UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
NÍVEL DOUTORADO

IDENTIFICAÇÃO DE CÉLULAS LEUCÊMICAS POR CITOMETRIA DE FLUXO UTILIZANDO LECTINAS CONJUGADAS

ELIZANGELA FERREIRA DA SILVA

RECIFE
2010

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Parecer da comissão examinadora da defesa da Tese de Doutorado de

ELIZANGELA FERREIRA DA SILVA

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A comissão examinadora, composta pelos professores abaixo, sob a presidência do primeiro, considera a doutoranda **ELIZANGELA FERREIRA DA SILVA** como:

APROVADA

Recife, 26 de fevereiro de 2010.

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RESUMO

Anticorpos são largamente utilizados para caracterizar células leucêmicas dentre seus estágios de diferenciação. O uso de lectinas, (glico)proteínas que reconhecem especificamente carboidratos, como sonda de análise da superfície celular fornece informações sobre carboidratos na membrana celular. A diferenciação das células e sua transformação maligna são caracterizadas por mudanças nos resíduos de carboidratos de glicoconjugados de membrana celular as quais podem ser detectadas por lectinas. Neste estudo, células de medula óssea de pacientes com leucemia mielóide aguda (LMA) e linfóide aguda (LLA), recém diagnosticados no Centro de Oncohematologia Pediátrica do Hospital Universitário Oswaldo Cruz/UPE e Fundação de Hematologia e Hemoterapia de Pernambuco (HEMOPE), foram avaliadas usando um painel com cinco lectinas conjugadas ao isotiocianato de fluoresceina, FITC (*fluorescein isothiocyanate*), Wheat germ agglutinin (WGA), *Lotus tetragonolobus* agglutinin (LTA), Concanavalina A (Con A), *Ulex europaeus* (UEA I) e *Arachis hypogaea* (PNA), de acordo com o protocolo de marcação utilizado para o diagnóstico de rotina com anticorpos monoclonais. A WGA, específica para N-acetilglicosamina, marcou intensamente as células leucêmicas de pacientes com LLA e LMA, mas não foi capaz de diferenciá-las. Entretanto, a UEA-I (L-fucose) mostrou maior especificidade para as células de linhagem mielóide (LMA) do que para LLA, sendo capaz de diferenciá-las. A LTA marcou tanto as células de LMA quanto de LLA, embora apresente também especificidade para o carboidrato L-fucose. Não foi observada marcação seletiva para as lectinas PNA (D-galactose específica) e Con A (D-glicose/D-manose) para as células de LLA e LMA. Estes achados demonstram que lectinas são potenciais sondas para detectar mudanças no perfil sacarídico de glicoconjugados de membrana de células hematopoiéticas imaturas, sendo mais uma ferramenta para o diagnóstico dos diferentes tipos de leucemia.

Palavras-chave: Lectinas, Leucemia, Citometria de Fluxo.

ABSTRACT

Antibodies are largely applied for the caractherization of leukemia cells in their differentiation stages. The use of lectins, (glyco)proteins that specifically recognize carbohydrates, as cell surface probes helps to understand the sugar content of cell membrane. Changes in saccharide moieties of glycoconjugates of cell membranes are features of cell differentiation and malign transformation which can be detected by lectins. In this study bone marrow cells from patients with acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) diagnosed at the Pediatric Oncohaemathology Centre of Oswaldo Cruz University Hospital – UPE and Fundation of Haemathology and Haemotherapy of the State of Pernambuco (HEMOPE) were evaluated with lectins conjugated to fluorescein isothiocyanate, FITC, Wheat germ agglutinin (WGA), *Lotus tetragonolobus* agglutinin (LTA), Concanavalina A (Con A), *Ulex europaeus* (UEA-I) e *Arachis hypogaea* (PNA), according to the protocol of routine diagnosis with monoclonal antibodies. WGA, N-acetyl-glucosamine specific, was positive to AML and ALL cells but failed to differentiate them. UEA-I (L-fucose) showed higher specificity for AML cell than to ALL being able to differentiate them. LTA (also L-fucose specific) recognized both leukemia cell lineages with the same pattern. It was not observed selective staining for AML and ALL by PNA and Con A, D-galactose and D-glucose/D-mannose specific, respectively. Results demonstrate that lectins are potential probes for the detection of changes in the saccharide profile in cell membrane glycoconjugates of immature haematopoietic cells being an auxiliary tool for the diagnoses of leukemia.

Keywords: Lectins, Leukemia, Flow Cytometry

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1 INTRODUÇÃO

Câncer representa um grupo heterogêneo de doenças que afeta humanos com alta frequência, e contribui significantemente para a morbidade e mortalidade (MARTINKOVA *et al.*, 2009). O câncer infanto-juvenil (abaixo de 19 anos) é considerado raro quando comparado com os tumores do adulto, correspondendo a 2-3% de todos os tumores malignos. Estimativa realizada pelo Intituto Nacional do Câncer (INCA) para o biênio 2008/09 revela que ocorreriam cerca de 9.890 casos por ano em crianças e adolescentes com até 18 anos de idade (MINISTÉRIO DA SAÚDE/INCA/SOBOPE, 2008).

A leucemia aguda é o tipo de câncer mais comum em crianças, correspondendo a um terço das doenças malignas. A leucemia linfoblástica aguda (LLA) e a leucemia mielóide aguda (LMA) representam um grupo heterogêneo de doenças, sendo a LLA mais frequente em crianças do que a LMA que é mais frequente em adultos. A expectativa de cura para a LLA tem sido em torno de 75-80%. Ao contrário da LLA a LMA é mais difícil de ser tratada (SHAH & AGARWAL, 2008).

O diagnóstico e a classificação das leucemias agudas baseavam-se, em grande parte, na análise morfológica e citoquímica das células neoplásicas. A falta de reprodutibilidade dessas análises e a dificuldade para classificar algumas leucemias incentivaram à busca de outros parâmetros. Assim o diagnóstico e a classificação das leucemias agudas apóiam-se, em grande parte, nos estudos imunofenotípicos por citometria de fluxo, permitindo avançar na identificação de determinados subgrupos dificilmente classificáveis do ponto de vista morfológico (FARIAS & CASTRO, 2004).

A imunofenotipagem por citometria de fluxo é essencial para estabelecer o correto diagnóstico da leucemia e definir a linhagem celular, sendo capaz de reconhecer LLA de

linhagem T, LLA de linhagem precursora de células B, LMA e leucemia aguda bifenotípica (RUIZ-ARGUELLES *et al.,* 2005; PUI *et al.,* 2008).

A classificação das leucemias agudas através da análise de marcadores de superfície é uma área de grande interesse na pesquisa e tem sido estimulada pela aplicação de anticorpos monoclonais (KALEEM *et al.,* 2003). Devido à falta de anticorpos específicos relacionados à diferenciação celular, com raras exceções, a classificação imunológica é usualmente baseada no padrão de reatividade de um painel (grupo selecionado) de anticorpos monoclonais associados à linhagem (DUNPHY, 2004).

Na prática laboratorial utilizam-se, rotineiramente, um painel de anticorpos monoclonais conjugados ao isotiocianato de fluoresceína (FITC - *fluorescein isothiocyanate*) ou ficoeritrina (PE - *phycoeritrin*), para o diagnóstico e classificação das leucemias. Uma alternativa ou uma abordagem complementar para estes biomarcadores (anticorpos) é o uso de lectinas conjugadas a marcadores fluorescentes, em citometria de fluxo.

Lectinas são proteínas de origem não imunológica, ubiquamente distribuídas na natureza, que reconhecem especificamente carboidratos e participam de uma variedade de processos celulares (GABIUS *et al.,* 2002; DAMODARAN *et al.,* 2007).

Como muitas lectinas ligam-se especificamente a carboidratos terminais de glicoproteínas e glicolipídeos presentes na membrana das células, elas podem ser usadas para caracterizar esses glicoconjugados (GORELIK *et al.,* 2001). Durante a diferenciação celular e transformação maligna, a biossíntese das cadeias de oligassacarídeos de glicoproteínas é freqüentemente alterada e esta alteração pode ser detectada pelas lectinas (GORELIK *et al.,* 2001; CASTILLO-VILLANUEVA *et al.,* 2005; DAMODARAN *et al.,* 2007).

Numerosos dados mostram que a transformação maligna está associada a várias alterações na expressão de carboidratos, indicando que os glicoconjugados desempenham um importante papel nesta transformação (SINHA *et al.,* 1999; GORELIK *et al.,* 2001; SHARON

& LIS, 2004, 2007). As lectinas muitas vezes tem maior preferência por células transformadas do que por células normais, por causa do grande número de receptores (açúcares) ou a distribuição alterada destes receptores na superfície das células cancerígenas (HEINRICH *et al.,* 2005; SHARON & LIS, 2007).

A reação de reconhecimento lectina-carboidrato é tão específica quanto a antígeno-anticorpo. A partir desta característica, as lectinas estão sendo utilizadas, experimentalmente, como sondas de sacarídeos de células neoplásicas da mama (BELTRÃO *et al.,* 1998, 2001), cérebro humano (BELTRÃO *et al.,* 2003), leucemia (PÉREZ-CAMPOS-MAYORAL *et al.,* 2008), entre outros.

As lectinas conjugadas ao FITC, como os anticorpos, reforçam a possibilidade de utilização desta classe de macromoléculas para identificação das células leucêmicas de linhagem mielóide e linfóide. O diagnóstico e a classificação das leucemias agudas são um argumento de contínua evolução, visto que permitem a identificação do tipo celular envolvido na leucemogênese, o que é essencial, pois orienta a terapêutica e determina, até certo ponto, o prognóstico (FARIAS & CASTRO, 2004; BASSO *et al.,* 2007).

2 OBJETIVOS

2.1 GERAL

Empregar lectinas conjugadas ao FITC para o reconhecimento de células blásticas de leucemia linfóide aguda (LLA) e leucemia mielóide aguda (LMA) através da citometria de fluxo.

2.2 ESPECÍFICOS

1. Utilizar as lectinas Wheat germ agglutinin (WGA), *Lotus tetragonolobus* agglutinin (LTA), Concanavalina A (Con A), *Ulex europaeus* (UEA I) e *Arachis hypogaea* (PNA) conjugadas ao FITC como sondas para carboidratos de superfície de células leucêmicas de LLA e LMA;
2. Avaliar a ligação da lectina *Ulex europaeus* (UEA I) a células leucêmicas de LMA e seus subtipos;
3. Comparar os resultados obtidos com os anticorpos CD45, CD34, CD10, CD19, CD3, CD7, CD13 and CD33 conjugados a PE ou ao FITC, utilizados na rotina diagnóstica, com as lectinas WGA, LTA, Con A, UEA I e PNA conjugadas com FITC.

3 REVISÃO BIBLIOGRÁFICA

3.1 LEUCEMIA

Hematopoiese é um sistema altamente organizado responsável pela produção das células sanguíneas. O controle da proliferação, diferenciação e maturação das células são feitos através de uma complexa interação molecular das células com o microambiente da medula óssea (SACHS, 1995). É um processo complexo regulado pela expressão coordenada de diversos fatores de transcrição, os quais são ativados ou inibidos de acordo com o produto final da hematopoiese (PUI *et al.,* 2004).

Leucemia é uma doença clonal de células precursoras hematopoiéticas e desenvolve-se através de uma série de mutações no clone. Resulta em uma progressiva diversificação genética seguida por uma "seleção natural" de subclones mutantes dominantes (GREAVES, 2002).

Evidências epidemiológicas sugerem que radiação ionizante; agentes químicos como pesticidas e benzeno; viroses, vírus tipo I da leucemia/linfoma de células T, vírus de Epstein-Barr e bactéria, como o *Helicobacter pylori,* podem estar envolvidos no desenvolvimento de alguns subtipos de leucemia e linfoma em adultos e crianças (ALEXANDER, 1995; GREAVES, 2002).

Leucemia aguda é o câncer mais comum em crianças representando cerca de metade de todos os tipos de câncer entre pessoas jovens menores que 15 anos, com uma taxa de incidência crescente nos últimos anos (BASSO *et al.,* 2007).

Devido a hematopoiese em vertebrados ser intensamente ativa durante o desenvolvimento fetal e nos primeiros anos de vida, é de se esperar que a leucemia seja mais comum em crianças, podendo afetar adultos também (PUI *et al.,* 2004). Na infância, a

incidência é um pouco maior em pessoas do sexo masculino e de cor branca (REDAELLI *et al.,* 2005).

No Brasil é também o tipo mais freqüente de câncer em crianças e adolescentes, alcançando uma média de 29% entre os casos nessa faixa etária. Em Recife, no período 1997-2001, ocorreu uma média de 50 casos, anualmente, por um milhão em meninos. Nas meninas, esse número é um pouco menor, 47 casos por milhão. Os dados constam de um estudo inédito feito pelo Instituto Nacional de Câncer (INCA) juntamente com a Sociedade Brasileira de Oncologia Pediátrica, SOBOPE (MINISTÉRIO DA SAÚDE/INCA/SOBOPE, 2008).

As leucemias são classificadas com base no tipo celular envolvido e no grau de maturação das células leucêmicas, podendo ser agudas ou crônicas. As leucemias crônicas são definidas como uma hiperplasia de elementos maduros onde a proliferação clonal não está associada inicialmente a um bloqueio maturativo. Assim a população celular diferencia-se e amadurece, embora haja graus variáveis de displasia, o que compromete funcionalmente a população afetada. Tendem a constituir distúrbios relativamente indolentes nos seus estágios iniciais, porém, tardiamente podem transformar-se em neoplasias agressivas, semelhantes às leucemias agudas (ANJOS *et al.,* 2000).

As leucemias agudas caracterizam-se pela proliferação clonal acompanhada de bloqueio maturativo variável, o que possibilita a existência de diferentes subtipos de leucemias. São reconhecidas duas variantes principais das leucemias agudas: as de origem linfóide, leucemia linfóide aguda (LLA), e as de origem mielóide, leucemia mielóide aguda (LMA) (ANJOS *et al.,* 2000).

A LLA e a LMA representam um grupo heterogêneo de doenças, que resultam de mecanismos diversos de leucemogênese. Leucemias agudas são definidas como uma doença genética, de fato, as anormalidades genéticas estão presentes em mais de 80% das LLAs e

mais de 90% das LMAs, e a maioria destas alterações genéticas são recorrentes (BASSO *et al.,* 2007).

A LLA é uma doença maligna derivada das células linfóides indiferenciadas (linfoblastos) que estão presentes em grande número na medula óssea, sangue e outros órgãos. Acumula-se grande quantidade de linfoblastos, pois os mesmos mantêm capacidade de multiplicação, mas não de diferenciação até formas maduras e normais (LORENZI, 2003; JABBOUR *et al.,* 2005).

Embora a LLA possa ocorrer em qualquer idade, sua incidência é maior entre crianças de 2 a 5 anos (PUI *et al.,* 2008), numa porcentagem de cerca de 70%, diminuindo entre adolescentes e adultos jovens, entre os quais a incidência das leucemias agudas é de 20% (LORENZI, 2003), voltando a crescer após os 60 anos de idade (FALCÃO *et al.,* 2002).

O evento preciso que leva ao desenvolvimento da leucemia linfóide aguda é desconhecido. Poucos casos (< 5%) estão associados com hereditariedade, síndromes com predisposição genética, como síndrome de Down, síndrome de Bloom, ataxia-telangiectasia, ou com irradiações ionizantes ou exposição a drogas quimioterápicas e específicas (JABBOUR *et al.,* 2005; PUI *et al.,* 2008).

O diagnóstico laboratorial da LLA é complexo e baseia-se em análises citomorfológica, imunológica, citogenética e molecular. A imunofenotipagem detalhada dos blastos leucêmicos distingue vários estágios de diferenciação de linfoblastos B ou T, podendo ser classificada como LLA de linhagem B ou T. As leucemias de linhagem B foram divididas de acordo com os estágios de diferenciação dos progenitores B na medula óssea, classificando-se em: pró-B, comum, pré-B e B-maduro. As LLAs de linhagem T dividem-se também de acordo com os níveis de diferenciação: LLA pró-T, pré-T, T-cortical e T-maduro (FALCÃO *et al.,* 2002; COOLS & VANDENBERGHE, 2009).

A LMA é uma doença clonal que se caracteriza pela proliferação anormal de células progenitoras da linhagem mielóide, ocasionando produção insuficiente de células sanguíneas maduras normais. A expansão clonal de vários tipos de células precursoras hematopoiéticas na medula óssea conduz a desorganização do delicado balanço entre auto-renovação e a diferenciação, que é característica da hematopoiese normal (MARTINS & FALCÃO, 2000; SILVA *et al.,* 2006).

O mecanismo que leva a célula progenitora da linhagem mielóide a perder o controle da proliferação celular, ocasionando a expansão do clone leucêmico, permanece incerto. Um aumento no número de aberrações genéticas, como translocações cromossômicas, que alteram a função dos fatores regulatórios de transcrição, tem sido identificado como a causa da LMA (ALTUCCI *et al.,* 2005).

A LMA é uma doença predominante em adultos mais velhos (acima de 60 anos de idade), em mais de 50% dos casos (LÖWENBERG, 2001; IOVINO & CAMACHO, 2003). É mais comum no sexo masculino do que no feminino (DOUER, 2003), representa cerca de 15-20% das leucemias agudas da infância e 80% das dos adultos (HALL, 2001; SILVA *et al.,* 2006).

O termo leucemia mielóide aguda é usado várias vezes para designar leucemias não linfóides, incluindo mielocítica (M0, M1, M2, M3), monocítica (M4, M5), eritróide (M6), e megacariocítica (M7) (CAMPANA & BEHM, 2000). É uma mistura de doenças distintas que diferem com relação a sua patogênese, anormalidades genéticas, características clínicas, resposta à terapia e prognóstico. Análise molecular e genética tem sido importante para identificação da doença dentre o grupo de subtipos de LMAs (LÖWENBERG *et al.,* 2003).

Em 1976, o grupo cooperativo Franco-Americano-Britânico (FAB) propôs um sistema de classificação para a LMA baseado nos aspectos morfológico e citoquímico. O grupo sugere que leucemia aguda com menos que 3% de blastos mieloperoxidase (MPO) e ou Sudan Black

(SBB) positivo são LLA, onde se maior ou igual a 3% de blastos positivos o diagnóstico é LMA (BENNETT *et al.,* 1976; STASI *et al.,* 1999).

A revisão desta classificação publicada em 1985 foi amplamente usada e reconhecida como a classificação padrão para LMA. Foi incluído na classificação FAB dois grupos que exibiam diferenciação monocítica, leucemia mielomonocítica (LMA-M4), e leucemia monocítica/monoblástica (LMA-M5) (BENNETT *et al.,* 1985; XU *et al.,* 2009). Em 1991, o grupo propôs critérios para designar a leucemia mielóide aguda minimamente diferenciada a LMA-M0 (STASI *et al.,* 1999).

Contudo, a classificação FAB está sendo substituída pela classificação da Organização Mundial da Saúde (OMS). Em 2001 a OMS em conjunto com a Sociedade de Hematopatologia e Associação Européia de Hematopatologia, publicou uma nova classificação para neoplasias mielóides. Ao contrário da classificação FAB (Tabela 1) a OMS inclue achados morfológicos, genéticos, imunofenotípicos, biológicos, e características clínicas para definir grupos específicos da doença (SHAH & AGARWAL, 2008).

Recentemente, uma revisão desta classificação foi publicada como parte da 4ª edição da série de monografias da OMS. O objetivo desta revisão foi incorporar novas informações científicas e clínicas para refinar os critérios de diagnóstico para neoplasias já descritas, e introduzir doenças recentemente reconhecidas (SWERDLOW *et al.,* 2008; VARDIMAN *et al.,* 2009). A Tabela 2 lista os subtipos da leucemia aguda de acordo com a classificação OMS.

Muitos casos de leucemia aguda podem ser classificados como mielóide e linfóide usando a classificação FAB e um painel de marcadores imunológicos. Entretanto, mesmo com morfologia, citoquímica e imunofenotipagem ainda é difícil determinar, em alguns pacientes, se as células leucêmicas são derivadas de linhagem mielóide ou linfóide. Estes casos são classificados como leucemia de linhagem ambígua de acordo com a classificação da OMS de

doenças hematológicas, incluindo leucemia aguda indiferenciada, leucemia aguda bilineal, e leucemia aguda bifenotípica, as quais representam cerca de 5% do total de leucemias agudas (XU *et al.*, 2009).

Tabela 1. Classificação FAB (Grupo Franco-Americano-Britânico) da leucemia mielóide aguda.

Subtipo	Classificação
M0	LMA com mínima diferenciação
M1	Leucemia mieloblástica sem maturação
M2	Leucemia mieloblástica com maturação
M3	Leucemia promielocítica aguda
M4	Leucemia mielomonocítica aguda
M5	Leucemia monoblástica/monocítica aguda
M6	Leucemia eritroblástica aguda
M7	Leucemia megacarioblástica aguda

(Fonte: SHAH & AGARWAL, 2008)

Leucemia aguda bifenotípica e bilineal são também conhecidas como leucemia de linhagem mista, nas quais ambas células mielóide e linfóide estão envolvidas. Por definição, leucemia bifenotípica significa uma linhagem de células leucêmicas, mas que expressam marcadores mielóide e linfóide. Por outro lado, leucemia aguda bilineal significa que há distintas populações (dois ou mais subtipos) de células expressando marcadores linfóides e mielóides, em um único paciente (RUBNITZ *et al.,* 2009; XU *et al.,* 2009).

Tabela 2. Classificação da leucemia aguda de acordo com a Organização Mundial da Saúde (OMS).

Leucemia mielóide aguda
Leucemia mielóide aguda com anormalidades genéticas recorrentes
LMA com t(8;21)(q22;q22); *RUNX1-RUNX1T1*
LMA com inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
LMA com APL with t(15;17)(q22;q12); *PML-RARA*
LMA com t(9;11)(p22;q23); *MLLT3-MLL*
LMA com t(6;9)(p23;q34); *DEK-NUP214*
LMA com inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
LMA (megacarioblástica) com t(1;22)(p13;q13); *RBM15-MKL1*
Entidade provisória: LMA com mutação NPM
Entidade provisória: LMA com mutação CEBPA
Leucemia mielóide aguda com alterações relacionadas a mielodisplasia
Neoplasia mielóide relacionada à terapia
Leucemia mielóide aguda (mesma classificação)
LMA com mínima diferenciação
LMA sem maturação
LMA com maturação
Leucemia mielomonocítica aguda
Leucemia monoblástica/monocítica aguda
Leucemia eritróide aguda
Leucemia eritróide pura
Eritroleucemia, eritróide/mielóide
Leucemia aguda megacarioblástica
Leucemia Basofílica aguda
Panmielose aguda com mielofibrose
Sarcoma mielóide
Proliferações mielóide relacionada com a síndrome de Down
Mielopoiese anormal transitória
Leucemia mielóide associada com a síndrome de Down
Neoplasia de células dendríticas blástica plasmocitóide
Leucemia aguda de linhagem ambígua

Leucemia aguda indiferenciada
Leucemia aguda com fenótipo misto com t(9;22)(q34;q11.2); *BCR-ABL1*
Leucemia aguda com fenótipo misto com t(v;11q23); rearranjo *MLL*
Leucemia aguda com fenótipo misto, B-mielóide
Leucemia aguda com fenótipo misto, T-mielóide
Entidade provisória: leucemia/linfoma linfoblástica de células natural killer (*NK*)
Leucemia/linfoma linfoblástica B
Leucemia/linfoma linfoblástica B
Leucemia/linfoma linfoblástica B, com alterações genéticas recorrentes
Leucemia/linfoma linfoblástica B com t(9;22)(q34;q11.2);*BCR-ABL 1*
Leucemia/linfoma linfoblástica B com t(v;11q23); rearranjo *MLL*
Leucemia/linfoma linfoblástica B com t(12;21)(p13;q22) *TEL-AML1* (*ETV6-RUNX1*)
Leucemia/linfoma linfoblástica B com hiperdiploidia
Leucemia/linfoma linfoblástica B com hipodiploidia
Leucemia/linfoma linfoblástica B com t(5;14)(q31;q32) *IL3-IGH*
Leucemia/linfoma linfoblástica B com t(1;19)(q23;p13.3);*TCF3-PBX1*
Leucemia/linfoma linfoblástica T

(Fonte: VARDIMAN *et al.,* 2009)

Uma classificação apropriada de LMA é importante para a clínica e permite que futuros estudos possam expandir e refinar a compreensão dessa doença (XU *et al.,* 2009), assim como uma adequada classificação da LLA, que atualmente apresenta um percentual de cura de 80%, devido à evolução das terapias, correspondendo a um grande sucesso na história da medicina moderna (PUI & JEHA, 2007).

O diagnóstico e a classificação das leucemias envolvem vários métodos incluindo morfologia, citoquímica, citogenética clássica e genética molecular, imunofenotipagem e biologia molecular. Estes métodos de diagnóstico são pré-requesitos para estratégias de tratamento individual e para avaliação da resposta ao tratamento, especialmente considerando que vários tipos de leucemia possuem terapia específica (BASSO *et al.,* 2007).

3.2 CITOMETRIA DE FLUXO

O diagnóstico e tratamento da leucemia dependem da detecção inequívoca da população de células leucêmicas e a identificação da linhagem hematopoética da qual a população se origina. Esta tarefa é melhor realizada pelo uso da análise imunológica contemporânea, a imunofenotipagem das doenças hematológicas por citometria de fluxo (CAMPANA & BEHM, 2000).

A citometria de fluxo é uma técnica multiparamétrica que utiliza anticorpos monoclonais conjugados a um composto fluorescente para analisar qualitativa e quantitativamente padrões de expressão de antígenos em populações celulares de interesse (RADCLIFF & JAROSZESKI, 1998; HARRIS *et al.,* 1999).

O equipamento utilizado para a técnica é denominado citômetro de fluxo que possui quatro componentes principais, incluindo um laser que incide sobre as células, fotodetectores de sinais, suspensões celulares marcadas com anticorpos fluorescentes e um computador ligado ao sistema. O computador elabora gráficos e histogramas, divididos em regiões de parâmetros de fluorescência denominadas FL1, FL2, FL3, FL4, etc., que possibilitam a análise de populações celulares diferentes, dentro de uma mesma amostra, desde que marcadas com fluorocromos distintos (Figura 1) (QUIXABEIRA & SADDI, 2008).

Os fluorocromos mais utilizados são o isotiocianato de fluoresceína (*fluorescein isothiocyanate* - FITC) e a ficoeritrina (*phycoerythrin* - PE), que emitem luz com diferentes comprimentos de onda. Outros fluocromos como PerCP (*peridinin chlorophyll protein complex*), APC (*allophycocyanin*), vermelho Texas e rodamina também são utilizados (CAMPANA & BEHM, 2000).

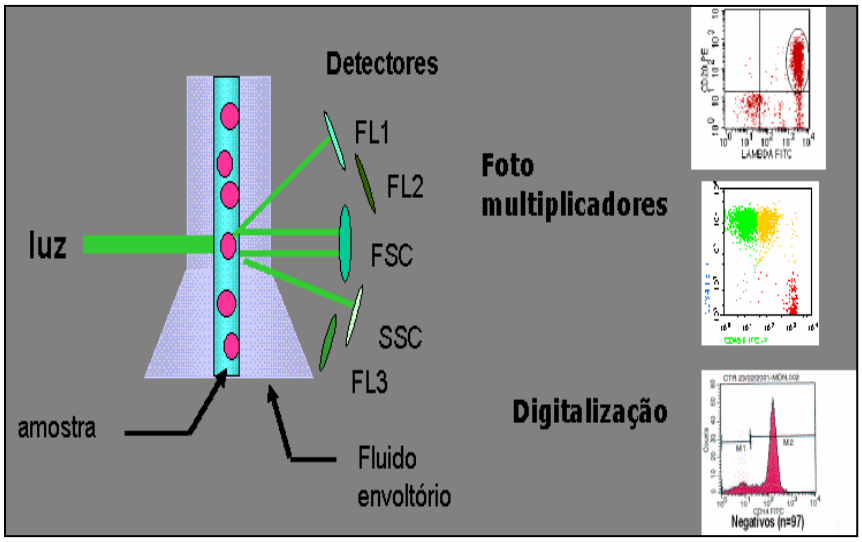


Figura 1: Representação esquemática do citômetro de fluxo.
(Fonte: http://www.fleury.com.br/.../figura3_capitulo2.gif)

Os parâmetros celulares avaliados pelo método podem ser divididos em dois principais grupos, os evidenciados pela luz dispersa (que refletem o tamanho celular e complexidade interna FSC- *forward scatter* e SSC- *side scatter*, respectivamente – Figura 2) e os associados à presença de um ou mais anticorpos conjugados com fluorocromos que reconhecem moléculas celulares específicas, que podem estar no interior da célula (antígenos citoplasmáticos ou nucleares) ou na sua superfície (LORENZI, 2003), refletindo características fenotípicas importantes, como grau de diferenciação celular, linhagem celular, ploidia, expressão de proteínas apoptóticas, dentre outras (ORFAO *et al.*, 1995; QUIXABEIRA & SADDI, 2008).

A citometria de fluxo é uma técnica que oferece objetividade, sensibilidade, rapidez e precisão na análise das características celulares, incluindo o conteúdo de DNA/RNA, a detecção e quantificação de antígenos celulares, a análise de resistência celular a drogas e a análise do conteúdo citoplasmático (QUIXABEIRA & SADDI, 2008).

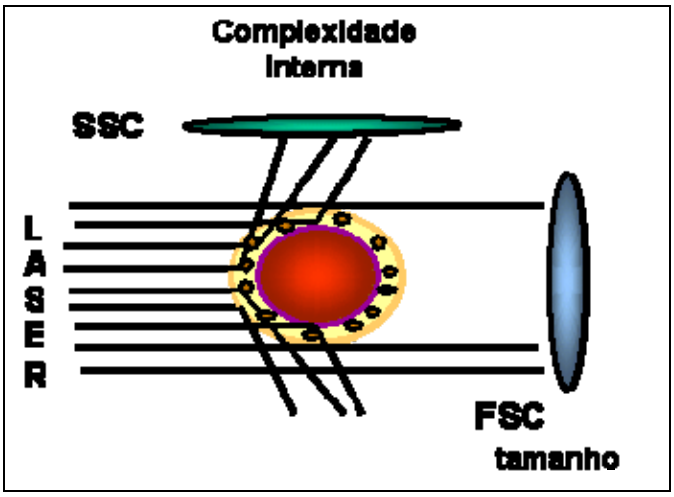


Figura 2: Análise das características físicas das células – tamanho e complexidade interna, no citômetro de fluxo. (Fonte: http://www.fleury.com.br/.../figura1_capitulo2.gif)

A combinação da alta afinidade, anticorpos monoclonais específicos juntamente com a sensibilidade, e processamento rápido da amostra resultam na obtenção de várias informações, que fazem da imunofenotipagem por citometria de fluxo um método de alta importância para o diagnóstico das leucemias (SAXENA & ANAND, 2008).

A citometria de fluxo tem sido largamente utilizada nos laboratórios clínicos e tornou-se uma poderosa ferramenta diagnóstica para caracterização imunofenotípica das leucemias e doenças linfoproliferativas crônicas, podendo ser utilizada para a identificação das linhagens mielóide e linfóide, detecção de doença residual mínima (MDR), e, mais recentemente, monitoramento de estratégias de tratamento baseadas em anticorpos (RUIZ-ARGUELLES *et al.,* 1998; SCHABATH *et al.,* 2003; VASCONCELOS, 2007).

Embora muitas leucemias possam ser identificadas morfologicamente, com ou sem análise citoquímica, a imunofenotipagem é indispensável para a identificação da leucemia mielóide aguda minimamente diferenciada (LMA-M0), eritroleucemia (LMA-M6), megacarioblástica (LMA-M7), leucemia bifenotípica mielóide-linfóide e a determinação da

linhagem de células B ou T nas leucemias linfoblásticas (GARAND & ROBILLARD, 1996; KALEEM *et al.,* 2003; SAXENA & ANAND, 2008).

Células precursoras de diferentes linhagens expressam diferentes tipos de moléculas na superfície, muitas delas definidas por antígenos CD (*Clusters of Differentiation* – "grupamentos" de diferenciação). Antígenos CD associados com a membrana plasmática de leucócitos podem ser moléculas envolvidas em uma variedade de funções como: interação célula-célula, receptores de citocinas, sinalização celular, canais iônicos, transportadores, enzimas, imunoglobulinas ou moléculas de adesão (BELOV *et al.,* 2001). O processo de categorizar as moléculas e epítopos associados a antígenos com leucócitos humanos data dos anos 80 e, até bem recentemente, mais de trezentas moléculas relacionadas aos leucócitos são conhecidas (VASCONCELOS, 2007).

A Figura 3 mostra o esquema de desenvolvimento das células B e T, com seus respectivos antígenos de acordo com os diferentes estágios de maturação celular.

Antígenos leucocitários são raramente de linhagem específica. Contudo, painéis de anticorpos monoclonais utilizados para detectar antígenos restritos de linhagem podem ser usados para identificar a linhagem de células leucêmicas na maioria dos casos (JANOSSY *et al.,* 1989; ROTHE & SCHMITZ, 1996; BOROWITZ *et al.,* 1997; CAMPANA & BEHM, 2000).

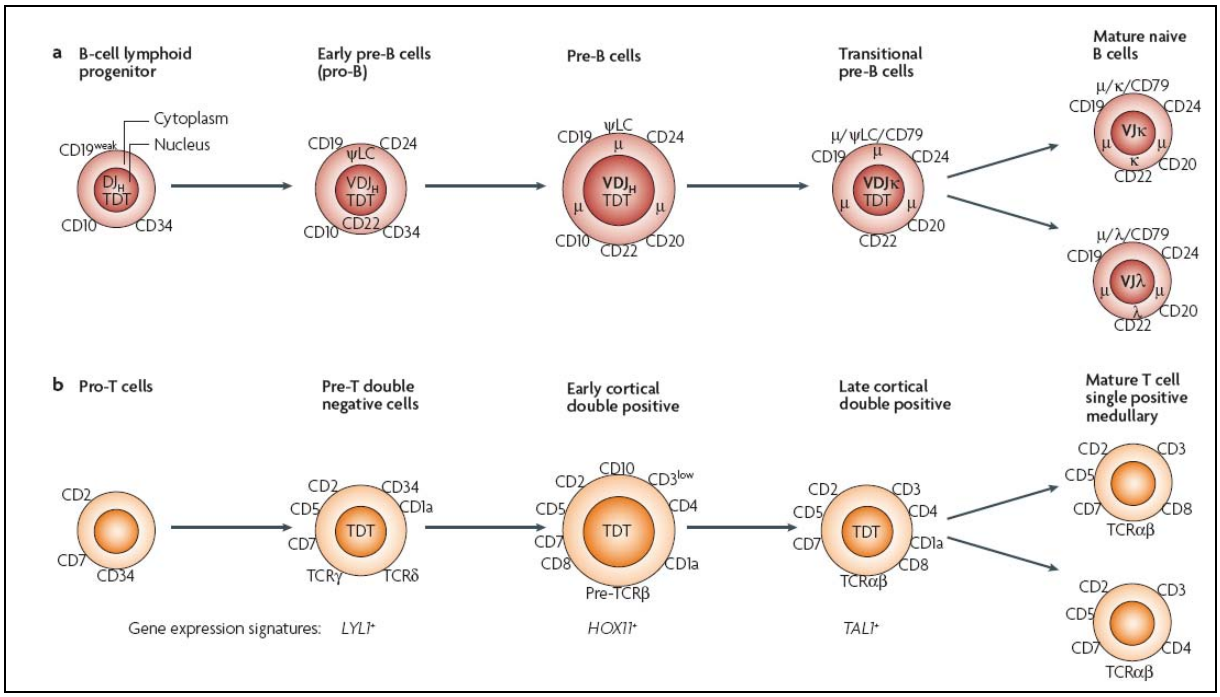


Figura 3: Esquema de desenvolvimento das células B e T, com seus respectivos antígenos de acordo com os diferentes estágios de maturação celular. (Fonte: PUI & JEHA, 2007)

O Grupo Europeu para Caracterização Imunológica das Leucemias (EGIL), em 1995, propôs critérios para a classificação imunológica das leucemias agudas com objetivo de estabelecer as diretrizes desta classificação baseado na expressão de marcadores, e uniformizar o diagnóstico dos vários tipos de doenças hematológicas nos diversos centros de diagnóstico (BENE *et al.*, 1995). O painel de marcadores recomendados pelo grupo EGIL para caracterizar as leucemias agudas está listado na Tabela 3.

Também foi proposto pelo EGIL critérios para o diagnóstico da leucemia aguda bifenotípica, que ainda é comumente utilizado. O sistema de pontuação (*scoring*) é basedo no número e grau de especificidade de certos marcadores para blastos mielóide ou linfóide B ou T (Tabela 4) (BENE *et al.*, 1995).

Tabela 3. Painel de marcadores utilizados para caracterizar leucemias agudas de acordo com grupo EGIL (Grupo Europeu para Caracterização Imunológica das Leucemias).

Primeira triagem	
Linfóide B	CD 19, CD22 citoplasmático, CD79a, CD10
Linfóide T	CD3 citoplasmático, CD2, CD7
Mielóide	anti-aMPO, CD13, CD33, CDw56, CD117
Não específico de linhagem	TdT, CD34, HLA-DR
Segunda triagem	
Se LLA de linghagem B	IgM citoplasmático, kappa, lambda, CD20, CD24
Se LLA de linghagem T	CD1a, CD3 membrana, CD4, CD5, CD8, anti-TCRαβ, anti-TCRγδ
Se LMA	anti-lisozima, CD14, CD15, CD41, CD61, CD64, anti-glicoforina A

(Fonte: BENE *et al.,* 1995)

Tabela 4. Sistema de pontuação para definição da leucemia aguda bifenotípica recomendado pelo grupo EGIL (Grupo Europeu para Caracterização Imunológica das Leucemias).

Pontuação	**Linhagem B**	**Linhagem T**	**Linhagem Mielóide**
2	CD79a, cIgM cCD22	cCD3/mCD3 anti-TCRαβ anti-TCRγδ	anti-aMPO (anti-lisozima)
1	CD19, CD10 CD20	CD2, CD5 CD8, CD10	CD13, CD33,CDw65
0,5	TdT, CD24	TdT, CD7 CD1a	CD14, CD15, CD64, CD117

Nota: Cada marcador corresponde à pontuação indicada; c (citoplasmático); m (membrana). (Fonte: BENE *et al.,* 1995)

O impacto da imunofenotipagem por citometria de fluxo no diagnóstico e acompanhamento da leucemia aguda tem sido rapidamente expandido. Este avanço pode ser atribuído ao resultado da inovação da ciência que levou a produção de centenas de anticorpos monoclonais e o avanço na tecnologia a laser (JENNINGS & FOON, 1997; SCHABATH *et al.,* 2003), e futuramente, a utilização das lectinas como mais uma ferramenta de identificação e monitoramento dos diferentes tipos de leucemia.

A citometria de fluxo também tem sido utilizada na pesquisa com lectinas conjugadas a fluocromos na determinação de glicoconjugados de superfície celular (MISLOVICOVÁ *et al.,* 2009). Omtoft e colaboradores (1988) quantificaram o conteúdo de DNA e a ligação de carboidratos em subpopulações celulares de carcinoma de células transicional. Langkilde e colaboradores (1989) estudaram a ligação das lectinas WGA e PNA a carcinomas de células transicional, correlacionando com a classificação histopatológia, invasão, e ploidia do DNA.

Sinhá e colaboradores (1999) utilizaram a lectina *Achatinina*$_H$ (ATN_H) como sonda para identificação de 9-O-acetil sialogliconjugados (9-OAcSGs) que podem servir como biomarcadores na detecção e monitoramento de linfoblastos na leucemia linfoblástica aguda em criança, utilizando a citometria de fluxo.

Mais recentemente, Pérez-Campos-Mayoral e colaboradores (2008) verificaram que a lectina *Macrobrachium rosenbergii* pode ser considerada como uma ferramenta útil no diagnóstico da leucemia linfoblástica aguda de células T, através da citometria de fluxo.

3.3 LECTINAS

No século XIX foi descoberta na natureza proteínas açúcar-específico, com atividade hemaglutinante, que foram posteriormente denominadas de lectinas (SHARON, 2007).

Do Latin *legere*, selecionar/escolher, as lectinas são uma classe de proteínas estruturalmente diversa, de origem não imunológica que compartilham a característica de ligar, de forma específica e reversível, a carboidratos (HAMELRYCK *et al.,* 1999; GORELIK et al 2001; CASTILLO-VILLANUEVA & ABDULLAEV, 2005). Ubiquamente distribuídas na natureza são encontradas não apenas em plantas, mas também em microorganismos, insetos, animais e humanos (GORELIK *et al.,* 2001; SHARON & LIS, 2007; DAMODARAN *et al.,* 2007).

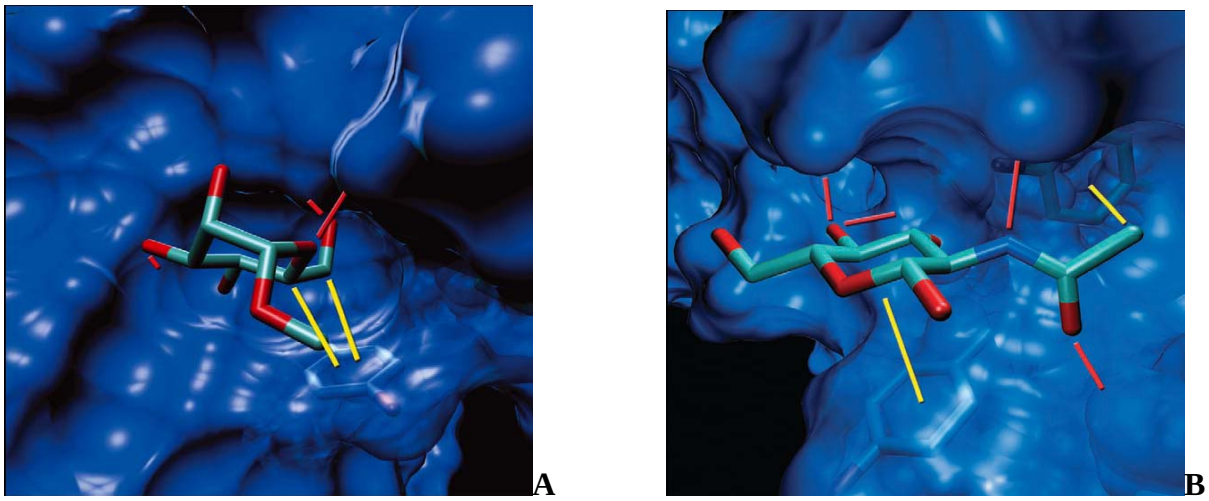
Lectinas apresentam especificidade definida para monossacarídeos e/ou oligossacarídeos, dentre elas podemos citar as lectinas de *Canavalia ensiformis* (Con A) específica para glicose/manose, *Triticum vulgaris* (WGA) específica para N-acetilglicosamina ou oligossacarídeos de N-acetilglicosamina e *Lotus tetragonolobus* agglutinin (LTA) específica para L-fucose (SHARON & LIS, 2004).

As lectinas são comumente utilizadas em bioquímica, biologia celular e histoquímica para caracterizar carboidratos de superfície e glicoproteínas, devido a sua habilidade de se ligarem a carboidratos na superfície das células. Elas requerem complementariedade estrutural e conformacional do açúcar (Figura 4) para a interação ocorrer. Nas últimas duas décadas centenas de estruturas tem sido estabelecida, e a maioria das lectinas contém tipicamente dois ou mais sítios de ligação a carboidrato (THOMAS & SUROLIA, 2000; NEUMANN *et al.,* 2004; SHARON & LIS, 2007).

Há diferentes famílias de lectinas que são diversas em sua seqüência, arquitetura do sítio de ligação a carboidrato, estrutura quaternária, afinidade por carboidrato, e especificidade, bem como desempenham diferentes papéis biológicos (CHANDRA *et al.,* 2006).

Nos últimos anos, tem havido um aumento no interesse em lectinas devido a sua atividade biológica, como citoaglutinação, atividade mitogênica, citoxicidade e sonda

histoquímica (SALLAY *et al.,* 2000; MORIWAKI *et al.,* 2000; OHBA & BAKALOVA, 2003, CAMPOS *et al.*, 2006; SOBRAL *et al.*, 2010).



A **B**

Figura 4. Domínio de reconhecimento de carboidrato da lectina. **A:** α-Metilmanose no sítio de ligação da ConA. **B:** N-acetilglicosamina no sítio de ligação da WGA. Interações hidrofóbicas são indicadas por linhas em amarelo, interações eletrostáticas e pontes de hidrogênio são ilustradas por linhas em vermelho. (Fonte: NEUMANN *et al.,* 2004)

Embora a pesquisa com lectinas tenha sido iniciada há mais de 100 anos atrás, é ainda repleta de novas descobertas. Alguns subgrupos de lectinas parecem ser instrumento da comunicação inter-celular, como as selectinas que são moléculas de adesão célula-célula envolvida na interação célula endotelial-leucócito solicitada para extravasamento da corrente sanguinea nos tecidos alvos, ou colectinas que desempenham um importante papel na imunidade inata sem envolvimento de anticorpos (SALLAY *et al.,* 2000; SHARON & LIS, 2007).

Na superfície celular elas participam de processos como adesão célula-célula ou célula-matriz extracelular bem como a proliferação celular e apoptose. Muitas lectinas são conhecidas como mediadores da inflamação e quimiotáxicas. Lectinas de macrófagos, células dendríticas e *natural killer* estão envolvidas na proteção antitumoral, antiviral e

antimicrobiana de organismo (HIGASHI *et al.,* 2002; McGREAL *et al.,* 2005; ARESCHOUG & GORDON, 2008; YOKOYAMA & RILEY, 2008; KURMYSHKINA *et al.,* 2009).

Um potencial uso de lectinas no diagnóstico envolve a determinação de alterações nos carboidratos em tecidos humanos, células, soro e outros fluidos biológicos (MISLOVICOVÁ *et al.,* 2009). Modificações estruturais de oligossacarídeos de glicoconjugados ocorrem durante o desenvolvimento normal das células sendo a glicosilação principal modificação postranslacional (LEHLE *et al.,* 2006). Entretanto, variação na glicosilação está associada com diferenciação e transformação maligna (Figura 5), de acordo com vários trabalhos publicados com tumores experimentais e humanos (DWEK *et al.,* 2001; CAMPOS *et al.*, 2006; HIRABAYASHI, 2008; TAO *et al.,* 2008; SOBRAL *et al.*, 2010).

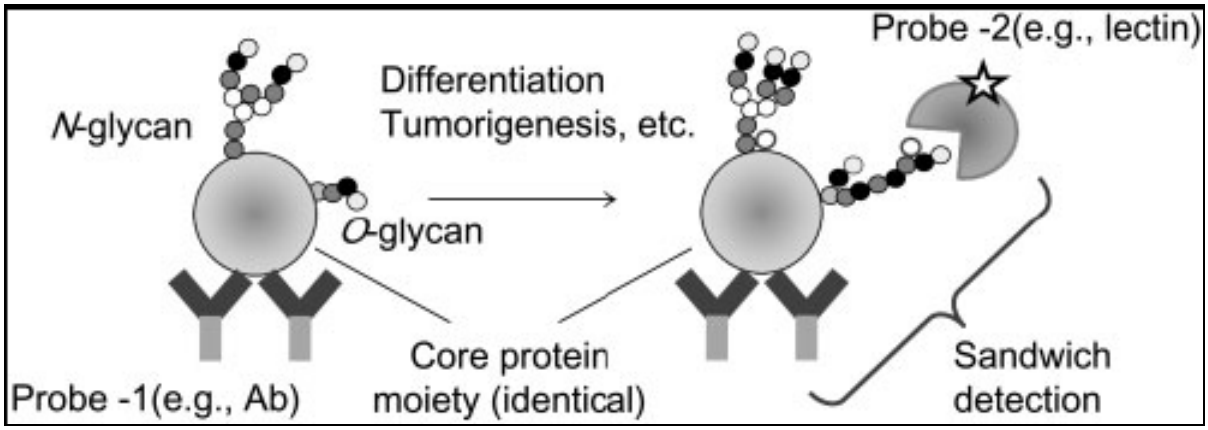


Figura 5. Princípio experimental de biomarcador de glicoproteína. Este esquema mostra que alterações significantes na glicosilação ocorrem de maneira associada com os vários estágios na célula, ex. no desenvolvimento, diferenciação e tumorogênese. (Fonte: HIRABAYASHI, 2008)

Células hematopoéticas normais e leucêmicas tem sido objeto de pesquisa utilizando lectinas, tendo em vista a variedade de células em seus diferentes estágios. A sensibilidade das lectinas de reconhecer resíduos de açúcares de antígenos de grupos sanguineos é comparável aos anticorpos (MATSUI *et al.,* 2001). Diversas lectinas como: *Dolichos biflorus*

agglutinin (DBA), *Vicia villosa* agglutinin (VVA), *Phaseolus lunatus* agglutinin (LBA), *Glycine max* agglutinin (SBA) e *Helix pomatia* agglutinin (HPA) reconhecem o antígeno A no grupo ABO usando o método de hemaglutinação. As lectinas *Griffonia simplicifolia* (GS I-B4) e *Ulex europeus* agglutinin (UEA-I) reconhecem no sangue os antígenos B e 0 (H) do grupo ABO, respectivamente (MATSUI *et al.,* 2001; SHARON & LIS, 2004).

Células mononucleadas derivadas da medula óssea possuem oligossacarídeos O-ligados a glicoproteinas, lectinas que mostram especificidade para este tipo de oligossacarídeos tem sido comumente utilizadas para fracionar subpopulações de timócitos e linfócitos (PORRAS *et al.,* 2000). A lectina HPA pode ser empregada para identificação e isolamento de células do tipo T (DE PETRIS & TACKARS, 1983), e VVA reconhece especificamente linfócitos CD8+ (citotóxico) (FORTUNE & LEHNER, 1988). Fracionamento seqüencial de linfócitos pelas lectinas SBA e PNA obteve uma fração enriquecida de células pluripotente stem cell, que foram transplantadas em pacientes (REISNER, 1983). Nagler e colaboradores (1999) também estudaram a possibilidade de se utilizar a aglutinina de soja (SBA) para fracionar células da medula óssea (CD 34+), na clínica do transplante de medula.

Ohba e colaboradores (2002) utilizou a lectina DBA em um método rápido de fracionamento de células leucêmicas do tipo T cultivadas, através da cromatografia de afinidade em coluna. Utilizando o mesmo procedimento, Bakalova e Ohba (2003) utilizaram as lectinas DBA e SBA para purificar linfócitos normais de células leucêmicas.

Várias técnicas tem sido utilizadas com lectinas para caracterizar células de acordo com o perfil de carboidratos, dentre elas (hema)aglutinação, histoquímica, ELLA (*enzyme linked lectin assay*), LAC (*lectin affinity chromatography*), western lectin blotting, CAIE (*crossed affinoimmunoelectrophoresis*) e SPR (*surface plasmon resonance*) (TAO *et al.,* 2008; MISLOVICOVÁ *et al.,* 2009).

A citometria de fluxo também tem sido empregada, como no trabalho descrito por Boldt e Nelson (1983) que utilizaram esta técnica para comparar a ligação das lectinas de germe de trigo (WGA) e *Lens culinaris* (Lcl) a linfócitos de doadores normais e pacientes com Leucemia Linfocítica Crônica (LLC). Mais recentemente, Pérez-Campos-Mayoral *et al.,* (2008) utilizou a lectina *Macrobrachium rosenbergii* como sonda de diagnósico da Leucemia Linfoblástica aguda de células do tipo T.

Várias pesquisas têm sido desenvolvidas no intuito de demonstrar a viabilidade e eficiência das lectinas como marcadores de células transformadas, verificando sutis diferenças em carcboidratos de glicoconjugados presentes na superfície de células não malignas e malignas (MODY *et al.,* 1995; BELTRÃO *et al.,* 1998, 2001, 2003; MORIWAKI *et al.,* 2000). Tais aplicações aumentam o campo de utilização desta família de (glico)proteínas.

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5 ARTIGO CIENTÍFICO I

LECTIN IN FLOW CYTOMETRY: HELPING TO DIAGNOSE LEUKEMIA

Artigo submetido ao **European Journal of Cell Biology**

LECTIN IN FLOW CYTOMETRY: HELPING TO DIAGNOSE LEUKEMIA

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ABSTRACT

Antibodies are largely used to characterize leukemia cells differentiating their stages. Lectins have been used as tools for cell surface glycoconjugate carbohydrates evaluation in cell differentiation and dedifferentiation which are characterized by changes in the number, disposition and type of saccharides found in their glycoproteins, mainly. In this study 88 bone marrow samples from patients with acute myeloblastic and lymphoblastic leukemia were evaluated with lectins conjugated to fluorescein isothiocyanate- FITC (Wheat germ agglutinin - WGA, *Lotus tetragonolobus* agglutinin - LTA, Concanavalin A - Con A, *Ulex europaeus* I - UEA-I and *Arachis hypogaea* - PNA). WGA stained both acute leukemia cells (myeloblastic and lymphoblastic) in 100% of samples. PNA and Con A stained similarly acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML) in 10% of cases. UEA-I presented a high specificity to cells of AML (57%) and low specificity to ALL (7%). LTA stained 50% of AML cases and 37% of ALL. These findings showed that lectins are promising and interesting tools to contribute to leukemia diagnosis in immunophenotypic analysis using flow cytometry.

Keywords: Lectins, Acute Leukemia, Flow Cytometry, Diagnosis.

INTRODUCTION

The classification of acute leukemia by cell surface marker analysis is an area of current increasing research and has been stimulated by the application of monoclonal antibody technology (Kaleem et al., 2003). With few exceptions cell-differentiation related antigens (Ags) lack absolute lineage specificity though immunologic classification of acute leukemia (AL) is usually based on reactivity patterns to a panel of lineage associated monoclonal antibodies (MoAbs) rather than to the presence or absence of a single antigen (Ag). As the number of MoAbs employed in immunophenotyping of AL grows, our ability to detect small-scale deviations from the normal scheme of B or T cell differentiation and to define immunologic diversity in acute lymphoblastic leukemia (ALL) has greatly expanded (Dunphy, 2004).

An alternative or potentially complementary approach to these biomarkers (antigens) is the use of specific carbohydrate-binding proteins, lectins, labeled with fluorescein isothiocyanate (FITC) in flow cytometry. So lectins, joining sugar-specific monoclonal antibodies, have been proposed as probes for the structure and composition of glycoconjugates carbohydrate chains which can phenotypicaly reflect distinct stages of differentiation of normal and neoplastic cells. Since most lectins react specifically with terminal, non-reducing sugars of glycoprotein and glycolipid in cell membrane, they can be used to characterize these surface glycoconjugates on the basis of the monosaccharides that inhibit their binding (Gabius et al., 1988; Gorelik et al., 2001).

The present study was designed to evaluate the application of FITC conjugated lectins as probes to differentiate pediatric myeloid and lymphoid acute leukemias in flow cytometry routine immunophenotyping method of diagnosis.

MATERIALS AND METHODS

Leukemia cell samples

The study used bone marrow (BM) aspirate samples from 88 patients admitted to the Pediatric Oncohemathology Centre of the Oswaldo Cruz University Hospital (HUOC) of the Pernambuco University (Brazil). Patients aged from four month-old to eighteen year-old (mean nine year-old). Sample immunophenotyping was performed by phenotypic characterization using commercial monoclonal antibodies by flow-cytometric analyses. This study was approved by Bioethical Commission of the Pernambuco University (N°258/03) with informed consent obtained from parents or child guardian.

Reagents

Wheat germ agglutinin (WGA), *Lotus tetragonolobus* agglutinin (LTA), Concanavalin A (Con A), *Ulex europaeus* agglutinin (UEA I) and *Arachis hypogaea* agglutinin (PNA) were obtained from Sigma (USA). All lectins used were fluorescein isothiocyanate (FITC) labeled and used at 100 μg/mL. Antibodies used were FITC or phycoerythrin (PE) labeled: CD45, CD34, HLA-DR, CD7, CD3, CD13, CD33, CD4, CD8, CD10, CD19, CD14, CD61 and cytoplasmic CD3, CD22, IgM and MPO (Becton Dickinson, USA). All reagents of analytical grade were obtained from Becton-Dickinson Co. and Sigma (USA).

Sample Preparation

Bone marrow samples were dilluted with RPMI 1640 medium (1:2) and mononuclear cells were separated by Histopaque (density =1,077) density-gradient centrifugation. After centrifugation at 1,500 rpm for 25 min, the middle layer containing enriched mononuclear cells was removed, washed two times and ressuspended in RPMI 1640 medium. Cell counting

was performed using an automatic blood cells counter.

Lectin-cell interaction

A volume of 5µL (100µg/mL) of each lectin (FITC-WGA, FITC-LTA, FITC-Con A, FITC-UEA, FITC-PNA) was separately added to the cell suspensions (1x 10^6 cells/mL) and incubation was carried out over 20 min at 25ºC in the dark before flow cytometric analysis. As negative control 5µL of FITC mouse immunoglobulin (IgG1) isotype control was added to the cell suspension and were used to determine background fluorescence. Samples were treated according the protocol utilized with conjugated MoAbs for immunophenotyping.

Flow cytometric analysis

Analyses were performed using a FACSCalibur Flow Cytometer (Becton-Dickinson, USA). The forward- and side-scatter parameters were adjusted to accommodate the cells within acquisition data. Labeled cells were analyzed after population was gated in a dot plot displaying of the cells. Dead cells were excluded from the analysis and 10,000 cells were counted. Data were collected and analyzed using BD CellQuest Software (Becton-Dickinson, USA). The percentage of cells expressing surface antigen/carbohydrate was considered positive when more than 20% of the cells were stained.

Fluorescence Microscopy

Fluorescence microscopy and image acquisition were performed with Olympus BX51 Microscope (Olympus Corporation, Tokio, Japan) and Cytovision software (Applied Imaging Corp., San Jose, California).

RESULTS AND DISCUSSION

Bone marrow aspirates from 88 leukemia patients diagnosed at HUOC were used: 60 cases of acute lymphoblastic leukemia (ALL) and 28 cases of acute myeloblastic leukemia (AML). Leukemia diagnosis was established by cytological examination of BM smears according to French-American-British Group recommendations and European Group for the Immunological Characterization of Leukemias -EGIL (Bene et al., 1995).

Lectin flow cytometry was performed according to routine phenotypic identification protocol. As shown in Table 1 WGA (N-acetylglucosamine specific) stained both acute leukemia cells (myeloblastic and lymphoblastic) in 100% of samples. PNA (D-galactose specific) and Con A (D-mannose/D-glucose) stained similarly ALL (12% and 11% of samples, respectively) and 10% of AML cases for both lectins. UEA-I (L-fucose) presented a high specificity to cells of AML in 57% of cases while for ALL only 7% of cases were stained by this lectin. LTA (also L-fucose specific) stained 50% of AML cases and 37% of ALL. Positivity for blastic cells was also evaluated and results are depicted in Table 2.

Figure 1 shows a representative profile of the flow cytometry of an AML patient characterized by 90% of leukemic blasts (morphological estimative) positive for WGA (99%) and UEA-I (55.7%). Results were presented as histogram which represents integral green fluorescence, generated by measuring 10,000 viable cells. Percentage of cells expressing surface antigen/carbohydrate was considered positive when more than 20% of total cells were stained. There was general consensus on the cut-off point to consider a marker positive and this was set up at 20% of cells stained with the monoclonal antibody according to Bene et al. (1995).

The staining of leukemia cells by FITC-WGA lectin was also visualized by fluorescent microscopy (Figure 2) where it was observed a homogeneous positive control using antibody (CD45-FITC) and a heterogeneous control for the lectin.

Glycosylation plays an important role in the biological activity of cell glycoconjugates involved in controlling cell differentiation. The carbohydrate profile, the glycocode, differs from the nucleotides in nucleic acids and amino acids in proteins. The monosaccharide units can attach to one another at multiple points forming branches (Sharon and Lis, 2004). Cell-surface carbohydrate structures have been a focus of many investigative efforts regarding their use as biomarkers in human pathologies (Beltrão et al., 2003; Sobral et al., 2010).

Lectins and their complementary carbohydrates are located on the surfaces of opposing cells, which can be of the same type or different types. Their interactions are required for cell differentiation, development and pathological states (Sharon and Lis, 2007). In this context lectins used in cyto- and histochemistry became probes to decipher the saccharide array in tumour cells regarding their ability of invasiveness, loss of adhesion, angiogenesis and apoptotic susceptibility. These processes are affected, directly or indirectly, by N- and/or O-glycosylation of functional proteins or lipids (Ono and Hakomori, 2004). So the characterization of the saccharide profile is indispensable to the understanding of many biological processes (Miyoshi et al., 2008).

The immunophenotypic analysis of acute leukemia using antibodies in flow cytometry has proved to be a powerful tool for the identification of leukemic lineage (Pérez-Campos-Mayoral et al., 2008). Our data, based on a nonselective leukemia patient group on pediatric age, indicate the use of lectins to investigate and/or help in the diagnosis of AML and ALL employing the same protocol for phenotypic identification. By using a one color flow cytometric approach, we investigated the expression of a panel of five sugars (N-

Acetylglucosamine, L-Fucose, D-Galactose, D-Glucose and D-Mannose) of cell surface molecules in leukemia cells.

Many lectins with their specific affinity for simple and complex sugars on tumor cell surface are found to recognize fine differences between leukemic and normal cells (Bakalova and Ohba, 2003). A strong interaction of WGA with leukemic cells was observed in flow cytometry and also by fluorescent microscopy. It appears that the leukemic cells are prone to be recognized by WGA in respect to the accessibility of N-acetyl-D-glucosamine/N-Acetyl-neuraminic acid on their cell surface glycoconjugates (mainly glycoprotein), resulting in no differentiation of leukemia cells from ALL and AML. Ohba and Bakalova (2003) observed that WGA usually interact with cultured leukemia cell lines to a higher degree without selectivity between leukemia cell lineages.

PNA and Con A recognized both AML and ALL cells in a similar pattern being positive for approximately 10% of the cases studied where such poor percent do not indicates their use as exclusive probes for AML or ALL. PNA was indicated as a prognostic marker in acute lymphoblastic leukemia T-lineage (Aller et al., 1996; Pérez-Campos-Mayoral et al., 2008). The reduced number of samples of ALL-T in our study did not allow inferring such finding but we also believe that the D-galactose residues in those cells can be “disguised”, for example, by terminal sialic acid in the carbohydrate moiety of glycoconjugates.

In contrast UEA-I showed to be highly selective recognizing cells of AML (57%) rather than of ALL (7%). On the other hand LTA, also specific to L-fucose as UEA-I, stained 50% of AML cases and 37% of ALL ones. The degree of UEA-I cell selectivity was higher than LTA such structural disposition of L-fucose allowed UEA-I to be more selective to blastic cells of myeloid origin. Despite this result the fluorescence histograms revealed that LTA interacts more strongly with AML cells. UEA-I and LTA bind to L-fucose with different

affinities and LTA exhibits approximately 3.5-fold higher affinity for the sugar over UEA-I (Thomas and Surolia, 2000).

The structural differences between UEA-I and LTA confer to them a specific disposition of L-fucose residues in the saccharide organization in glycoconjugates. Thomas and Surolia (2000) described that UEA-I presents a modified binding pocket to L-fucose and the gap of four residues between the aliphatic hydrophobic residue and the invariant asparagine residue in UEA-I carbohydrate recognition domain would necessarily present a shallower binding cleft as compared to that in LTA. This probably might account for the different affinities exhibited by these two lectins for the sugar ligand.

Variations in lectin structure have significant functional implications for the binding of multivalent ligands on tumor cell surfaces and the fine differences in their quaternary structures seems to be very important for the strength of lectin-saccharide in cell binding (Ohba and Bakalova, 2003). This differentiated reactivity can also be an indicative of differences among subsets of myeloid blasts which cannot be evaluated in our study since the number of cases was limited. We are now analyzing whether a similar analysis could be useful to select lectins of interest as additional probes for leukemia diagnosis besides the information gained using antibodies.

In conclusion our findings showed that lectins are promising tools to contribute to leukimia diagnosis in flow cytometry. This strategy would reduce costs of immunophenotypic analysis and help in conflicting or doubtful diagnosis.

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Table 1 - Lectins activity toward leukemia cells.

LECTINS		LEUKEMIAS (lectin positivity/total cases)	
	Sugar specificity	ALL	AML
Wheat germ agglutinin (WGA)	N-acetylglucosamine	60/60 (100%)	28/28 (100%)
Lotus tetragonolobus agglutinin (LTA)	L-fucose	22/59 (37%)	13/26 (50%)
Concanavalin A (Con A)	D-mannose/D-glucose	7/60 (11%)	3/28 (10%)
Ulex europaeus (UEA-I)	L-fucose	3/39 (7%)	11/19 (57%)
Arachis hypogaea (PNA)	D-galactose	5/39 (12%)	2/19 (10%)

Table 2 - Percentage of reactivity of blast cells to lectins.

	WGA		LTA		Con A		UEA-I		PNA	
Leukemia	nº cases positive	Mean/ Range	nº cases positive	Mean/ Range	nº cases positive	Mean/ Range	nº cases positive	Mean/ Range	nº cases positive	Mean/ Range
ALL	60	95% 90-100	22	64% 24-98	7	49% 21-94	3	38% 21-55	5	50% 21-90
AML	28	95% 90-100	13	66% 22-97	3	46% 32-54	11	44% 25-88	2	53% 40-67

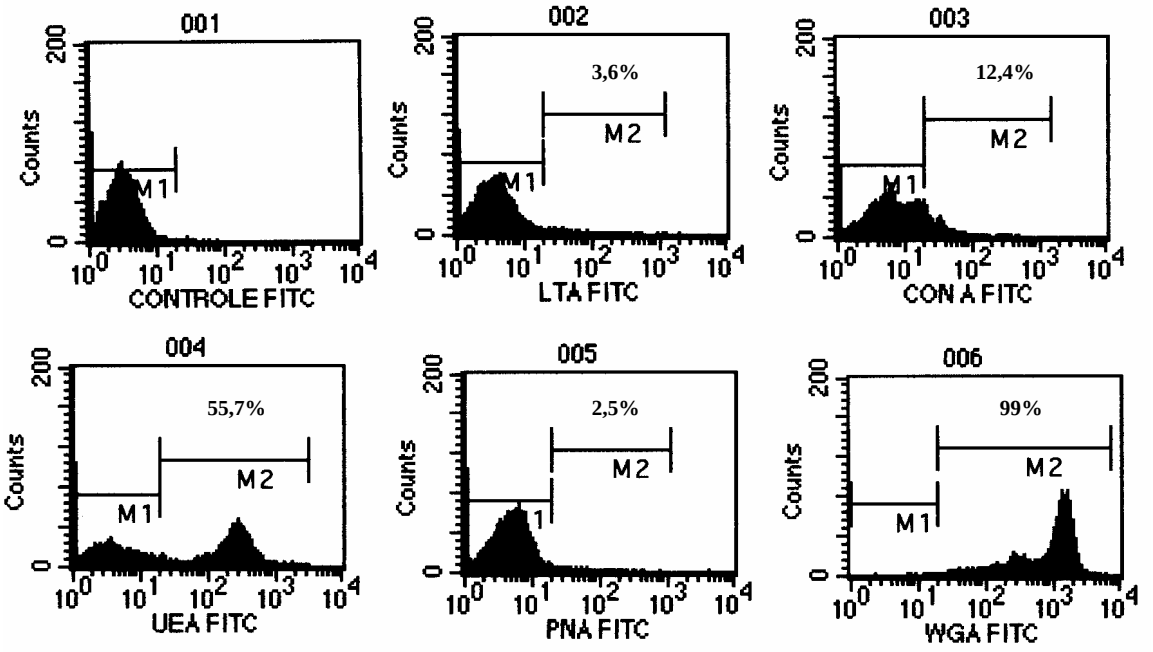

Figure 1: Flow cytometric analysis of leukemic blasts from a patient expressing (002) FITC-LTA, (003) FITC-ConA, (004) FITC-UEA, (005) FITC-PNA and (006) FITC-WGA, specific carbohydrates L-Fucose, D-Glucose/D-Mannose, L-Fucose, D-Galactose and N-Acetylglucosamine, respectively. Results were presented as histogram which represents integral green fluorescence, generated by measuring 10,000 viable cells, and the percentage of cells expressing surface carbohydrate was considered positive when more than 20% of the cells were stained, so only lectins UEA (55,7%) and WGA (99%) were considered positive.

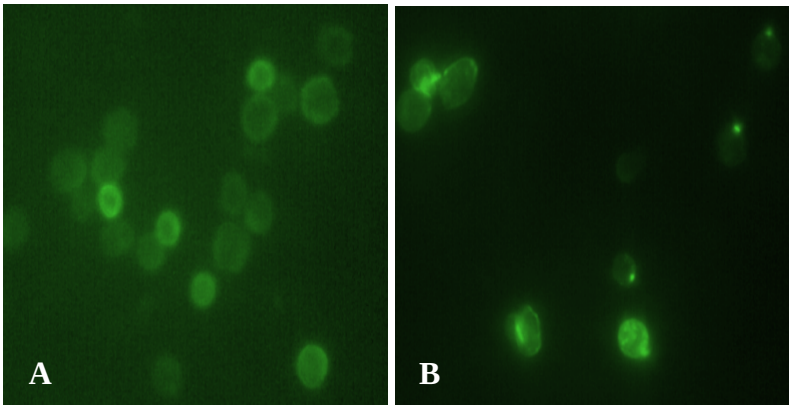

Figure 2: Fluorescent microscopy analysis of leukemic blasts positive to antibody control FITC-CD45 (A) and to lectin FITC-WGA (B). Note variation in staining intensity among cells.

6 ARTIGO CIENTÍFICO II

Ulex europeus I AS AUXILIARY TOOL FOR THE DIAGNOSE OF ACUTE MYELOID LEUKEMIA IN FLOW CYTOMETRY

Artigo submetido a **BIOTECHNOLOGY LETTERS**

Ulex europeus I AS AUXILIARY TOOL FOR THE DIAGNOSE OF ACUTE MYELOID LEUKEMIA IN FLOW CYTOMETRY

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ABSTRACT The term acute myeloid leukemia (AML) collectively refers to a mixture of distinct diseases that differ between them regarding their pathogenetic evolution, genetic abnormalities, clinical features, response to therapy and prognosis. The presence of specific sugar (L-fucose) receptors for lectin *Ulex europeus* I (UEA-I) was identified on leukemic cells from AML using flow cytometry. The reactivity was determined in 40 patients using fluorescein isothiocyanate (FITC) conjugated UEA-I. It was observed that 55% of all subtypes of AML (n = 22), analysed together, were positive for UEA-I. It is likely that analysis of UEA-I binding to blasts may be a useful adjunct to the diagnosis of AML using flow cytometry.

Keywords: Lectins, Leukemia, Flow Cytometry, Diagnosis.

INTRODUCTION

The term acute myeloid leukemia (AML) collectively refers to a mixture of distinct diseases that differ between them regarding their pathogenetic evolution, genetic abnormalities, clinical features, response to therapy, and prognosis (Löwenberg et al. 2003). Leukemic blasts from patients with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) commonly express cell markers of more than one lineage besides retaining characteristics that demonstrate a strong commitment to a single lineage (Rubnitz et al. 2009).

The distinction of acute leukemia as myeloid or as lymphoid is important in terms of therapeutic approach and prognostic evaluation. Immunophenotypic analysis has a central role in distinguishing between minimally differentiated acute myeloid leukemia and acute lymphoblastic leukemia and in the recognition of acute megakaryoblastic leukemia (Schabath et al. 2003).

Lectins are carbohydrate-binding proteins, ubiquitously distributed in nature, that specifically recognize diverse sugar structures and mediate a variety of cellular processes providing biological scripts to decipher the complex information embedded in the glycome. The involvement of lectins in processes such as cell–cell and host–pathogen interactions, serum-glycoprotein turnover and innate immune responses are of particular relevance to tumour growth and metastatic spread (Damodaran et al. 2007). It is well known that lectins often interact better with cancer cells than with normal cells because of greater numbers of cell surface lectin receptors or because of altered distributions of these receptors on the surfaces of the cancer cells (Heinrich et al. 2005).

The aim of the present study was evaluate the binding of *Ulex europaeus* agglutinin (UEA-I), an L-fucose specific lectin, to myeloid origin leukemic cells, evaluating their subtypes (FAB classification) and their maturation stage by flow cytometry.

MATERIALS AND METHODS

Leukemia cell samples

Forty bone marrow (BM) aspirate samples from patients admitted to the Pediatric Oncohemathology Centre of the Oswaldo Cruz University Hospital (HUOC) of the Pernambuco University, (n = 20, mean age of 10 year-old) and Fundação de Hematologia e Hemoterapia de Pernambuco (HEMOPE) *(*n = 20, mean age of 50 year-old) were selected. All cases were classified according to French-American-British Cooperative Group (FAB) criteria and the sample immunophenotyping was performed by phenotypic characterization using commercial monoclonal antibodies by flow-cytometric analyses. This study was approved by Bioethical Commissions of the Pernambuco University and HEMOPE with informed consent obtained from parents or child guardian (pediatric samples) or patient consent (adult samples).

Reagents

Fluorescein isothiocyanate (FITC) conjugated *Ulex europaeus* agglutinin (UEA-I) was obtained from Sigma (USA) and used at 100 μg/mL. Antibodies used were FITC or phycoerythrin (PE) labeled: CD45, CD34, HLA-DR, CD7, CD13, CD33, CD14, CD4, CD61 and cytoplasmic MPO (Becton-Dickinson, USA).

Flow Cytometry

Mononuclear cells from bone marrow aspirates were isolated using Histopaque (density =1,077) and stained with various combinations of fluorescein isothiocyanate (FITC) and/or phycoerythrin (PE) labeled monoclonal antibodies for CD45, CD34, HLA-DR, CD7, CD13, CD33, CD14, CD4, CD61 and cytoplasmic MPO (Becton-Dickinson, USA), in accordance

with the manufacturer's recommendations. A volume of 5µL (100µg/mL) of FITC-UEA-I was added to the cell suspensions ($1x10^6$ cells/mL) and incubation was carried out over 20 min at 25ºC in the dark before flow cytometric analysis. As negative control, 5µL of FITC mouse immunoglobulin (IgG1) isotype control was added to cell suspensions and were used to determine background fluorescence. Samples were treated according the protocol utilized with conjugated MoAbs for immunophenotyping according to manufacturer. Two color flow cytometric immunophenotyping was performed on FACSCalibur Flow Cytometer (Becton-Dickinson, USA) by collecting 10,000 ungated list mode events, selecting an appropriate blast gate on the combination of forward and side scatter, and analyzing cells with the most appropriate blast gate. Data were collected and analyzed by using BD CellQuest Software (Becton-Dickinson, USA) and data were presented as a dot plot of FITC-fluorescence and PE-fluorescence with quadrant markers drawn to distinguish FITC- and PE-labeled cells and histograms for lectins. An antigen/lectin was considered positively expressed when at least 20% of the gated cells were positive.

RESULTS

The cases included 20 children (mean age of 10 years) and 20 adults (mean age of 50 years) diagnosed with acute myeloid leukemia (AML) according to FAB classification and also by positivity for CD13, CD33 and aMPO by flow cytometric analysis. Figure 1 shows the morphologic characteristics of blast cells of AML-M7, corresponding to the last subtype (M7) in the eight FAB classification subtypes (M0, M1, M2, M3, M4, M5, M6 e M7).

The expression of UEA-I specific sugar (L-fucose) was observed in 55% of all cases. The blasts of 11 out of 20 cases (55%) of myelocytic leukemia (M0/M1/M2) were reactive, whereas in 3 out of 8 cases (37%) promyelocytic leukemia (M3), 5 in 9 cases

(55%) of myelomonocytic and monocytic (M4 e M5), and 3 in 3 cases (100%) of megakaryoblastic leukemia, the blasts cells showed significant UEA binding (Table 1). Results suggest a correlation between the AML blast cells staining by UEA-I and cytologic maturation. No AML cells classified as M6 were examined in this study.

Lectin flow cytometry was performed according routine phenotypic identification protocol. Results are shown in Figure 2. Flow cytometric analysis of leukemic blasts UEA-I positivity was 44% which means that these patients present accessible L-fucose in their cell surface glycoconjugates of blast cells.

Table 1 – Binding of UEA-I-FITC in relation to FAB classification of AML.

Classification	Positive	Negative
M0/M1/M2 (n= 20)	11	9
M3 (n= 8)	3	5
M4/M5 (n= 9)	5	4
M7 (n= 3)	3	0
TOTAL n= 40	22	18

UEA-I, *Ulex europaeus* agglutinin; FITC, fluorescein isothiocyanate; FAB, French-American-British group; AML, acute myeloid leukemia.

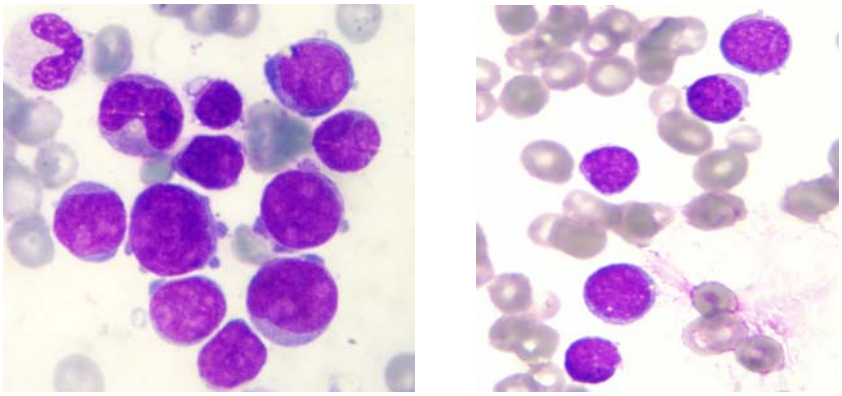

Figure 1: Morphologic aspect of bone marrow aspirates containing leukemic blasts of acute myeloid leukemia (AML-M7).

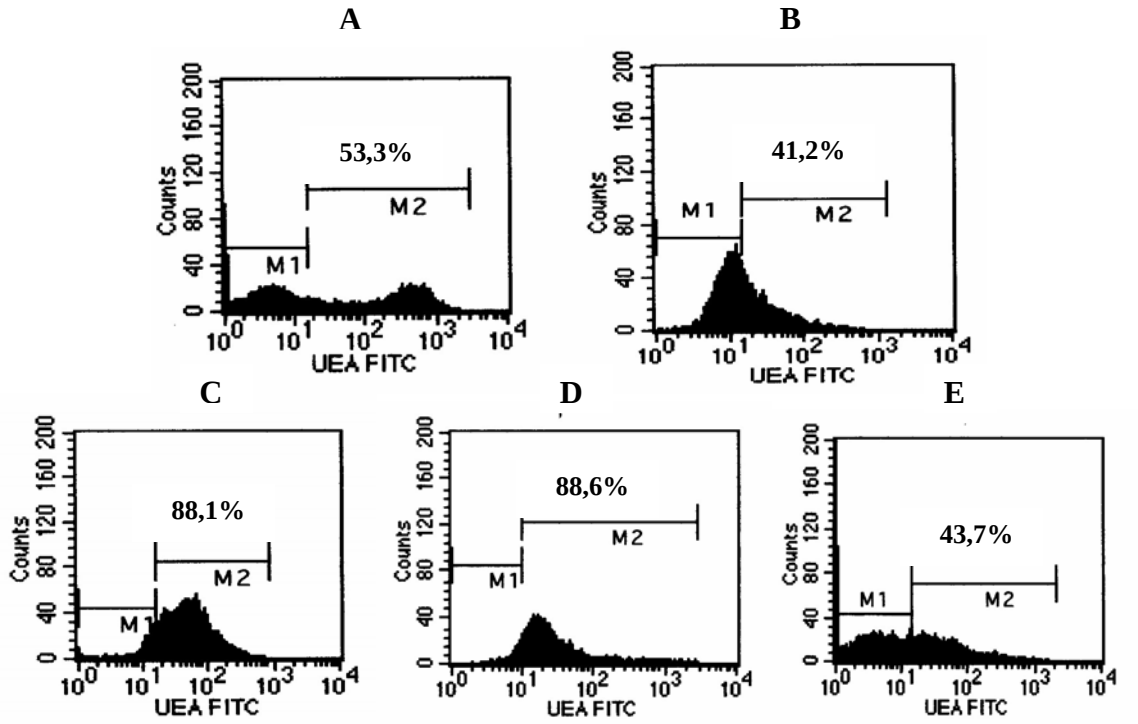

Figure 2: Flow cytometric analysis of leukemic blasts from patients expressing UEA-FITC. **A**: AML-M1; **B**: AML-M3; **C**: AML-M4; **D**: AML-M5; **E**: AML-M7. Results were presented as histogram which represents integral green fluorescence, generated by measuring 10,000 viable cells, and the percentage of cells expressing surface carbohydrate was considered positive when more than 20% of the cells were stained.

DISCUSSION

The diagnosis and management of acute leukemia largely depends on the detection, identification and characterization of leukemic cells. Traditionally, applications of flow cytometry in leukemia diagnosis have relayed on the assignment of the blast cell lineage (Vecchio et al. 2004). During the last 20 years, the diagnosis of acute leukemias emerged from cytomorphology alone to a comprehensive bundle of different methods that are necessary not only for the diagnosis and classification but also for individual treatment decisions (Haferlach et al. 2005).

A variety of new markers for different acute leukemia categories to facilitate a correct diagnosis and define the critical role of flow cytometry has been investigated. In the present study UEA-I was used in the diagnosis of leukemia based on the binding of this lectin to AML blast cells. Such reactivity was also correlated to morphological classification according to the criteria of the French-American-British Cooperative Group (FAB). The classification of acute myeloid leukemia has evolved from the primarily morphologic and cytochemical system of the early FAB proposal and this classification has been the major system used by hematopathologists for more than 20 years (Arber et al. 2003).

Changes in carbohydrate composition (alteration of sialylation, branching, fucosylation and type and/or number of sugar residues) are applicable for cancer diagnostic and therapeutic purposes. The altered lectin binding and appearance of new glycoconjugates or modified level of existing glycoconjugates associated with disease progression can be detected by lectin diagnostics (Wearne et al. 2006; Mislovicová et al, 2009).

The application of lectins for cell surface characterization seems, therefore, mainly useful for cell line characterization. Our results suggest a correlation between the capability of blasts cells in pediatric and adult AML to bind UEA-I and cytologic maturation. Similar

results were observed in adults by Delwel et al in 1986 but authors did not correlated to FAB M3 and M7 subtypes.

In the present study a correlation is suggested as regards the capacity of cells to have their L-fucose residues recognized by UEA-I and their morphology, mainly AML-M7, since in all cases the blasts cells were positive for this lectin.

The diagnosis of AML-M7 can be a challenge given the fact that most of these cases have extensive bone marrow fibrosis that causes pancytopenia with only a few circulating blasts (Brunning, 2001). The diagnostic difficulty can be compounded by the fact that in several cases the megakaryoblasts are small, resembling lymphoblasts or even lymphocytes (Figure 1). In such cases, flow cytometric immunophenotyping can identify circulating rare ‘‘atypical’’ cells as megakaryoblasts expressing CD41 and/or CD61 but not MPO. A diagnosis of AML-M7 should not shy away from this just because the peripheral blood does not meet the 20% blast criterion as in frequent cases of AML-M7. The expression of CD41 and CD61 was observed in otherwise typical cases of AML M1/M2 that do not qualify as AML-M7 but such a distinction is often of academic rather than routine diagnosis interest (Kaleem et al 2003).

In conclusion the analysis of UEA-I receptors on AML blasts showed to be a useful adjunct to the diagnosis in flow cytometry, specially megakaryoblastic leukemia (AML-M7). The application of lectins for cell surface characterization seems, therefore, to be useful for cell line characterization.

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7 ARTIGO CIENTÍFICO III

COMPARATIVE ANALYSIS OF LEUKEMIA DIFFERENTIATION STAGES BY CELL SURFACE CARBOHYDRATES USING LECTINS

Artigo a ser submetido

COMPARATIVE ANALYSIS OF LEUKEMIA DIFFERENTIATION STAGES BY CELL SURFACE CARBOHYDRATES USING LECTINS

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ABSTRACT The immunophenotypic analysis of acute leukemia by flow cytometry has become a powerful tool for proper identification of myeloid or lymphoid lineage and in this capacity has enjoyed widespread use in clinical laboratories. Acute leukemia displays characteristic expression patterns of surface antigen, which facilitate their identification and proper classification and hence play an important role in instituting proper treatment plans. Acute leukemias can also be identified via multiparameter flow cytometric analysis using lectins, ubiquitous carbohydrate recognizing proteins. Samples of bone marrow from patients diagnosed with acute myeloblastic and lymphoblastic leukemias were evaluated with lectins conjugated to fluorescein isothiocyanate - FITC (Wheat germ agglutinin - WGA, *Lotus tetragonolobus* agglutinin - LTA, Concanavalin A – Con A, *Ulex europaeus* I – UEA-I and *Arachis hypogaea* agglutinin - PNA) and the results were compared to those obtained with routine monoclonal antibodies (CD 45, CD 34, CD 10, CD19, CD 3, CD7, CD13 and CD33). The results indicate that lectins are promising auxiliary tools to contribute to leukemia diagnosis in flow cytometry.

Keywords: Lectins, Antibodies, Leukemia, Flow Cytometry.

INTRODUCTION

Acute leukemia displays characteristic expression patterns of surface antigen, the clusters of differentiation (CD antigens), which facilitate their identification and proper classification and hence play an important role in adopting the proper treatment plans. In addition to cytochemical analysis, multiparameter flow cytometric analysis has become common place in most laboratories for that purpose (Kaleem et al., 2003).

As cells differentiate along particular lineages, expression of CD antigens changes; *e.g.,* as myeloid stem cells expressing CD34 differentiate down the granulocyte lineage, they begin to express CD13 and CD33 and down regulates CD34. Mature neutrophils express CD11b, CD13, and CD15, with the loss of expression of CD33. The expression of CD antigens on leukocytes is currently determined by flow cytometry, which is expensive and labor-intensive, requiring 5–20μL of fluorescently labeled antibodies (10–500 mg/mL) and allowing concurrent analysis for a limited number of CD antigens, usually three to four (Belov et al., 2001).

An alternative or potentially complementary approach to these biomarkers (antigens) is the carbohydrates of cell surface which can be recognized by lectins, specific carbohydrate-binding proteins, conjugated to fluorescein isothiocyanate in flow cytometry. Lectins interact with specific carbohydrate residues of glycoproteins and glycolipids on the tumor cell surface, and have been widely used for isolation, identification and characterization of tumor cell surface receptors (Ohba et al., 2002; Campos et al., 2006).

The present study aimed to evaluate the cell surface carbohydrates on T, B and myeloid origin of leukemic cells using lectins by flow cytometry and to compare with routine monoclonal antibodies (CD 45, CD 34, CD 10, CD19, CD 3, CD7, CD13 and CD33).

MATERIALS AND METHODS

Leukemia cell samples

Bone marrow (BM) aspirate samples (n = 88) were obtained from patients admitted to the Pediatric Oncohemathology Centre of the Oswaldo Cruz University Hospital (HUOC) of the Pernambuco University (Brazil). Patients aged from four month-old to eighteen year-old (mean nine year-old). Immunophenotyping assay was performed using commercial monoclonal antibodies in flow cytometric analyses. This study was approved by Bioethical Commission of the Pernambuco University (N°258/03) with informed consent obtained from parents or child guardian.

Reagents

Lectins from Wheat germ agglutinin (WGA), *Lotus tetragonolobus* agglutinin (LTA), Concanavalin A (Con A), *Ulex europaeus* agglutinin (UEA I) and *Arachis hypogaea* agglutinin (PNA) were obtained from Sigma (USA). All lectins used were FITC-conjugated and used at 100 μg/mL. Antibodies used were fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugted: CD45, CD34, HLA-DR, CD7, CD3, CD13, CD33, CD4, CD8, CD10, CD19, CD14, CD61 and cytoplasmic CD3, CD22, IgM and MPO (Becton Dickinson, USA). All reagents of analytical grade were obtained from Becton Dickinson Co. and Sigma (USA).

Flow Cytometry

After sampling bone marrow aspirates were immediately dilluted with RPMI 1640 medium (1:2) and mononuclear cells were isolated using Histopaque (density =1,077) and stained with various combinations of fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)– conjugated

monoclonal antibodies against the following antigens: CD45, CD34, HLA-DR, CD7, CD3, CD13, CD33, CD4, CD8, CD10, CD19, CD14, CD61 and cytoplasmics CD3, CD22, IgM and MPO (Becton Dickinson, USA), in accordance with the manufacturer's recommendations. A volume of 5μL (100μg/mL) of each lectin (FITC-WGA, FITC-LTA, FITC-Con A, FITC-UEA-I, FITC-PNA) was separately added to the cell suspensions (1x 10^6 cells/mL) and incubation was carried out over 20 min at 25ºC in the dark before flow cytometric analysis. As negative control, 5μL of FITC conjugated mouse immunoglobulin (IgG1) isotype was added to the cell suspension and it was used to determine background fluorescence. Samples were treated according the protocol utilized with conjugated MoAbs for immunophenotyping. Analyses were performed using a FACSCalibur Flow Cytometer (Becton-Dickinson, USA). The forward- and side-scatter parameters were adjusted to accommodate cells within acquisition data. Labeled cells were analyzed after population was gated in a cell dot-plot displaying. Dead cells were excluded from the analysis and 10,000 cells were counted. Data were collected and analyzed using BD CellQuest Software (Becton-Dickinson, USA). The percentage of cells expressing surface antigen/carbohydrate was considered positive when more than 20% of the cells were positive.

RESULTS

Acute leukemia (AL) was classified on the basis of standard morphological and cytochemical criteria of the French-American-British (FAB) cooperative study group. Immunological classification of AL was assessed using a panel of MoAbs for phenotypic characterization of leukemic blast cells.

Bone marrow aspirates from 88 leukemia patients were diagnosed as 52 cases of B-cell lineage ALL, 8 cases of T-cell ALL, and 28 cases of AML. In AML, CD45 was expressed in 100% of samples while CD34 was only in 53% of all cases. In ALL, expression of CD45 was observed in 77% and CD34 in 63% of the 88 cases studied. The expression of CD13 and CD33 was positive in 85% and 92%, respectively, of all subtypes of AML. In B-ALL, CD13 and CD33 expression was positive in 23% and 15% of 52 cases, respectively. Only 2 of 8 cases of T-ALL showed positivity for CD13 and none expressed CD33. CD7 and CD4 were positive in 100% and 62%, respectively, of T-ALL diagnosis. Their expressions, however, was also found in 13% and 2%, respectively, of B-ALL cases and 32% of AML cases. Only 18% of AML diagnosis was positive for CD4 and only 1 of 52 of B-ALL cases was positive for this antigen.

Almost all cases of B-ALL showed expression of pan B-cell markers CD19 (50/52) and CD10 (57/60). In AML 7% and 3% of cases were CD19 and CD10 positive, respectively. None of T-ALL samples was positive for CD19 and only one case was positive for CD10. Monoclonal antibodies results are depicted in Table 1.

WGA (N-Acetyl-glucosamine specific) was positive in 100% of all ALL and AML cases. In AML LTA recognized L-fucose residues in 50% while UEA-I (also L-fucose specific) was in 57% of all cases. In ALL, LTA positivity was observed in 37%, where subtype T-ALL and B-ALL was positive in 50% and 35%, respectively. UEA-I in lymphoid leukemias was positive in 6% of B-ALL samples and in one case of T-ALL. Con A (D-glucose/D-mannose specific) and PNA (D-galactose) were both positive in 10% of AML cases of all subtypes. In B-ALL, Con A and PNA positivity was observed in 11% and 9% cases, respectively. Only 1 out of 8 cases of T-ALL showed Con A positivity. PNA was positive in 33% cases of T-ALL. Lectin flow cytometry results are summarized in Table 2.

The flow cytometric analysis of leukemic blasts from a T-ALL patient is presented in Figure 1 where it can be seen a positivity for all lectins but UEA-I and Figure 2, comparison of lectin and CD7 flow cytometric analysis for leukemic blasts.

Another T-ALL patient positive for CD34, CD7, CD45, cytoCD3 and CD13 was positive only for UEA-I and WGA when analised regarding his leukemic blasts (Figure 3).

Table 1 – Immunophenotypes of acute lymphoblastic and myeloblastic leukemia.

Leukemia	**Leukocyte antigen expression (% of positive cases)**							
	CD45	**CD34**	**CD10**	**CD19**	**CD3**	**CD7**	**CD13**	**CD33**
ALL	77	63	95	83	10	25	23	13
B-ALL	73	69	94	96	2	13	23	15
T-ALL	100	25	12	0	62	100	25	0
AML	100	53	3	7	18	32	85	92

Table 2 - Lectins activity toward leukemia cells.

Leukemia	**WGA**		**LTA**		**Con A**		**UEA-I**		**PNA**	
	positive cases	%	positive cases	%	positive cases	%	positive cases	%	positive cases	%
ALL	60/60	100	22/59	37	7/60	11	2/39	5	5/39	12
B-ALL	52/52	100	18/51	35	6/52	11	1/33	3	3/33	9
T-ALL	8/8	100	4/8	50	1/8	12	1/6	16	2/6	33
AML	28/28	100	13/26	50	3/28	10	11/19	57	2/19	10

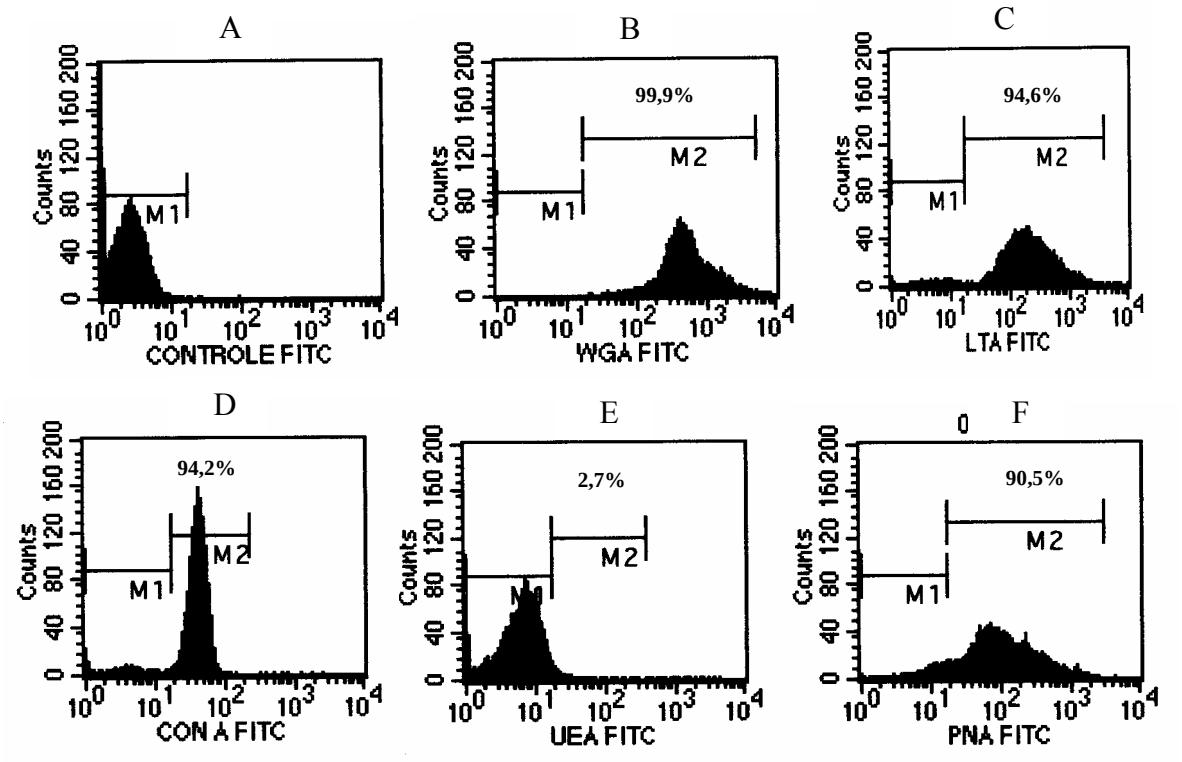

Figure 1: Flow cytometric analysis of leukemic blasts from a patient with T-ALL. A: CONTROL FITC; B: WGA-FITC (+); C: LTA-FITC (+); D: Con A-FITC (+); E: UEA-FITC (-); F: PNA-FITC (+). Results are presented as histogram which represents integral green fluorescence, generated by measuring 10,000 viable cells, and the percentage of cells expressing surface carbohydrate was considered positive when more than 20% of the cells were positive.

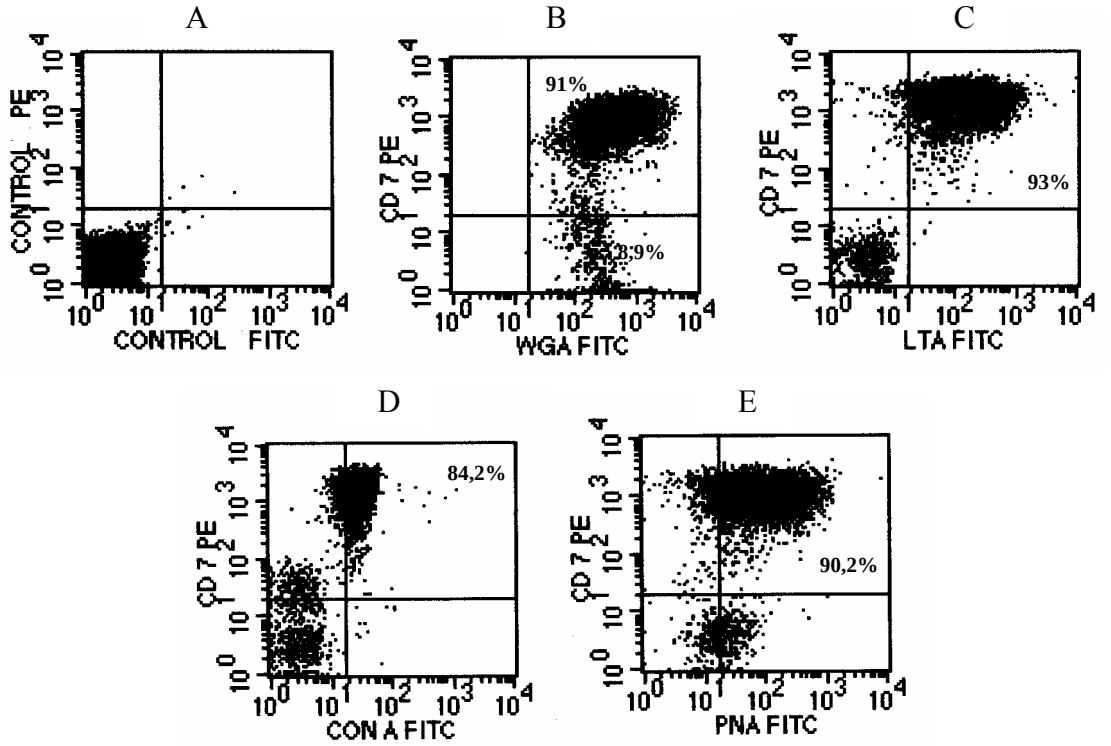

Figure 2: Flow cytometric analysis of leukemic blasts from a patient with T-ALL expressing CD7. A: control of FITC/PE; B: WGA-FITC/CD7PE(+); C: LTA-FITC/CD7PE(+); D: ConA-FITC/CD7PE(+); E: PNA-FITC/CD7PE(+).Results are presented as dot-plot generated by measuring 10,000 viable cells, and the percentage of cells expressing surface carbohydrate was considered positive when more than 20% of the cells were positive.

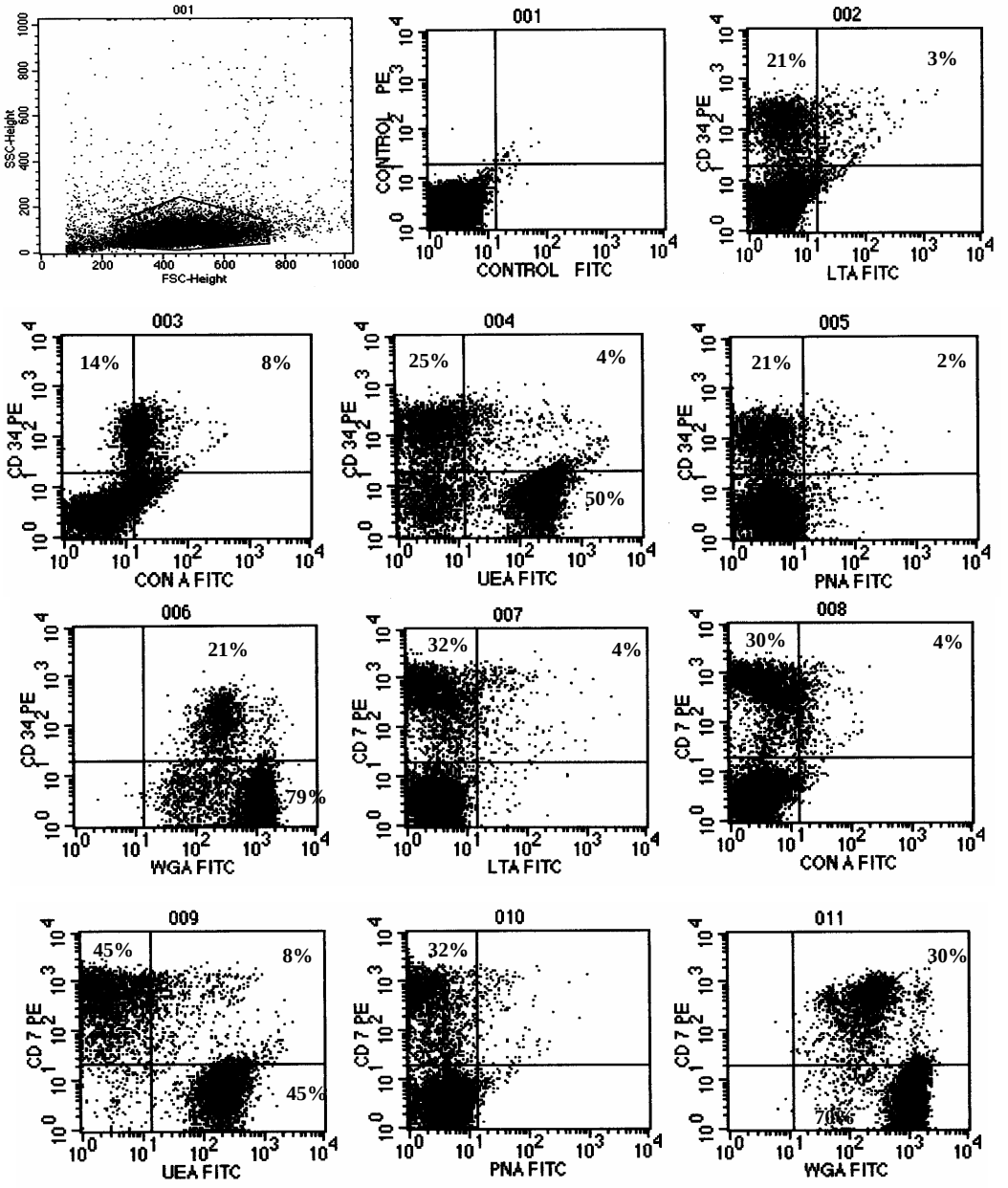

Figure 3: Flow cytometric analysis of leukemic blasts from a patient with T-ALL. MoAbs against CD34, CD7, CD45, CD13 and cytoCD3 were positive. UEA-I and WGA were positive. Results are presented as dot-plot generated by measuring 10,000 viable cells, and the percentage of cells expressing surface carbohydrate was considered positive when more than 20% of the cells were positive.

DISCUSSION

Contemporary treatment plans needs acute leukemia to be distinguished as being of lymphoid or myeloid origin and lymphoid leukemia is further identified as precursor B-cell or T-cell origin. In most clinical situations, this can be done using morphologic and enzyme cytochemical analysis alone. There are, undoubtedly, some cases that defy correct identification based on these simple techniques and require immunophenotyping for proper characterization. These are the situations where multiparametric flow cytometry plays an indispensable role (Kaleen et al., 2003).

Our findings using MoAbs are in accordance with routine diagnosis, as described by Paredes-Aguilera et al. (2001) where CD45, CD34, CD10, CD19, CD3, CD7, CD13 and CD33 are the earliest identifiable markers, CD10 and CD19 are B-ALL specific, T-ALL are confirmed with CD3 and CD7; and CD13 and CD33 are myeloid markers (Campana and Behm, 2000; Paredes-Aguilera et al., 2001).

Glycosylation is a major form of posttranslational modification and variation in glycosylation is associated with cell differentiation and malignant transformation. Such cell mechanism makes carbohydrates potentially valuable diagnostic indicators of cellular diversity and (de)differentiation. Specific surface glycosylation patterns are characteristic features of certain cell types, such as embryonic stem cells, and whole organs, such as the kidney and ABO blood group antigens (Tao et al., 2008). Recently, a growing body of evidence has indicated that changes in the expression of sugar-bearing molecules may actively direct a variety of cell biological processes (Van Dyken et al., 2007).

The structural diversity of a cell surface glycome with biological significance allows a rapid detailed analysis. In this context lectins, which are glycan binding (glyco)proteins, have long been used in techniques to characterize serum or cells by focusing on individual glycans

(Ferguson et al. 2005). Lectin-based approaches are particularly advantageous because of their ability to discriminate sugar isomers and given the commercial availability of a large number of lectins with a variety of saccharide specificities, their use provide a more accurate and detailed "cell surface glycan signatures" evaluation (Tao et al., 2008).

Our results showed that PNA was specific for T-ALL indicating that this lineage expresses a high content of D-galactose in its blasts as also observed by Pérez-Campos-Mayoral et al. (2008). The number of positive cases might be higher if we take account that D-galactose residues are not masked by sialic acid residues, being, then, reachable by PNA. This lectin cannot be compared to CD3 or CD7 (T-ALL specific) as marker. Our findings for PNA can be related to be specific for the most immature type of T-ALL which can be classified into differentiated immunophenotyping stages (Campana and Behm, 2000).

AML is characterized by positivity for CD13 and CD33, mainly. In this context of myeloid markers we found that L-fucose is a biomarker for this type of leukemia as observed by UEA-I positive binding. This lectin was specific for AML even the positivity for only one sample of B-ALL and T-ALL, each. Such feature regarding to UEA-I positivity can be related to aberrant expression for CD13 (Rubnitz et al., 2009) in these both samples.

LTA, other L-fucose specific lectin used in this study, was also positive for AML as well as for T-ALL. Different lectins with the same carbohydrate specificity can present different binding ability since their tridimensional structure imposes different accessibility to saccharide in their carbohydrate recognition domain (Thomas and Surolia (2000). Such feature explains why LTA was positive for the major cases of T-ALL differently of UEA-I (one case only).

B-ALL was not specifically recognized by any lectin used not being possible comparation with its specif MoAbs (CD10 and CD19). WGA was considered a biomarker similar to CD45 based on its behavior of recognizing all types of leukemia.

The relation of lectins with leukemia is far closer than we may speculate. Of note, CD33 is a 67-kd glycoprotein found predominantly on myeloid cells, including early myeloid precursors in the bone marrow and certain subsets of mature circulating myeloid cells. Although initially described as a marker for normal and leukemic myeloid progenitor cells, it has received renewed interest due to its demonstrated lectin activity for α-2-6– and α-2-3–enlinked sialylated oligosaccharides expressed on red blood cells and certain myeloid cells (Grobe & Powel, 2002). These observations established CD33 as a member of a group of lectins termed Siglecs, a family that includes sialoadhesin, CD22, myelin associated glycoprotein 1, MAG-1 (Grobe & Powel, 2002).

Our results indicates the possibility of using lectins as auxiliary tools for leukemia diagnosis but quite works are to come until the day these versatile proteins are included in the routine diagnosis of this pathology. It seems particularly important that the cells can be measured by flow cytometry which primary aim is to establish relative binding patterns for different lectins. These patterns are characteristic for the cell lines. The application of lectins for cell surface characterization seems, therefore, mainly useful for cell line characterization. Minor modifications in the carbohydrate cell surface structure that occur on malignant transformation may be monitored by flow cytometry, as demonstrated in this study. In this sense the characterization of cell by lectins seems a useful supplement to the available antibody methodology.

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8 CONCLUSÕES

A) Avaliando os tipos de leucemias classificadas como LLA e LMA, sem levar em consideração subtipagem ou qualquer outra característica, concluímos:

- WGA não conseguiu diferenciá-las, não sendo uma sonda específica;
- PNA e Con A não apresentaram positividade seletiva, não sendo também sondas específicas;
- UEA-I mostrou-se ser sonda específica para LMA;
- LTA reconheceu células da LMA a LLA inespecificamente.

B) Avaliando apenas a LMA com relação a sua população celular característica, concluímos:

- Apenas a UEA-I foi capaz de reconhecer especificamente os blastos em 55% das amostras de AML (inclindo todos os seus subtipos), indicando ser uma sonda específica para esta leucemia.

C) Avaliando os subtipos de LLA, de linhagem B e T, concluímos:

- LTA mostrou ser uma possível sonda para o subtipo LLA-T;
- PNA mostrou ser sonda específica apenas para o subtipo LLA-T;
- As demais lectinas não apresentaram especificidade exclusiva.

D) Avaliando a positividade dos anticorpos monoclonais e lectinas, concluímos:

- WGA foi considerada ser uma sonda similar ao CD45, reconhecendo todos os tipos de leucemia;
- A positividade da UEA-I pode está relacionada à expressão do CD13;
- As demais lectinas não apresentaram correlação com os anticorpos avaliados.

9 ANEXOS

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EJCB European Journal of Cell Biology

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Recife, 09 de fevereiro de 2004.

PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
GERÊNCIA DE PROJETOS DE PESQUISA

O COMITÊ DE ÉTICA EM PESQUISA DA UPE

O Comite em reunião do dia **02/02/04** considerou em **APROVADO**, o Projeto de Nº **258/03**, intitulado:

IDENTIFICAÇÃO DE CÉLULAS LEUCÊMICAS POR CITOMETRIA DE FLUXO UTILIZANDO LACTINAS MARCADAS, que tem como pesquisadora principal

Profª **MARIA DO SOCORRO DE MENDONÇA CAVALCANTI.**

RESUMO DO COMITÊ DE ÉTICA

O estudo não apresenta maiores riscos de agravos Éticos e está em consonância com as Resoluções do Conselho Nacional da Saúde, referentes às pesquisas que envolvem seres humanos, com a Declaração do Helsinque e com o Código de Nurenberg.

Atenciosamente,

Profª. Drª. Aronita Rosenblatt
Presidente do Comitê de Ética da UPE

Prof. Dr. Aurélio Molina
Coordenador da CPQ e CEP/UPE

Av. Agamenon Magalhães s/n Santo Amaro - Recife - PE
CEP - 50100-010 - Fone (81) 3416 4000 - site www.upe.br
CNPJ 11 022 897/0001-91

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

DADOS DE IDENTIFICAÇÃO:

Título do Projeto: Identificação de Células Leucêmicas por Citometria de Fluxo utilizando Lectinas Conjugadas.

Pesquisador Responsável: Luis Bezerra de Carvalho Júnior

Instituição a que pertence o Pesquisador Responsável: UFPE

1. Afirmação introdutória

O senhor(a) ou o paciente que acompanha está sendo convidado(a) a participar de um estudo a nível de laboratório e não implicará em modificação no tratamento preconizado pelo médico assistente. Para decidir se deseja ou não participar, você precisa entender os benefícios e riscos do mesmo, a fim de formar a sua opinião.

2. Objetivo do estudo

O médico diagnosticou uma doença conhecida como leucemia, que é caracterizada por uma produção anormal de glóbulos brancos no sangue e acúmulo de células jovens anormais na medula óssea. Neste estudo, não serão administrados medicamentos para esta doença, mas através da coleta de amostra da medula óssea, poderão ser investigados determinados marcadores celulares que podem estar associados ao tipo da doença e estas alterações a nível celular poderão estar ligadas a resposta ao tratamento. Farão parte deste estudo adultos, crianças e adolescentes que apresentem Leucemia linfóide aguda (LLA) ou Leucemia mielóide aguda (LMA).

3. Procedimentos a serem seguidos

Após você ter assinado o Consentimento Informado, será utilizada a amostra de medula óssea que foi colhida para o diagnóstico da leucemia que se encontra no laboratório de imunofenotipagem. O paciente continuará a receber a assistência que já vinha sendo oferecida, sem prejuízo ou interrupção de medicamentos que por ventura já esteja fazendo uso.

4. Desconforto e riscos

Nenhum novo medicamento ou forma de tratamento será testado e não determinará qualquer risco, podendo ocasionar apenas desconforto durante a coleta da medula óssea que é obtida através de punção no osso esterno ou em crista ilíaca, cujo procedimento já é realizado rotineiramente durante o diagnóstico e tratamento. Portanto, o paciente não será submetido a qualquer procedimento que não faça parte do acompanhamento normal da doença.

5. Exclusões

Se você não entender alguma palavra do texto, peça explicações ao seu médico ou ao pesquisador que acompanha o projeto.

6. Benefícios aos participantes

A participação não trará qualquer benefício direto, mas ao aceitar participar deste trabalho você estará colaborando para um maior conhecimento médico e científico sobre a doença, e com isto, possibilitará que novas formas de diagnóstico possam ser utilizadas.

7. Confidencialidade

A menos que sejam requeridos por lei, apenas os investigadores e os membros da Comissão de Ética irão ter acesso a dados confidenciais os quais identificam o paciente pelo nome.

A apresentação dos resultados desta pesquisa fica restrita a apresentação em reuniões médicas e científicas ou em publicações especializadas, sem que você seja identificado nestas apresentações. Todo o material biológico coletado neste estudo será exclusivamente utilizado para as pesquisas.

8. Pessoas para contato

O pesquisador ou seu substituto responderá a todas as perguntas formuladas. Se você tiver mais questões a fazer, no decorrer deste estudo sobre a pesquisa ou seus direitos como um participante da mesma, poderá dirigi-las ao comitê de ética em pesquisa do Hospital Universitário Oswaldo Cruz pelo fone 21011536 e aos pesquisadores: Maria Socorro M. Cavalcanti (Fone:99756644), Elizangela Ferreira da Silva (Fone: 99770390) e Cíntia G. Faria Machado (Fone:31824655).

9. Participação voluntária

Você está participando voluntariamente deste estudo. Pode se recusar de participar do mesmo a qualquer momento, sem penalidades nem perda dos benefícios que ela já tem direito.

10. Consentimento Informado

Li o texto acima e estou ciente do conteúdo deste formulário de consentimento. Minhas perguntas foram respondidas. Aceito participar do estudo. Recebi uma cópia deste formulário de consentimento.

Nome do paciente: _____

Assinatura do responsável: _____

data:__/__/__

Assinatura da testemunha: _____

data:__/__/__

Pessoa que conduziu o Termo de Consentimento: _____

Assinatura: _____

data:__/__/__