globin primer.

Amplification conditions were as follows: "hot start" of 94°C for four minutes, followed by 40 cycles of 95°C for one minute, 55°C for one minute, then 72°C for one minute. The final extension was for five minutes at 72°C. The amplicons were visualised on a 3% agarose gel stained with ethidium bromide. The anticipated size of the DNA fragment was 268 bp.

Three replicate assays were performed for each case.

Results

HHV-8 DNA sequences were not detected in the five follicular dendritic cell tumours, including the tumour from the patient diagnosed 5.5 years previously as having Castleman's disease (hyaline vascular subtype). In addition, tissue from the latter lymph node, showing morphological evidence of the hyaline vascular subtype of Castleman's disease, did not contain HHV-8 DNA sequences. The Kaposi's sarcoma positive control contained HHV-8 DNA, and the negative controls did not contain HHV-8 DNA (fig 1). Each of the specimens demonstrated DNA for the β globin

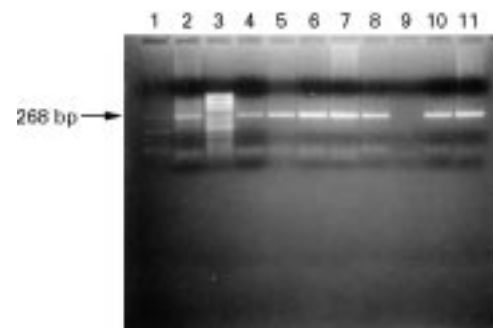


Figure 2 Gel electrophoresis of PCR amplified products corresponding to β globulin gene (268 bp). Lane 1, negative control; lane 2, positive control; lane 3, molecular weight marker; lane 4, case 1; lane 5, case 1 (second biopsy); lane 6, case 2; lane 7, case 3 (lymph node with Castleman's disease); lane 8, case 3 (follicular dendritic cell tumour); lane 9, blank; lane 10, case 4; lane 11, case 5.

chain, indicating that the DNA was preserved adequately (fig 2).

Discussion

Follicular dendritic cell tumours are very rare malignant tumours derived from the antigen presenting follicular dendritic cells, integral to the lymphoid follicle and the immune response. These tumours may show a spectrum of biological behaviour, from low grade malignancy through to high grade aggressive behaviour and rapid death of the patient.³ These tumours are almost certainly under recognised.

We tested the hypothesis that HHV-8 might play a role in the aetiopathogenesis of these tumours, in view of the well documented instances of follicular dendritic cell tumours arising from pre-existing Castleman's disease, as well as the occurrence of the latter condition in association with HHV-8.

Our data indicate that HHV-8 is not associated with follicular dendritic cell tumours. The link with Castleman's disease and HHV-8 is an important one because follicular dendritic cell tumours have been associated with the hyaline vascular subtype, and HHV-8 DNA sequences have been demonstrated in all the variants of multicentric Castleman's disease.⁴ To our knowledge, such a study has not been performed previously in follicular dendritic cell tumours.

HHV-8 DNA sequences have also been demonstrated in bone marrow dendritic cells in multiple myeloma.¹⁴ It is postulated that HHV-8 might play a role in the progression of monoclonal gammopathy of undetermined significance into myeloma.

In summary, HHV-8 plays no role in the aetiology of follicular dendritic cell tumours, and the aetiopathogenesis of this unusual neoplasm remains obscure, with only a few cases exhibiting a predisposing condition such as Castleman's disease or Epstein-Barr virus infection. Although our series is small, other causative models need to be explored.

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Telomerase activity might persist in the human thymus throughout life

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Abstract

Telomerase activity has been demonstrated at low levels in peripheral blood lymphocytes but at high levels in germinal centre B cells and thymocyte subpopulations. This study shows that telomerase is activated in the normal human thymus at different times of life. Telomerase activity was detected in thymic protein extracts from two newborn babies and from a 12 year old boy, as well as in extracts from two of six adult patients. The two positive cases were patients aged 54 and 66 years. These results suggest strongly that the thymus can remain functional despite involution in elderly patients.

(*J Clin Pathol: Mol Pathol* 1998;51:170-173)

Keywords: thymus; telomerase; human; elderly

Human telomerase is a ribonucleoprotein,¹⁻⁴ which is active in germ line cells and in cancer cells but not in somatic and differentiated cells.³⁻⁶ However, telomerase activity has been detected at low levels in peripheral blood lymphocytes (PBLs), as well as in the bone marrow.⁷⁻⁹ An increase in telomerase activity is seen after in vitro activation of B and T cells purified from PBLs.⁹ Recently, high levels of telomerase activity have been demonstrated in hyperplastic tonsils and lymph nodes.¹⁰⁻¹¹ In these lymphoid tissues, the main sources of telomerase activity are germinal centre B cells,¹⁰ most of which subsequently die from apoptosis. In germinal centres, the activation of telomerase seems to be an early event, followed by positive or negative selection of B cells.¹⁰

In the thymus, maturation and selection of T cell lymphocytes occurs through contact in-

volving the T cell receptor (TCR) of CD4⁺/CD8⁺ thymocytes and major histocompatibility complex (MHC) molecules expressed on the epithelial cell surface.¹² The production of mature T cells in the thymus occurs during embryonic development and infancy. Then, the thymus undergoes a progressive involution during late adolescence and it is not known whether the organ is still functional in adults. The mechanisms that control the negative selection of T cells in the thymus involve apoptosis or programmed cell death. There are several similarities between the selection of antigen specific T cells and antigen specific B cells within germinal centres of lymphoid follicles. Two of the mechanisms that allow a selected B or T cell to survive and replicate after positive selection are: (1) the expression of anti-apoptotic molecules, such as Bcl-2,¹³ and (2) the elongation of telomeres by telomerase.¹⁰⁻¹⁴ The latter is thought to occur in the thymus because after several rounds of replication clonally expanded CD28⁻/CD8⁺ T cells have shorter telomeres than precursor CD28⁺/CD8⁺ T cells.¹⁵ In this study, we looked for telomerase activity in protein extracts from thymic tissues obtained from patients at different ages to determine whether the thymus is still functional during involution.

Materials and methods

CONTROL CELL LINES FOR TELOMERASE ACTIVITY
Lymphoma cell lines (T cell leukaemia CEM and Ichikawa cell lines) were used as positive controls. Aliquots of 10⁶ cells were harvested and pelleted at 15 000 ×g for four minutes at 4°C, resuspended in 200 µl of ice cold lysis buffer (0.5% CHAPS, 10 mM Tris HCl (pH 7.5), 1 mM EGTA, 0.1 mM AEBFS,

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Accepted for publication
10 March 1998



Figure 1 Results of the TRAP assay in control samples. Lane O, normal thyroid tissue (negative control); lane P, telomerase activity of 10^6 cells of the Ichikawa cell line (positive control); lane R, RNase control; lane I, thyroid carcinoma used for comparison.

1 mM $MgCl_2$, 5 mM β mercaptoethanol), and kept on ice for 30 minutes.^{5 6 11} The resulting protein extract was centrifuged for 20 minutes at 4°C at 16 000 $\times g$ and 160 μ l of the supernatant was collected and snap frozen in liquid nitrogen before being stored at -80°C.

THYMIC TISSUE SAMPLES

Eleven fresh tissue samples were obtained from patients with different pathological states. Two samples were from newborn babies (3 and 6 months old) undergoing postmortem for sudden death. One sample was from a child with an Epstein-Barr virus associated lymphoproliferative disorder. In the latter sample, there was no localisation of the disease to the thymus, as assessed on immunomorphological grounds and by in situ hybridisation with Epstein-Barr encoded RNA (EBER) probes (not shown). Six samples were from adult patients undergoing surgery for thyroid tumours; in all cases the lack of thymic involvement by cancer cells was shown by careful immunomorphological examination. In two additional cases, thymic tissue specimens were from patients affected with myasthenia gravis. In these two patients, histopathological examination showed large numbers of lymphoid follicles within the thymic tissue.

All frozen tissue specimens were processed as described by Kim and colleagues⁵ and Piatyszek *et al.*,⁶ with some modifications.¹¹ Briefly, 40 cryostat sections of 5 μ m thickness (corresponding to ~ 50 mg of tissue) were collected in Kontes homogenisation tubes. Cryostat blades were changed for each tissue sample. Tissue sections were suspended in 200 μ l of ice cold lysis buffer and homogenised using matching disposable pestles rotated at 450 rpm by a drill. This step was repeated twice during the 30 minute incubation.

Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, California, USA), using bovine serum albumin as the internal control. The results were similar for cultured cell lines and for frozen tissues and varied from 1.5 mg/ml to 8 mg/ml of protein. Samples were aliquoted at 1.5 mg/ml and 3 mg/ml.

TELOMERASE (TRAP) ASSAY

The procedure was similar to that described by Kim and colleagues⁵ and Piatyszek and colleagues,⁶ with some minor modifications.¹¹ Briefly, step 1 consisted of extension of a telomerase substrate oligomer (5'-AATCCGTCGAGCAGAGTT-3') and step 2 consisted of a "hot start" PCR amplification of the product using a reverse CX primer (5'-(CCCTTA)₃CCCTAA-3'). CHAPS extracts (2-4 μ l; 6 μ g protein) were tested in a polymerase chain reaction (PCR) tube in 50 μ l reaction buffer containing 68 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris HCl (pH 8.3), 1 mM EGTA, 0.05% Tween 20, 0.5 μ M T4 gene 32 protein (Boehringer, Mannheim, Germany), 50 μ M each dNTP (dATP, dTTP, dGTP, dCTP), 0.25 μ l [α -³²P]dCTP, 0.25 μ l [α -³²P]dTTP (10 μ Ci/ μ l each), 2.5 U Taq

Table 1 Telomerase activity in the thymus samples tested in this study

Patient	Age (years)	Associated pathology	Lymphoid follicles	Telomerase activity
1	<1	Sudden death	No	+
2	<1	Sudden death	No	+
3	12	EBV-LPD	No	+
4	38	Myasthenia	Yes	+
5	41	Myasthenia	Yes	+
6	54	Thyroid tumour	No	+
7	66	Thyroid tumour	No	+
8	21	Thyroid tumour	No	-
9	35	Thyroid tumour	No	-
10	64	Thyroid tumour	No	-
11	60	Thyroid tumour	No	-

EBV-LPD, Epstein-Barr virus associated lymphoproliferative disorder.

DNA polymerase, 0.1 μ g primer TS, and 0.1 μ g primer CX.

The tube was incubated for 30 minutes at 23°C to allow for the extension of the TS primer by the telomerase. After elongation, the sample was heated at 94°C for two minutes. The PCR assay consisted of 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds. Controls were included in each assay as described previously¹¹: (1) protein extracts from a positive control (lymphoma cell line) predigested with RNase A (0.5 μ g for each 10 μ l extract, 20 minutes at 37°C), (2) no protein, and (3) no CX primer (fig 1). To assess the sensitivity of the TRAP assay and to rule out the presence of Taq polymerase or telomerase inhibitors, protein extracts from the positive control (Ishikawa lymphoma cell line) were diluted 1/10, 1/100, 1/250, 1/500, 1/750, and 1/1000 in protein extracts from negative cases.¹¹

PCR amplification products were analysed on a 10% polyacrylamide gel (non-denaturing) in 1 \times Tris-borate-EDTA buffer at 50 V overnight. The gels were fixed in 0.5 M NaCl, 40 mM sodium acetate (pH 4.2), and 50% absolute ethanol for 45 minutes, sealed in a plastic bag, and exposed directly to an autoradiographic film (X-OMAT; Kodak, Rochester, New Jersey, USA), using intensifying screens.

IMMUNOHISTOCHEMICAL STAINING

All cases analysed in this study were immunostained with antibodies against: B cell antigens (CD20 and CD79a; Dako, Copenhagen, Denmark), T cell associated antigens (CD2, CD3, CD4, CD5, and CD8; Dako and Becton-Dickinson, Mountain View, CA, USA), Bcl-2 protein (Dako), and Ki-67 proliferating associated antigen (using MIB1 antibody; Immunotech, Marseille, France). Anticytokeratin antibodies (KL-1 and MNF116; Immunotech and the authors' laboratories, respectively) and anti-epithelial membrane antigen (EMA; Dako) were used to exclude a possible involvement by metastatic carcinoma. The epitopes detected by most of these antibodies are denatured by most fixatives and immunostaining on paraffin wax embedded sections was performed following the antigen retrieval method

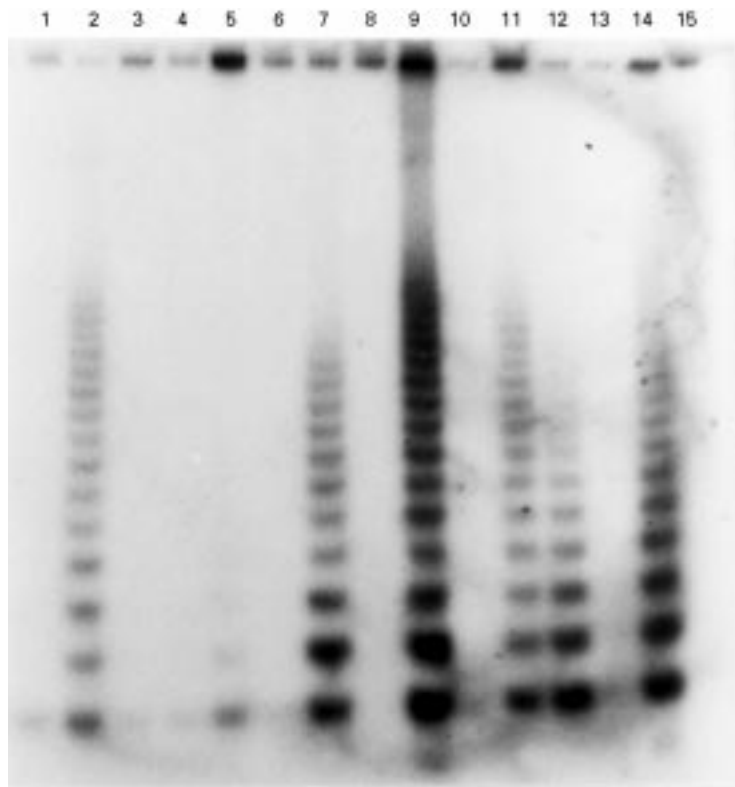


Figure 2 Results of the TRAP assay in thymus samples. Lane 1, no protein extract (negative control); lane 2, clear activity seen in the sample from the 12 year old boy with Epstein-Barr virus associated lymphoproliferative disorder; lanes 3–5, three adult thymus specimens, negative in lanes 3 and 4 but weakly positive in lane 5 (patient with myasthenia gravis); lanes 6 and 8, no protein extract (negative controls); lanes 7 and 9, telomerase activity seen in the thymus of the two newborn babies; lanes 10–13, telomerase activity in thymus specimens from adult patients, negative in lanes 10 and 13 and positive in lanes 11 (54 year old patient) and 12 (66 year old patient); lane 14, patient with myasthenia gravis; lane 15, blank—it is difficult to determine the origin of the activity because the samples contained lymphoid follicles with germinal centres.

described by Shi *et al*,¹⁶ with some modifications.¹⁷ Staining was performed by the streptavidin–biotin–peroxidase complex method using the Dako StreptABCComplex/HRP Duet (mouse/rabbit) kit (K492; Dako).¹⁷

Results

All results are summarised in table 1. Telomerase activity was detected in the two thymus samples obtained from newborn babies and in the thymus of the 12 year old boy (fig 2). These three thymic specimens were normal and non-involutive on morphological and immunohistochemical evaluation, but lacked lymphoid follicles. The possibility of contamination of tissue extracts by adjacent lymph nodes was ruled out by histopathological examination of cryostat sections before protein extraction.

The six samples of normal thymus obtained from adult patients showed various degrees of thymic involution. Telomerase activity was detected in only two of these normal thymic specimens (fig 2). These two positive cases contained relatively large amounts (10–15%) of lymphoid tissue and were from patients aged 54 and 66 years, whereas the four TRAP negative cases contained 1–5% of lymphoid tissue. Because of the sensitivity of the TRAP method, the lack of telomerase activity in the latter cases might be explained by both the scarcity of lym-

phoid tissue remnants and the lack of telomerase positive T cells within these remnants.

Telomerase activity was also detected in protein extracts from the two glands associated with myasthenia gravis (fig 2). These tissues contained numerous lymphoid follicles with large germinal centres that could account for the telomerase activity detected in these specimens.

Immunohistochemical investigations with the MIB1/Ki-67 antibody, which reacts with cycling cells, and the anti-Bcl-2 antibody revealed that these two antigens were mutually exclusive in most tissue samples. Indeed, a high percentage of MIB1/Ki-67 positive/Bcl-2 negative cells was found among cortical thymocytes, whereas the opposite staining pattern (high numbers of MIB1/Ki-67 negative/Bcl-2 positive cells) was seen in the thymic medulla.

Discussion

In this study, we show that the human thymus can activate telomerase in thymocytes throughout life. The detection of such an enzymatic activity in protein extracts from the thymus of newborns or children was expected, but the results seen in two of six adult patients raise several questions. First, it is well established that the thymus undergoes a progressive involution that varies from one individual to another. This involution might not be associated with the loss of function of the thymus regarding T cell maturation and selection. Thus, several reports have suggested that the thymic factor thymulin is still detectable in peripheral blood in humans over 90 years of age and that the secretion of thymulin is strongly dependent on microenvironmental factors such as zinc ions.^{18 19} In our study, telomerase activity was found in thymus protein extracts from adult patients (over 50 years old). These results suggest an active role of the thymus in selecting T cells throughout life. The lack of activity noted in four adult patients might be explained by the depth of involution of their thymus glands. Even if the TRAP assay used in this study is able to detect the activity of 10/10 000 cells,¹¹ it could be that in the TRAP negative cases telomerase positive cells were under the threshold of detection. In addition, the loss of telomerase activity after a few years of storage of the frozen tissues cannot be excluded.⁶ Indeed, some samples were retrieved from our tissue bank after four to six years of storage. Of note, in telomerase negative cases, there was no evidence of telomerase or Taq polymerase inhibitors (data not shown).

Weak but detectable telomerase activity has been seen in haematopoietic progenitors, and in normal leucocytes from blood, cord blood, and bone marrow; high levels of telomerase activity have been found in activated B and T cells.^{6–9} Recently, we¹¹ and others¹⁰ have detected high levels of telomerase activity in reactive hyperplastic lymph nodes and tonsils. Noorback and colleagues¹⁰ have demonstrated clearly that among B cells the highest telomerase activity is seen in germinal centre cells. In contrast to peripheral lymphoid tissue, studies of telomerase activity in the thymus are scarce. The recent description of shorter telomeres in

peripheral CD28⁻/CD8⁺ T cells, compared with their CD28⁺/CD8⁺ counterparts,¹⁵ indicates that telomerase is activated in precursor T cells during thymic selection. One can assume that there are strong similarities between telomerase activation in cortical thymocytes and in germinal centre B cells, most of which die from apoptosis, as do cortical thymocytes. The recent demonstration of telomerase activity in human thymocyte subpopulations in children⁴ indicates that the same mechanism occurs in cortical thymocytes undergoing intrathymic selection. As shown in the present study, similar to germinal centre cells, cortical thymocytes are highly proliferating cells (they have a high percentage of Ki-67⁺ cells) and are negative for the anti-apoptotic protein Bcl-2. Conversely, thymocytes in the medulla no longer proliferate and become protected from apoptosis by the steady expression of Bcl-2. This might indicate that telomerase is highly activated in thymocytes from the cortex compared with thymocytes from the medulla. Nevertheless, the recent report by Weng and colleagues¹⁴ is in keeping with the persistence of high levels of telomerase activity in CD4⁻/CD8⁻, CD4⁺/CD8⁺, and CD4⁺/CD8⁻ thymocyte subpopulations, where the proportion of cell cycling is relatively low.

In conclusion, our results confirm that intrathymic maturation and selection of T cells is associated with telomerase activity. This step allows T cells to acquire telomere sequences long enough to undergo several rounds of replication. The persistence of telomerase activity in thymic tissue from elderly patients strongly supports the theory that in humans the thymus is capable of selecting T cells throughout life.

This work was funded by grants from the Association pour la Recherche sur le Cancer (ARC), the Ligue Nationale Contre le Cancer (Comité de la Haute Garonne), the Délégation à la Recherche Clinique (DRC), and the Institut Universitaire de France.

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Mol Path 1998 51: 168-170

doi: 10.1136/mp.51.3.168

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