Brief Definitive Report

CHARACTERIZATION OF CD3$^+$, CD4$^-$, CD8$^-$ CLONES EXPRESSING THE PUTATIVE T CELL RECEPTOR γ GENE PRODUCT

Analysis of the Activation Pathways Leading to Interleukin 2 Production and Triggering of the Lytic Machinery

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The surface molecules that serve as receptor for antigen/MHC recognition are composed of a disulfide α/β heterodimer noncovalently associated with CD3, a multipolypeptide cell membrane complex (1, 2). However, a minor cell subset has recently been identified that does not express α/β molecules in association with CD3 (3). In addition to the lack of reactivity with the WT31 mAb (specific for a framework determinant of the α/β heterodimer), these cells do not express CD4 or CD8 differentiation antigens (3), which are expressed by distinct subsets of conventional CD3$^+$ cells. A number of CD3$^+$, WT31$^-$ cells, including cell lines, from immunodeficiency patients (4), clones derived from fetal blood (5), or thymus (6), and a leukemia (7) were recently found to express CD3-associated molecules, different from α/β molecules, which may represent the putative T cell receptor (TCR)-γ gene product. Thus far, little is known on the functional activities of CD3$^+$, WT31$^-$ cells and on the surface molecules and mechanisms for activating these lymphocytes to express their functional program(s).

In this study, we analyzed four CD3$^+$, WT31$^-$ clones derived from CD3$^+$, 2$^+$, 4$^-$, 8$^-$ normal peripheral blood populations. The expression of a major CD3-associated 45 kD molecule, together with the detection of γ mRNA and the lack of mature α and β mRNA suggested that these clones may express the TCR-γ gene product. In addition, we show that, like CD3$^+$, WT31$^+$ cells, signalling via either CD3 or CD2 surface molecules activated CD3$^+$, WT31$^-$ clones to produce lymphokines or to mediate cytolytic activity. Therefore, molecules that function as surface receptors for activation in typical CD3$^+$ cells (equipped with an α/β TCR) can also activate in CD3$^+$, 4$^-$, 8$^-$ cells expressing the putative TCR-γ gene product.

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Materials and Methods

Isolation and Cloning of $CD3^+$, $CD4^-$, $CD8^-$ Lymphocytes. Peripheral blood lymphocytes from normal volunteers were isolated by Ficoll-Hypaque gradients and then separated into E rosetting–positive and –negative populations. E-rosetting cells were then stained with a mixture of anti-CD4 and anti-CD8 mAbs followed by treatment with rabbit complement for 1 h at 37°C. Viable cells were cultured in limiting-dilution conditions in the presence of autologous irradiated feeder cells and a source of exogenous IL-2 derived from spleen cells stimulated with PHA as described (8). Cell clones were screened directly by FACS analysis for the presence of surface CD3 and the simultaneous lack of CD4 or CD8 antigens. The mAbs used in these experiments were Leu-4 (anti-CD3) (Becton Dickinson and Co., Basel, Switzerland), CD2-1, CD2-9, and MAR 206 (all directed to CD2), B9.4 (anti-CD8), CK.79 (anti-CD4), and CK.248 (anti-T44) were isolated in our laboratory as previously described (9). FACS analysis was also performed after staining with the WT31 mAb directed to a framework of the α/β T cell receptor. An additional anti-T44 mAb (9.3) was provided by Dr. J. Hansen (Fred Hutchinson Cancer Research Center, Seattle, WA).

Functional Analysis of T Cell Clones. Production of IL-2 from clones was assessed by culturing 10^5 cloned cells/well in the presence of autologous irradiated feeder cells and either one of the following stimuli: anti-CD3 mAb (10 μl of culture supernatant), anti-T44 mAb (10 μl of culture supernatant), PHA (1% vol/vol) or a mixture of two anti-CD2 mAbs (CD2-9 + CD2-1) that were previously shown to be stimulatory on resting as well as on activated T cells (9). After 24 h, culture supernatants were removed and each tested in an indicator cell system consisting of the IL-2-responsive murine CTLL cell line as described previously (10). The cytolytic activity of the clones was tested in a 4-h ^{51}Cr-release assay in which 1.5×10^4 cloned cells were used as effector cells against different types of target cells: the NK-sensitive K562 cell line, the hybridoma producing anti-CD3 mAb and the two hybridoma producing two distinct stimulatory anti-CD2 mAb. All these target cells were used at 5×10^3 cells/well for a final effector/target cell ratio of 3:1. Percent specific lysis was determined as previously described (8).

Immunoprecipitation. Cloned cells (5×10^6) were radioiodinated by the lactoperoxidase technique and then intact cells were crosslinked by incubation in 600 μl PBS (pH 8) containing 25 μg dithiobis-succinimidyl propionate (DSP) for 45 min at room temperature as previously described (2). Cells were then solubilized in buffer containing 1% NP-40 and immunoprecipitated as described (11). After immunoprecipitation samples were examined by SDS-PAGE under reducing conditions using 5% 2-ME, which cleaves both disulfide bond between protein subunits and the chemical crosslinker.

Northern Analysis. RNA was extracted from frozen cell pellets by the guanidium thiocyanate method and purified by centrifugation through a CsCl gradient. RNA was size-fractionated by electrophoresis in a 15% agarose gel containing formaldehyde and was transferred by capillary suction to a charged Nylon membrane (Gene-Screen Plus, New England Nuclear, Boston, MA) in 1.5 M NaCl, 0.15 M sodium citrate. Hybridization of the filters with ^{32}P-labeled DNA probes and the washing of the filters were performed as described (12). Human Tα, Tβ, and Tγ cDNA probes (kindly provided by Dr. Tak Mak, Ontario Cancer Institute, Toronto, Canada) have been previously described (13, 14).

Results and Discussion

Clones were derived by limiting dilution from E-rosetting cells from which $CD4^+$ and $CD8^+$ cells were removed by treatment with mAb + C depletion. Four clones that expressed the $CD2^+$, 3^+, 4^-, 8^-, $WT31^-$ surface phenotype were further expanded in IL-2-containing media.

Surface labelling followed by crosslinking and immunoprecipitation with anti-CD3 mAbs showed (Fig. 1) that, in all four clones, CD3-associated molecules consisted of a major 45 kD band and a slightly lower band of ~43 kD (under

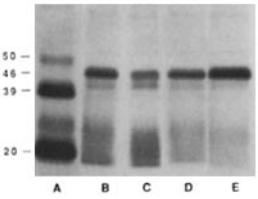

FIGURE 1. Immunoprecipitation and SDS-PAGE analysis of the CD3-associated molecules expressed on $CD3^+$, $WT31^-$ clones. The four $CD3^+$, $WT31^-$ clones (lanes *B–E*) and one $CD3^+$, $WT31^+$ (lane *A*) were labeled with ^{125}I, intact cells were crosslinked with DSP, and immunoprecipitated with anti-CD3 mAb as described in Materials and Methods. Immunoprecipitates were analyzed using 11% acrylamide gels under reducing conditions.

TABLE I
Cytolytic Activity of $CD3^+$, $WT31^-$ Clones

Clone	Percent ^{51}Cr release from labeled target cells:					
	K562	CD3*	CD2-1	CD2-9	CD2-1 + CD2-9	T44
A17	43	62	3	1	48	3
S5	57	43	5	6	67	7
S11	38	67	2	2	46	2
S12	20	61	2	1	39	4

* CD3, CD2-1, CD2-9, and T44 refer to hybridomas producing mAb to these antigens. Anti-CD2 hybridoma were used either alone or in combination. In the latter case only one of the two hybridomas was ^{51}Cr-labeled. In the data reported for the combined use of CD2-1 + CD2-9, only the CD2-9 hybridoma was labeled; no significant changes were observed when the ^{51}Cr-labeled hybridoma was CD2-1.

reducing conditions). Similar CD3-associated molecules were also observed by Moingeon et al. (5) in immunoprecipitates from a $CD3^+$, 8^+, $WT31^-$ clone derived from fetal lymphocytes. On the other hand, a $CD3^+$, 4^-, 8^-, $WT31^-$ clone derived from human thymus, expressed, together with a 44 kD molecule, a heavier 62 kD molecule associated with CD3 (6). Different molecular masses (55 and 40 kD) for CD3-associated molecules in $CD3^+$, $WT31^-$ cell lines, derived from immunodeficiency patients, have been reported by Brenner et al. (4). These authors first proposed that the 55 kD molecule may represent the putative surface product of the TCR-γ genes. This possibility was also supported by the observation that these cells expressed mRNA for the γ chain, while mRNA for α and β chains were missing. We further investigated the presence of mRNA for different TCR chains in three clones. Northern blot analysis demonstrated that mRNA for the γ chain was expressed at high levels. In contrast, no mRNA could be detected for the α chain, whereas a defective mRNA for the β chain (1 kb instead of 1.3 kb) was present (data not shown). This finding, together with the results of immunoprecipitation experiments, supports the notion that the γ gene molecular product, rather than the α or β chain were associated to CD3 molecules in our clones.

Next, clones were characterized for their functional capabilities. As shown in Table I, all four clones lysed the NK-sensitive K562 target cells, although with different efficiencies. Because conventional $CD3^+$, $WT31^+$ CTLs and $CD2^+$, $CD3^-$ NK cells can be induced to lyse target cells after stimulation with mAbs directed to the CD3/TCR molecular complex (15) or to CD2 molecules (16), respectively, we investigated whether similar mechanisms of activation of the lytic machinery were also functioning in $CD3^+$, 2^+, $WT31^-$ cells. To this end, we used as triggering targets hybridomas producing mAbs directed to CD3 or CD2 molecules (15). As shown in Table I, all the clones efficiently lysed the anti-CD3 mAb–producing hybridoma. On the other hand, control hybridomas pro-

TABLE II
IL-2 Production by CD3$^+$, WT31$^-$ Clones

Clone*	Surface phenotype	[^{3}H]TdR uptake (cpm × 10^{-3}) by cells cultured in presence of:‡				
		Medium	PHA	Anti-CD3	Anti-CD2	Anti-T44
A17	CD3$^+$, 4$^-$, 8$^-$, WT31$^-$	0.3	24.8	28.3	26.1	0.4
S5	CD3$^+$, 4$^-$, 8$^-$, WT31$^-$	0.7	19.0	28.9	24.2	1.4
S11	CD3$^+$, 4$^-$, 8$^-$, WT31$^-$	1.2	41.7	66.2	72.3	1.3
S12	CD3$^+$, 4$^-$, 8$^-$, WT31$^-$	1.4	22.9	17.4	18.3	ND
R7	CD3$^+$, 4$^-$, 8$^+$, WT31$^+$	0.7	0.8	0.7	ND	0.9
R14	CD3$^+$, 4$^-$, 8$^+$, WT31$^+$	0.8	28.4	14.2	11.4	15.1

* Clones A17, S5, S11, and S12 are CD3$^+$, WT31$^-$ whereas R7 and R14 are two CD3$^+$, CD8$^+$, WT31$^+$ clones showed for comparison. One of the CD8$^+$ clones produced IL-2, whereas the second did not.

‡ All the various stimuli were given in the presence of irradiated autologous feeder cells. The stimulatory combination of anti-CD2 mAb used was represented by CD2-1 + CD2-9 mAb, as previously described (12). IL-2 production by the different clones was assessed as indicated in Materials and Methods.

ducing mAbs directed against a number of surface molecules, were not lysed (not shown). In Table I, the hybridoma producing anti-T44 mAb is shown, mAbs to T44 molecules are known to mediate T cell activation (11, 17). Interestingly enough, all the clones studied (as well as the starting CD3$^+$, 4$^-$, 8$^-$, WT31$^-$ cell population) neither expressed the T44 surface molecule nor lysed the anti-T44 producing hybridoma. Therefore, a putative alternative pathway of T cell activation is missing in this T cell subset. Anti-CD2-secreting hybridomas as triggering targets have been used to study whether induction of cytolytic activity could occur also via CD2 surface molecules. It is now well established that appropriate combinations of mAbs directed to distinct epitopes of CD2 molecule can trigger conventional T cells to express their functional program (9). Therefore, we used a combination of hybridomas producing anti-CD2 mAbs which, when used together, were shown to activate CD3$^+$, WT31$^+$ cells (9). Neither one of the two anti-CD2 hybridomas was lysed when used alone, whereas efficient killing occurred when they were used in combination (Table I).

We have shown that most CD3$^+$, 4$^-$, 8$^-$ clones produced IL-2 and IFN-γ following stimulation with PHA (18). As shown in Table II, all of the clones analyzed released IL-2 after PHA stimulation, moreover, efficient stimulation could be achieved by using anti-CD3 mAbs or a suitable combination of anti-CD2 mAbs. The release of IL-2 was comparable in magnitude to that of a representative CD3$^+$, WT31$^+$ clone. Note also that only the latter clone was responsive to stimulation with an anti-T44 mAb. Although not shown, all the clones produced IFN-γ as well. Our present data indicate that CD3$^+$, 4$^-$, 8$^-$, WT31$^-$ cells display multiple functional capabilities that can be triggered via the CD3/TCR complex, as well as via CD2 molecules. Thus, these cells appear to use for cell activation surface structures similar to those used by CD3$^+$, WT31$^+$ T lymphocytes. The question remains as to how antigen recognition via γ chain molecules occurs. The lack of CD4 and CD8 surface antigen may suggest that TCR γ–positive cells bind target cells without the need for recognition of MHC molecules.

The differences in the molecular mass reported by different groups for the CD3-associated molecules in WT31$^-$ cells may reflect (*a*) different techniques used for immunoprecipitation and/or crosslinking, (*b*) a different degree of

glycosylation in cells derived from various $CD3^+$, $WT31^-$ cell populations (including thymus, fetal tissue, cells from immunodeficiency patients, and leukemic cells). Note however that no major differences in molecular mass could be detected in different clones all derived from peripheral blood of healthy donors. It is also of note that CD3-associated molecules precipitated from the same clones under conditions that preserve the TCR-CD3 association (7) showed a molecular mass of 80 kD under nonreducing conditions, thus indicating they are part of a disulfide-linked dimer (not shown). The finding of a minor 43 kD band in addition to the major 45 kD molecule raises the question of whether this molecule may represent a differently glycosylated form of the 45 kD molecule, or rather the molecular product of different gene(s).

Summary

Four clones were derived from human peripheral blood T lymphocytes from which $CD4^+$ and $CD8^+$ cells had been removed by treatment with specific mAbs and complement. All expressed the $CD2^+$, 3^+, 4^-, 8^-, $T44^-$ phenotype, and did not react with the WT31 mAb, which is specific for a framework determinant of the CD3-associated α/β heterodimer which serves as receptor for antigen on most human T lymphocytes. Surface iodination followed by crosslinking with dithiobis-succinimidyl propionate (DSP) and immunoprecipitation with anti-CD3 mAbs indicated that, in all four clones, the CD3-associated molecules consisted of a major 45 kD band and a minor band of 43 kD. Northern blot analysis showed that mRNA for the γ chain was expressed at high levels, whereas mRNA for the α chain was missing; β chain mRNA was present in a defective form (1 kb instead of 1.3 kb). These data support the concept that these clones may express, in association with CD3, the molecular product of the T cell receptor γ genes instead of the typical α/β heterodimer. $CD3^+$, $WT31^-$ clones lysed the NK-sensitive K562 target cells and produced IL-2 upon stimulation with PHA. In addition, they released IL-2 after triggering with soluble anti-CD3 mAbs or with an appropriate combination of anti-CD2 mAbs (in the presence of adherent cells). When $CD3^+$, $WT31^-$ clones were incubated with an anti-CD3 producing hybridoma as triggering target, the latter was efficiently lysed. Target cell lysis also occurred when a suitable combination of anti-CD2 mAbs-producing hybridomas was used. Therefore, $CD3^+$, $WT31^-$ cells appear to use two pathways of cell activation that function also in conventional $CD3^+$, $WT31^+$ T cells, but they lack a third putative pathway initiated by T44 surface molecules.

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