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Membrane Marker Studies With Anti-T, Anti-Ia (DR) and Anti-myeloblast Antisera in Acute Leukaemias

ANNE-MARIE LEBACQ,* PIERRE GOFFINET, MARC DE BRUYERE, JEAN RODHAIN and GÉRARD SOKAL
Unité de Recherches sur les Maladies du Sang, University of Louvain, Belgium

Abstract—*Hetero-antisera reacting specifically with all cells of the T lineage, with Ia-like (DR) antigens or with myeloblasts were used as diagnostic aids in the classification of 64 acute leukaemias. The anti-myeloblast proved particularly useful in discriminating early myeloblasts from atypical lymphoblasts and undifferentiated blasts.*

INTRODUCTION

MEMBRANE markers and enzyme assays have become an essential tool in the diagnosis of hemopoietic malignancies (reviewed in [1]). They are particularly useful for determining the cell lineage and hence the treatment of unclassified or controversially-classified acute leukaemias [2, 3].

Thus, in the absence of typical morphological and cytochemical features, acute lymphoid leukaemias can nevertheless be identified by the presence of one of the following markers: the DNA polymerase terminal deoxynucleotidyl transferase [4], the common ALL antigen [5], intracytoplasmic μ chains [6, 7] and T cell lineage antigens [8]. Sheep erythrocyte receptors [9] and membrane immunoglobulins [10] are usually associated with recognizable lymphoid cells.

By contrast, acute myeloid leukaemias are only identified when expressing characteristic morphological and cytochemical features [11]. The allo- and hetero-antisera reported to react specifically with myeloblasts and with differentiating granulocytes do not unequivocally distinguish early myeloblasts from non-lymphoid, undifferentiated blasts [12–14]. However, one of these antisera proved of great diagnostic value by detecting small bone marrow mononuclear cells that increased in numbers prior to acute myeloblastic leukaemia relapse [13].

The aim of our work was to produce useful immunodiagnostic reagents to assist the classification of acute leukaemias. In addition to antisera reacting specifically with all cells of the T lineage and with Ia-like antigens, we obtained an antiserum that not only labelled the majority of blasts in 20/21 well-characterized acute myeloblastic leukaemias, but also discriminated between early myeloblasts and early lymphoblasts in a few cases difficult to classify by conventional diagnostic methods.

MATERIALS AND METHODS

Patients and initial diagnosis

Blood and bone marrow aspirates were taken from leukaemic patients at admission. The initial diagnosis was based upon conventional morphology and cytochemistry, which included reactions for PAS, peroxidases, chloroacetate esterases, naphthol acetate esterases and aspecific naphthol acetate esterases [15].

Biological samples

Normal blood was drawn from healthy volunteers. Normal bone marrow came from a marrow graft. Thoracic duct lymph was drained from future kidney graft recipients. Normal spleens came from kidney donors. Thymic biopsies were from children undergoing cardio-vascular surgery. Tonsils were recovered after tonsillectomy. Out-of-date

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*Correspondence to: Dr Anne-Marie Lebacq, Unité de Recherches sur les Maladies du Sang, Clos Chapelle-aux-Champs, 30, UCL 3052, 1200 Brussels, Belgium.

human red blood cells and platelets were given by the Blood Bank.

Cell suspensions

Organs were teased into cell suspensions in 2 mM Hepes–0.5% BSA Eagle's medium. Thymocytes, thoracic duct lymphocytes and tonsil lymphocytes were used without purification. Blood, bone marrow and spleen mononuclear cells were separated by density centrifugation on Ficoll-Isopaque [16]. Cells not used immediately were adjusted to 20,000/mm^3 in 2 mM Hepes–1% BSA and 7.5% DMSO Eagle's medium and progressively frozen (−1°C/min) in liquid nitrogen. When required, cells were thawed rapidly and washed in 2 mM Hepes–0.5% BSA medium. Nucleated cells were counted in a Coulter Counter (Coulter Electronics, DN model). Differential nucleated cell counts were done on May–Grünwald and Giemsa-stained cytocentrifuge cell smears.

Sheep erythyrocyte rosettes

One hundred microliters of cells at 5000/mm^3 in Eagle's medium containing 2 mM Hepes and 50% FCS (Gibco) were mixed for 15 min at 37°C with 20 μl of AET-treated sheep red blood cells [17]. Cells were centrifuged for 5 min at 60 ***g*** and kept for 1 hr at 4°C. Rosettes were counted after gentle resuspension, dilution and addition of Toluidine Blue.

Complement-dependent cytotoxicity

Series of identical Terasaki plates were stored at −20°C with serial dilutions of antisera. When thawed, they were incubated for 30 min at room temperature with 1 μl of a source of rabbit complement (Biotest). Negative controls (antiserum alone and complement alone) and a positive control (polyvalent anti-WBC antiserum with complement) were included into each plate. Dead cells were revealed by the addition of 1 μl of 5% eosine and scored on an inverted microscope. Each sample was titrated at least three times in duplicate. Cytotoxic titers correspond to the last dilution killing at least 80% of the cells, with negative controls below 20%.

Immunofluorescence

Membrane immunoglobulin-positive cells (mIg$^+$) were assayed by direct immunofluorescence, using a commercial goat anti-human Fab fluoresceinated conjugate (GAHu-FITC, Nordic Pharmaceuticals).

Other membrane markers were determined by indirect immunofluorescence. Twenty-five microliters of cells at 20,000/mm^3 in 2 mM Hepes–Eagle's medium were incubated on ice with 25 μl of rabbit antiserum (see below) or with 25 μl of normal rabbit serum (NRS) adjusted to the same immunoglobulin concentration. Cells were washed in cold medium supplemented with 0.2% NaN_3 and 0.1% BSA, resuspended in ± 50 μl and incubated with 10 μl of an undiluted goat anti-rabbit IgG fluoresceinated conjugate (GAR-FITC, Nordic Pharmaceuticals). After further washes, cytocentrifuge cell smears were prepared and examined under a Leitz Dialux fluorescence microscope equipped with an epi-illuminator and with phase-contrast optics. Alternatively, samples were analysed in a Fluorescence-Activated Cell Sorter at the ICRF, London (FACS I, Becton Dickinson, Mountain View, California) [14]. The percentage of specifically labelled cells was obtained by subtracting the percentage of cells labelled by NRS from the percentage of cells labelled by an antiserum.

Antisera

The preparation of anti-T lymphocytes (anti-T), anti-hairy cells (anti-Ia) and anti-myeloblast (anti-M) antisera is summarized in Table 1.

Anti-T was raised in 2 rabbits by 13 i.m. injections of 5×10^7 cryopreserved thoracic duct lymphocytes (≥95% T lymphocytes) given within 10 months. Complete Freund's adjuvant was added to the first 5 injections. The animals were bled twice, 7 and 20 days after the 11th and the 13th injections.

Anti-Ia was raised in 2 rabbits by 8 i.m. injections of 5×10^7 hairy cells purified from a leukaemic spleen and coated with rabbit anti-WBC antiserum [5]. The latter was taken from the anti-T rabbits 10 days after the 7th injection and absorbed with 6 vol of packed AB$^+$ red blood cells. Injections were given within 5 months, only the first one including complete Freund's adjuvant. The animals were bled twice after the 6th and the 8th injections.

Anti-M was raised in 2 rabbits by 6 weekly i.m. injections of 10^8 cryopreserved blood MN cells obtained from a patient with acute myeloblastic leukaemia (≥90% myeloblasts) and coated with anti-WBC antiserum. Only the first injection was given with adjuvant. The animals were bled 14 days after the 6th injection and 7 and 20 days after a last boost given 20 weeks later.

All sera harvested from homologous rabbits were pooled, heat-inactivated (30 min at 56°C) and extensively absorbed, as indicated in Table 1. Fully-absorbed antisera were centrifuged (1 hr at 40,000 ***g***), filtered on 45 μ-Millipore® filters and stored in small quantities at −80°C.

Table 1. *Preparation of rabbit antisera*

Antisera	Immunizing cells	Number and timing in weeks of i.m. injections*	Number of absorptions† Ig	RBC	PLT	BCL	THY	TON
Anti-WBC	5×10^7 Thoracic duct lymphocytes (95% T cells)	7 $0^+, 4^+, 8^+, 12^+, 15^+$, 17, 19	—	6	—	—	—	—
Anti-T	5×10^7 Thoracic duct lymphocytes (95% T cells)	13 22, 24, 25, 26, 41, 44	1	12	6	1	—	—
Anti-Ia	5×10^7 Splenic hairy cells + anti-WBC	8 0^+, 2, 3, 4, 5, 6, 21, 24	1	9	3	—	—	—
Anti-M	10^8 AML Blasts + anti-WBC	7 0^+, 2, 3, 4, 5, 6, 29	1	13	—	—	4	1

*i.m. = Intra-muscular; + = injections with complete Freund's adjuvant.

†Absorptions of 1 vol of antiserum with 1 vol of packed cells; Ig = crude human immunoglobulins cross-linked to AH-Sepharose 4B; RBC = AB^+ red blood cells; absorptions were carried out until antisera failed to agglutinate trypsinized RBC; PLT = pooled human platelets; BCL = B cell line Raji; THY = human thymocytes; TON = tonsil lymphocytes.

F(ab')2 fragments were prepared by digesting antisera with 2 mg/ml of pepsin (Sigma) for 20 hr at 37°C [14]. The efficiency of the digestion was controlled by SDS-polyacrylamide gel electrophoresis [18].

Monoclonal antibody J 5

Typing with monoclonal antibody anti-cALL [19] was performed on leukaemias referred to M. F. Greaves at the ICRF, London.

RESULTS

Cytotoxicity of antisera for lymphoid and myeloid cell samples

Antisera were first titrated by cytotoxicity on lymphoid and myeloid cell samples (Table 2). The anti-M killed nothing but leukaemic myeloblasts in 5/5 acute myeloblastic leukaemias (≥75% myeloblasts). Conversely, the anti-T killed T but neither B nor myeloid cells, and the anti-Ia lysed its targets (hairy cells) but not T cells.

Immunofluorescent binding of antisera to subpopulations of human leucocytes

Antisera were further tested by indirect immunofluorescence on well-characterized cell samples (Table 3).

The anti-T labelled specifically a percentage of lymphoid cells that corresponded to the percentage of rosette-forming cells. It labelled neither the B cell line RAJI nor $cALL^+$ lymphoblasts nor myeloblasts. Its F(ab')2 fragment stained 78% of blood MN cells, thus confirming its absence of specific binding to B lymphocytes and to monocytes.

The anti-Ia labelled specifically all FcR^- cells tested, namely the B cell line RAJI, $cALL^+$ lymphoblasts and leukaemic myeloblasts. It also labelled most FcR^+ cells far above background. Its specific binding to monocytes, B lymphocytes, B ALL blasts, hairy cells and leukaemic monoblasts was confirmed by the use of its F(ab')2 fragment.

The anti-M labelled only leukaemic myeloblasts. Since its F(ab')2 fragments were too weakly active, we had to use the whole anti-M and FACS analysis to check its lack of specific attachment to FcR^+ cells. In these conditions, anti-M did not label tonsil lymphocytes nor B-CLL lymphocytes. Furthermore, its analytical absorption with an equal volume of packed tonsil lymphocytes did not alter its binding to leukaemic myeloblasts. It also labelled specifically a proportion of cells of the promyelocytic line HL-60 [20] and 7–13% of normal bone marrow mononuclear cells.

Immunodiagnostic studies with antisera in acute leukaemias

Antisera were further used as diagnostic aids in the classification of 64 acute leukaemias and blast crises (Table 4). Samples containing less than 75% blasts were excluded from this study.

The anti-T reacted only with T lymphoblasts (E^+, mIg^-, Ia^-) and identified 2 cases of 'pre-T' ALL (i.e., E^-, mIg^-, Ia^-) in the group of non-T non-B ALL.

Table 2. Cytotoxicity of antisera

Cell samples*	No.	Composition†	Median cytotoxic titer‡ of: Anti-T	Anti-Ia	Anti-M
Lymphoid samples					
Thymocytes	2	⩾95% T lymphocytes	64	—§	—
T.D. lymphocytes	2	⩾90% T lymphocytes	16	—	—
P.B.MN cells	3	60–80% T lymphocytes	8	(2)‖	—
T-ALL	2	>80% T lymphoblasts	16.8	—	—
Sezary cells	1	83% T lymphocytes			—
B-ALL	1	99% B lymphoblasts	—		—
CLL	9	>90% Lymphocytes	—		—
LSCL	1	>90% Lymphocytes			—
HCL	1	95% Hairy cells		8	—
ALL	4	>90% Lymphoblasts			—
AML remission	7	>85% Lymphocytes			—
AMoL remission	1	90% Lymphocytes			—
Myeloid samples					
AML	5	>75% Myeloblasts	—		8
AMoL	1	90% Monoblasts			—
P.B. PMN	2	>80% Granulocytes	—		—

*T.D. = Thoracic duct; P.B.MN and PMN cells = peripheral blood mononuclear or polymorphonuclear cells; ALL = acute lymphoblastic leukaemia; CLL = chronic lymphatic leukaemia; LSCL = lymphosarcoma cell leukaemia; HCL = hairy cell leukaemia; AML = acute myeloblastic leukaemia; AMoL = acute monoblastic leukaemia.
†T Cells = sheep erythrocyte rosette-forming cells; B cells = membrane immunoglobulin-bearing cells; other cells determined by morphology and cytochemistry.
‡Last dilution killing at least 80% of cells; negative controls below 20%; each sample tested at least 3 times in duplicate wells.
§— = Undiluted antiserum kills no cells above background.
‖(2) = 1/2 Antiserum kills a minor fraction of cells above background.

Table 3. Immunofluorescent binding of antisera

Cell samples*	Composition†	Percentage of specifically-labelled cells Anti-T	Anti-Ia	Anti-M
Thymocytes	99% T lymphocytes	98 (90)‡	1+§	<1
T.D. lymphocytes	96% T lymphocytes	95	5+	<1
P.B.MN cells	80% T lymphocytes	95 (78)	20+(15)	<1
Tonsil lymphocytes (FACS)	85% T + B lymphocytes	—	26	<1
T-ALL	80% T lymphoblasts	83	1	<1
T line MOLT	100% T lymphoblasts	100	1	<1
B-ALL	99% B lymphoblasts	NI‖	100+(90)	NI
B-CLL (FACS)	79% B lymphocytes		85	<1
B line RAJI	100% B lymphoblasts	1	100	<1
C-ALL	90% Blasts 53% CALL$^+$	2	70+	<1
Ly-BC	96% Blasts 95% CALL$^+$	1	85+	<1
AMoL	95% Monoblasts	NI	100+(92)	NI
AML	85% Myeloblasts	5	33	80%
HL-60 line (FACS)	>75% Promyelocytes		12%	28–38%

*As in Table 2—Ly – BC = lymphoid blast crisis; FACS = measured by FACS analysis at the ICRF, London.
†As in Table 2; cALL$^+$ cells determined at the ICRF, London.
‡( ) = Percentage of cells labelled by F(ab')2 fragment of antisera.
§+ Denotes a bright labelling.
NI = Not interpretable due to high background.

Table 4. Classification of 64 acute leukaemias

Conventional diagnosis*	Membrane phenotype of blasts†	No. of cases	Classification
ALL	E^-, mIg^-, T^-, Ia^+	19	Non T non B ALL
	$E^-, mIg^-, T^-, Ia^+, M^-, cALL^+$	4	Common ALL
	E^-, mIg^-, T^+, Ia^-	2	Pre-T ALL
	$E^+, mIg^-, T^+, Ia^-, M^-$	4	T ALL
	E^-, mIg^+, Ia^+	2	B ALL
CML—lymphoid BC	$E^-, mIg^-, T^-, Ia^+, M^-, cALL^+$	3	Lymphoid BC
AML (>5% CAE^+ blasts)‡	$E^-, mIg^-, T^-, Ia^{+}_{-}, M^+$ (4 $cALL^-$)	20	AML
AML? (1–2% CAE^+ blasts)	$E^-, mIg^-, T^-, Ia^+, M^+$	2	AML§
AEL	$E^-, mIg^-, T^-, Ia^-, M^-$	3	AEL
AUL	$E^-, mIg^-, T^-, Ia^+, M^-, cALL^-$	2	AUL
	$E^-, mIg^-, T^-, Ia^+, M^-$	2	AUL or ALL?
	$E^-, mIg^-, T^-, Ia^-, M^-$	1	AUL

*ALL = Acute lymphoid leukaemia; AML = acute myeloblastic leukaemia; CML – BC = chronic myeloid leukaemia in blast crisis; AEL = acute erythroleukaemia; AUL = acute undifferentiated leukaemia.
†E^* = Forming sheep erythrocyte rosettes; mIg^+, T^+, Ia^+, M^+, $cALL^+$: specifically labelled by anti-immunoglobulin, anti-T, anti-Ia, anti-M and anti-cALL.
‡CAE^+ = chloroacetate esterase-positive cells.
§AML confirmed by electron microscopy.

The anti-Ia labelled all leukaemic blasts except those from T ALL pre-T ALL, AEL and from some AUL; it usually stained a fraction of, but rarely all, AML blasts.

The anti-M labelled specifically the majority of leukaemic blasts in 20/21 well-characterized AML (at least 5% of chloroacetate-positive blasts). Interestingly, the anti-M also reacted with atypical blasts of two acute leukaemias. Careful cytochemical examination disclosed 1–2% of chloroacetate-positive blasts, and electron microscopy eventually confirmed the identification of early myeloblasts. Conversely, the anti-M failed to react with 3 lymphoid blast crises that responsed to vincristine–prednisone therapy and that were eventually typed as $cALL^+$ blast crises at the ICRF, London. Four $cALL^+$ were also found to be negative with the anti-M.

DISCUSSION

We obtained 3 hetero-antisera that reacted with all cells of the T lineage, with Ia-like antigens and with myeloblasts, and used them as diagnostic aids in the classification of 64 acute leukaemias.

Several anti-T antisera have been reported in the literature (reviewed in [21]), but none were raised against thoracic duct lymphocytes nor rendered T cell-specific by a single absorption with B cells. The latter was done after extensive absorptions with red blood cells and with platelets (Table 1) which diluted at least 20 times the native antiserum and which removed most, if not all, cross-reacting antibodies like anti-i and anti-HLA-A, -B, -C antibodies. The anti-T probably identifies most, if not all, cells of the T lineage, including 'pre-T' cells that do not express sheep erythrocyte receptors [8]. Such a pan-T reagent is quite useful for the determination of the T cell lineage prior to subtyping with OKT monoclonal antibodies [22]. Many of the latter are indeed T subset-specific, but neither pan-T nor T lineage-specific [23].

The anti-Ia was raised against hairy cells and reacted with all cells known to express Ia-like/HLA-DR antigens [1]. No attempts were made to dissect its activity by analytical absorptions with B cells or with monocytes.

The anti-M reacted specifically with myeloblasts and with a minor fraction of the promyelocytic line HL-60. The latter data suggest that it does not bind to the more mature granulocytic cells, a feature that distinguishes it from the anti-M described by Roberts and Greaves [14].

FACS analysis showed that the anti-M labelled specifically 7–13% of normal bone

marrow mononuclear cells; their unequivocal identification is waiting for a cell-sorting experiment. Our anti-M appears similar to the mouse anti-myeloblast described by Baker *et al.* [13]. However, we have not yet attempted to predict AML relapse by monitoring the reactivity of bone marrow cells from patients in remission.

The interest of our anti-M resided in the identification of atypical myeloblasts in two cases of acute leukaemias, in which less than 2% of the blasts displayed chloroacetate esterase activity, but most of them appeared as early myeloblasts by electron microscopy. Conversely, poorly differentiated blasts eventually typed as cALL$^+$ lymphoblasts were not labelled by the anti-M.

In conclusion, these 3 hetero-antisera proved useful in the classification of acute leukaemias. They are now being used to prepare the corresponding monoclonal antibodies.

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