

NATURAL KILLER CLONES DERIVED FROM FETAL
(25 wk) BLOOD

Probing the Human T Cell Receptor With WT31 Monoclonal Antibody

BY A. NOWILL, P. MOINGEON, A. YTHIER, M. GRAZIANI, F. FAURE,
L. DELMON, M. RAINAUT, F. FORRESTIER, C. BOHUON, AND
TH. HERCEND

From the Unité de Biologie Cellulaire, Institut Gustave-Roussy, 94805 Villejuif, France

Tax et al. have recently described a T cell-specific mAb (1) termed WT31; this reagent is mitogenic for resting T lymphocytes in the presence of functional macrophages and blocks cytotoxicity mediated by alloantigen-specific T cell clones (2, 3). Binding of WT31 is blocked by preincubation of target cells with OKT3, showing that both antibodies define either an identical or closely related antigenic determinant (1). However, biochemical studies have suggested that WT31 may not be directed at the low molecular mass (25 kD and 20 kD) T3 proteins. Rather, it was postulated (3, 4) that the determinant recognized by WT31 is borne by a constant region of the T3-associated, 90 kD heterodimeric structure that functions as a specific receptor for antigen recognition (5, 6). This hypothesis is based upon the finding that WT31 precipitates predominantly high mol. mass material (90 kD under nonreducing conditions), while additional antibodies with apparently similar specificity, such as Leu-4, precipitate predominantly low mol. mass proteins (25–20 kD). Because these differences are only relative, immunoprecipitations strongly suggested, but did not formally prove, that WT31 has a unique specificity compared with conventional anti-T3 antibodies.

In this study, we show that a number of cytotoxic clones derived from circulating blood of a human fetus aged 25 wk express T3 proteins defined by the well characterized DFCI 2d2, OKT3, Leu-4, or UCHL1 antibodies. Yet, only a fraction of these cloned cell lines reacts with WT31 mAb. Furthermore, surface expression of WT31 structure correlated with the capacity for effector cells to recognize alloantigens, while NK reactions appeared to be WT31-independent.

Material and Methods

Human Fetal Cloned Cell Lines. We obtained normal fetal peripheral blood by using novel sampling methods aimed at prenatal diagnosis (7, 8). Blood (0.5 ml) of the fetus studied here was sampled by direct puncture of the cord because the mother presented a toxoplasmosis seroconversion; there was no biological evidence for infection of the child.

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Fetal PBMC (FPBMC) were extracted by Ficoll-Hypaque density gradient centrifugation (2×10^6 cells/ml). FPBMC were cocultured with irradiated (6,000 rad) LAZ 388 EBV-transformed B lymphocytes. Responder lymphocytes were restimulated two times (day 8 and 12). On day 19 bulk culture cells were cloned by limiting dilution at 0.66 cells/well and remaining cells were frozen in several aliquots. For cloning, feeder layer consisted of irradiated LAZ 388 cells (5,000 rad) plus irradiated allogeneic adult lymphocytes (3,000 rad). Cultures were fed every 3 d with lymphocyte conditioned medium (LCM) containing IL-2, prepared as described previously (9). Culture medium was DME supplemented with 15% human AB serum. Further propagation of individual cell lines was performed by diluting lymphocytes (~ 1000 cells/well in the same type of 96 V-bottomed microtiter plates used for initial cloning) repeatedly on irradiated feeder layer cells and adding LCM every 3 d until they reached a concentration of 2×10^6 cells/ml. mAbs used to phenotype the clones were: Coulter clones T4 and T8; anti-T3 (DFCI 2ad2), OKT3, Leu-4, UCHL1; WT31 (1); we also employed the "Pan NK" anti-NKH1A mAb (10).

Phenotypic analysis was performed on an Epics C (Coulter Electronics, Inc., Hialeah, FL) after indirect immunofluorescence assays, as previously described (9). 10,000 cells were analyzed in each sample; histograms shown in Fig. 1 display number of cells vs. intensity fluorescence measured on a log scale. Negative control was the irrelevant anti-B1 mAb used at saturating concentration.

Cytotoxicity Assays. Cytotoxicity assays were performed according to a standard chromium release method described previously (9). All experiments were done in triplicate using V-bottomed microtiter plates. Assays were generally performed for 3 h at 37°C using 5,000 target cells per well. Medium was RPMI plus 5% human AB serum.

Cell Sorting Experiments. To purify WT31⁺ cells from bulk culture lymphocytes, cells were stained by indirect immunofluorescence using WT31 ascites at saturating concentrations plus FITC-conjugated goat anti-mouse F(ab')₂ immunoglobulin (Coulter clone). WT31⁺ and WT31⁻ sorted cells were collected in RPMI 1640 containing 5% normal human serum, washed twice, and resuspended in fresh medium for functional assays. Trypan blue dye exclusion was performed on both sorted populations, and viability was always >95%. Purity of sorted cells was >95% in each fraction ($\geq 95\%$ WT31⁺ cells in the positive fraction, $\leq 5\%$ WT31 cells in the negative fraction).

Results and Discussion

To study NK active lymphocytes as early as possible during normal human development, we have recently generated cytotoxic clones derived from peripheral blood of one healthy fetus aged 25 wk. A first series of clones (Ythier et al., manuscript submitted for publication) was obtained after stimulation of fetal lymphocytes by an allogeneic, EBV-transformed B lymphoblastoid cell line termed LAZ 388. When tested for cytotoxic activity, all cloned lymphocytes were found to lyse the NK susceptible target cell line K562, but none killed LAZ 388 immunizing cells. Yet, T3 proteins were expressed on these fetal effector cells and it was questioned whether they possess (in addition to T3) appropriate receptor structures to recognize alloantigens. Genes encoding Ti molecules were studied in one clone termed F6C7. Clonal rearrangements of Ti β genes were found in F6C7 cells that expressed corresponding messenger RNA. However, we could not detect any Ti α transcripts, suggesting that there was no construction of a conventional [T3/Ti α - β] receptor on the surface of the fetal lymphocytes.

In light of these results, further cloning procedures were performed here starting from bulk cultured cells that had been frozen after three (day 0, 8, and 12) stimulations by irradiated LAZ 388 B lymphoblastoid cells. A total of 19 clones was obtained that displayed sufficient proliferative capacity to be assayed at least two times for both phenotype and functional activity. They are listed in Table I, which shows their reactivity with anti-T3, WT31, T4, T8, and NKH1A

TABLE I
Reactivity of Cloned Cells with mAbs

Clone	Phenotype					Cytotoxic function [‡]	
	T3	WT31	T4	T8	NKH1A	K562	LAZ 388
F6A4	+	-	-	+	+	+++	-
F6A5	+	-	-	+	+	+++	-
F6A7	+	-	-	+	+	+++	-
F6B9	+	+	+	-	-	-	-
F6B10	+	-	-	+	+	++	-
F6C7	+	-	-	+	+	+++	-
F6D7	+	-	-	+	+	++	-
F6D10	+	+	+	-	-	-	-
F6D12	+	-	-	+	+	+++	-
F6E6	+	+	+	-	-	-	-
F6F5	+	-	-	+	+	++	-
F6F7	+	-	-	+	+	++	-
F6F8	+	-	-	+	+	++	-
F6F9	+	-	-	+	+	++	-
F6G3	+	+	-	+	+	+++	+++
F6G7	+	-	-	+	+	++	-
F6G10	+	+	-	+	+	+++	+++
F6H1	+	-	-	+	+	++	-
F6H2	+	+	-	+	+	+++	+++

Reactivity was assayed by indirect immunofluorescence and subsequent cytofluorometric analysis.

* Most cells in a given clone appear to be positive, but only weak fluorescence is detected.

[‡] Cytotoxicity assays were performed for 3 h at generally 6:1 E/T cell ratio. Results represent mean cytotoxicity for triplicate culture. - < 5%; 5% ≤ + < 25; 25 ≤ ++ < 50; +++ ≥ 50%. + ≥ 90/cells stained by corresponding MAb; - ≤ 5 cells stained with corresponding antibodies.

mAbs, as well as their ability to lyse either immunizing LAZ 388 or K562 target cells.

Three colonies (F6B9, F6D10, and F6E6) were found to be noncytotoxic and to express a conventional T3⁺, WT31⁺, T4⁺, T8⁻, NKH1A⁻ "inducer" phenotype. The great majority of the clones (16 out of 19) reacted with anti-T3, T8, and NKH1A mAbs. A comparable phenotype has been previously described in studies of adult peripheral blood. It is represented by a number of clones, such as JT9 (9, 11), which are derived from a minor population of circulating T3⁺NKH1A⁺ lymphocytes (10). JT9 cells have been extensively studied; they were found to transcribe both *Ti α* and *Ti β* genes and to surface express a conventional 49/43 kD receptor structure termed NKTa (11, 12). Moreover anti-NKTa mAb was shown to block non-MHC-restricted cytotoxicity displayed by JT9 cells against various NK susceptible tumor cell lines. However, 13 clones (represented by F6C7 cells on Fig. 1A) described here are phenotypically distinct from JT9 (Fig. 1C) because they do not express WT31, while reacting normally with several well characterized antibodies specific for T3 structures such as DFCl 2ad2, Leu-4, OKT3, or UCHT1 (Fig. 1A). This finding indicates that the antigenic determinant recognized by WT31 is not borne by the 20–25 kD proteins. In light of previous biochemical data (3, 4) obtained with this mAb, we can conclude that WT31 recognizes an invariant epitope of the human T cell receptor. Moreover, because *Ti α* transcripts could not be detected in F6C7 cells, it is reasonable to postulate that WT31 mAb is directed at either the α chain or, alternatively, at an antigenic configuration whose construction depends upon the presence of both α and β chains. Several studies have suggested an ordered expression of γ , β , and finally α chains during T cell ontogeny (13, 14); thus,

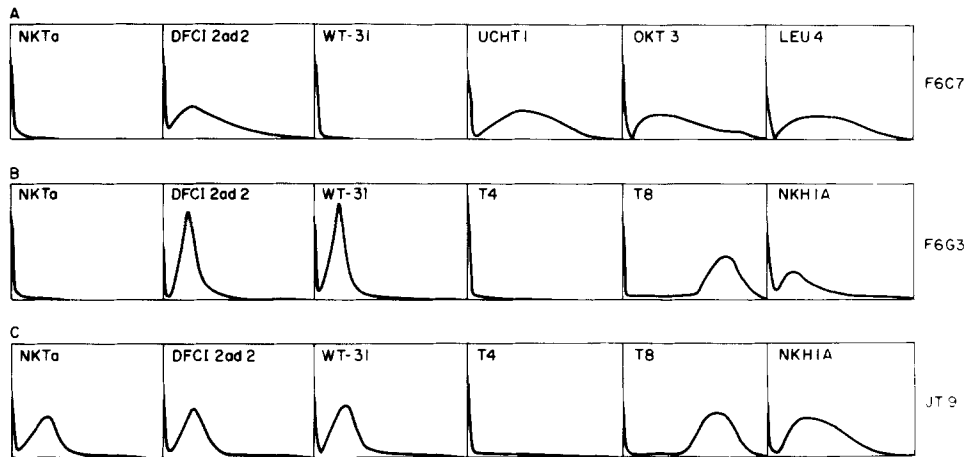


FIGURE 1. Indirect immunofluorescence analysis of NK clones. Saturating concentration of all mAbs used in these assays was predetermined on appropriate antigen-bearing cells. Negative control was anti-B1 mAb. Reactivity of anti-B1 was superimposable to that of WT31 and NKTa on F6C7 cells. Histograms presented here display number of cells (Y) versus intensity of fluorescence (X) on a logarithm scale. Reactivity of all reagents with cloned cells was $>90\%$ or $<5\%$.

TABLE II
Compared Cytotoxic Activity of WT31⁺ and WT31⁻ Fractions

Cells used	Percent reactivity WT-31*	Target cells	
		K562 [‡]	LAZ 388
F6 unsorted cells	62	41	54
F6 WT-31 ⁺ sorted cells	>90	25	62
F6 WT-31 ⁻ sorted cells	≤ 5	42	9

* Determined by indirect immunofluorescent assays.

[‡] E/T ratio, 15:1; 3-h assay; numbers represent mean of triplicate cultures; SD $\leq 5\%$.

WT31 may represent a unique tool to easily assess the differentiation stage of a given T cell by means of simple surface analysis.

Three additional clones F6G3, F6G10, and F6H2 expressed T3, WT31, T8, and NKH1A antigens. Representative immunofluorescence analysis of F6G3 lymphocytes is presented in Fig. 1B. These clones were found to kill immunizing cells, as well as the NK target cell line K562, suggesting a positive correlation between surface expression of WT31 and the capacity to recognize alloantigens. If this was correct, most if not all cytotoxic activity against LAZ 388 cells should be found in the WT31⁺ subpopulation of the cell line used for cloning experiments. To address the point, bulk culture cells were separated into WT31⁺ and WT31⁻ fractions (representing respectively 62 and 38% of the cells) by cell sorting. Cytotoxic activity against either alloantigen-bearing cells or K562 was then compared using unsorted lymphocytes vs. WT31⁺ and WT31⁻ effector cells. Both bulk culture and WT31⁺ sorted lymphocytes were found to kill LAZ 388, as well as K562 cells. In contrast, there was little if any killing (Table II) against LAZ 388 in the WT31⁻ subpopulation that mediated strong NK activity, correlating with that of T3⁺ WT31⁻ clones.

These results support the view that recognition of MHC gene products may require the presence of WT31 determinant, that is of either the α chain or of both α and β chains. In contrast, recognition of NK target structures appears to be WT31-independent. Yet, a $Ti\alpha/\beta$ heterodimer (NKTa) previously identified on JT9 cells (11) was shown to be involved in NK-mediated lysis against K562 and a variety of additional genetically unrelated tumor cell lines. This apparent discrepancy leads us to postulate that certain NK reactions are mediated through Ti molecules, but do not, in fact, necessitate the construction of a complete conventional T cell receptor. Note that a putative NK receptor is likely to be related to T3 proteins when they are surface expressed. Indeed anti-T3 blocks cytotoxic function of F6C7 cells (Ythier et al., manuscript submitted for publication) and, more generally, of all $T3^+ WT31^-$ NK clones described here (not shown). Further studies will be conducted to search for the presence of a T3-associated (physically, or at least functionally) molecule on $WT31^-$ cells and assess its possible role in NK target cell recognition.

The diversity of NK active lymphocytes is now well established, whereas receptor structures used by most of these cytotoxic cells are not yet characterized. This situation has created considerable confusion. In particular, it is not known whether the distinct lymphocyte subsets (for example: $T11^-T3^-WT31^-NKH1A^+$, $T11^+T3^-WT31^-NKH1A^+$, $T11^+T3^+WT31^-NKH1A^+$, and $T11^+T3^-WT31^+NKH1A^+$) that display apparently similar cytotoxic activity against conventional target cells such as K562 are related or if they evolve from distinct differentiation pathways. The present experiments suggest that relevant cloning strategies may contribute to definitions of intermediate stages of T cell maturation, one of them being possibly reflected here by fetal $T3^+WT31^-$ lymphocytes. Hopefully, a better characterization of these early T cell differentiation steps will help to clarify the origin of the various "NK" lymphocyte populations.

Summary

We have conducted a phenotypic and functional analysis of 19 cloned cell lines generated after allogeneic stimulation of circulating lymphocytes from a normal human fetus aged 25 wk. Using a limited series of mAbs (Anti-T3, WT31, T4, T8, and NKH1A), cloned cells were found to fall in three groups. Three clones have a conventional "inducer" phenotype. Three clones have a phenotype ($T3^+$, $WT31^+$, $T8^+$, and $NKH1A^+$) similar to that of certain NK active mature T lymphocytes present in adult peripheral blood. In contrast, 13 cell lines display surface characteristics that have not been described previously. Indeed, they express T3 proteins but not the WT31 determinant. In light of previous studies, these results show that WT31 mAb is a unique reagent directed at an invariant epitope of the human T cell receptor that is not present on all circulating $T3^+$ fetal lymphocytes. Functionally the $T3^+$, $WT31^+$, and $NKH1A^+$ clones were found to kill immunizing LAZ 388 cells, as well as K562, while $T3^+$, $WT31^-$, and $NKH1A^+$ clones display NK-like function exclusively. Moreover, only $WT31^+$ lymphocytes present in the cell line used for cloning experiments have the capacity to recognize alloantigen-bearing cells. Together, these data suggest that expression of WT31 may be necessary for recognition of alloantigens, while NK reactions mediated by $T3^+$ lymphocytes are WT31-independent.

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