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Structural and Functional Aspects of the T-Cell Differentiation Antigens T3, T6, and T8

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A. Introduction

The progressive diversification of T-lymphocytes begins within the thymus gland. Since cell-cell interactions may play a major role in this process, the study of the expression of thymic surface markers would aid our understanding of thymic differentiation. On murine thymocytes, selective expression of genes coding for cell surface markers has been studied with alloantisera [1]. More recently, monoclonal antibodies have made possible the study of selective expression of cell surface glycoproteins on human thymocytes. Thus, as in the mouse, early and late events in the thymic differentiation have been recognized [2]. Although the precise function of these thymic differentiation antigens remains to be determined, it seems plausible that they may govern associative recognition among cooperative cells sets. One could therefore surmise that inappropriate expression of some of the thymic differentiation antigens may play a role in leukemogensis.

The majority of human thymocytes (75%–80%) express the markers T4, T6, and T8, whereas only the most mature thymocytes (15%) can be decorated with the monoclonal reagent anti-T3 tagged with fluorescein-isothiocyanate [2]. T6 has never been found on peripheral blood T cells, whereas T3 is present on all T-lymphocytes. T4 and T8 are present on different subsets of T cells and on $T3^+$ $T6^-$ thymocytes [2]. As the thymocyte is exported into the peripheral T-cell compartment, it is again involved in a variety of cell-cell interactions. Interestingly, the T-cell markers T3 and T8 appear to serve functions which are specific for interactions of the cell type which expresses them.

Here we discuss the preliminary structural analysis of T3, T6, and T8 and the possible role of T3 and T8 in the recognition of target cells by cytotoxic T-lymphocytes. Studies involving the glycoprotein structure, biosynthesis, and membrane insertion of T3, T6, and T8 will, in our opinion, facilitate investigations that develop our understanding of the differentiative pathways of human thymus-derived lymphocytes; aid in the molecular description of T-cell functions; and make further classification of T-cell leukemias possible.

B. The Thymic Differentiation Antigen T6

The T6 antigen is a marker which is found primarily on human thymocytes and thymus-derived leukemias [2, 3]. The only nonthymic cells which have shown positive reactivity with the anti-T6 monoclonals [OKT6(2), NA1/34(3), and IIC7(7)] are Langerhans cells in the skin and in lymph nodes [4].

SDS-polyacrylamide gel electrophoresis revealed that the T6 antigen is a protein with an apparent molecular weight of 49K under both reducing and nonreducing conditions [5]. If the thymocytes were radioactively labeled with tritiated sodium borohydride after mild treatment with 1 m*M* sodium periodate, a similar band of 49K daltons was observed after SDS-polyacrylamide gel electrophoresis of the immunoprecipitate, indicating that the target antigen for anti-T6 is a glycoprotein [5]. A

smaller protein of 12K daltons was detected in experiments starting with ^{125}I-labeled lysates. This protein was not seen after tritium labeling and was subsequently identified as β2 microglobulin [5–7]. Both in mice and humans, β2 microglobulin is a protein found to be associated with class I products of the major histocompatibility complex. Based on tissue distribution and molecular weights, T6 may be considered the human homologue of the mouse TL (thymus leukemia antigens). Using isoelectric focusing as an analysis of ^{125}I-labeled T6 antigens from over 20 thymus preparations and four T leukemic cell lines, we detected no charge heterogeneity between T6 antigens from different individuals. With the exception of T6 from the leukemic cell line, MOLT-4 (15 bands) T6 from all individuals was found in twelve bands [6]. However, upon treatment with neuraminidase, this pattern was reduced to four bands in all cases. As the MOLT-4 T6 contained more sialic acids, and since the MOLT-4 T6 contained forms with a higher molecular weight, we suspected that MOLT-4 T6 could have an extra oligosaccharide sidechain [6].

Further studies using the enzyme endoglycosidase-F, which was found to remove most complex N-linked oligosaccharide chains [7], showed quite surprinsingly that the protein backbone of T6 was 34K. This was supported by deglycosilation experiments using acid hydrolysis with trifluoromethane sulfonic acid, which also resulted in a 34K product [7]. The unglycosilated form of T6 MOLT-4 was also found to be 34K, which suggestes strongly that the MOLT-4 T6 may contain an extra oligosaccharide sidechain. Whether this means that MOLT-4 T6 is an "alien histocompatibility antigen" will need protein sequence analysis.

The difference in glycosylation between HLA-A/B (10%) and T6 (30%–35%) could explain why biosynthetic labeling of T6 was found to be far more difficult than in the case of HLA-A/B antigens [7]. Perhaps, the posttranslational modifications are a rate-limiting step in this process. Quite surprisingly, the expression of β2m-associated proteins, but not T6, can be enhanced by interferon-containing lymphocyte-conditioned medium [7]. Whether this is a reflection of the role of T6 in the thymocyte differentiation process remains to be determined. More importantly, as in the case of TL antigens [8], only a restricted group of thymocytes in the appropriate differentiation stage may be able to express the T6 gene.

The function of the T6 antigen is unknown. It may be a receptor for a thymic hormone or this structure may govern associative interactions between thymocytes. It is of interest to note that T6$^-$ thymocytes strongly express HLA-A/B antigens, whereas T6$^+$ thymocytes stain only weakly with fluoresceinated anti-HLA-A/B reagents [2]. Perhaps these two class I M.H.C. antigens may fulfill a similar function in distinct stages of differentiation of the thymus. Knowledge of the structure and function of T6 will therefore undoubtedly aid in our understanding of thymic differentiation and of leukemogenesis.

C. Complexity of the T-Lymphocyte Cell Surface Antigen T3

The human cell surface marker, recognized by the monoclonal reagent anti-T3 (OKT3 or anti-Leu-4), was found most strongly on mature thymocytes. This is exemplified by the finding that only T3-positive thymocytes were responders in a mixed lymphocyte reaction [2, 9]. All human T-lymphocytes were reactive with the monoclonal antibody anti-T3. Thus far, only cells from the human thymus lineage have been found to be reactive with this monoclonal reagent [2].

The target antigen for anti-T3 appears to be involved in most of the proliferative functions of human T-lymphocytes. Interestingly, anti-T3, and to a much lesser extent the monovalent *Fab* fragment, induced DNA synthesis in human peripheral blood T-lymphocytes. Maximal mitogenesis occurred at concentrations of about 10^{-12} *M* (10). When added in higher concentrations of about 10^{-8} *M*, the antibody (and its *Fab* fragment) blocked proliferative responses to soluble and cell surface antigens, inhibited the generation of cytotoxic T-lymphocytes in a mixed lymphocyte culture, and abrogated the ability of T cells to help B cells in antibody production

[11]. These studies suggest that the T3 antigen is involved in cell-cell interaction and/or receptor functions.

Several glycoproteins were found in the immunoprecipitates prepared with a monoclonal reagent anti-T3 [12]. In addition to the major glycoprotein complex of 20K, glycoproteins of 25–28K, 37K, and 44K were detected in immunoprecipitates. The charge heterogeneity of the 20K and 25–28K proteins was caused by variable sialic acid content. The 20K T3 glycoprotein carried several "complex-type" and endoglycosidase-H sensitive sugar moieties. We found that the 20K protein could be labeled with ^{125}I-iodonapthylazide, a reagent that reacts with hydrophobic areas of proteins [12]. Charge shift electrophoresis experiments showed that the 20K, 25–28K, 37K, and 44K glycoproteins are associated in a detergent-soluble complex [14].

Biosynthetic studies and experiments using endoglycosidases demonstrated that:

1. The nonglycosylated precursor of 20K T3 is 14K [13].
2. The nonglycosylated precursor of the 25–28K protein is 16K [13].
3. A nonglycosylated 20K T3 exists which is labeled strongly with ^{125}I-iodonaphthylazide.

Analysis by two-dimensional gel electrophoresis provided evidence that the 20K and 25–28K T3 from resting T-lymphocytes, PHA-activated T-lymphocytes, CTL clones, and a T leukemic cell line HPB-ALL are virtually identical [11, 20].

D. Structure of the T-cell Subset Antigen T8

The target structures recognized by the monoclonal antibodies anti-T5 (OKT5), anti-T8 (OKT-8), or anti-Leu-2A on human T-lymphocytes are borne by a dimer of two sulfhydryl-bridged 33K glycoproteins. Upon reduction and alkylation of NP-40 solubilized membrane proteins, only OKT-8 precipitates the 33K monomer, whereas the target structures of OKT5 and anti-Leu-2A are probably borne by the dimeric structure only. In SDS gels, the dimer migrates either as a 67K protein or a 76K protein. As no other component was found upon reduction than the 33K glycoprotein, and since peptide maps of the 76K form were identical to those of the 67K form, we assume that this phenomenon is caused by differences in protein folding [15, 16].

Peptide mapping and two dimensional gel electrophoresis detected no structural differences between the target antigens for OKT-5, OKT-8, and anti-Leu-2A on T cells or thymocytes. For reasons of simplicity this glycoprotein will be termed T8, throughout this paper.

On thymocytes, the 33K is associated with a 45K glycoprotein via sulfhydryl bridges. Upon SDS-gel electrophoresis under nonreducing conditions, multimers of 140K, 150K, 200K, 220K, and 260/280K were found in addition to the 67K and 76K dimers [17]. When T8 was precipitated from detergent-solubilized thymocyte membrane protein preparations after reduction and alkylation, only the 33K band was found. This indicated that the T8 determinant was expressed on the 33K glycoprotein only. Whether T8 is associated with the 45K protein on all thymocytes or on $T6^+$ thymocytes is currently being investigated.

Attempts to remove glycan sidechains of T8 with endoglycosidase-H and endoglycosidase-F were unsuccessful [16]. After treatment with trifluoromethane sulfonic acid, the molecular weight of the 33K glycoprotein was reduced to 30K. This would allow for one *N*-linked oligosaccharide or several *O*-linked glycan sidechains. However, this 30K form may still contain carbohydrate [16], and the molecular weight of the T8 polypeptide chain is therefore unknown.

Studies using the hydrophobic-labeling reagent ^{125}I-iodonaphthylazide demonstrated that T8 contains a hydrophobic pocket. This suggested that T8 is an integral membrane glycoprotein.

Taken together, the tissue distribution of T8 [2] and the preliminary biochemical data suggest thatT8 is a human homologue of the murine Lyt 2, 3 antigens. The finding that T8 (see below) and Lyt 2, 3 antigens [18] are involved in the target recognition by cytotoxic T-lymphocytes supports that idea.

E. The Role of T3 and T8 in Antigen-Specific Cytotoxicity Mediated By $T8^+$ and $T4^+$ Human Cytotoxic T-Cell Clones

The nature of the antigen recognition structure(s) on human cytotoxic thymus-derived T-lymphocytes (CTL) is still an unresolved issue. The availability of clones of human CTL will facilitate the analysis of individual CTL target cell interactions on a functional and molecular level. We previously demonstrated that stable CTL clones could be derived from tertiary MLC in which cells of an Epstein Barr virus transformed B-cell line (JY) were used as stimulator cells [19, 20]. More recently, four such clones, which have different antigenic specificities and phenotypes, were used to investigate the role of T-cell antigens T3 and T8 in effector/target cell interaction [20, 21]. Four clones are described in Table 1 which differ in phenotype and specificity. The T8-positive clones HG-31 and JR-2-16 were shown to react only with target cells carrying the serologically defined HLA B7 and HLA A2 determinants respectively, confirming the results of a recent study in which these clones were tested on a large panel of HLA-typed lymphocytes [22]. The T4-positive CTL clone HG-38 was strongly cytotoxic for JY cells only. The T4-positive CTL clone JR-2-19 was cytotoxic for cells expressing HLA DR-2 and HLA DR-6. This reactivity pattern cannot be attributed to a mixture of a HLA DR-2 specific and a HLA DR-6 specific clone since subclones of JR-2-19 reacted in a similar way. It may be possible that clone JR-2-19 recognizes a determinant shared by the DR-2 and DR-6 antigens. Alternatively, this clone may recognize an antigenic determinant on a molecule which is distinct from DR-2 and DR-6, like the recently described DC1 specificity [23] that has been shown to be a molecular complex dinstinct from DR-1, -2, and -6, but which is in linkage disequilibrium with these specificities.

Two monoclonal reagents, OKT-8 and anti-Leu-2A, inhibited the cytotoxic reactions of the $T8^+$ clones HG-31 and JR-2-16 [20, 21], whereas these antibodies did not affect the cytotoxic reactions mediated by the $T4^+$ clones HG-38 and JR-2-19. Anti-T3 antibodies were found to inhibit the cytotoxic reactivity of all four CTL clones. In contrast, OKT-1 and OKT-4 had no effect on the lytic activity of the CTL clones. The inhibitory effect of the monoclonal antibodies anti-T3 and anti-T8 could not be attributed to a functional inactivation of the CTL clones, since ConA overcame the inhibition of cytotoxicity without affecting the binding of the monoclonal antibodies to the clones. These findings indicate that the monoclonal antibodies anti-T3 and an-

Table 1. The cytotoxic activity of the CTL clones JR-2-16, JR-2-19, HG-31, and HG-38 against 12 EBV transformed lymphoblastoid B-cell line cells and one Burkitt lymphoma cell line (Daudi)

Target cell	HLA phenotype				% specific lysis CTL clone			
	A	B	C	DR	JR-2-16	JR-2-19	HG-31	HG-38
JY	2	7		4, 6	80.0	49.8	47.4	46.2
Daudi	–	–		6	2.2	42.8	4.4	10.1
HHK	3	7		6	10.6	46.4	48.3	6.4
EKR	3	7		7	15.9	−1.5	42.2	−0.4
NOB	3	7		2	2.3	69.9	62.6	0.1
PHS	3	7		2	11.3	68.2	52.2	−2.8
SB	1, 2	17, w44		2, 3	79.3	46.7	14.3	10.1
HAR	1	8		7	−0.2	2.2	3.7	2.1
MvL	w32	27	w2	1	−1.7	3.9	2.0	3.2
APD	1	40		6	NT	54.8	7.0	16.1
CJO	11	w35	w4	1	1.7	−0.8	−1.8	−2.6
OOS	26	w22	w2	1	NT	3.8	8.5	5.7
WT	2, 11	27, 55		1	82.5	2.8	0	−0.1

CTL clone	Trypsin treatment	% specific lysis			
		Directly after trypsin treatment		After 24 h incubation at 37° C	
		2.5[a]	5	2.5	5
JR-2-16	Yes	3.9	15.2	72.0	100.1
	No	39.3	81.8	56.0	88.0
JR-2-19	Yes	42.3	50.9	41.5	60.8
	No	54.8	59.8	57.5	69.7
HG-31	Yes	2.9	6.1	59.7	85.9
	No	42.0	56.0	55.7	89.4
HG-38	Yes	13.3	32.7	13.4	41.8
	No	18.4	45.3	15.4	50.1

Table 2. The cytotoxic activity of the CTL clones on JY cells after trypsin treatment

[a] Effector/target cell ratio

ti-T8 blocked the recognition process, but not the cytotolytic effector mechanism itself.

In an attempt to study the role of T3 and T8 in the CTL effector function in more detail, a series of experiments was conducted using proteolytic enzymes. An example is shown in Table 2. Trypsinization of CTL clones JR-2-16 and HG-31 during 10 min at 37 °C resulted in a complete abrogation of the cytotoxic activity of these clones, whereas the cytotoxic activity was fully restored after incubation in IL-2 containing medium at 37 °C and 5% CO_2 overnight. In contrast, the cytotoxic activity of clones HG-38 and JR-2-19 was minimally affected by trypsinization. The disappearance of cytotoxicity after trypsin treatment correlated with the removal of the T1 and T8 determinants. However, the expression of T3, T4, HLA/A/B/C heavy chain and HLA DR remained unaltered. Both the expression of T8 and T1 determinants was restored after overnight incubation at 37 °C and 5% CO_2 (Table 2).

More detailed analysis of the effects of trypsin treatment on the cytotoxic activity of the CTL clones revealed that the cytotoxic activity of the clones HG-31 and JR-2-16 was already abolished after a trypsin treatment of 1 min (Table 3). At that time, the OKT-8 and Leu-2A determinants were still present, although the fluorescence intensity as measured with the FACS IV was decreased [21].

Trypsin treatment of the CTL clones during 2.5 min completely removed the Leu-2A and OKT-8 determinants. In contrast, the lectin-dependent cytotoxicity mediated by the clones HG-31 and JR-2-16 was found to be still intact after a trypsin treatment of 30 min (Table 4). This latter observation indicates that the general ability to lyse target cells is not lost by the trypsin treatment, but suggests that trypsin treatment affects the recognition phase of CTL clone target cell interaction in which T8 is involved.

These results show that the recognition unit for class I antigens on the $T8^+$ CTL is more sensitive to trypsin than the receptor(s) for class II antigen on the $T4^+$ clones. Hydrolysis of the T8 glycoprotein is synchronous with the disappearance of the target recognition by the $T8^+$ clones. Apparently trypsin cleaves off the determinant involved in cytotoxicity before it removes the T8 epitope. Whether the T8 antigen interacts directly with the class I antigen on the target cell or via an associated protein remains to be determined. These studies also do not exclude the possibility that the trypsin sensitivity of T8 expression and cytotoxicity are purely coincidental.

The role of the T3 glycoproteins in the cytotoxic function remains unclear. The inhibition of cytotoxicity of CTL clones by OKT3 described here confirmed previous reports in which "bulk" CTL cultures were used, but thus far it has not been reported

Table 3. The expression of cell surface antigens after trypsin treatment of the CTL clones HG-31 and HG-38

Monoclonal antibody	% of the cells reacting with monoclonal antibody					
	HG-31			HG-38		
	Control	Trypsin treated	Trypsin treated and incubated for 24 h	Control	Trypsin treated	Trypsin treated and incubated for 24 h
a HLA A, B, and C (w6/32)	100 (+ +)	100 (+ +)		100 (+ +)	100 (+ +)	
a HLA DR (OKIa 1)	100 (+ +)	100 (+ +)		100 (+ +)	100 (+ +)	
OKT-1	80 (+)	<1	100 (+ +)	95 (+ +)	<1	100 (+ +)
OKT-3	100 (+ +)	100 (+ + +)		100 (+ +)	100 (+ + +)	
OKT-4	<1	<1		95 (+ +)	95 (+ +)	
OKT-8	100 (+ + +)	<1	100 (+ +)	<1	<1	
Leu 2a	100 (+ + +)	<1	100 (+ +)	<1	<1	
Leu 4	95 (+ +)	100 (+ +)		95 (+ +)	95 (+ +)	

(+), (+ +), or (+ + +) indicate the relative fluorescence intensity

Table 4. The effect of trypsin treatment on the expression of the monoclonal antibodies OKT-8 and Leu 2a and the cytotoxic activity of the CTL clones HG-31 and JR-2-16 against JY in the presence and absence of ConA

Trypsin treatment during	% of cells reacting with		% specific lysis			
	OKT-8	Leu 2a	HG-31		JR-2-16	
			JY	JY + ConA	JY	JY + ConA
0 min	97% (+ + +)	96% (+ + +)	36.8	37.0	54.1	62.3
1 min	81% (+)	82% (+)	1.0	28.7	10.2	59.1
2.5 min	<1% (−)	<1% (−)	0.4	40.1	1.8	58.6
5 min	<1%	<1%	0.6	41.9	2.0	55.1
10 min	<1%	<1%	0.1	39.9	3.1	50.2
30 min	1%	1%	−0.2	36.4	2.0	53.4

(−), (+), or (+ + +) indicate the relative fluorescence intensity

that Leu-4 can also inhibit cytotoxicity. The neutralizing effect of ConA on the inhibition by Leu-4 and OKT3 suggests that this antigen is involved in some stage of the recognition process. But, the trypsin experiments showed that T3 must be involved in a different phase of the target cell recognition than T8 [21]. This has also been reported by other [24]. Chang et al. [25] raised the possibility that the T3 molecular complex is part of the T-cell antigen receptor.

However, several findings argue against this possibility. Firstly, although the cytotoxicity of the clones HG-31 and JR-2-16 was highly susceptible to trypsin treatment, the expression of OKT-3 remained unaltered. Moreover, trypsin treatment did not influence the cytotoxicity of the clones HG-38 and JR-2-19, which indicates that

the antigen recognition structure of clones HG-31 and JR-2-16 is different from that on HG-38 and JR-2-19. Secondly, if the T3 complex itself is the antigen receptor, it would be expected that there is a structural difference in the antigen specificity. However, the two-dimensional electrophoretic patterns of the OKT-3 precipitated material from both clones were identical [21]. Taken together, these findings suggest that the 20K T3 and 25–28K T3 itself may not serve as the T-cell receptor for alloantigens. However, the T-cell receptors could be associated with the T3 structure perhaps through interactions with hydrophobic nonglycosylated 20K T3. Further biochemical studies including cross-linking around T3 and lysostripping experiments with anti-T3 using several different CTL clones derived from one individual and directed at different class I and class II antigens will probably provide evidence for associations between T-cell receptors and the T3 complex.

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References

1. Owen JTT, Raff MC (1970) J Exp Med 132:1216–1232
2. Reinherz EL, Schlossman SF (1980) Cell 19:821–827
3. McMichael AJ, Pilch J, Galfre G, Mason DY, Fabre JW, Milstein C (1979) Eur J Immunol 9:205–210
4. Poppema S, Bahn AK, Reinherz EL, Mc Cluskey RT, Schlossman SF (1981) J Exp Med 153:30–41
5. Terhorst C, Van Agthoven A, LeClair K, Snow P, Reinherz EL, Schlossman SF (1981) Cell 23:771–780
6. Van Agthoven A, Terhorst C (1982) J Immunol 128:426–432
7. Lerch P, Van de Rijn M, Schrier P, Terhorst C (to be published) Human Immunol
8. Rothenberg E (1982) J Exp Med 155: 140–154
9. Kung PC, Goldstein G, Reinherz EL, Schlossman SF (1979) Science 206:347–349
10. Van Wauwe JP, DeMey JR, Goossens JG (1980) J Immunol 124:2708–2713
11. Reinherz EL, Hussey RE, Schlossman SF (1980) Eur J Immunol 10:758–761
12. Borst J, Prendiville MA, Terhorst C (1982) J Immunol 128:1560–1565
13. Borst J, Alexander S, Elder J, Terhorst C (to be published) J Immunol
14. Borst J, Prendiville MA, Terhorst C (to be published) J Biol Chem
15. Terhorst C, Van Agthoven A, Reinherz E, Schlossman SF (1980) Science 209:520–523
16. Snow p, Spits H, De Vries J, Terhorst C (to be published) Hybridoma
17. Snow P, Terhorst C (to be published) J Immunol
18. Nakayama E, Shiku H, Stockert E, Oettgen HF, Old LJ (1978) Proc Natl Acad Sci USA 78:1977–1981
19. Spits H, De Vries J, Terhorst C (1981) Cell Immunol 59:435–447
20. Spits H, Ijssel H, Terhorst C, De Vries J (1982) J Immunol 128:95–99
21. Spits H, Borst J, Terhorst C, De Vries J (to be published) J Immunol
22. Spits H, Breuning M, Ivany P, De Vries J (to be published) Immunogenetics
23. Tosi R, Tanyaki N, Centis D, Ferrara GB, Pressman D (1978) J Exp Med 148: 1592–1587
24. Landegren U, Ramstedt U, Axberg I, Ullberg M, Jondal M, Wigzell H (1982) J Exp Med 155:1579–1584
25. Chang TW, Kung PC, Gingras SP, Goldstein G (1981) Proc Natl Acad Sci USA 78:4500–4506