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CARACTÉRISATION PHÉNOTYPIQUE ET FONCTIONNELLE DES LYMPHOCYTES B DANS LA LYMPHOCYTOSE POLYCLONALE CHRONIQUE B

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Résumé

La lymphocytose polyclonale chronique B (LPCB) est un syndrome peu connu caractérisé par une élévation polyclonale du nombre de lymphocytes B et de l'IgM sérique. Elle se distingue des pathologies lymphoïdes classiques par son origine polyclonale, sa grande stabilité ainsi que sa symptomatologie discrète et affecte majoritairement des femmes fumeuses. La présence de caractéristiques morphologiques et cytogénétiques distinctives, notamment cellules binucléées et anomalies génétiques (réarrangements *bcl2/Ig* multiples, isochromosome +i(3q)), guide le diagnostic initial. Ces particularités associées à un processus de transformation maligne contrastent avec l'apparente bénignité de la LPCB. Néanmoins, elles n'ont pas permis la délimitation précise de la population B impliquée dans la lymphocytose. Nos travaux avaient pour but d'identifier la population et les mécanismes impliqués dans l'émergence du syndrome, et éventuellement d'estimer les risques de progression clinique. En premier lieu, l'évaluation détaillée du profil immunologique des lymphocytes sanguins chez plusieurs patientes nous a permis de circonscrire formellement la lymphocytose aux cellules B $IgD^{+}IgM^{+}CD27^{+}$. Mettant à profit les récentes avancées techniques et théoriques concernant la biologie du développement chez le lymphocyte B mature, nous avons entrepris l'analyse moléculaire des régions variables des gènes des immunoglobulines. Ces investigations ont confirmé le statut mémoire des cellules B en expansion dans la LPCB. Elles n'ont toutefois pas révélé la signature moléculaire résultant de sélection antigénique, processus central de la réponse immunitaire T-dépendante. Parallèlement, nos études fonctionnelles ont attesté de l'intégrité des molécules CD40 et AID, deux régulateurs clés de la maturation chez le lymphocyte B. Il ressort de nos analyses qu'un défaut dans la régulation de la réponse immunitaire, permettant le contournement de la sélection antigénique dans les centres germinatifs, plutôt qu'un blocage de différenciation cellulaire, serait probablement à l'origine de la lymphocytose. Alternativement, ces cellules pourraient être dérivées d'une population nouvellement caractérisée, les lymphocytes B mémoires de la zone marginale splénique, aussi retrouvés dans le sang, provenant présumement d'une voie de diversification indépendante des centres germinatifs. En conclusion, nos résultats ont permis de préciser le portrait diagnostique de la LPCB et de délimiter de nouvelles pistes de recherche touchant tant les aspects cliniques que la biologie fondamentale du syndrome.

Abstract

Persistent polyclonal B cell lymphocytosis (PPBL) is an unusual haematological disorder, mainly detected in adult female smokers, that shares features of both benignity (polyclonal expansion, polyconal IgM secretion, lack of clinical symptoms, stable and mostly uneventful course); and features of malignancy (atypical binucleated cells, multiple *bcl-2/Ig* translocations, chromosome 3 anomalies, bone marrow involvement). Still, these morphological and clonal genetic anomalies have not been restricted to a distinctive B cell subset, and the apparent heterogeneity of the involved cellular population has long impeded further characterization of the syndrome. The aim of our research was to formally identify the population involved in the lymphocytosis, to gain some insight into the mechanisms at play in its development and to evaluate the risk for subsequent transformation in patients. Over the recent years, technical inputs from the molecular field have largely contributed to a better discrimination of the various B cells subsets and, by extension, of B cell lymphoid disorders. Thus, detailed immunophenotypic studies conducted in numerous PPBL patients allowed us to definitely circumscribe the disorder to $IgD^+IgM^+CD27^+$ B lymphocytes, whereas exhaustive molecular analysis of immunoglobulin genes' variable regions has corroborated the memory status of these cells. Yet, molecular signature of the antigenic selection process, the characteristic of a T-dependent immune response, was not detected. Sequencing of the CD40 and AID genes, key regulators in the diversification and affinity maturation of the immunoglobulin receptor, was additionally carried out and expression of both molecules was assessed. No anomaly was evidenced for either gene. In light of those observations, we conclude that a differentiation block in PPBL B lymphocytes is unlikely. Rather, we propose that defects in the affinity maturation process, namely impairment of the antigenic selection mechanism, allows the survival of low affinity $IgD^+IgM^+CD27^+$ memory B lymphocytes in PPBL patients. Conversely, these cells could be related to the as yet scantily characterized $IgD^+IgM^+CD27^+$ memory B cell subset from the splenic MZ, also found in the blood, and presumably derived from a germinal centre independent diversification pathway. Altogether, our results contributed to the elaboration of an accurate clinical definition for PPBL, and delineated avenues for future investigations regarding both the pathological aspects of the disorder and its purely fundamental biologic ramifications.

Avant-Propos

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Contributions

Chapitre I : Lack of CD40-dependent B cell proliferation in B lymphocytes isolated from patients with persistent polyclonal B-cell lymphocytosis.

Auteurs: Marguerite Massinga Loembé, Josée Lamoureux, Noella Deslauriers, André Darveau et Robert Delage.

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Aucune modification.

Rédaction et expérimentations : Marguerite Massinga Loembé, analyse moléculaire du gène CD40 effectué sous la supervision technique de Josée Lamoureux.

Directrice : Noella Deslauriers.

Codirecteurs : André Darveau et Robert Delage.

Chapitre 2 : Analysis of Expressed V_H Genes in Persistent Polyclonal B Cell Lymphocytosis Reveals an Absence of Selection in $IgM^+IgD^+CD27^+$ Memory B Cells.

Auteurs : Marguerite Massinga Loembé, Sonia Néron, Robert Delage et André Darveau.

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Modifications mineures apportées au tableau 6 (les séquences non mutées, soit 1 mutation ou moins, ont été exclues du tableau) et le texte a été modifié en conséquence (la mention 26/29 séquences mutées a été remplacée par 21/29 séquences mutées).

Rédaction et expérimentations: Marguerite Massinga Loembé, immunophénotypage des cellules réalisé en collaboration avec Sonia Néron, chercheur à Héma-Québec, conception des modèles mathématiques et d'analyse de séquence effectuée avec l'aide d'André Darveau.

Directeur : André Darveau.

Codirecteur : Robert Delage.

Chapitre III: Predominant IgM secretion with normal AID gene status further substantiates a marginal zone origin for expanding $IgD^{+}IgM^{++}$ memory B cell subset in persistent polyclonal B cell lymphocytosis.

Auteurs: Marguerite Massinga Loembé, Jessie Farah Fecteau, Sonia Néron, Robert Delage et André Darveau.

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Rédaction et expérimentations : Marguerite Massinga Loembé, suivi de l'expression des récepteurs membranaires (analyse cytofluorométrique) effectué par Jessie Farah Fecteau étudiante au doctorat chez Sonia Néron à Héma-Québec.

Directeur : André Darveau.

Codirecteur : Robert Delage.

Discussion générale: Persistent Polyclonal B Cell lymphocytosis: The Making of a Lymphoma? An immunological perspective.

Auteurs: Marguerite Massinga Loembé, Robert Delage et André Darveau.

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Rédaction : Marguerite Massinga Loembé.

Directeur : André Darveau.

Codirecteur : Robert Delage.

A Gouled, mon acolyte de tous les moments, et Jélany le petit moustique, preuve que la joie de vivre et le papotage sont des traits héréditaires.

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Liste des abréviations

a.a. : acide aminé.
Ab ou Ac : *antibody* ou anticorps.
Ag : antigène.
AID : *activation-induced cytidine deaminase* ou cytidine désaminase induite par l'activation cellulaire.
ALL : *acute lymphoblastic leukemia* ou leucémie lymphoblastique aiguë.
ALPS : *autoimmune lymphoproliferative syndrome* ou syndrome lymphoprolifératif autoimmun.
AP-1 : *activating protein 1*
APC : allophycocyanine.
APRIL : *apoptosis-inducing ligand.*
BAFF : *B cell activation factor.*
BCMA : *B cell maturation antigen.*
BCR *: B cell receptor* ou récepteur des lymphocytes B.
BL : *Burkitt lymphoma* ou lymphome de Burkitt.
BSAP: *B cell specific activator protein.*
C: *constant region*, région constante des gènes des immunoglobulines.
CDR : *complementarity determining regions* ou régions déterminant la complémentarité, hypervariables.
CLL : *chronic lymphocytic leukemia* ou leucémie lymphoïde chronique.
CMHII : complexe majeur d'histocompatibilité de classe II.
CTAR : *carboxy-terminal activating region* ou région activatrice carboxy-terminale.
CS: *class-switching* ou commutation isotypique.
D: *diversity region*, région de diversité des gènes des immunoglobulines.
DLBCL *: diffuse large B cell lymphoma* ou lymphome B diffus à grandes cellules.
DZ : *dark zone* ou zone foncée des centres germinatifs.
EBV : *Epstein-Barr virus* ou virus Epstein Barr.
FACS : *fluorescence activated cell sorter* ou cytométrie en flux.
FADD : *fas associated death domain* ou domaine de mort associé à fas.
FBS *: fetal bovine serum*, serum de veau foetal.
FDC : *follicular dendritic cell* ou cellule folliculaire dendritique.
FITC : fluorescéine.
FL *: follicular lymphoma* ou lymphome folliculaire.
FM : *follicular mantle zone* ou zone du manteau folliculaire.
FR : *framework regions* ou régions charpente.
H: *heavy chain*, chaîne lourde des immunoglobulines
HBLD : *hairy B cell lymphoproliferative disorder* ou désordre lymphoprolifératif polyclonal avec tricoleucoytes.
HBV *: Hepatitis B virus* ou virus de l'hépatite B.
HCL : *hairy cell leukemia* ou leucémie à tricholeucocytes.
HIGM : syndrome d'hyper IgM.
IL : interleukine.
Ig : immunoglobuline.

J: *jonction region*, région de jonction des gènes des immunoglobulines.
JAK : Janus kinase.
JNK : c-jun kinase.
L: *light chain*, chaîne légère des immunoglobulines.
LT-α: lymphotoxine α.
LZ : *light zone* ou zone claire des centres germinatifs.
MAPK : *mitogen activated protein kinases* ou protéines kinases activées par les mitogènes.
MBR : *major breakpoint region*
mcr : *minor cluster region.*
MMR: *mismatch repair* machinery, machinerie de réparation des mésappariements nucléotidiques.
MZ : *marginal zone* ou zone marginale.
NHEJ *: non homologous ends joining machinery* ou machinerie de réparation des coupures d'ADN avec brins non homologues.
NFAT: *nuclear factor of activated T-cells.*
NFκB: *nuclear factor κB.*
NHL *: Non Hodgkin lymphoma* ou lymphome non hodgkinien.
NIK: *NFκB inducing kinase*.
PCC : *premature chromosome condensation*, condensation prématurée des chromosomes.
PBMC: *peripheral blood mononuclear cell* ou cellule mononucléée du sang périphérique.
PE : phycoérythrine.
PLL : *prolymphocytic leukemia* ou leucémie prolymphocytaire.
PPBL : *persistent polyclonal B cell lymphocytosis* ou lymphocytose polyclonale chronique des lymphocytes B (en français LPCB).
R : mutation de remplacement.
RAR: *retinoic acid receptor*, récepteur de l'acide rétinoique.
RSS *: recombination signal sequence* ou séquences signal de recombinaison.
S : mutation silencieuse.
SAPK : *stress activated protein kinases* ou protéines kinases activées par le stress.
SH : *somatic hypermutation* ou hypermutation somatique.
STAT : *signal transducers and activators of transcription.*
TACI: *transmembrane activator and CAML interactor*.
TCR *: T cell receptor* ou récepteur des lymphocytes T.
TdT : *terminal deoxynucleotidyl transferase*, ou désoxynucléotidyl transférase terminale.
TI : antigène T-indépendant.
TNF : *tumor necrosis factor*.
TNF-R: *TNF-receptor.*
TRADD: *TNF-R adaptor death domain protein.*
TRAF: *TNF-receptor associated factors.*
UNG : *uracil-ADN-glycosylase*.
V: *variable region*, région variable des gènes des immunoglobulines.

Introduction

Système immunitaire et notion d'homéostasie.

L'immunité, ou ensemble des mécanismes permettant à l'organisme de se défendre contre les agressions, particulièrement celles représentées par les microorganismes pathogènes, est assurée grâce aux interactions multiples prenant place entre plusieurs types de cellules spécialisées. Ces interactions sont notamment assurées par le biais de récepteurs variés (récepteur antigénique, molécules CD…) et de médiateurs solubles de nature diverse (cytokines, chimiokines, molécules du complément…). En raison de la complexité des mécanismes impliqués, et des répercussions encourues au plan clinique, le système immunitaire a suscité, et suscitera sûrement encore longtemps, l'intérêt de scientifiques et cliniciens issus de domaines variés. De nombreux efforts ont particulièrement été investis dans la caractérisation des étapes impliquées dans le développement de la réponse immunitaire qui mène de précurseurs cellulaires relativement inoffensifs aux cellules effectrices éminemment compétentes de cette réponse. Aux cours des dernières années, une évidence s'est cependant imposée : les mécanismes qui permettent l'élaboration d'une immunité efficace peuvent également être à l'origine de dérèglements cellulaires, faisant pour l'organisme une menace de ces mêmes cellules censées assurer sa défense. Cela est notamment le cas avec les syndromes inflammatoires aigus ou chroniques, les maladies autoimmunes ou encore certains syndromes immunoprolifératifs. Avec cette thèse, nous avons tenté d'élucider si cela n'était possiblement pas également le cas avec un syndrome immunoprolifératif relativement peu connu : la lymphocytose polyclonale chronique des lymphocytes B (*persistent polyclonal B cell lymphocytosis* ou PPBL, en français LPCB).

Parmi les acteurs de la réponse immunitaire, une distinction est classiquement faite entre les cellules responsables de la réponse spontanée, ou naturelle, assurant la défense de première ligne (monocytes, macrophages, neutrophiles, NK), et celles impliquées dans l'immunité adaptative, que ce soit les médiateurs de l'immunité cellulaire (lymphocytes T) ou de la réponse humorale (lymphocytes B). L'immunité adaptative se distingue par sa spécificité pour l'agent pathogène rencontré et sa capacité à générer une mémoire immunitaire dirigée contre ce dernier. Elle est aussi désignée par le terme d'immunité

acquise car elle représente en fait un reflet de l'expérience individuelle, l'exposition aux agents pathogènes et à leurs constituants structuraux ou toxines (antigènes, ou Ag) variant d'une personne à l'autre. La reconnaissance spécifique des Ag pathogènes est assurée par le biais de récepteurs cellulaires, que ce soit chez le lymphocyte T (*T cell receptor* ou TCR) ou chez le lymphocyte B (*B cell receptor* ou BCR). De plus, dans le cas particulier des lymphocytes B, la sécrétion de molécules spécifiques, les anticorps (*antibodies* ou Ab, Ac en français), permet la neutralisation des particules antigéniques en circulation. Chaque individu possède une variété innombrable de cellules, chacune dotée d'un récepteur différent ou d'une spécificité antigénique distincte (clones), dont l'ensemble (répertoire) lui offre le potentiel de répondre à une multitude d'Ag différents. Toutefois, selon la théorie développée à l'origine par Macfarlane Burnet (Burnet 1959), les seuls clones qui prendront de l'expansion, se différencieront, et éventuellement se diversifieront, pour mener à la réponse immunitaire hautement spécifique et au développement de la mémoire, seront ceux qui pourront lier l'Ag : il s'agit du principe de la **sélection clonale de l'immunité acquise**. En parallèle, l'organisme a développé des mécanismes pour éliminer les clones potentiellement dangereux (délétion clonale des cellules auto-réactives), limiter l'expansion des cellules effectrices une fois la menace antigénique écartée et, ainsi, éviter les effets nuisibles d'une réponse immune chronique (auto-immunité, proliférations malignes). La combinaison de ces mécanismes de régulation des différents compartiments cellulaires du système immunitaire mène à un équilibre dynamique, autrement connu sous le nom d'homéostasie.

Pour une personne en santé, et malgré des variations inter-individuelles dues à des facteurs variés (âge, sexe, activité physique…), des normes ont été établies déterminant les valeurs quantitatives pour chacun des types cellulaires retrouvés dans le sang. Ainsi, chez un adulte, la quantité normale de leucocytes sanguins est évaluée à $7x\ 10^9$ cellules/l (Theml 2000). De ces derniers, ~30% seront des lymphocytes soit, en majorité, des lymphocytes T (~75% des lymphocytes totaux) et, en minorité, des lymphocytes B (~10% des lymphocytes totaux) (voir tableau 1 ci-après pour valeurs exactes). En dehors de ces normes, le contrôle homéostatique cellulaire sera considéré comme rompu.

Tableau 1 : Numération lymphocytaire normale du sang périphérique chez l'adulte (Révillard 2001, Theml 2000).

	Pourcentage (%)	*Valeur absolue (10^9/L)*
Lymphocytes B CD19$^+$	11.8 (5-25)*	0.2 (0.06-0.5)
Lymphocytes T CD3$^+$ CD4$^+$	45 (30-61)	0.8 (0.5-1.6)
Lymphocytes T CD3$^+$ CD8$^+$	28 (13-43)	0.5 (0.2-1)
Lymphocytes NK CD3$^-$CD16$^+$CD56$^+$	14.6 (3-36)	0.3 (0.05-0.7)
Lymphocytes totaux		2.5 (1.5-4)

*Moyenne et limites de la norme à 95%.

Observé parfois chez l'adulte, mais plus souvent chez le jeune enfant, un bris de l'homéostasie des compartiments cellulaires lymphoïdes sanguins avec augmentation du nombre de lymphocytes, ou hyperlymphocytose, est synonyme d'un processus réactionnel enclenché en réponse à un agent infectieux (ex : primo-infection virale et voir tableau ci-après). L'hyperlymphocytose est alors accompagnée de fièvre et d'une augmentation de la taille des organes lymphoïdes secondaires (adénopathie, splénomégalie). Elle est caractérisée par l'expansion de cellules morphologiquement atypiques ou activées et est, essentiellement, de nature transitoire (Hoffbrand, *et al* 2001, Theml 2000).

Tableau 2 : Causes communes de l'hyperlymphocytose chez l'humain (Hoffbrand*, et al* 2001, Theml 2000).

Infections	Transitoires : • Mononucléose infectieuse • Rubéole • Oreillons • Coqueluche • Lymphocytose infectieuse • Hépatites • Primo-infection par cytomégalovirus, VIH Chroniques : • Tuberculose • Toxoplasmose • Brucellose • Syphilis
Proliférations malignes	Leucémie lymphoïde chronique (CLL) Leucémie prolymphocytaire (PLL) Leucémie à tricholeucocytes (HCL) Leucémie lymphoblastique aiguë (ALL) Phase leucémique de certains lymphomes non hodgkiniens.
Génétiques	Syndrome lymphoprolifératif autoimmun (mutation de CD95/Fas)

En contre-partie, une hyperlymphocytose persistante correspondra plutôt à un syndrome lymphoprolifératif malin, notamment à une leucémie lymphoïde (expansion incontrôlée de lymphocytes dans le sang) ou encore à la dissémination d'un lymphome non hodgkinien [*Non Hodgkin lymphoma* ou NHL] (expansion incontrôlée de lymphocytes au sein d'un organe lymphoïde). Dans les deux cas, on distingue les entités hautement malignes, avec une évolution rapide (leucémie lymphoblastique aiguë [*acute*

lymphoblastic leukemia ou ALL], lymphome B diffus à grandes cellules [*diffuse large B cell lymphoma* ou DLBCL], lymphome de Burkitt [*Burkitt lymphoma* ou BL]), et les entités de malignité faible ou intermédiaire dont la progression est plus lente (leucémie lymphoïde chronique [*chronic lymphocytic leukemia* ou CLL], leucémie à tricholeucocytes [*hairy cell leukemia* ou HCL], la plupart des NHL) (Hoffbrand, *et al* 2001). Il n'est toutefois pas exclu qu'un syndrome lymphoprolifératif initialement indolent évolue vers un phénotype plus agressif. C'est notamment ce qui observé dans le cas du lymphome folliculaire (*follicular lymphoma* ou FL) qui évolue fréquemment vers un DLBCL, apparemment à la suite de l'enrichissement en anomalies génétiques (mutations des gènes p53, *bcl6*, ou *myc*)(Stamatopoulos, *et al* 2000). Ces réalités cliniques illustrent bien la théorie selon laquelle les désordres lymphoïdes seraient en fait des pathologies clonales, consécutives à un événement génétique initial qui, en allongeant la survie d'une cellule normale, la rend susceptible à l'accumulation d'anomalies génétiques subséquentes menant à une transformation maligne irréversible (Dolcetti and Boiocchi 1996). On parle alors du mécanisme de lymphomagenèse comme d'un processus à étapes multiples (***the multi-step theory of lymphomagenesis***).

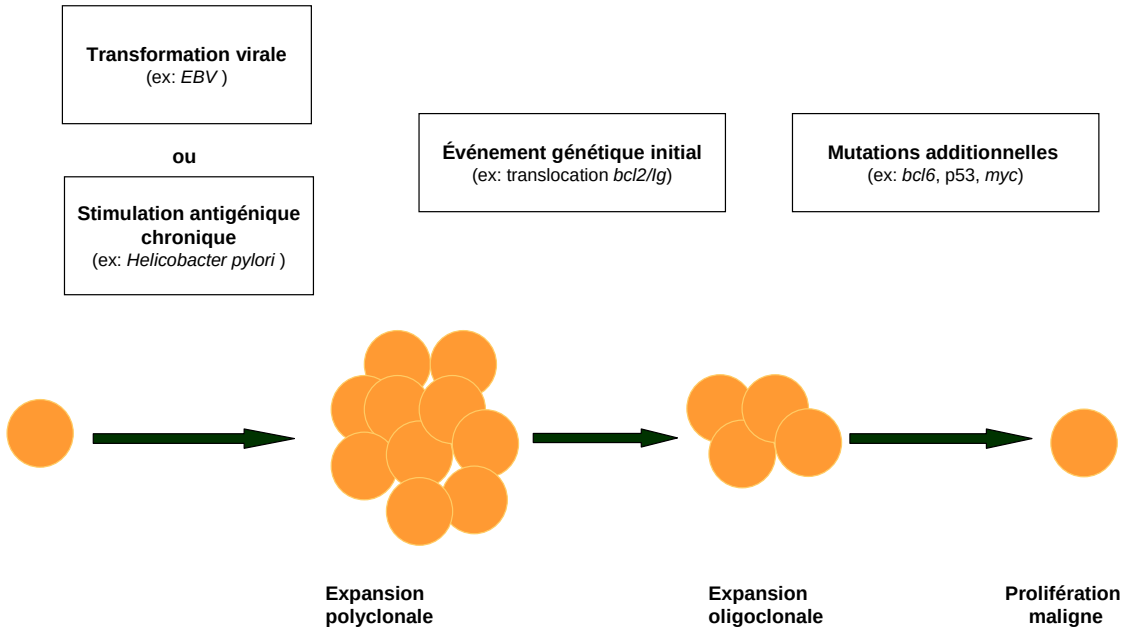


Figure 1 : La lymphomagenèse, un processus à étapes multiples.

Diversification du répertoire des immunoglobulines et genèse des désordres lymphoïdes B.

Mise en contexte.

Comme cela a été mentionné précédemment, et bien qu'il ne faille pas ignorer l'influence jouée par les facteurs environnementaux (ex : exposition aux radiations ou à des substances chimiques), les indices expérimentaux se sont accumulés qui soulignent l'importance du rôle joué par les mécanismes de diversification de la réponse immune dans l'émergence des syndromes immunoprolifératifs. Cela affecterait particulièrement les lymphocytes B dont le développement est jalonné de nombreuses phases de maturation impliquant divers remaniements du génome cellulaire. Cependant, contrairement aux lymphocytes T, les lymphocytes B ont initialement peu suscité l'intérêt des scientifiques : les mécanismes de différenciation et de diversification de la réponse humorale, et les différents stades de développement du lymphocyte B sont longtemps demeurés obscurs. Au cours des dernières années, la situation a cependant connu un revirement considérable et la recherche consacrée à la biologie du développement chez le lymphocyte B a connu un essor fulgurant. Ce regain d'intérêt est grandement redevable aux avancées techniques dans le domaine de la biologie moléculaire, illustrées par l'avènement des micro-puces à ADN (*DNA miccroarrays*) ou encore l'analyse des gènes des immunoglobulines (Ig) à l'échelle de la cellule individuelle (*single-cell PCR*). L'identification de nouveaux marqueurs phénotypiques (molécules CD) permettant la discrimination des stades ponctuant la différenciation du lymphocyte B en périphérie et dans les organes lymphoïdes secondaires, notamment le marqueur des cellues B mémoires CD27 (Agematsu, *et al* 2000), et, enfin, la découverte de régulateurs enzymatiques contrôlant la diversification du répertoire des Ig, spécialement la protéine clé AID (Muramatsu, *et al* 1999, Revy, *et al* 2000), sont parmi les avancées majeures de la dernière décennie.

Ces avancées dans le domaine de la recherche fondamentale ont, par ricochet, influencé le domaine clinique, notamment en ce qui concerne l'identification des différentes entités

pathologiques lymphoïdes et le choix d'une approche thérapeutique adaptée. Les méthodes de classification classiques, tels le système de Rappaport (1970), la *Working Formulation* (USA, 1982) ou le système de Kiel (Europe, 1988), basées principalement sur des critères histologiques (lymphomes à petites ou grandes cellules, architecture folliculaire ou diffuse) et sur des données phénotypiques et cytogénétiques succinctes, manquaient de flexibilité et menaient parfois à l'amalgame de certaines entités pathologiques distinctes. Ces méthodes ont depuis cédé la place aux classifications REAL (*Revised European American Lymphoma*, 1994) et de l'OMS (Organisation mondiale de la santé, 2001) qui, en intégrant les connaissances récentes regardant la biologie du développement du lymphocyte B, dressent un portrait détaillé de la cellule maligne (morphologie, phénotype, données cytogénétiques et de biologie moléculaire [configuration des gènes des Ig, présence de translocations erronées]), et permettent dans la majorité des cas d'en spécifier le précurseur non transformé (Isaacson 2000). Il est ainsi devenu possible de retracer les étapes de différenciation subies par la cellule maligne, de conjecturer les mécanismes ayant contribué à sa transformation et, parfois même, d'anticiper le comportement clinique de la maladie. La connaissance plus approfondie des processus de différenciation et de maturation du lymphocyte B permet désormais aux chercheurs de mieux appréhender les mécanismes physiopathologiques impliqués dans la genèse de prolifération lymphoïdes B malignes.

Le répertoire primaire : la mise en place d'une défense de première ligne.

Le développement des lymphocytes B est jalonné de plusieurs cycles de maturation se déroulant dans les compartiments lymphoïdes centraux et périphériques. Ces différents cycles sont tous caractérisés par des modifications moléculaires séquentielles de l'immunoglobuline (Ig) de surface. Celle-ci, à la différence de la forme sécrétoire, est ancrée à la membrane cellulaire et est associée aux chaînes α et β, constituant le récepteur du lymphocyte B (*B cell receptor* ou BCR). Grâce à dernier, le lymphocyte B peut reconnaître un antigène donné de façon spécifique.

Structurellement, l'Ig est un tétramère composé de deux chaînes lourdes (*heavy chains* ou H) et deux chaînes légères (*light chains* ou L) comportant chacune un domaine constant

(C) impliqué dans la structure de l'anticorps et lui conférant classe (IgA, IgD, IgE, IgG ou IgM) et fonctionnalité biologique adaptée au type de réponse immune requise (ex : réponse primaire ou secondaire, forme sérique ou sécrétoire); et un domaine variable (V), site de reconnaissance de l'antigène. Chez l'humain, l'information nécessaire à la synthèse des Ig est retrouvée sur les chromosomes 14 (chaîne lourde), 2 et 22 (chaînes légères κ et λ). En particulier, le domaine variable des chaînes H et L est codé par différents segments non adjacents et présents en copies multiples sur le chromosome : V (variabilité), D (diversité, spécifique à la chaîne H) et J (jonction) (Cook et Tomlinson 1995, Matsuda, *et al* 1998). L'expression d'une molécule complète d'Ig à la surface de la cellule est tributaire de l'assemblage, vraisemblablemement aléatoire, de ces différents segments via un processus de recombinaison de l'ADN germinal : **le réarrangement V(D)J**, puis de leur jonction à un domaine constant des chaînes H (Cμ pour IgM ou Cδ pour IgD) et des chaînes L (Cκ et Cλ). A ce stade de la différenciation cellulaire, les autres classes de la chaîne H, situées en aval du chromosome, ne sont pas exprimées.

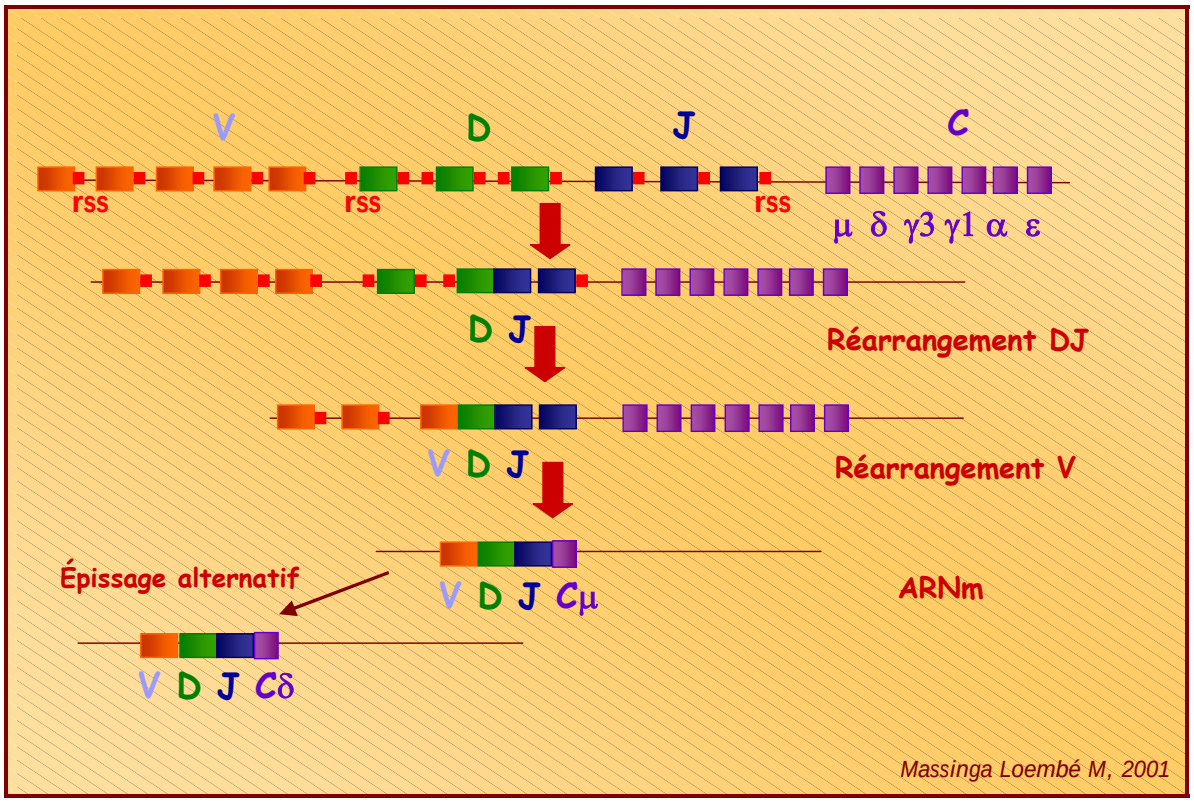


Figure 2 : Le réarrangement V(D)J : recombinaison de l'ADN germinal et génération du répertoire primaire des anticorps.

RSS : séquences signal de recombinaison, consistant en un heptamère et un nonamère de séquence conservée séparés selon le cas par des espaceurs de 12 ou 23 nucléotides non conservés.

Les effecteurs enzymatiques du réarrangement VDJ, de même que les principales phases du processus de recombinaison, sont relativement bien définis et ont fait l'objet de nombreuses revues détaillées (Bassing, et al 2002, Gellert 1997). Ainsi, la destinée du lymphocyte B s'enclenche avec l'expression de RAG1 et RAG2 (recombination activating genes) chez un précurseur lymphoïde B de la moelle osseuse ou du foie foetal. Ces enzymes initient le processus de recombinaison par le clivage double brin de l'ADN cible et la formation de structures en tête d'épingle. À ce stade sont recrutées les protéines du complexe de réparation des coupures d'ADN avec brins non homologues (non homologous ends joining, ou NHEJ) dont l'expression cellulaire est ubiquitaire. Ces dernières seront responsables de la jonction des brins générés par les RAG. Le complexe comprend les trois sous-unités de la protéine kinase ADN dépendante (Ku70, Ku 80 et unité catalytique), la protéine Artémis, nouvellement identifiée et apparemment dotée d'une activité endonucléase permettant l'ouverture des structures en tête d'épingle (Ma*, et al* 2002), et enfin l'ADN ligase IV et la protéine XRCC4 responsables de la fusion finale de la coupure. L'enzyme TdT (*terminal deoxynucleotidyl transferase*) ajoute à la diversité combinatoire en catalysant l'addition aléatoire de nucléotides au site de jonction, engendrant ainsi la diversité jonctionnelle. La recombinaison V(D)J étant un processus ordonné, la cellule procède dans un premier temps au réarrangement de la chaîne lourde puis, en cas de succès, à celui de la chaîne légère. En effet, en raison des contraintes du code génétique (respect nécessaire des cadres de lecture, création possible de codons Stop), la plupart des réarrangements obtenus seront non productifs. Le répertoire primaire est donc généré au hasard dans le seul but d'accroître la diversité des anticorps produits par l'organisme : il contribue à la réponse immunitaire naturelle, de première ligne et de faible affinité qui précède l'exposition à l'antigène.

Le répertoire secondaire : l'adaptation.

Le répertoire secondaire, au contraire, découle de l'exposition à l'antigène et des processus de diversification des Ig et de maturation d'affinité des Ac : c'est la réponse immunitaire adaptative, taillée sur mesure pour l'organisme, variant d'un individu à l'autre. Cette phase de développement, dite dépendante de l'antigène, se déroule pour l'essentiel dans les centres germinatifs (GC) des organes lymphoïdes secondaires (pour

revues générales, consulter : Defrance, *et al* 2002, MacLennan 1994, MacLennan, *et al* 2003).

À leur sortie de la moelle osseuse, les lymphocytes B matures mais encore vierges ($IgM^+IgD^+CD38^-$) transitent par la voie sanguine et vont coloniser les follicules primaires des organes lymphoïdes secondaires (rate, amygdales, ganglions lymphatiques, tissus lymphoïdes non encapsulés) entre lesquels ils re-circulent activement via le sang et la lymphe (Butcher et Picker 1996). Morphologiquement, ces lymphocytes B sont de petite taille et quiescents (Pascual, *et al* 1994).

Quand, en conjonction avec un lymphocyte T activé spécifique, les B rencontrent l'antigène reconnu par l'immunoglobuline membranaire (Lanzavecchia 1985), ils deviennent eux-mêmes activés (suite à l'interaction entre la molécule de surface CD40 et son ligand CD154 exprimé par le lymphocyte T, voir détails plus loin), prolifèrent (Liu, *et al* 1991), et voient alors deux options se présenter. D'une part, ils peuvent former localement un foyer de prolifération extra-folliculaire où ils se différencient rapidement en plasmocytes à courte vie, sécrétant des anticorps de faible affinité (Jacob et Kelsoe 1992, Liu, *et al* 1991). Dans ces foyers B-blastiques, les cellules peuvent être soumises au processus de **commutation isotypique** (*class-switching*, ou CS) des chaînes lourdes des Ig voir figure 4B, grâce auquel devient possible la substitution de la chaîne lourde (C_H) des Ig, donc la sécrétion d'anticorps de classe et de fonctionnalité différente (IgG, IgA, IgE), sans altération toutefois de la spécificité pour l'antigène (Toellner, *et al* 1996). D'un autre côté, une minorité de lymphocytes B activés retournent dans un follicule, déclenchant la formation d'un GC qui permettra la génération de cellules B mémoires et de plasmocytes à longue vie, sécréteurs d'anticorps de haute affinité (Jacob et Kelsoe 1992, Liu, *et al* 1991). Le follicule devient alors un follicule secondaire (voir figure 3), doté d'un GC, d'un manteau folliculaire (*follicular mantle* ou FM) et d'une zone extra folliculaire, à la face interne de laquelle se trouve la zone marginale (*marginal zone* ou MZ).

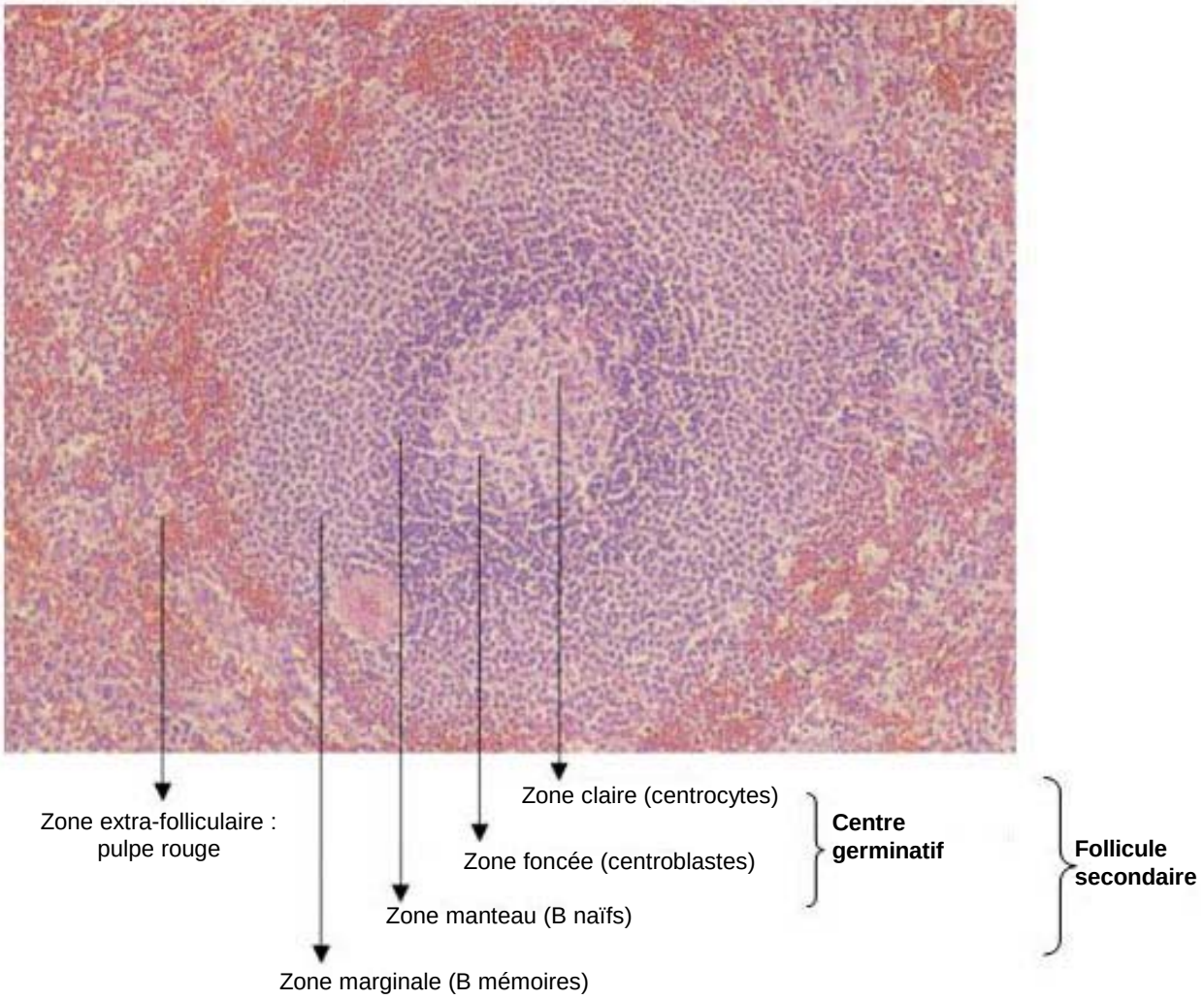


Figure 3 : Structure micro-anatomique d'un follicule secondaire splénique. Adapté de Sagaert et De Wolf-Peeters 2003.

Structurellement chez l'humain, les centres germinatifs se divisent en deux compartiments principaux (Feuillard, *et al* 1995, Kuppers, *et al* 1993, Pascual, *et al* 1994) :

- La zone foncée (*dark zone* ou DZ) où les lymphocytes B fondateurs ayant été activés par l'antigène ($IgM^+IgD^+CD38^+$) (Lebecque, *et al* 1997) se différencient en centroblastes ($IgD^-CD38^+CD77^+CD44^-$) et sont soumis à un cycle d'expansion clonale intense (Liu, *et al* 1991). À ce stade est activé le processus **d'hypermutation somatique** (*somatic hypermutation*, ou SH), lequel mène à l'introduction aléatoire et progressive de mutations ponctuelles dans les régions variables (V_H et à un moindre degré V_L (Klein, *et al* 1998a)) des gènes codant les immunoglobulines (voir figure 4A). Ainsi, à partir de précurseurs B dotés d'une séquence germinale d'immunoglobuline, sera générée une progéniture de clones pourvus de séquences mutées présentant une affinité variable pour l'antigène ayant initié la formation du GC (Jacob, *et al* 1991, Kuppers, *et al* 1993) . En effet, ces clones auront soit amélioré, baissé ou perdu leur spécificité pour l'Ag initiateur.

- La zone claire (*light zone*, LZ), pourvue d'un réseau de cellules folliculaires dendritiques et de quelques lymphocytes T $CD4^+$, où migrent les clones mutants ayant achevé le cycle de prolifération cellulaire et subséquemment différenciés en centrocytes ($IgD^-CD38^+CD77^-$) (MacLennan 1994). C'est alors qu'intervient le phénomène de la **sélection antigénique**, opérant en faveur des clones présentant une affinité accrue pour l'antigène et aux dépens de ceux à l'affinité amoindrie ou potentiellement auto-réactifs (Liu, *et al* 1989). En effet, chez les lymphocytes B des centres germinatifs, on observe une régulation négative de la protéine de survie Bcl-2 accompagnée d'une hausse de l'expression de gènes pro-apoptotiques tels que CD95 (*fas*) et *bax* (Martinez-Valdez, *et al* 1996). La sélection repose sur la capacité du lymphocyte B à 1) lier efficacement l'antigène présenté par la cellule folliculaire dendritique, 2) dégrader et présenter ce dernier à un lymphocyte T $CD4^+$, et, grâce à l'interaction ensuivant entre CD40 et CD154, échapper à l'induction de l'apoptose présumément induite par

l'engagement du récepteur CD95 par son ligand (Liu, *et al* 1989 et description détaillée dans la discussion générale). En contre-partie, la reconnaissance d'un antigène via l'immunoglobuline membranaire en absence d'aide fournie par le T $CD4^+$ (autoréactivité) ou l'interaction avec un T en absence de reconnaissance de l'antigène (perte d'affinité) entraîneront l'élimination de la cellule (Defrance, *et al* 2002). À cette étape, le centrocyte est également soumis au processus de CS (Liu, *et al* 1996) et peut achever sa différenciation vers le stade de cellule effectrice, soit plasmocyte ou B mémoire, et quitter le centre germinatif. Alternativement, il peut aussi retourner dans le GC et être soumis à une nouvelle ronde de diversification (Casamayor-Palleja, *et al* 1996, Kepler et Perelson 1993).

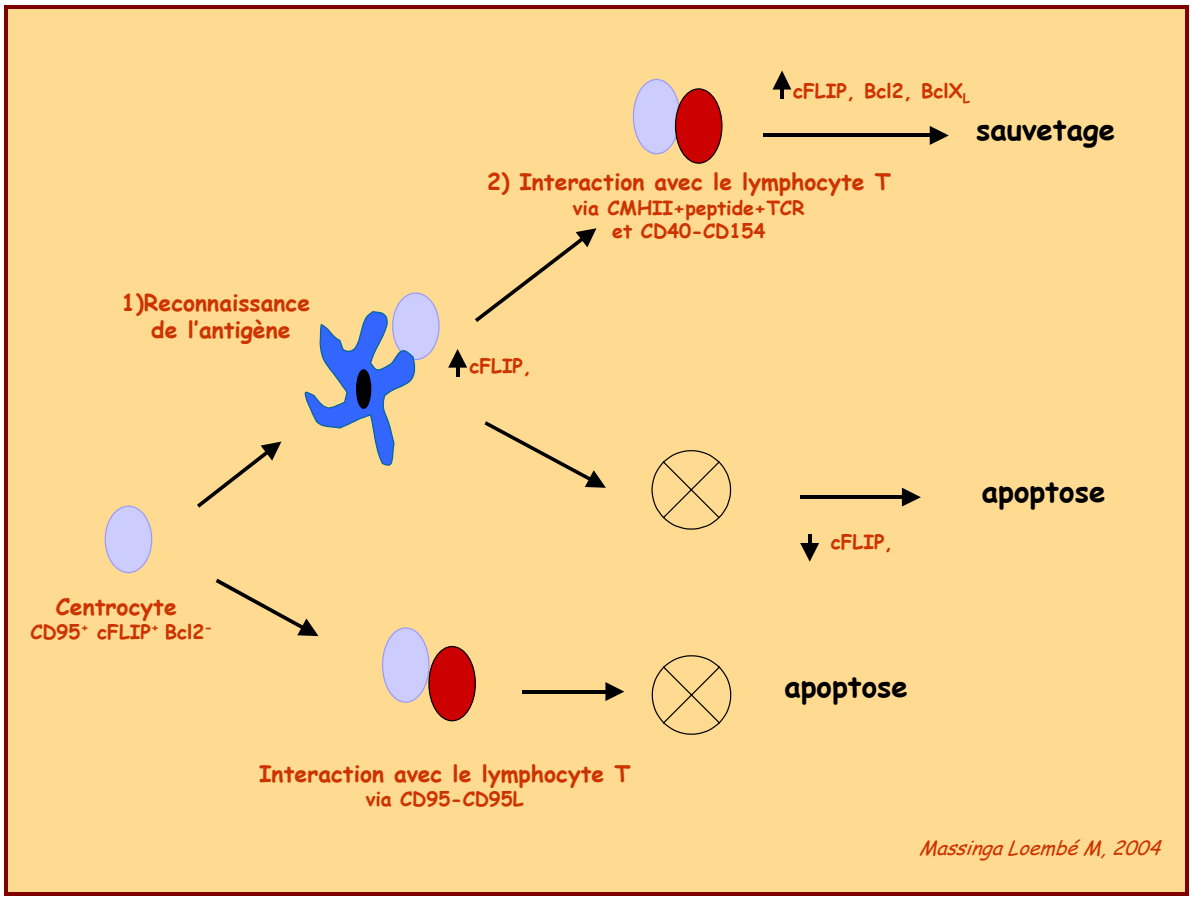


Figure 4 : La sélection antigénique dans les centres germinatifs.

Ironiquement, ce serait donc l'antigène lui-même qui dirige à la fois la génération et la sélection des anticorps mutants de haute affinité qui constituent le répertoire secondaire et permettent à l'organisme de se défendre efficacement.

Il y a quelques années, Chang et Casali (Chang et Casali 1994) ont élaboré une méthode statistique, par la suite raffinée par Lossos et collaborateurs (Lossos, *et al* 2000), pour mettre en évidence la pression sélective exercée par l'antigène sur une séquence donnée d'immunoglobuline. Cette méthode est basée sur le postulat selon lequel les sous-régions de la portion variable des gènes des immunoglobulines présentent une susceptibilité différentielle à l'accumulation de mutations de remplacement lors du processus d'hypermutation somatique. Ainsi les régions hypervariables ou CDR (*complexity determining regions*), impliquées dans la reconnaissance seraient une cible préférentielle pour les mutations de remplacement génératrices de diversité. L'accumulation de sigificative de mutations de remplacement dans les CDR (relativement à celles générées par le simple effet du hasard) était donc associée à une pression sélective visant à accroître l'affinité de l'Ac pour l'Ag. Toutefois, cette hypothèse a depuis été remise en question à cause notamment de la mise en évidence de points chauds de mutation, concentrés surtout dans les CDR (Dunn-Walters, *et al* 2001). Par contre, chez les régions de structure ou FR (*frameworks regions*), une contre-sélection des mutations de remplacement a été observée car ces dernières sont potentiellement délétères pour la structure de l'anticorps et pourraient mener à une perte complète de reconnaissance (Dorner, *et al* 1997, Klein, *et al* 1998a). Par conséquent, la proportion R:S (ratio : mutations de remplacement / mutations silencieuses) sera inférieure pour la séquence V_H d'un lymphocyte B ayant subi la sélection par l'antigène (ex. : B mémoire) comparativement à la séquence d'un B non sélectionné (ex. : B naïf) possédant des mutations générées au hasard. Cette propriété est maintenant communément utilisée pour mettre en évidence la pression sélective exercée sur un clone B de séquence V_H déterminée (voir : Klein, *et al* 1998a).

Au contraire du processus de réarrangement VDJ, les mécanismes de SH et de CS ne sont que très peu définis. Une étape considérable a néanmoins été franchie avec l'identification de la protéine AID (*activation induced deaminase*). Cependant, son ou

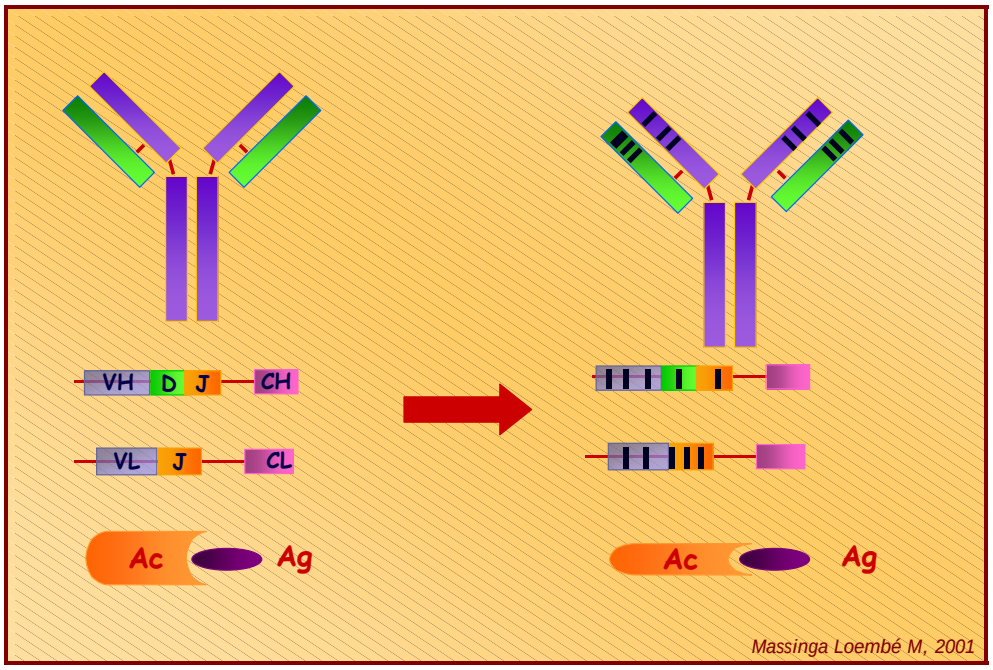
ses modes d'action demeurent pour l'instant relativement imprécis et suscitent bien des débats comme en témoigne un survol rapide de la littérature scientifique des cinq dernières années.

Le rôle prépondérant d'AID dans la maturation d'affinité des anticorps a été mis en évidence chez l'humain à la suite d'études menées auprès de patients atteints du syndrome de l'hyper-IgM (HIGM). Ce syndrome regroupe un ensemble de déficits immunitaires transmis génétiquement, soit liés au chromosome X, soit autosomiques, caractérisés par une diminution de toutes le fractions d'Ig excluant l'IgM et, fréquemment, par l'absence de mutations somatiques dans les gènes des Ig et l'absence de GC. Les patients atteints présentent de plus une grande susceptibilité aux infections bactériennes et opportunistes. Tous ces facteurs témoignent d'un grave défaut dans la maturation des lymphocytes B, lié dans la majorité des cas à un défaut dans la voie d'activation de CD40 et de son ligand CD154 (voir section suivante). Une variante du syndrome, à transmisson autosomique récessive, ou HIGM2, présentant la plupart des critères susmentionnés mais se distinguant par la présence de GC souvent hypertrophiés, a été formellement associée à des mutations invalidantes de la protéine AID (Revy, *et al* 2000). AID est exprimée spécifiquement chez les lymphocytes B des GC ou ceux activés *in vitro* par l'intermédiaire de CD40 (Muramatsu, *et al* 1999). Son expression est nécessaire et suffisante à l'induction des processus de CS et de SH, comme en atteste l'activation des deux mécanismes lorsque l'enzyme est transfectée dans des fibroblastes (Yoshikawa, *et al* 2002). Structurellement, AID présente une homologie de séquence avec la protéine APOBEC-1, laquelle est responsable de l'édition de l'ARNm encodant l'apolipoprotéine B par le biais d'une activité cytidine désaminase (Navaratnam, *et al* 1993, Teng, *et al* 1993). En analogie, AID pourrait donc diriger l'édition et l'activation d'une endonucléase impliquée dans la génération des bris d'ADN, événement initiatiateur de la SH et la CS (Honjo, *et al* 2002, Muramatsu, *et al* 1999). La démonstration de la nécessité d'une synthèse protéique *de novo*, préalable à son action, soutient cette hypothèse (Doi, *et al* 2003). Alternativement, AID pourrait avoir une action directe sur l'ADN, et provoquer la désamination de la désoxycytidine suivie de sa conversion en désoxyuridine, introduisant de fait des mésappariements nucléotidiques qui nécessiteraient l'intervention de machineries cellulaires de réparation de l'ADN

(Petersen-Mahrt, *et al* 2002). La pertinence physiologique des modèles expérimentaux procaryotiques utilisés lors de ces observations (*E.coli*) a toutefois été mise en question. Il n'en demeure pas moins que chez la souris et l'humain, l'inactivation de la protéine UNG (uracil-ADN-glycosylase), impliquée dans l'excision des mésappariements C-U et la génération de coupures d'ADN simple brin, interfère avec la SH et inhibe partiellement la CS (Imai, *et al* 2003, Rada, *et al* 2002). La controverse demeure donc entière.

Quel que soit son mode d'action, AID intervient en aval de la transcription de l'ADN cible, étape préalable commune à la SH et la CS (Honjo, *et al* 2002). Cependant, les deux mécanismes seraient régulés de manière distincte. Ainsi dans la SH, les bris d'ADN générés sont préférentiellement simple brin, nécessitent l'intervention de polymérases non fidèles et, subséquemment, de la machinerie de réparation des mésappariements nucléotidiques (*mismatch repair* ou MMR) menant ainsi à la création de mutations ponctuelles dans l'ADN (Faili, *et al* 2002, Honjo, *et al* 2002, Reynaud, *et al* 2003). De son côté, la CS implique plutôt la formation de coupures d'ADN double-brin et le recrutement de la machinerie de recombinaison NHEJ, avec un mécanisme d'action similaire à celui décrit pour le réarrangement V(D)J (Honjo, *et al* 2002, Kenter 2003). Enfin la CS requière apparemment la présence de co-facteurs spécifiques encore non identifiés (Ta, *et al* 2003), et le type de transcrit germinal stérile induit, donc de classe d'anticorps produit, sera modulé par la nature des cytokines auxquelles la cellule sera simultanément exposée (Armitage, *et al* 1993).

A)



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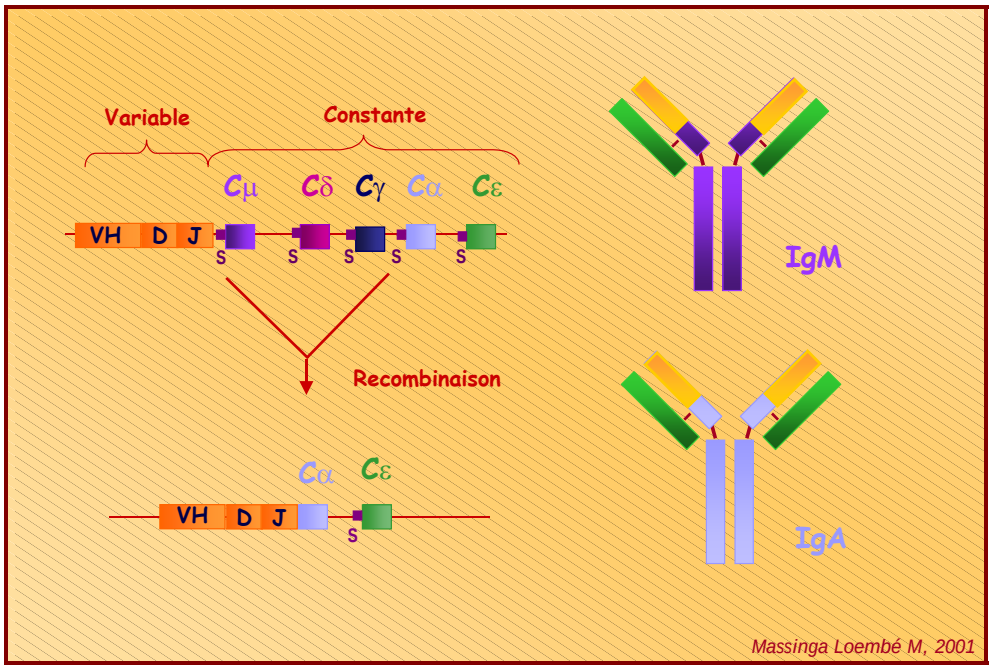


Figure 5 : Modifications du locus des Ig et génération du répertoire secondaire des anticorps.

Signalisation, diversification et maturation d'affinité chez le lymphocyte B.

Lorsque le lymphocyte B reconnaît spécifiquement l'antigène via le BCR, il procède à son internalisation, sa dégradation et peut alors présenter les peptides antigéniques par l'intermédiaire du CMH de classe II, ou CMHII (Lanzavecchia 1985). Le complexe antigène-CMH-II résultant permettra la coopération consécutive du lymphocyte B avec un lymphocyte T activé spécifique et la mise en place d'interactions membranaires directes de type ligand-récepteur qui enclencheront le processus de diversification des gènes des Ig, la maturation d'affinité et le développement d'une réponse humorale de haute efficacité (Bishop et Hostager 2001).

Les protéines appartenant à la superfamille du TNF (*tumor necrosis factor*) et de ses récepteurs TNF-R (*TNF-receptor*) sont des acteurs essentiels de cette coopération B-T. Particulièrement, la paire formée par CD40, un récepteur membranaire de type I d'environ 280 a.a., exprimé constitutivement par le lymphocyte B, et son ligand CD154, molécule membranaire de type II d'environ 260 a.a, exprimée par le lymphocyte T activé, jouent un rôle central (Bishop et Hostager 2003). L'implication clé de CD40 dans le développement d'une réponse humorale de haute affinité, essentiellement dans la formation des GC et la diversification du répertoire des Ac, a été mise en évidence chez les patients souffrant du syndrome HIGM, subséquemment à des mutations délétères du ligand CD154 (HIGM-1) (Korthauer, *et al* 1993) ou de la molécule CD40 elle-même (HIGM-3) (Ferrari, *et al* 2001). Chez ces patients, on constate ainsi l'absence de centres germinatifs dans les organes lymphoïdes secondaires et l'inhibition des processus de SH et de CS. Il en résulte un blocage dans la sécrétion d'anticorps d'isotypes autres que l'IgM, l'absence de mutations somatiques dans les régions V_H des gènes d'Ig, et enfin l'absence de cellules mémoires $CD27^+$ (la population $IgD^+IgM^+CD27^+$ constitue une exception qui est abordée en détail dans la discussion générale). Ces carences attestent du rôle joué par le récepteur dans les nombreux processus physiologiques qui découlent de l'activation du lymphocytes B, soit : l'expansion clonale et la différenciation, l'induction ou l'optimisation de l'expression des molécules d'adhésion homotypique (CD54 ou ICAM-1, CD11a/CD18 ou LFA-1, CD23), d'activation cellulaire (CD38, CD80, CD86, CD95, CMH-II) et des protéines régulatrices (Bcl-X_L, AID), la sécrétion

des Ac et l'induction de la CS, la sécrétion de cytokines et de chimiokines, le sauvetage des cellules B de haute affinité lors de la sélection antigénique et le développement de la mémoire immunitaire humorale (Arpin, *et al* 1995, Bishop et Hostager 2003, Grammer et Lipsky 2000, Liu, *et al* 1989, Muramatsu, *et al* 1999, Zhou, *et al* 2003). Plusieurs de ces effets sont de plus amplifiés en présence de cytokines (Lipsky, *et al* 1997).

Les effets pléiotropiques induits à la suite de l'engagement de CD40 par son ligand coïncident avec l'activation de nombreux facteurs nucléaires activateurs de transcription dont, entre autres, NFκB (*nuclear factor κB*), NFAT (*nuclear factor of activated T-cells*), BSAP (*B cell specific activator protein*), AP-1 (*activating protein 1*), et les membres de la famille des STAT (*signal transducers and activators of transcription*). L'activation de ces facteurs de transcription est elle-même reliée au déclenchement préalable de voies de signalisation impliquant des protéines kinases au nombre desquelles se trouvent les SAPK (*stress activated protein kinases*) p38 et JNK (*c-jun kinase*), des MAPK (*mitogen activated protein kinases*) comme NIK (*NFκB inducing kinase*) et les protéines tyrosines kinases Lyn, Syk et Fyn (Bishop et Hostager 2001, Bishop et Hostager 2003, Faris, *et al* 1994). Les mécanismes exacts à partir desquels CD40 initie ces différentes voies de signalisation demeurent cependant largement spéculatifs. Le récepteur est dépourvu d'activité enzymatique intrinsèque et assure la propagation de son signal grâce au recrutement de protéines adaptatrices, les TRAF (*TNF-receptor associated factors*, au nombre de 6) en analogie avec les autres membres de la famille des TNF-R, de même qu'en interagissant avec la tyrosine kinase JAK3 (*janus kinase*) (Bishop et Hostager 2001, Grammer et Lipsky 2000, Lam et Sugden 2003). Ainsi, une fois engagé par son ligand, CD40 forme un complexe homotrimérique et provoque le recrutement des TRAF du cytoplasme vers la membrane cellulaire par l'intermédiaire des radeaux lipidiques (Bishop et Hostager 2003). Deux motifs de fixation des TRAF ont été identifiés dans la queue cytoplasmique de CD40. Ainsi, un motif PXQXT, situé dans la partie distale de la queue cytoplasmique (a.a. 250 à 254) permet l'interaction directe avec TRAF1, TRAF2 et TRAF3, tandis qu'un second motif situé plus près de la membrane (a.a. 231 à 238) permet la fixation de TRAF6. TRAF5 serait recrutée indirectement en formant des hétéro-oligomères avec TRAF3 (Pullen, *et al* 1998). La voie de signalisation de CD40 la

mieux caractérisée à ce jour est sûrement celle menant de l'engagement du récepteur à l'activation du facteur de transcription NFκB (Bishop et Hostager 2003). Ce dernier est notamment responsable de l'induction de la protéine CD80 et, à un moindre degré, de CD23, CD95 et CD54 et de la sécrétion d'Ig. Son activation résulterait de l'interaction de CD40 avec TRAF2, TRAF5, TRAF6 (Rothe, *et al* 1995, Tsukamoto, *et al* 1999) et possiblement aussi de la participation de la MAPK NIK (Garceau, *et al* 2000). D'un autre côté, l'association constitutive de CD40 avec JAK3, permettant l'activation des STAT, est impliquée dans l'induction de CD23, CD54 et la sécrétion de LT-α (lymphotoxine α) (Hanissian et Geha 1997). L'issue de la signalisation via CD40 varie cependant selon le stade de différenciation de la cellule B. Ainsi une inhibition dans la sécrétion des Ac ou parfois même l'apoptose peuvent résulter de l'engagement du récepteur, notamment chez les B mémoires (Fecteau et Neron 2003, Miyashita, *et al* 1997).

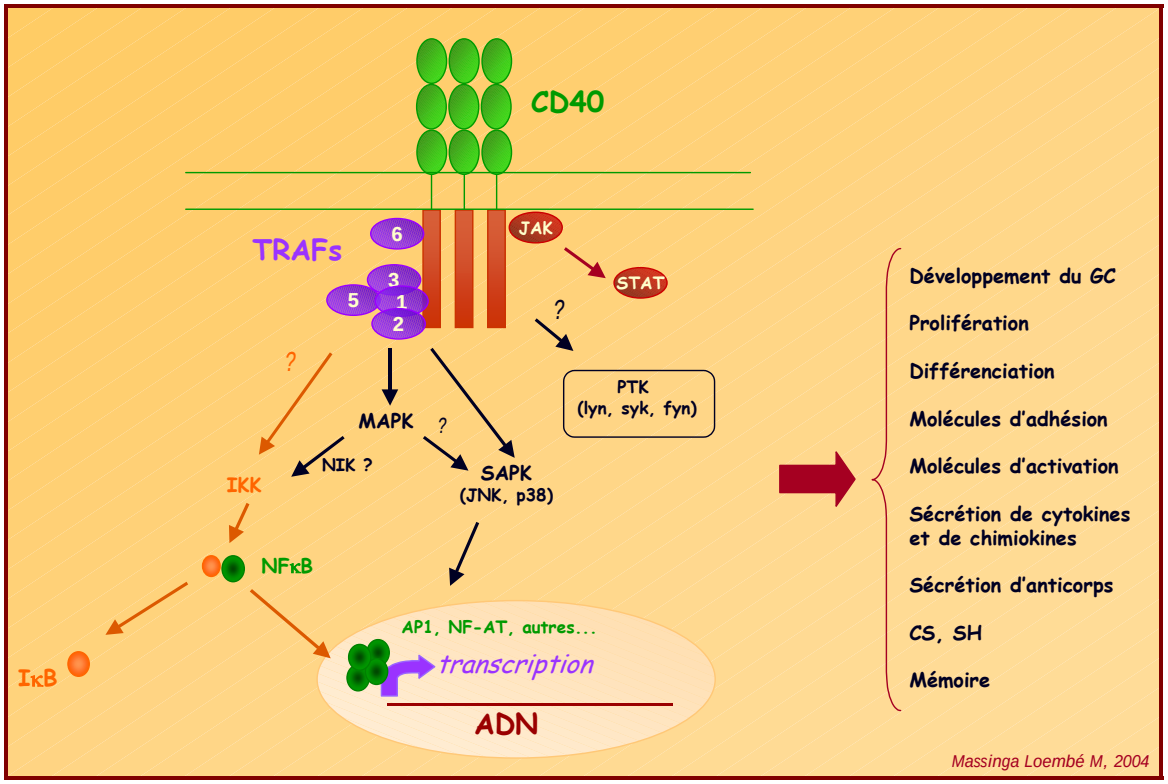


Figure 6 : Cascade de signalisation déclenchée par l'interaction de CD40 avec son ligand CD154.

D'autres molécules, membres de la famille du TNF ou de ses récepteurs, sont impliquées dans les phases complémentaires de la différenciation du lymphocyte B. C'est le cas, comme on l'a déjà mentionné, de la molécule CD95 et de son ligand, qui interviennent plus particulièrement dans le processus de sélection antigénique des lymphocytes B mémoires de haute affinité (voir discussion générale). Le récepteur cellulaire CD27, membre de la famille des TNF-R à l'instar de CD40 joue, comme ce dernier, un rôle pivot dans la différenciation terminale du lymphocyte B à l'issue de la réaction des GC. CD27 est exprimé spécifiquement à la surface des lymphocytes B dotés de gènes des Ig hypermutés et, à ce titre, il a été désigné comme un marqueur spécifique des B mémoires (Agematsu, *et al* 2000, Klein, *et al* 1998b). Similairement à CD40, l'engagement de CD27 par son ligand, CD70, retrouvé à la surface des lymphocytes T mais aussi sur les lymphocytes B, favorise la sécrétion importante d'Ig de toutes classes confondues (Agematsu, *et al* 1997). Toutefois, alors que CD40 induit préférentiellement la différenciation vers la stade de B mémoire (Arpin, *et al* 1995), CD27 préside à différenciation plasmocytaire (Jacquot, *et al* 1997). Cet effet serait amplifié en présence d'IL-10 (Agematsu, *et al* 1998a). Parallèlement, la paire formée par CD134 (OX40) exprimé par les lymphocytes T et son ligand CD134L (OX40L) joue aussi un rôle dans la différenciation plasmocytaire, mais intervient plus spécifiquement lors de la formation des foyers de prolifération B-blastiques dans la zone extra folliculaire (Stuber et Strober 1996).

Dernièrement, les chercheurs se sont attardés à la caractérisation de deux nouveaux membres de la famille du TNF, BAFF (*B cell activation factor* aussi appelé Blys ou TALL1) et APRIL (*apoptosis-inducing ligand*) tous deux exprimés par la majorité des PBMC, à l'exclusion des lymphocytes B, et capables de lier communément les récepteurs BCMA (*B cell maturation antigen*) et TACI (*transmembrane activator and CAML interactor*) et, spécifiquement pour BAFF, le récepteur BAFF-R, tous exprimés par le lymphocyte B (pour une revue consulter : Mackay et Ambrose 2003). Le rôle de BAFF et APRIL dans le développement des lymphocytes B n'est pas encore clairement élucidé, mais ils semblent promouvoir la survie cellulaire. Une observation particulièrement remarquable découle du fait que ces deux molécules sont apparemment capables d'induire la CS indépendamment de CD40 (Litinskiy, *et al* 2002). Ce phénomène serait

important pour assurer une réponse efficace contre les antigènes T-indépendants (TI), tels les polysaccharides ou les lipopolysaccharides bactériens.

Les stades de différenciation du lymphocyte B et leurs équivalents néoplasiques présumés.

Système de classification des populations de lymphocytes B.

Au cours des ans, différents modèles de classification ont été élaborés afin de pouvoir discriminer les populations de lymphocytes B. Ces systèmes étaient basés sur une variété de critères de classification différents soit : la lignée d'origine, la localisation topographique au sein des zones B-dépendantes des organes lymphoïdes secondaires, ou encore les propriétés fonctionnelles et le stade développemental de la cellule.

Le système B1-B2 : la notion de lignées développementales fonctionnellement distinctes.

Ce système de classification, d'abord élaboré chez les modèles expérimentaux (souris, rats), a par extension été appliqué chez l'humain (Sagaert et De Wolf-Peeters 2003, Youinou, *et al* 1999). Il a pour prémisse l'existence de deux lignées distinctes de lymphocytes B. La première, composée des cellules B1-a (ou $CD5^+$) et B1-b ($CD5^-$ mais CD5 $ARNm^+$), détectée tôt dans l'ontogénie, serait impliquée dans la réponse immunitaire humorale naturelle ou TI. Cette réponse est caractérisée par la sécrétion d'anticorps de faible affinité et polyréactifs (surtout d'isotype IgM, mais aussi IgA et IgG_3) dirigés principalement contre des antigènes bactériens de type lipopolysaccharides (antigènes T-indépendants de type 1 ou TI-1) ou polysaccharides (antigènes T-indépendants de type 2, ou TI-2, caractéristiques des bactéries encapsulées) et contre des auto-antigènes. Typiquement, ces cellules ont un phénotype de surface $IgM^{++}IgD^+$. Chez la souris, les cellules B1 sont majoritairement retrouvée dans les cavités péritonéale et pleurale tandis que chez l'homme adulte, on les retrouve dans la zone du manteau folliculaire des organes lymphoides secondaires et, en faible proportion (~15% des lymphocytes B totaux), dans le sang périphérique (Klein, *et al* 1998b). Initialement

générée à partir de précurseurs situés dans le foie fœtal et la moelle osseuse, la lignée B1 aurait la propriété de s'auto-renouveler grâce à la division des cellules B1 matures pré-existantes. Prédominante chez le fœtus et le nouveau-né, la lignée B1 cèderait progressivement la place à la lignée B2, $CD5^-$ ou conventionnelle, impliquée dans la réponse humorale adaptative ou T-dépendante. Contrairement à la précédente, la lignée B2 est générée tout au long de l'existence de l'individu à partir de précurseurs de la moelle osseuse et suit le sentier classique de diversification somatique du répertoire primaire des Ig assuré par la réaction des GC.

La pertinence de ce système de classification chez l'humain est toutefois remise en question. En effet, des donnés expérimentales récentes indiquent que, chez ce dernier, la réponse T-indépendante, spécifiquement celle dirigée contre des antigènes TI-2, serait assurée non par les B $CD5^+$, mais plutôt par une sous-population de lymphocytes B de la MZ (Dono, *et al* 2003, Kruetzmann, *et al* 2003). Similairement aux cellules B1 de souris, ces cellules B de la MZ démontrent un phénotype de surface $IgM^{++}IgD^+$. Par contraste toutefois, elles utilisent un répertoire d'anticorps parfois hypermutés. Il est spéculé que ces mutations somatiques découleraient non pas d'une diversification T-dépendante conventionnelle passant par les GC, mais plutôt d'un processus de diversification pré-immun et indépendant des GC (Fagarasan et Honjo 2000, Weller, *et al* 2004, Weller, *et al* 2001). Argument supplémentaire : les cellules $CD5^+$ du manteau folliculaire chez l'humain expriment le marqueur CD23, ce qui est le cas ni des cellules B1 chez la souris, ni des lymphocytes B $IgM^{++}IgD^+$ de la MZ (Dono, *et al* 2003). Conséquemment, chez l'homme, les cellules $IgM^{++}IgD^+CD23^-$ de la MZ représenteraient l'équivalent approprié de la lignée B1 tandis l'expression de CD5 ne définirait à ce stade aucune population cellulaire fonctionnellement distincte (Chiorazzi et Ferrarini 2003).

Le système Bm1-Bm5 : quand la localisation définit la fonction.

Ce système de classification, élaboré par Pascual et collaborateurs (Pascual, *et al* 1994), récapitule en grande partie le développement T-dépendant du lymphocyte B mature à la suite de la reconnaissance antigénique et du passage par les GC tel que présenté

précédemment (voir également figure 7). En effet, ces auteurs ont su tirer profit du fait que chacune des phases de développement reliées au processus de maturation d'affinité (B naïf, centroblaste, centrocyte, B mémoire) se définit par une ségrégation du lymphocyte B dans des compartiments distincts du follicule secondaire (FM, DZ, LZ, MZ) et correspond à une apparence morphologique caractéristique (soit petit lymphocyte ou lymphoblaste) assortie d'un profil d'expression distinctif des marqueurs de surface. Notamment, selon ce système, l'expression de l'IgD membranaire est associée au stade du B naïf. Ces caractéristiques rendent alors possible la discrimination physique des populations de lymphocytes B impliquées dans la récation de GC, que ce soit par micro-dissection ou tri cytofluorométrique. La justesse de cette approche a été confirmée par le biais d'études consacrées à l'analyse individuelle des gènes codant les portions V_H des Ig chez les cellules correspondant à chacun des stades ainsi définis. Ainsi du statut non hypermuté chez le B naïf ou stade Bm1-2, on assiste progressivement à l'accumulation de mutations somatiques dans les régions variables des Ig au cours des stades subséquents, accompagnée par une restriction de la diversité clonale à partir du stade de centrocyte ou Bm4, ce qui reflète le processus de sélection par l'antigène des clones dotés d'une Ig membranaire de haute affinité (Kuppers, *et al* 1993, Pascual, *et al* 1994). Bien que cette approche se soit révélée très utile pour l'analyse des populations cellulaires isolées de spécimens cliniques provenant d'organes lymphoïdes secondaires tels les amygdales ou la rate, elle s'est heurtée à des limitations considérables en ce qui concerne l'analyse de la population hétérogène des lymphocytes B du sang périphérique. En effet, contrairement aux lymphocytes B folliculaires, la population sanguine comporte un pourcentage considérable de lymphocytes B exprimant une IgD membranaire et cependant dotés de gènes V_H hypermutés (Klein, *et al* 1998b), ce qui rend caduque l'expression différentielle de ce marqueur comme critère de discrimination des lymphocytes B mémoires et naïfs. De plus, la compartimentation morphologique ne peut par définition s'appliquer au réservoir sanguin. Par conséquent, il n'était donc pas possible d'appliquer la classification Bm1-5 aux lymphocytes B du sang périphérique sur la seule base de l'expression différentielle des marqueurs alors disponibles.

La découverte des marqueurs du lymphocyte B mémoire CD27, CD148 et l'atteinte d'un consensus.

Ces difficultés ont été résolues avec l'identification de marqueurs spécifiques aux lymphocytes B mémoires soit CD27 (Agematsu, *et al* 2000, Klein, *et al* 1998b) et, plus tard, CD148 (Tangye, *et al* 1998). La découverte du premier marqueur découla des observations faite chez les patients atteints de la forme d'HIGM liée au chromosome X (HIGM1), incapables de former des GC du fait de mutations dans CD40L et par extension dépourvus de lymphocytes B mémoires, chez lesquels on constata que l'expression de CD27 par les lymphocytes B du sang périphérique était nulle (Agematsu, *et al* 1998b). L'analyse moléculaire des régions V_H après tri des lymphocytes B $CD27^+$ et $CD27^-$ du sang périphérique permit de confirmer que l'expression du marqueur était restreinte aux seules cellules ayant subi la diversification somatique du répertoire (Klein, *et al* 1998b). CD148 fut ensuite identifié sur la base de critères similaires, mais cette fois chez les cellules spléniques. Par conséquent, quelle que soit la cellule isolée, et sur la base de l'expression différentielle des marqueurs phénotypiques et la configuration des gènes des Ig, il est désormais possible d'établir précisement son stade de développement et les événements moléculaires qui ont jalonné sa différenciation.

Comme mentionné précédemment, ces avancées théoriques sont largement redevables aux progrès considérables des techniques d'analyse de la cellule au cours de dernières années (séquençage complet du répertoire des V_H [ADN germinal] chez l'humain (Cook et Tomlinson 1995, Suzuki, *et al* 1995), l'analyse moléculaire détaillée des V_H à l'échelle de la cellule individuelle [*single-cell PCR*] (Brezinschek, *et al* 1995), et amélioration technologique des méthodes de tri des sous-populations cellulaires découlant de l'identification progressive de nouveaux marqueurs membranaires [*fluorescence-activated cell sorting* ou FACS, microdissection]). Un degré supplémentaire de précision a été atteint avec la mise au point de la technologie des micro-puces à ADN, laquelle permet l'analyse simultanée de milliers de gènes, générant ainsi un profil d'expression génétique exhaustif et distinctif pour chacun des stades de différenciation du lymphocyte B (King et Sinha 2001, Klein, *et al* 2003).

Classification des désordres immunoprolifératifs : les nouvelles normes.

Avec une meilleure connaissance des étapes de son développement, il a été possible d'établir des parallèles entre les phases de différenciation du lymphocyte B et le processus de lymphomagenèse. Ainsi, le principe de base sous-tendant les nouvelles méthodes de classification qui ajoutent l'étude de la configuration des gènes des Ig, l'évaluation du patron d'expression des molécules membranaires et, lorsque que possible, le profilage génétique aux critères classiques d'analyse, veut que la prolifération maligne résulte de l'expansion incontrôlée d'une cellule ayant échappé au processus normal d'élaboration de la réponse immunitaire. Conséquemment, les propriétés fonctionelles de la cellule transformée reflètent au moins partiellement celles de son précurseur normal, sur la base duquel des études comparatives peuvent être effectuées. De plus, en regard des propriétés du précurseur désigné, il devient possible d'évaluer les mécanismes dirigeant normalement la différenciation cellulaire qui auraient présumément contribué au processus de transformation maligne ("l'histoire naturelle" de la prolifération lymphoïde). Il est même devenu envisageable d'anticiper le comportement clinique de la maladie (Hummel et Stein 2000, Shaffer, *et al* 2002). Cela a particulièrement bien été illustré dans le cas de la CLL (voir : Chiorazzi et Ferrarini 2003).

En tenant compte des critères additionnels de classification énoncés ci-haut, les proliférations lymphoïdes clonales sont désormais réparties en trois grandes catégories (Hummel et Stein 2000) :

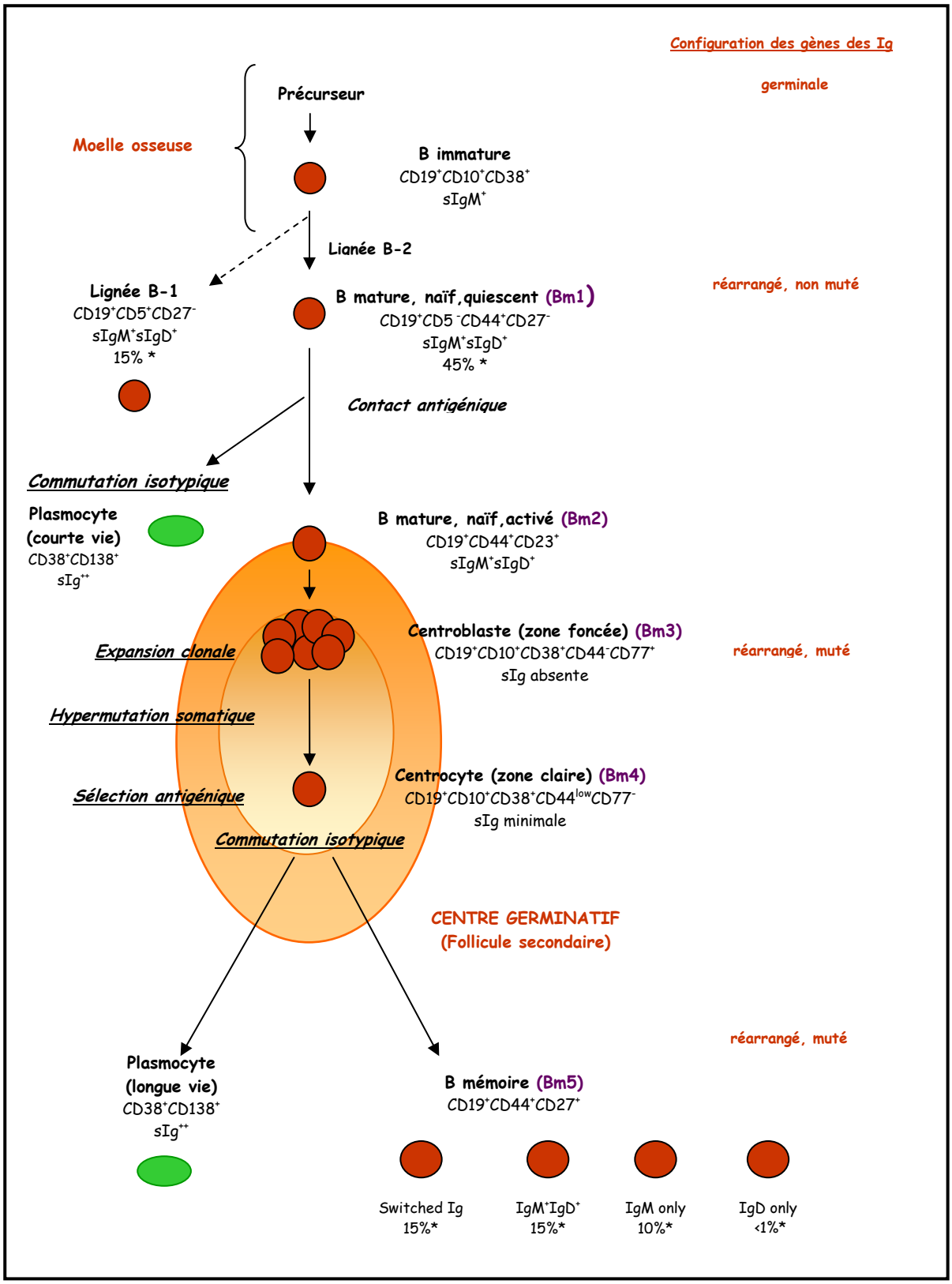
- Origine pré-GC , regroupant les pathologies caractérisées par des gènes Ig réarrangés mais non mutés (ex : certains CLL, lymphome du manteau [*mantle cell lymphoma* ou MCL])

- Origine GC , regroupant les pathologies caractérisées par des gènes Ig réarrangés, mutés et présentant une diversité intraclonale indicatrice d'un processus actif de SH (ex : FL, certains DLBCL)

- Origine post-GC , regroupant les pathologies caractérisées par des gènes Ig réarrangés, mutés et sans diversité intraclonale (ex : certains CLL, HCL, BL, les lymphomes de la zone marginale [*marginal zonel lymphoma* ou MZL]).

Figure 7 : La classification des sous populations B selon la lignée, l'immunophénotype et la configuration des gènes des Ig.

Les principaux mécanismes associés à la diversification des gènes des Ig sont également présentés.


Configuration des gènes des Ig
germinale
Précurseur
Moelle osseuse
B immature
CD19+CD10+CD38+
sIgM+
Lianée B-2
Lignée B-1
CD19+CD5+CD27-
sIgM+sIgD+
15% *
B mature, naïf,quiescent (Bm1)
CD19+CD5-CD44+CD27-
sIgM+sIgD+
45% *
réarrangé, non muté
Contact antigénique
Commutation isotypique
Plasmocyte
(courte vie)
CD38+CD138+
sIg++
B mature, naïf,activé (Bm2)
CD19+CD44+CD23+
sIgM+sIgD+
Centroblaste (zone foncée) (Bm3)
CD19+CD10+CD38+CD44-CD77+
sIg absente
réarrangé, muté
Expansion clonale
Hypermutation somatique
Centrocyte (zone claire) (Bm4)
CD19+CD10+CD38+CD44lowCD77-
sIg minimale
Sélection antigénique
Commutation isotypique
CENTRE GERMINATIF
(Follicule secondaire)
réarrangé, muté
Plasmocyte
(longue vie)
CD38+CD138+
sIg++
B mémoire (Bm5)
CD19+CD44+CD27+
Switched Ig
15%*
IgM+IgD+
15%*
IgM only
10%*
IgD only
<1%*

Les désordres lymphoprolifératifs : un contrecoup de la diversification du répertoire immunitaire.

En raison de leur nature hautement mutagène, les étapes de diversification des Ig constituent un risque hypothétique considérable de générer des cellules mutantes pouvant subséquemment évoluer vers une prolifération néoplasique. C'est pourquoi l'organisme a développé des mécanismes de régulation étroits servant à cibler spécifiquement la diversification du patrimoine génétique cellulaire. Parmi ces mécanismes, on note l'expression restreinte des facteurs enzymatiques impliqués dans le remaniement de l'information génomique. Ainsi, et quoique cette hypothèse soit maintenant remise en question, on a longtemps admis que l'expression de la TdT et des RAG était limitée aux seuls précurseurs lymphoïdes. A cela vient s'ajouter la présence de séquences spécifiques, soit les séquences signal de recombinaison (*recombination signal sequence*, ou RSS) et les séquences S (*switch sequences*), situées en amont de chacun des gènes C_H des Ig, qui sont ciblées spécifiquement par la machinerie de recombinaison VDJ et de la CS respectivement.

Cependant, un ensemble de données expérimentales récentes tend à prouver que ces mécanismes de protection du génome cellulaire ont des failles qui interviendraient principalement lors de la réaction du GC. Premièrement, une réexpression des enzymes RAG1, RAG2, et TdT a été détectée dans les centrocytes et, de façon inégale, dans les centroblastes des GC (Giachino, *et al* 1998, Girschick, *et al* 2001). Au plan physiologique, cette ré-induction serait associée au phénomène de révision du récepteur prenant place à ce stade précis de la différenciation des lymphocytes B, supposément suite à la pression exercée par la sélection antigénique sur les lymphocytes d'affinité faible ou nulle (Meffre, *et al* 1998, Nemazee et Weigert 2000, Wilson, *et al* 2000). L'expression des RAG, combinée au fort taux de prolifération prenant place dans les GC prédisposerait cependant les cellules à l'acquisition d'anomalies génétiques, notamment à des translocations erronées. En effet, en addition aux séquences RSS régulatrices retrouvées dans les locus des gènes des Ig, il pourrait y avoir jusqu'à $10x10^6$ quasi-séquences RSS dissimulées à travers le génome humain (Lewis, *et al* 1997). Les RAG seraient de plus capables de reconnaître des quasi-séquences χ (activateur de

recombinaison procaryote), présentes, elles aussi, dans le génome humain (Davila, *et al* 2001, Willis et Dyer 2000). À cela vient finalement s'ajouter une activité transposase latente, démontrée *in vitro*, et représentant probablement un reliquat de l'évolution (Hiom, *et al* 1998). Bien que la contribution exacte de ces mécanismes à la génération de translocations demeure à être évaluée, elle n'est pas pour autant négligeable (voir discussion par Davila, *et al* 2001ainsi que Shaffer, *et al* 2002).

Les translocations erronées ne seraient pas les seules aberrations génétiques possiblement générées au sein des GC. Des mutations au sein de proto-oncogènes, plus rarement des additions ou délétions, résulteraient de l'activité de SH. Comme cela à été mentionné, les étapes du processus de SH sont encore largement spéculatives et, par extension, il en va de même des mécanismes qui en assurent la régulation. Toutefois, il semble que le ciblage de la machinerie enzymatique impliquée dépende de la présence d'éléments régulateurs agissant en *cis*, notamment les *enhancers* des gènes des Ig, et du niveau de transcription des gènes visés. Cependant, ces mécanismes ne sont apparemment pas exempts de failles puisque la présence de mutations somatiques a pu être détectée en dehors des locus des Ig chez des cellules isolées des GC d'individus sains, précisément dans les gènes des protéines régulatrices que sont Bcl6 (Pasqualucci, *et al* 1998) et CD95 (Muschen, *et al* 2000) fortement exprimées à cette étape du développement. Ces mutations somatiques étaient absentes chez les cellules B naïves des mêmes individus, mais présentes chez les cellules mémoires, appuyant l'idée selon laquelle elles résulteraient du processus de diversification génétique des Ig. De plus elles présentaient les mêmes caractéristiques que les mutations générées dans les régions V_H, à savoir : prédilection pour les substitutions uniques de nucléotides (en opposition aux délétions ou additions), concentration dans les premiers 2 kb situés en aval du site d'initiation de la transcription et, spécifiquement dans le cas de Bcl6, ciblage préférentiel de points chauds (motifs RGYW, avec R = A ou G, Y = C ou T, W= A ou T) (Storb, *et al* 2001). Leur fréquence était plus basse que celle observée dans les régions V_H (bcl6 = 5 x 10^{-4} / pb, CD95 = 2.5 x 10^{-4} /pb, comparativement à V_{H4} IgG = 12 x 10^{-2} / pb, Storb, *et al* 2001), mais considérablement plus élevée que le taux spontané de mutation du génome humain (~ 2.5 X10^{-8} / pb, Nachman et Crowell 2000). Les deux protéines susmentionnées jouent un rôle non négligeable dans la régulation des compartiments lymphoïdes B : CD95

induit l'apoptose cellulaire alors que Bcl6 réprime spécifiquement l'expression de certains gènes et contrôle de ce fait la transition vers le stade de plasmocyte. Il s'ensuit donc que des mutations au sein de ces deux proto-oncogènes peuvent avoir de sérieuses répercussions sur les risques d'émergence de prolifération lymphoïdes (voir revue par Shaffer, *et al* 2002).

Les lymphocytes B, qui ne constituent qu'une minorité des lymphocytes totaux en circulation, sont pourtant à l'origine de la majorité des prolifération lymphoïdes. Additionnellement, parmi les leucémies et NHL de type B, la plupart présentent un phénotype de type GC ou post-GC. En comparaison, les lymphocytes T, qui possèdent eux aussi un récepteur de surface spécifique à l'antigène, mais uniquement soumis processus de recombinaison des segments V, D, J et C (Révillard 2001), ne sont responsables que d'une minorité de NHL. Toutes ces observations appuient donc l'hypothèse faisant de la réaction des GC un intervenant majeur dans le processus à étapes multiples de lymphomagenèse.

Enfin, le cas, rapporté par Matolcsy et collaborateurs (Matolcsy, *et al* 1999), d'un FL présentant une hétérogénéité intra-clonale au niveau des régions V_H et donc supposément capable de diversification somatique, ayant ensuite évolué vers un DLBCL exprimant une séquence V_H unique, et indétectable parmi les cellules du lymphome initial, indique qu'en cas de prolifération préexistante, le GC pourrait additionnellement jouer un rôle dans la génération de variants dotés d'un phénotype plus agressif.

Un cas particulier : la lymphocytose polyclonale chronique des lymphocytes B ou LPCB.

Perspective historique.

Il y a maintenant vingt ans, une première publication par l'équipe de Gordon et collaborateurs (Gordon, *et al* 1982) faisait état d'un syndrome lymphoprolifératif inhabituel que les auteurs allaient baptiser la lymphocytose polyclonale chronique des lymphocytes B (LPCB). Initialement diagnostiqué chez trois patientes adultes fumeuses,

le syndrome se caractérisait par une hyperlymphocytose (>4x10^9 cellules/L) impliquant des lymphocytes B (~50% ou plus des lymphocytes sanguins totaux), plusieurs atypiques, de taille moyenne, dotés d'un cytoplasme dense et modérément basophile, et d'un noyau bilobé ou totalement binucléé (voir figure 8). Cette hyperlymphocytose s'accompagnait d'une élévation polyclonale du taux d'IgM sérique et d'une légère baisse de l'IgA et de l'IgG. À la différence de la majorité des syndromes lymphoprolifératifs de type B, ayant souvent une origine monoclonale et une évolution maligne, la LPCB se distinguait par sa nature polyclonale, attestée par l'expression concomitante des chaînes légères κ et λ au sein de la population de lymphocytes B, de même que par son extrême stabilité avec des suivis allant jusqu'à vingt ans. Peu de signes cliniques étaient associés au syndrome : on faisait surtout état chez ces patientes d'hyperplasies lymphoïdes modérées et occasionnelles (splénomégalies) et d'infections bénignes des voies aériennes supérieures, secondaires à l'utilisation du tabac. Les auteurs soulignaient cependant l'expression commune du HLA-DR7, fait marquant étant donné la fréquence peu élevée de ce phénotype (~20%) au sein de la population caucasienne (Linet, *et al* 1988) et suggéraient une prédisposition génétique. La morphologie particulière des lymphocytes B, quant à elle, évoquait l'implication possible d'un agent infectieux dans la pathogenèse du syndrome. Toutefois, aucun n'était mis en évidence suite à l'analyse par microscopie électronique, malgré une sérologie indiquant une infection antérieure par le virus EBV, le cytomégalovirus et l'herpès virus chez toutes les patientes. Selon l'avis des auteurs, la LPCB constituait une entité hématologique nouvelle, distincte des néoplasies B typiques, probablement indolente, mais dont l'étiologie restait à élucider.

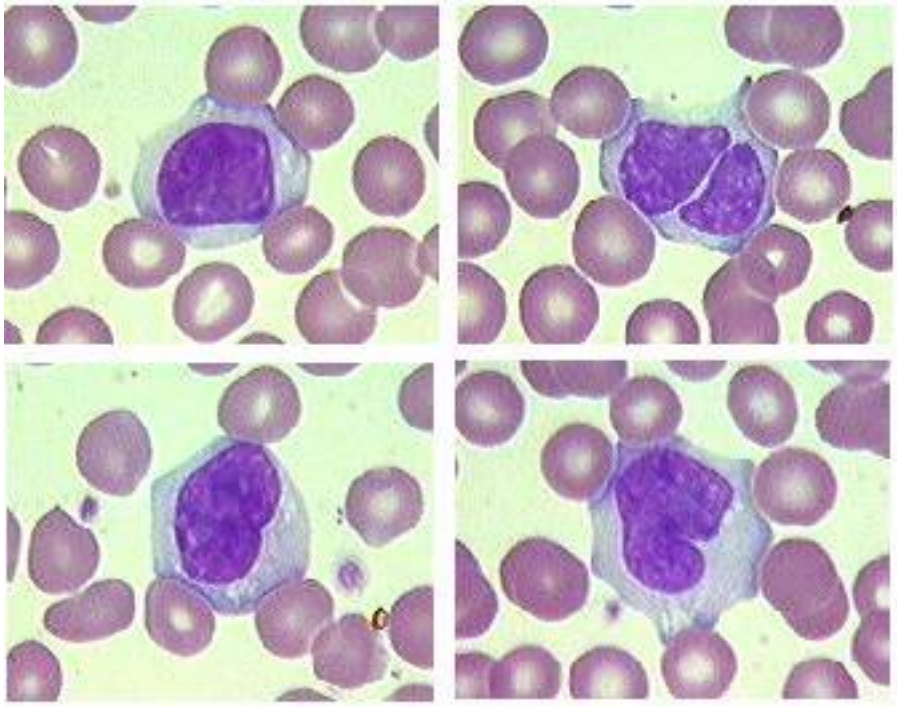


Figure 8 : Morphologie hétérogène des lymphocytes B atypiques dans la LPCB.
Les cellules peuvent présenter un noyau élargi (A), de forme soit peu à très clivée (B, C) ou complètement bi-nucléée (D).

En raison principalement du manque de manifestations cliniques, la LPCB fut initialement considérée comme un désordre hématologique rarissime. La présence de lymphocytes B binucléés, observation récurrente chez les patientes, devint vite la signature diagnostique de la LPCB. En effet ce type de morphologie n'est observé qu'exceptionnellement chez des individus normaux, suite à une irradiation à faible dose (Roy-Taranger, *et al* 1965), ou dans de rares cas de CLL (Samson, *et al* 2002). L'examen vigilant de frottis sanguins et la détection de ces lymphocytes B atypiques menèrent à l'identification de cas additionnels de LPCB (un total de 118 cas rapportés entre 1982 et 2004, voir tableau 3). Avec le recul des années, on peut déclarer que ce désordre est plus prévalent dans la population générale que ne le laissait croire les estimations initiales : ainsi la cohorte de patientes suivies par notre équipe dans la seule région de Québec comporte à présent dix-sept personnes. De plus, comme le démontre une analyse rapide des cas rapportés dans la littérature (voir tableau 3), le rythme des diagnostics de LPCB s'est accéléré grâce à la caractérisation toujours plus raffinée du syndrome

(immunophénotype et cytogénétique, voir plus loin) et sa popularisation progressive auprès de la communauté médicale. Ces nouveaux cas de LPCB ont permis de confirmer la prédominance du sexe féminin (104/118), de l'allèle HLA-DR7 (81/118), d'une sérologie EBV positive (52/82 cas testés) et de l'usage de la cigarette (113/118) chez les patients atteints de LPCB, les désignant comme facteurs de prédisposition potentiels. En contrepartie, le syndrome a aussi été diagnostiqué chez quelques hommes (13/118) et un nouveau-né (Gomez, *et al* 2000), de même que chez des individus non fumeurs (4/118) ou dépourvus de l'allèle HLA-DR7 (18/118). La contribution exacte des facteurs de risque susmentionnés à la pathogenèse de la LPCB demeure à ce jour encore largement spéculative, bien qu'ils aient tous été les objets de diverses investigations et sources de nombreuses hypothèses.

Immunophénotype et génotype dans la LPCB.

Le principal défi que chercheurs et cliniciens ont tenté de relever au cours des vingt dernières années a été de découvrir l'identité de la population B en expansion chez les patients atteints de LPBC : est ce que le désordre résultait de la dérégulation globale de l'homéostasie des lymphocytes B ou seulement de celle d'une sous population B particulière? Les premières études qui s'attaquèrent à la question utilisaient l'approche diagnostique traditionnellement utilisée pour la caractérisation des prolifération malignes : étude morphologique, immunophénotypage et analyse cytogénétique. L'expression invariable de marqueurs spécifiques à la lignée lymphoïde B comme CD19, CD20, CD24 et FMC7, a permis de confirmer l'origine présumée de la lymphocytose. L'expression de CD5 (marqueur des cellules B1), de CD23 (marqueur d'activation des lymphocytes B naïfs) et de CD38 (marqueur des B immatures et des GC), tous utilisés dans le diagnostic différentiel de la CLL (Chiorazzi et Ferrarini 2003), était négative. Il en allait de même pour l'expression de CD10 (marqueur des B des GC). Le marqueur CD103, surexprimé dans les cas de HCL et son éventuelle variante polylconale (le désordre lymphoprolifératif polyclonal avec tricoleucoytes [*hairy B cell lymphoproliferative disorder*, ou HBLD] (Machii, *et al* 1997), n'étaient pas détectés. Un second marqueur spécifique de ces désordres, CD11c, était, quant à lui, détecté de manière variable. On notait une forte expression d'IgM et d'IgD membranaires chez la

population lymphocytaire B ce qui, selon le système de classification Bm1-Bm5 (Pascual, *et al* 1994), correspondait à un phénotype Bm1 ou naïf. Par contre, les caractéristiques morphologiques concordaient avec une activation cellulaire : un pourcentage important de cellules (jusqu'à 50% selon Gordon et collaborateurs) présentaient une zone cytoplasmique dense et basophile, un noyau élargi, crénelé et parfois bilobé ou complètement binucléé (voir figure 8). L'expression des marqueurs d'activation CD25 (récepteur α de l'IL2) et de CD21 (récepteur du complément et du virus EBV) était également rapportée chez certains patients. Enfin, l'accumulation d'immunoglobulines au niveau du cytoplasme, observée chez quelques patientes (Feugier, *et al* 2004, Gordon, *et al* 1982, Ide, *et al* 2002), suggérait un phénotype plasmoctytaire précoce. Le manque d'uniformité de toutes ces observations découlait du principal obstacle à la méthode diagnostique utilisée, à savoir la nature polyclonale du syndrome et l'implication d'une population hétérogène de lymphocytes B.

Étonnamment, en dépit de cette polyclonalité, le diagnostic cytogénétique dévoilait tout de même la présence d'anomalies chromosomiques clonales restreintes aux lymphocytes B dans la LPCB. D'abord constaté chez un cas isolé en 1989 (Perreault, *et al* 1989), un isochromosome +i(3q), consistant en une duplication partielle du chromosome 3, était rapporté comme étant une observation récurrente chez une série de 25 patients suivies par Mossafa et collaborateurs (Mossafa, *et al* 1999, Mossafa, *et al* 1996, Troussard, *et al* 1997a) et, simultanément, chez trois patients suivis par l'équipe de Callet-Bauchu et collaborateurs (Callet-Bauchu, *et al* 1999, Callet-Bauchu, *et al* 1997). Chez certains patients, cet isochromosome était par ailleurs accompagné d'un phénomène de condensation précoce des chromosomes (PCC). Ces résultats furent par la suite confirmés par d'autres équipes chez plusieurs autres patients (voir tableau 3, colonne 8). Peu après, notre propre équipe mettait à jour la présence de réarrangements entre le locus de la chaîne lourde des Ig et le proto-oncogène *bcl2* (t(14;18) ou *bcl2/Ig*) chez onze patientes de la cohorte québécoise. Ces réarrangements, à la différence de ceux parfois détectés chez les individus normaux (particulièrement les fumeurs et les personnes âgées), étaient multiples et impliquaient à la fois le MBR (*major breakpoint region*) et le mcr (*minor cluster region*), sauf chez une des patientes (Delage, *et al* 1997, Delage, *et al*

1998). De plus, à une valeur de $1/10^2$-$1/10^3$ cellules positives, leur fréquence était environ mille fois plus élevée que celle détectable chez des individus sains. Bien qu'une surexpression parallèle de BCL2 ait été constatée lors de cette étude, des publications subséquentes, tout en corroborant la présence de ces réarrangements multiples, ne purent mettre en évidence une telle hausse (Himmelmann, *et al* 2001a, Lancry, *et al* 2001). Fait notable, ces anomalies chromosomiques étaient ici encore réparties de façon hétérogène au sein de la population B et indépendamment de la morphologie nucléaire. Par conséquent, la lymphocytose ne pouvait être circonscrite à une population morphologiquement ou génotypiquement distincte.

Facteurs de prédisposition putatifs dans la LPCB.

Facteur environnemental : le tabac.

La très haute prédominance de fumeurs parmi les patients atteints de LPCB (114/118 cas, voir tableau 3, colonne 2) éveilla tôt des soupçons quant à une implication possible du tabac dans l'étiologie de ce syndrome. Cette hypothèse se trouva confortée par l'observation d'une patiente chez laquelle l'arrêt du tabac coïncida avec une normalisation de la lymphocytose, puis, à la reprise de la cigarette, avec une nouvelle augmentation du niveau de lymphocytes sanguins (Carstairs, *et al* 1985). Avec l'identification de cas supplémentaires de LPCB, une normalisation de la lymphocytose et du taux d'IgM sériques a similairement été identifiée chez d'autres patients à l'arrêt du tabac (Bain, *et al* 1998, Rodriguez, *et al* 1996, Tonelli, *et al* 2000). Conséquemment, le tabac fut désigné un facteur de prédisposition pour ce syndrome. Toutefois, l'arrêt du tabac n'a pas toujours coïncidé avec une réduction du nombre de lymphocytes B et les anomalies génétiques spécifiques de la LPCB semblaient persister (nos propres observations et Mossafa, *et al* 1999). Les B binucléés atypiques quant à eux demeuraient détectables chez les ex-fumeurs, ce, même en absence d'une lymphocytose absolue (Bain, *et al* 1998, Himmelmann, *et al* 2001b, Rodriguez, *et al* 1996). Cet état de fait est remarquable puisqu'il indique que le syndrome peut fort bien exister chez des individus sans pour autant être détectable, d'autant plus que le diagnostic initial découle dans la majorité des cas d'une numération lymphocytaire élevée. En contraste, le dépistage

contrôle de fumeurs n'a pu mettre en évidence la présence de B binucléés caractéristiques (Troussard et Flandrin 1996) ou de réarrangements *bcl2/Ig* multiples (Delage, *et al* 2001). Additionnellement, l'usage intensif du tabac chez des individus en bonne santé est associé avec une réduction des niveaux sériques d'IgM (Mili, *et al* 1991, Moszczynski, *et al* 2001), faisant de la cigarette une suspecte peu probable pour expliquer l'augmentation observée chez les patients atteints de LPCB (voir discussion par (Vignes, *et al* 2000)). Enfin, la LPCB ayant également été diagnostiquée chez des non fumeurs, il semblerait donc que, bien qu'il puisse exacerber l'ampleur de la lymphocytose, le tabac ne constitue pas pour autant un facteur déterminant dans l'étiologie du syndrome.

Influence génétique : une apparente prédisposition familiale.

La présence du HLA-DR7, présent de façon homozygote ou hétérozygote chez la plupart des patients diagnostiqués (99/118 cas, voir tableau 3, colonne 5), était intrigante étant donnée sa faible prédominance dans la population caucasienne générale (~20%), et suggérait une prédisposition génétique. Notablement, l'allèle HLA-DR7 a été associé à l'infection chronique par le virus de l'hépatite B (*Hepatitis B virus,* ou HBV), consécutivement à une possible incapacité des T helper à reconnaître le peptide viral présenté par cet allèle particulier du CMH et stimuler la réponse humorale des lymphocytes B. L'hypothèse a donc été émise par certains auteurs que ce phénomène s'appliquerait pareillement à la LPCB, mais dans le cadre d'une infection chronique par le virus EBV. La découverte de B binucléés chez la parenté d'un des patients et, de façon plus frappante, le diagnostique du syndrome chez deux jumelles identiques, a renforcé la notion d'une composante génétique. Cette notion s'est par la suite trouvée confirmée par les données expérimentales recueillies par deux équipes de recherche. D'une part on peut citer l'étude de Delage et collaborateurs (Delage, *et al* 2001), qui a su tirer partie de la famille élargie d'une patiente comportant neuf parents au premier degré, quatorze au second degré, couvrant ainsi trois générations. Cette étude a permis de démontrer que certains des critères associés à la LPCB telles l'élévation du taux d'IgM et du nombre de B circulants mais, plus particulièrement, la présence de réarrangements multiples *bcl2/Ig*, étaient des observations fréquentes chez les membres de la famille. Cette étude a ainsi mené à la détection de deux cas supplémentaires parmi les sœurs de la patiente. Fait

notable, l'allèle HLA-DR7 n'était cependant pas présent chez cette famille. La seconde étude, présentée par Himmelmann et collaborateurs (Himmelmann, *et al* 2001b), fut menée auprès des quatre membres d'une même famille présentant tous les facteurs de prédisposition potentiels associés à la LPCB à savoir : sérologie EBV positive, expression de l'allèle HLA-DR7 et utilisation du tabac. Ici encore, l'étude mena à l'identification d'un cas de LPCB supplémentaire, en la personne d'un des frères de la patiente index. Toutefois, l'observation de deux autres membres de la famille demeurant malgré tout non affectés par la LPCB soulève la perspective de facteurs de prédisposition différents ou supplémentaires œuvrant dans l'étiologie du syndrome. Dans les deux études précitées, les membres de la famille ayant reçu un diagnostic de LPCB ne présentaient toutefois qu'une lymphocytose légère ou même un décompte normal de lymphocytes totaux. Ce constat illustre encore une fois le fait que le nombre officiel de cas de LPCB rapportés dans la littérature, d'ailleurs souvent diagnostiqués à la suite d'une élévation de la numération lymphocytaire, ne constitue qu'une modeste approximation de la fréquence réelle du syndrome dans la population générale.

Facteur infectieux : le virus Epstein-Barr.

La morphologie particulière des lymphocytes B observés dans la LPCB suggérant une infection virale, les premiers efforts de recherche ont été consacrés plus particulièrement à l'identification des facteurs infectieux possiblement impliqués dans la pathogenèse du syndrome. Rapidement, les soupçons se sont tournés en direction du virus EBV. En effet, si aucune particule virale n'avait pu être détectée par microscopie électronique, il n'en demeurait pas moins que la majorité des patients atteints de LPCB présentaient une sérologie EBV positive, indicatrice d'une infection antérieure par ce virus (voir tableau 3, colonne 7). Cependant, il faut souligner, comme l'ont fait remarquer Wyatt et Coyle (Wyatt et Coyle 1991), qu'en absence de contrôles appropriés, une mesure individuelle d'un titre antiviral ne certifiait pas pour autant son implication dans l'étiologie du syndrome. De plus, cette observation peut attester simplement du fait que 90% de la population adulte est porteuse du virus sous forme latente et asymptomatique. En contrepartie, la détection effective du génome viral chez les patients atteints de LPCB, que ce soit par hybridation *in situ* ou amplification PCR, a été irrégulière (voir tableau 3,

colonne 7). Nonobstant ces considérations, EBV constituait le candidat le plus plausible étant donné sa capacité à transformer les lymphocytes B et subséquemment induire leur expansion polyclonale *in vitro* et *in vivo*, ainsi que son association étiologique fréquente avec des proliférations lymphoïdes, notamment chez les individus immunodéprimés (post-transplantation, SIDA). L'inférence d'une chaîne causale dans la LPCB était par ailleurs tentante, considérant l'association apparente entre le tabac, inducteur putatif de la phase lytique d'infection par EBV, et la prédominance de l'haplotype HLA-DR7, possiblement impliqué dans un phénomène d'évasion de la reconnaissance immunitaire. Cette hypothèse est cependant contestable car usage du tabac et haplotype HLA-DR7, bien que dominants, ne sont pas observés chez tous les patients atteints de LPCB (voir tableau 3). De plus, alors qu'aucun effet notable sur l'infection lytique par EBV n'a pu être mise en évidence suite à l'exposition aux constituants chimiques du tabac *in vitro*, l'évasion immune par ce même virus a été reconnue, mais en relation avec l'allèle HLA-A11 toutefois.

Une infection lytique chronique par EBV a cependant été concrètement observée chez une patiente, et une lignée lymphoblastoïde stable établie à partir de laquelle des études fonctionnelles ont été conduites (Mitterer *et al*, 1995, Larcher *et al*, 1997). La question demeure cependant quant à la pertinence de ces résultats concernant la LPCB puisque la lignée n'exprimait pas l'IgD membranaire, contrairement à la population en expansion chez les patients. Des résultats significatifs ont toutefois découlé de l'analyse moléculaire de la protéine virale LMP1 chez cette patiente. En effet, ces analyses ont mené à l'identification d'une délétion unique de 69 pb dans la région carboxy-terminale de la protéine. Chez les membres de la famille de la patiente index, une délétion récurrente de 30pb et des mutations ponctuelles étaient détectées, reflétant le polymorphisme naturel de la protéine au sein de la population générale, mais, remarquablement, la délétion de 69 pb n'était pas mise en évidence. De par sa localisation dans le domaine de la protéine LMP1 liée à l'activation du facteur de transcription NF-κB, cette délétion pouvait avoir des répercussions importantes sur la cascade de signalisation induite par cet homologue viral de la protéine cellulaire CD40, et oncoprotéine reconnue. En effet, il a été établi que LMP1 constitue un analogue de la

protéine CD40, capable d'activer constitutivement les lymphocytes B et soutenir leur prolifération. LMP1 ne nécessite pas d'interaction avec un ligand pour s'auto-aggréger et initier une cascade de signalisation dont les effets physiologiques sur le lymphocyte B recoupent partiellement ceux découlant de l'engagement de CD40 par son ligand (Gires, *et al* 1997). La queue cytoplasmique de LMP1 possède un domaine CTAR1 (*carboxy-terminal activating region*) qui comprend un motif concensus PXQXT permettant à la molécule de recruter TRAF1, TRAF2 et TRAF3 (Huen, *et al* 1995, Lam and Sugden 2003). Un site de fixation de JAK3 est aussi présent (Gires, *et al* 1999). Toutefois à la différence de CD40, LMP1 ne possède pas de motif de fixation pour TRAF6 qu'elle recruterait cependant indirectement (Lam et Sugden 2003). En fait, un domaine CTAR2 est plutôt impliqué dans l'interaction avec TRADD (*TNF-R adaptor death domain protein*) et est notamment responsable à 60-70% de l'activation de NFκB par l'intermédiaire de TRAF2, le pourcentage résiduel de l'activation du facteur de transcription étant assuré conjointement par CTAR1 (Eliopoulos et Rickinson 1998, Lam et Sugden 2003).

Selon la théorie avancée par les auteurs, cette forme variante de la protéine LMP1 chez une patiente atteinte de LPCB, pourrait possiblement, en modifiant la physiologie des lymphocytes B, être à l'origine de la LPCB. Selon notre expérience, l'amplification et le séquençage de la région carboxy-terminale chez neuf patientes suivies dans la cohorte de Québec a mené à l'identification de mutations ponctuelles indépendantes et de la délétion de 30 pb mise en évidence chez les parents de la patiente index. La délétion de 69 pb n'a toutefois jamais été détectée (Carle Ryckman, résultats non publiés). Ces résultats ne soutiennent donc pas l'hypothèse de l'implication d'une forme variante de EBV qui déclencherait la LPCB par le biais d'une interférence dans la cascade de signalisation induite par LMP1.

En conclusion, les études menées au cours des vingt dernières années concernant la LPCB, ont permis d'accumuler des preuves en faveur d'une origine génétique au syndrome. Cependant la nature précise de la population en expansion, les causes exactes de la prolifération de même que les facteurs ou la combinaison de facteurs impliqués dans la physiopathogenèse du syndrome demeurent pour l'instant largement spéculatifs.

Tableau 3 : La LPCB, une récapitulation sur vingt ans.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Auteurs	***Cas : sexe (âge)***	***Tabac***	***Symptômes et conditions associés***	***IgM/IgA/ IgG sériques***	***HLA-DR7***	***Cellules binucléées (%)***	***Sérologie EBV***	***Anomalies génétiques***	***Marqueurs de surface***
Gordon, *et al* 1982	3F (41-54)	3/3	AM (1/3) IR	+M/-A,G	3/3	+ (24-29)	+ (passée) 3/3	/	sIgM, sIgD
Carstairs, *et al* 1985 et Chan, *et al* 1990	4F (31-52)	4/4	IR	+M	4/4	+	/	réarrangement D_HJ_H anormal 2/2 t	/
Casassus, *et al* 1987	1F (26)	1/1	aucun	+M/-A,G	1/1	+ (3-8) **avec poches nucléaires**	/	Caryotype normal	/
Perreault, *et al* 1989	6F (28-47)	/	SM (3/6) AM (2/6) HL (1/6)	+M/- or =A,G	4/6	+ (1-30)	+ (passée) 5/6 t	+i(3q) 1/6	sIgM $CD19^+$
Lawlor, *et al* 1991	1F (43)	1/1	SM AM IR	+M/=A,G	1/1	/	+ (passée)	/	sIgM $CD19^+$, 20^+ $FMC7^+$
Tissot, *et al* 1991	1F (54)	1/1	SM IR	+M/=A,-G	1/1	3-5%	-	/	sIgM, sIgD $CD19^+$
Chow, *et al* 1992	1F (47)	1/1	aucun	/	1/1	+ (<1)	+ (passée) PCR+ HIS+	/	$CD20^+$
Delannoy, *et*	4F	4/4	SM (3/4)	+M/=A,G	2/3 t	+	-	Caryotype	/

al 1993	(27-44)					(<5)		normal	
Agrawal, *et al* 1994	1F	1/1	SM	+M/-A,G	1/1	+ (30) certaines trinucléées	+ (passée)	Anomalies caryotypiques isolées	$CD22^+$, 37^+ $FMC7^+$ $CD23^-$
Troussard, *et al* 1994	6F (33-46)	5/6	SM (3/6)	+M/- or =A,G	6/6	+ aussi chez des parents	+ (passée) 6/6 HIS-	/	$CD11b^+$, 19^+, 20^+, 21^+(2/3 t), 22^+, 24^+, 25^+(2/6) $FMC7^+$ (3/4 t) $CD10^-$,$11c^-$, 38^-, 23^-
Mitterer, *et al* 1995 et Larcher, *et al* 1997	1F (47)	1/1	Fatigue chronique **Mononucléose**	+M	1/1	+ (8-25)	+ (active) PCR+ délétion de 69 pb du gène LMP1	/	sIgM, sIgD $CD19^+$, 20^+, 21^+, 22^+, 25^+, 54^+ $FMC7^+$ $CD5^-$,10^-, 23^-
Rodriguez, *et al* 1996	1F (35)	1/1	aucun	+M/-A,G	1/1	+ (5)	+ (passée)	/	$CD19^+$, 20^+, 21^+, 22^+
Troussard et Flandrin 1996 et Mossafa, *et al* 1996 et Troussard, *et al* 1997al et Troussard, *et al* 1997b et Mossafa, *et al* 1999	22F, 3M* (31-55)	23/25	SM (7/25) HM (2/25) AM (3/25)	+M	19/20 t	+ (1.5-9)	- 17/22 t	CPC 11/22 t +i(3q) 17/22 t Les deux 9/22 Autres anomalies caryotypiques isolées	$CD19^+$ $FMC7^+$ (13/16 t) $CD23^-$
Delage, *et al* 1997 et Delage, *et al*	11F (38-64)	11/11	SM **Lymphome non**	+M	8/11	+	+ (passée) 7/7 t§	*bcl2/Ig* mutiple 10/11 *bcl2/Ig* simple	sIgM **et** sIgD (4/4t) $CD19^+$, 20^+

1998 et Roy, *et al* 1998 et Loembe, *et al* 2002			**Hodgkinien** (1/11)					1/11	(1/11 t), 27^+ (4/4t) $CD5^-$
Bain, *et al* 1998	1F (49)	1/1	aucun	+M/-A/=G	/	+	/	/	$CD19^+$, 22^+, 37^+
Callet-Bauchu, *et al* 1997 et Callet-Bauchu, *et al* 1999	1F, 3M (38-57)	4/4	SM 4/4 **Lymphome MALT** (1/3)	+M/-A	3/3 t	+ (2-9)	/	+i(3q) 4/4 +3 2/4 dup(3)(q26q29) 1/4 *bcl2/Ig* mutiple 2/3 t	$CD19^+$, 20^+ $CD5^-$
Carr, *et al* 1997	2F **jumelles** (33)	2/2	SM (2/2)	+M/-A/=G	2/2	+	+ (passée) 2/2	/	$CD19^+$ $FMC7^+$ $CD25^-$(1/1 t)
de Jaureguiberry, *et al* 1997	1M (46)	1/1	SM IR	+M/=A,G	1/1	+	+ (passée)	/	/
Granados, *et al* 1998	1F (24)	1/1	aucun	+M/-A,G	1/1	+ (2)	+ (passée) PCR-	*bcl2/Ig* mutiple	$CD19^+$, 20^+ $CD5^-$, 10^-, 25^- 103^-
Reeder et al, 1999	1M (43)	1/1	aucun	+M/=A,G	0/1	+	+ (passée)	Caryotype normal	$CD19^+$, **$CD5^+$**
Woessner, *et al* 1999 et Espinet, *et al* 2000	1F (44)	1/1	aucun	+M/- or =A,G	1/1	+ (10) **avec poches nucléaires**	+ (passée)	+i(3q) der(3) +3 *bcl2/Ig* mutiple	$CD19^+$, 25^+ $CD5^-$, 23^-,
Tonelli, *et al* 2000	1F (43)	1/1	aucun	+M/-A/=G	0/1	+ (5)	+ (passée) PCR+	+3 *bcl2/Ig* mutiple	sIgM, sIgD $CD19^+$
Vignes, *et al* 2000	3F, 1M (36-66)	4/4	SM (3/4) IR (2/4) HL (2/4)	+M/- or =A/=G	2/3 t	+ (1-10)	/	+22 1/3 t	/

Reimer, *et al* 2000	1M (35)	1/1	SM	+M/=A,G	1/1	+ (10)	+ (passée) HIS-	/	$CD19^+$ $CD5^-$, 10^-, 23^-
Gomez, *et al* 2000	1NN (3 months)	0/1	SM	+M/- or =A/=G	0/1	-	+ (passée) PCR-	Caryotype normal Aucun *bcl2/Ig*	$CD19^+$, 22^+, 23^+, 25^+. 38^+ HC2+, **$CD5^+$** $FMC7^+$ $CD2^-$,$11c^-$, 103^-
(Gil-Fernandez, *et al* 2001)	1F (37)	1/1	SM	+M	1/1	+	+ (passée)	*bcl2/Ig* mutiple	$CD19^+$, 20^+, 22^+, $45RA^+$, $79\beta^+$ $FMC7^+$
Himmelmann, *et al* 2001b et Himmelmann, *et al* 2001b	4F, 1M **incluant 1 frère et 1 soeur** (34-53)	2/2 t	SM (2/2 t)	+M	5/5	+	+ (passée) 5/5	*bcl2/Ig* mutiple 5/5	sIgD $CD25^+$, 27^+, 148^+ $CD5^-$,23^{low}, 10^-, 38^-
Delage, *et al* 2001	3F* **soeurs** (36-50)	3/3	/	+M/- or =A,G	0/3	+	/	*bcl2/Ig* mutiple 3/3	/
Lancry, *et al* 2001	7F, 1M * (25-50)	8/8	SM (3/8)	+M/- or =A/=G	5/5 t	+ (1-5)	+ (passée) 3/3 t	+i(3q) 3/5 t CPC 1/5 t *bcl2/Ig* mutiple 3/8 *bcl2/Ig* simple $5/8^{£}$	$CD19^+$, 20^+, 22^+
Ide, *et al* 2002	1F (29)	1/1	aucun	+M	0/1	+ (4.5)	+ (passée)	Caryotype normal	sIgM, sIgD $CD19^+$, 20^+, 21^+, 22^+ $CD23^{low}$ $FMC7^+$ $CD5^-$,10^-

Salcedo, *et al* 2002	3F (41-47)	3/3	/	/	3/3	+	/	+i(3q) 3/3 6q- 1/3	$CD19^+$, 21^+, 24^+, 25^+, 79^+, 95^+, $11c^+$, $CD5^-$,23^-
Granel, *et al* 2002	3F (42-43)	3/3	aucun	+M **IgM anti-cofacteur aPa**	0/3	+ (6-11)	/	Caryotype normal	$CD19^+$ $CD5^-$,23^-
Schonermarck, *et al* 2003	1F (54)	1/1	AM	+M/=A,G	/	+ (2)	+ (passée)	*bcl2/Ig*£ simple	$CD19^+$ $CD5^-$,10^-
Vincenot-Blouin, *et al* 2003	1F (31)	1/1	AM SM	+M	/	+	+ (passée)	CPC -X +8	$CD19^+$, 20^+ $CD5^-$,10^-
Feugier, *et al* 2004	7F, 1M (25-56)	8/8	SM (5/8)	+M	2/3 t	+ (1-25)	- PCR-HIS-	+3 1/7 t del 6 1/7 t bcl2/Ig mutiple 4/6 t *bcl2/Ig* simple 1/6 t£	sIgM (6/7 t), and sIgD (3/6) $CD19^+$, 22^+, 79^+ $CD11c^+$(3/6 t) $CD38^{low}$ (3/7 t) $FMC7^+$(6/7 t) $CD5^-$,10^-, 54^-, $62E^-$, $62P^-$

Abréviations: / aucune information, F (féminin), M (masculin), NN (nouveau-né), AM (adénomégalie), HM (hépatomégalie), SM (splénomégalie), IR (infections bénignes des voies respiratoires), HL (herpes labial) concentration des Ig (+: augmentée, -: baissée, =: normal), t: testé, aPa (*anti-phospholipid cofactor antibody*), HIS (hybridation *in situ*), pb (paires de bases), CPC (condensation prématurée des chromosomes).

* Certains des cas peuvent avoir déjà été présentés dans les articles précédents de la même équipe de recherche ou des mêmes auteurs.

§ Voir texte, la section "Facteurs infectieux: virus Epstein Barr" (page 41), pour description détaillée.

£ Analyse de la région MBR uniquement.

Hypothèses et objectifs du projet de recherche.

L'objectif global de ce projet de recherche est d'élucider la nature de la population en expansion dans la LPCB, plus précisément de caractériser le stade de différenciation des cellules impliquées dans l'augmentation du compartiment lymphoïde B et, le cas échéant, de retracer l'équivalent normal de ces cellules. Le stade de différenciation sera défini par les trois critères suivants : propriétés fonctionnelles des lymphocytes B circulants chez les patients, immunophénotype détaillé et, finalement, détail de la configuration des gènes codant les V_H des Ig. Toutes les études décrites dans la présente thèse seront conduites chez des patientes atteintes de LPCB suivies au Service d'hématologie et d'immunologie clinique de l'hôpital Saint-Sacrement (Québec). Afin d'augmenter la validité des expérimentations et des conclusions qui en seront tirées, les expériences seront effectuées simultanément chez plusieurs patientes et incluront toujours l'analyse parallèle d'au moins un contrôle normal (volontaire anonyme en bonne santé).

Étude fonctionnelle des lymphocytes B.

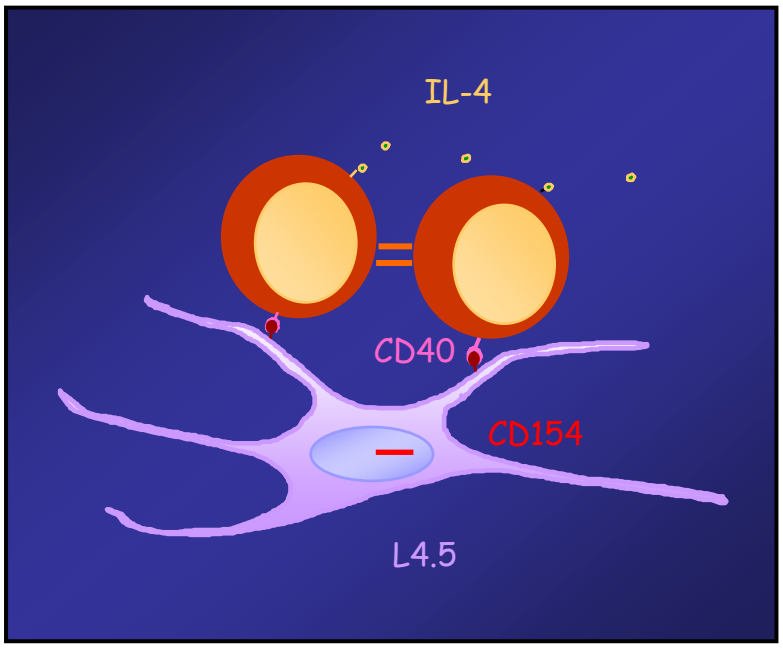
La lymphocytose, qui constitue un bris de l'homéostasie, présuppose une anomalie dans les mécanismes de régulation des différents compartiments lymphoïdes B. Ainsi, la LPCB pourrait être le reflet secondaire des effets d'un facteur externe comme, par exemple, un agent pathogène causant une réponse immune donc une activation cellulaire chronique ou encore la transformation cellulaire tel que cela a été proposé pour l'EBV. Alternativement, elle pourrait être d'origine intrinsèque et refléter une incapacité des lymphocytes B à répondre aux signaux physiologiques responsables de la transition entre les différents stades du développement chez ce type cellulaire. Comme l'ont suggéré certains auteurs (Gordon, *et al* 1982), la prédominance de l'IgM sérique chez les patients atteints de LPCB pourrait indiquer un défaut dans les mécanismes de diversification du répertoire des anticorps et/ou de la différenciation cellulaire. Afin de gagner plus d'information quant au stade de développement et aux capacités fonctionnelles des lymphocytes B dans la LPCB, nous avons décidé d'utiliser un système de culture basé sur l'activation cellulaire via CD40. Ce dernier permet de recréer *in vitro* les interactions

physiologiques prenant place *in vivo* entre les lymphocytes B et T lors de la réponse immunitaire dépendante de l'antigène dans les organes lymphoïdes secondaires. Il est basé sur le protocole expérimental originellement décrit par Banchereau et collaborateurs (Banchereau et Rousset 1991), avec quelques adaptations (Neron, *et al* 1996). Ce système présente l'avantage d'utiliser le ligand naturel de CD40, CD154, et non un anticorps qui résulterait possiblement dans l'induction d'effets distincts (Baccam et Bishop 1999). De plus, l'expression du ligand à la surface des fibroblastes permet à ce dernier d'adopter sa conformation physiologique, ce qui n'est pas toujours le cas des ligands CD154 solubles. Divers aspects liés au récepteur membranaire et à la réponse cellulaire à la suite de son engagement seront étudiés : séquence et expression du gène CD40, cascade de signalisation, prolifération cellulaire, sécrétion d'anticorps.

Dans un deuxième temps, l'évaluation du statut du gène AID (séquence et expression), permettra d'explorer plus en détail le processus de CS chez les lymphocytes B de patientes. Il sera ainsi possible de déterminer si une anomalie reliée à des mutations et/ou à un défaut d'expression du gène n'est pas associée à la hausse du compartiment $IgD^{+}IgM^{+}$ et la sécrétion préférentielle d'IgM sérique, en analogie avec le phénotype observé chez les patients souffrant du syndrome HIGM2.

Enfin, nous procéderons à l'analyse de la molécule CD95. Cette dernière joue en effet un rôle important dans le contrôle de la survie cellulaire au sein des GC, vraisemblablement en déclenchant l'apoptose chez les clones B de faible affinité pour l'antigène (Defrance, *et al* 2002, Mizuno, *et al* 2003). Ce rôle est attesté au plan clinique chez les individus atteints du syndrome lymphoprolifératif autoimmun (*autoimmune lymphoproliferative syndrome* ou ALPS) chez lesquels des mutations du récepteur sont associés à la présence de lymphocytoses touchant entre autres les cellules B (Straus, *et al* 1999). Conséquemment, en complément aux études des molécules CD40 et AID, nous procèderons à l'évaluation de la séquence et de l'expression de CD95.

A)



B)

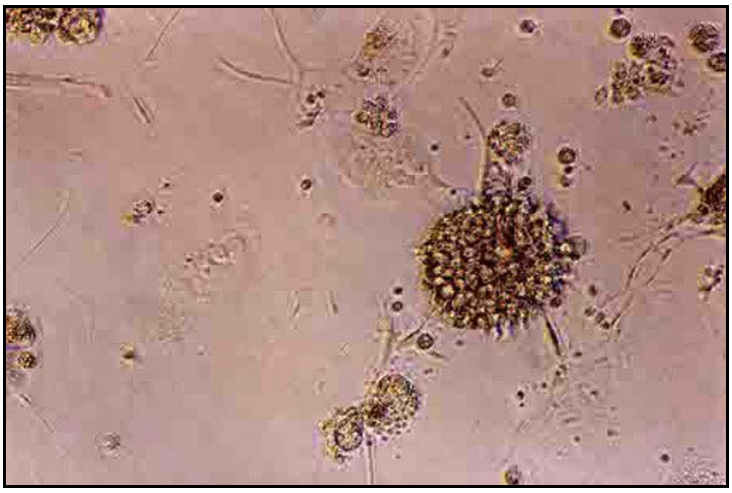


Figure 9 : Le sytème de culture *in vitro* dépendant de CD40.

Les lymphocytes B sont mis en culture en présence de fibroblastes de souris transfectés avec le gène du CD154 murin. La culture est effectuée en présence de cytokines (IL-4, IL-2 ou IL-10 selon les effets désirés) et de facteurs de croissance (insuline, transferrine). A) représentation schématique. B) Photo prise après quelques jours de culture en présence d'IL-4.

Profil immunophénotypique.

Le phénotype de surface des lymphocytes B dans la LPCB rend difficile la détermination de leur stade de développement, puisque la forte expression d'IgM et d'IgD détectée chez la majorité des patientes peut être associée aussi bien à une expansion de cellules naïves que mémoires, ou à un encore à un mélange des deux. Comme cela est mentionné au début de cette introduction, de nombreuses équipes ont entrepris l'étude de marqueurs de surface supplémentaires variés chez les patients atteints LPCB. Toutefois, ces analyses, effectuées par le biais de la cytofluorométrie (*fluorescence activated cell sorting* ou FACS), reposaient sur des protocoles variés qui n'étaient pas toujours indiqués avec précision (expression de chaque marqueur évaluée soit individuellement sur l'ensemble des PBMC, soit conjonction avec d'autres marqueurs, par exemple de façon à permettre la restriction de l'analyse aux lymphocytes B $CD19^{+}$). Il était donc difficile de tirer des conclusions définitives des diverses observations rapportées. L'avènement de CD27 en tant que marqueur des lymphocytes B mémoires a ouvert la voie à la discrimination des différentes sous-populations lymphocytaires B du sang périphérique (voir figure 10 et Klein, *et al* 1998b). Notre intention est donc de mettre à profit l'expression conjointe de ce marqueur et des différentes classe de récepteurs antigéniques (IgD, IgG, IgM) chez les lymphocytes B sanguins pour déterminer la composition détaillée du compartiment cellulaire B périphérique chez des patientes atteintes de LPCB. De plus, en utilisant le FACS en trois ou quatre couleurs, il nous sera possible d'évaluer l'expression simultanée de molécules clés (tel CD95), en établissant des fenêtres d'analyse autour de chacune des sous-populations définies. Afin de pouvoir tirer des conclusions significatives de ces analyses, les profils et niveaux d'expression pour chacune des molécules étudiées seront comparés avec ceux observés chez des témoins normaux. Les résultats ainsi générés pourront servir à préciser et affiner le diagnostic de la LPCB et mèneront éventuellement à l'identification précise de la ou des sous-populations B impliquées dans ce syndrome.

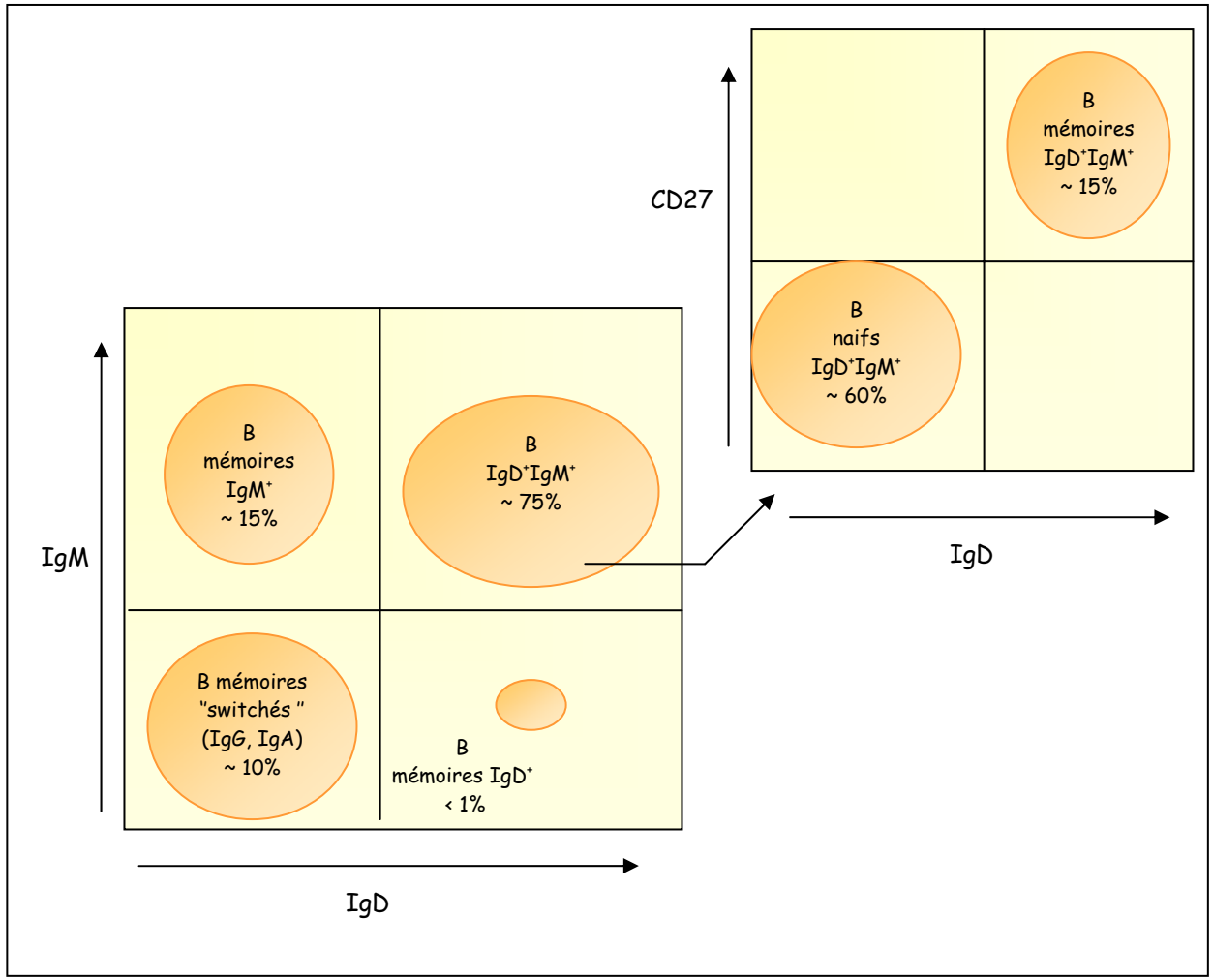


Figure 10 : Sous-populations lymphocytaires B du sang périphérique, détectées par analyse cytofluorométrique en trois couleurs (selon l'expression de IgM, IgD et CD27).

Analyse moléculaire des régions V_H chez les gènes des Ig.

En similitude avec la démarche entreprise pour l'étude des désordres lymphoïdes malins, nous entreprendrons l'analyse moléculaire détaillée des régions V_H chez les lymphocytes B sanguins isolés de patientes. Dans un premier temps, nous déterminerons le profil d'utilisation des différents gènes V_H. En effet, nous avons précédemment fait mention de la possibilité d'une stimulation chronique par un agent infectieux dans la LPCB. Cette hypothèse pourrait se concrétiser par une restriction dans l'utilisation des différentes familles de V_H comme cela a été observé à la suite de l'infection par certains pathogènes (Adderson, *et al* 1991, Wisnewski, *et al* 1996). Dans un deuxième temps, nous estimerons la fréquence des mutations somatiques dans les régions V_H afin de gagner un aperçu du stade de différenciation chez les lymphocytes B dans la LPCB. L'analyse de la répartition des mutations silencieuses et de remplacement selon la méthode de Lossos et collaborateurs (Lossos, *et al* 2000) nous permettra d'estimer la probabilité que ces cellules aient pris une part active à une réponse immunitaire T-dépendante. Enfin, en regard des résultats obtenus, il nous sera possible de déterminer les processus de diversification auxquels ces lymphocytes B auront été soumis au cours de leur développement.

En résumé, en combinant les résultats générés par ces différentes analyses peut être sera-t-il possible d'extrapoler et de gagner une meilleure compréhension des mécanismes en jeux dans la pathogenèse du syndrome ainsi que d'estimer les risques de progression subséquente vers un phénotype plus agressif. Ce dernier aspect doit particulièrement être pris en conséquence quand vient le temps d'étudier un désordre hématologique dont l'évolution paraît au premier abord plutôt indolente, telle la LPCB. La question se pose en effet de savoir si une transformation successive vers une forme plus agressive n'est pas la suite logique d'un dérèglement initial et prolongé de l'homéostasie des compartiments lymphoïdes. Le but ultime de ces analyses sera donc d'évaluer si la LPCB constitue la première phase dans un processus de lymphomagenèse à étapes multiples.

Chapitre I

Évaluation des propriétés fonctionnelles des lymphocytes B dans la lymphocytose polyclonale chronique B consécutivement à la stimulation *in vitro* du récepteur CD40.

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Lack of CD40-dependent B cell proliferation in B lymphocytes isolated from patients with persistent polyclonal B-cell lymphocytosis.

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Résumé

La lymphocytose polyclonale chronique B (LPCB) est un désordre hématologique apparemment bénin, diagnostiqué principalement chez des femmes fumeuses. Elle est caractérisée par une élévation polyclonale des lymphocytes B circulants, ainsi que du taux d'IgM sérique. Morphologiquement, cette population B se distingue par la présence de lymphocytes binucléés. Des anomalies génétiques (réarrangements multiples *bcl2*/Ig et d'une trisomie partielle +i(3q)) ont de plus été mises en évidence, indiquant que la lymphocytose pourrait constituer la première étape d'une évolution progressive vers une prolifération maligne. Les propriétés fonctionnelles de ces lymphocytes B n'ont toutefois pas encore fait l'objet d'investigations poussées. Le but de notre étude était d'aborder cet aspect grâce à l'utilisation *in vitro* du système de culture dépendant de CD40. Cette molécule de la famille des récepteurs du TNF, exprimée à la surface des lymphocytes B, joue un rôle clé dans l'activation et la différenciation de ces cellules *in vivo*. Nous avons observé que, contrairement aux lymphocytes B normaux, les cellules de patientes ne proliféraient pas en réponse à l'engagement du récepteur, indiquant un défaut possible dans la cascade d'activation de CD40 dans la LPCB. L'amplification PCR et le séquençage de CD40 chez des patientes, de même que l'évaluation du récepteur par cytofluorométrie, ont éliminé l'hypothèse d'un défaut de structure ou d'expression de la molécule. De plus, le patron de phosphorylation des résidus tyrosine, événement précoce dans la cascade de signalisation de CD40, et révélé par analyse Western, était similaire chez patientes et chez témoins normaux. Ces résultats nous amènent à la conclusion que l'absence de réponse constatée chez les patientes atteintes de LPCB est probablement causée par défaut situé en aval de la cascade de signalisation de CD40.

Abstract

Persistent B cell lymphocytosis (PPBL) is an haematological disorder diagnosed primarily in female smokers, which is characterised by a polyclonal increase in peripheral blood B lymphocytes and a moderate elevation of serum IgM. B lymphocyte-associated cellular abnormalities such as the occurrence of multi-lobed nuclei, increased *bcl2*/*Ig* gene rearrangements and the identification of an extra long-arm chromosome (i3)(q10) in the B cell population, indicate that PPBL could be part of multi-step process leading to the emergence of a malignant B lymphoproliferation. However, the resulting impact on cellular functional properties remains to be elucidated. Our goal was to address that aspect via the study of B-cell activity following stimulation through CD40, a key molecule of the TNF receptor superfamily involved in B lymphocytes development. We report that, in contrast to normal B cells, PPBL B lymphocytes were unable to respond to the proliferative signal delivered *in vitro* by CD40, indicating a defect in the CD40 activation pathway. PCR amplification and sequencing of the receptor as well as FACScan analysis of patient B lymphocytes dismissed the possibility of a defect in either CD40 structure or expression. Moreover, Western blot analysis of tyrosine phosphorylation, an early event in CD40-signaling cascade, was similar in patients and controls, leading to the conclusion that the defect affecting B lymphocytes in PPBL patients is likely to be located downstream of that signalling cascade.

Keywords: lymphocytosis, B lymphocytes, CD40, TNF-R, B cell development.

Running title: CD40-dependent B cell proliferation in PPBL.

Introduction

Persistent polyclonal B cell lymphocytosis is a non-malignant lymphoid disorder, which was first described by Gordon *et al.* in 1982. It is characterized by an increase in the number of peripheral blood B lymphocytes and an elevation of serum IgM. That latter is polyclonal as indicated by the normal expression of both κ and λ light chain isotypes (Gordon *et al*., 1982). This disease has been mostly associated with female smokers of a variable age range, although a few men have also been diagnosed recently (Callet-Bauchu *et al*., 1997; Troussard *et al*., 1997b). Patients predominantly express the HLA-DR7 phenotype although this allele usually occurs in only 26 % of the normal Caucasian population (Agrawal *et al*., 1994).

Over the years, efforts have been mainly directed towards the morphological and genotypic characterization of B lymphocytes in PPBL patients. Thus it is now well established that those cells are atypical, with an abundant cytoplasm, and, in variable proportion (1-9 %), binucleated (Troussard *et al*., 1997a). Despite the polyclonal nature of the B cell proliferation, the frequency of rearrangements between the *bcl-2* and the immunoglobulin (Ig) heavy chain genes are increased a hundred fold compared with normal B cells (Delage *et al*., 1997). In addition, patients present in a large proportion multiple *bcl-2/Ig* gene rearrangements. More recent reports also indicated that an extra chromosome 3 long arm and premature chromosome condensation (PCC) were present in some B cells of most individuals affected by the lymphocytosis (Troussard *et al*., 1997a). Those genetic aberrations were found to be restricted to the B lymphocytes, indicating the presence of a contrasting clonal cytogenetic population in PPBL patients (Callet-Bauchu *et al*., 1997). This confirms that some B cells in this disorder are distinct from their normal counterparts. However, no information is as yet available on the functional properties of B lymphocytes in PPBL. Their ability to respond to physiological signals, such as those responsible for the maintenance of the homeostasis in the immune system or the development of an immune response has not been evaluated.

Functional studies conducted on B lymphocytes often involve triggering of the CD40 receptor. CD40 is a member of tumour necrosis factor superfamily of receptors (TNF-R), which comprises among others TNF-R1, TNF-R2, CD95 (Fas), CD30 and CD27. As such, it is involved in the development of lymphocytes and is responsible for the regulation of the immune response. CD40 is principally implicated in the delivery of a survival signal (Kehry, 1996; Law *et al*., 1996). More exactly, interaction between CD40 and its ligand CD154, which is expressed on T lymphocytes, is implicated in the antigen-dependent maturation of B cells. *In vivo*, this interplay leads to the formation of germinal centres in lymphoid organs and the terminal differentiation of B lymphocytes to either plasma or memory cells. *In vitro*, effects induced by the ligation of CD40 include: tyrosine phosphorylation, NFκ-B activation, cellular proliferation, increased expression of cell surface markers and adhesion molecules, cytokines and antibodies secretion and Ig class-switch (Law *et al*., 1996). However, these effects might vary according to the nature of the cells tested. Hence, some B lymphoma cell lines and EBV-infected lymphoblastoid B cell lines (LCL) will undergo growth arrest or apoptosis instead of proliferation (Heath *et al*., 1993; Henriquez *et al*., 1999). Triggering of CD40 is usually achieved by using monoclonal anti-CD40 antibodies, albeit the resulting signal may be weak without fixation or cross-linking (Faris *et al*., 1994). To avoid these disagreements, we developed a culture system based on the utilisation of murine L fibroblasts transfected with the cDNA encoding the CD40 ligand (Néron *et al*., 1996). These CD154 expressing cells act in synergy with IL-4 to induce B lymphocytes activation and proliferation, thus allowing the evaluation of their functional properties.

The goal of this study was to further characterize B cells in PPBL by using the CD40-dependent culture system. Results obtained with B lymphocytes isolated from PPBL patients differed significantly from those seen with normal cells. Indeed, patient cells did not proliferate in the culture system, indicating a possible defect in CD40 signalling. However, tyrosine phosphorylation, which is an early event in CD40 signalling(Faris *et al*., 1994), took place in cells from both patients and normal controls. Therefore, the defect observed in PPBL patients is probably located downstream of the CD40 signalling cascade.

Methods

Patients.

This study was conducted on four female patients (CL3, CL4, CL7 and CL8) ranging from 40 to 63 years of age and referred by the St-Sacrement Hospital. Clinical diagnosis of PPBL was based on the following criteria: a) persistent $CD19^+$ $CD5^-$ B cell lymphocytosis (longer than 6 months) with a normal κ/λ ratio; b) polyclonal increase in serum IgM concentration; c) presence of binucleated lymphocytes. Blood was collected from all patients after informed consent was obtained, and further characterization of the B lymphocyte population was determined by flow cytofluorometry.

B lymphocyte isolation.

Peripheral blood samples were obtained from patients and healthy donors after obtaining informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Pharmacia Biotech, Baie d'Urfé, Qc) density gradient centrifugation, suspended in RPMI medium (Gibco-BRL, Burlington, ONT) supplemented with 20% heat-inactivated FBS (Hyclone, Logan, UT) and 5% DMSO, then frozen in liquid nitrogen.

CD40-dependent cell culture system.

After thawing, B lymphocytes were purified and cultured in the CD40-dependent system as described before (Néron *et al*., 1996). Briefly, T cells were depleted by adsorption to CD2-coated magnetic beads (Dynal, Lake Success, NY). Purified B cells thus obtained were seeded at $2.5X10^4$ cells/well in 96 wells plates containing $1X10^4$ irradiated L4.5 (murine fibroblasts expressing the CD40 ligand, CD154) cells/well. Culture were grown in 200 μl Iscove medium (Gibco-BRL) supplemented with 5% FBS, 5 μg/ml bovine insulin (Sigma), 5 μg/ml human transferrin (Sigma), 100 U/ml penicillin (GibcoBRL), 100 μg/ml streptomycin (GibcoBRL) and 100 U/ml IL-4 (Genzyme, Cambridge, MA). For long-term stimulation, half of the medium was replaced every 3 days and irradiated L4.5 were changed every 6 days.

Proliferation assays.

For short-term stimulation (9 days), B lymphocytes proliferation was monitored using the colorimetric conversion of MTT to formazan. 20 μl of MTT (2.5 mg/ml, Sigma) was added to 80 μl of cell culture in triplicate and incubated for 3 hours before addition of an extraction buffer (20% SDS, 50% DMF, 0.04N HCl). Optical densities (550 nm, reference 690 nm) were determined on an ELISA plate reader after an overnight incubation period.

For long-term stimulation (20 days), B cells were grown until culture density reached $2X10^5$ cells/well. B lymphocytes concentration was then brought back to its initial value by diluting the cells in culture medium and transferring them to a 96 wells plate containing freshly irradiated L4.5 cells. Meanwhile, proliferation was evaluated by direct cell count under light microscopy using a haemocytometer.

Sequence analysis of the CD40 gene.

Total RNA was extracted from patient PMBC and conversion of CD40 mRNA to cDNA was performed using murine Moloney leukemia virus reverse transcriptase (GibcoBRL) with oligo dT as a primer. 2 μg of cDNA was amplified by PCR using the 5'-GGGAATCACCATGGTTCGTCTGCC-3' sense primer and the 5'-GAAGATCTGCAGCCTCACTGTCTC-3' antisense primer and Taq DNA polymerase (GibcoBRL). The amplified products were then concentrated with the Quiagen DNA extraction kit (Mississauga, ONT) and subsequently used for cloning with the TOPO-cloning kit (InVitrogen, Carlsbald, Ca). Relevant clones were selected and used for sequencing with an automated DNA sequencer. Sequences were then analysed using the GCG database.

Tyrosine phosphorylation.

B lymphocytes isolated from patients and healthy donors were stimulated either for 45 min using an anti-CD40 soluble monoclonal antibody (G28.5, courtesy of W. Mourad, CHUL, Qc, 5 μg/ml) in the presence of 100U/ml IL-4 or for 2 hours and 72 hours in the CD40 system. Total cellular proteins were then extracted as described elsewhere (Lapointe *et al*., 1996). Briefly, cells were suspended in a lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM

NaCl, 10 % (w/v) glycerol, 1 % (w/v) Triton X-100, 2mM EDTA, 1mM Na_3VO_4, 1 mM PMSF, 2 μM pepstatin A) and incubated at 4°C for 20 min. Protein concentration was evaluated by Bradford and 40 μg were separated by SDS-PAGE on a 10 % polyacrylamide gel and then transferred to a PVDF membrane. Tyrosine–phosphorylated proteins were detected using the murine 4G10 monoclonal antibody (1μg/ml, Upstate Biotechnology Inc., Lake Placid, NY) then revealed with a biotin-conjugated secondary antibody (goat anti-murine IgG, A, M, diluted 1/1000, Sigma) and the streptavidin alkaline-phosphatase system (BRL, Gaithersburg, MD).

Results

Patients' profiles and phenotypic characterization of circulating B lymphocytes.

The patients in this study all displayed the clinical signs associated with PPBL: a lymphocytosis (n=1.5-3.0 X 10^9/l) resulting from an increase in B cell numbers (n= 5-15%), and accompanied by a marked increase in serum IgM (n=1.5-3.0 g/l) (Table 4). The κ/λ ratio indicated a polyclonal origin for the lymphoproliferation in all cases. Co-stimulatory molecules are often differentially expressed on B lymphocytes of a malignant origin. As an example, CD27 a cell surface molecule of the TNF family of receptors, and a general marker for memory B lymphocytes, has been shown to be up-regulated in B-cell lymphoproliferative disorders (Trentin *et al*., 1997). To confirm the non-malignant nature of PPBL we therefore decided to address this issue. Surprisingly however, cytofluorometric characterization of the B cell population regarding the expression of CD27 indicated that there was a major difference between normal controls and PPBL patients (see

Table 5). Accordingly with what has been published by others, the $CD19^+CD27^+$ B-cell subset was evaluated to an average of 11 % in our controls (Agematsu *et al*., 2000; Klein *et*

al., 1998). In PPBL patients, this value was estimated to an average of 90%, and therefore represented a significant increase (

Table 5). It will be interesting to determine whether this expansion is resulting from an up-regulated expression of CD27 by individual B lymphocytes, or whether it is the consequence of an augmentation in the number of memory B cells in PPBL patients.

Defective response to CD40 ligation.

Interactions between the B cell surface receptor CD40 and its ligand CD154 expressed on T lymphocytes plays a central role in the T cell-dependent maturation phase of B lymphocytes and the regulation of the immune response. Therefore, triggering of the CD40 molecule is now regularly used to study the functional properties in B lymphocytes. Using a CD40-dependent cell culture system, investigation of CD40-induced activation was conducted with B cells isolated from patients CL4, CL7 and CL8 as well as with B lymphocytes from 2 normal controls (40, 42). B cells were placed in 96-wells micro-plates containing irradiated L4.5 fibroblasts expressing the CD154 ligand in the presence of IL-4. Cellular proliferation was then monitored using either the colorimetric conversion of MTT to formazan (for 9 days stimulation periods) or direct cell count with a haemocytometer (for 21 days stimulation periods). Events resulting from CD40 ligation in B cells included the rapid formation of cell clumps due to the up-regulation of cellular adhesion molecules such as ICAM-1and LAF-1 (Néron *et al*., 1996), and the initiation of an exponential growth phase detectable after 5 days of culture (Figure 11A). In contrast, patients' B lymphocytes failed to respond to the activation stimulus delivered by the receptor and cellular growth following CD40 ligation remained stagnant. Even after a stimulation period of 21 days, total B cells number did not exceed $8X10^4$/well in patients, that is 50 times less than controls' (Figure 11B). This distinctive inhibition of the proliferative response to CD40 stimulation was observed in every patient tested thus indicating a possible defect in the CD40-induced B cell activation pathway in PPBL.

Study of CD40 cDNA encoding sequence and expression of the receptor in PPBL patients.

Since a faulty CD154 ligand could not be responsible for the inhibition of proliferation in the CD40 system, the defect had to be intrinsic to patients' B lymphocytes. We thus decided to verify whether the inhibition of CD40 activation in B lymphocytes from PPBL patients could result from a defect in the CD40 molecule itself. CD40 mRNA was isolated from two patients (patient CL4 and CL3); the CD40 cDNA-encoding region was amplified by PCR and was subsequently used for sequencing. Those sequences appeared to be normal when compared with the wild-type human CD40 sequence using the GCG database. Similarly, cytoflorometric assessment of CD40 expression in B lymphocytes was found to be normal (data not shown). Those results indicated that the CD40 receptor in PPBL patient was indeed capable to interact with its ligand *in vivo* and *in vitro* and to transmit an activation signal.

Study of early events in the CD40 activation cascade.

To verify if the CD40 molecule was indeed apt to transmit a signal, we evaluated early events in the CD40-induced activation cascade. Engagement of the receptor by its ligand CD154 is responsible for the rapid phosphorylation of protein tyrosine kinases (PTK) such as Fyn, Lyn and Syk as well as for the activation of serine threonine kinases such as the MAPK. PTK phosphorylation was therefore assessed by Western analysis in B lymphocytes isolated from patient CL8, after various stimulation periods in the CD40 culture system (45 min, 2hr, 72hr). The phosphorylation patterns were then compared to that observed in a normal control (T44). As its shown in Figure 12, no significant difference could be detected between patient and control.

Tables

Table 4: Clinical data of PPBL patients.

Patient	Age (years)	Lymphocytosis ($x10^9/l$)	Serum IgM (g/l)	$CD19^+$ subset (%)	$CD19^+ \kappa / CD19^+ \lambda$ ratio
CL3	47	4.5	6.7	62	0.88
CL4	42	7.15	10.2	72	0.97
CL7	63	5.23	9.5	67	1.77
CL8	40	3.36	7.9	53	1.09

Table 5: Analysis of CD27 expression in PPBL patients.

	B lymphocyte population			
	$CD19^+$ subset (%)	**$CD19^+$ $CD27^+$subset (%)**	**Percent of the B lymphocyte population represented by CD27+ cells (%)**	**Average (%)**
Patients	52	47	90	**93 +/- 2.65**
	51	48	94	
	65	62	95	
Controls	7	2	28	**11.2 +/- 9.98**
	16	1	6	
	18	2	11	
	11	1	9	
	20	4	2	

Figures

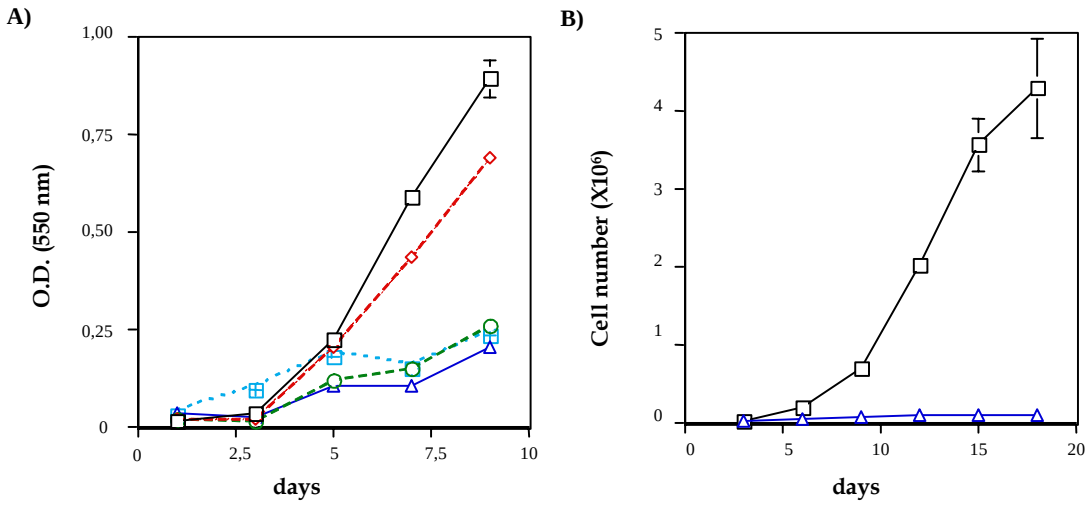

Figure 11: Inhibition of CD40 dependent cellular proliferation in PPBL patients' B lymphocytes.

Following purification, B lymphocytes from PPBL patients CL4 (—△—), CL7 (- - -⊞- - -), CL8 (····○····), and healthy donors T40 (—□—), T42 (·····◇·····) were cultured in the CD40 system. Cellular proliferation was assessed using the colorimetric MTT conversion assay (A) and by direct cell count (B).

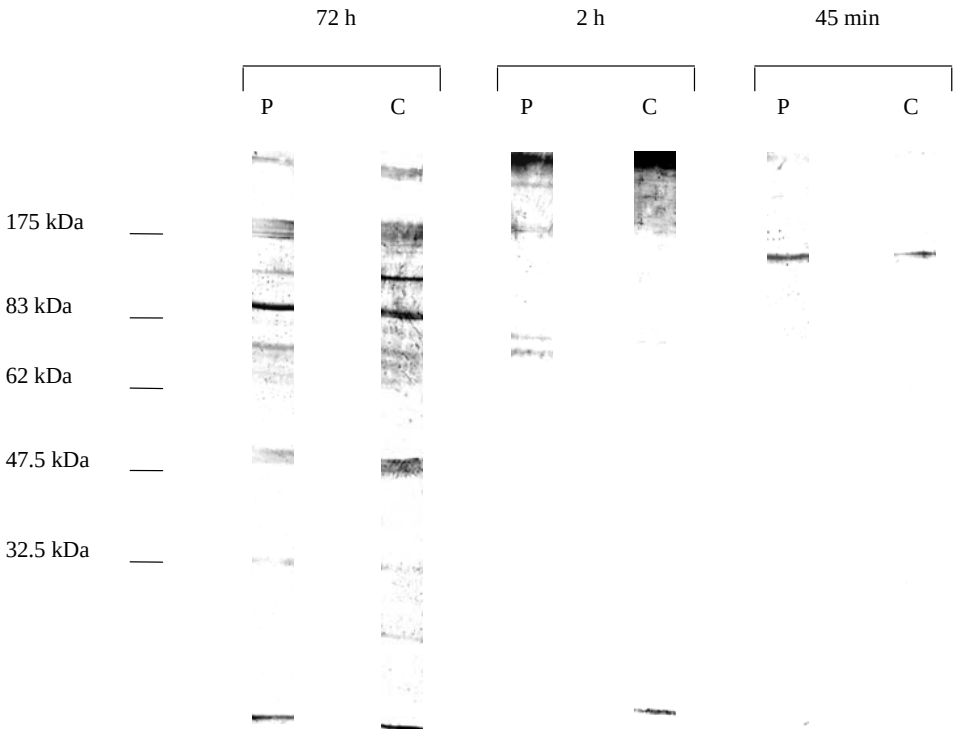

Figure 12: CD40-induced tyrosine phosphorylation in B lymphocytes.

Purified B lymphocytes isolated from patient CL8 (P) and healthy control T44 (C) were stimulated for various periods of time using either the anti-CD40 G28.5 soluble antibody (45 min) in the presence of IL-4 or the CD40 system (2hr, 72hr). Cellular proteins were extracted and 40 g were separated by SDS-PAGE and transferred to a PVDF membrane. Tyrosine phosphorylation was subsequently revealed using the 4G10 anti-phosphotyrosine monoclonal antibody.

Discussion

The CD40 molecule plays a crucial role in B cells development *in vivo*. This is well illustrated by the X-linked hyper-IgM syndrome, which is the outcome of an inadequate interaction between the CD40 molecule expressed by B lymphocyte and its ligand, CD154, presented by T cells. This disorder is characterized by a defect in immunoglobulin isotype switching resulting in excessive IgM secretion with impaired IgA and IgG production. Moreover, patients have lymphoid organs that are devoid of germinal centres, and they are unable to develop memory B cells in response to T–dependent antigens (Vogel & Noelle, 1998). Affected individuals are consequently predisposed to recurrent bacterial and viral infections. The characteristics associated with this disorder are reminiscent of that observed in those patients affected with persistent polyclonal B cell lymphoproliferation. Hence in PPBL, patients have elevated serum IgM, polyclonal B cell proliferation and an association has been established with a latent infection by Epstein-Barr virus. Although many cases of hyper-IgM syndrome are related to genetic abnormalities in CD154 preventing interaction with the receptor (Allen *et al*., 1993; DiSanto *et al*., 1993; Korthäuer *et al*., 1993), in a subset of patients the disorder rather results from defects in the CD40-induced B cell activation pathway (Conley *et al*., 1994; Durandy *et al*., 1997). Indeed, when B lymphocytes isolated from patients are stimulated *in vitro* in the CD40-dependent cell culture system in the presence of an intact ligand, they are still unable to proliferate and undergo immunoglobulin isotype switching. Our results have demonstrated that, likewise, B lymphocytes isolated from PPBL patients did not respond to the expansion signal delivered through CD40. Plus, this defect appeared to be homogenous among the complete B cell population since no proliferation could be detected even after a 21 days stimulation period in the CD40 system. Thus for the first time, a functional defect is described in those cells that could possibly account for their apparent incapacity to respond to physiological signals controlling the homeostasis of the immune system. However, what exactly is the cause for the disruption of the CD40-induced cascade in PPBL patients is yet unclear. It cannot be the result of a defective receptor as both the CD40-encoding sequence and the receptor expression on the cell surface were found to be normal in the patients we tested.

Moreover, an early component of signal transduction in the CD40 generated cascade, PTK activation, was also analysed and no difference could be identified between the tyrosine phosphorylation patterns obtained with patients and controls. It is therefore likely that the defect observed in PPBL B lymphocytes following CD40 cross-linking would be localized downstream of the CD40-induced signalling cascade. We are presently studying those events downstream of the CD40 activation pathway such as nuclear transcription factor NF-kB activation, immunoglobulin isotype switching and secretion, and up-regulation of the cell surface markers CD95, CD80 and CD23. This should allow us to better focus the level at which disruption in the CD40 cascade is occurring and will eventually lead us to the characterization of the exact molecular mechanisms responsible for the functional defect observed in PPBL patients.

Since no enzymatic activity is intrinsic to the receptors of the TNF-R super-family (Baker & Reddy, 1998; Gravestein & Borst, 1998), signal transduction following CD40 receptor cross-linking is generated through the interaction of its cytoplasmic tail with adapters proteins called TRAFs (TNF receptor-associated factors). More exactly, CD40 is capable of direct association with TRAF2, 3, 5 and 6 and indirect association with TRAF1 (through TRAF2) (Pullen *et al*., 1999; Vogel & Noelle, 1998). These interactions lead to the formation of a membrane signalling complex that allows subsequent recruitment of other enzymatic proteins and culminates in the activation of cellular transcription factors such as c-jun and NF-κB. That last factor is responsible for CD40-induced antibody secretion as well as for the up-regulation adhesion markers ICAM-1 and LAF-1 and activation markers CD95, CD23 and CD80 (Hsing & Bishop, 1999; Lee *et al*., 1999). Although the precise mechanisms triggering biological effects engendered by CD40 yet remain to be resolved, the chain of events leading to the activation of the NF-κB transcription factor is now fairly well described. In B lymphocytes, NF-κB activation is mainly mediated trough CD40 interaction with TRAF2 (Kosaka *et al*., 1999; Lee *et al*., 1999). Moreover signals that alter the cytosolic concentration of TRAF2 relative to TRAF3 could be modifying the outcome of NF-κB activation, hence the cellular response to CD40 stimulation (Kuhne *et al*., 1997; Rothe *et al*., 1996). As an example, over-expression of TRAF3 has been shown to block

CD40-induced NF-κB activation (Rothe *et al*., 1996). It would therefore be interesting to study the exact composition of the CD40-associated signalling complex in PPBL patients.

One factor that could be held accountable for a modification in the CD40-associated signalling cascade in PPBL patients is the EBV latent protein LMP-1. Epstein-Barr virus is carried asymptomatically by nearly 95 % of the adult population, and about 1 in 10^6 B lymphocyte is latently infected by the virus. However, as demonstrated in previous studies done in our laboratory, EBV proteins that are expressed during latency are not detectable by NESTED-PCR in the general population. In contrast, the latent protein LMP-1 was detected in every PPBL patient examined in our study. Because of its capacity to bind the adapter proteins TRAF1, 2, 3, 5, and 6 and to induced biological effects similar to those induced by CD40, LMP-1 has been described as a constitutively active viral homologue of this receptor (Eliopoulos & Rickinson, 1998; Kilger *et al*., 1998). The scenario that we propose to explain LMP-1 involvement in CD40 cascade is based upon the hypothesis that, similarly to what has been conjectured for other members of the TNF-R family, LMP1 association with TRAF2 and TRAF3 would result in their sequestration in its own signalling complex (Duckett & Thompson, 1997) and/or would alter the cytosolic TRAF2/TRAF3 ratio (Kuhne *et al*., 1997). These molecules no longer available to bind to CD40, this would then result in a desensitization to CD40-mediated B lymphocyte activation and account for the incapacity of PPBL B cells to proliferate in the CD40 system.

Analysis of CD27 expression on peripheral blood lymphocytes demonstrated a higher proportion of CD27+ B cells in PPBL patients. Previous studies have linked CD27 expression on B lymphocytes to memory B cells (Agematsu *et al*., 1997; Klein *et al*., 1998). It is not clear at this point whether the augmentation in the number of circulating B lymphocytes in PPBL patients can be largely attributed to the increased level of memory B cells. Similarly, the proliferative capacity of memory B cells in the CD40 system has not been clearly established. Is there a connection between the higher proportion of memory B cells in PPBL patients and the impaired *in vitro* CD40-dependent proliferation of their circulating B lymphocytes? Further characterization of the various B cell subpopulations will be essential to understand the various mechanisms at the origin of PPBL.

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Chapitre II

Immunophénotypage et analyse moléculaire détaillée des régions variables (V_H) des gènes des immunoglobulines dans la lymphocytose polyclonale chronique B.

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Analysis of Expressed V_H Genes in Persistent Polyclonal B Cell Lymphocytosis Reveals an Absence of Selection in $IgM^+IgD^+CD27^+$ Memory B Cells.

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Résumé

Contrairement aux pathologies lymphoïdes B classiques, la lymphocytose polyclonale chronique B (LPCB) se distingue par sa symptomatologie discrète, sa grande stabilité, et la polyclonalité de la population cellulaire impliquée. Cependant, des anomalies génétiques récurrentes sont détectées (réarrangements *bcl2/Ig* multiples et trisomie partielle +i(3q)). La présence de cellules atypiques binucléées est caractéristique. Jusqu'à présent, ces anomalies génétiques et morphologiques n'ont pu être circonscrites à une sous population lymphocytaire B distincte. Afin de déterminer le stade précis de maturation de la population en expansion, nous avons entrepris l'immunophénotypage détaillé des lymphocyte B circulants chez quatre patientes et l'analyse moléculaire exhaustive des régions variables de la chaîne lourde des Ig (V_H). Nos résultats ont démontré une augmentation substantielle des lymphocytes B IgD^+IgM^+ chez toutes les patientes. L'utilisation des familles de gènes V_H ne signalait aucun biais dans le répertoire des Ac. Le clonage et le séquençage des séquences V_H ont révélé la présence de mutations somatiques chez la majorité des clones (21/29), corroborant l'expression élevée de CD27, marqueur de lymphocytes B mémoires détecté à la surface de la majorité des cellules B IgD^+IgM^+ chez les patientes. Dans le centre germinatif (GC), la sélection des clones B mutés dotés d'une affinité accrue pour l'Ag constitue une étape clé de la maturation d'affinité. Cependant, l'analyse multinomiale de la répartition des mutations S et R dans les régions de structure FR (*framework*) n'a pas mis en évidence de pression pour la conservation, empreinte putative de la sélection antigénique. En regard de ces résultats, nous concluons que la LPCB résulte de l'expansion du compartiment des cellules B mémoires $IgD^+IgM^+CD27^+$, expansion vraisemblablement causée par une réponse déficiente aux signaux physiologiques responsables de l'élimination des lymphocytes B de faible affinité au sein des GC.

Abstract

Persistent polyclonal B-cell lymphocytosis (PPBL) is an haematological disorder diagnosed predominantly in women, characterized by a polyclonal increase in the number of peripheral blood B lymphocytes. Abnormality of the B-cell population was evidenced by the finding of multiple *bcl-2/Ig* gene rearrangements and an additional long arm chromosome within a significant proportion of B cells. To gain further insight about the developmental status of B lymphocytes in PPBL, analysis of cell surface immunoglobulin receptors was undertaken. An important expansion of the $CD27^{+}IgM^{+}IgD^{+}$ B cell population was noted in PPBL patients (n=4). When investigated by PCR, pattern of V_H genes usage in patients (n=6) was shown to be similar to that observed in healthy individuals (n=4). In depth investigation was then conducted through cloning and sequencing of individual V_H genes in 3 of those patients. They were mostly found to be mutated (21/29), correlating with the observed increase in CD27 expression, a marker of memory B cells. Altogether, these data clearly point out to the exact nature of the expanding B subset in patients. Finally, analysis of the repartition of R versus S mutations in framework regions (FR) of immunoglobulin genes showed no evidence of positive antigenic selection following somatic hypermutation. Thus, we suggest that a lack of response to physiological signals responsible for the elimination of low affinity memory $IgM^{+}IgD^{+}$ B cells in germinal centers could play an important role in the development of PPBL.

Keywords: Persistent polyclonal B cell lymphocytosis, memory B lymphocytes, CD27, somatic hypermutation.

Running title: Molecular analysis of $CD27^{+}IgM^{+}IgD^{+}$ B cells in PPBL.

Introduction

Disturbance in the homeostasis of the immune system, accompanied by a strong increase in B cell numbers, can be the sign of a malign proliferation resulting from the expansion of a transformed clone. Persistent polyclonal B cell lymphocytosis (PPBL) constitutes an exception to that general rule. Hence, this syndrome results from a polyclonal increase of peripheral B lymphocytes, and a polyclonal elevation of serum IgM as indicated by a normal expression of κ and λ light chains isotypes. This indolent lymphoid disorder affects mostly smoking women of a variable age range, most of whom express the HLA-DR7 genotype and present few clinical symptoms (Agrawal, *et al* 1994, Callet-Bauchu, *et al* 1997, Gordon, *et al* 1982, Troussard, *et al* 1997b). PPBL is markedly stable over the years and no evidence of a malignant evolution has been established as yet for the patients.

Over the years, efforts have been made to characterize the B cell population in PPBL, and studies have mainly assessed the morphological and genotypic properties of these cells. B lymphocytes in PPBL are atypical with an abundant cytoplasm and many are binucleated (ranging from 1 to 9%) (Troussard, *et al* 1997a). Moreover, despite the polyclonal nature of PPBL, rearrangements between the *bcl-2* and the immunoglobulin (Ig) genes are multiple and their frequency is a hundred times higher than that sometimes observed in healthy individuals (Delage, *et al* 1997). Cytogenetic abnormalities restricted to the B cell population, namely an extra chromosome 3 long arm and a premature chromosome condensation (PCC) phenomenon, have also been associated with PPBL (Callet-Bauchu, *et al* 1997, Troussard, *et al* 1997a). Additional studies, rather focusing on the functional attributes of B lymphocytes in patients, disclosed a failure to respond to the proliferative signal delivered by CD40, a key molecule involved in B cell differentiation and maturation *in vivo* (Loembe, *et al* 2001). All those elements indicate the presence of a distinct B cell population in PPBL whose exact origin remains to be elucidated.

We, as well as others, recently reported an important increase in the number of cells expressing CD27 in the peripheral blood of PPBL patients (Himmelmann, *et al* 2001, Loembe, *et al* 2001). Notably, that increase was circumscribed to the B cell population.

CD27, a member of the TNF family of receptors, is a type I cell surface glycoprotein that was recently designed as a marker for memory B lymphocytes (Agematsu 2000, Agematsu, *et al* 2000). This postulation was largely based on the observation that CD27 expressing B lymphocytes possessed features usually associated with memory function such as an abundant cytoplasm, mutations of Ig heavy chain variable regions (V_H), and an enhanced capacity to secrete antibodies. B lymphocyte ontogeny in human can be divided into two distinct phases. The first one, which is antigen (Ag) independent, takes place in the bone marrow and consists in the rearrangement of the heavy and light Ig chains (V(D)J recombination) leading to the generation of a functional Ag receptor. Out of the forty-four functional V_H genes segments, which are grouped in seven families, a single one is chosen per Ig gene (Cook and Tomlinson 1995, Matsuda, *et al* 1998). This gives rise to the primary antibody (Ab) repertoire. This phase is then followed by a second maturation cycle ensuing encounter with the antigen, in the course of which naive B lymphocytes enter the germinal centres and differentiate along either the memory or the plasma cell pathway. In the germinal centres, nucleotide substitutions are introduced in the V_H region of the Ig gene locus through the process of somatic hypermutation. Those cells with the highest affinity for the Ag are then selected, generating the secondary Ab repertoire, whereas the cells with low or no affinity for the antigen are eliminated (Liu, *et al* 1997). Therefore, somatic hypermutation constitutes a definitive marker for memory B lymphocytes that have undergone the germinal centre reaction (Agematsu, *et al* 1998, Klein, *et al* 1998b). Accordingly, analysis of the mutational status of V_H genes can be helpful when determining the developmental stage of B lymphocytes. This is particularly true for the characterization of B-cell lymphomas as they often express a single sIg, which results in the over-representation of a particular V_H gene or family in patients (Klein, *et al* 1998a). Similarly, chronic stimulation by a given Ag can cause a non random use of V_H genes leading to a restriction of the V_H repertoire (Dunn-Walters, *et al* 2001, Klein, *et al* 1998a, Kuppers, *et al* 1999).

To gain insights about the developmental status of B lymphocytes in PPBL, in depth analysis of cell surface Ig receptors was carried out by cytofluorometry. Along with the increased expression in CD27 that we previously reported, an important expansion of the $CD27^+IgM^+IgD^+$ B cell population was noted in all patients studied. The pattern of V_H

gene usage was then investigated in six patients and compared to that observed in healthy individuals. Further investigation of the mutation status for used V_H gene segments was carried on in three patients through exhaustive sequence analysis. Correlating with the increased CD27 expression, they were almost exclusively found to be mutated (21/29), however, no evidence of positive selection by an antigen was apparent as indicated by the multinomial analysis of mutation distribution in FRs.

Material and Methods

Patients' material.

All PPBL patients were diagnosed and followed at the Saint-Sacrement Hospital. Criteria used for the clinical diagnosis consisted of: a) a persistent CD19+CD5- B cell lymphocytosis (longer than 6 months) with a normal κ/λ ratio; b) a polyclonal increase in serum IgM concentration; c) the presence of binucleated B lymphocytes. Blood samples from both patients and healthy donors were collected after informed consent was obtained. Mononuclear cells were isolated from heparinized peripheral blood by Ficoll-Paque density gradient centrifugation (Amersham Pharmacia Biotech, Baie D'Urfé, Qc), suspended in freezing medium (Iscove (Invitrogen Life Technologies, Burlington, ON) + 20% heat-inactivated FBS (Hyclone, Logan, UT)+ 5% DMSO) and frozen in liquid nitrogen. Purified B cells were later obtained from thawed cryo-preserved PBMC using the Stemsep (Stemcell Technologies, Vancouver, BC) purification system for B lymphocytes.

Cytofluorometric analysis of B lymphocytes.

CD27 expression on B lymphocytes was assessed by two-colour FACScan analysis using a FITC-labelled anti-CD27 ($IgG1_K$) and a PE-labelled anti-CD19 ($IgG1_K$). Further monitoring of sIgM and sIgG expression on $CD27^+$ B lymphocytes was conducted by three-colour FACScan analysis following staining with a PE-labelled anti-CD27 ($IgG1_K$), an APC-labelled anti-CD19 ($IgG1_K$), and either a FITC-labelled anti-IgG (IgG1) or a

FITC-labelled anti-IgM (IgG1). Finally, the evaluation of $CD27^{+}IgM^{+}$ B lymphocytes that were co-expressing IgD was carried out: gated CD27-IgM-positive B cells (detected as described formerly) were analyzed for IgD expression by staining with a biotin-labelled anti IgD ($IgG2_{aK}$), followed by APC conjugated to strepavidin (antibodies and reagents were all obtained from BD Pharmingen, Mississauga, ON). All analyses were collected using a FACScalibur flow cytometer (Becton-Dickinson, Oakville, ON) and antibodies were always used at saturating concentration.

RNA isolation and cDNA synthesis.

Total cellular RNA was isolated from cryopreserved PBMC or isolated B lymphocytes using TRIZOL reagent. Conversion to cDNA was then performed using 1μg of RNA. The reaction mixture contained 1μl murine Moloney leukemia virus reverse transcriptase (200U/μl, Invitrogen Life Technologies), 10mmol/L dithiothreitol (DTT), 1 mmol/L of each dNTP, 0.5 μmol/L oligo(dT), 4 μl 5X first strand buffer in a final volume of 20μl. Incubation was then carried on at 42°C for 45 min.

PCR amplification of V_H genes.

For each cDNA sample, the G3PDH gene was amplified and served as a positive control. 5 μl of each cDNA sample was amplified using 30 pmol of either one of the V_H-family specific leader primer (see Table 6) combined with 30 pmol of a J_H consensus reverse primer. The PCR reaction mixture was performed in the presence of 0.5 μl Taq DNA polymerase (5U/μl, Invitrogen Life Technologies) and 5 μl 10X PCR buffer with 15 mmol/L $MgCl_2$ in a final volume of 50 μl. The amplification consisted of 30 cycles of 30 seconds at 94°C, 40 seconds at 55°C and 60 seconds at 72°C and it was terminated by a final elongation period of 10 minutes at 72°C. Each round of amplification was completed with a negative control containing no cDNA template to exclude the possibility of cross contamination. The PCR products were analyzed on a 0.8% agarose gel.

Cloning and sequencing of PCR products.

For each patient tested, a mix combining 5 μl of each PCR product obtained following amplification of the six V_H families (final volume 30 μl) was run on a 0.8% agarose gel.

The band comprised between 400 and 500 bp was excised and the DNA was purified using the QIAquick gel extraction kit (Qiagen, Mississauga, ON). 1 to 4 μl of the recovered DNA was then used for ligation in the pCRII-TOPO vector (TOPO TA cloning kit, Invitrogen Life Technologies) and cloned following the manufacturer's instructions. Ten clones per patient were subsequently used for sequencing with an automated DNA sequencer (ABI 373, Perkin-Elmer Applied Biosystems, Foster City, CA). For the V_H2 and V_H6 family however, 30 μl of their respective PCR amplification product was used for subsequent analysis in order to obtain at least one positive clone. To determine the error rate intrinsic to Taq polymerase, the G3PDH gene was amplified and cloned using the same procedure as that described for V_H genes, and three clones were randomly used for sequencing. The Taq polymerase error frequency was thus evaluated at 0.14%, which amounts to 0.4 mutation per V_H clone.

Mutation analysis of V_H genes

Mutations were assigned to sequenced V_H gene segments using the IMGT database on the internet (IMGT, the international ImMunoGeneTics database http://imgt.cines.fr:8104, Initiator and coordinator: Marie-Paule Lefranc, Montpellier, France (Lefranc 2001, Lefranc, *et al* 1999, Ruiz, *et al* 2000)): sequences were aligned with the germline gene presenting the highest homology and the number of somatic mutations was determined. Mutations at the last nucleotide position were excluded to rule out the possibility of deletions at the joining site. Sequence homology values were determined from the alignment of sequences going from the beginning of FR1 to the end of FR3.

To resolve whether the presence of somatic mutations in rearranged V_H genes was the result of antigen selection, we used the mutinomial model of Losso *et al* (Lossos, *et al* 2000b). Multinomial P value for each mutated V_H gene were determined using the JAVA applet available at http://www-stat.stanford.edu/immunoglobulin.

Results

Analysis of CD27 expression.

Mature B cell subsets in humans can be characterized according to the expression of cell surface Ig and CD27, a newly described marker for memory B lymphocytes. In healthy individuals, $CD27^+$ memory B lymphocytes represent 40% of the total peripheral B cell population. Memory B cells can be further subdivided into class-switched, IgM only , IgM^+IgD^+ subsets (Agematsu, *et al* 1997, Klein, *et al* 1998a, Klein, *et al* 1998b) (about 30-40% each). Comparison of CD27 expression in two PPBL patients (CL4 and CL5) and in one healthy donor (T44), as revealed by two-colour FACS analysis of peripheral blood lymphocytes (PBL), showed a significant increase in the number of circulating $CD27^+$ B lymphocytes (Figure 13A). Two additional patients (CL10 and CL11) were evaluated and displayed similar results (not shown), indicating that this phenomenon was particular to PPBL patients. Total B cell numbers were evaluated at 5148/μl in patient CL4 and 2404/μl in patient CL5, 80% of which were expressing CD27, yielding a value of 4118/μl and 1923/μl $CD27^+$ B lymphocytes in patient CL4 and CL5 respectively. Comparatively, total B cell numbers in healthy adults can be estimated at 150-400/μl, 40% of which are $CD27^+$, yielding a mean value of 110/μl $CD27^+$ B lymphocytes. $CD27^+$ B lymphocyte numbers were therefore raised by an average factor of 20 in patients (patient CL4: 17, CL5: 37). Additional characterization of $CD27^+$ B cells in PPBL (Figure 13B and Figure 13C) revealed that they were almost exclusively IgM^+IgD^+ co-expressing memory B lymphocytes (CL4: 91.5%, CL5: 84.3% of $CD27^+$ memory B lymphocytes), which was in sharp contrast to what is usually observed in healthy individuals (30-40%). The expansion of the B lymphocyte population seen in PPBL could therefore largely be accounted for by the considerable augmentation in the number of $IgM^+IgD^+CD27^+$ memory B cells. These results were consistent with the elevation in polyclonal IgM usually associated with PPBL.

Molecular study of V_H gene segments in PPBL.

PCR amplification of six V_H families allowed us to evaluate the V_H repertoire amid six healthy donors and six PPBL patients. Whereas amplification was readily obtained in PPBL patients, prior purification of B lymphocytes was necessary to achieve similar results in healthy donors (Figure 14). That was probably a consequence from the increased B cell proportion in patients' samples when compared to donors. While evidences have been found for a restricted use of V_H gene segments in healthy adults (Brezinschek, *et al* 1995, Kraj, *et al* 1997, Rao, *et al* 1999, Suzuki, *et al* 1995), distribution of V_H families still correlates with the number of functional genes they each contain (Brezinschek, *et al* 1995, Matsuda, *et al* 1998). Thus, although usage of V_H gene families, as revealed by PCR amplification, proved to be diverse among three healthy donors, the largest V_H3, V_H4 and V_H1 families were always amplified. On the other hand, the less abundant V_H2, V_H5 and V_H6 families each occurred less frequently. Still, they were all amplified in at least one donor. Similar results were obtained following analysis of six PPBL patients, although the V_H5 family was never observed. Therefore, when compared to healthy donors, no obvious bias in the V_H gene repertoire of PPBL patients could be detected following PCR amplification alone. More thorough analysis of V_H, J_H and D_H genes segments usage involving cloning and sequencing of PCR products was therefore undertaken and allowed us to gain supplementary information regarding this aspect. The data presented in table 1 confirmed that V_H gene usage in patients was not uniform and that the V_H1, 3 and 4 families were most frequently used in patients. Additionally, when comparing the frequency of usage for the V_H1, 3 and 4 families in patients preferential usage of the V_H4 family was noticed in patient CL4 (55%) and especially in patient CL8 (90%) (Table 8). D_H and J_H gene segments usage was diversified. Notably, no members of the smallest V_H2 and V_H6 families could be cloned despite their former amplification by PCR (Figure 14), probably as a result of their lower abundance. Consequently, to confirm the amplification and mutational status of the V_H2 and V_H6 families, the PCR product for either families was analyzed individually (see Material and Methods) and they were found to be mutated without exception (results not shown).

Study of V_H genes mutation patterns in patients.

A total of 29 V_H clones were obtained from three patients as described in the Material and methods section, their mutation analysis is summarized in Table 7. Only 3 clones had unmutated germline sequence while the remaining 26 V_H sequences showed from 97.3 to 99.8% homology with the corresponding most similar germline genes. As the Taq DNA polymerase spontaneous error rate was estimated at only 0.14% in our experimental design, the clones with less than 99.8% homology (i.e.: more than one mutation per V_H gene, 21/29 sequences) were considered to be somatically mutated. The multinomial distribution model of Lossos *et al* (Lossos, *et al* 2000b) was used to evaluate the probability of antigenic selection pressure (Table 9). Scarcity of R mutations in the FR, which suggests antigenic pressure to conserve these regions in the Ig molecule and maintain binding to an antigen, was significant ($p<0.05$) in 4 of 21 mutated V_H sequences.

Tables

Table 6: Primers used for V_H gene amplification.

Name	Sequence (5’ to 3’)
J_H consensus	TGAGGATCCGGTGACCAGGGTGCCTTGGCCCCAG
V_H1	ACCATGGACTGAAGCTTGAGGATCCTCTTCTTGGTG
V_H2	ACCATGGACAGCTTTGTTCCACGCTCCTGCTACTG
V_H3	ACCATGAAGCTTGGGCTGAGCTGGGTTTTCTTGTT
V_H4	AACATGAAGCTTCTGTGGTTCTTCCTCCTGCTGGTG
V_H5	AAGATGAAGCTTACCGCCATCCTCGCCCTCCTCCTG
V_H6	ACAATGTAAGCTTCCTTCCTCATCTTCCTGCCCGTG

Table 7: Analysis of immunoglobulin heavy chain genes in PPBL.

V_H clone	Germline gene with highest similarity	n	% homology	D_H	J_H
CL4.3	3-7	3	99.3	4-23	4
CL4.4	3-66	0	100	2-2 inv	4
CL4.6	3-13	5	98.8	6-13	6
CL4.8	4-59	8	98	5-24 inv	4
CL4.9	4-59	4	99	4-23	4
CL4.12	4-4	3	99.3	/	/
CL4.14	1-8	2	99.5	6-6	4
CL4.15	4-34	8	98	4-17	4
CL4.18	4-39	2	99.5	1-26	4
CL5.1	3-74	1	99.8	/	/
CL5.2	1-18	0	100	2-15	6
CL5.3	3-66	1	99.8	6-19	4
CL5.4	1-2	4	99	2-15 inv	4
CL5.5	1-18	2	99.5	1-14 inv	4
CL5.6	4-30-2	7	98.3	3-16	3
CL5.7	4-30-4	2	99.5	2-8 inv	4
CL5.8*	4-30-4	2	99.5	2-8 inv	4
CL5.9	3-33	1	99.8	2-15	6
CL5.10	3-33	1	99.8	2-15	6
CL8.2	4-59	11	97.3	1-26	4
CL8.7	4-59	4	99	6-25	4
CL8.10	4-59	6	98.6	2-15	4
CL8.11	4-4	7	98.3	2-15	4
CL8.13	3-49	7	98.3	3-22	4
CL8.14*	4-59	6	98.6	2-15	4
CL8.15	4-39	5	98.8	5-18	4
CL8.17	4-30-4	4	99	5-18	2
CL8.19	4-59	0	100	6-19	4
CL8.20	4-59	1	99.8	3-22	5

a)**: V_H clone represented more than once.

b) n: number of mutations, D_H: Ig_H diversity gene, J_H: Ig_H junction gene.

Table 8: V_H gene segments usage in PPBL patients.

Family	CL4	CL5	CL8	Overall
V_H1	11%	33%	/	13.8%
V_H3	33%	44%	10%	27.6%
V_H4	55%	22%	90%	58.6%

Table 9: Molecular analysis of mutations in framework regions of V_H genes.

V_H clone	Observed R/S	Inherent R:S	P value
CL4.3	2/1	2,94	0,439
CL4.6	1/2	2,86	0,062
CL4.8	2/2	2,80	0,032*
CL4.9	1/2	2,80	0,136
CL4.12	2/0	2,82	0,439
CL4.14	0/2	3,18	0,134
CL4.15	5/2	2,94	0,281
CL4.18	2/0	2,77	0,368
CL5.4	3/0	3,27	0,376
CL5.5	1/1	3,10	0,466
CL5.6	2/3	2,78	0,073
CL5.7	0/2	2,81	0,153
CL5.8**	0/2	2,81	0,153
CL8.2	4/4	2,80	0,055
CL8.7	0/4	2,80	0,020*
CL8.10	1/4	2,80	0,029*
CL8.11	2/1	2,82	0,063
CL8.13	4/1	2,99	0,287
CL8.14**	1/4	2,80	0,029*
CL8.15	2/0	2,77	0,224
CL8.17	1/2	2,81	0,146

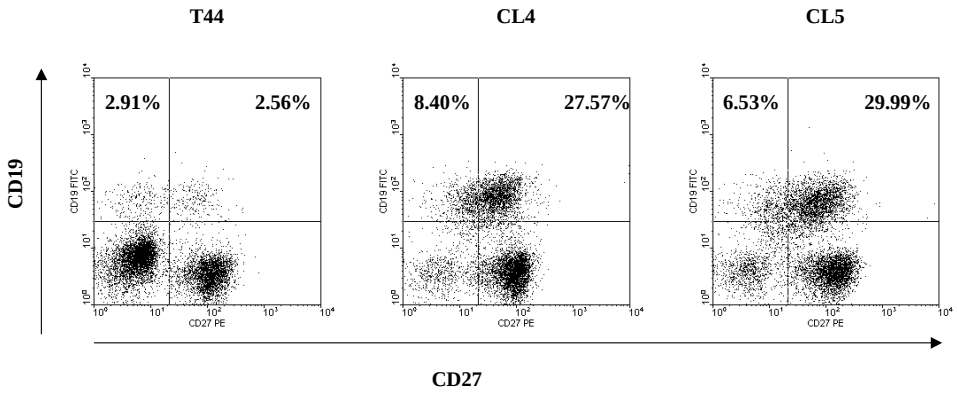
a) **Abbreviations:** R: replacement mutation, S: silent mutation, inherent R:S: mutation ratio, quotient of total possible R to total possible S mutations, P: probability that scarcity of R mutations in FR occurred by chance.

b)*: statistically significant,

c)**: V_H clone represented more than once.

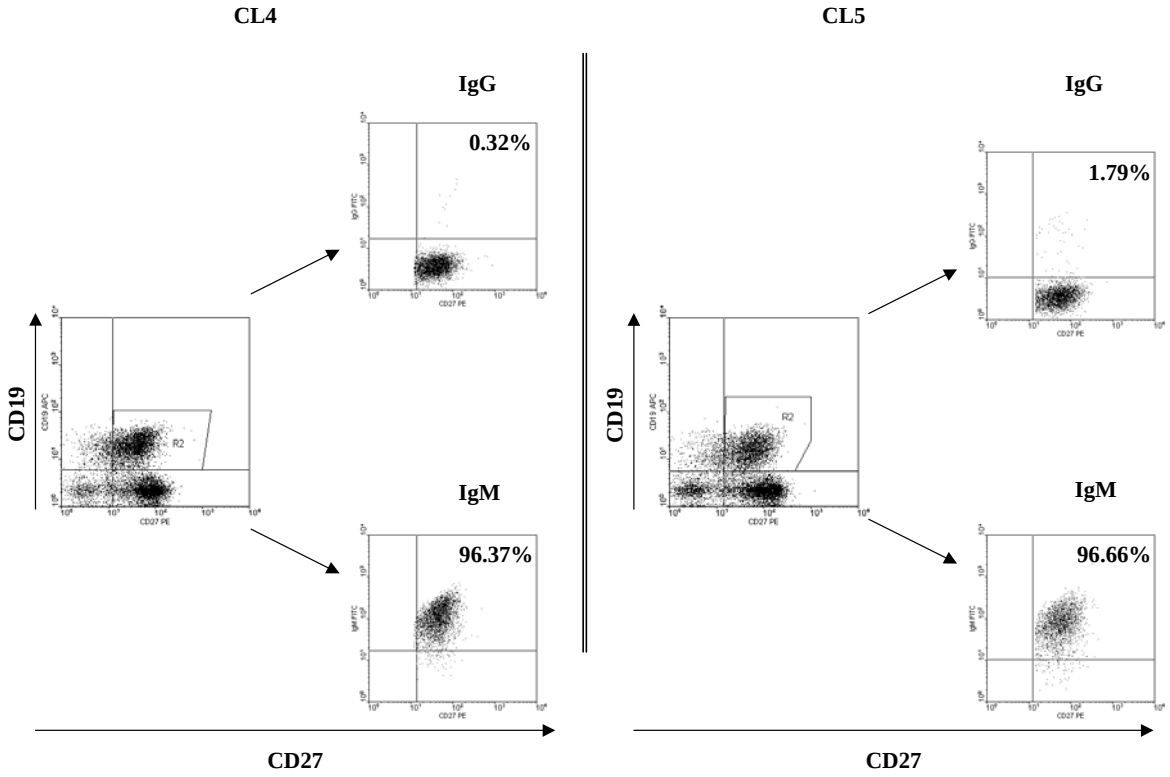
Figures

A)



$CD27^+$ B lymphocytes	**46.8%**	**76.7%**	**82.1%**

B)



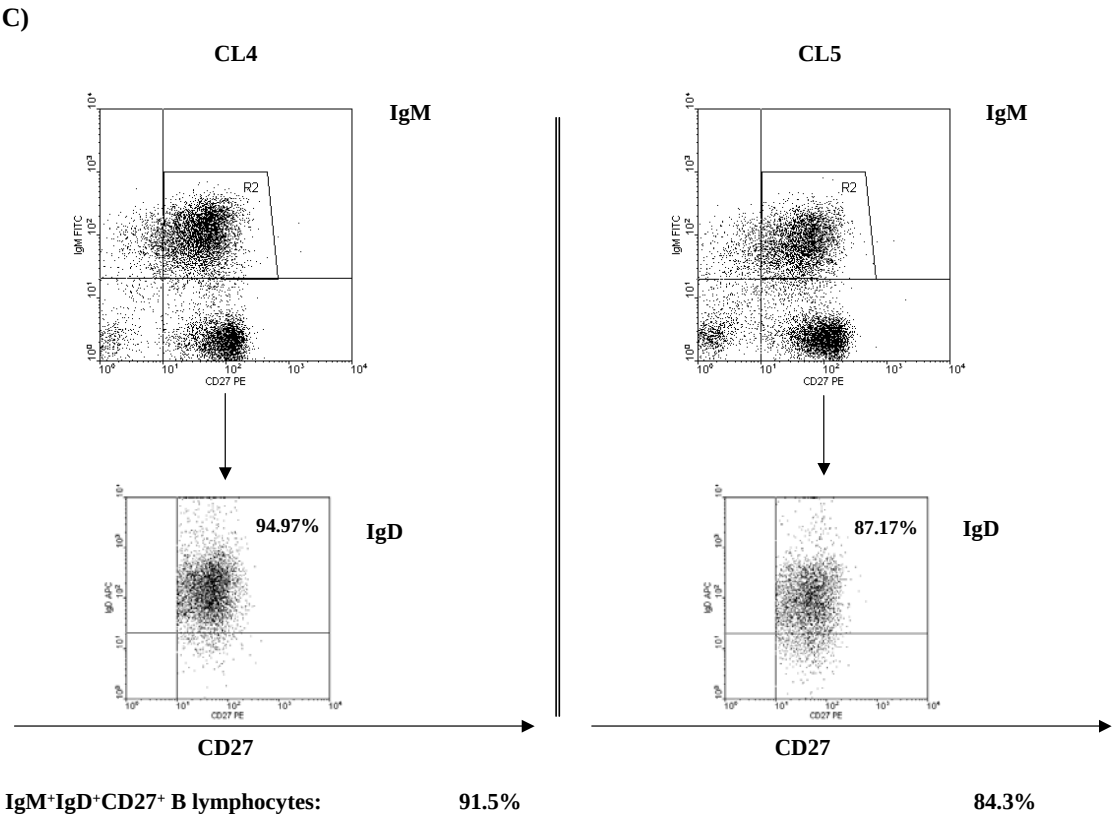


Figure 13: Fluorescence analysis of CD19+ B lymphocytes from PPBL patients with regard to the expression of CD27, sIgM, sIgG and sIgD.

A) Comparative analysis of CD27 expression in one healthy donor (T44) and two patients (CL4 and CL5) by two-colour cytofluorometry. B) Further analysis of sIgM and sIgG expression in patients CL4 and CL5 by three-colour cytofluorometry. C) Evaluation of IgD expression on $CD27^+IgM^+$ B lymphocytes in patients CL4 and CL5 by three-colour cytofluorometry. In all cases PBMC were isolated from peripheral blood then stained with antibodies as described in the text. For patients, windows were set first around the $CD27^+CD19^+$ B cell population (B), next around $CD27^+IgM^+$ positive B cells (C) to further analyze sIgM and sIgG expression then IgD expression respectively. Frequencies for the different B cells subsets are indicated.

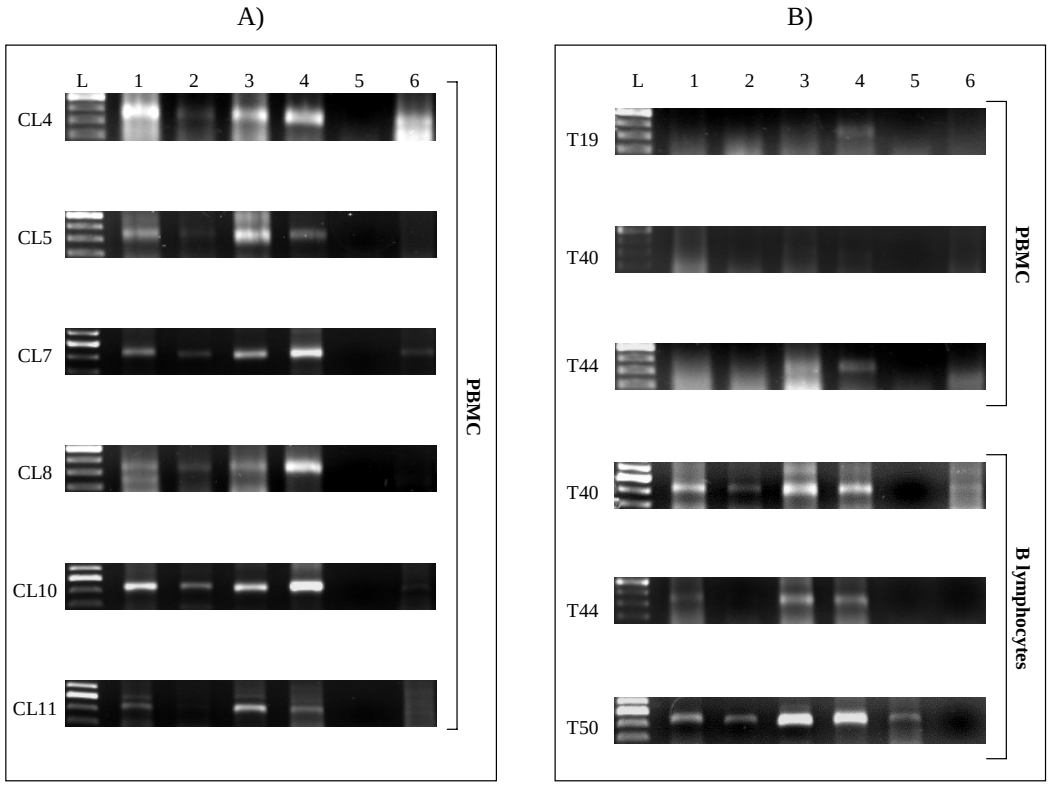


Figure 14: VH families usage In PPBL patients determined by PCR amplification.

V_H gene families 1 to 6 were amplified in six patients (CL4, CL5, CL7, CL8, CL10, CL11) from PBMC only (panel A) and three healthy donors from either PBMC (T40, T44, T19) or purified B cells (T40, T44, T50). For each sample, total cellular RNA was isolated, converted to cDNA and subjected to PCR amplification (primers see table 4). PCR products were analyzed on 0.8% agarose gel. A control (no cDNA) was included with each amplification and was always negative (not shown). Lane L: 100 bp DNA ladder 9 ranging from 300 to 600 bp), lanes1-6: V_H family 1 to 6 respectively.

Discussion

Over the past few years, much progress has been achieved with regard to the characterization of the various B cell subsets. The designation of CD27 as a memory B cell marker has allowed a more accurate classification of the different memory subsets in humans (Agematsu 2000, Agematsu, *et al* 2000, Klein, *et al* 1998b). At the same time, improvement of the methods used to study Ig V_H gene rearrangements and mutational load did permit to establish the lineage of proliferating cell clones in many B cell malignancies and to refine both the diagnosis and prognosis of these diseases (Dunn-Walters, *et al* 2001, Kuppers, *et al* 1999, Naylor and Capra 1999). The B cell population in PPBL however has never been submitted to such a systematic characterization, and the identification of the proliferating subset in this syndrome remained to be established.

Preliminary studies done in our laboratory having revealed that the expression of CD27 was greatly enhanced in PPBL patients, we decided to further establish the developmental status of B lymphocytes in PPBL. More elaborate study of surface antigen markers has revealed that the $CD27^+$ subset was greatly over-represented in patients: while the total B cell population was about 10 times more abundant than in healthy individuals, the $CD27^+$ subset was increased on average by a factor of 20 in patients. In addition, assessment of cell surface IgM, IgD and IgG isotype receptors indicated that these $CD27^+$ cells almost exclusively co-expressed IgM and IgD contrarily to an observed frequency of only about 30%-40% in $CD27^+$ B lymphocytes in normal adult donors (Agematsu, *et al* 1997, Klein, *et al* 1998b). Interestingly, a morphological attribute of $CD27^+$ memory B cells, namely the presence of an enlarged cytoplasm, has also been associated with B lymphocytes in PPBL (Agematsu, *et al* 1997). It therefore appears that the expansion of memory $CD27^+IgM^+IgD^+$ B cell plays an important role in PPBL and that it accounts for most of the observed lymphocytosis. These results are in accordance with the recent observations of others (Himmelmann, *et al* 2001, Salcedo, *et al* 2002), and substantiate the fact that the presence of an increased $CD27^+IgM^+IgD^+$ B cell pool is a key feature that should now be taken into account when establishing a PPBL diagnosis.

Concomitant increase in CD27 and CD70 expression has been linked to malignant lymphoproliferative diseases by some (Loenen 1998, Ranheim, *et al* 1995). However, only marginal CD70 expression could be detected in PPBL patients $CD27^+$ B lymphocytes (data not shown). Nonetheless, the polyclonal nature of PPBL is well established. PCR analysis of the Ig V_H gene usage demonstrated accordingly that the relative abundance of each V_H gene family was similar to that observed in healthy donors, and that each family was used in a heterogeneous fashion among patients. We proceeded with the study of the mutational status of Ig genes in three patients in order to exclude the possibility of an up-regulation of the CD27 receptor at the individual B cell level. We also expected to gain a better estimation of the maturation stage of PPBL B lymphocytes and of their exact pattern of V_H genes usage. The results obtained following cloning and sequencing of 29 V_H genes (10 each for patients CL5 and CL8, 9 for patient CL4) were consistent with the observed increase in CD27 expression as most genes were mutated (21/29). The average mutational frequency was 1.39% (CL4: 1.52%, CL5: 0.52%, CL8: 1.82%), which is close to the 2% value reported for IgM^+ memory B cells, and favours a (post) GC origin for the $CD27^+IgM^+IgD^+$ population in PPBL (Klein, *et al* 1998a). Although V_H genes usage between patients again appeared to be diversified, a bias toward the V_H4 family was noted in patient CL4 and in patient CL8. In CL8, the V_H4-59 gene was especially over-represented (6/10), although the presence of different V-D-J junctions excludes the possibility of a specific clonal expansion. However, V_H genes families usage can vary among healthy individuals and the V_H4 (V_H4-34 and V_H4-59) family is naturally over-represented in the repertoire of the normal population (Kraj, *et al* 1997, Rao, *et al* 1999, Suzuki, *et al* 1995). Further studies, particularly quantitative analysis of V_H gene usage in additional patients, will be needed to determine the exact signification of those observations.

We previously reported similarities between PPBL and the recessive form of the hyper-IgM syndrome (non-XL-HIM) (Loembe, *et al* 2001) namely increase in serum IgM and in IgD^+IgM^+ B lymphocytes that are unable to undergo CD40-induced cell proliferation. Recently, Revy *et al* (Revy, *et al* 2000) demonstrated that, in non-XL-HIM patients, IgD^+IgM^+ B lymphocytes were mostly unmutated or, if so, to a degree inferior to 2%. This was apparently caused by mutations in the AID (activation-induced cytidine deaminase)

gene, an enzyme implicated in class switching and somatic hypermutation. In regard with our results, it would seems that PPBL and non-XL-HIM are separate entities, though analysis of the AID gene in PPBL patients would be required to definitively rule out any connection between both syndromes.

According to the current knowledge of the germinal centre (GC) reaction, it is believed that upon antigen encounter, B lymphocytes undergo somatic hypermutation of their Ig receptors, generating a progeny of related B cells expressing variably mutated Ig genes. Among this diverse population, only those cells with high affinity for Ag will then be selected for survival and entry in either the memory or plasma B lymphocyte pool through the process known as affinity maturation (Klein, *et al* 1998a, Wagner and Neuberger 1996). The imprint of somatic mutation can be indicative of such antigen selection. Comparative studies of the hypermutation pattern in productively and non-productively rearranged human V_H genes have demonstrated a counter-selection of replacement mutations in the FR regions of expressed Ig genes (Dorner, *et al* 1997, Dorner, *et al* 1998, Dunn-Walters, *et al* 2001, Klein, *et al* 1998a). This suggested a pressure to maintain integrity of the Ab molecule. As a result, one observed in the FR a lower R/S ratio than expected if mutations had happened by chance only (a value of about 3.0). Since most lymphoproliferations are derived from GC, they are consistently often reported to be Ag selected. Some even depend on a specific antigenic stimulation to ensure their progression (Dunn-Walters, *et al* 2001, Kuppers, *et al* 1999). In the case of PPBL patients however, analysis of the 21 hypermutated V_H sequences showed evidence of selection by an Ag in only 4 clones. It therefore appears that PPBL consists mainly in the expansion of memory $CD27^+IgM^+IgD^+$ B cells that do not appear to have been submitted to a selection based on better binding to the Ag. These results do not support the theory of a chronic antigenic stimulation in PPBL that would particularly implicate the Epstein-Barr virus (EBV) often associated with the syndrome. Rather, the association with EBV would be the consequence of the lymphoproliferation, as it is now established that the memory B cell compartment is the site of long-term viral persistence. Interestingly however, the virus was demonstrated to be restricted to IgD^- memory B cells (Joseph, *et al* 2000), which is in opposition with what is seen in our patients.

The question remains unresolved as to what causes the expansion of $CD27^+IgM^+IgD^+$ B lymphocytes in PPBL patients. Are those cells resistant to physiological signals responsible for the elimination of memory B cells with low or no affinity for the Ag in GC, such as the triggering of CD95? The implications would be multiple on the clinical level. It will be crucial to determine whether the $CD27^+IgM^+IgD^+$ B lymphocytes in PPBL are capable to resist to apoptosis inducing signals: the fact that they possibly continue to undergo somatic mutations, could put them at higher risk of accumulating genetic defects. Indeed, the mutational machinery involves the creation of DNA strand breaks, and as a result mediates the translocation of non Ig genes (i.e.: *c-myc*, *bcl-2* and *bcl-6*) in the Ig locus (see review in Kuppers *et al* (Kuppers, *et al* 1999)). In line with this view, we demonstrated previously that the frequency of *bcl-2* translocations was greatly enhanced in patients. Moreover, the hypermutation process is not restricted to Ig genes as commonly believed, and it can cause the introduction of point mutations in critically important genes such as the proto-oncogene *bcl-6* (Pasqualucci, *et al* 1998, Storb, *et al* 2001) or the tumour suppressor CD95 (Muschen, *et al* 2000). These transforming events often constitute the first steps of lymphomagenesis as exemplified by diffuse large B cell lymphoma (DLBCL), recently shown to originate from a heterogeneous B cell population with evidence of progressively accumulated mutations (Alizadeh, *et al* 2000, Lossos, *et al* 2000a). Unfortunately, it will be clinically difficult to ensure a follow-up of the mutational status of individual B cells in patients due to the polyclonal nature of PPBL and the impossibility to isolate a single proliferating clone. Nevertheless, careful monitoring of patients remains recommended.

One can't help but wonder at the exact origins of $CD27^+IgM^+IgD^+$ B lymphocyte population in patients. Although it possesses many characteristics of GC-derived B cells, the possibility of a different lineage cannot be completely excluded. In their article, Klein *et al* propose that this memory subset could descent from rare tonsillar IgM^+IgD^+ B lymphocytes on the premise that both populations express the CD70 marker (Klein, *et al* 1998b). However as mentioned earlier, no such expression was detected in PPBL patients, hence this conjecture does not seem plausible here. Additionally, Weller *et al* (Weller, *et al* 2001) as well as Agematsu *et al* (Agematsu, *et al* 1998) reported the presence of mutated $CD27^+IgM^+IgD^+$ B in XHIM (X-linked hyper-IgM syndrome) patients, which are not able

to form GC, suggesting a possible GC-independent diversification pathway in those individuals. If Weller *et al* hypothesis proves true, it could be interesting in the future to evaluate whether the proliferating $CD27^{+}IgM^{+}IgD^{+}$ population in PPBL patients does originate from such a GC-independent maturational pathway but which would be deregulated.

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Chapitre III

Analyse moléculaire la molécule AID et évaluation de la sécrétion d'immunoglobulines chez des patientes atteintes de la lymphocytose polyclonale chronique B.

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Predominant IgM secretion with normal AID gene status further substantiates a marginal zone origin for expanding $IgD^{+}IgM^{++}$ memory B cell subset in persistent polyclonal B cell lymphocytosis.

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Résumé

L'identification récente de CD27 comme marqueur des lymphocytes B mémoires a démontré qu'une proportion considérable des B IgD^+IgM^+ circulants, jusque là présumés naïfs, ont formellement connu la diversification des gènes des Ig. La lymphocytose polyclonale B chronique (LPCB), est une prolifération lymphoïde impliquant ce sous-compartiment cellulaire. Elle est caractérisée par une élévation polyclonale de l'IgM sérique et des anomalies cytogénétiques caractéristiques (réarrangements *bcl2/Ig* multiples, isochromosome +i(3q)). Une prédisposition familiale a de plus été établie. Le syndrome d'hyper-IgM (HIGM) regroupe des déficits immunitaires caractérisés par une synthèse élevée d'IgM, une baisse ou absence d'IgA et d'IgG et une susceptibilité accrue aux infections bactériennes ou opportunistes. Une inhibition des mécanismes de maturation du lymphocyte B est à l'origine de ce syndrome. Une variante à transmission autosomique récessive, soit le syndrome HIGM2, est causée par des mutations du gène AID, régulateur clé des processus d'hypermutation somatique (SH) et de commutation isotypique (CS). Il a été suggéré qu'un blocage similaire au niveau de la différenciation cellulaire soit impliqué dans la LPCB. Cette proposition a donc été explorée expérimentalement chez des patientes atteintes de la lymphocytose. La sécrétion des différentes classes d'Ig a été estimée suite à la stimulation *in vitro* via CD40 en présence de cytokines inductrices de CS. De plus, le statut moléculaire de la molécule AID a été évalué. Bien que le profil de sécrétion d'Ac chez les patientes ait été dominé par l'IgM, aucune anomalie affectant soit la séquence, soit l'expression du gène AID n'a pu être détectée. Ces résultats n'appuient pas l'hypothèse d'une inhibition de la CS consécutivement à un déficit de l'enzyme AID et indiquent que LPCB et HIGM2 constituent deux entités distinctes. Les propriétés fonctionnelles observées sont plutôt évocatrices du phénotype des lymphocytes mémoires B de la zone marginale (MZ), récemment associés à la population des lymphocytes B $IgD^+IgM^+CD27^+$ du sang périphérique.

Abstract

Persistent polyclonal B cell lymphocytosis (PPBL) is a lymphoid disorder consisting in an expansion of $IgD^+IgM^+CD27^+$ B lymphocytes with concomitant serum IgM elevation. Genetic predisposition is suggested by familial occurrence of the syndrome and B cell genetic aberrations. The hyper-IgM syndrome is a genetically heterogeneous condition characterized by elevated serum IgM, low to absent serum IgG, IgA, and IgE, increased susceptibility to bacterial and, in some cases, opportunistic infections, which originates in total or partial impairment of antibody maturation in B cells. One form of the disease is the autosomal recessive HIGM syndrome (HIGM-2), caused by deleterious mutations of the activation-induced cytidine deaminase (AID) gene, a key regulator for the processes of somatic hypermutation and class switch recombination. The theory of a similar differentiation block in PPBL B cells was investigated. Ig secretion, following CD40 stimulation in the presence of class-switch inducing cytokines, and the status of AID molecule were assessed. Whereas IgM secretion remained highly predominant in PPBL B cells, AID gene sequence and expression were normal, indicating that this disorder and HIGM-2 are definitely distinct entities. Thus, impairment of class switching as a result of an AID-dependent mechanism is unlikely in PPBL patients. Rather, observed features would be consistent with the phenotype of marginal zone memory B lymphocytes, a recently proposed counterpart for circulating $IgD^+IgM^+CD27^+$ B lymphocytes.

Introduction

PPBL is an unusual haematological disorder, mostly diagnosed in female smokers presenting few symptoms, except for occasional splenomegaly and benign upper respiratory track infections (Gordon, *et al* 1982). It is characterized by a polyclonal and stable increase in B lymphocytes and elevated serum immunoglobulin (Ig) M levels, with low to normal IgG and IgA. Due to the few clinical manifestations, PPBL is often detected following routine blood testing, and most of the time diagnosis has been one of exclusion. However, the past years have witnessed notable progresses regarding characterization of this syndrome. The expanding population has been defined as consisting of somatically mutated $IgD^+IgM^+CD27^+$ memory B lymphocytes (Loembe, *et al* 2002). Hallmark morphological (binucleated cells) and cytogenetic (multiple *bcl-2*/*Ig* gene rearrangements, distinctive chromosome 3 aberrations) features have been uncovered, and evidences have accumulated for a familial inheritance pattern (Delage, *et al* 2001, Delage, *et al* 1998, Mossafa, *et al* 1999). These elements have largely contributed to the elaboration of a more refined diagnosis in PPBL. Nevertheless, the precise mechanisms responsible for IgD^+IgM^+ memory B cells expansion and increase in IgM secretion remain undefined.

Predominance of IgD^+IgM^+ cells, with or without somatic mutations, accompanied with increased IgM levels, is usually associated with the Hyper-IgM syndrome (HIGM), a rare and genetically heterogeneous condition characterized by impairment in the class-switching recombination and somatic hypermutation processes, leading to variably severe immunodeficiencies (review in (Durandy, *et al* 2004)). Defects in the CD40 molecule (HIGM3) or its ligand (HIGM1), indispensable to the formation of germinal centres (GC), as well as mutations of the AID gene (HIGM2), a key regulator of somatic hypermutation and class-switching in B cells, are responsible for the absent or diminished expression of switched Ig isotypes in patients, and the resulting increase in circulating IgD^+IgM^+ cells. HIGM-1 inheritance is sex-linked, and dominant, whereas HIGM2 and 3 are transmitted following an autosomal recessive mode. It was suggested early on that a block in differentiation, similar to that seen with HIGM, could play a role in PPBL and account for

elevated IgM secretion in patients (Gordon, *et al* 1982), and similitude has been suggested between PPBL and HIGM-2 (Loembe, *et al* 2001, Loembe, *et al* 2002). As a way to clarify these issues, evaluation of the class-switching capacity to downstream isotypes and assessment of the AID gene status in PPBL B lymphocytes were carried out. IgM secretion remained predominant in patients following CD40 stimulation in the presence of class-switch inducing cytokines. Nevertheless, AID gene sequence and expression were normal, dismissing a link between PPBL and HIGM2. On the other hand, these phenotypic features were reminiscent of those reported for marginal zone (MZ) memory B lymphocytes, recently proposed as counterparts for circulating $IgD^{+}IgM^{+}CD27^{+}$ B cells (Weller, *et al* 2004). Our results would therefore provide additional evidence in support of such a lineage for the expanding subset in PPBL, as was formerly proposed by Salcedo et al (Salcedo, *et al* 2002).

Material and methods

B cells preparation.

Blood samples were collected from healthy volunteers (T0, T1, T2) and PPBL patients (CL3, CL4, CL5, CL7, CL10 and CL12) with informed consent. PBMC were isolated by Ficoll-Paque (Pharmacia Biotech, Baie d'Urfé, Qc Canada) density gradient centrifugation. B cells were negatively selected with Stemsep purification system (Stemcell technologies, Vancouver, BC, Canada) according to supplied instructions.

Evaluation of immunoglobulin secretion.

Cultures were performed in ninety-six wells cell culture plates using $1x10^5$ B cells and $1x10^4$ irradiated, CD40L-transfected murine L4.5 fibroblasts in a final volume of 0.2 ml/well. Culture medium consisted of Iscove (Invitrogen Life Technologies, Burlington, ON, Canada) supplemented with 5% heat inactivated foetal bovine serum (Hyclone, Logan, UT, USA), 5 μg/ml human transferrin (Sigma-Aldrich, Oakville, ON, Canada), 5 μg/ml bovine insulin (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

Either 100 U/ml IL-4 (Genzyme, Cambridge, MA, USA), 50 U/ml IL-2 or 50 ng/ml IL-10 (Biosource, Camarillo, CA, USA) was added to cultures. After ten days incubation at 37° C with 5% CO_2, supernatants were collected and Ig secretion was quantified in a standard Elisa assay. IgM, IgA and IgG isotypes were detected using human μ, α, and γ heavy chains specific monoclonal antibodies (all, Sigma). Bound Igs were revealed with peroxidase-conjugated goat antiserum (Sigma) using ortho-phenylenediamine (OPD, Sigma) as a substrate. O.D. was measured at 490/630 nm.

AID gene sequence analysis.

Total RNA was extracted from patients' purified B cells (CL-5, CL-7) following CD40 plus IL-4 stimulation. Extraction was performed in TRIzol reagent (Invitrogen), according to the manufacturer's instructions. 1μg RNA was used for reverse transcription to cDNA using 10U/μl Moloney leukemia virus reverse transcriptase (MMLV, Invitrogen) and 0.5μmol/l oligo (dT) as a primer in a total volume of 20 μL. PCR amplification was carried out using 2.5 μl cDNA and 2.5U Taq DNA polymerase (New England Biolabs, Mississauga ON, Canada) in a final volume of 50 μL (35 cycles, 94°C, 1min; 55°C, 1min and 72°C, 1 min). The primers used were directed at non coding sequences situated upstream (primer C: 5'-GAG GCA AGA AGA CAC TCT GG) and downstream (primer D: 5'-GTG ACA TTC CTG GAA GTT GC) of AID cDNA. Sequencing in both directions was performed directly from PCR products using an automated DNA sequencer (3100 Genetic Analysis, Hitachi, Applied Biosystems, Foster City, CA, USA). Obtained sequences were then aligned with wild type AID gene mRNA (accession number: AB040431) to detect the presence of mutations.

Induction of AID expression in PBBL B lymphocytes.

Patients' (CL10, CL12) and healthy controls' (T0, T1, T2) B cells were stimulated for 0, 2 and 4 days with CD40L-expressing L4.5 cells and IL-4. Cells were collected, total RNA was extracted in TRIzol reagent (Invitrogen), using 5 μg tRNA as a carrier, and suspended in DEPC water (10μl/1x10^5 initial cells). To reduce the risk of cross-contamination between samples, RT-PCR amplification of AID gene was carried out using Titan one step

RT-PCR (reverse transcription: 55°C, 30min, denaturation: 94°C, 3min followed by 35 cycles: 94°C, 30 sec; 57°C, 45 sec and 72°C, 45 sec). For each sample, 5µl of RNA was used per reaction. Upstream primer C and downstream primer B (5'-CAA AAG GAT GCG CCG AAG CTG TCT GGA G) were used. Amplification of the retinoic acid receptor (RAR) gene, using primers RAR-C (5'-CAG CAC CAG CTT CCA GTT AG) and RAR-I (5'-CCC GGT GAC ACG TGT ACA CC), served as an internal control. Negative controls consisted of RT-PCR reagents with either no RNA and or RNA isolated from L4.5 cells, whereas RNA isolated from the Ramos cell line, which constitutively express AID, was used as a positive control. PCR products were visualized on a 3 % agarose gel stained with ethidium bromide (BioRad, Mississauga, ON, Canada).

Results

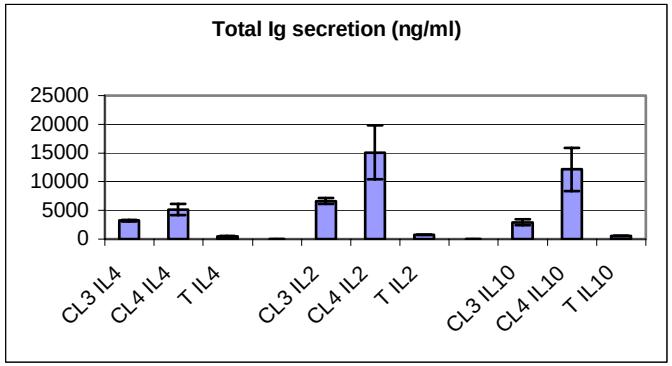
As a way to reproduce T-B cells interactions that preside to the formation of germinal centres and induction of class-switching *in vivo*, PPBL B cells were stimulated *in vitro* using CD40L, expressed on the surface of murine fibroblasts, in the presence of class-switch inducing cytokines, in similitude to the CD40 system first described by Banchereau et al (Banchereau and Rousset 1991), with minor adaptations (Neron, *et al* 1996). As presented in Figure 15A, total Ig secretion was most important in the presence of IL-2 and, on a lesser extent, IL-10 in both patients and healthy control. Strikingly, total Ig production was much higher in PPBL patients, regardless of the nature of the stimulation provided, suggesting that the potential to respond to antigenic challenge via robust Ig production was present in PPBL B cells, and compatible with their memory status. When detailed, Ig secretion in patients consisted almost exclusively in IgM (Figure 15B), in great contrast to the control where, under all culture conditions, approximately half of the total Ig production was accounted for by switched IgA and IgG isotypes (Figure 15C-D). Still, total IgG production in patient CL4 in the presence of CD40+IL-4 (140 ng/ml, ± 5 ng/ml) and IL-2 (377 ng/ml, ± 2 ng/ml) was equivalent to that detected in the control (105 ng/ml, ± 3 ng/ml and 205 ng/ml, ± 6 ng/ml respectively). The same was true for IgA secretion in the presence of CD40+Il-2 (CL4: 109 ng/ml, ± 10 ng/ml versus control: 170 ng/ml, ± 25 ng/ml)

In patient CL3, IgG secretion was detectable in the presence of CD40+IL-2, albeit at lower levels than control (89 ng/ml, ± 8 ng/ml). These observations indicated that, under certain culture conditions, the apparent defect in switched isotypes secretion rather reflected increased IgM production. Nevertheless, at this point, impairment in class-switching could not be dismissed altogether and additional investigations were carried out to further clarify the question. We have already demonstrated that expression and sequence of the CD40 molecule in PPBL patients are normal (Loembe, *et al* 2001). Lack of proliferative response to CD40 ligation has been observed, but it has since been demonstrated that this functional trait could represent a normal feature of $CD27^{+}$ memory B cells following long-term stimulation (Fecteau and Neron 2003). In line with this idea, monitoring of either CD40-L and IL-4, or CD40-L and IL-2+IL-10 stimulated B cells, by means of fluorescence-activated cell sorting (FACS) analysis, revealed an almost complete disappearance of the CD27-expressing population in two tested PPBL patients after 5 days (CL10 and CL12, results not shown). To examine the possibility of an AID-dependent class switch defect in PPBL, we thus proceeded with the analysis of the AID molecule in two patients (CL10 and CL12) and healthy controls (T1 and T2). AID mRNA induction was monitored by RT-PCR, following *in vitro* stimulation with CD40 (Figure 16). In both patients and healthy controls, AID transcripts were induced in a time-dependent fashion with increased expression at day four. Sequencing of the complete AID cDNA was additionally carried out in two patients (CL5, CL7). Contrarily to what is observed in HIGM-2, no mutations were detected in any of the patients tested.

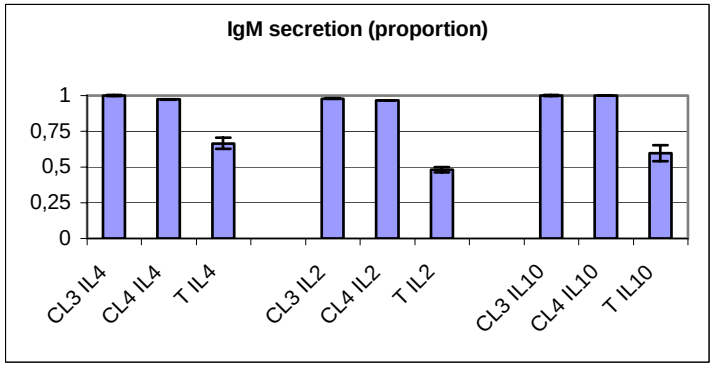
Figures

Figure 15: Evaluation of Ig secretion in CD40-stimulated PPBL B lymphocytes.
Purified peripheral blood B cells, isolated from PPBL patients (CL3 and CL4) and one healthy adult control (T0) were cultured in the CD40-dependent cell culture system as described in the text in the presence of either IL-4, IL-2 or IL-10 soluble cytokines. Ig secretion was evaluated by standard ELISA assay. A): total Ig secretion. B), C) and D): relative percentage of IgM, IgG and IgA secretion respectively.

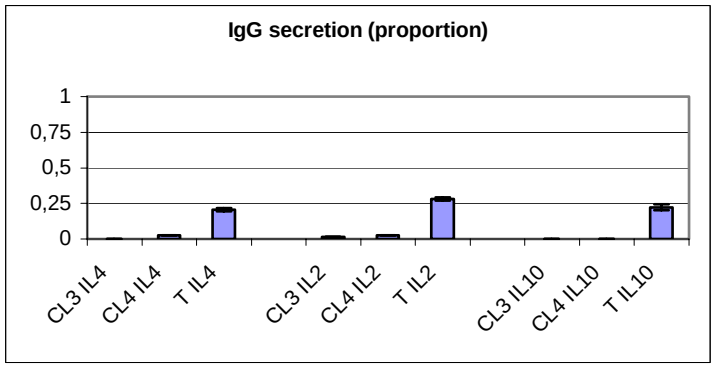
A)


Total Ig secretion (ng/ml)
25000
20000
15000
10000
5000
0
CL3 IL4
CL4 IL4
T IL4
CL3 IL2
CL4 IL2
T IL2
CL3 IL10
CL4 IL10
T IL10

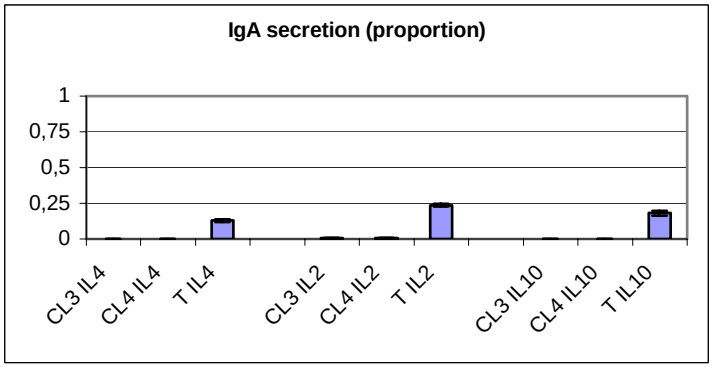
B)


IgM secretion (proportion)
1
0,75
0,5
0,25
0
CL3 IL4
CL4 IL4
T IL4
CL3 IL2
CL4 IL2
T IL2
CL3 IL10
CL4 IL10
T IL10

C)


IgG secretion (proportion)
1
0,75
0,5
0,25
0
CL3 IL4
CL4 IL4
T IL4
CL3 IL2
CL4 IL2
T IL2
CL3 IL10
CL4 IL10
T IL10

D)


IgA secretion (proportion)
1
0,75
0,5
0,25
0
CL3 IL4
CL4 IL4
T IL4
CL3 IL2
CL4 IL2
T IL2
CL3 IL10
CL4 IL10
T IL10

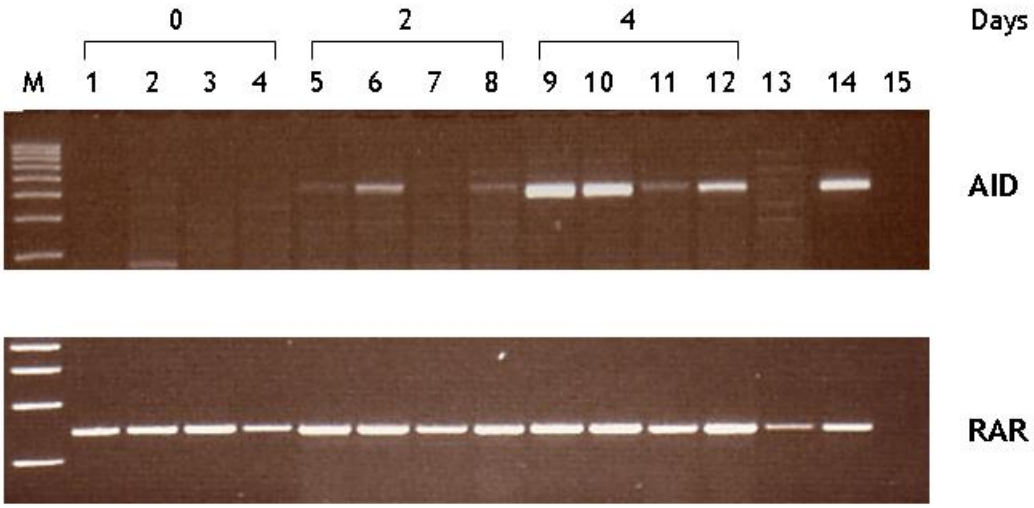


Figure 16: Induction of AID mRNA in B cells after *in vitro* CD40 stimulation.

AID transcripts induction in purified B cells was evaluated after 0, 2 and 4 days stimulation with CD154 and IL-4 as described in Material and methods. Healthy controls T1 (lanes 1, 5, 9), T2 (lanes 2, 6, 10), patients CL 10 (lanes 3, 7, 11) and CL12 (lanes 4, 8, 12) are presented. M: 100pb DNA ladder. Lane 13 and 15: negative controls (L4.5 fibroblasts RNA, or no RNA respectively). Lane 14: positive control (Ramos cell line).

Discussion

As is attested by the experimental results reported herein, PPBL patients have an intact, CD40-inducible AID gene, indicating that a deficiency in this particular molecule is unlikely to result in impaired switching to downstream isotypes. In this regard, we can therefore conclude that PPBL and HIGM-2 are distinct haematological entities. Nevertheless, as IgM is predominantly secreted in PPBL patients, even in the presence of class-switch inducing cytokines IL-2 and IL-10, hypothesis of a defective class-switch recombination mechanism cannot be completely dismissed in this disorder.

Interestingly, the existence of a fourth subtype of HIGM (HIGM4), resulting from an AID and CD40/CD40-L independent mechanism, has recently been reported (Imai, *et al* 2003). Similarly to PPBL patients, HIGM4-affected individuals show normal AID expression and presence of somatic hypermutation $IgD^{+}IgM^{+}CD27^{+}$ B cells at levels comparable to that detected in healthy individuals. Moreover, a familial inheritance pattern is also suspected. However, contrarily to PPBL, HIGM4 is more frequent in male than female patients, IgA and IgG isotypes are low to undetectable, autoimmune manifestations and lymphoid organs hyperplasia are common. Circulating B cell numbers and CD27 expression are normal. These differences in clinical phenotype do not support a relationship between HIGM4 and PPBL. Uncovering of HIGM4 has provided clinical evidences to reinforce experimental data suggesting a differential regulation of the SH and CSR processes by AID, and pointing to the existence of CSR-specific cofactors (Ta, *et al* 2003). Conversely, as AID-induced DNA double strand breaks can be detected in HIGM4 patients, it has been suggested that the observed CSR defect would rather be situated downstream of AID function (Imai, *et al* 2003). In any event, formal identification and characterization of these cofactors or additional steps required for completion of CSR will be needed to definitely rule out the possibility of a differentiation block in PPBL B lymphocytes.

The presence of somatically mutated memory cells among the IgD^{+} IgM^{+} B lymphocyte compartment has only recently been acknowledged and has particularly been highlighted with the demonstration that these cells express the memory marker CD27. Moreover,

evidences have been accumulating lately which indicate that $IgD^+IgM^+CD27^+$ B lymphocytes could actually belong to a separate developmental lineage. Firstly, they are the only memory subset detectable in HIGM-1 patients which, as a result of a defective CD40-L molecule, are unable to develop GC, therefore suggesting that these cells would derive from a GC-independent diversification pathway (Weller, *et al* 2001). Secondly, detailed experimental data (immunophenotyping, gene expression profiling and CDR3 spectratyping) have shown that peripheral blood $IgD^+IgM^+CD27^+$ B lymphocytes are likely to be the circulating counterparts of splenic MZ B cells, a population that plays a significant role in T-independent immune responses (Weller, *et al* 2004). As peripheral blood $IgD^+IgM^+CD27^+$ B lymphocytes had previously been assimilated to classical GC-derived, switched B lymphocytes, they have not been the objects of many functional studies as a distinct memory subset. Nevertheless, reports have mentioned a poor switch capacity, as well as preferential and vigorous IgM production both in splenic and peripheral blood $IgD^+IgM^+CD27^+$ B lymphocytes (Shi, *et al* 2003, Tangye, *et al* 1998). The functional properties of PPBL B lymphocytes reported herein reflect these aforementioned phenotypic features. Consequently, our experimental data, combined with the exhaustive analysis of cell surface markers in PPBL B cells by Salcedo *et al* (Salcedo, *et al* 2002), would substantiate the assertion that this disorder results from an expansion of MZ memory B cells. Furthermore, it has been proposed that $IgD^+IgM^+CD27^+$ B lymphocytes would provide an immediate, T-independent, line of defence against encapsulated bacteria through the secretion of pre-diversified, natural IgM antibodies (Kruetzmann, *et al* 2003, Weller, *et al* 2004). This hypothesis would be in accordance with the demonstration that no antigenic selection pressure could be evidenced following molecular analysis of mutated Ig variable regions in B cells isolated from PPBL patients (Loembe, *et al* 2002). Finally, in a recent report by Feugier *et al* (Feugier, *et al* 2004), altered expression of adhesion molecule has been identified in five PPBL patients. Levels of expression were generally found to be lower than that expected in healthy individuals. Thus, improper homing to lymphoid compartments could play a significant role in the preferential accumulation of $IgD^+IgM^+CD27^+$ B lymphocytes within the peripheral blood in PPBL patients.

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Discussion générale

La lymphocytose polyclonale chronique est-elle une étape initiale dans la genèse d'un lymphome ? Synthèse des données immunologiques récentes.

Manuscrit non publié

Persistent Polyclonal B Cell lymphocytosis: The Making of a Lymphoma? An immunological perspective.

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Résumé

Décrite pour la première fois il y a vingt ans, la lymphocytose polyclonale B chronique (LPCB) a suscité bien des questionnements parmi les chercheurs et les cliniciens. Diagnostiqué principalement chez des fumeuses adultes, ce syndrome inhabituel présente des critères de bénignité (expansion polyclonale, accumulation polyclonale d'IgM sérique, symptomatologie discrète, stabilité clinique) contrastant avec des attributs possiblement indicateurs de progression maligne (cellules morphologiquement atypiques, translocations *bcl2/Ig* multiples, anomalies du chromosome 3, infiltration de la moelle osseuse et, dans de rares cas, néoplasie lymphoïde ultérieure ou simultanée) et constitue donc une entité hématologique singulière. La nature polyclonale de la LPCB et l'apparente hétérogénéité de la population cellulaire impliquée ont longtemps entravé les efforts de caractérisation phénotypique et fonctionnelle. Cependant, les dernières années ont connu un influx remarquable de données expérimentales concernant la biologie du développement du lymphocyte B. Nombre de mécanismes jalonnant la maturation d'affinité ont été élucidés et des facteurs enzymatiques clés ont été identifiés. Ces avancées ont jeté un nouvel éclairage sur le processus de lymphomagenèse chez le lymphocyte B. Cette revue illustre l'impact de ces avancées théoriques et techniques dans le cas concret de la LPCB. Une rétrospective est faite des différentes avancées qui ont mené à l'identification de l'équivalent cellulaire normal dans la lymphocytose et qui ont contribué à l'élaboration d'une définition clinique plus exhaustive du syndrome. Diverses théories pouvant expliquer les origines de la lymphocytose sont explorées et les indices éventuels de progression à long terme sont discutés. En l'absence d'une réponse définitive concernant les risques de transformation maligne subséquente dans la LPCB, de nouvelles avenues de recherche sont suggérées et des recommandations faites quant au suivi des patients.

Abstract

First described twenty years ago, persistent polyclonal B cell lymphocytosis (PPBL) has since stirred much perplexity among researchers and clinicians. Diagnosed principally in adult female smokers, this unusual haematological disorder shares features of both benignity (polyclonal expansion, polyclonal IgM secretion, lack of significant clinical symptoms, stable and mostly uneventful course) and malignancy: (morphologically atypical binucleated cells, *bcl-2/Ig* genes translocations, chromosome 3 anomalies, bone marrow involvement and, in rare patients, concurrent occurrence of neoplasia). As explained in the present review, the polyclonal nature of PPBL and the apparent heterogeneity of the involved cellular population long impeded phenotypic and functional characterization of the disorder. The last years however have witnessed tremendous progresses in the field of B lymphocyte immunobiology. Technical inputs from the molecular field have lead to a better discrimination of developmentally distinct B cells subsets and, by extension, of B cell lymphoid disorders. Numerous molecular mechanisms and enzymatic actors involved in peripheral B cell maturation have been uncovered. Altogether, these achievements yielded a better understanding of the lymphomagenesis process. As far as PPBL is concerned, formal characterization of the expanding IgM^+IgD^+ memory B lymphocyte subset has ensued and a better delineation of its clinical definition has emerged. Indeed, deceitful cytogenetic and histologic features in PPBL have formerly exposed some patients to unneeded intrusive therapy, emphasizing the requirement for proper diagnosis of this syndrome. Still, it is unclear at this point whether progression to malignancy represent the long-term issue in PPBL, making attentive monitoring of patients mandatory. As testified by is virtual absence from haematology manuals and by the lack of recent comprehensive review, PPBL has not yet gained mainstream recognition as a distinct physiopathological entity. With this paper, we aim to provide a retrospective view of the research realizations during these last twenty years and an up-to-date picture of PPBL.

Keywords: PPBL, memory B cell, marginal zone B cell, affinity maturation, antigenic selection, lymphomagenesis.

Molecular analysis of Ig genes and PPBL: what we have learned.

Supplementary immunophenotyping of peripheral B lymphocytes in PPBL, using the newly defined memory marker CD27, and molecular analysis of Ig variable regions were undertaken in order to gain some insight in the developmental status of circulating B lymphocytes in patients. The memory subset, which represents 40% of peripheral B lymphocytes in healthy individuals, has thus been found to be increased up to 80% in PPBL patients. Noticeably, the memory compartment almost exclusively consists in $IgM^{++}IgD^{+}$ coexpressing B lymphocytes (Himmelmann, *et al* 2001, Loembe, *et al* 2002, Salcedo, *et al* 2002), a population that only accounts for about one third of $CD27^{+}$ memory B cells in healthy individuals, alongside class-switched, IgM-only, and a few IgD-only B lymphocytes (Klein, *et al* 1998). This raise in CD27 expression is corroborated by a parallel elevation in CD148 expression, a second memory B cell marker, originally identified on somatically mutated splenic marginal zone (MZ) B cells (Himmelmann, *et al* 2001, Tangye, *et al* 1998). Taking into account the observed lymphocytosis, this amounts to a twenty-fold increase of the specific $IgM^{++}IgD^{+}CD27^{+}$ population in patients. Those observations have simultaneously led us, and another team, to proceed with the in depth molecular analysis of immunoglobulin genes in patients, since this strategy had proven so useful for investigation and classification of neoplastic lymphoid disorders (Loembe, *et al* 2002, Salcedo, *et al* 2002). Both studies have generated concordant results: in an average proportion of 73%, V_H genes have been found to be somatically mutated. No bias in the Ig repertoire has been detected: V_H genes usage among PPBL patients was heterogeneous and reflected the relative abundance of each V_H gene family. Between both studies, the mutation frequency varied between 0.52% and 3%, with a mean value of 1.85%. Statistical analysis of mutations distribution in V_H genes was also undertaken. Evidence for antigen-driven negative selection, specifically suppression of replacement mutations in FR, has not been found, except in a few mutated sequences (Loembe, *et al* 2002: 4/21, Salcedo, *et al* 2002: 12/51). It therefore seems PPBL results from the preferential expansion of the memory $IgM^{++}IgD^{+}CD27^{+}$ B lymphocyte population. Intriguingly, the majority of these

cells do not display the imprint of antigen selection that is considered to be the hallmark of the affinity maturation process in germinal centres.

$IgM^{++}IgD^{+}CD27^{+}$ memory B cells in PPBL, a GC derived subset…

Presence of somatic hypermutations in V_H genes, CD27 expression, and absence of GC associated markers CD38 and CD10, all suggests a post-germinal centre origin for the expanding $IgM^{++}IgD^{+}$ B cell population in PPBL. In human, hypermutated memory $IgM^{+}IgD^{+}$ populations have been identified in the bone marrow (Paramithiotis and Cooper 1997), peripheral blood (Agematsu, *et al* 1997, Klein, *et al* 1998), and among the MZ B cells (Dono, *et al* 2000, Tangye, *et al* 1998). The latter two subsets especially have multiple phenotypical features in common with PPBL including: CD27 expression, high levels of sIgM, large abundant cytoplasm and, specifically for MZ B lymphocytes, irregular nuclear morphology with frequent expression of CD21 and CD25. Mutation frequencies observed in V_H genes in PPBL (~1.85%) are closest to that seen in marginal zone and bone marrow $IgM^{+}IgD^{+}$ memory B subsets (see Table 10), and are definitely lower than that reported in class-switched memory B cells (Pascual, *et al* 1994).

The marginal zone is a micro-anatomically defined structure of the spleen, defined as the outermost margin of the follicular mantle zone. It is mainly populated by B cells displaying a phenotype intermediate between small resting lymphocytes and activated plasmablasts: the MZ B cells (Dono, *et al* 1996, Hsu 1985). This lymphoid compartment's function as a reservoir for memory B cells involved in the response to TD and T-independent type 2 (TI-2) antigens was first extensively described in the rat (MacLennan and Liu 1991). In humans, the term marginal zone is now commonly used in a larger sense not only to refer to the extrafollicular areas of the spleen but also to similar micro-anatomical regions in other lymphoid tissues, namely: the subepithelial zone of the tonsils, the dome of the Peyer's patches, the subcapsular areas of the lymph nodes, and extranodal reactive mucosa-associated lymphoid tissues (MALT) (Dono, *et al* 2003). Typically, MZ B cells are positioned at the front line of potential antigenic assault. B cells in this subset share common morphological attributes (see Table 10), are usually non cycling and express high level of IgM with no or low IgD (Dono, *et al* 1996, Hsu 1985). Molecular studies, on

the other hand, have revealed quite a level of heterogeneity among MZ B cells within a single anatomical site, with some expressing unmutated and others mutated V_H genes. Those cells with mutations may or not present evidence of antigen-driven selection (Dono, *et al* 2000, Dunn-Walters, *et al* 1995, Tierens, *et al* 1999). It is conventionally believed that the non-cycling hypermutated MZ B cells are the progeny of adjacent GC (Liu, *et al* 1988). Functional diversity in MZ B lymphocytes is further attested by the differential expression of cell surface markers according to their anatomic location: CD21 expression is high in splenic MZ B cells but low in their tonsillar counterparts (Dono, *et al* 2003), whereas the newly discovered IRTA-1 receptor is selectively expressed by tonsillar, MALT, and Peyer's patches MZ B lymphocytes (Falini, *et al* 2003). Moreover, IgA and IgG expression is mostly restricted to splenic MZ B lymphocytes (Dono, *et al* 1996, Tangye, *et al* 1998). Differences in the type of antigenic challenge (TD versus TI-2) and in the level of antigen exposure could largely account for this heterogeneity in MZ B lymphocytes. The particular $IgM^{++}IgD^{+}$ MZ B cells subset has been investigated in the tonsils (Dono, *et al* 1996, Dono, *et al* 2003) and in the spleen (Tangye, *et al* 1998). This population is chiefly comprised of memory B cells as attested by CD27 and CD148 expression, presence of somatic mutations in V_H genes and, in the tonsil subset, evidence for antigen-driven selection (Table 10). Functionally, in analogy to animal models, these cells take part in both TD and TI-2 immune responses (Dono, *et al* 2003). Furthermore, following CD40 engagement, splenic $IgM^{++}IgD^{+}$ MZ B cells secrete both IgM and switched isotypes. In the rat, MZ B cells do not recirculate, except for their recruitment to the follicules upon recall immune reponses (Liu, *et al* 1988). Contrastingly in humans, this concept is challenged by the presence of B cells with characteristics similar to MZ B lymphocytes in the blood and to a lesser degree in the bone marrow (Table 10 and (Weller, *et al* 2004)). Some authors have consequently proposed that, following antigenic stimulation and somatic diversification, either in a TD or TI fashion, MZ B cells could transit trough peripheral blood before homing to the marginal zone of neighbouring lymphoid organs. They could also migrate to the bone marrow where local environmental signals would promote terminal differentiation to high affinity plasma cells (Paramithiotis and Cooper 1997, Tierens, *et al* 1999).

Ensuing the extensive cytofluorometric assessment of cell surface molecules expression in PPBL B lymphocytes, complemented by the molecular analysis of Ig genes, Salcedo and collaborators have designated memory cells from the marginal zone compartment as normal counterparts for circulating $IgM^{++}IgD^{+}CD27^{+}$ B cell in patients (Salcedo, *et al* 2002). Although this hypothesis seems highly plausible, supplementary immunophenotyping and functional studies are needed to warrant definitive validation of the developmental lineage in PPBL B lymphocytes.

…Or a separate, GC- independent, memory B cell lineage?

Recently, somatically mutated $IgM^{+}IgD^{+}CD27^{+}$ memory B cells were identified in the peripheral blood of hyper IgM (HIGM1) patients who cannot form GC because of an invalidating mutation in CD154, the CD40 ligand. Conversely, and in accordance with their GC origin, class-switched and IgM only $CD27^{+}$ memory B cells were not detected (Weller, *et al* 2001). This observation has raised a serious debate about the notion of a GC-dependent origin in $IgM^{+}IgD^{+}CD27^{+}$ memory B lymphocytes. Weller and collaborators, the team at the origin of this significant discovery, suggested that peripheral blood $IgM^{+}IgD^{+}CD27^{+}$ memory B cells are the circulating counterparts of MZ $IgM^{++}IgD^{+}CD27^{+}$ B cells. Furthermore, these cells would be involved in TI responses, particularly TI-2 responses (Kruetzmann, *et al* 2003, Weller, *et al* 2004), implying that somatic diversification of the Ig receptor in HIGM1 patients would arise from GC-independent developmental pathway (Weller et al 2003). In line with this view, cycling ($KI\text{-}67^{+}$) MZ B cells with a memory phenotype were identified in nodal tissues sections and they displayed no clonal relationship to proximate GC, suggesting that these lymphocytes had possibly mutated *in situ* independently of the GC environment (Tierens, *et al* 1999). In similitude to mice B1 B cells, antigen exposure would be a prerequisite to specific antibody production by $IgM^{++}IgD^{+}CD27^{+}$ B cells (Fagarasan and Honjo 2000, Kruetzmann, *et al* 2003). In this sense, this subset would not represent the "true", GC-derived, memory pool, but rather mediate a natural yet specific immunity, at the junction between the innate and the T-dependent, adaptive immune response, insuring the first line of defence against encapsulated bacteria (Kruetzmann, *et al* 2003, Weller, *et al* 2004). The fact that deregulation of homeostasis observed in PPBL patients solely affects the $IgM^{++}IgD^{+}CD27^{+}$

lymphoid compartment makes a distinct origin for this particular memory subset a conceivable hypothesis. This disorder could thus add to the emerging picture of a GC-independent developmental pathway for $IgM^{+}IgD^{+}CD27^{+}$ B cells.

Is a deregulated memory response the foundation for PPBL?

On a practical level, addressing the phenotype and lineage of the expanding B cell population in PPBL patients has had appreciable repercussions for clinicians as it provided them with additional and valuable diagnostic tools. But the investigative objectives pursued by researchers were primarily to uncover the cause(s) behind the disorder and to predict its evolution, if any was to be expected. And, in this regard, no clear-cut answer has been offered as yet. Two scenarios have nevertheless been advanced to explain polyclonal amplification of the $IgM^{++}IgD^{+}CD27^{+}$ B cell lymphoid compartment in patients: either 1) an increase in memory B cells production (as a result of chronic antigenic stimulation) or 2) a decrease in their developmentally programmed elimination (due to impairment of apoptosis).

The first supposition is based on several lines of evidence demonstrating a connection between infectious agents and the emergence of various B cell lymphoid disorders (Dolcetti and Boiocchi 1996). The cause-effect relationship between B cell-tropic viruses with transforming capacities, such as EBV, and lymphoma in immunosupressed individuals is a well-documented example (Crawford 2001). More recently, evidences have also accumulated for the indirect implication of pathogens in B cell lymphoproliferations, specifically those originating from the marginal zone, as a result of sustained antigenic stimulation. Such is the case for *Helicobacter pylori* infection and gastric mucosa associated lymphoid tissue (MALT) lymphoma where pathogen eradication by antibiotherapy frequently leads to disease regression (Wotherspoon, *et al* 1993). Epidemiological studies suggest a similar cause-effect relationship between splenic marginal zone lymphoma with villous lymphocytes (SLVL) and the hepatitis C virus (Hermine, *et al* 2002). As far as PPBL is concerned, a role for EBV in the natural history of the disease has long been postulated as formerly discussed. Although direct B cell infection and transformation by a virus variant could not be formally evidenced, the

possibility remains that an as yet unidentified (environmental?) factor could drive chronic reactivation of lytic infection in patients, generating a persistent memory $IgM^{++}IgD^{+}$ B cell immune response, in either a GC-dependent or GC-independent pathway. Coincidentally, one of the anticipated anatomical location for $IgM^{++}IgD^{+}CD27^{+}$ memory cells, namely the subepithelial zone of the tonsils, was also proposed as the primary site for EBV infection in healthy individuals (Faulkner, *et al* 2000). Repeated virus production in this site could evoke a sustained immune response in resident $IgM^{++}IgD^{+}CD27^{+}$ memory cells, in similitude to what is observed in MALT lymphoma. On the other hand, no restriction of the immunoglobulin V_H gene repertoire was evidenced in PPBL patients. Contrastingly, this phenomenon has been reported in lymphoma derived from antigen-experienced memory B cells (Fais, *et al* 1998, Weng and Levy 2003), or even after *Haemophilus influenzae* immunization in healthy individuals (Adderson, *et al* 1991). In addition, the lack of evidence for antigenic selection pressure in expressed V_H gene regions further challenges the model of chronic antigenic selection in PPBL.

As we already pointed out, maintenance of homeostasis in the B cell lymphoid compartment is dependent upon stringent regulation mechanisms that insure control of the molecular processes involved in somatic diversification of the antibody repertoire. In the GC, balanced survival and elimination of mutated clones, through the process of antigen-driven selection, is necessary to delete potentially harmful autoreactive clones. It also prevents the feeding of superfluous low-affinity mutants in the memory pool. Thereby, only the best-fitted antigen-specific mutants are allowed to differentiate to memory B cells. The fact that $IgM^{++}IgD^{+}CD27^{+}$ B cells in PPBL patients showed no evidence for antigen-driven selection, as indicated by the distribution of replacement versus silent mutations in Ig V_H genes, was intriguing given that both their immunophenotype and the molecular configuration of their Ig genes were otherwise indicative of a post-GC origin. Lack of evidence for antigen-driven selection can signify that the process of affinity maturation, the hallmark of a TD immune response, is altered in PPBL patients. In light of those observations, we have put forward a second scenario whereby impairment of the antigen-driven selection mechanism would enable the survival of low affinity mutants B cells within GC in PPBL, allow their recruitment into the memory B cell pool, and subsequently cause expansion of this compartment in the periphery. This hypothesis appears to be

corroborated by several experimental facts. First, expression the Bcl-2 and the Bcl-x_L anti-apoptotic proteins have been reported to be upregulated in PPBL. The interplay between differential expression of key pro and anti-apoptotic genes in GC B cells is crucial to the antigen-driven selection process. Tonsillar GC B cells accordingly express an apoptosis-sensitive phenotype, namely high expression of the pro-apoptotic CD95, Bax, Bak, Bim, and c-Myc proteins combined with low expression of the survival protein Bcl-2 (Liu and Arpin 1997, Yokoyama, *et al* 2002). This expression pattern apparently predisposes them to deletion through CD95/Fas-mediated killing, unless they can be rescued by efficient binding of immunizing Ag on the surface of follicular dendritic cells (FDC) and interaction with antigen specific CD40-L expressing T cells in the light zone of GC (Liu, *et al* 1989). CD95-induced cellular apoptosis can utilize two different signalling pathways (Mizuno, *et al* 2003). Type I apoptosis proceeds through association of a death inducing complex (DISC) where CD95 death domain (DD) recruits the CD95/Fas containing associated death-domain containing adapter protein (FADD) and procapase 8, later leading to the activation of caspase 8 and the ensuing apoptotic signalling cascade. In type II or mitochondrion-dependent apoptosis, an amplification step is necessary which involves mitochondrial release of cytochrome C, apoptosome assembly and activation of capase 9. Members of the Bcl-2 protein family can specifically block this latter type of apoptosis. Which type of Fas-induced apoptosis is predominant in GC B cells is still subjected to debate. Some experimental data indicates that both types I and II apoptosis could contribute to the process of antigen-driven selection. In point of fact, animals with decreased CD95 expression (*lpr/lpr* phenotype) (Takahashi, *et al* 2001), as well as those possessing a transgene for either Bcl-2 or Bcl-x_L constitutive expression (Smith, *et al* 2000, Takahashi, *et al* 1999), all display a distortion of affinity maturation that leads to the accumulation of low affinity memory B cells. Contrarily to Bcl-2, Bcl-xL expression is high in GC B cells (Tuscano, *et al* 1996). Moreover, rescue from CD95-mediated apoptosis, mimicked *in vitro* by CD40 stimulation and Ig cross-linking, correlates with up-regulation of Bcl-x_L (Zhang, *et al* 1996), suggesting an active regulatory role for this anti-apoptotic protein. In the same way, increased Bcl-x_L expression in PPBL patients could confer resistance to CD95-mediated apoptosis. However, one cautionary note regarding this conclusion resides in the fact that Bcl-2 and the Bcl-x_L expression levels in patients

were compared to protein expression in total B cells, which comprised a majority (~60%) of naïve B cells. Whereas Bcl2 protein expression is similar in naïve and memory B cells, Bcl-x_L mRNA is about 8-fold higher in the memory compartment (Bovia, *et al* 1998). The apparent up-regulation of this protein in patients could thus be a mere reflect of an increased proportion in memory B cells. Nevertheless, *in vitro* resistance to Fas-mediated apoptosis has concomitantly been reported in PPBL B lymphocytes (Roussel, *et al* 2003). Again, normal controls consisted not in memory B cells, but rather in the Ramos cell line, with a GC phenotype and a conceivably differential expression of apoptosis-related genes. Nevertheless, since PPBL B lymphocytes displayed apoptotic features when treated with the anti-neoplastic etoposide reagent, the observed resistance appeared to be specific to the CD95-induced apoptotic pathway. CD95 has been described as a tumour suppressor gene, as loss of susceptibility to CD95-mediated killing often correlates with tumour progression. Notably, impairment of CD95 function would allow the survival of GC generated autoreactive or pre-malignant B clones that would otherwise be eliminated by antigen-driven selection. Support for this role is provided by studies in animal models with a *lpr/lpr* phenotype that display increased susceptibility for B cell malignancies (Davidson, *et al* 1998). The same holds true for CD95 deficient human patients with the autoimmune lymphoproliferative syndrome (ALPS) who lack a functional CD95 protein (Straus, *et al* 2001). Down-regulation of CD95 protein expression, disruption of the CD95 signalling cascade, or somatic mutations of the CD95 encoding gene, particularly those affecting the death domain (DD) region, are all mechanisms that have been identified in lymphoid malignancies and which could promote lymphomagenesis (for a detailed review of CD95 resistance mechanisms and their contribution to lymphomagenesis please see Mizuno, *et al* 2003 and Muschen, *et al* 2002). With regards to PPBL, CD95 expression in $IgM^{++}gD^{+}CD27^{+}$ memory B lymphocytes is similar or superior to that detected in healthy controls (Salcedo, *et al* 2002 and our own observations), therefore the observed resistance cannot be related to negative modulation of the receptor in patients. Still, mutations in CD95 DD could be present that would allow cell surface expression of the protein but interfere with signalling. Presence of the DISC components FADD and caspase 8 has been detected (Roussel, *et al* 2003), but it is not known at this point if they assemble properly. Moreover, expression of FLIP or FAIM, known mediators of CD95 resistance (Schneider,

et al 1999, Wang, *et al* 2000), has not yet been investigated. The postulate of a deficient antigen-driven selection mechanism in PPBL has recently been complemented by the uncovering of IgM anti-phospolipid antibodies (apA/cofactor) in patients despite the absence of noticeable autoimmune disease (Granel, *et al* 2002). Hence, propensity for increased survival of auto-reactive and low affinity B cells clones seems to exist in PPBL patients. We ergo propose that a deregulation of the physiological processes implicated in the naïve to memory B cell transition could contribute significantly to the disruption of homeostasis in PPBL patients.

And what can we anticipate?

Genetic instability and the risk of malignant transformation in PPBL.

Although the majority of patients have had uneventful follow-ups amounting to more than 25 years in some cases, there is a persistent concern that the disruption of homeostasis observed in PPBL could in fact represent the first stage in a multi-step progression toward a more aggressive proliferation. Accordingly, in two patients, PPBL diagnosis has been associated with the occurrence of non-Hodgkin lymphomas: DLBCL (19 years after diagnosis) (Roy, *et al* 1998), and MALT lymphoma (concomitantly with diagnosis) (Callet-Bauchu, *et al* 1999). Clonal or oligoclonal V_H genes rearrangements have been detected in four patients, yet no further evolution was reported for these cases (Chan, *et al* 1990, Delage, *et al* 1997, Feugier, *et al* 2004). These observations nevertheless emphasize the notion that emergence of a predominant, and potentially malignant, clone is a likely outcome in PPBL. Bone marrow B cell intra-vascular infiltration was recently reported to be a recurrent finding in PPBL patients (Feugier, *et al* 2004). Moreover, careful microscopic observation has also revealed the presence of nuclear pockets in atypical B lymphocytes, a feature which is usually witnessed in pre-leukemic or leukemic leucocytes (Casassus, *et al* 1987, Espinet, *et al* 2000, Woessner, *et al* 1999). The discovery of clonal genetic abnormalities in PPBL B cells, contrasting with the polyclonal nature of the proliferation, illustrates the risk of subsequent transformation in this disorder. As mentioned previously, isochromosome (+i3q), first documented as an isolated event in

1989 (Perreault, *et al* 1989), has since been demonstrated to be a recurrent finding among PPBL patients (Callet-Bauchu, *et al* 1997, Mossafa, *et al* 1996): it was observed in 29/41 cases tested (see table 3, column 8). Additional numerical aberrations involving chromosome 3, namely trisomy 3, partial duplication 3q, and derivative 3, were also sporadically reported (table 3, column 8). Unlike PCC, whose occurrence has been linked to the presence of multinucleated cells (Mossafa, *et al* 1999), the (+i3q) aberration is apparently not restricted to a morphologically distinct B cell population and both events are independent of the light chain isotype (Callet-Bauchu, *et al* 1997, Espinet, *et al* 2000). Interestingly, observations made at referral and then at 2 years follow-up seem to indicate an accumulation of these chromosomal anomalies in at least one patient (Callet-Bauchu, *et al* 1999). Among non-Hodgkin lymphoid disorders, chromosome 3 aberrations are more frequently identified in MZL, more precisely in the extranodal (MALT), nodal (monocytoid B cell lymphoma or MCBL) and splenic marginal zone B cell lymphoma (or SMZL, including splenic lymphoma with villous lymphocytes [or SLVL]) types (Dierlamm, *et al* 1996). Consistent with the postulated origin for $IgM^{++}IgD^{+}CD27^{+}$ B lymphocytes in PPBL, SMZL shares many additional features with this disorder:

-Generally slow clinical progression.
-A heterogeneous morphological composition involving a mixture of plasma, blast-like and small cells, occasionally presenting a cleaved nucleus.
-Peripheral blood and bone marrow involvement (Thieblemont, *et al* 2003).
-Lack of expression of CD5, CD10 and CD23.
-Heterogeneous utilisation of V_H genes among cases, and presence of somatic hypermutations without evidence of antigenic selection in most cases (Dierlamm, *et al* 1996).

Unlike PPBL however, SMZL are clearly monoclonal with detectable IgH rearrangements. In addition, no *bcl2* gene rearrangement can be observed, which is in sharp contrast to what is observed in PPBL.

In eleven patients of the cohort followed by our team in Quebec City, nested-PCR amplification has allowed the detection of multiple distinct *bcl-2/Ig* genes rearrangements (up to seven), involving both the mcr (minor cluster region) and MBR (major breakpoint region), in all but one case (only one rearrangement) (Delage, *et al* 1997, Delage, *et al* 1998). These observations were later reproduced in additional patients, and the frequency of the t(14;18) translocation was estimated between $1/10^2$ and $1/10^4$ cells (see table 3, column 8). In analogy to the +i(3q) chromosomal anomaly, there appears to be an accumulation of the *bcl-2/Ig* rearrangements in some patients (Delage, *et al* 1998). *bcl-2/Ig* genes rearrangements are also reported in approximately 50% of healthy individuals, however their frequency is lower ($1/10^5$ to $1/10^6$) and multiple rearrangements are only revealed with very sensitive detection methods (Ji, *et al* 1995). Bcl-2 oncogenic potential is illustrated by the occurrence of the t(14;18) translocation in 80% of cases of follicular lymphoma (FL), the most frequent human B cell malignancy. A positive correlation was observed between age or tobacco usage and the presence of *bcl-2/Ig* rearrangements in healthy individuals (Bell, *et al* 1995, Liu, *et al* 1994). As the incidence of non-Hodgkin lymphoma is also increased in aged people and smokers, authors have accordingly proposed that the presence of *bcl-2/Ig* rearrangements reflects an individual's risk for developing a subsequent lymphoid malignancy.

At the cellular level, the t(14;18) chromosomal translocation produces a *bcl-2*-immunoglobulin fusion gene and, owing to the resulting proximity with the Ig transcriptional enhancer, leads to an overexpression of the Bcl-2 protein (Graninger, *et al* 1987). Furthermore, this deregulated expression interferes with apoptosis, though it does not promote cellular proliferation (Hockenbery, *et al* 1990). Interestingly, mice models bearing a *bcl-2/Ig* minigene initially display an indolent lymphoid hyperplasia consisting of polyclonal IgM^+IgD^+ resting B cells. Slow progression to clonal DLCL, apparently as the result of secondary genetic alterations (*c-myc* translocation), ensues in these mice (McDonnell and Korsmeyer 1991). Thus *bcl-2* acts as a proto-oncogene who, once translocated, promotes B cell survival and increases the risk for subsequent tumorigenic genome alterations leading to the emergence of neoplasia. In humans, the similitude with PPBL is remarkable, and that a similar outcome could occur in patients is indeed a troubling probability. Still, at the physiological level, presence of *bcl2* gene translocations

doesn't always translate into increased protein expression, especially at the relatively low translocation frequency observed in PPBL patients. Accordingly, reports of Bcl2 protein upregulation in PPBL patients have been negative in most cases (Delage, *et al* 1998, Himmelmann, *et al* 2001, Lancry, *et al* 2001). Nevertheless, cytoplasmic expression of the protein has been detected by immunocytochemistry in both binucleated and non-binucleated B cells (Feugier, *et al* 2004, Lancry, *et al* 2001). Again, utilisation of the corresponding normal memory cellular subset as basis for comparison could provide a definitive answer regarding Bcl-2 expression levels in patients.

Similarly to animal models, progression from low (i.e.: FL) to high (i.e.: DLBCL) grade disease apparently requires additional genetic mutations secondary to the FL-characteristic *bcl-2/Ig* translocation. Oncogenic lesions affecting genes with growth promoting (*c-myc*), differentiation blocking (*bcl-6, pax5*) or apoptosis blocking (*bcl-*x_L, NF-κB activators) properties can presumably synergize with Bcl-2 to promote progression from low to high-grade lymphoma (Shaffer, *et al* 2002). Mutations in the open reading frame of the *bcl-2* gene itself have been correlated with morphologic transformation form FL to DLBCL (Matolcsy, *et al* 1996). Since they are restricted to the GC and post-GC compartments, these genetic alterations apparently accumulate as by-products of affinity maturation molecular machinery (Shaffer, *et al* 2002). Which bring us back to PPBL: although they preferentially differentiate to plasma cell upon recall responses, memory B cells can also participate to further rounds of GC-dependent affinity maturation (Liu 1997). Higher frequency in memory B cells could thus increase the risk for genomic instability in patients. A presumably deficient antigen-driven selection in PPBL patients could substantially contribute to the emergence of clonal proliferations. To this day, no molecular analysis of those genes related with low to high-grade disease progression was conducted in PPBL patients, with the exception of studies regarding *bcl-2* translocations. *bcl-2* mutational status per se has not been ascertained however. A special focus on lymphoma-associated oncogenes, aiming at the estimation of both gene expression and gene structure, is an avenue that was only scarcely explored in PPBL and which need to be further investigated in the near future as it could prove extremely useful to predict the outcome of this disorder.

Atypical persistent polyclonal B cell lymphocytosis.

Throughout this review, we have purposely left out the atypical PPBL case diagnosed in a newborn child by Gomez *et al* in 2000. That case undeniably stands apart among classical PPBL reports as far as the age of the patient is concerned: this is the only diagnosis that has been described in a child during the last twenty years. More importantly, atypical B lymphocytes display an unusual morphology with no binucleated cells, and the occasional presence of cytoplasmic villi or a lymphoplasmacytoid appearance. None of the prospective PPBL predisposition factors (positive EBV serology, HLA-DR7 haplotype), or characteristic genetic instability (*bcl-2/Ig* genes rearrangements, +i(3q)) have been identified. IgM levels are increased, but only slightly. Finally, B cells display a distinctive surface immunophenotype with expression of CD23, CD25, CD38, CD103, CD5 and no expression of CD11c (Gomez, *et al* 2000 and personal communication). These clinical features are rather reminiscent of those seen in hairy B cell lymphoproliferative disorder (HBLD), the alleged polyclonal counterpart of the Japanese variant of HCL, which however is negative for CD25 and positive for CD11c (Machii, *et al* 1997). To our knowledge, $CD5^+$ persistent polyclonal B cell lymphocytosis has only been reported once in a male adult (Reeder and Conley 1999). In neither of those two cases was the cell surface expression of IgM, IgD and CD27 assessed. Configuration of Ig V_H genes in these patients has not been determined either. Until these elements are evaluated, it will not be possible to definitely resolve whether this atypical $CD5^+$ PPBL is a variant form of classic PPBL or whether it is a separate entity as its distinct immunophenotype seems to indicate.

Future prospects

In order to better understand the aetiology of PPBL and estimate the risks of malignant progression in patients, it will be mandatory to refine the characterization of the expanding $IgM^+IgD^+CD27^+$ subset. Supplementary immunophenotyping, particularly exploring those markers such as IRTA-1, or the newly described CD1c, which are specifically expressed among MZ B cell subsets (Falini, *et al* 2003, Weller, *et al* 2004), could be useful to confirm whether PPBL B lymphocytes actually are a MZ derived population. If access to secondary lymphoid organ specimens should ever become possible, it would be very interesting to conduct *in situ* studies in order to retrace the exact anatomical origin for $IgM^+IgD^+CD27^+$ B lymphocytes in patients. Molecular analysis of individually picked B cells, isolated from the distinct areas of secondary follicules (dark zone, light zone, mantle and marginal zone), as was elegantly presented by Küppers et al (Kuppers, *et al* 1993), may allow researchers to establish the genealogy between GC founders and hypermutated memory B cells. This could well represent an authoritative answer in the debate regarding the GC origin of $IgM^+IgD^+CD27^+$ B lymphocytes not only in patients but also in healthy individuals. If indeed they originate from a distinct lineage, molecular profiling and the establishment of the gene signature of $IgM^+IgD^+CD27^+$ B lymphocytes both in patients and healthy individuals, might provide an effective way to uncover the possible physiological deregulation present in PPBL.

Very few functional studies have been conducted on PPBL B lymphocytes (see Table 11). The polyclonal nature of the population under scrutiny, and the lack of an accurately identified normal counterpart, long impeded such experiments. The results, when obtained, were difficult to interpret. Nonetheless, early functional studies gave clear indication of the functional distinctiveness of PPBL B cells (Loembe, *et al* 2001, Reimer, *et al* 2000). This already suggested that the majority of peripheral blood B lymphocytes in patients presented a different developmental status relative to healthy individuals. The subsequent characterization of the expanding $IgM^+IgD^+CD27^+$ population, corroborated those preliminary observations and, at least in theory, greatly reduced the obstacles to further

functional studies. However, one was still confronted with the scarcity of comprehensible information regarding peripheral blood memory B lymphocytes, notably unswitched, hypermutated IgM^+IgD^+ cells, long considered a naïve subset. As this field is now rapidly being uncovered, it should be expected that additional investigations will be undertaken in the coming years. It will then be possible to gain a better understanding of the physiological function of the $IgM^+IgD^+CD27^+$ B cell population in patients, notably the type of immunity it mediates (TD versus TI), and its capacity to participate to recall immune responses. In this regard, it will be interesting to determine the role played by those cells as far as antibodies production is concerned. Assessment of the differentiation capacity in PPBL $IgM^+IgD^+CD27^+$ B lymphocytes might ascertain the hypothesis, advanced by some authors, that impaired maturation would be accountable for the selective expansion of this subset, yielding the high serum IgM levels observed in patients.

Definitive confirmation of the preliminary results hinting at a defect in the antigen-driven selection process will require *in vitro* studies. The pattern of pro-and anti-apoptotic genes expression in PPBL patients (especially regarding Bcl-x_L) as well as the dynamics of CD95 DISC assembly and its ensuing signalling cascade will need to be evaluated. It will be however mandatory that the normal counterpart, i.e.: $IgM^+IgD^+CD27^+$ memory B cell population from healthy donors, be used for comparison. Moreover, to more closely recreate the GC microenvironment, stimulation systems should include FDC, a cellular type that has been shown to play a preponderant part in the antigen-driven selection process, along with CD40-L and CD95-L expressing T lymphocytes (Li and Choi 2002).

The frequent involvement of chromosome 3 numerical anomalies could equally prove of clinical significance. Thus, in depth genetic investigation are required to 1) locate regions bearing genes of prospective interest on this chromosome 2) determine if the function of those genes could be significant to the pathogenesis of PPBL.

Finally, a freshly published paper has reported defective expression of adhesion molecules in PPBL (Feugier, *et al* 2004), highlighting a possible role for improper homing in this disorder that could cause accumulation of $IgM^+IgD^+CD27^+$ memory B cell in the peripheral blood. Surely, studies in additional patients that would combine analysis of cellular adhesion and chemotaxis could be yet another interesting avenue that would benefit from

deeper investigation and might provide considerable insight into the genesis of this disorder.

Concluding remarks

PPBL is an unusual haematological disorder, sharing attributes of both indolent proliferation (polyclonality, stability, lack of significant symptoms) and malignant proliferation (atypical cellular morphology, chromosomal anomalies, bone marrow infiltration). As such, it has long elicited puzzlement among the medical and scientific communities. Paralleling the significant achievements made with regards to the understanding of B cell immunobiology, a clearer clinical definition of the disorder has notwithstanding been emerging in the recent years. The proliferating subset has been clearly delineated, specific genetic anomalies have been uncovered, and a familial predisposition has been evidenced. Nevertheless, general awareness about this disorder is still lagging behind, inasmuch as it is not yet listed as a distinct pathological entity in recent haematology manuals.

In our opinion, two principal reasons justify bringing this disorder to the forefront. Firstly, despite its apparent rarity, PBBL could be relatively common, especially among family members of established cases. The repeated detection of patients lacking any clinical evidence of the disorder, other than occasional presence of circulating binucleated B lymphocytes, emphasizes the fact that PPBL does not always manifest itself as an overt leucocytosis. More cases could thus go unrecognized in the general population. It is necessary that the clinical picture of PPBL, especially its indolent progression, be widely acknowledged so these prospective cases are not submitted to unnecessary aggressive therapies, as were some former patients (Perreault, *et al* 1989). Secondly, as the risk for malignant evolution in PPBL cannot be dismissed altogether at this point, vigilant long-term monitoring should be advised and special efforts should be invested so that patients do not become lost to follow-up.

On a more fundamental level, advances regarding the fundamentals of B cell developmental biology and technical inputs from the molecular field have made it possible to gain remarkable insight into the genesis of lymphoid disorders in general, and PPBL in particular. This clinical model clearly illustrates the multi-step theory of lymphomagenesis. Disruption of peripheral lymphoid homeostasis and genetic instability in patients are presumably preliminary steps towards malignant progression (see Figure 17). Apparently however, they are insufficient to drive definitive clonal transformation, and/or specific safeguard mechanisms operate in PPBL. Surely future investigations will grant a better understanding of the lymphoid transformation process, or lack of thereof, in patients, and by extension in the general population.

Tables

Table 10: Human somatically mutated $IgM^{+}IgD^{+}$ memory B cell subsets.

	Blood (Agematsu 2000, Klein, *et al* 1998)	***Bone marrow*** (Paramithiotis and Cooper 1997)	***Marginal zone and its equivalents***. (Dono, *et al* 2000, Tangye, *et al* 1998)
Morphology	Large, abundant cytoplasm	Large, non dividing	Medium sized, irregular or indented nuclei, abundant cytoplasm either proliferating or not
Mutation frequency	V_H1,3,4: 4.86%	V_H5: 3.3 ± 2.9%	V_H4: 2.07% (tonsil) V_H5: 1.9% (spleen) V_H6: 2.5% (spleen)
Antigenic selection	Present†	Present* Clonally related cells	Present†. Clonally related cells.
Antibody production upon stimulation	IgM (SAC+IL-2)	IgM (SAC+IL-2)	IgM (SAC+IL-2) IgM, IgA, IgG (αCD40+IL-2 +IL-10)
Cell surface markers	$IgM^{++}IgD^{+}$ $CD27^{+}$ $CD23^{-}CD70^{+}$	$IgM^{+}IgD^{+}$ $CD27^{+}$? $CD71^{+}CDw75^{+}CD23^{+}$ $CD38^{+}CD95^{+}CD22^{+}$ $CD10^{-}CD25^{-}$	$IgM^{++}IgD^{+}$ $CD27^{+}$ $CD23^{-}CD25^{+/-}$ $CD38^{-}CD21^{-}$(tonsil) $CD148^{+}CD21^{+}CD95^{+}$(spleen)

*: as estimated by analysis of mutations distribution in CDR.

†: as estimated by analysis of mutations distribution in FR.

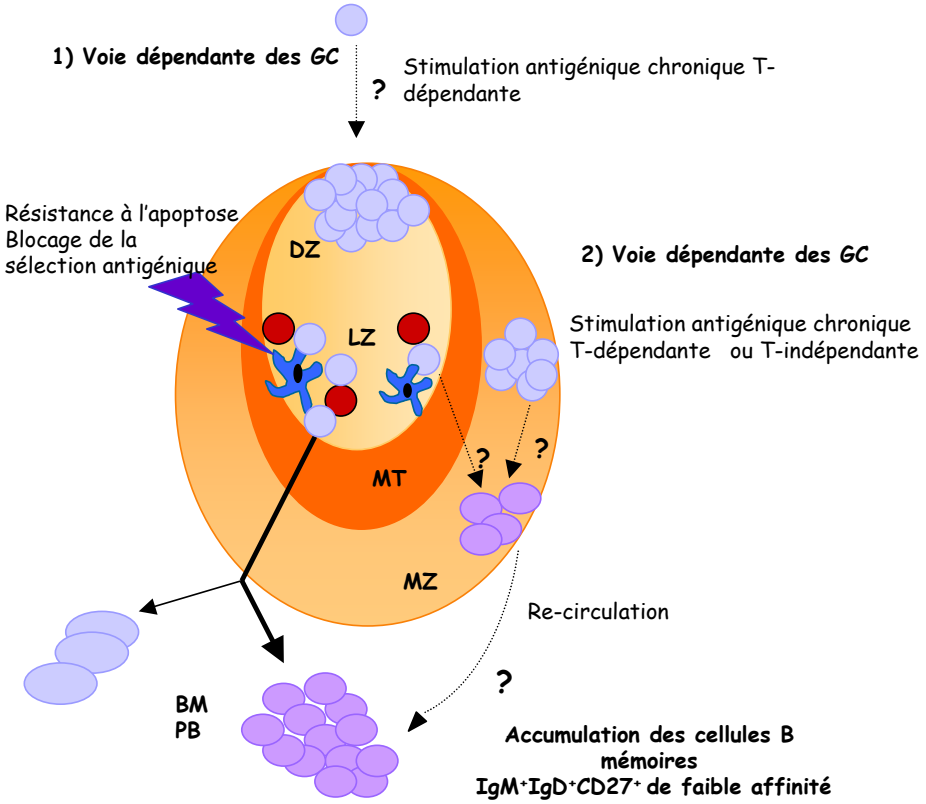
Table 11: Assessment of functional properties in PPBL B lymphocytes.

Authors	*Experiment*	*Observations**
Reimer, *et al* 2000	-sIgM + IL-4-induced proliferation. -sIgM and/or IL-4-induced HLA-DR expression. - sIgM and/or IL-4-induced CD23 expression.	-No proliferative response. -No induction of HLA-DR or CD23 following IL-4 stimulation specifically.
Loembe, *et al* 2001	- Cytofluorometric assessment of CD40 expression. - CD40 gene sequencing. -CD40-induced proliferation. -Western blot assessment of CD40-induced tyrosine phosphorylation.	Despite normal expression of an intact CD40 molecule, and induction of CD40 signalling cascade proximal events, no proliferative response.
Himmelmann, *et al* 2001	-IL-2/SAC stimulation (patients vs healthy controls).	Elevated IgM secretion but marginal, diminished IgG secretion.
Roussel, *et al* 2003	-Cytofluorometric assessment of CD95 expression. -CD95-induced apoptosis. -Western blot assessment of FADD and caspase 8 expression.	Despite normal expression of the CD95 molecule, and presence of the DISC components FADD and caspase 8, no induction of apoptosis.

*As compared to purified B lymphocytes from healthy controls (Himmelmann, *et al* 2001, Loembe, *et al* 2001, Reimer, *et al* 2000) or the Ramos B cell line (Roussel, *et al* 2003).

Figure

Figure 17: PPBL, past and future: the aetiopathology of PPBL and its prospective clinical evolution.



Rondes de maturation d'affinité dépendante ou indépendante des GC

Événement génétique secondaire
(*bcl2*/Ig, mutation de Bcl2 ou de CD95, anomalies du chromosome 3)

Progression vers une prolifération lymphoïde maligne ?

FDC

lymphocyte T

BM: moelle osseuse

PB: sang périphérique

lymphocyte B IgM+IgD+CD27- naïf

lymphocyte B IgM+IgD+CD27+ mémoire

plasmocyte

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Conclusion

Comme nous l'avons signalé à maintes reprises au cours de cette thèse, la recherche touchant à la biologie du développement du lymphocyte B s'est particulièrement distinguée au cours des cinq dernières années et a permis d'approfondir considérablement la connaissance des mécanismes de mise en place de la réponse immunitaire de haute affinité, définie classiquement comme T-dépendante et dérivée de la réaction des GC. De même, nous avons mentionné les répercussions considérables encourues au niveau clinique en ce qui touchait à la compréhension du processus de lymphomagenèse ainsi qu'à l'approche et la prise en charge des anomalies du système lymphoïde. Nos études portant sur la caractérisation de la LPCB, un syndrome alors méconnu, ont illustré concrètement l'impact de cette évolution dans le domaine hématologique. Ainsi, afin de surmonter les limites imposées par des approches de classification traditionnelles, basées principalement sur des critères morphologiques et cytogénétiques ayant pour objet la cellule à l'échelle individuelle, nous avons opté pour une approche moléculaire plus globale qui, en abordant les caractéristiques cellulaires à l'échelle populationelle, a mené à l'identification formelle du compartiment lymphocytaire B à l'origine du syndrome. Le cas particulier de la LPCB a permis de démontrer comment l'approche exploratoire aux syndromes lymphoprolifératifs ne se limite plus uniquement à l'analyse d'un clone isolé, mais prend plutôt en considération la cellule comme une entité ayant atteint un stade précis de développement, déterminé par un ensemble de caractéristiques (morphologie, profil immunologique membranaire, configuration des gènes des Ig [mutés ou non], expression et/ou répression concertée d'un ensemble de gènes), définition pouvant de fait être élargie à une sous-population lymphocytaire B complète, clonale ou non.

En établissant *de facto* un équivalent cellulaire présumé pour chaque entité ainsi définie, il est devenu possible d'orienter plus précisément les efforts de recherche. À la lumière de nos résultats, confirmés conjointement par les observations de deux autres équipes de recherche (Himmelmann, *et al* 2001a, Salcedo, *et al* 2002), nous pouvons formellement conclure que la cellule d'origine dans la lymphocytose est représentée par le B mémoire de phénotype $IgD^{+}IgM^{+}CD27^{+}$. Des indices additionnels pointent d'ailleurs spécifiquement vers la population B mémoire de la MZ splénique. Des études immunophénotypiques plus poussées, spécifiquement l'analyse du marqueur CD1c, spécifique aux lymphocytes B de la MZ (Weller *et al*, 2004), ou encore la détermination de la signature moléculaire de cette

population lymphocytaire par analyse avec micro-puces à ADN, devraient permettre de confirmer définitivement cette hypothèse. De même, en dressant des parallèles avec des pathologies lymphoïdes de phénotype semblable et d'évolution similairement indolente, il nous a été possible de conjecturer les mécanismes à l'origine de la lymphocytose. Cependant, il n'est pas possible à ce stade de résoudre de la nature exacte des processus physiologiques causant l'expansion préférentielle des lymphocytes B $IgD^+IgM^+CD27^+$ dans la LPCB. S'agit-il d'un mécanisme extrinsèque (ex : stimulation antigénique chronique), dont la lymphocytose ne serait que le reflet et dont l'élément initiateur demeurerait à être identifié ? Le manque de biais dans le répertoire des Ac chez les patientes atteintes de LPCB que nous avons étudiées, et plus significativement, l'absence d'une signature moléculaire indicatrice de sélection antigénique argumentent contre cette hypothèse. Alternativement, la LPCB pourrait résulter d'un dérèglement physiologique intrinsèque, causant soit une inhibition dans les mécanismes d'élimination des B IgD^+IgM^+ périphériques ou encore un blocage dans le processus de différenciation lymphocytaire du B. Nos expériences ont généré des résultats qui supportent le premier scénario, soit une survie indue des lymphocytes B $IgD^+IgM^+CD27^+$ de basse affinité, mais n'apportent pour l'instant pas de crédit à la seconde hypothèse (voir chapitre III). Enfin comme l'a souligné une étude récente (Feugier, *et al* 2004), un défaut dans la localisation (*homing*) d'une sous-population B autrement normale pourrait également être impliquée. Les propriétés migratoires des cellules lymphoïdes et les fondements biologiques de leur compartimentation morphologique au cours de la réponse immunitaire commencent seulement à être explorés (Cyster 1999, Moser, *et al* 2004) et constituent une avenue potentiellement intéressante pour l'investigation future de la LPCB.

La probabilité d'une transformation maligne subséquente dans la LPCB demeure spéculative. Les avancées récentes ont clairement attesté du rôle actif joué par le GC dans la genèse et la progression des syndromes lymphoprolifératifs. À la lumière de ces observations, il semble que la notion d'un "simple" dérèglement de l'homéostasie des compartiments lymphoïdes B oeuvrant dans la LPCB soit erronée par essence. En effet, au moins une proportion des lymphocytes B mémoires $IgD^+IgM^+CD27^+$ pourraient, en théorie, transiter à plusieurs reprises via les GC en réponse à un challenge antigénique secondaire (Arpin, *et al* 1997) et encourir des rondes supplémentaires de diversification, augmentant

conséquemment les risques de transformation. La fréquence accrue des anomalies génétiques détectables chez les patients atteints de LPCB étaye d'ailleurs cette hypothèse. Le défi posé aux chercheurs sera donc de déterminer si la fréquence à laquelle ces anomalies sont générées élève significativement le risque de transformation maligne chez les patients comparativement à la population générale. En toute éventualité, un suivi médical attentif est recommandé.

En résumé, nos études ont permis, dans un premier temps, de cerner la population cellulaire à la base de la LPCB, préciser la définition clinique du syndrome et établir des balises pour guider de futures démarches expérimentales. Notamment, un équivalent cellulaire normal est maintenant établi sur la base comparative duquel devrait procéder toute future évaluation des proprietés fonctionnelles et phénotypiques des B dans la LPCB. Dans un deuxième temps, l'analyse moléculaire détaillée des gènes des Ig chez les patientes atteintes de la LPCB a permis de gagner une meilleure compréhension des mécanismes l'origine du syndrome et d'anticiper son évolution. Bien que les hypothèses qui en aient découlé demandent pour la plupart des vérifications expérimentales, des pistes ont néammoins été défrichées qui serviront à guider les futurs efforts de recherche. Enfin, nous avons illustré comment l'intérêt de la LPCB ne se limite pas au modèle clinique qu'elle représente. En effet, au niveau plus fondamental, la LPCB, en affectant spécifiquement les B mémoires $IgD^{+}IgM^{+}CD27^{+}$, offre une illustration concrète de la singularité fonctionnelle et phénotypique de cette sous-population cellulaire. Tout progrès dans la compréhension de la biologie des B dans la LPCB s'ajoutera aux récentes découvertes touchant à ce compartiment mémoire particulier qui semble se situer à la marge de l'immunité acquise et l'immunité innée ou naturelle (Kruetzmann, *et al* 2003, Weller, *et al* 2004), indépendante des GC et de l'aide du lymphocyte T (du moins selon la conception traditionnelle de l'interaction B-T médiée par le CMH (Fairhurst, *et al* 1998)). Jusqu'à présent, l'immunité naturelle a été relativement délaissée tant les projecteurs se sont concentrés sur la réponse immunitaire de haute affinité, présumément dérivée des GC. Dans un futur proche, il se pourrait néanmoins que l'on démontre hors de tout doute que la relation exclusive classiquement établie entre réponse de haute affinité et réaction des GC ne soit après tout sujette à des exceptions.

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Annexes

Annexe 1 : complément au chapitre II.

Détail de l'analyse des séquences des gènes V_H des immunoglobulines chez trois patientes atteintes de LPCB.

Tableau 12: Détail de l'analyse des mutations R et S des gènes V_H chez trois patientes.

	Clone	*Séquence germinale avec la plus grande homologie*	*FR1*	*CDR1*	*FR2*	*CDR2*	*FR3*
Patiente CL4	CL4.3	3-7	1R				1R/1S
	CL4.4	3-66					
	CL4.6	3-13		2R			1R/2S
	CL4.8	4-59			1R/1S	4R	1R/1S
	CL4.9	4-59				1R	1R/2S
	CL4.12	4-4				1R	2R
	CL4.14	1-8	2S				
	CL4.15	4-34				1R	5R/2S
	CL4.18	4-39	1R				1R
Patiente CL5	CL5.1	3-74					1S
	CL5.2	1-18					
	CL5.3	3-66				1R	
	CL5.4	1-2	1R			1R	2R
	CL5.5	1-18			1S		1R
	CL5.6	4-30	1S		2S	1R,1S	2R
	CL5.7	4-30					2S
	CL5.8	4-30					2S
	CL5.9	3-33	1S				
	CL5.10	3-33	1S				
PatienteCL8	CL8.2	4-59	1R	1R		2R	3R/4S
	CL8.7	4-59			1S		3S
	CL8.10	4-59			1R/1S	1R	3S
	CL8.11	4-4		1R/1S	1R	2R	1R/1S
	CL8.13	3-49	1S	1R	1R	1S	3R
	CL8.14	4-59			1R/1S	1R	3S
	CL8.15	4-39		2R	1R	1R	1R
	CL8.17	4-30-4		1R			1R/2S
	CL8.19	4-59					
	CL8.20	4-59					1S

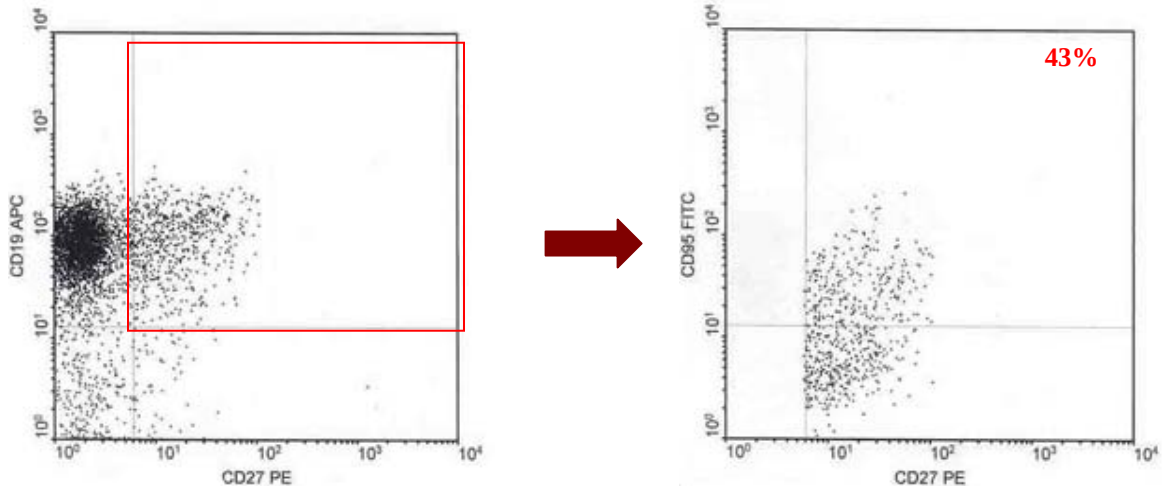
Annexe 2 : complément à la discussion générale.

Analyse de l'expression de CD95 chez les lymphocytes B $CD27^+$ dans la LPCB.

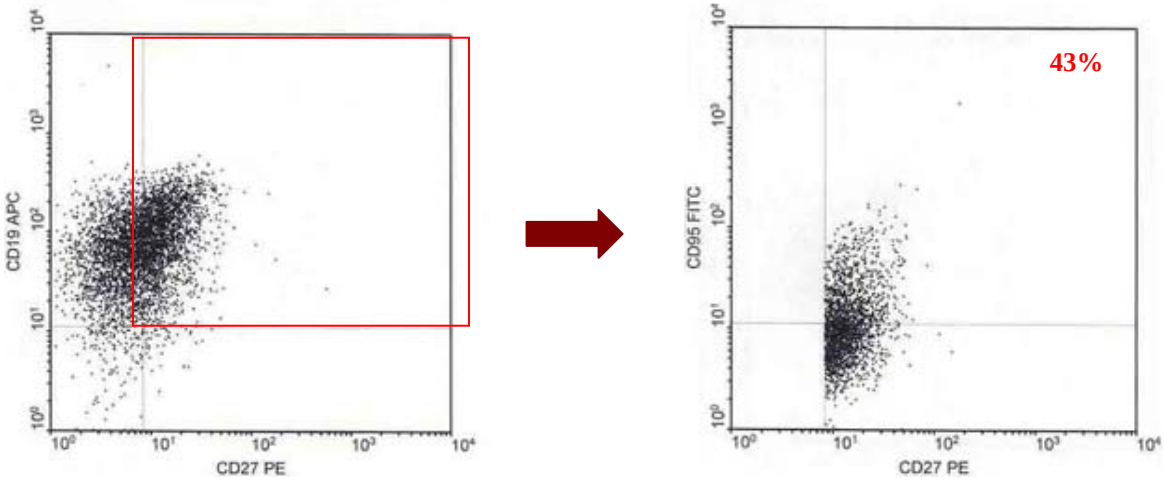
Figure 18 : Évaluation de l'expression de CD95 chez les lymphocytes B $CD27^+$ par analyse cytofluorométrique en trois couleurs.

Des PBMC ont été isolés chez deux patientes (B :CL4 et C : CL7) et un donneur contrôle (A : T52) par centrifugation sur gradient de Ficoll. Le marquage a été effectué en trois couleurs en utilisant un anticorps monoclonal anti-CD27 couplé à la phycoérythrine (CD27-PE), un anticorps monoclonal anti-CD19 couplé à l'allophycocyanine (CD19-APC), et un anticorps monoclonal anti-CD95 d'isotype IgG_1 (DX2) suivi d'un anti-IgG_1 couplé à la fluorescéine (CD95-FITC) (tous BD Bioscience, sauf DX2 provenant de Biosource International). Un premier temps l'analyse en fonction des marqueurs CD27 et CD19 a permis de circonscrire la population des lymphocytes B mémoires. Par la suite une fenêtre a été établie permettant l'évaluation spécifique de la sous-population de lymphocytes B de phénotype $CD27^+CD95^+$ (pourcentage indiqué sur chaque figure).

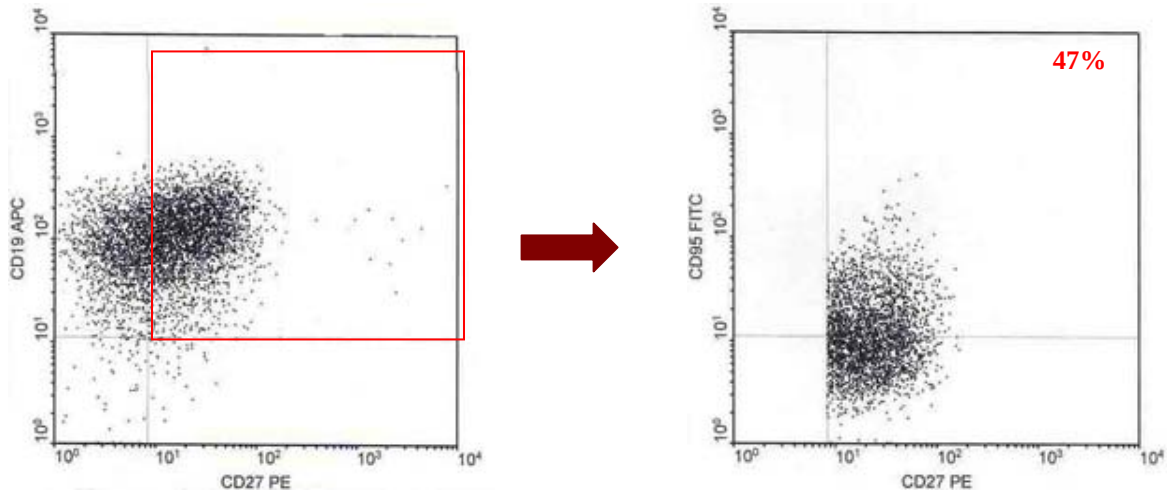
A)


CD19 APC
CD27 PE
CD95 FITC
CD27 PE
43%

B)


CD19 APC
CD27 PE
CD95 FITC
CD27 PE
43%

C)


CD19 APC
CD27 PE
CD95 FITC
CD27 PE
47%

Annexe 2 : complément à la discussion générale.

Persistent Polyclonal B Cell lymphocytosis: The Making of a Lymphoma? An immunological perspective.

(Version intégrale)

Persistent Polyclonal B Cell lymphocytosis: The Making of a Lymphoma? An immunological perspective.

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Abstract

First described twenty years ago, persistent polyclonal B cell lymphocytosis (PPBL) has since stirred much perplexity among researchers and clinicians. Diagnosed principally in adult female smokers, this unusual haematological disorder of B lymphocytes shares both features of benignity (polyclonal expansion, polyconal IgM secretion, lack of significant clinical symptoms, stable and mostly uneventful course) and features of malignancy: (morphologically atypical binucleated cells, *bcl-2/Ig* genes translocations, chromosome 3 anomalies, bone marrow involvement and, in rare patients, concurrent occurrence of neoplasia). As explained in the present review, the polyclonal nature of PPBL and the apparent heterogeneity of the involved cellular population has long impeded phenotypic and functional characterization of the disorder. The last years however have witnessed tremendous progresses in the field of B lymphocyte immunobiology. Technical inputs from the molecular field have lead to a better discrimination of developmentally distinct B cells subsets and, by extension, of B cell lymphoid disorders. Numerous molecular mechanisms and enzymatic factors involved in peripheral B cell maturation have been uncovered. These achievements have yielded a better understanding of the lymphomagenesis process. As far as PPBL is concerned, formal characterization of the expanding $IgM^{+}IgD^{+}$ memory B lymphocyte subset has ensued and a better delineation of its clinical definition has emerged. Indeed, deceitful cytogenetic and histologic features in PPBL have formerly exposed some patients to unneeded intrusive therapy, emphasizing the requirement for proper diagnosis of this syndrome. Moreover, it is still unclear at this point whether progression to malignancy represent the long term issue in PPBL, making attentive monitoring of patients mandatory. Nevertheless, as testified by is virtual absence from recent haematology manuals, or by the lack of recent comprehensive review on the subject, PPBL has not yet gained mainstream recognition as a distinct physiopathological entity. In this paper, our aim is to provide a retrospective view of the research realizations during these last twenty years and an up-to-date picture of PPBL.

Historical overview.

Nearly two decades ago, Gordon and collaborators were the first to report an unusually stable proliferative disorder of B lymphocytes, which they termed persistent polyclonal lymphocytosis of B lymphocytes, or PPBL (Gordon, *et al* 1982). The syndrome, observed in three adult female smokers, consisted in a marked elevation of peripheral lymphocytes numbers. PPBL involved mainly atypical B lymphocytes, medium sized, with an abundant cytoplasm and indented to fully bilobulated nuclei, and it was accompanied by a rise in serum IgM, with low IgG and IgA. Lymphocytosis is typically a transient event in healthy adults, often related to benign causes such as infectious reactions (see Granados, *et al* 1998 for a review). Persistent lymphocytosis, on the other hand, is frequently the sign of a neoplastic condition, with a clonal origin and a malignant evolution (i.e.: B-chronic lymphocytic leukaemia or B-CLL, hairy cell leukaemia or HCL, prolymphocytic leukemia or PLL, Waldenstrom's macroglobinaemia, the leukemic phase of some non Hodgkin lymphoma) (Hoffbrand, *et al* 2001). Yet, unlike most persistent lymphoproliferations of B cells, PPBL was remarkable by its polyclonal nature, evidenced by the expression of both λ and κ immunoglobulin light chains in the B cell population, and its extreme stability, with up to 25 years follow-up. Few clinical signs were associated with the syndrome, except for occasional lymphoid hyperplasia (mostly splenomegaly) and tobacco related respiratory track irritation or minor infections. The fact that all three patients in the study expressed the HLA-DR7 antigen, only observed in ~20% of the Caucasian population (Linet, *et al* 1988), was especially noteworthy, and hinted at a genetic predisposition to the syndrome. Moreover, the atypical lymphocyte morphology suggested the involvement of an infectious agent in the aetiology of PPBL. No virus could be detected by means of electron microscopy, nevertheless there were serological evidences of past infection with the Esptein-Barr virus (EBV), cytomegalovirus (CMV) and herpes simplex virus (HSV) in all patients. According to the authors, this lymphocytosis constituted a new disorder, most unusual among typically malignant B cell lymphoproliferations, probably benign, but which cause still needed to be fully elucidated.

Owing mostly to the lack of physical symptoms, PPBL was at first seldom diagnosed and considered a rare entity. The presence of binucleated B lymphocytes, a regular observation in patients, exceptional in normal individuals, soon became a hallmark of the lymphocytosis, and enabled the identification of several new cases by way of blood smear examination (see figure 19). The years have subsequently proven PPBL to be more prevalent than initially thought: as of 2004, approximately 118 cases have been described in the literature worldwide (see table 13 for an overview). These additional reports have confirmed the marked predominance of the feminine gender (104/118), positive EBV serology (52/82 tested), HLA-DR7 (81/118) phenotype and smoking in PPBL (113/118), making them all prospective susceptibility factors. Nevertheless, the syndrome was also diagnosed in a few men (13/118) and one infant (Gomez, *et al* 2000), in non-smoking patients (4/118) and in individuals negative for the HLA-DR7 haplotype (18/118) (see table 13, column 2 and 5). The extent to which each of the aforementioned features contributes to the pathogenesis of PPBL has been the subject of investigations and debates. However, as we discuss in the first part of this review, evidences that have accumulated since the first PPBL case reports only allow a partial resolution of the enigma.

Despite the fact that B lymphocytes account for the origin of 80% of all lymphoma types (Isaacson 2000), study of the different B cell subsets, particularly the mature pool, has long remained an uncharted territory. Accordingly, early lymphoma classification systems, such as the Rappaport classification (1970) or the Working Formulation (1982), relied mainly on the histological properties of malignant cells and clinical progression for the identification of B cell haematological disorders. However, owing to an inherent lack of flexibility, these methods did not always allow proper discrimination between distinct neoplastic entities (Isaacson 2000). The incorporation of basic phenotypic data (cell surface receptors), as illustrated in the Kiel Classification (1988), and of disease specific genetic features (chromosomal anomalies) later offered a supplementary level of analysis. Contrastingly, the recent years have witnessed a tremendous influx of scientific data regarding B cell developmental biology. Technical advances in the field of molecular genetics (i.e.: exhaustive Ig heavy chain variable region [V_H] analysis at the single cell

level, microarrays technology), along with the discovery of new cell surface markers, have had a considerable impact on the categorisation of the different human B cell compartments. Uncovering of enzymatic actors directing B cell maturation (exemplified lately by the exciting discovery of the AID cytidine deaminase) and clarification of implicated molecular processes have yielded tremendous insights into B cell immunobiology. Establishing a complete phenotypic and molecular fingerprint for a monoclonal lymphoid disorder is now feasible. At the same time, the notion that lymphoid malignancies are in a large part genetic disorders, resulting from the stepwise accumulation of anomalies in developmentally crucial genes, has gained wider acceptance among the scientific and medical communities (Dolcetti and Boiocchi 1996). As a result, elaborate immunophenotyping and molecular analysis are steadily becoming mainstream tools for the scientific investigation of lymphoid disorders, a reality clearly attested by a rapid overview of recent publications in this domain. With the lineage of a particular malignant clone, and provided with detailed molecular analysis of the B cell antigen receptor and/or molecular profiling, further attempts can be made to retrace the natural history of the neoplasm and even, to a certain level, to predict its clinical evolution according to the functional properties of the proposed normal cellular progenitor. This has been well illustrated with B-CLL, were distinct diagnostic categories were shown to be directly correlated with the molecular status (mutated versus unmutated) of V_H genes. The clinical relevance of this analytical approach has been further demonstrated by the elaboration of new systems, namely the REAL (1994) and WHO (2001) lymphoma classifications, which rely not only on morphologic and clinical features, but further encompass immunophenotypic and molecular properties in the definition of a lymphoid neoplasm (Isaacson 2000). Contrarily to their predecessors, these methods have achieved an international consensus and demonstrated an appreciable efficiency.

The second part of this review will focus on the immunological basis for the phenotypic and molecular fingerprinting of a given B cell clone, and illustrate how this approach led to the discovery that the expanding B cell population in PPBL consisted in polyclonal, somatically mutated $IgM^+IgD^+CD27^+$ memory B cells. The hypothesis that can be put forward regarding the lineage of these cells and the possible cause(s) behind the

lymphocytosis will be explored. Finally a survey will be made of the avenues that can tentatively be offered to answer the question which probably is of most concern to patients, clinicians and scientists alike: despite an apparent indolent course, is there a risk for long term malignant evolution in PPBL patients?

Immunophenotype and genotype of PPBL B cells.

The main challenge facing researchers and clinicians was the identity of the expanding B cell population in PPBL: was the lymphocytosis the result of a global expansion of B lymphocytes or only that of a specific subset? Evaluation of cellular morphology and immunophenotyping, a classical approach for the characterisation of malignant clones, was undertaken (table 13, column 9). Steady expression of the pan B cell markers CD19, CD20 CD22, CD24 and FMC7 corroborated the B lineage of the lymphocytosis. Expression of CD5 (B1 B cell marker), CD23 (naïve B cell activation marker) and CD38 (immature and germinal center [GC]B cell marker), often used for the differential diagnosis of CLL (Chiorazzi and Ferrarini 2003), was negative, as did expression of GC marker CD10. When tested, the CD103 marker, specific for both HCL and a polyclonal counterpart (hairy B cell lymphoproliferative disorder or HBLD) (Machii, *et al* 1997), was negative. CD11c, a second HCL and HBLD specific marker, was variably expressed. Most B lymphocytes displayed surface IgM and IgD which, according to the Bm1-5 classification system of B lymphocytes subsets (Liu and Arpin 1997, Pascual, *et al* 1994), pointed to a Bm1 or naïve phenotype. Contrastingly, features consistent with cellular activation were also reported: a high percentage of cells (up to 50%) presented with an abundant, basophilic cytoplasm, enlarged, often indented nucleus or apparently two completely separated nuclei (figure 19). Expression of activation marker CD25 (IL-2 receptor α) and of CD21 (EBV and complement receptor) was frequently observed. In addition, cytoplasmic immunoglobulin positivity, suggesting an early plasma cell phenotype, was documented (Feugier, *et al* 2004, Gordon, *et al* 1982, Ide, *et al* 2002). The lack of consistency of those observations illustrates the limitations resulting from the polyclonal nature of the disorder, and the probable involvement of a heterogeneous B cell population. Karyotyping and molecular analysis have further disclosed genetic anomalies within the B cell compartment in PPBL (table 13, column 8): premature

chromosome condensation (PCC), isochromosome +i(3q) and multiple *bcl-2/Ig* genes rearrangements, were recurring findings in most cases tested. Yet again, these two last chromosomal anomalies were distributed randomly in the B lymphocyte population, regardless of the morphological aspect of the cell, whether binucleated (Callet-Bauchu, *et al* 1999, Callet-Bauchu, *et al* 1997, Espinet, *et al* 2000, Lancry, *et al* 2001, Mossafa, *et al* 1999). Thus, at that point, the exact identity of the expanding subset in PPBL remained elusive.

Potential predisposition factors in PPBL.

Environmental factors : tobacco.

The high predominance of smokers among PPBL patients (114/118, table 13, column 2) has raised questions about the possible involvement of tobacco in the aetiology of the syndrome. These suspicions appeared to be supported early on, after the lymphocytosis had apparently resolved in one patient when she ceased smoking, but subsequently returned upon tobacco reintroduction (Carstairs, *et al* 1985). As additional PPBL cases were identified, normalization of the lymphocytosis, as well as diminution of serum IgM levels, was correspondingly observed in other patients after they quit smoking (Bain, *et al* 1998, Rodriguez, *et al* 1996, Tonelli, *et al* 2000). As a result, tobacco has been designated a causal factor for the disorder. However, tobacco cessation has not always yielded decreased B cell counts, and cytogenetic abnormalities have persisted (our own unpublished informations in three patients and Mossafa, *et al* 1999). Furthermore, atypical bilobulated B lymphocytes have remained present in ex-smoking patients, even in the absence of measurable absolute lymphocytosis (Bain, *et al* 1998, Himmelmann, *et al* 2001b, Rodriguez, *et al* 1996). This is especially noteworthy, as it indicates that the syndrome may well be present but goes unnoticed in many individuals. Meanwhile, control screenings of asymptomatic heavy smokers could not reveal the presence of characteristic bilobulated cells (Troussard and Flandrin 1996) or multiple *bcl-2/Ig* gene rearrangements (Delage, *et al* 2001). Finally, heavy smoking in healthy individual is significantly associated with lowered IgM levels (Mili, *et al* 1991, Moszczynski, *et al* 2001), making tobacco an unlikely suspect to explain the IgM elevation constantly

observed in PPBL (Vignes, *et al* 2000). As the disorder has also been observed in non smokers, it therefore seems that, though tobacco undoubtedly exacerbates the distinctive PPBL associated polyclonal B lymphocyte expansion, it is probably not a key factor in the aetiology of the disease.

Genetic factors: familial occurrence of PPBL.

Detection of the HLA-DR7 haplotype, present homozygously among the first three identified PPBL cases (Gordon, *et al* 1982), and either homozygously or heterozygously in most of the later cases (99/118, table 13, column 5), was intriguing given the low predominance of this antigen in the Caucasian population (~20%). It suggested a genetic predisposition to the disease. Interestingly, the HLA-DR7 has been associated with persistent infection with hepatitis B virus (Almarri and Batchelor 1994), possibly due to inadequate recognition by cytotoxic T cells, a phenomenon which, according to some authors, could likewise contribute to the apparent implication of EBV in PPBL (Chow, *et al* 1992, Mitterer, *et al* 1995). The finding of bilobulated lymphocytes in two of one patient's relatives (Troussard, *et al* 1994) and, more notably, the occurrence of PPBL in identical twins (Carr, *et al* 1997), has reinforced the notion of a genetic component in the disease. Ensuing investigations have provided decisive arguments in favour of a familial inheritance pattern in PPBL. The study by Delage *et al* (Delage, *et al* 2001), that took advantage of a patients' large family of nine first degree relatives, fourteen second degree relatives, and span three generations overall, has established that PPBL-associated criteria such as elevated serum IgM and B cells numbers, but particularly multiple *bcl-2/Ig* genes rearrangements, are more frequent among family members. As a matter of fact, it has led to the identification of two more PPBL cases among the patient's siblings. Singularly, the HLA-DR7 haplotype was absent in this family. A second study was conducted in a family of four siblings, which all presented PPBL associated risk factors (EBV positive serology, HLA-DR7 antigen expression, tobacco usage) (Himmelmann, *et al* 2001b). A second study was conducted in a family of four siblings, which all displayed PPBL associated risk factors (EBV positive serology, HLA-DR7 antigen expression, tobacco usage) (Himmelmann, *et al* 2001b). Again, two of the siblings presented with the disorder, emphasizing the plausibility of a familial inheritance pattern. Nevertheless, the

fact that two individuals in this family have remained unaffected raises the possibility that different or additional factors could be involved in the pathogenesis of PPBL. In both studies, relatives affected by PPBL either had a normal total lymphocytes count or a mild lymphocytosis. This again stresses the fact that the number of officially reported PPBL cases, which were referred and diagnosed as a result of elevated cell counts, only give a conservative estimate of the actual frequency of this disorder in the general population.

Infectious factors: Epstein Barr virus.

As the peculiar B cell morphology in PPBL suggested a viral infection, attempts were rapidly made to ascertain which infectious factors could play a role in its pathogenesis. Although, as mentioned, no viral agent had been detected by electron microscopy, serologic assays indicated past EBV infection in a majority of reported cases (52/82 tested, see table 13, column 7). Nevertheless, in the absence of appropriate controls, individual measurements of positive EBV serology did not warrant the implication of the virus in the disorder (Wyatt and Coyle 1991). Moreover, this observation is in line with the fact that the virus is carried asymptomatically by more than 90% of the adult population as a lifelong persistent infection (Crawford 2001) . Meanwhile, detection of viral genomes by *in situ* hybridation and PCR amplification in PPBL patients has been inconsistent (see table 13, column 7). Still, EBV was considered the most plausible candidate, owing to its capacity to drive B cell polyclonal proliferation and its frequent aetiological association with lymphoproliferative diseases, notably in immunosuppressed individuals (post-transplant and AIDS patients) (Crawford 2001, Thorley-Lawson and Gross 2004). The apparent association between PPBL, tobacco, a suspected inducer of EBV active infection, and the HLA-DR7 phenotype, involved in the immune evasion of virally infected cells, has reinforced the notion of a causal chain leading to the emergence of the lymphocytosis (Mitterer, *et al* 1995). This hypothesis is nonetheless challenged by the fact that, as we previously pointed out, tobacco usage and the HLA-DR7 haplotype are not shared by all PPBL patients. Moreover, when assayed *in vitro,* tobacco constituents have had no detectable effect upon active EBV replication (Jenson, *et al*

1999), whereas immune evasion of EBV has only been reported in relation with the HLA-A11 haplotype (de Campos-Lima, *et al* 1993).

Actual chronic active EBV infection was reported in one patient (Mitterer, *et al* 1995), from which a stable lymphoblastoid cell line was derived (Larcher, *et al* 1995) and functional studies conducted. Whether this cell line is representative of the disorder can be questioned, as it does not express surface IgD, contrarily to the expanding B cell subset in PPBL patients. Potentially more significant results arose from the molecular analysis of the carboxy-terminal region of the virus latent LMP1 gene. It indeed led to the identification of a unique 69 bp deletion that was not observed in four of the patient's siblings, despite evidence of past EBV infection. In these healthy relatives, distinct 30 bp deletions and point mutations were observed, reflecting the natural polymorphism of the LMP1 gene in the general population (Khanim, *et al* 1996). The 69 bp deletion, located within LMP1 NF-kB activation domain, was nonetheless susceptible to profoundly alter the signalling properties of this CD40 analog (Kilger, *et al* 1998). The authors have accordingly proposed that the variant form of the LMP1 oncoprotein, through its effect on B cell physiology in infected patients, could lead to PPBL (Larcher, *et al* 1997). In our experience, amplification and sequencing of LMP1 carboxy-terminal region in nine PPBL patients has disclosed the presence of independent point mutations in every one. Moreover, in four of them, the 30 bp deletion detected in relatives of the index case was also identified. In opposition, the 69 bp mutation was never found (Carle Ryckmann, unpublished results). Altogether, these results challenge the hypothesis of an infection with a specific variant EBV strain that could be implicated in the pathogenesis PPBL by way of impaired LMP1 signalling.

Following primary infection, EBV persists in the host through latent infection of peripheral blood memory B cells. The virus is narrowly detected in the $IgD^{-}CD27^{+}$ memory B lymphocytes pool (Joseph, *et al* 2000) and latent gene expression is null, excepted for the EBNA1 protein in dividing cells. Conversely, LMP1 latent protein expression is reported in naïve and activated GC B cells in the lymph nodes (Thorley-Lawson and Gross 2004). When tested, amplification of the latent viral genes LMP1 and EBNA1, using a sensitive nested RT-PCR technique, invariably led to their amplification

from PBMC in PPBL patients (LMP1:,7/7, EBNA1:7/7), whereas they were seldom detected in healthy controls (LMP1: 0/7, EBNA1:1/7) even when using DNA from purified B cells (Carle Ryckmann, unpublished results). It can be argued that expansion of the $CD27^+$ memory B cell subset in PPBL patients could explain the preferential detection of the virus in PPBL patients. However some questions remain unanswered. It is somewhat puzzling that patients, who display an expansion of the IgD^+ memory fraction, which allegedly excludes latent infection, show a parallel increased expression of latency associated viral genes. Moreover, it is not clear at this point what the significance of LMP1 expression in this specific memory compartment is. Subsetting memory B cells based on surface Ig expression, and further molecular investigations will undoubtedly help clarify the meaning of these observations.

Overall, the various studies conducted over the last twenty years have led to the accumulation of evidences for a genetic basis in PPBL. However, the precise mode of transmission, as well as the exact factor or combination of factors required for full expression of the disease, are still the subject of speculations and, for the most part, remained to be elucidated.

Lymphoid disorders as side effects of normal B cell development.

B cell maturation is punctuated by developmental checkpoints aiming at the expression of a functional receptor on the cell surface and its subsequent remodelling in order to provide the organism with the means to respond efficiently to antigenic challenge. At the molecular level, B cell maturation is reflected by sequential molecular modifications of the Ig gene locus. At the immunophenotypic level, differential expression of CD cell surface markers provides supplementary means to characterize developmentally distinct B cells.

B cell ontogeny in humans is classically divided into the antigen-independent and the antigen-dependent developmental stages (overview provided in Duchosal 1997). The antigen-dependent stage takes place in the foetal liver and the bone marrow. It allows the

successful assembly of the structural elements composing the Ig molecule, and its ensuing expression at the cell surface as part of the B cell receptor (BCR). In particular, elements of the Ig heavy (V_H) and light (V_L) chains variable domain are first brought together before being joined to a constant domain. These elements, namely the variable (V), diversity (D, V_H only) and joining (J) gene segments, are scattered on non adjacent parts of the chromosome. Accordingly, expression of a functional Ig receptor is contingent upon germline DNA rearrangement, or V(D)J gene segments recombination. Introduction of DNA double strand breaks, by the recombination activating enzymes RAG1 and RAG2, initiates the V(D)J recombination process which is next resolved by enzymes from the ubiquitous non-homologue ends-joining repair apparatus. The key to antibody diversity lays in the fact that V, D and J segments are encoded by multiple distinct gene copies which are assembled in a purely random fashion. Further variation is introduced by nucleotides additions or deletions at junction sites between segments as a result of the terminal deoxynucleotidyl transferase (TdT) enzyme activity. Hence, each B cell clone is provided with a unique Ig gene combination and the capacity to specifically recognise a singular antigenic determinant. The immune repertoire thus generated is referred to as the primary immune repertoire.

Mature naïve B cells, the outcome of the antigen-dependent differentiation stage, represent ~60% of circulating B lymphocytes, express surface IgM and IgD with (~45%) or without (~15%) CD5 (see figure 20 and Klein, *et al* 1998). The $IgM^+IgD^+CD5^+$ subset (or B-1 cells) represent a presumably distinct lineage that is associated with the natural, T-independent (TI), immune response (Youinou, *et al* 1999). $IgM^+IgD^+CD5^-$, on the other hand, recirculate actively between the follicules of secondary lymphoid organs until antigen encounter, interaction with an antigen-specific T cell, and initiation of the antigen-dependent development stage. Once activated, B cells either migrate to the T-cell zone of lymphoid organs (the extra-follicular region) and differentiate to low affinity short-lived plasmocytes, or they seed a follicule and give rise to a germinal centre (GC) (Jacob and Kelsoe 1992, Liu, *et al* 1991). Surrounding unactivated naïve B lymphocytes within the follicule are displaced to the periphery and form the follicular mantle zone (Liu, *et al* 1991). Inside the GC specialized microenvironment, B cells will be submitted to affinity maturation of their Ig receptor, and the secondary immune repertoire will be

generated. Activated naïve B lymphocytes first undergo massive clonal expansion and differentiate to centroblasts, forming the GC dark zone. During this stage, the Ig receptor is diversified by somatic hypermutation (SH), which introduces random point mutations within the variable domains of the Ig H and, to a lesser extent, L chains (Jacob, *et al* 1991, Kuppers, *et al* 1993). Thereafter, centroblasts differentiate to centrocytes and migrate to the light zone of GC. SH operates at random, and yields B cell mutants with either improved, lessen or lost affinity for the Ag. Correspondingly, an antigen-driven selection mechanism, whose specifics will be discussed in more details in a latter section, ensures that further centrocyte differentiation and entry into the effector compartment (plasma or memory B cells) is restricted to mutants with increased Ag-binding capacity. Imprint of such a selection process is visible at the molecular level as replacement mutations within the framework regions (FR) of V segments that are essential to antibody structure will be counter-selected. Silent mutations on the other hand will be favoured, yielding a lower R/S ratio than that expected by chance only (statistical methods for analysis of mutations distribution within V domains of Ig genes are presented in Lossos, *et al* 2000). Some mutants B cells will additionally switch to the expression of alternative (downstream) constants domains, conferring the Ig receptor new functional properties while maintaining its Ag-binding specificity (Liu, *et al* 1996). Effector B cells lastly quit the GC and migrate either to the marginal zone of the secondary lymphoid organs, peripheral blood or the bone marrow (Duchosal 1997, Klein, *et al* 1998, Liu, *et al* 1988). In the peripheral blood, the secondary immune repertoire will hence be comprised of somatically mutated memory B cells with switched (IgG and IgA principally, ~15%), $IgD^{+}IgM^{+}$ (~15%), IgM only (~10%), and a minority of IgD only (<1%) isotype (Klein, *et al* 1998). Contrarily to the antigen-dependent phase, enzymatic effectors and mechanisms involved in the affinity maturation of the Ig receptor were very only poorly defined. The recent discovery of the activation induced cytidine deaminase AID, a key molecule in the class-switch (CS) and SH processes, has however lifted a corner the veil and allowed for a better comprehension of the GC reaction (Muramatsu, *et al* 1999, Okazaki, *et al* 2003).

Various systems have been elaborated over the years to categorize developmentally distinct B lymphocytes. One, mentioned previously, segregates lymphocytes according

to surface expression of CD5, with $CD5^+$ or B-1 B cells mediating the natural or TI response and $CD5^-$ or B-2 cells mediating the conventional T-dependent (TD) response. However the pertinence of using CD5 as an indicator for cell lineage, although common in the mouse, has been debated in humans (Sagaert and De Wolf-Peeters 2003, Youinou, *et al* 1999). Another approach, the Bm1-Bm5 classification, rather focuses on the differentiation status of B lymphocytes (naïve, centroblast, centrocyte, memory) as indicated by morphology and differential expression of phenotypic markers (sIg, CD38, CD10, CD44 and CD77) and their corresponding location within discrete micro-anatomical structures of the GC (follicular mantle, dark zone, light zone, marginal zone) (Pascual, *et al* 1994). This system has proven very useful for the characterization of B cells subsets isolated from human secondary lymphoid clinical specimens (tonsils, lymph nodes, spleen sections). Nevertheless, it has had serious limitations as far as categorization of the heterogeneous peripheral blood B cell population has been concerned. Contrarily to follicular B cells, a significant proportion of sIgD-expressing circulating B lymphocytes carries hypermutated Ig genes (Klein, *et al* 1998), making IgD expression an improbable indicator for naïve B cells. As a consequence, discrimination of memory and naïve subsets has not been possible based on morphological features and phenotypic markers alone. These difficulties have been resolved with the identification of CD27 as a marker for somatically mutated memory B lymphocytes (Agematsu 2000, Klein, *et al* 1998). Consequently, Ig configuration, along with differential expression of phenotypic markers, now provides any isolated B cell clone with a unique fingerprint that makes it possible to establish its developmental status. Using above criteria, clonal lymphoid disorders can thus be separated in three broad categories (Hummel and Stein 2000):

-Pre-GC origin B cells, with rearranged but unmutated Ig genes (ie: some CLL, mantle cell lymphoma or MCL).

-GC origin B cells, with rearranged, mutated Ig genes and intra-clonal diversity indicative of ongoing SH (ie: follicular lymphoma or FL, some diffuse large B cell lymphomas or GC-like DLBCL).

-Post-GC origin B cells, with rearranged, mutated Ig genes and no intra-clonal diversity (ie: some CLL, marginal zone lymphoma or MZL, HCL, Burkitt lymphoma or BL).

An ultimate level of refinement has been brought to this classification system with the advent of the DNA micrroarrays technology which, by allowing the simultaneous analysis of thousand of genes, yields an extremely precise gene-expression signature for each distinct stage in B cell development, particularly those involved in the GC reaction (Klein, *et al* 2003).

In recent years, evolution of molecular biology techniques has made analysis of chromosomal anomalies and Ig genes configuration accessible procedures and, in turn, has had a considerable impact on the clinical management of lymphoid disorders. The biologic properties of a malignant cell are partly dependent on the differentiation status of its normal cellular progenitor. Thus, molecular tools, which make it possible to retrace the non-transformed counterpart for a given clonal lymphoid proliferation, allow inferences into the natural history of the disease, but also predictions about its clinical behaviour and prognosis. Additionally, classification of lymphoma based on the molecular structure of Ig genes has made it evident that most lymphoid disorders had a GC or post-GC origin, suggesting an active role for the GC reaction in the generation of malignant cells.

This notion has recently been backed-up by a consistent body of experimental evidences which go far beyond the scope of this review, but has been the object of excellent analysis in recent publications (Davila, *et al* 2001, Shaffer, *et al* 2002). Briefly, though those molecular mechanisms involved in the maturation of the Ig receptor are by essence highly mutagenic, strict regulatory mechanisms had been identified that were thought to circumscribe danger for the organism. Among them, the specific targeting to the Ig locus directed by the presence of specific genes sequences such as recombination signal sequences (RSS) and switch region (SR), or the restricted expression of enzymatic effectors such as the RAG proteins, to a narrowly defined stage of B cell development (B cell precursors). But recently, analysis of GC derived cells in healthy individuals have made it clear that the SH machinery was acting outside the Ig gene locus and targeting

potential proto-oncogenes such as the BCL-6 or CD95 proteins (Muschen, *et al* 2000, Pasqualucci, *et al* 1998). Similarly, RAG proteins have been found to be re-expressed in GC (Girschick, *et al* 2001) and are believed to play an active role in the generation of presumably transforming chromosomal translocations such as the *bcl2/Ig* or *c-myc/Ig* translocations (Davila, *et al* 2001). Thus there are many reasons to believe that malignant cells are in fact generated as by-products of the GC reaction.

Molecular analysis of Ig genes and PPBL: what we have learned.

Supplementary immunophenotyping of peripheral B lymphocytes in PPBL, using the newly defined memory marker CD27, and molecular analysis of Ig variable regions were undertaken in order to gain some insight in the developmental status of circulating B lymphocytes in patients. The memory subset, which represents 40% of peripheral B lymphocytes in healthy individuals, has thus been found to be increased up to 80% in PPBL patients. Noticeably, the memory compartment almost exclusively consists in $IgM^{++}IgD^{+}$ coexpressing B lymphocytes (Himmelmann, *et al* 2001a, Loembe, *et al* 2002, Salcedo, *et al* 2002), a population that only accounts for about one third of $CD27^{+}$ memory B cells in healthy individuals, alongside class-switched, IgM-only, and a few IgD-only B lymphocytes (Klein, *et al* 1998). This raise in CD27 expression is corroborated by a parallel elevation in CD148 expression, a second memory B cell marker, originally identified on somatically mutated splenic marginal zone (MZ) B cells (Himmelmann, *et al* 2001a, Tangye, *et al* 1998). Taking into account the observed lymphocytosis, this amounts to a twenty fold increase of the specific $IgM^{++}IgD^{+}CD27^{+}$ population in patients. Those observations have simultaneously led us, and another team, to proceed with the in depth molecular analysis of immunoglobulin genes in patients, since this strategy had proven so useful for investigation and classification of neoplasic lymphoid disorders (Loembe, *et al* 2002, Salcedo, *et al* 2002). Both studies have generated concordant results: in an average proportion of 73%, V_H genes have been found to be somatically mutated. No bias in the Ig repertoire has been detected: V_H genes

usage among PPBL patients was heterogeneous and reflected the relative abundance of each V_H gene family. Among both studies, the mutation frequency varied between 0.52% and 3%, with a mean value of 1.85%. Statistical analysis of mutations distribution in V_H genes was also undertaken. Evidence for antigen-driven negative selection, specifically suppression of replacement mutations in FR, has not been found, except in a few mutated sequences (Loembe *et al*: 4/21, Salcedo *et al*: 12/51). It therefore seems PPBL results from the preferential expansion of the memory $IgM^{++}IgD^{+}CD27^{+}$ B lymphocyte population. Intriguingly, the majority of these cells do not display the imprint of antigen selection that is considered to be the hallmark of the affinity maturation process in germinal centres.

$IgM^{++}IgD^{+}CD27^{+}$ memory B cells in PPBL, a GC derived subset…

Presence of somatic hypermutations in V_H genes, CD27 expression, and absence of GC associated markers CD38 and CD10, all suggests a post-germinal centre origin for the expanding $IgM^{++}IgD^{+}$ B cell population in PPBL. In human, hypermutated memory $IgM^{+}IgD^{+}$ populations have been identified in the bone marrow (Paramithiotis and Cooper 1997), peripheral blood (Agematsu, *et al* 1997, Klein, *et al* 1998), and among the MZ B cells (Dono, *et al* 2000, Tangye, *et al* 1998) (table 14). The latter two subsets especially have multiple phenotypical features in common with PPBL including: CD27 expression, high levels of sIgM, large abundant cytoplasm and, specifically for MZ B lymphocytes, irregular nuclear morphology with frequent expression of CD21 and CD25. Mutation frequencies observed in V_H genes in PPBL (~1.85%) are closest to that seen in marginal zone and bone marrow $IgM^{+}IgD^{+}$ memory B subsets (see table 14), and are definitely lower than that reported in class-switched memory B cells (Pascual, *et al* 1994).

The marginal zone is a micro-anatomically defined structure of the spleen, defined as the outermost margin of the follicular mantle zone. It is mainly populated by B cells displaying a phenotype intermediate between small resting lymphocytes and activated plasmablasts: the MZ B cells (Dono, *et al* 1996, Hsu 1985). This lymphoid compartment's function as a reservoir for memory B cells involved in the response to TD

and T-independent type 2 (TI-2) antigens was first extensively described in the rat (MacLennan and Liu 1991). In humans, the term marginal zone is now commonly used in a larger sense not only to refer to the extrafollicular areas of the spleen but also to similar micro-anatomical regions in other lymphoid tissues, namely: the subepithelial zone of the tonsils, the dome of the Peyer's patches, the subcapsular areas of the lymph nodes, and extranodal reactive mucosa-associated lymphoid tissues (MALT) (Dono, *et al* 2003). Typically, MZ B cells are positioned at the front line of potential antigenic assault. B cells in this subset share common morphological attributes (see table 14), are usually non cycling and express high level of IgM with no or low IgD (Dono, *et al* 1996, Hsu 1985). Molecular studies, on the other hand, have revealed quite a level of heterogeneity among MZ B cells within a single anatomical site, with some expressing unmutated and others mutated V_H genes. Those cells with mutations may or not present evidence of antigen-driven selection (Dono, *et al* 2000, Dunn-Walters, *et al* 1995, Tierens, *et al* 1999). It is conventionally believed that the non cycling hypermutated MZ B cells are the progeny of adjacent GC (Liu, *et al* 1988). Functional diversity in MZ B lymphocytes is further attested by the differential expression of cell surface markers according to their anatomic location: CD21 expression is high in splenic MZ B cells but low in their tonsillar counterparts (Dono, *et al* 2003), whereas the newly discovered IRTA-1 receptor is selectively expressed by tonsillar, MALT, and Peyer's patches MZ B lymphocytes (Falini, *et al* 2003). Moreover, IgA and IgG expression is mostly restricted to splenic MZ B lymphocytes (Dono, *et al* 1996, Tangye, *et al* 1998). Differences in the type of antigenic challenge (TD versus TI-2) and in the level of antigen exposure could largely account for this heterogeneity in MZ B lymphocytes. The particular $IgM^{++}IgD^{+}$ MZ B cells subset has been investigated in the tonsils (Dono, *et al* 1996, Dono, *et al* 2003) and in the spleen (Tangye, *et al* 1998). This population is chiefly comprised of memory B cells as attested by CD27 and CD148 expression, presence of somatic mutations in V_H genes and, in the tonsil subset, evidence for antigen-driven selection (table 14). Functionally, in analogy to animal models, these cells take part in both TD and TI-2 immune responses (Dono, *et al* 2003). Furthermore, following CD40 engagement, splenic $IgM^{++}IgD^{+}$ MZ B cells secrete both IgM and switched isotypes. In the rat, MZ B cells do not recirculate, except for their recruitment to the follicules upon recall immune

reponses (Liu, *et al* 1988). Contrastingly in humans, this concept is challenged by the presence of B cells with characteristics similar to MZ B lymphocytes in the blood and to a lesser degree in the bone marrow (table 14 and Weller, *et al* 2004). Some authors have consequently proposed that, following antigenic stimulation and somatic diversification, either in a TD or TI fashion, MZ B cells could transit trough peripheral blood before homing to the marginal zone of neighbouring lymphoid organs. They could also migrate to the bone marrow where local environmental signals would promote terminal differentiation to high affinity plasma cells (Paramithiotis and Cooper 1997, Tierens, *et al* 1999).

Ensuing the extensive cytofluorometric assessment of cell surface molecules expression in PPBL B lymphocytes, complemented by the molecular analysis of Ig genes, Salcedo and collaborators have designated memory cells from the marginal zone compartment as normal counterparts for circulating $IgM^{++}IgD^{+}CD27^{+}$ B cell in patients (Salcedo, *et al* 2002). Although this hypothesis seems highly plausible, supplementary immunophenotyping and functional studies are needed to warrant definitive validation of the developmental lineage in PPBL B lymphocytes.

…Or a separate, GC- independent, memory B cell lineage ?

Recently, somatically mutated $IgM^{+}IgD^{+}CD27^{+}$ memory B cells were identified in the peripheral blood of hyper IgM (HIGM1) patients who cannot form GC because of an invalidating mutation in CD154, the CD40 ligand. Conversely, and in accordance with their GC origin, class-switched and IgM only $CD27^{+}$ memory B cells were not detected (Weller, *et al* 2001). This observation has raised a serious debate about the notion of a GC-dependent origin in $IgM^{+}IgD^{+}CD27^{+}$ memory B lymphocytes. Weller and collaborators, the team at the origin of this significant discovery, suggested that peripheral blood $IgM^{+}IgD^{+}CD27^{+}$ memory B cells are the circulating counterparts of MZ $IgM^{++}IgD^{+}CD27^{+}$ B cells. Furthermore, these cells would be involved in TI responses, particularly TI-2 responses (Kruetzmann, *et al* 2003, Weller, *et al* 2004), implying that somatic diversification of the Ig receptor in HIGM1 patients would arise from a GC-independent developmental pathway (Weller et al 2003). In line with this view, cycling

(KI-67^{+}) MZ B cells with a memory phenotype were identified in nodal tissues sections and they displayed no clonal relationship to proximate GC, suggesting that these lymphocytes had possibly mutated *in situ* independently of the GC environment (Tierens, *et al* 1999). In similitude to mice B1 B cells, antigen exposure would be a prerequisite to specific antibody production by IgM^{++}IgD^{+}CD27^{+} B cells (Fagarasan and Honjo 2000, Kruetzmann, *et al* 2003). In this sense, this subset would not represent the "true", GC-derived, memory pool, but rather mediate a natural yet specific immunity, at the junction between the innate and the T-dependent, adaptive immune response, insuring the first line of defense against encapsulated bacteria (Kruetzmann, *et al* 2003, Weller, *et al* 2004). The fact that deregulation of homeostasis observed in PPBL patients solely affects the IgM^{++}IgD^{+}CD27^{+} lymphoid compartment makes a distinct origin for this particular memory subset a conceivable hypothesis. This disorder could thus add to the emerging picture of a GC-independent developmental pathway for IgM^{+}IgD^{+}CD27^{+} B cells.

Is a deregulated memory response the foundation for PPBL?

On a practical level, addressing the phenotype and lineage of the expanding B cell population in PPBL patients has had appreciable repercussions for clinicians as it provided them with additional and valuable diagnostic tools. But the investigative objectives pursued by researchers were primarily to uncover the cause(s) behind the disorder and to predict its evolution, if any was to be expected. And, in this regard, no clear-cut answer has been offered as yet. Two scenarios have nevertheless been advanced to explain polyclonal amplification of the IgM^{++}IgD^{+}CD27^{+} B cell lymphoid compartment in patients: either 1) an increase in memory B cells production (as a result of chronic antigenic stimulation) or 2) a decrease in their developmentally programmed elimination (due to impairment of apoptosis).

The first supposition is based on several lines of evidence demonstrating a connection between infectious agents and the emergence of various B cell lymphoid disorders (Dolcetti and Boiocchi 1996). The cause-effect relationship between B cell-tropic viruses with transforming capacities, such as EBV, and lymphoma in immunosupressed individuals is a well documented example (Crawford 2001). More recently, evidences

have also accumulated for the indirect implication of pathogens in B cell lymphoproliferations, specifically those originating from the marginal zone, as a result of sustained antigenic stimulation. Such is the case for *Helicobacter pylori* infection and gastric mucosa associated lymphoid tissue (MALT) lymphoma where pathogen eradication by antibiotherapy frequently leads to disease regression (Wotherspoon, *et al* 1993). Epidemiological studies suggest a similar cause-effect relationship between splenic marginal zone lymphoma with villous lymphocytes (SLVL) and the hepatitis C virus (Hermine, *et al* 2002). As far as PPBL is concerned, a role for EBV in the natural history of the disease has long been postulated as formerly discussed. Although direct B cell infection and transformation by a virus variant could not be formally evidenced, the possibility remains that an as yet unidentified (environmental ?) factor could drive chronic reactivation of lytic infection in patients, generating a persistent memory $IgM^{++}IgD^{+}$ B cell immune response, in either a GC-dependent or GC-independent pathway. Coincidentally, one of the anticipated anatomical location for $IgM^{++}IgD^{+}CD27^{+}$ memory cells, namely the subepithelial zone of the tonsils, was also proposed as the primary site for EBV infection in healthy individuals (Faulkner, *et al* 2000). Repeated virus production in this site could evoke a sustained immune response in resident $IgM^{++}IgD^{+}CD27^{+}$ memory cells, in similitude to what is observed in MALT lymphoma. On the other hand, no restriction of the immunoglobulin V_H gene repertoire was evidenced in PPBL patients. Contrastingly, this phenomenon has been reported in lymphoma derived from antigen-experienced memory B cells (Fais, *et al* 1998, Weng and Levy 2003), or even after *Haemophilus influenzae* immunization in healthy individuals (Adderson, *et al* 1991). In addition, the lack of evidence for antigenic selection pressure in expressed V_H gene regions further challenges the model of chronic antigenic selection in PPBL.

As we already pointed out, maintenance of homeostasis in the B cell lymphoid compartment is dependent upon stringent regulation mechanisms which insure control of the molecular processes involved in somatic diversification of the antibody repertoire. In the GC, balanced survival and elimination of mutated clones, through the process of antigen-driven selection, is necessary to delete potentially harmful autoreactive clones. It also prevents the feeding of superfluous low-affinity mutants in the memory pool.

Thereby, only the best-fitted antigen-specific mutants are allowed to differentiate to memory B cells. The fact that $IgM^{++}IgD^{+}CD27^{+}$ B cells in PPBL patients showed no evidence for antigen-driven selection, as indicated by the distribution of replacement versus silent mutations in Ig V_H genes, was intriguing given that both their immunophenotype and the molecular configuration of their Ig genes were otherwise indicative of a post-GC origin. Lack of evidence for antigen-driven selection can signify that the process of affinity maturation, the hallmark of a TD immune response, is altered in PPBL patients. In light of those observations, we have put forward a second scenario whereby impairment of the antigen-driven selection mechanism would enable the survival of low affinity mutants B cells within GC in PPBL, allow their recruitment into the memory B cell pool, and subsequently cause expansion of this compartment in the periphery. This hypothesis appears to be corroborated by several experimental facts. First, expression the Bcl-2 and the Bcl-x_L anti-apoptotic proteins have been reported to be up-regulated in PPBL. The interplay between differential expression of key pro and anti-apoptotic genes in GC B cells is crucial to the antigen-driven selection process. Tonsillar GC B cells accordingly express an apoptosis-sensitive phenotype, namely high expression of the pro-apoptotic CD95, Bax, Bak, Bim, and c-Myc proteins combined with low expression of the survival protein Bcl-2 (Liu and Arpin 1997, Yokoyama, *et al* 2002). This expression pattern apparently predisposes them to deletion through CD95/Fas-mediated killing, unless they can be rescued by efficient binding of immunizing Ag on the surface of follicular dendritic cells (FDC) and interaction with antigen specific CD40-L expressing T cells in the light zone of GC (Liu, *et al* 1989). CD95-induced cellular apoptosis can utilize two different signalling pathways (Mizuno, *et al* 2003). Type I apoptosis proceeds through association of a death inducing complex (DISC) where CD95 death domain (DD) recruits the CD95/Fas containing associated death-domain containing adapter protein (FADD) and procapase 8, later leading to the activation of caspase 8 and the ensuing apoptotic signalling cascade. In type II or mitochondrion-dependent apoptosis, an amplification step is necessary which involves mitochondrial release of cytochrome C, apoptosome assembly and activation of capase 9. This latter type can specifically be blocked by members of the Bcl-2 protein family. Which type of Fas-induced apoptosis is predominant in GC B cells is still subjected to

debate. Some experimental data indicates that both types I and II apoptosis could contribute to the process of antigen-driven selection. In point of fact, animals with decreased CD95 expression (*lpr/lpr* phenotype) (Takahashi, *et al* 2001), as well as those possessing a transgene for either Bcl-2 (Smith, *et al* 2000) or Bcl-x_L (Takahashi, *et al* 1999) constitutive expression, all display a distortion of affinity maturation which leads to the accumulation of low affinity memory B cells. Contrarily to Bcl-2, Bcl-xL expression is high in GC B cells (Tuscano, *et al* 1996). Moreover, rescue from CD95-mediated apoptosis, mimicked *in vitro* by CD40 stimulation and Ig cross-linking, correlates with up-regulation of Bcl-x_L (Zhang, *et al* 1996), suggesting an active regulatory role for this anti-apoptotic protein. In the same way, increased Bcl-x_L expression in PPBL patients could confer resistance to CD95-mediated apoptosis. However, one cautionary note regarding this conclusion resides in the fact that Bcl-2 and the Bcl-x_L expression levels in patients were compared to protein expression in total B cells, which comprised a majority (~60%) of naïve B cells. Whereas Bcl2 protein expression is similar in naïve and memory B cells, Bcl-x_L mRNA is about 8-fold higher in the memory compartment (Bovia, *et al* 1998). The apparent up-regulation of this protein in patients could thus be a mere reflect of an increased proportion in memory B cells. Nevertheless, *in vitro* resistance to Fas-mediated apoptosis has concomitantly been reported in PPBL B lymphocytes (Roussel, *et al* 2003). Again, normal controls consisted not in memory B cells, but rather in the Ramos cell line, with a GC phenotype and a conceivably differential expression of apoptosis-related genes. Nevertheless, since PPBL B lymphocytes displayed apoptotic features when treated with the anti-neoplastic etoposide reagent, the observed resistance appeared to be specific to the CD95-induced apoptotic pathway. CD95 has been described as a tumour suppressor gene, as loss of susceptibility to CD95-mediated killing often correlates with tumour progression. Notably, impairment of CD95 function would allow the survival of GC generated autoreactive or pre-malignant B clones that would otherwise be eliminated by antigen-driven selection. Support for this last role is provided by studies in animal models with a *lpr/lpr* phenotype who display increased susceptibility for B cell malignancies (Davidson, *et al* 1998). The same holds true for CD95 deficient human patients with the autoimmune lymphoproliferative syndrome (APLS) who lack a functional CD95 protein (Straus, *et al*

2001). Down-regulation of CD95 protein expression, disruption of the CD95 signalling cascade, or somatic mutations of the CD95 encoding gene, particularly those affecting the death domain (DD) region, are all mechanisms that have been identified in lymphoid malignancies and which could promote lymphomagenesis (for a detailed review of CD95 resistance mechanisms and their contribution to lymphomagenesis please see Mizuno, *et al* 2003 and Muschen, *et al* 2002) With regards to PPBL, CD95 expression in $IgM^{++}gD^{+}CD27^{+}$ memory B lymphocytes is similar or superior to that detected in healthy controls (Salcedo, *et al* 2002 and our own observations), therefore the observed resistance cannot be related to negative modulation of the receptor in patients. Still, mutations in CD95 DD could be present that would allow cell surface expression of the protein but interfere with signalling. Presence of the DISC components FADD and caspase 8 has been detected (Roussel, *et al* 2003), but it is not known at this point if they assemble properly. Moreover, expression of FLIP or FAIM, known mediators of CD95 resistance (Schneider, *et al* 1999, Wang, *et al* 2000), has not yet been investigated. The postulate of a deficient antigen-driven selection mechanism in PPBL has recently been complemented by the uncovering of IgM anti-phospolipid antibodies (apA/cofactor) in patients despite the absence of noticeable auto-immune disease (Granel, *et al* 2002). Hence, propensity for increased survival of auto-reactive and low affinity B cells clones seems to exist in PPBL patients. We ergo propose that a deregulation of the physiological processes implicated in the naïve to memory B cell transition could contribute significantly to the disruption of homeostasis in PPBL patients.

And what can we anticipate?

Genetic instability and the risk of malignant transformation in PPBL.

Although the majority of patients have had uneventful follow-ups amounting to more than 25 years in some cases, there is a persistent concern that the disruption of homeostasis observed in PPBL could in fact represent the first stage in a multi-step progression toward a more aggressive proliferation. Accordingly, in two patients, PPBL diagnosis has been associated with the occurrence of non Hodgkin lymphomas: DLBCL

(19 years after diagnosis) (Roy, *et al* 1998), and MALT lymphoma (concomitantly with diagnosis) (Callet-Bauchu, *et al* 1999). Clonal or oligoclonal V_H genes rearrangements have been detected in four patients, yet no further evolution was reported for these cases (Chan, *et al* 1990, Delage, *et al* 1997, Feugier, *et al* 2004). These observations nevertheless emphasize the notion that emergence of a predominant, and potentially malignant, clone is a likely outcome in PPBL. Bone marrow B cell intra-vascular infiltration was recently reported as recurrent finding in PPBL patients (Feugier, *et al* 2004). Moreover, careful microscopic observation has also revealed the presence of nuclear pockets in atypical B lymphocytes, a feature which is usually witnessed in pre-leukemic or leukemic leucocytes (Casassus, *et al* 1987, Espinet, *et al* 2000, Woessner, *et al* 1999). The discovery of clonal genetic abnormalities in PPBL B cells, contrasting with the polyclonal nature of the proliferation, illustrates the risk of subsequent transformation in this disorder. As mentioned previously, isochromosome (+i3q), first documented as an isolated event in 1989 (Perreault, *et al* 1989), has since been demonstrated to be a recurrent finding among PPBL patients (Callet-Bauchu, *et al* 1997, Mossafa, *et al* 1996): it was observed in 29/41 cases tested (see table 13, column 8). Additional numerical aberrations involving chromosome 3, namely trisomy 3, partial duplication 3q, and derivative 3, were also sporadically reported (table 13, column 8). Unlike PCC, whose occurrence has been linked to the presence of multinucleated cells (Mossafa, *et al* 1999), the (+i3q) aberration is apparently not restricted to a morphologically distinct B cell population and both events are independent of the light chain isotype (Callet-Bauchu, *et al* 1997, Espinet, *et al* 2000). Interestingly, observations made at referral and then at 2 years follow-up seem to indicate an accumulation of these chromosomal anomalies in at least one patient (Callet-Bauchu, *et al* 1999). Among non Hodgkin lymphoid disorders, chromosome 3 aberrations are more frequently identified in MZL, more precisely in the extranodal (MALT), nodal (monocytoid B cell lymphoma or MCBL) and splenic marginal zone B cell lymphoma (or SMZL, including splenic lymphoma with villous lymphocytes [or SLVL]) types (Dierlamm, *et al* 1996). Consistent with the postulated origin for $IgM^{++}IgD^{+}CD27^{+}$ B lymphocytes in PPBL, SMZL shares many additional features with this disorder:

-Generally slow clinical progression.

-A heterogeneous morphological composition involving a mixture of plasma, blast-like and small cells, occasionally presenting a cleaved nucleus.

-Peripheral blood and bone marrow involvement (Thieblemont, *et al* 2003).

-Lack of expression of CD5, CD10 and CD23.

-Heterogeneous utilisation of V_H genes among cases, and presence of somatic hypermutations without evidence of antigenic selection in most cases (Dierlamm, *et al* 1996).

Unlike PPBL however, SMZL are clearly monoclonal with detectable IgH rearrangements. In addition, no *bcl2* gene rearrangement can be observed, which is in sharp contrast to what is observed in PPBL.

In eleven patients of the cohort followed by our team in Quebec City, nested-PCR amplification has allowed the detection of multiple distinct *bcl-2/Ig* genes rearrangements (up to seven), involving both the mcr (minor cluster region) and MBR (major breakpoint region), in all but one case (only one rearrangement) (Delage, *et al* 1997, Delage, *et al* 1998). These observations were later reproduced in additional patients, and the frequency of the t(14;18) translocation was estimated between $1/10^2$ and $1/10^4$ cells (see table 13, column 8). In analogy to the +i(3q) chromosomal anomaly, there appears to be an accumulation of the *bcl-2/Ig* rearrangements in some patients (Delage, *et al* 1998). *bcl-2/Ig* genes rearrangements are also reported in approximately 50% of healthy individuals, however their frequency is lower ($1/10^5$ to $1/10^6$) and multiple rearrangements are only revealed with very sensitive detection methods (Ji, *et al* 1995). Bcl-2 oncogenic potential is illustrated by the occurrence of the t(14;18) translocation in 80% of cases of follicular lymphoma (FL), the most frequent human B cell malignancy. A positive correlation was observed between age or tobacco usage and the presence of *bcl-2/Ig* rearrangements in healthy individuals (Bell, *et al* 1995, Liu, *et al* 1994). As the incidence of non Hodgkin lymphoma is also increased in aged people and smokers, authors have accordingly proposed that the presence of *bcl-2/Ig* rearrangements reflects an individual's risk for developing a subsequent lymphoid malignancy.

At the cellular level, the t(14;18) chromosomal translocation produces a *bcl-2*-immunoglobulin fusion gene and, owing to the resulting proximity with the Ig transcriptional enhancer, leads to an overexpression of the Bcl-2 protein (Graninger, *et al* 1987). Furthermore, this deregulated expression interferes with apoptosis, though it does not promote cellular proliferation (Hockenbery, *et al* 1990). Interestingly, mice models bearing a *bcl-2/Ig* minigene initially display an indolent lymphoid hyperplasia consisting of polyclonal IgM^+IgD^+ resting B cells. Slow progression to clonal DLCL, apparently as the result of secondary genetic alterations (*c-myc* translocation), ensues in these mice (McDonnell and Korsmeyer 1991). Thus *bcl-2* acts as a proto-oncogene who, once translocated, promotes B cell survival and increases the risk for subsequent tumorigenic genome alterations leading to the emergence of neoplasia. In humans, the similitude with PPBL is remarkable, and that a similar outcome could occur in patients is indeed a troubling probability. Still, at the physiological level, presence of *bcl2* gene translocations doesn't always translate into increased protein expression, especially at the relatively low translocation frequency observed in PPBL patients. Accordingly, reports of Bcl2 protein upregulation in PPBL patients have been negative in most cases (Delage, *et al* 1998, Himmelmann, *et al* 2001a, Lancry, *et al* 2001). Nevertheless, cytoplasmic expression of the protein has been detected by immunocytochemistry in both binucleated and non binucleated B cells (Feugier, *et al* 2004, Lancry, *et al* 2001). Again, utilisation of the corresponding normal memory cellular subset as basis for comparison could provide a definitive answer regarding Bcl-2 expression levels in patients.

Similarly to animal models, progression from low (ie: FL) to high (ie: DLBCL) grade disease apparently requires additional genetic mutations secondary to the FL-characteristic *blc-2/Ig* translocation. Oncogenic lesions affecting genes with growth promoting (*c-myc*), differentiation blocking (*bcl-6, pax5*) or apoptosis blocking (*bcl-*x_L, NF-κB activators) properties can presumably synergize with Bcl-2 to promote progression from low to high grade lymphoma (Shaffer, *et al* 2002). Mutations in the open reading frame of the *blc-2* gene itself have been correlated with morphologic transformation form FL to DLBCL (Matolcsy, *et al* 1996). Since they are restricted to the GC and post GC compartments, these genetic alterations apparently accumulate as by-products of affinity maturation molecular machinery (Shaffer, *et al* 2002). Which

bring us back to PPBL: although they preferentially differentiate to plasma cell upon recall responses, memory B cells can also participate to further rounds of GC-dependent affinity maturation (Liu 1997). Higher frequency in memory B cells could thus increase the risk for genomic instability in patients. A presumably deficient antigen-driven selection in PPBL patients could substantially contribute to the emergence of clonal proliferations. To this day, no molecular analysis of those genes related with low to high grade disease progression was conducted in PPBL patients, with the exception of studies regarding *bcl-2* translocations. *bcl-2* mutational status per se has not been ascertained however. A special focus on lymphoma-associated oncogenes, aiming at the estimation of both gene expression and gene structure, is an avenue that was only scarcely explored in PPBL and which need to be further investigated in the near future as it could prove extremely useful to predict the outcome of this disorder.

Atypical persistent polyclonal B cell lymphocytosis.

Throughout this review, we have purposely left out the atypical PPBL case diagnosed in a newborn child by Gomez *et al* in 2000. That case undeniably stands apart among classical PPBL reports as far as the age of the patient is concerned: this is the only diagnosis that has been described in a child during the last twenty years. More importantly, atypical B lymphocytes display an unusual morphology with no binucleated cells, and the occasional presence of cytoplasmic villi or a lymphoplasmacytoid appearance. None of the prospective PPBL predisposition factors (positive EBV serology, HLA-DR7 haplotype), or characteristic genetic instability (*bcl-2/Ig* genes rearrangements, +i(3q)) have been identified. IgM levels are increased, but only slightly. Finally, B cells display a distinctive surface immunophenotype with expression of CD23, CD25, CD38, CD103, CD5 and no expression of CD11c (Gomez, *et al* 2000 and personal communication). These clinical features are rather reminiscent of those seen in hairy B cell lymphoproliferative disorder (HBLD), the alleged polyclonal counterpart of the Japanese variant of HCL, which however is negative for CD25 and positive for CD11c (Machii, *et al* 1997). To our knowledge, $CD5^{+}$ persistent polyclonal B cell lymphocytosis has only been reported once in a male adult (Reeder and Conley 1999). In

neither of those two cases was the cell surface expression of IgM, IgD and CD27 assessed. Configuration of Ig V_H genes in these patients has not been determined either. Until these elements are evaluated, it will not be possible to definitely resolve whether this atypical $CD5^+$ PPBL is a variant form of classic PPBL or whether it is a separate entity as its distinct immunophenotype seems to indicate.

Future prospects

In order to better understand the aetiology of PPBL and estimate the risks of malignant progression in patients, it will be mandatory to refine the characterisation of the expanding $IgM^+IgD^+CD27^+$ subset. Supplementary immunophenotyping, particularly exploring those markers such as IRTA-1, or the newly described CD1c, which are specifically expressed among MZ B cell subsets (Falini, *et al* 2003, Weller, *et al* 2004), could be useful to confirm whether PPBL B lymphocytes actually are a MZ derived population. If access to secondary lymphoid organ specimens should ever become possible, it would be very interesting to conduct *in situ* studies in order to retrace the exact anatomical origin for $IgM^+IgD^+CD27^+$ B lymphocytes in patients. Molecular analysis of individually picked B cells, isolated from the distinct areas of secondary follicules (dark zone, light zone, mantle and marginal zone), as was elegantly presented by Küppers et al (Kuppers, *et al* 1993), may allow researchers to establish the genealogy between GC founders and hypermutated memory B cells. This could well represent an authoritative answer in the debate regarding the GC origin of $IgM^+IgD^+CD27^+$ B lymphocytes not only in patients but also in healthy individuals. If indeed they originate from a distinct lineage, molecular profiling and the establishment of the gene signature of $IgM^+IgD^+CD27^+$ B lymphocytes both in patients and healthy individuals, might provide an effective way to uncover the possible physiological deregulation present in PPBL.

Very few functional studies have been conducted on PPBL B lymphocytes (see table 15). The polyclonal nature of the population under scrutiny, and the lack of an accurately identified normal counterpart, long impeded such experiments. The results, when obtained, were difficult to interpret. Nonetheless, early functional studies gave clear

indication of the functional distinctiveness of PPBL B cells (Loembe, *et al* 2001, Reimer, *et al* 2000). This already suggested that the majority of peripheral blood B lymphocytes in patients presented a different developmental status relative to healthy individuals. The subsequent characterisation of the expanding $IgM^+IgD^+CD27^+$ population, corroborated those preliminary observations and, at least in theory, greatly reduced the obstacles to further functional studies. However, one was still confronted with the scarcity of comprehensible information regarding peripheral blood memory B lymphocytes, notably unswitched, hypermutated IgM^+IgD^+ cells, long considered a naïve subset. As this field is now rapidly being uncovered, it should be expected that additional investigations will be undertaken in the coming years. It will then be possible to gain a better understanding of the physiological function of the $IgM^+IgD^+CD27^+$ B cell population in patients, notably the type of immunity it mediates (TD versus TI), and its capacity to participate to recall immune responses. In this regard, it will be interesting to determine the role played by those cells as far as antibodies production is concerned. Assessment of the differentiation capacity in PPBL $IgM^+IgD^+CD27^+$ B lymphocytes might ascertain the hypothesis, advanced by some authors, that impaired maturation would be accountable for the selective expansion of this subset, yielding the high serum IgM levels observed in patients.

Definitive confirmation of the preliminary results hinting at a defect in the antigen-driven selection process will require *in vitro* studies. The pattern of pro-and anti-apoptotic genes expression in PPBL patients (especially regarding Bcl-x_L) as well as the dynamics of CD95 DISC assembly and its ensuing signalling cascade will need to be evaluated. It will be however mandatory that the normal counterpart, ie: $IgM^+IgD^+CD27^+$ memory B cell population from healthy donors, be used for comparison. Moreover, to more closely recreate the GC microenvironment, stimulation systems should include FDC, a cellular type which has been shown to play a preponderant part in the antigen-driven selection process, along with CD40-L and CD95-L expressing T lymphocytes (Li and Choi 2002).

The frequent involvement of chromosome 3 numerical anomalies could equally prove of clinical significance. Thus, in depth genetic investigation are required to 1) locate

regions bearing genes of prospective interest on this chromosome 2) determine if the function of those genes could be significant to the pathogenesis of PPBL.

Finally, a freshly published paper has reported defective expression of adhesion molecules in PPBL (Feugier, *et al* 2004), highlighting a possible role for improper homing in this disorder that could cause accumulation of $IgM^{+}IgD^{+}CD27^{+}$ memory B cell in the peripheral blood. Surely, studies in additional patients that would combine analysis of cellular adhesion and chemotaxis could be yet another interesting avenue that would benefit from deeper investigation and might provide considerable insight into the genesis of this disorder.

Concluding remarks

PPBL is an unusual haematological disorder, sharing attributes of both indolent proliferation (polyclonality, stability, lack of significant symptoms) and malignant proliferation (atypical cellular morphology, chromosomal anomalies, bone marrow infiltration). As such, it has long elicited puzzlement among the medical and scientific communities. Paralleling the significant achievements made with regards to the understanding of B cell immunobiology, a clearer clinical definition of the disorder has notwithstanding been emerging in the recent years. The proliferating subset has been clearly delineated, specific genetic anomalies have been uncovered, and a familial predisposition has been evidenced. Nevertheless, general awareness about this disorder is still lagging behind, inasmuch as it is not yet listed as a distinct pathological entity in recent haematology manuals.

In our opinion, two principal reasons justify bringing this disorder to the forefront. Firstly, despite its apparent rarity, PBBL could be relatively common, especially among family members of established cases. The repeated detection of patients lacking any clinical evidence of the disorder, other than occasional presence of circulating binucleated B lymphocytes, emphasizes the fact that PPBL does not always manifest itself as an overt leucocytosis. More cases could thus go unrecognized in the general

population. It is necessary that the clinical picture of PPBL, especially its indolent progression, be widely acknowledged so these prospective cases be not submitted to unnecessary aggressive therapies, as were some former patients (Perreault, *et al* 1989). Secondly, as the risk for malignant evolution in PPBL cannot be dismissed altogether at this point, vigilant long-term monitoring should be advised and special efforts should be invested so that patients do not become lost to follow-up.

On a more fundamental level, advances regarding the fundamentals of B cell developmental biology and technical inputs from the molecular field have made it possible to gain remarkable insight into the genesis of lymphoid disorders in general, and PPBL in particular. This clinical model clearly illustrates the multi-step theory of lymphomagenesis. Disruption of peripheral lymphoid homeostasis and genetic instability in patients are presumably preliminary steps towards malignant progression (see figure 21). Apparently however, they are insufficient to drive definitive clonal transformation, and/or specific safeguard mechanisms operate in PPBL. Surely future investigations will grant a better understanding of the lymphoid transformation process, or lack of thereof, in patients, and by extension in the general population.

Figures

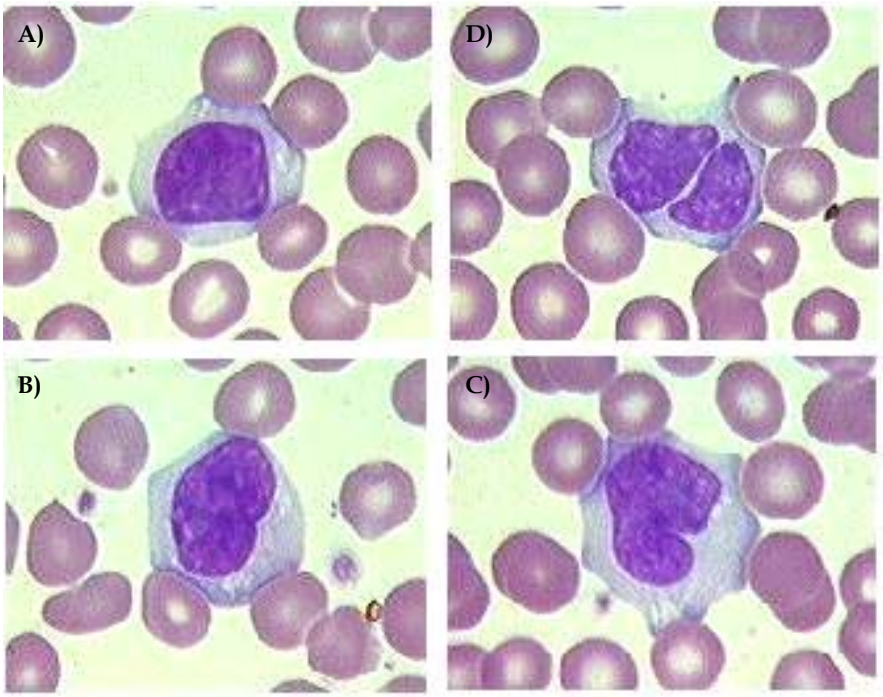


Figure 19 : Morphology of atypical lymphocytes in PPBL.

Pictures illustrating the heterogeneous aspect of atypical B lymphocytes in PPBL which can present with an enlarged (A), either slightly (B) to deeply indented (C), or fully binucleated (D) nucleus.

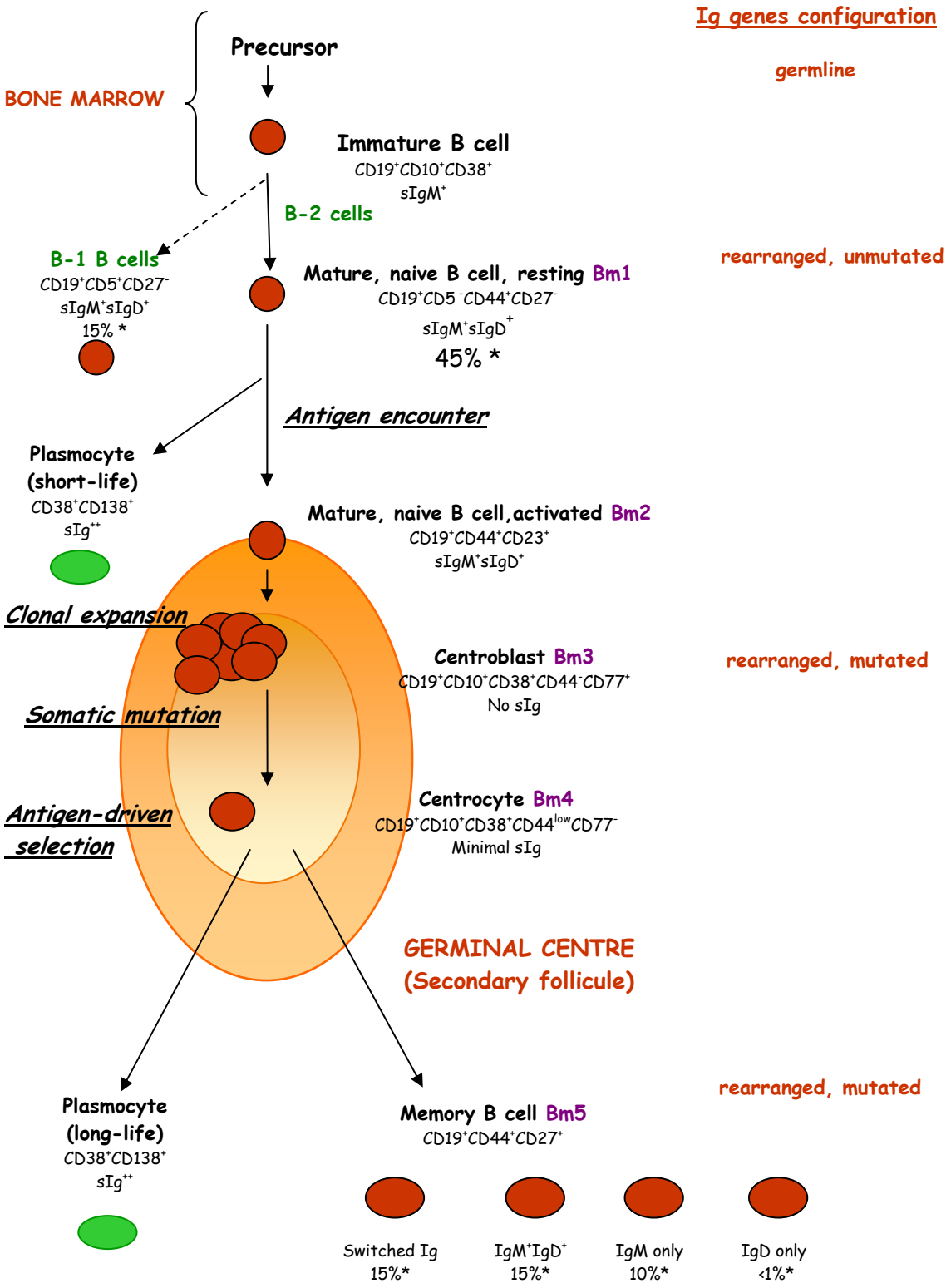
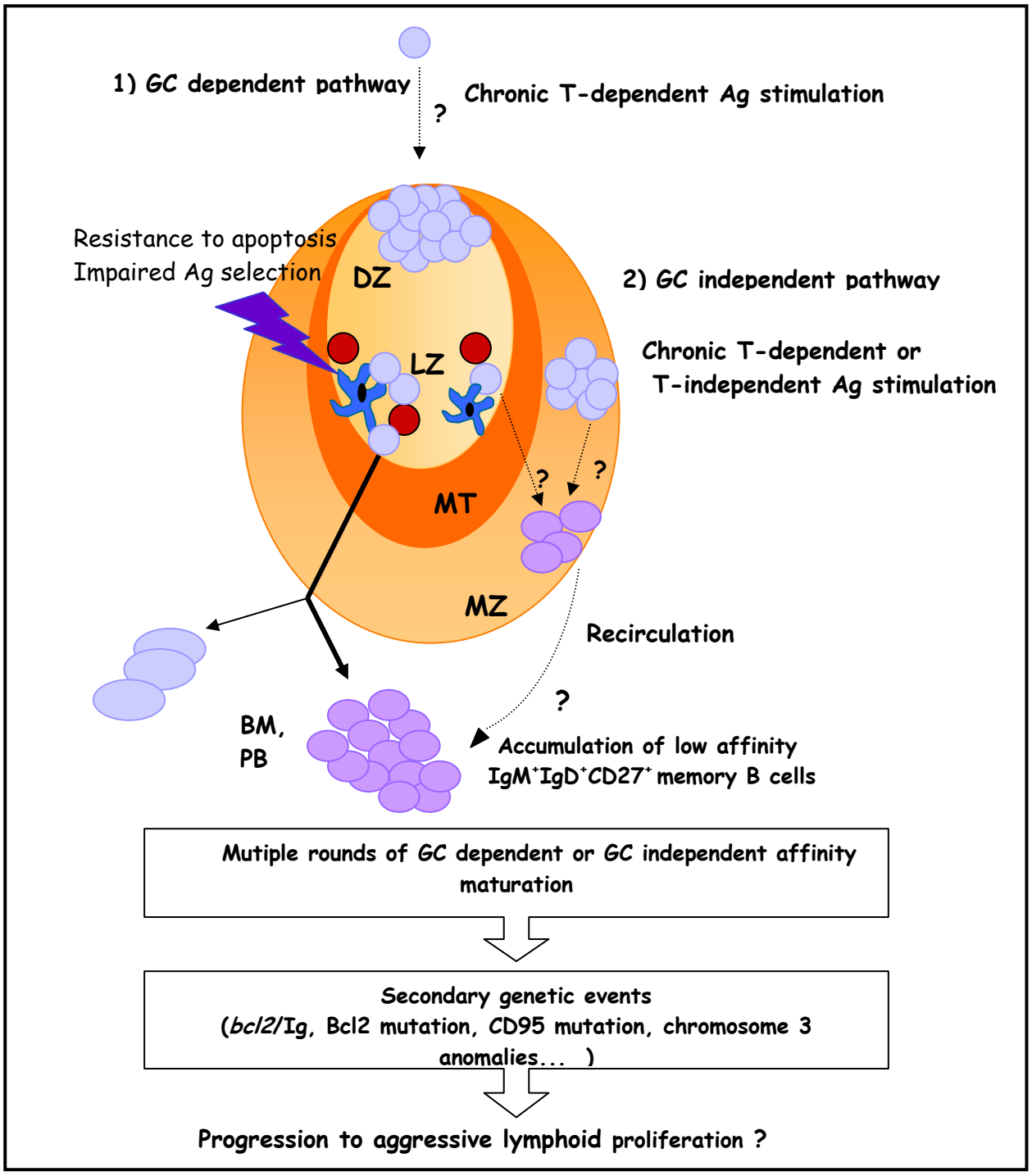
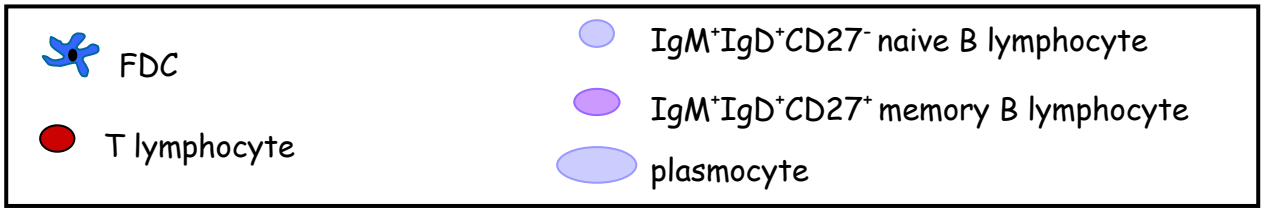


Figure 20 : Peripheral B cell development as indicated by Ig genes configuration and cell surface immunophenotype.* see (Klein, *et al* 1998).

Figure 21 : PPBL, past and future: the aetiopathology of PPBL and its prospective clinical evolution.


1) GC dependent pathway
?
Chronic T-dependent Ag stimulation
Resistance to apoptosis
Impaired Ag selection
DZ
LZ
MT
MZ
2) GC independent pathway
Chronic T-dependent or
T-independent Ag stimulation
?
?
Recirculation
?
BM,
PB
Accumulation of low affinity
IgM+IgD+CD27+ memory B cells
Mutiple rounds of GC dependent or GC independent affinity maturation
Secondary genetic events
(bcl2/Ig, Bcl2 mutation, CD95 mutation, chromosome 3 anomalies...)
Progression to aggressive lymphoid proliferation ?


FDC
T lymphocyte
IgM+IgD+CD27- naive B lymphocyte
IgM+IgD+CD27+ memory B lymphocyte
plasmocyte

Tables

Table 13 : PPBL, a twenty years recapitulation.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Authors	***Cases (age)***	***Tobacco***	***Symptoms/Associated conditions***	***Serum IgM/IgA/ IgG***	***HLA-DR7***	***Binuclated cells (%)***	***EBV serology***	***Cytogenetics***	***Surface markers***
Gordon, *et al* 1982	3F (41-54)	3/3	AM (1/3) RI	+M/-A,G	3/3	+ (24-29)	+ (past)	/	sIgM, sIgD
Carstairs, *et al* 1985, and Chan, *et al* 1990	4F (31-52)	4/4	RI	+M	4/4	+	/	Abnormal clonal D_HJ_H rearrangement 2/2 t	/
Casassus, *et al* 1987	1F (26)	1/1	none	+M/-A,G	1/1	+ (3-8) **with nuclear pockets**	/	Normal karyotype	/
Perreault, *et al* 1989	6F (28-47)	/	SM (3/6) AM (2/6) HL (1/6)	+M/- or =A,G	4/6	+ (1-30)	+ (past) 5/6 t	+i(3q) 1/6	sIgM $CD19^+$
Lawlor, *et al* 1991	1F (43)	1/1	SM AM RI	+M/=A,G	1/1	/	+ (past)	/	sIgM $CD19^+$, 20^+ $FMC7^+$
Tissot, *et al* 1991	1F (54)	1/1	SM RI	+M/=A,-G	1/1	3-5%	-	/	sIgM, sIgD $CD19^+$

Chow, *et al* 1992	1F (47)	1/1	none	/	1/1	+ (<1)	+ (past) PCR+ ISH+	/	$CD20^+$
Delannoy, *et al* 1993	4F (27-44)	4/4	SM (3/4)	+M/=A,G	2/3 t	+ (<5)	-	Normal karyotypes	/
Agrawal, *et al* 1994	1F	1/1	SM	+M/-A,G	1/1	+ (30) some trinucleated	+ (past)	Abnormal karyotypes	$CD22^+$, 37^+ $FMC7^+$ $CD23^-$
Troussard, *et al* 1994	6F (33-46)	5/6	SM (3/6)	+M/- or =A,G	6/6	+ also present in one patient's relatives	+ (past) ISH-	/	$CD11b^+$, 19^+, 20^+, 21^+(2/3 t), 22^+, 24^+, 25^+(2/6) $FMC7^+$ (3/4 t) $CD10^-$,$11c^-$, 38^-, 23^-
Mitterer, *et al* 1995, and Larcher, *et al* 1997	1F (47)	1/1	Chronic fatigue **Mononucleosis**	+M	1/1	+ (8-25)	+ (active) PCR+ 69 bp deletion in LMP1 gene	/	sIgM, sIgD $CD19^+$, 20^+, 21^+, 22^+, 25^+, 54^+ $FMC7^+$ $CD5^-$,10^-, 23^-
Rodriguez, *et al* 1996	1F (35)	1/1	none	+M/-A,G	1/1	+ (5)	+ (past)	/	$CD19^+$, 20^+, 21^+, 22^+
Troussard and Flandrin 1996, Mossafa, *et al* 1996, Troussard,	22F,3M * (31-55)	23/25	SM (7/25) HM (2/25) AM (3/25)	+M	19/20 t	+ (1.5-9)	- 17/22 t	PCC 11/22 t +i(3q) 17/22 t both 9/22 other minor abnormal karyotypes	$CD19^+$ $FMC7^+$ (13/16 t) $CD23^-$

et al 1997a, Troussard, *et al* 1997b and Mossafa, *et al* 1999									
Delage, *et al* 1997, Delage, *et al* 1998, Roy, *et al* 1998 and Loembe, *et al* 2002	11F (38-64)	11/11	SM **Non Hodgkin lymphoma** (1/11)	+M	8/11	+	+ (past) 7/7 t§	Mutiple *bcl2/Ig* 10/11 Single *bcl2/Ig* 1/11	sIgM **and** sIgD (4/4t) $CD19^+$, 20^+ (1/11 t), 27^+ (4/4t) $CD5^-$
Bain, *et al* 1998	1F (49)	1/1	none	+M/-A/=G	/	+	/	/	$CD19^+$, 22^+, 37^+
Callet-Bauchu, *et al* 1997, Callet-Bauchu, *et al* 1999	1F, 3M (38-57)	4/4	SM 4/4 **MALT lymphoma** (1/3)	+M/-A	3/3 t	+ (2-9)	/	+i(3q) 4/4 +3 2/4 dup(3)(q26q29) 1/4 Mutiple *bcl2/Ig* 2/3 t	$CD19^+$, 20^+ $CD5^-$
Carr, *et al* 1997	2F **twins** (33)	2/2	SM (2/2)	+M/-A/=G	2/2	+	+ (past)	/	$CD19^+$ $FMC7^+$ $CD25^-$(1/1 t)
de Jaureguiberry, *et al* 1997	1M (46)	1/1	SM RI	+M/=A,G	1/1	+	+ (past)	/	/
Granados, *et al* 1998	1F (24)	1/1	none	+M/-A,G	1/1	+ (2)	+ (past) PCR-	Mutiple *bcl2/Ig*	$CD19^+$, 20^+ $CD5^-$, 10^-, 25^- 103^-
Reeder et al, 1999	1M (43)	1/1	none	+M/=A,G	0/1	+	+ (past)	Normal karyotype	$CD19^+$, **$CD5^+$**

Woessner, *et al* 1999 and Espinet, *et al* 2000	1F (44)	1/1	none	+M/- or =A,G	1/1	+ (10) **with nuclear pockets**	+ (past)	+i(3q) der(3) +3 Mutiple *bcl2/Ig*	$CD19^+$, 25^+ $CD5^-$, 23^-,
Tonelli, *et al* 2000	1F (43)	1/1	none	+M/-A/=G	0/1	+ (5)	+ (past) PCR+	+3 Mutiple *bcl2/Ig*	sIgM, sIgD $CD19^+$
Vignes, *et al* 2000	3F, 1M (36-66)	4/4	SM (3/4) RI (2/4) HL (2/4)	+M/- or =A/=G	2/3 t	+ (1-10)	/	+22 1/3 t	/
Reimer, *et al* 2000	1M (35)	1/1	SM	+M/=A,G	1/1	+ (10)	+ (past) ISH-	/	$CD19^+$ $CD5^-$, 10^-, 23^-
Gomez, *et al* 2000	1NB (3 months)	0/1	SM	+M/- or =A/=G	0/1	-	+ (past) PCR-	Normal caryoptype No *bcl2/Ig*	$CD19^+$, 22^+, 23^+, 25^+. 38^+ HC2+, **$CD5^+$** $FMC7^+$ $CD2^-$,$11c^-$, 103^-
Gil-Fernandez, *et al* 2001	1F (37)	1/1	SM	+M	1/1	+	+ (past)	Mutiple *bcl2/Ig*	$CD19^+$, 20^+, 22^+, $45RA^+$, $79\beta^+$ $FMC7^+$
Himmelmann, *et al* 2001b and Himmelmann, *et al* 2001b	4F, 1M includes 2 **siblings** (34-53)	2/2 t	SM (2/2 t)	+M	5/5	+	+ (past)	Mutiple *bcl2/Ig* 5/5	sIgD $CD25^+$, 27^+, 148^+ $CD5^-$,23^{low}, 10^-, 38^-
Delage, *et al* 2001	3F* siblings (36-50)	3/3	/	+M/- or =A,G	0/3	+	/	Mutiple *bcl2/Ig* 3/3	/
Lancry, *et*	7F, 1M	8/8	SM (3/8)	+M/- or	5/5 t	+	+	+i(3q) 3/5 t	$CD19^+$, 20^+,

al 2001	* (25-50)			=A/=G		(1-5)	(past) 3/3 t	PCC 1/5 t Mutiple *bcl2/Ig* 3/8 Single *bcl2/Ig* 5/8[£]	22^{+}
Ide, *et al* 2002	1F (29)	1/1	none	+M	0/1	+ (4.5)	+ (past)	Normal karyotype	sIgM, sIgD $CD19^{+}$, 20^{+}, 21^{+}, 22^{+} $CD23^{low}$ $FMC7^{+}$ $CD5^{-}$,10^{-}
Salcedo, *et al* 2002	3F (41-47)	3/3	/	/	3/3	+	/	+i(3q) 3/3 6q- 1/3	$CD19^{+}$, 21^{+}, 24^{+}, 25^{+}, 79^{+}, 95^{+}, $11c^{+}$, $CD5^{-}$,23^{-}
Granel, *et al* 2002	3F (42-43)	3/3	none	+M **aPa/cofactor IgM**	0/3	+ (6-11)	/	Normal karyotypes	$CD19^{+}$ $CD5^{-}$,23^{-}
Schonermarck, *et al* 2003	1F (54)	1/1	AM	+M/=A,G	/	+ (2)	+ (past)	Single *bcl2/Ig*[£]	$CD19^{+}$ $CD5^{-}$,10^{-}
Vincenot-Blouin, *et al* 2003	1F (31)	1/1	AM SM	+M	/	+	+ (past)	PCC -X +8	$CD19^{+}$, 20^{+} $CD5^{-}$,10^{-}
Feugier, *et al* 2004	7F, 1M (25-56)	8/8	SM (5/8)	+M	2/3 t	+ (1-25)	- PCR-ISH-	+3 1/7 t del 6 1/7 t Mutiple *bcl2/Ig* 4/6 t Single *bcl2/Ig* 1/6 t[£]	sIgM (6/7 t), and sIgD (3/6) $CD19^{+}$, 22^{+}, 79^{+} $CD11c^{+}$(3/6 t) $CD38^{low}$ (3/7 t) $FMC7^{+}$(6/7 t) $CD5^{-}$,10^{-}, 54^{-}, $62E^{-}$, $62P^{-}$

Abbreviations: F (female), M (male), NB (newborn), AM (adenomegaly), HM (hepatomegaly), SM (splenomegaly), RI (upper respiratory track infections), HL (Herpes labialis) Ig concentrations (+: raised, -: lowered, =: normal), t: tested, aPa/cofactor Ab (anti phospholipids/cofactor antibody), ISH (*in situ* hybridation), PCC (premature chromosome condensation), bp (base pairs).

* Some of these cases may have been the object of previous report by the same team/authors. § See text, section Infectious factors: Epstein Barr virus, for description. £ Only the MBR was analysed.

Table 14 : Human somatically mutated IgM^+IgD^+ memory B cell subsets.

	Blood (Agematsu 2000, Klein, *et al* 1998)	***Bone marrow*** (Paramithiotis and Cooper 1997)	***Marginal zone and its equivalents***. (Dono, *et al* 2000, Tangye, *et al* 1998)
Morphology	Large, abundant cytoplasm	Large, non dividing	Medium sized, irregular or indented nuclei, abundant cytoplasm either proliferating or not
Mutation frequency	V_H1,3,4: 4.86%	V_H5: 3.3 ± 2.9%	V_H4: 2.07% (tonsil) V_H5: 1.9% (spleen) V_H6: 2.5% (spleen)
Antigenic selection	Present†	Present* Clonally related cells	Present†. Clonally related cells.
Antibody production upon stimulation	IgM (SAC+IL-2)	IgM (SAC+IL-2)	IgM (SAC+IL-2) IgM, IgA, IgG (αCD40+IL-2 +IL-10)
Cell surface markers	$IgM^{++}IgD^+$ $CD27^+$ $CD23^-CD70^+$	IgM^+IgD^+ $CD27^+$? $CD71^+CDw75^+CD23^+$ $CD38^+CD95^+CD22^+$ $CD10^-CD25^-$	$IgM^{++}IgD^+$ $CD27^+$ $CD23^-CD25^{+/-}$ $CD38^-CD21^-$(tonsil) $CD148^+CD21^+CD95^+$(spleen)

*: as estimated by analysis of mutations distribution in CDR.

†: as estimated by analysis of mutations distribution in FR.

Table 15 : Assessment of functional properties in PPBL B lymphocytes.

Authors	*Experiment*	*Observations**
Reimer, *et al* 2000	-sIgM + Il-4-induced proliferation. -sIgM and/or Il-4-induced HLA-DR expression. - sIgM and/or Il-4-induced CD23 expression.	-No proliferative response. -No induction of HLA-DR or CD23 following IL-4 stimulation specifically.
Loembe, *et al* 2001	- Cytofluorometric assessment of CD40 expression. - CD40 gene sequencing. -CD40-induced proliferation. -Western blot assessment of CD40-induced tyrosine phosphorylation.	Despite normal expression of an intact CD40 molecule, and induction of CD40 signalling cascade proximal events, no proliferative response.
Himmelmann, *et al* 2001a	-Il-2/SAC stimulation (patients vs healthy controls).	Elevated IgM secretion but marginal, diminished IgG secretion.
Roussel, *et al* 2003	-Cytofluorometric assessment of CD95 expression. -CD95-induced apoptosis. -Western blot assessment of FADD and caspase 8 expression.	Despite normal expression of the CD95 molecule, and presence of the DISC components FADD and caspase 8, no induction of apoptosis.

*As compared to purified B lymphocytes from healthy controls (Himmelmann, *et al* 2001a, Loembe, *et al* 2001, Reimer, *et al* 2000) or the Ramos B cell line (Roussel, *et al* 2003).

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