T CELL ANTIGEN RECEPTOR-$\gamma\delta$ LYMPHOCYTES

STRUCTURES AND FUNCTIONS

ELS STURM

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CIP GEGEVENS KONINKLIJKE BIBLIOTHEEK, 'S-GRAVENHAGE

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aan mijn ouders

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CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

The vertebrate immune system can eliminate and neutralize virtually any foreign invader and distinguish such antigens from the body's own. There are two major classes of immune responses, the humoral and cellular response respectively. The humoral response is mediated by antibodies that are produced by B lymphocytes upon specific antigen recognition. The binding of antibody to antigen facilitates the elimination of the invader by other cells present in the immune system. The cellular response involves the production of specialized cells such as T lymphocytes and non-specific natural killer (NK) cells, monocytes, macrophages and granulocytes.

T lymphocytes can be divided into two mutually exclusive populations. The majority of T lymphocytes recognizes antigens via their T cell receptor (TCR) which is composed of an α and β protein (1-3). The antigens are specifically recognized when presented by accessory cells in the context of major histocompatibility complex (MHC) antigens (4-6). TCR$\alpha\beta$ lymphocytes can be divided in helper/ inducer and cytotoxic T lymphocytes (CTL) (7,8). After recognition of antigen, helper/inducer lymphocytes become activated and secrete a variety of growth and differentiation factors, to facilitate an antibody and/or a CTL response (9-11). CTL are involved in the elimination of host cells expressing non self antigens (5,12-14).

Only 3-10% of the total T lymphocyte population expresses a TCR, composed of a γ and δ protein (15,16). This population of T lymphocytes exerts MHC unrestricted cytolysis when activated in vitro and produces lymphokines (17-21). However, a specific biological role of the TCR$\gamma\delta$ lymphocytes remains still enigmatic.

Another important mediator of cell mediated immunity is the NK cell (22). These cells can lyse tumor target cells in vitro wihout prior activation and this cytotoxicity is not restricted by MHC antigens (23,24). A specific NK cell receptor has not as yet been identified.

In this chapter, the different types of cytotoxic lymphocytes and their activation pathways will be described. Finally, this chapter will focus on TCR$\gamma\delta$ lymphocytes, their structures and functions.

NK cell and CTL mediated cytotoxicity

NK cell and CTL mediated cytotoxicity results from membrane interactions between effector and target cells. The first step involves binding and recognition and requires the divalent cation Mg^{++} (25-27). The second step is programming for lysis and delivery of the lethal hit (28). Cytotoxic granules, composed of perforins, granzymes, and proteinglycans, are released by the CTL and NK cell. These poreforming molecules bind to the target cell and

induce its destruction (29-33). Subsequently, the effector cell will detach from the dying target cell and may bind to a new one, recycling.

The initial binding of the effector cell to the target cell is mainly mediated by antigen non-specific, non-polymorphic membrane molecules. The CD2 and CD11/18a molecules expressed by the CTL, bind to their counterpart structure on the target cell, CD58 and CD54 respectively (34-36). Then, the CTL specific conjugation with the target cells occurs via binding of its TCR to the antigen on the target cell. The MHC molecule expressed by the target cell presents the antigenic peptides via the peptide binding groove located in the extracellular domain of the molecule (37,38). The TCR binds simultaneously to the MHC molecule and the presented antigen, thereby promoting CTL- target cell adhesion (39,40). The CD4 or CD8 antigens serve as co-receptors in the antigen specific MHC restricted cytolysis (41-44). The putative receptors involved in MHC unrestricted cytolysis displayed by NK cells and TCR$\gamma\delta$ lymphocytes have not as yet been identified. It has been suggested that multiple receptors, which provide target cell selectivity, are involved in MHC unrestricted cytolysis (multi-receptor model). In this model (Fig.1) lysis of target cells by activated lymphocytes can be triggered by the interplay of signals transduced by several cell surface molecules that interact with their respective ligands on the target cell (45-47).

Multi receptor model for antigen non-specific recognition.

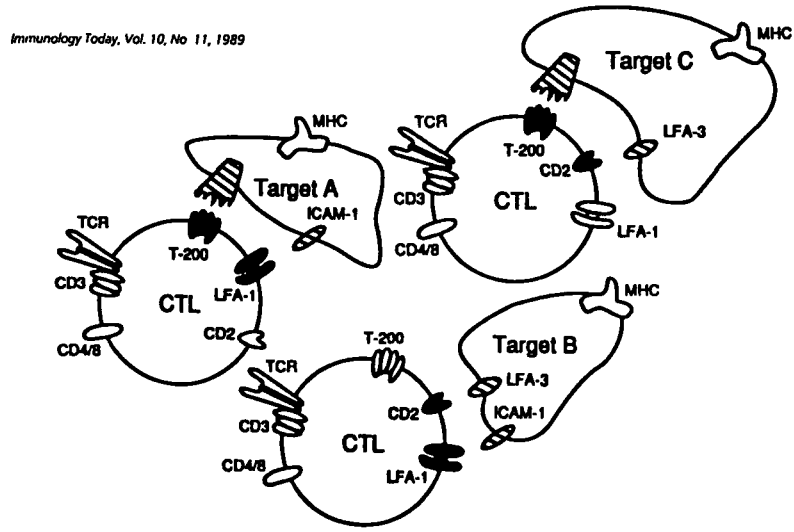

Fig. 1. Triggering of MHC-unrestricted cytotoxicity by interactions of multiple cell surface molecules on effector cells and common cell surface ligands on target cells, from Thiele, D.L. and P.E. Lipsky. 1989. Immunol.Today 10:375.

Signal transduction in T lymphocyte activation

Activation of T lymphocytes requires binding of an agonist to its receptor (48,49). Receptor activated G proteins are thought to directly interact with phospholipase C (PLC) and increase its affinity for Ca++, thereby activating PLC (50,51). Stimulation of PLC leads to the hydrolysis of phosphatidyl inositol 4,5 biphosphate (PIP_2) to yield inositol triphosphate (IP_3) and diacylglycerol (DG) (52,53). DG activates the enzyme protein kinase C (PKC) (54). PKC is a serine/threonine kinase, dependent upon calcium and phospholipid (55) (Fig.2). PKC can also be directly activated by non physiological compounds like phorbol esters e.g. phorbol myristate acetate (PMA) (56,57). Activation of PKC is associated with its translocation from the cytosol to the membrane. PKC activation leads to an increase in intracellular pH. This alkalinization is caused by an increased exchange of intracellular H+ for extracellular Na+, mediated by an unique transmembrane protein the Na+/H+ antiporter (58). PKC catalyses the phosphorylation of protein substrates, e.g. the CD3γ chain on its serine residue (58,59).

Signal transduction in T cell activation.

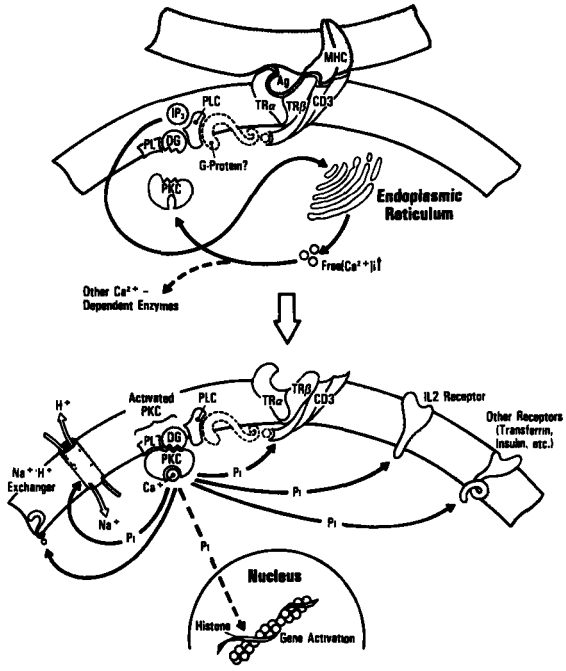

Fig.2. A model of signal transduction during antigen-mediated T cell activation, from Isakov. et. al., 1987 Immunol. Rev. 95:89, For explanations see text.

The second product of PIP_2 hydrolysis, IP_3 releases Ca^{++} from intracellular stores, probably the endoplasmic reticulum (48,60). Calcium ionophores have been shown to directly increase the intracellular Ca^{++} concentration, thereby circumventing PKC activation. The initial rise in intracellular Ca^{++} concentration may then induce a Ca influx from the environment (56).

When T lymphocytes are activated, the increase of Ca^{++} and the subsequent PKC activation induces transcription of the IL-2 and IL-2R genes about the same time (61,62). The mechanism underlying the regulation of the transcription of the IL-2 gene has recently been identified. Transcriptional enhancer regions have been found upstream from the IL-2 gene. The regulatory sites in the IL-2 enhancer are regulated by nuclear DNA binding proteins (63,64). Binding of these proteins upregulate the production of IL-2 by induction of the IL-2 promotor. In addition, a denovo induced repressor protein appears to downregulate IL-2 production by shutting off the transcription of the IL-2 gene (65). The transcriptional regulatory sequence in the IL-2 promotorregion consists of multiple binding sites. As an example of these, the lymphoid specific enhancer NF-kB is induced by a posttranslational mechanism that involves the phosphorylation of the specific cytoplasmatic inhibitor I-kB. This process results in the translocation of NF-kB to the nucleus (66,67). The inducebility of NF-kB in T lymphocytes and its negative regulation by I-kB suggests that the corresponding enhancer element plays a critical role in T lymphocyte activation.

TCR αβ lymphocytes

The availability of anti-CD3/TCR monoclonal antibodies (mAb) has facilitated the elucidation of the protein structure of this receptor complex (68-71). The TCR α and β genes encode transmembrane glycoproteins whose sequences are homologous to those of immunoglobulins. Together, the TCR α and β chains determine the specificity of the TCRαβ lymphocytes (72,73). The TCR α and β polypeptides contain a single transmembrane spanning region, and a very short (12 aminoacids) cytoplasmatic domain, suggesting that the TCR itself is not involved in signal transduction (74,75). The TCR molecules are non-covalently associated with the CD3 molecular complex, which is thought to be the signal transducing structure (76-78). The CD3 complex consists of five distinct polypeptides, γ, δ, ε, ζ and η .The γ and δ chains are glycosylated while the ε , ζ , and η are non-glycosylated. These proteins range in molecular weight between 16 and 28 kD. Approximately 90% of ζ chain is found as a homodimer and 10% forms a heterodimer with the η chain. The transmembrane domains of all subunits contain a centrally located acidic residue. Together with the basic residues found in the corresponding domains of the TCR subunits, these charged residues may form saltbridges which stabilize the non-covalent

association between the CD3 and TCR complexes (79-84). The cytoplasmatic domain of the ζ chain contains seven tyrosine residues that are potential substrates for tyrosine protein kinases (TPK). Indeed the tyrosine phosphorylation of this ζ subunit has been shown after activation via the CD3 or CD2 antigens (85,66).

The TCRαβ lymphocytes recognize antigen peptides which are presented in the peptide binding groove of the MHC molecule. (Fig.3). Anti-CD3/TCR mAb can activate T lymphocytes (87). For activation to occur, the CD3/TCR complexes need to be crosslinked, while similar mAb, presented in soluble form, block the activation of T lymphocytes (88,89). Crosslinking of the CD3/TCR complex, either by antigen specific stimulation or by anti-CD3/TCR mAb (90), initiates signal transduction and leads to full T cell activation; expression of the IL-2 receptor (IL-2R), production of lymphokines, cytolytic activity, and proliferation.

Most TCRαβ lymphocytes express either the CD4 or CD8 molecules. Helper/inducer functions are exhibited by CD4+ lymphocytes, whereas CD8+, and a minority of CD4+, lymphocytes exert cytolytic activities (8,91,92).

Dual recognition of antigen and the presenting MHC molecule

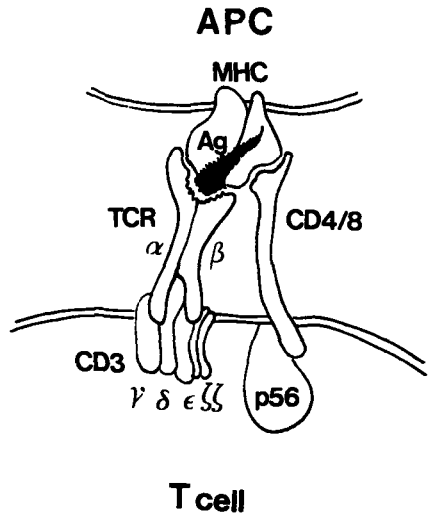

Fig.3. Presentation of an antigenic peptide (Ag, in black) in the context of MHC molecules to a T lymphocyte. Association of the CD4 or CD8 antigen with MHC molecules during Ag presentation brings the $p^{56\ lck}$ into proximity of the receptor complex, where it can phosphorylate substrates on tyrosine residues, from Altman et.al., 1990. Adv.Immunol. 48:227

Antigen specific recognition by CD4+ lymphocytes is restricted by MHC class II antigens, whereas the CD8+ lymphocytes are restricted by MHC class I molecules (39,47). The CD4/CD8 glycoproteins have distinct functions in T cell activation. They stabilize and increase the avidity of the interaction of the CD3/TCR complex and the Ag-MHC by binding to the same MHC molecule that presents the antigen. As shown in co-immunoprecipitation studies, this binding physically links the CD4/CD8 molecules to the CD3/TCR complex (41,44,93-95) (Fig.3). The CD4/CD8 molecules also play an important role as an independent signal transducing molecule. The intracellular domains of CD4 and CD8 are physically associated with lymphocyte specific tyrosine protein kinase (TPK), $p^{56\ lck}$ (96,97). This TPK seems to play a critical role in T cell development and activation, by phosphorylating the ζ chain of the CD3 molecular complex. The phosphorylation of the ζ chain is thought to be essential for signal transduction (85,97).

In addition to the CD3/TCR complex, the CD2 molecules also serve as a functionally important interaction structure on TCRαβ lymphocytes. The CD2 molecule was originally identified as a Sheep Red Blood Cell receptor (SRBC) (98). CD2 mediates the adhesion of the effector and target cell via binding to its natural ligand, leucocyte function associated antigen-3 (LFA-3)(CD58), that is expressed by the target cells, thereby contributing to the antigen non-specific T cell activation (99). Three functionally important epitopes, T11.1, T11.2 and T11.3 have been identified on CD2 (100). The T11.1 epitope is associated with the SRBC binding site, whereas the T11.2 and T11.3 epitopes are not. Combinations of mAb directed against the CD2 epitopes T11.2 and T11.3 can induce IL-2 dependent TCRαβ cell proliferation, provide help for antibody reponses, and induce antigen non-specific cytolytic activity by CTL (36,101,102). Single anti-CD2 mAb usually block TCRαβ cell activation and proliferation (103,104). Moreover, the CD2 and CD3/TCR antigens can synergize in T cell activation. Triggering via CD2 induces phosphorylation of the CD3 γ chain, and the tyrosine phosphorylation of the CD3 ζ chain respectively (86,105,106). Recently it has been shown that upon T cell activation CD2 is not only functionally but also physically associated with the CD3 molecular complex (107). Upon activation by anti-CD2 mAb, the ζ chain is phosphorylated. This phosphorylation is regulated by the CD45 tyrosine phosphatase (108), which is an important molecule in the regulation of T cell activation. CD45 is a family of surface glycoproteins, expressed by non-erythroid haematopoietic cells. The four members of this family vary in molecular weight between 180 and 220 kD. The different isoforms arise through alternate RNA splicing (109-112). The CD45RO antigen, identified by the mAb UCHL-1, is expressed by all activated lymphocytes (TCRαβ, TCRγδ and NK cells) and represents a marker for 'primed' lymphocytes (113,114, chapter V). The expression of the CD45RA antigen, identified by mAb Leu18, decreases with lymphocyte

activation and is not expressed by NK and T cell clones. Therefore CD45RA is considered as a marker for 'naive' lymphocytes (115,116). Blocking studies with anti-CD45 mAb showed a variety of immunological activities, a; blocking of T cell mediated cytolysis, b; inhibition of B cell proliferation and differentiation, and c; inhibition of NK cell function (117). The cytoplasmatic domain of the CD45 isoforms contains potential phosphorylation sites for protein kinase C (PKC), and has tyrosine phosphatase activity (118). CD45 is a potent regulator of signal transduction and can affect lymphocyte activation in multiple pathways such as the CD2, CD3 and CD28 signal transduction pathways. Crosslinking of CD2, CD3, or CD28 antigens by mAb induces an increase in cytoplasmatic Ca^{++} (119). This increase is abolished when CD45 is crosslinked together with the CD2, CD3 or CD28 antigens, thereby inhibiting signal transduction (108,120,121). The co-clustering of CD45 with CD4 or CD8 leads to dephosphorylation of p^{56lck} and inhibits in vitro kinase activity. This suggests that $p^{56\ lck}$ is an in vivo substrate of CD45 (122).
Protein phosphorylation is a basic mechanism for the modification of protein function in eukaryotic cells. Protein kinases are counteracted by protein phosphatases that dephosphorylate substrate proteins. The physiological role of the various de- and phosphorylating mechanisms is not as yet unraveled.

Natural killer cells

NK cells are lymphoid cells with a typical "large granular lymphocyte" (LGL) morphology and represent approximately 10% of peripheral blood lymphocytes (PBL). NK cells are functionally defined by their ability to display cytotoxicity without in vitro activation against various target cells (22-24). They do not productively transcribe TCR genes nor do they express CD3γ and CD3δ transcripts (123,124). Therefore their lytic activity is by definition MHC unrestricted and they do not show a memory response. In addition to their cytotoxic activities, a wide variety of non-cytotoxic functions have been identified, e.g. the production of lymphokines in response to stimulation with lectins, viruses, bacteria or NK susceptible target cells. The lymphokines produced by NK cells include interferon-α and -γ (IFN α, IFN γ), IL-1, IL-2, natural killer cytotoxic factor (NKCF), lymphotoxin (LT) and colony stimulation factor (CSF). The capacity to produce a pleiora of lymphokines suggests an important immunoregulatory role for NK cells (24, 125-128).
NK cells commonly express CD2, CD16 and NKHI (CD56) membrane antigens (129). In contrast to TCRαβ lymphocytes, single anti-CD2 mAb preparations can induce cytotoxicity by NK cells in particular target cell combinations, indicating that this alternative activation pathway is also functional in NK cells (102,103).
The CD16 (FcγRIII) molecule represents the low affinity receptor for the Fc fragment of

IgG, and mediates antibody dependent cellular cytotoxicity (ADCC). The ability to mediate ADCC via CD16 indicates that this structure is also involved in the triggering of the cytolytic mechanism (90,130,131). In NK cells, the ζ chain is present and associated with the CD16 molecule. The ζ chain may be neccesary for efficient cell surface expression of this CD16 antigen complex (132-134). Indeed, activation with anti-CD16 mAb results in the tyrosine phosphorylation of the ζ chain (135). Therefore, it seems likely that the NK cell ζ containing complex may be functionally analogous to the T lymphocyte antigen recognition complex (136). The 200 kD NKHI (CD56) molecule functions as an adhesion molecule in NK-target cell interactions (137).

TCRγδ lymphocytes

The isolation of the γ gene and the biochemical characterization of the corresponding TCR-γ protein, that is expressed on the cell surface together with the polymorphic TCR-δ chain, raised questions about the possible function of the T lymphocytes expressing this novel kind of receptor (15,16). In the next paragraphs the genomic organization, the distribution of TCRγδ lymphocytes among various tissues, and their possible functions will be described.

Genomic organization of the TCRγ and TCRδ loci

The TCR γ and δ genes undergo rearrangements like the TCR α and β genes. The TCRγ locus has been mapped at chromosome 7 and comprises fourteen variable gene segments (V), five joining segments (J), and two constant gene segments (C) (Fig.4). The fourteen Vγ genes which include six pseudogenes and eight potentially active genes are divided into four subgroups. Subgroup I comprises nine Vγ genes, five functional genes, Vγ2, Vγ3, Vγ4, Vγ5 and Vγ8, and four pseudogenes, Vγ1, Vγ5P, Vγ6 and Vγ7. Subgroups II-IV are formed by Vγ9, Vγ10 and Vγ11 respectively. Finally two pseudogenes, VγA and VγB, belong to none of these subgroups (Reviewed in 138).

The Cγ1 gene segment is composed of three exons. Exon-1 encodes the Ig like domain, exon-2 encodes a 16 aminoacid connector peptide that includes a cysteine residue. This cysteine residue is likely to be involved in the interchain disulphide bond. Exon-3 encodes the transmembrane and cytoplasmatic regions of the TCR-γ chain. The Cγ2 gene segment contains two or three copies of exon-2, this in addition to exon-1 and -3 (139,140). The cysteine residue present in exon-2 is conserved in the Cγ1 gene, whereas this residue is not conserved in exon-2 of the Cγ2 gene.

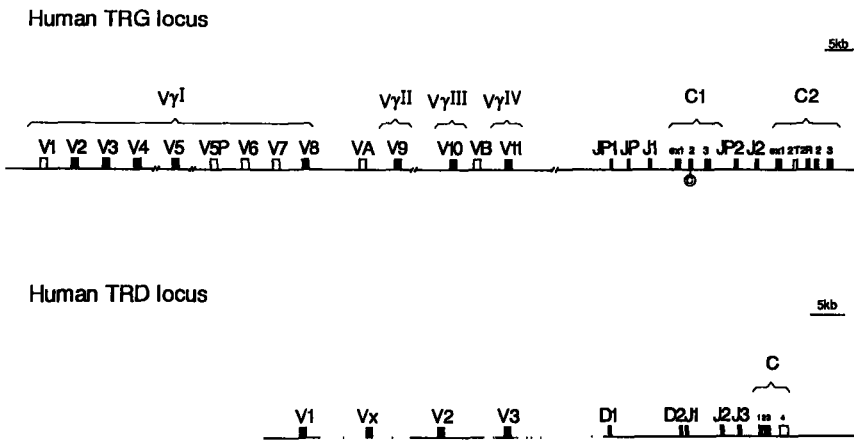

Fig.4. (A) Organization of the V, J and C regions of the human TCR genes, from Lefranc. et. al., 1989. Eur. J. Immunol. (B). Organization of the V, D, J, and C regions of the TCR δ locus.

These differences correspond to three different types of TCR-γ proteins at the cell surface of TCR γδ lymphocytes (Fig.5). The 40 kD disulphide Cγ1 encoded TCR-γ chain, the 40 and 44 kD non-disulphide Cγ2 encoded TCR-γ chain with a duplicated exon-2 and the 55kD non disulphide linked TCR-γ chain encoded by a triplicated exon-2 in the Cγ2 gene (141,142).

The human TCR-δ gene locus is embedded in the TCR-α locus, between the Vα and Jα elements. This locus comprises eight variable, three joining, three diversity (D) gene segments and a unique constant gene segment (138,143,144) (Fig.4).

The combinatorial diversity is a consequence of the number of V, D and J gene segments, which is in comparison to the TCRαβ germline repertoire relatively small (145,146). The diversity is restricted by the limited combinatorial use of distinct V gene elements. However, by the random addition of nucleotides at the V-J, V-D, D-D and D-J regions (N-linked diversity), a large receptor repertoire is generated (48,147,148).

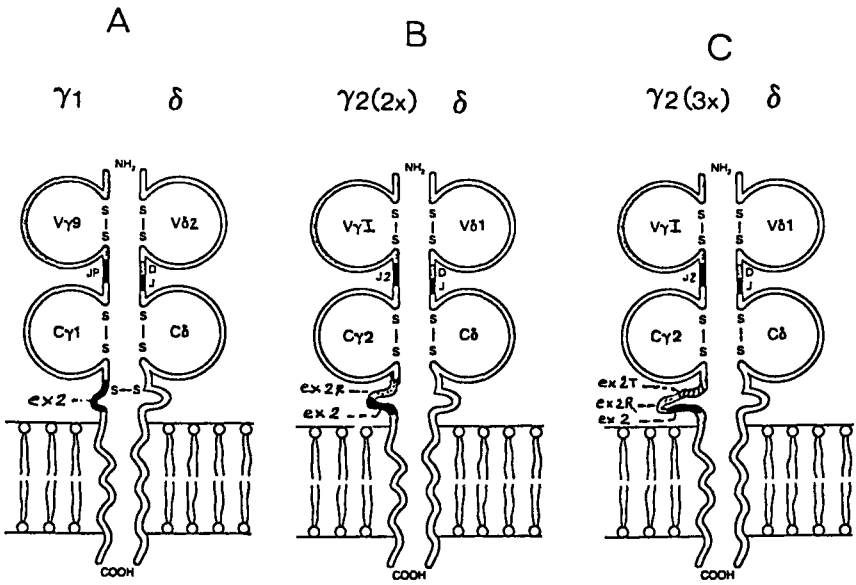

Fig.5. Depending on the TCRγ chain, there are three types of receptors: (A) The TCR-Cγ1, TCR-δ disulphide linked receptor, (B) The TCR-Cγ2, with a duplicated exon-2, TCR-δ non-disulphide linked receptor and (C). The TCR-Cγ2, with a triplicated exon-2, TCR-δ non-disulphide linked receptor, from Lefranc and Rabbitts. 1989.Trends Biochem. Sci. 14:214.

Ontogeny of TCRγδ lymphocytes

Studies in the mouse showed that the initial waves of TCRγδ development occur prior to TCRαβ development. In the mouse only a small percentage of TCRγδ lymphocytes are found in lymphoid tissues. In contrast, the TCRγδ lymphocytes reside mainly in epithelial surfaces of intestine, lung, skin, and reproductive tract (149,150). During the ontogeny of the mouse TCRγδ lymphocytes, TCR-γ and TCR-δ gene rearrangements occur in a orderly fashion. The first TCRγδ lymphocytes to leave the thymus are the Vγ1-Vδ3 lymphocytes, which preferentially localize in epithelial layers, the socalled dentritic epidermal cells (DEC) (151). The Vγ4-Vδ1 lymphocytes home in the second wave to the reproductive tract and finally the Vγ5-Vδ4 lymphocytes home to the gut (152-154). The latter are the intestinal epidermal lymphocytes (IEL). TCRγδ lymphocytes destined to home in the periphery leave

the thymus also in a orderly fashion, first the Vγ1-Cγ2-Vδ4/5, than the Vγ1-Cγ2-Vδ5/6 and last the Vγ1-Cγ4-Vδ5/6 lymphocytes. The presence of the Vγ1-Cγ4 lymphocytes in the periphery correlates with the onset of immunocompetence in normal and Vγ1-Cγ4 transgenic mice (154-156).

The initial rearrangements at the human TCR-δ locus join Vδ2 to Dδ3 and the initial TCR-γ rearrangements join the Vγ8 and Vγ9 gene-elements to Jγ1 and Cγ1 (157-158). No counterparts of the murine DEC cells have been found in humans. In postnatal thymus the Vδ1 gene segment is preferentially used, whereas in the periphery the Vγ9 and Vδ2 gene-elements are dominantly used (159). Also at later times a switch in the rearrangement pattern of the TCR-γ locus is observed. Upstream Vγ gene-elements in the VγI family are joined to the Jγ2 cluster. This indicates that also in human ontogeny TCRγδ gene rearrangements occur in a orderly fashion (146,157,158).

Phenotype and distribution of TCRγδ lymphocytes

In man TCRγδ lymphocytes were initially identified as T cells that lacked reactivity with mAb against TCRαβ. TCRγδ lymphocytes are mainly double negative CD4- and CD8- (160,161). The availability of mAb specific for the particular TCR-Vγ and Vδ gene products allowed a detailed characterization of the TCRγδ population (20,162-165). Human TCR γδ lymphocytes, have been found in all lymphoid tissues and gut epithelium (166,167).

The TCRγδ population in peripheral blood can be divided into two mutually exclusive subsets (168,169). The majority expresses the disulphide linked receptor, encoded by the Vγ9 and Vδ2 gene segments, and can be identified by mAb TiγA and BB3 respectively. A minor population expresses a non-disulphide linked receptor which is encoded by a VγI subgroup gene segment in association with the Vδ1 encoded determinant, as identified by the mAb δTCS-1. The percentage Vγ9-Vδ2 lymphocytes in PBL rises with age (Fig.6), whereas the percentage Vδ1 lymphocytes decreases. In postnatal thymus however, the Vδ1 lymphocyte population represents the majority of the TCRγδ lymphocyte population (170,171).

Like TCRαβ, TCRγδ gene products are non-covalently associated with the CD3 molecular complex (15,16). The CD8 antigen is differentially expressed on Vδ1+ lymphocytes (172). CD2 is expressed on all TCRγδ lymphocytes at levels comparable to the CD2 expression by TCRαβ lymphocytes (173). Some TCRγδ clones express like NK cells the FcγRIII receptor, CD16 (17,174).

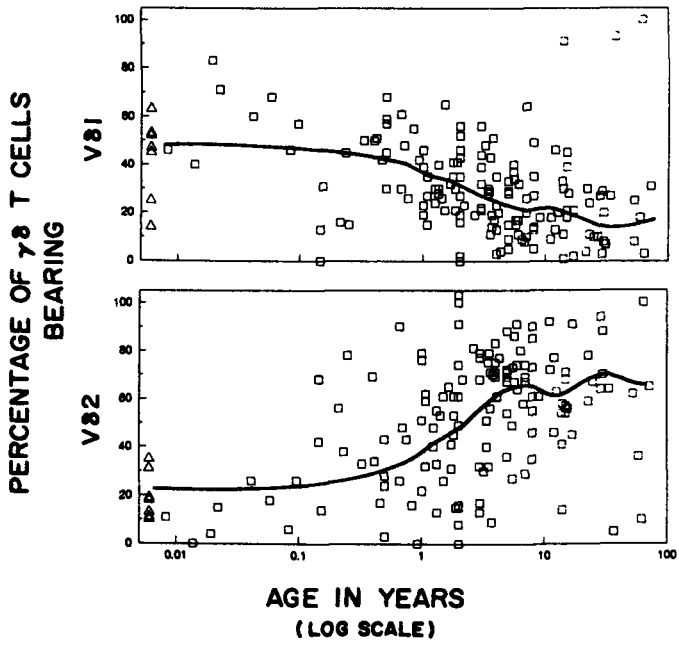

Fig.6. From Parker. et. al., 1990. J. Exp. Med. 171:1597.
Age-related variation in the percentage of TCRγδ lymphocytes that bear Vδ1 or Vδ2-encoded receptors in peripheral blood. The percentage of peripheral blood TCRγδ lymphocytes expressing Vδ1 or Vδ2 was determined by single color FACS analysis and plotted versus age. Umbilicord blood determinations are represented by open triangles adjacent to the y-axis and adult samples by open squares.

Freshly isolated Vγ9-Vδ2 lymphocytes express the CD45RO antigen, and lack expression of the CD45RA antigen. The Vδ1 lymphocytes however express the CD45RA marker and lack expression of the CD45RO antigen (175,176). After activation in vitro, Vδ1 lymphocytes lose expression of the CD45RA antigen and acquire expression of CD45RO (177). Several TCRγδ clones express the NKHI (CD56) antigen, which serves as an adhesion molecule in antigen non-specific recognition. CD56+ TCRγδ clones display a specific pattern of cytolysis against various tumor target cell lines (178).

Functions of TCRγδ lymphocytes

Freshly isolated TCRγδ lymphocytes do not lyse NK susceptible target cells. Upon in vitro activation, TCRγδ lymphocytes can exert MHC unrestricted cytolysis towards a variety of tumor cells of distinct histologic origin, which does not involve the TCR (17-20,179). MAb mediated blocking or modulation of the TCR complex does not only inhibit TCR mediated lysis but also MHC unrestricted cytolysis, possibly through the delivery of an overall negative signal to the lymphocyte resulting in inhibition of cytolysis (17). Antigen specific TCRγδ lymphocytes however have also been identified. Some of the identified antigens are; the CD1c antigen expressed on Molt-4 cells, and T cell target 1 (TCT-1) widely distributed in the haematopoietic system (180,181). Moreover, TCRγδ lymphocytes have been characterized that recognize MHC antigens. These include HLA A2, A23, DR7 and DQw6 antigens (21,182-184). The finding that TCRγδ lymphocytes can recognize tetanus toxoid in a HLA-DR4 restricted way, supports the notion that at least some TCRγδ lymphocytes are capable of recognizing antigenic peptides in the context of MHC (185). Antigen specific activition of TCRγδ lymphocytes induces secretion of BLT serine esterase (benzyloxycarbinyl L-lysine-thiobenzylester), whereas in IL-2 induced activition such secretion has not been found (186). The predominant receptor type used by antigen specific TCRγδ lymphocytes is Vδ1 (187).

The recognition of Staphylococcal enterotoxin A (SEA) is dictated by the Vγ9 gene element, requires MHC class II expression and is specific (188). Therefore this SEA reactivity is reminiscent of a superantigen response as earlier defined for TCRαβ lymphocytes (189,190). Mycobacterial antigens, heat shock proteins and the human B cell line Daudi also elicit specific responses of the entire Vγ9-Vδ2 lymphocyte population, however without MHC restriction (191-193, chapters III and IV). Mycobacterial antigens are potent activators of both murine and human TCRγδ lymphocytes. Mycobacterial reactive TCRγδ lymphocytes have been found not only in immunized mice but also in lesions of leprosy patients and in the synovial fluid of arthritic patients. Many of these TCRγδ lymphocytes react with an unusually low molecular weight mycobacterial antigen. These findinigs led to the idea that TCRγδ lymphocytes or a subset of them are specialized for mycobacterial immunity (194).

The expression of CD45RO, the CD45 isoform associated with prior activation by antigen on the entire Vγ9-Vδ2 lymphocyte subset in peripheral blood, favors an in vivo activation of this lymphocyte subset. The Vδ1 lymphocyte subset however expresses the CD45 isoform, CD45RA associated with a naive phenotype. Cloned Vδ1 lymphocytes express the activation marker, CD45RO. Therefore, the CD45 isoforms are genuine activation markers for TCRγδ lymphocytes (175,176, Chapter V).

When TCRγδ lymphocytes are stimulated with anti-CD3/TCR, anti-CD2 mAb or PHA, a rapid increase in intracellular Ca++ concentration is detected. Moreover, increased levels of inositoltriphosphate are observed after activation. Therefore the inositol lipid pathway plays a functional role in the activation of TCRγδ lymphocytes (195,196). Anti-CD3/TCR mAb can efficiently trigger the lytic of TCRγδ lymphocytes by binding to IgG Fc receptors appropriate target cells (90). Triggering via anti-CD3/TCR mAb results also in a proliferative response of TCRγδ clones, which is accompanied by IL-2 production. In contrast to TCRαβ clones, anti-CD2 mAb induced lysis by TCRγδ clones requires triggering via one epitope (T11.1) only (173,197). These observations reveal a fundamental difference between cloned TCRγδ and TCRαβ lymphocytes regarding the triggering of the CD2 dependent activation pathway. TCRγδ clones are able to secrete a variety of lymphokines e.g., IL-2, IFNγ, tumornecrosisfactor-α (TNFα) and granulocyte- macrophage colony stimulating factor (GM-CSF) (198,199).

TCRγδ lymphocytes and disease

Increased percentages of TCRγδ lymphocytes have been observed in several immunodeficient patients; Wiskott-Aldrich syndrome, severe combined immune deficiency and DiGeorge syndrome patients (200,210). The increased levels of TCRγδ lymphocytes in PBL may be related to the defects in development and/or maturation of TCRαβ lymphocytes.

A number of studies suggest a role for TCRγδ lymphocytes in infectious disease. TCR γδ lymphocytes have been found in mycobacterial infection, tuberculosis, leprosis, leishmanias, influenza and parasite infections such as malaria and trypanosomiasis (202-205). The presence of TCRγδ lymphocytes, predominantly the Vγ9-Vδ2 subset, at inflammation sites is probably related to their reactivity with 'stressed' cells, which express heat shock proteins (191,206). This reactivity can lead to the destruction of the stressed cells or the production of lymphokines by TCRγδ lymphocytes to elicit a CTL response.

TCRγδ lymphocytes may be autoreactive in immune disorders (207). The elevated levels of Vδ1 lymphocytes in peripheral blood and synovial fluid of patients with rheumatoid arthritis (RA) together with the reported mycobacterial reactivity of TCRγδ clones obtained from RA synovial fluid suggests a pathogenic role for the Vδ1 lymphocytes in RA (207). The Vδ1 lymphocytes may react against unknown elements of the cartilage within the joint, leading to the destruction of the cartilage (208).

Objective of this study

The ultimate aim of immunotherapy of cancer is the eradication of the tumor. This requires detailed knowledge of the immune network mechanism, and the development of strategies to direct the immune system. Therefore, the study of different lymphocyte populations, their activation sites for signaltransduction together with accessory molecules involved in immune lymphocyte-tumor cell interactions will provide information about how distinct subpopulations of lymphocytes function within the immune network. In the last decade, the phenotypes and functions in the immune network of TCRαβ lymphocytes and NK cells have been well characterized. The discovery of the TCRγδ lymphocyte population has raised new questions about their immunological role within the immune network. Therefore, this study focuses on the structures and functions of this minor population of T lymphocytes. Chapter II describes the preferential usage of particular variable gene elements within the TCRγδ lymphocyte population in peripheral blood. The TCRγδ receptor protein structures are related to the dichotomy in TCRγδ population. Also the dichotomy of the distinct TCRγδ lymphocyte subsets in PBL is discussed. Chapters III and IV deal with the specific immune reactivities of TCRγδ lymphocytes in relation to their role within the immune network. The differential expression of the CD45 antigen isoforms on TCRγδ lymphocytes and NK cells is described in chapter V. Chapter VI discusses the question: Are TCRγδ lymphocytes important mediators within the human immune system, or a remnant of evolution?
Finally, in chapter VII, the data presently available on the TCRγδ lymphocyte population, their origin, functional properties in vitro, and their putative immunological role in vivo will be discussed.

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CHAPTER II

COORDINATED Vγ9 AND Vδ GENE-SEGMENT REARRANGEMENTS IN HUMAN γδ + LYMPHOCYTES

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SUMMARY

Monoclonal antibodies (mAbs) were used to characterize a panel (n=46) T cell receptor (TCR)γδ+ T cell clones. Three of these antibodies have been described to react with specific variable region encoded protein products and can therefore be used to detect functional gene rearrangements. The majority of peripheral blood derived clones (43 out of 45) expressed the epitopes recognized by mAb BB3, encoded by the Vδ2 gene segment and mAb TiγA, encoded by the Vγ9 gene segment. These clones lacked the antigenic determinant recognized by mAb δTCS-1, encoded by the Vδ1 gene segment. The other two peripheral blood derived clones and an ascites derived clone were TiγA-, BB3- and δTCS-1+. Biochemical analysis revealed that all TiγA+, BB3+ T cell clones expressed the disulphide linked form of the receptor. The two PBL derived δTCS-1+ T cell clones expressed the non-disulphide linked form. Whereas the ascites derived δTCS-1+ clone, AK119 expressed the disulphide linked form of the TCRγδ heterodimer. This indicates that Vδ1 encoded δ chains can be associated either with a Cγ1 or a Cγ2 encoded γ chain. The preferential use of certain Vγ and Vδ gene segments suggests the existence of a limited combinatorial diversity in TCRγδ heterodimers, i.e. TiγA+ (Vγ9), BB3+ (Vδ2) and δTCS-1- disulphide linked heterodimers and TiγA-, BB3- and δTCS-1+ (Vδ1) disulphide or non-disulphide linked forms.

INTRODUCTION

Human T lymphocytes express receptors for antigen that are non-covalently associated in a molecular complex to CD3 surface proteins. In most CD3+ cells this T cell receptor (TCR) consists of disulphide linked α and β chains (1). This TCR αβ dimer recognizes antigen in the context of MHC molecules (2). A minor population of the CD3+ cells, generally lacking CD4 and CD8 molecules, expresses a heterodimer composed of a TCR γ and a TCR δ chain on their surface (3,4). Although TCRγδ+ cells may show MHC restricted recognition (5), the exact function and ligand of this receptor are still unknown.

Unlike immunoglobulin, the TCR does not undergo substantial somatic mutation (6). This means that monoclonal antibodies (mAbs) raised against epitopes of the variable (V) region of the TCR can identify the presence of functionally rearranged variable TCR genes. The human TCRγ gene locus (7) comprises two constant region gene segments Cγ, and at least 14 variable region gene segments Vγ, belonging to four subgroups (7-9). Structural differences exist between the two constant region gene segments: Cγ1 encodes a highly conserved cysteine residue, involved in the interchain disulphide bridge, whereas this residue is absent in the coding sequence of the Cγ2 gene segment (10). The human TCRδ

gene locus is embedded in the TCRα locus between the Vα and Jα gene segments (11-13). It comprises a unique constant region gene segment Cδ preceeded by at least three joining J and two diversity segments D. The number of the δ gene segments seems limited. So far six of them have been sequenced from cDNA clones (14). Here we analyzed the TCR-γ and TCR–δ chain diversity in a panel of human TCRγδ+ T cell clones, expressing either the disulphide or one of the nondisulphide forms of the TCRγδ heterodimer. Phenotypical, biochemical and molecular analysis revealed the coordinated expression of the antigenic determinants encoded by Vδ2 and Vγ9 gene segments which are exclusively present on disulphide linked TCRγδ+ cells. This coordinated expression of Vδ2 and Vγ9 was mutually exclusive with the expression of the antigenic determinant encoded by the Vδ1 gene segment which can be expressed by both non-disulphide and disulphide linked TCRγδ heterodimers.

MATERIALS & METHODS

Cells

Cloned TCRγδ+ lymphocytes were generated from peripheral blood lymphocytes (PBL) obtained from healthy individuals (n=44). From pleura exudate of a patient with mamma carcinoma, clone AK119; from PBL of a patient with subacute sclerosing panencephalitis, clone WiK (15) kindly provided by Dr. D.A. Hafler, Boston, USA. All clones were cultured as described previously (16). The human T cell leukemic cell line Peer (17) was kindly provided by Dr. J. Borst, the Netherlands.

Monoclonal antibodies + immunofluorescence analysis

Five mAbs specifically reactive with the TCRγδ complex have been used: TCRγδ-1, IgG1 (18), TiγA, IgG2a (19), δTCS-1, IgG1 (20), TCRδ-1, IgG1 (21) and BB3, IgG1 (22). For immunoprecipitation studies anti-CD3 mAb CLB T3/4 (IgG2a) (23) was used. Cell surface expression was analyzed on a FACScan (Becton Dickinson, Sunnyvale, USA.) after indirect labeling using FITC conjugated goat anti-mouse Ig (GAMIg) (Nordic, Tilburg, the Netherlands) at a dilution 1:40 as described previously (16).

Probes and Southern blot analysis

TCR-Jγ1 probe pH60 (7) is a 700 bp HindIII-EcoRI fragment isolated from M13H60 and containing the Jγ1 gene segment. This probe cross-hybridizes to the Jγ2 containing fragment. TCR-Jδ1 probe, clone JδS16 (24), is a 1.5 kb SacI fragment containing the region 3' of the Jδ1 gene segment. TCR-Jδ2 probe R21XH (25) is a 2.8 kb XhoI-HindIII

fragment containing the Jδ2 gene segment. TCR-Vδ1 probe is a 240 bp EcoRI-ScaI fragment isolated from the O-240/38 cDNA clone (11). TCR-Vδ2 probe is a 250 bp HindIII-PvuI fragment isolated from the X13 cDNA clone (26). DNA was extracted and digested with BamHI, EcoRI, HindIII, KpnI and XbaI. DNA (10 μg) was electrophoresed through 0.8% agarose and transferred to nitrocellulose filters as described by Southern (27). Hybridizations were carried out at 42 °C overnight in 5 x SSPE, 50% formamide, 5x Denhardts, 0.5% SDS, 5% dextransulphate, 50 μg/ml herring sperm DNA and 10 ng/ml of probe ^{32}P dCTP labeled by random priming (28). Filters were washed 5 min at roomtemperature in 2x SSC, 0.1% SDS, then for 2 hours at 65 °C in 0.1x SSC containing 0.5% SDS and subjected to autoradiography.

Radiolabeling and immunoprecipitation of the TCRγδ complex

Fifteen x 10^6 cells were surface iodinated with ^{125} I (Amersham Corp. Buckinghamshire, UK) using the lactoperoxidase method (29). The cells were then lysed in buffer, containing either 1% NP40 or 1% digitonin (Sigma St. Louis, USA). Supernatants were precleared two times and specific immunoprecipitations were carried out with anti-CD3 mAb CLB T3/4 or the anti-TCRγδ mAbs: BB3, TCRδ-1 or δTCS-1, which were coated to Staphylococcus Aureus for 1 hour at 0°C. Subsequently the pellets were washed five times with lysis buffer containing 1% NP40 in case of anti-TCRγδ mAb precipitations or 1% digitonin in case of precipitations with anti-CD3 mAb and analyzed under nonreducing conditions by 12% SDS poly acryl amide gel electrophoresis (SDS-PAGE). For autoradiography, KODAK X-AR-5 films were used.

RESULTS

Coordinated expression of Vγ and Vδ epitopes recognized by mAbs BB3 and TiγA

The reactivity of five anti-TCRγδ mAbs: TCRγδ-1, TCRδ-1, δTCS-1, TiγA and BB3, was determined by immunofluorescence analysis on a number of human T cell clones (n=46) expressing different molecular forms of the TCRγδ heterodimer. All clones reacted withTCRγδ–1 and TCRδ–1 which each recognize a common epitope on TCRγδ+ T cells (18,21). A representative selection is shown in Table 1. As described recently (19), TiγA recognizes the γ chain encoded by the Vγ9 gene segment. BB3 specifically detects the δ chain encoded by the Vδ2 gene segment (this paper and ref. 26). All TCRγδ+ clones which reacted with TiγA also reacted with BB3. Interestingly, none of the TiγA+, BB3+ clones reacted with δTCS-1. Three clones (AK119, WiK and FKCL2), and the cell line Peer

reacted with δTCS-1, revealing that these clones express the protein product encoded by the Vδ1 gene segment. These three δTCS-1+ clones, and Peer were BB3-, TiγA- suggesting that the expression of the epitopes recognized by these mAbs is generally mutually exclusive.

TCRγδ anti-family mAbs recognize functional Vγ and Vδ rearrangements

The Vγ and Vδ rearrangements of two clones, each representing one of the major subsets of TCRγδ+ T cells described above, i.e. AK119 (BB3-, TiγA-, δTCS-1+) and 1012 (BB3+, TiγA+, δTCS-1-) were analyzed by Southern blotting. The Vγ rearrangements were studied using the pH60 probe. It allows the detection and assignment of all Vγ rearrangements involving Jγ1 or Jγ2 when hybridized to BamHI, EcoRI and HindIII digests (9), and the detection of those involving the Jγ gene segments JγP, JγP1, and JγP2 when hybridized to KpnI digests (30).

Table 1. Reactivity of TCRγδ+ T cell clones with five anti-TCRγδ mAb

TCRγδ + clones	TCRγδ-1	TCRδ-1	δTCS-1	TiγA	BB3
N-4	+	+	-	+	+
615	+	+	-	+	+
1003	+	+	-	+	+
1004	+	+	-	+	+
1005	+	+	-	+	+
1011	+	+	-	+	+
1012	+	+	-	+	+
1015	+	+	-	+	+
1019	+	+	-	+	+
1042	+	+	-	+	+
AK119	+	+	+	-	-
WiK	+	+	+	-	-
FKCL2	+	+	+	-	-
T cell line					
PEER	+	+	+	-	-

Binding of mAb was determined by immunofluorescence. Cells were incubated with TCRγδ-1 culture supernatant, TCRδ1 1:100 dilution of ascites, δTCS-1 3.75 μg/ml, TiγA 1:500 dilution of ascites and BB3 1:100 dilution of ascites followed by incubation of FITC conjugated goat anti-mouse Ig.

Assignments of the TCRγ rearrangements to given Vγ and Jγ segments on basis of size of the rearranged bands detected in Southern blot analysis is according to reference 9. One allele of the TCRγδ + clone AK119 has a Vγ5Jγ1 rearrangement, whereas the other allele is in germline configuration (Fig.1.) TCRγδ+ clone 1012 displays two rearrangements: the first one is a Vγ9JγP rearrangement visualized only after hybridization of the pH60 probe to KpnI digests, which detects a 12 kb KpnI rearranged fragment (30). This rearrangement is transcribed in all TiγA+ lymphocytes as previously shown by Northern blot analysis using the VγII probe (31). The second rearrangement represents Vγ10Jγ1.

The Jδ and Vδ rearrangements of clones AK119 and 1012 were studied using the Jδ1 (JδS16) (24), Jδ2 (R21XH) (25), TCR-Vδ1 (from 0-240/38) (11) and TCR-Vδ2 (from X13) (26) probes. As shown in Fig. 2 TCRγδ+ clone AK119 displays a 3.2 kb EcoRI, a 10 kb HindIII and a 5.9 kb XbaI rearranged band hybridizing to both TCR-Jδ1 and TCR-Vδ1 probes, and therefore demonstrates a Vδ1Jδ1 rearrangement (Table 2).

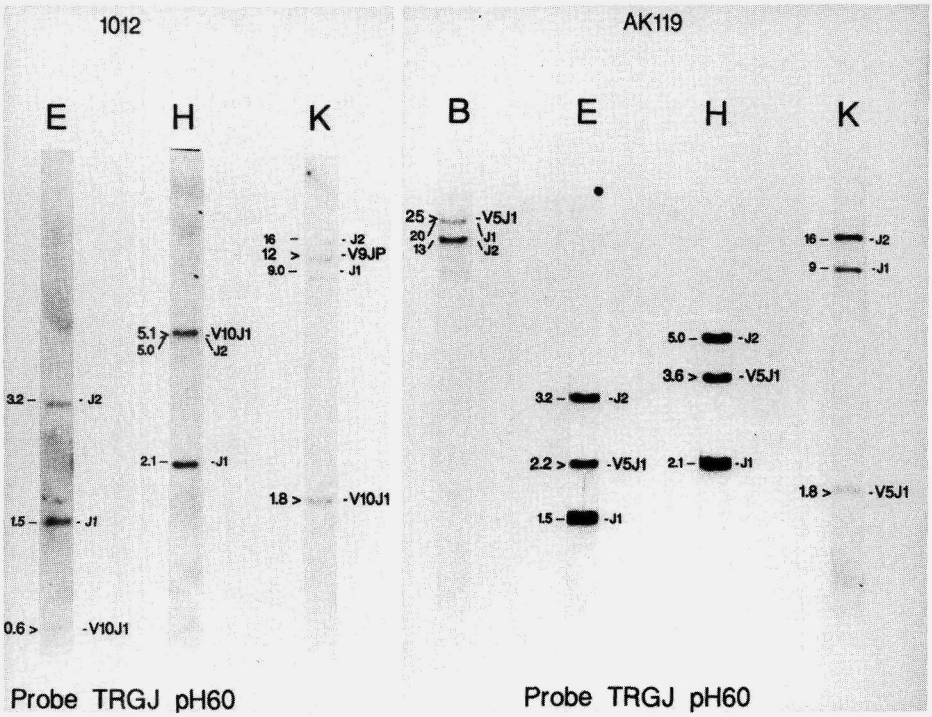

Figure 1: Southern hybridization to TCRγ probe pH60 (7) was hybridized to BamHI (B), EcoRI (E), Hind III (H) and KpnI (K) digested DNA from clones AK119 and 1012. Assignments of the TCRγ rearrangements to given V and J segments is according to ref. 9.

TCRγδ+ clone 1012 shows a 5.2 kb EcoRI, a 5.8 kb HindIII and a 4.0 kb XbaI rearranged band hybridizing to both the TCR-Jδ1 and Vδ2 probes and therefore has a Vδ2-Jδ1 rearrangement (Table 2). Six other δTCS-1- clones, analyzed by Southern blot analysis, also hybridized with a TCR-Vδ2 specific probe (data not shown).

Clones AK119 and 1012 display a second rearrangement defined by a 2.6 kb EcoRI, a 4.7 kb HindIII and a 2.8 kb XbaI fragment, which do not hybridize to Vδ1 or Vδ2. These bands correspond to a third Vδ gene segment designated Vδ3 in Table 2. These data confirm that the TiγA+, BB3+ clones use the Vγ9 and the Vδ2 rearrangements, whereas the δTCS-1+ clones use the Vδ1 rearrangement.

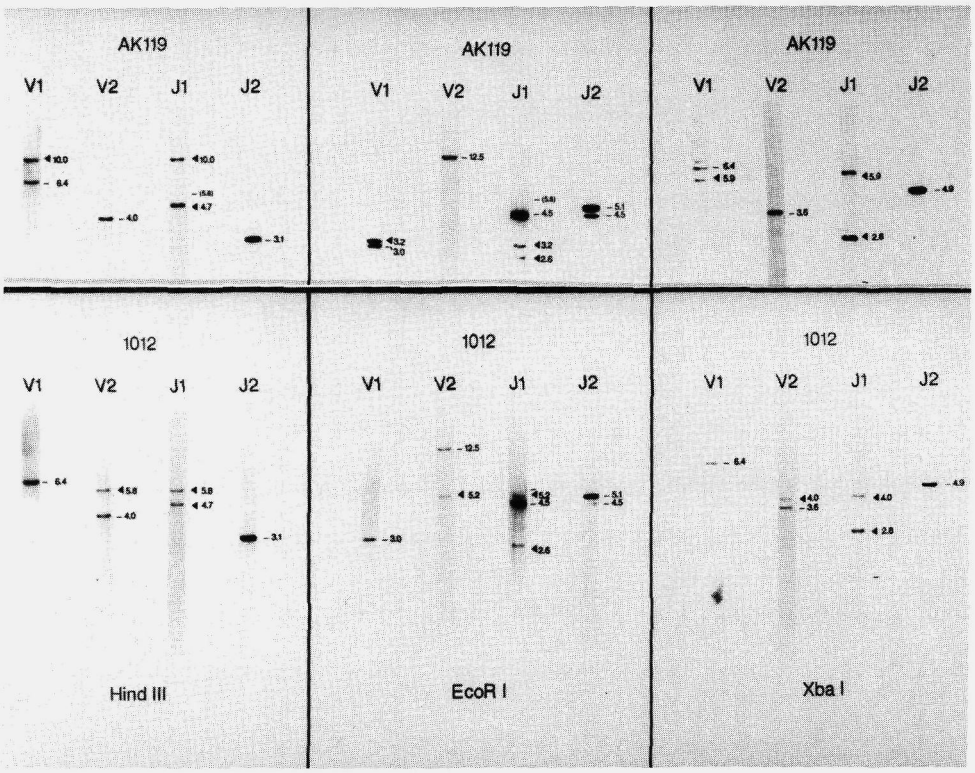

Figure 2: Southern hybridization to TCRδ probes. Vδ1 from 0-240/38 (11), Vδ2 from X13 (26), Jδ1 (JδS16) (30) and Jδ2 (R21XH) (25) probes were hybridized to HindIII, EcoRI, and XbaI digests from clones AK119 and 1012. Dashes indicate germline bands whereas the black triangles indicate rearranged bands. Both TCRγδ+ clones AK119 and 1012 have two TCRδ rearrangements.

Although Southern blot analysis does not reveal whether a rearrangement is functional, the correlation between the expression of a TCRγδ anti-family mAb specific epitope and usage of the corresponding Vγ or Vδ rearrangement suggests that reactivity with anti-family mAbs can be used to determine functional rearrangements.

Table 2. Assignment of the TCRδ rearrangements[a]

	EcoRI	Hind III	XbaI
Germline J1	5.8[b]	5.8	1.7
V1-D-J1	3.2	10.0	5.9
V2-D-J1	5.2	5.8	4.0
V3-D-J1	2.6	4.7	2.8

a) The TCR-Jδ1 (JδS16) (13) probe detects all rearranged bands mentioned in this table whereas the Vδ1 (from 0-240/38) (11) and Vδ2 (from X13) (26) probes only detect the fragment specific of the corresponding Vδ gene (see Fig.2). "D" represents either D1 or D2.
b) The size of the restriction fragments is given in kb.

δTCS-1+ cells express either a disulphide or a non-disulphide linked TCRγδ heterodimer.

TCRγδ+ cells can be divided into two subsets: one expresses a disulphide linked heterodimer, the other a non-disulphide linked TCRγδ complex. It was examined whether the dichotomy on basis of disulphide linkage correlates with the division on basis of the mutually exclusive expression of either Vγ9 and Vδ2 epitopes, or the expression of a Vγ associated with the Vδ1 gene product. Therefore SDS-PAGE analysis was performed using radiolabeled immunocomplexes precipitated either by anti-CD3 mAb from digitonin cell lysates or by three anti-TCRγδ mAb, i.e. TCR δ-1, δTCS-1, BB3, from NP40 cell lysates In Fig. 3, like anti-CD3 mAb (lanes A-C), TCRδ-1 precipitated a 80 kD disulphide linked TCRγδ heterodimer from clones N-4 and AK119 and a non-disulphide linked heterodimer from clone Wik (lanes D-F). δTCS-1 does not react with clone N-4 but precipitates either a disulphide linked heterodimer from clone AK119 or a non-disulphide linked heterodimer from clone WiK (lanes G-I). BB3, precipitates a disulphide linked heterodimer from clone N-4 but does not react with clones AK119 and WiK (lanes J-L). Like N-4, all other TiγA+,

BB3+ clones analyzed (n=9), expressed a disulphide linked heterodimer. Whereas the δTCS-1+ lymphocytes may express either a disulphide linked or a non-disulphide linked TCRγδ heterodimer.

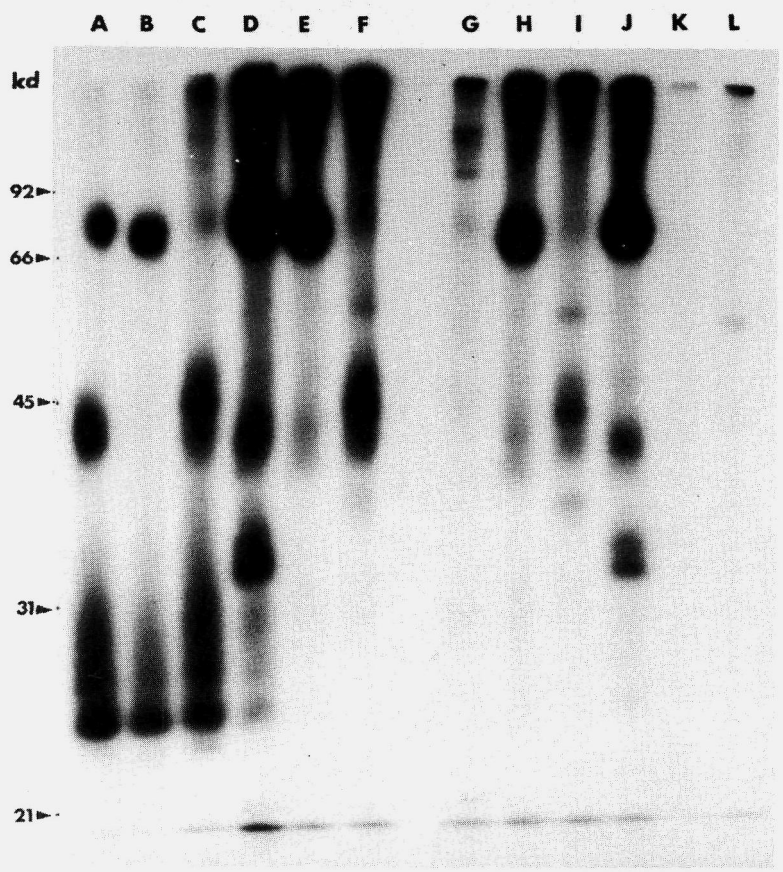

Figure 3: SDS-PAGE analysis of surface molecules immunoprecipitated from TCRγδ+ clones N-4 (lanes, A,D,G,J,), AK119 (lanes B,E,H,K,), and WiK (lanes C,F,I,L). Fifteen x 10^6 cells were surface iodinated, one fourth of the cells were lysed in buffer containing 1g/100 ml digitonin and the remaining cells were lysed in a buffer containing 1% NP40. Digitonin lysates were precipitated with CLB T3/4 (anti-CD3) (lanes A-C), whereas NP40 lysates were precipitated with TCRδ-1 (lanes D-F), δTCS-1 (lanes G-I) and BB3 (lanes J-L)

DISCUSSION

The diversity of both TCRγ and δ peptides expressed at the cell surface of a panel of TCRγδ+ T cell clones was analyzed. Because the Vγ and Vδ gene segments do not undergo substantial somatic mutation (8), [M.-P.L. unpublished data], reactivity with mAbs directed against distinct epitopes on the variable region of the gamma and delta chains of the TCR can identify the presence of functionally rearranged variable TCR genes.

Immunofluorescence and molecular analysis revealed a coordinated expression of the antigenic determinants encoded by the Vγ9 and Vδ2 gene segments, recognized by TiγA and BB3. All TiγA+, BB3+ heterodimers were disulphide linked. The expression of BB3 and TiγA specific epitopes was mutually exclusive with the expression of the epitope encoded by Vδ1, recognized by δTCS-1. The reciprocal reactivity of mAb TiγA and δTCS-1 observed in our TCRγδ+ clones is in accordance with a recent report of T. Hercend et.al. (32) with the reported exception of one TiγA+, δTCS-1+ clone. These data suggest a limited combinatorial diversity in TCRγδ heterodimers, despite the large potential diversity at the genomic level. The limited diversity does not appear to be donor specific since the clones analyzed were derived from seven idividuals. It is tempting to speculate on the mechanisms underlying this coordinated expression of certain Vγ and Vδ gene segments. The functional rearrangement of either the TCRγ or TCRδ gene may dictate the rearrangement of the other TCR gene. In our case, a rearranged Vδ2 gene segment would dictate the rearrangement of the Vγ9 gene segment (VγII subgroup) which rearranges to Cγ1 or vice versa. Interestingly, the Vδ1+ (δTCS-1+) clone AK119 and Vδ1+ cell lines Peer and Molt express Vγ5, Vγ8 and Vγ1 (17) respectively, all belonging to the VγI subgroup. Thus, functional rearrangement of Vδ1 may evoke the rearrangement of a VγI subgroup gene segment or vice versa, irrespective as to whether the VγI subgroup gene segment rearranges to Cγ1, clone AK119 or to Cγ2, clone WiK. Alternatively, physiological constraints prevent the association of Vδ1 encoded delta chains with Vγ9 encoded gamma chains. The demonstration that transfection of a Cγ1 containing TCRγ cDNA into cell lines that express the Cγ2 encoded TCRγ chain results in the expression of both disulphide and non-disulphide linked TCRγδ heterodimers argues against an exclusion model based on physiological constraints (Dr. M. Brenner, personal communication). Finally the coordinated expression of certain Vγ and Vδ gene products may result from (thymic) selection. All but one clone tested were derived from PBL. Thus, the predominant subset of TCRγδ+ cells (TiγA+, BB3+) may be restricted to the periphery. Moreover, the TCRγδ lymphocyte diversity and subset composition may differ between various tissue locations. The δTCS-1+ (Vδ1) lymphocytes comprise a minor subset in peripheral blood but are

abundantly expressed within the thymus. Both, disulphide and non-disulphide linked TCRγδ heterodimers are present within the δTCS-1+ thymic subset (33). Within our panel the PBL derived δTCS-1+ clones expressed a non-disulphide linked heterodimer. The pleura exudate derived δTCS-1+ clone AK119 is unique in expressing a disulphide linked TCRγδ heterodimer. Therefore, this thus far unique clone AK119 might descend directly from an intrathymic precursor.

The limited use of certain Vγ and Vδ gene segments does not necessarily reflect a limited variability of the ligands recognized. The diversity of nucleotides (N regions) at the V-(D)-J junction may contribute considerably to the conformational structure of the TCRγδ complex (34) and therefore to the diversity of ligands. The possibility to assign the use of certain Vγ and Vδ genes in functional rearrangements with the use of anti-family mAbs allows the analysis of the diversity of functionally rearranged Vγ and Vδ gene segments at the single cell level.

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CHAPTER III

HUMAN Vγ9-Vδ2 T CELL RECEPTOR-γδ LYMPHOCYTES SHOW SPECIFICITY TO DAUDI BURKITT'S LYMPHOMA CELLS

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SUMMARY

Peripheral blood TCRγδ cells with different functional Vγ or Vδ gene rearrangements represent two non-overlapping subsets. The major subset uses the Vγ9 and the Vδ2 genesegments and the minor subset the Vδ1 genesegment in its functional TCR rearrangement. Upon in vitro activation, these T cell receptor (TCR)γδ lymphocytes display MHC unrestricted lytic activity, against a wide variety of tumor cells of distinct histologic origin. Here we show that fresh TCRγδ lymphocytes that express a Vγ9-Vδ2 encoded TCR display a specific proliferative response to Daudi, Burkitt's lymphoma cells. Moreover, cloned Vγ9-Vδ2 lymphocytes show the capacity to lyse Daudi cells, whereas none of the cloned Vδ1 TCRγδ lymphocytes shows such specificity. Nucleotide diversity at the V-D-J junction of the TCR-Vδ2 gene did not contribute to this Daudi cell specificity. Comparison of the MHC unrestricted cytolytic capacities of the Vγ9-Vδ2 and the Vδ1 clones using a panel of distinct types of tumor target cells showed that on avarage, the level of MHC unrestricted lysis of Vγ9-Vδ2 clones against these tumor cells exceeded that of Vδ1 clones. However, in contrast to all these tumor cell lines, only the Daudi cells showed such an absolute distinction in susceptibility to lysis by Vγ9-Vδ2 and Vδ1 clones. Vγ9-Vδ2 clones which were generated with a stimulator cell other than Daudi did not lyse their stimulator cells but nevertheless showed specific cytolysis of Daudi cells. The specific proliferation to and cytolysis of Daudi cells of the entire Vγ9-Vδ2 subpopulation of TCRγδ lymphocytes is reminiscent of a superantigen response.

INTRODUCTION

The antigen receptor expressed on the majority of human T lymphocytes is a disulphide-linked heterodimer composed of an α and β chain, which is non-covalently associated with the CD3 complex [1]. Lymphocytes bearing the T cell receptor (TCR)αβ recognize antigen in a major histocompatibility complex (MHC) restricted manner [2]. A minor portion of T lymphocytes expresses the TCRγδ instead of the TCRαβ heterodimer [3-4]. The majority of these TCRγδ lymphocytes lacks the expression of the CD4 and CD8 molecules [5-6]. Recently, we and others showed that human TCRγδ lymphocytes in the periphery mainly consist of two non-overlapping subsets, which are defined on basis of reactivity with anti-TCRγδ mAb, that selectively recognize protein products of particular functional TCR gene rearrangements [7-9]. The major subset expresses the Vγ9-Vδ2 encoded TCRγδ, whereas the minor uses the Vδ1 gene segment for its functional TCR rearrangement. TCRγδ lymphocytes freshly isolated from peripheral blood do not display substantial cytolytic

activities against K562 target cells [10]. After activation or culture in vitro, these lymphocytes acquire MHC unrestricted cytolytic activity against a wide variety of tumor cells of distinct histologic origin [3, 11-15]. It is unclear whether the broad spectrum of cytotoxicity mediated by in vitro activated TCRγδ lymphocytes reflects their in vivo activity and whether the TCR complex is involved in this MHC unrestricted cytolytic process [16]. Although recently reports of Ag-specific and/or MHC-restricted TCRγδ lymphocytes have appeared [17-21], it is still enigmatic whether this Ag-specificity is a hallmark of TCRγδ bearing lymphocytes. The present study compares the proliferative and cytolytic capacities of fresh and cloned TCRγδ lymphocytes of the Vγ9-Vδ2 and Vδ1 subsets. The results show that only those fresh or cloned TCRγδ lymphocytes, that express a Vγ9-Vδ2 encoded TCR, specifically proliferate to or lyse Daudi Burkitt's lymphoma cells.

Table I: Immunophenotypic analysis of TCRγδ clones

TCRγδ clone	stimulator cells in primary culture	Anti-TCRγδ mAb				
		TCRγδ -1 (All)	TCR δ1 (All)	δTCS-1 (Vδ1)	BB3 (Vδ2)	TiγA (Vγ9)
ER22	APD/BSM	+	+	+	-	-
ER25	APD/BSM	+	+	+	-	-
ER44	APD/BSM	+	+	+	-	-
WiK	none	+	+	+	-	-
AK119	none	+	+	+	-	-
N-4	none	+	+	-	+	+
1042	none	+	+	-	+	+
1005	none	+	+	-	+	+
KOBA70	Daudi	+	+	-	+	+
KOBA72	Daudi	+	+	-	+	+
PG60	APD/BSM	+	+	-	+	+
PJ4	none	+	+	-	-	-
ThyCl-5	none	+	+	+	-	-
ThyCl-10	none	+	+	+	-	-

LEGEND table I: Binding of the mAb was determined by immunofluorescence.T cell clones were incubated with TCRγδ -1 culture supernatant, TCRδ1 1:100 dilution of ascites, δTCS-1, 3,75 μgr/ml, TiγA 1:500 dilution of ascites and BB3 1:1000 dilution of ascites followed by incubation with FITC conjugated goat anti-mouse-Ig.

This Daudi cell specificity appears independent of the nucleotide sequence diversity at the V-D-J junction of the TCR-Vδ2 chain. This also implies that the Vγ9-Vδ2 population showing this response is polyclonal. All TCRγδ clones exert MHC unrestricted lysis, when tested against a variety of distinct tumor target cells. The overall level of cytolytic activity of Vγ9-Vδ2 clones exceeds that of Vδ1 clones. In the present study we conclusively demonstrate that Daudi cells are lysed by all Vγ9-Vδ2 clones but by none of the Vδ1 clones. The data obtained with fresh as well as cloned TCRγδ lymphocytes documents that a Vγ9-Vδ2 encoded TCR in itself may be sufficient to impart proliferative or cytolytic specificity for Daudi cells. We therefore suggest that the Vγ9-Vδ2 lymphocytes display a response to an antigenic determinant, expressed on Daudi cells which is reminiscent of a superantigen response.

MATERIALS & METHODS

Isolation and culture of TCRγδ clones.

Peripheral blood lymphocytes (PBL) of several healthy donors were cultured in 24 wells tissue culture plates (COSTAR, Cambridge, Mass. USA) in a volume of 1 ml/well at $3x10^5$ cells per well together with $2x10^5$ irradiated feeder cells either Daudi cells, (a Burkitt lymphoma derived B-lymphoblastoid cell line (B-LCL)) or a mixture of $1x10^5$ APD with $1x10^5$ BSM, both in vitro Epstein barr virus (EBV) transformed B-LCL. Cells were cultured in RPMI 1640 (Flow-laboratories, UK) supplemented with 10 % pooled human serum, 4 mM L-glutamin, and 100 IU penicillin/streptomycin. Thirty units recombinant human interleukin-2 (rIL-2) (Cetus Inc. Emmeryville, CA, USA) were added on day four, (when thawed PBL were used, 30 units rIL-2 were added directly), and fresh rIL-2 supplemented medium was added two times a week. Fresh feeder cells ($2x10^5$) were added to $3x10^5$ responder cells at 14 day intervals. After four weeks of culture, responder cells were cloned by limiting dilution in the presence of irradiated APD/BSM feeder cells as described previously [22].

Clones analysed in this study are listed in Table I. KOBA clones were obtained from PBL primed with Daudi cells, whereas the PG clones were derived from PBL from another donor primed with APD/BSM cells. ER clones were obtained from the same primed culture as the PG clones but were positively selected for reactivity with the δTCS-1 mAb (T-Cell Sciences Cambridge USA) on a FACS III (Becton and Dickinson, Mountain View, USA). Clone PJ4 was obtained after positive selection with the TCRδ-1 mAb from a third donor without priming. Clones N-4 [23], 1005, 1012 [24] and 1042 were derived after limiting dilution from PBL without priming. Clone AK119 [3] was derived from pleura exudate of a

patient with mamma carcinoma without priming. Clone WiK was obtained from a patient with subacute sclerosing panencephalitis (kindly provided by Dr. D. A. Hafler, Boston, MA, USA) also without priming [25]. Clones ThyCL5 and ThyCL10 were thymus derived (kindly provided by Dr. F. Koning, Leiden, The Netherlands) [26]. Expansion of the clones was routinely done in RPMI 1640; 10 % human serum; 4 mM L-glutamin; 100 IU penicillin/streptomycin; 1 ng/ml indomethacine; 1 μgr/ml leucoagglutin (purified PHA, Pharmacia, Upssala, Sweden), and 25 U/ml rIL-2. Irradiated (25 GY) feeder cells were added weekly [22].

Cytotoxicity assays

Cytotoxic activity was measured in a standard 3 hr ^{51}Cr release assay. Briefly, varying numbers of effector cells were added in triplicate to 96 wells microtiter plates. MAb were added to the effector cells at the indicated concentrations 30 minutes before addition of a fixed amount (n= 2000) of ^{51}Cr labeled target cells. Supernatants were collected using a Skatron harvesting system (Skatron, Lier, Norway) and counted in a gamma counter. Percentage specific lysis was calculated as described [27].

Target cells

The following tumor cell lines were used as target cells in cytotoxicity assays; K562, an erythromyeloid derived cell line; Daudi, a Burkitt lymphoma derived cell line; AVL and BSM, two EBV transformed B-LCL; Molt-4, a leukemic T cell line; IGROV, an ovarium carcinoma derived cell line (kindly provided by Dr. Bernard, Paris, France); U937, a monocytic cell line; IgR37, a melanoma derived cell line, and GLC-2E, a small cell lung carcinoma derived cell line. K562 and Daudi were also used in conjugate formation studies.

MAb and Immunofluorescence studies

Anti-CD3 mAb (CLB-T3/4.1, CLB-T3/4.2a) [28] and OKT3 (hybridoma culture supernatant) were used in concentrations as indicated. Five mAb reactive with the TCRγδ complex, have been used; TCRγδ-1 [29], and TCRδ1 [30], both reactive with all TCRγδ lymphocytes, TiγA, specific for the Vγ9 encoded epitope [31], BB3, specific for the Vδ2 encoded epitope [32], and δTCS-1, specific for the Vδ1 encoded epitope [33] (T- Cell Sciences, Cambridge, USA).

Cell surface expression was analysed by flow cytometry on a FACScan, after indirect labeling, using 1:40 dilution of fluorescein isothiocyanate conjugated (FITC) goat anti-mouse Ig (GAMIg) (Nordic, Tilburg, The Netherlands) [27]. For two color fluorescence studies, PBL were stained with either TiγA, (IgG2a), followed by IgG2a subclass specific

goat anti-mouse GAMγ2a conjugated with phycoerythrin (Southern Biotech. Inc. Birmingham, USA) or BB3 (IgG1) or δTCS-1,(IgG1), followed by IgG1 subclass specific goat anti-mouse GAMγ1 conjugated with FITC. After washing once, the lymphocytes were incubated with excess purified mouse IgG to block free GAMγ2a or GAMγ1 binding sites. Subsequently, lymphocytes were stained with TCRγδ-1 conjugated with either FITC (TiγA) or PE (BB3, δTCS-1). Two colour flow cytometry was performed on a FACScan. To analyse Vγ9 (TiγA), Vδ2 (BB3), and Vδ1 (δTCS-1) profiles of the TCRγδ subset, a gate was set on FL-1 or FL-2 fluorescence such that only data on TCRγδ lymphocytes were acquired. Green/red fluorescence histograms, showing the Vγ9, Vδ2, and Vδ1 profiles of the gated lymphocytes were made using the FACScan software.

Conjugate formation assay

Spontaneous and mAb-induced conjugate formation of effector cells and target cells (K562 and Daudi) was assessed by FACScan analysis [34]. Effector cells ($5x10^6$) were labeled intracellular with 40 μg/ml hydroethidine (HE) in a volume of 0.5 ml at 37 °C for 30 min. Target cells ($5x10^6$) were labeled the same way with 0.05 μg/ml carboxy fluorescein diacetate (CFDA). The labeled effector and target cells were washed twice in PBS and diluted to 10^7 cells/ml in RPMI 1640; 10 % FCS before use. When indicated, 20 μl effector cells were preincubated with anti-CD3 mAb (CLB-T3/4.1, at saturating concentration as determined by fluorescence analysis) at 21°C for 20 min before addition of an equal number labeled target cells. The final volume was adjusted to 100 μl with RPMI 1640; 10 % FCS; 4 mM L-glutamine, and 100 IU penicillin/ streptomycin. Conjugates were allowed to form for 15 min at 21 °C. Conjugate formation was stopped by the addition of 0.5 ml ice cold PBS/1 % BSA and samples were directly analysed on a FACScan. Ten-10^3 events were collected and the % conjugates was calculated by dividing the number of events simultaneously emitting red and green fluorescence (=number of conjugates) by the total events emitting red fluoresence (total number of effector cells).

DNA sequencing

Approximately 300 bp of genomic DNA encompassing the V-J junction of a TCR-δ gene rearrangement was isolated from 3 Vγ9-Vδ2 clones, 1004, 1018, and 1019 [24] by the PCR method [35] and subcloned in a Bluescript vector (Stratagene) after digestion with EcoRI and HindIII.

The primers used were AAGCTTCCAAACAGTGCCTGTGTCAATAGGGGTCCC (Vδ2) and TTCCACAGTCACACGAATTCCTTTTCC (Jδ1). The Jδ1 primer has two base substitutions so as to generate an EcoRI site for cloning purposes. In addition, a cDNA

library was generated of the Vγ9-Vδ2 clone, N-4. The cDNA sequences were cloned in the phage vector λgt10 [36]. The cDNA library was screened for TCR-δ cDNA using a ^{32}P labeled nick translated 1.5 kb EcoRI fragment, isolated from the TCR-δ cDNA clone O-240 [37]. The longest hybridizing cDNA clones were selected for sequence analysis by the dideoxy chain termination method [38].

RESULTS

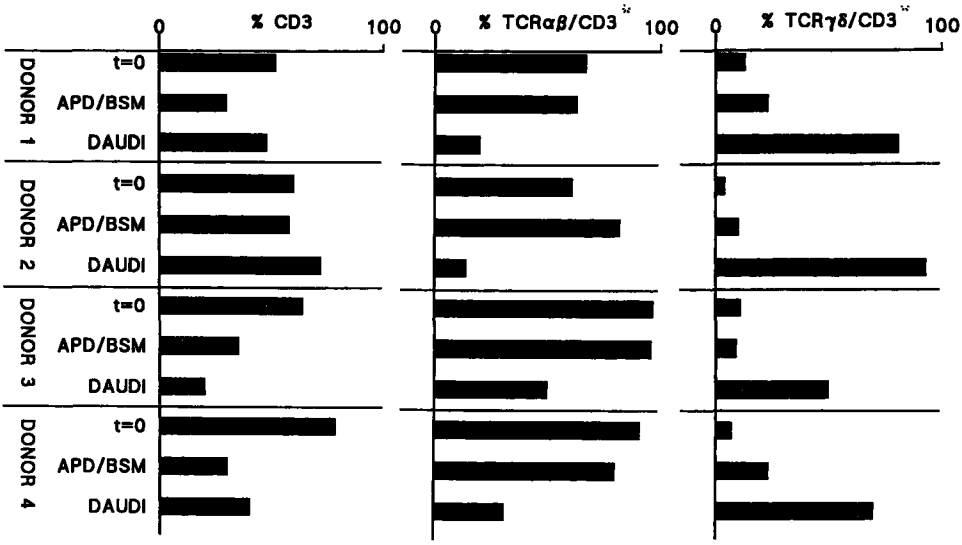
Selective outgrowth of TCRγδ versus TCRαβ lymphocytes upon stimulation with Daudi cells

It is known that certain cell lines, including B lympho blastoid cell lines (B-LCL), sustain proliferation of purified TCRγδ populations and TCRγδ clones [15,23]. PBL from individual healthy donors were cultured in the presence of various irradiated B-LCL. As shown in Fig.1a, stimulation of PBL with Daudi B-LCL feeder cells, but not other lines of B-LCL, induced increased percentages of TCRγδ versus TCRαβ lymphocytes within a 7 day culture period. This result suggests an antigen-specific stimulation of TCRγδ lymphocytes by Daudi cells. Because the total lymphocyte yields obtained after Daudi cell and other B-LCL stimulation were similar, the Daudi cells must selectively induce proliferation of TCRγδ lymphocytes. This selective outgrowth of TCRγδ lymphocytes is not related to the absence of HLA-class I antigens on Daudi cells since stimulation with HLA-class I negative K562 cells did not show such selective outgrowth of TCRγδ lymphocytes, although K562 cells could stimulate proliferation of CD3- lymphocytes (data not shown). Moreover Daudi cells transfected with β2-microglobulin cDNA, which restored the MHC class I expression could selectively stimulate TCRγδ lymphocytes.

Only Vγ9-Vδ2 lymphocytes show a specific proliferative response to Daudi cells

Human TCRγ lymphocytes in peripheral blood contain only limited combinatorial diversity and comprise mainly two distinct non-overlapping subsets. The major subset expresses the Vγ9-Vδ2 encoded TCRγδ whereas the minor expresses the Vδ1 encoded TCRγδ [7-9]. Stimulation of unfractionated PBL with the APD/BSM B-LCL, results in an expansion of the total T lymphocyte population (TCRαβ and TCRγδ lymphocytes) after a 7 day culture period. No selective outgrowth of TCRγδ lymphocytes is observed, both the Vγ9-Vδ2 and the Vδ1 subsets are equally stimulated within the TCRγδ lymphocyte population. However, stimulation of unfractionated PBL with Daudi cells, for 7 days results in a selective outgrowth of TCRγδ lymphocytes which is restricted to the Vγ9-Vδ2 subset (Fig.1b). In

three out of four donors, Vγ9-Vδ2 lymphocytes represented more than 90% of all TCRγδ lymphocytes. In a fourth donor however, Vδ1 lymphocytes comprised approximately 25-30% of the total TCRγδ population in PBL. Also in this donor only the Vγ9-Vδ2 subset showed selective proliferation to Daudi cells, whereas both the Vγ9-Vδ2 and the Vδ1 subset showed proliferation when stimulated with the APD/BSM B-LCL.



LEGEND fig.1a: Daudi cells specifically stimulate proliferation of TCRγδ lymphocytes. PBL were isolated from 4 healthy donors, and directly stained (t=0) with anti-CD3, anti-TCRαβ or anti-TCRγδ mAb. These PBL were also cultured during a 7 day period with either irradiated Daudi cells or a mixture of APD/BSM feeder cells after which the cells were stained with identical mAb as used at day 0.
* : % TCRαβ/CD3 and % TCRγδ/CD3 was determined by dividing % TCRαβ or %TCRγδ stained lymphocytes by %CD3 positive lymphocytes from the same culture.

Immunophenotypic analysis of Vγ9-Vδ2 and Vδ1 cloned lymphocytes

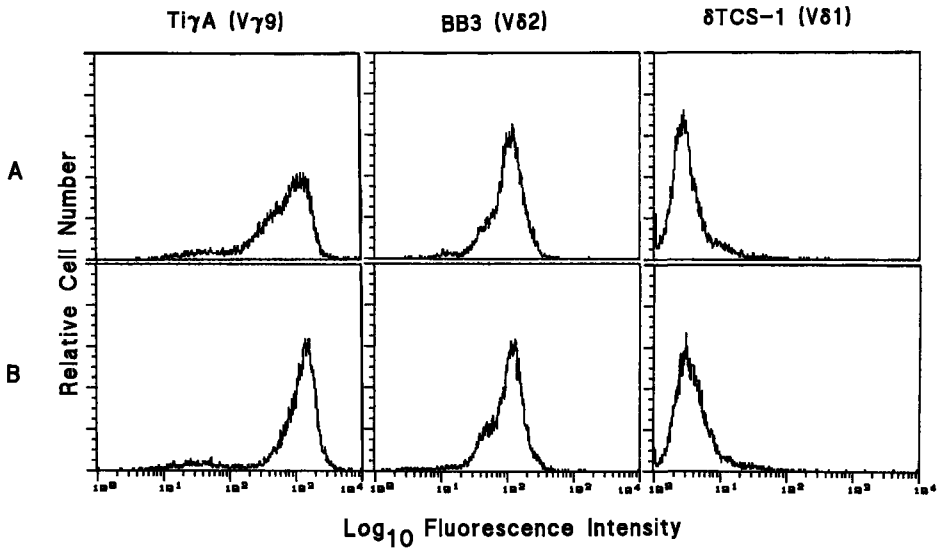
To exclude the possibility that the selective proliferation of Vγ9-Vδ2 lymphocytes to Daudi cells resulted from the antigen-driven proliferation of only a few precursor lymphocytes, we generated cloned TCRγδ lymphocytes from PBL and thymus, obtained from seven healthy individuals, and two patients (Table I). The Vγ9-Vδ2 clones KOBA70 and 72 were derived from a TCRγδ lymphocyte population which selectively proliferated to Daudi cells. The Vγ9-Vδ2 clones N-4, 1042, and 1005 were generated without specific stimulation. The Vγ9-Vδ2 clone PG60 was derived from a TCRγδ lymphocyte population, stimulated with a mixture of EBV-BLCL (APD/BSM). Clones ThyCl5 and ThyCl10 were thymus derived and expressed a Vγ9-Vδ1 encoded TCR, and PJ4 reacted with none of the V region specific mAb. All TCRγδ clones expressed CD2, 3, 11a, 18, 25, and 26 in similar densities. The level of expression of CD16 and CD56 varied between individual TCRγδ clones, irrespective of V region usage or their origin. The CD8 antigen was expressed at low density on some Vδ1 clones, as reported [39].

The use of clones obtained from nine individuals and the use of distinct stimulator cells to generate these TCRγδ clones excludes the possibility that the selective proliferation of Vγ9-Vδ2 lymphocytes upon Daudi cell stimulation is a result of an antigenic proliferation of a single precursor cell.

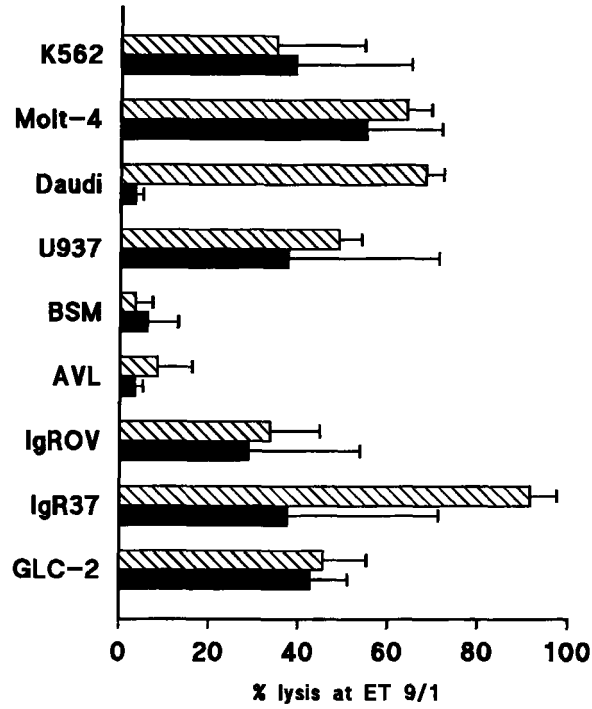
Coordinated expression of the Vγ9-Vδ2 gene-segments correlates with the capacity to lyse Daudi cells

It has been reported that TCRγδ clones can mediate MHC unrestricted cytolysis when activated in vitro [3,11-15]. Therefore we analysed the cytolytic reactivities of Vγ9-Vδ2 and Vδ1 clones against a panel of tumor target cell lines of distinct histologic origin. In Fig.2 the mean percentage of MHC unrestricted lysis exerted by a representative panel of TCRγδ clones against a number of tumor cell types is shown. All the TCRγδ clones exerted MHC unrestricted cytolytic activity, although, the level of MHC unrestricted lysis of different tumor cell lines varied between individual TCRγδ clones. Cytolytic activity against EBV transformed B-LCL was relatively weak. Overall, Vδ1 clones displayed a lower MHC unrestricted cytolytic activity than Vγ9-Vδ2 clones [13-14]. Noteworthy, Daudi cells were exclusively lysed by all Vγ9-Vδ2 clones but were not lysed by any of the Vδ1 clones. The Vγ9-, Vδ2-, Vδ1- clone PJ4 and the thymus derived Vγ9+-Vδ1+ clones ThyCl5 and ThyCl10 did not lyse Daudi cells (Fig. 3). No other tumor cell line tested (out of a panel of eighteen) showed such an absolute distinction in susceptibility to lysis by Vγ9-Vδ2 and Vδ1 clones.

The presence or absence of specific lysis of Daudi cells appeared independent of the type of stimulator cells used to generate and maintain the Vγ9-Vδ2 clones. Thus, the Vγ9-Vδ2 clones that were never stimulated with Daudi cells before the cytotoxicity assay, specifically lysed Daudi cells equally well as Daudi cell primed Vγ9-Vδ2 clones, e.g. clones N-4, 1042, PG60, and KOBA70, KOBA72 respectively (Fig. 3).

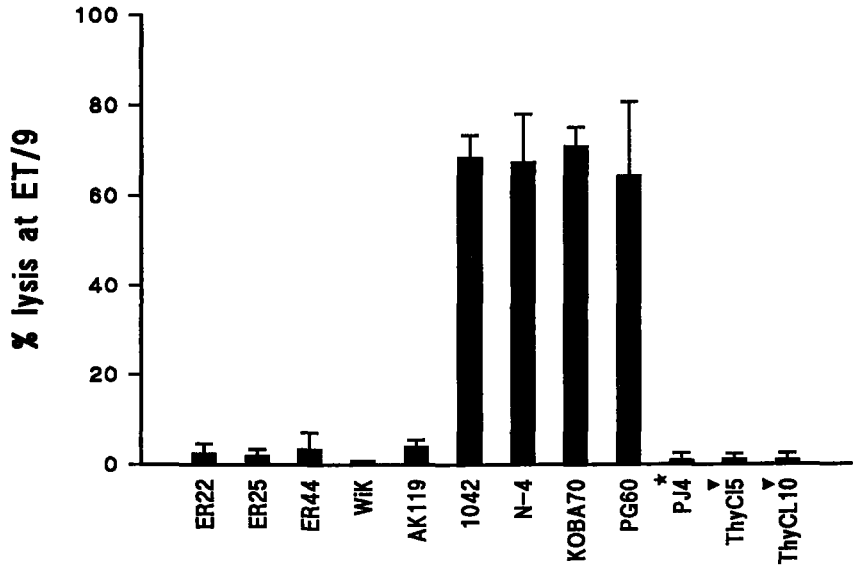


LEGEND fig 1b: Daudi cell induced proliferation of fresh TCRγδ lymphocytes is confined to the Vγ9-Vδ2 subset.
Donor A: On day 0, before Daudi cell stimulation, the total TCRγδ lymphocytes population contained 25% Vδ1 (δTCS-1), and 75% Vγ9 (TiγA) -Vδ2 (BB3) positive lymphocytes. Donor B: On day 0, before Daudi cell stimulation, % Vγ9 (TiγA) -Vδ2 (BB3) positive lymphocytes comprised more than 90% of the total TCRγδ lymphocytes population. Unfractionated PBL were cultured during a 7 day period with irradiated Daudi cells and thereafter stained with either TiγA (Vγ9), BB3 (Vδ2) or δTCS-1 (Vδ1) followed by subclass specific GAMIg conjugated with either PE or FITC. Subsequently, lymphocytes were stained with TCRγδ-1 (reactive with all TCRγδ lymphocytes) conjugated with either FITC (TiγA) or PE (BB3, δTCS-1). Green/ red fluorescence histograms representing the Vγ9-Vδ2 and Vδ1 profiles of the gated lymphocytes (TCRγδ) are shown.



LEGEND fig.2: MHC unrestricted cytotoxic activity of a panel of TCRγδ clones.
▨- Mean % lysis ± SD of five Vγ9-Vδ2 clones in the same experiment.
■- Mean % lysis ± SD of five Vδ1 clones in the same experiment.
* Cytolytic activity was measured in a standard 3 hr ^{51}Cr release assay.

Resistance of Daudi cells to lysis by Vδ1 clones is not due to the absence of HLA-class I molecules on Daudi cells, because of a defective β2m synthesis. This can be concluded because Daudi cells transfected with β2m cDNA, which restored HLA class I expression [40], were also resistant to lysis by Vδ1 clones (data not shown). Moreover, other HLA class I negative cell lines, e.g. K562 and GLC-2E, were susceptible to MHC unrestricted lysis by Vδ1 clones (Fig. 2).



LEGEND fig.3: MHC unrestricted lysis of Daudi cells by TCRγδ clones.
Vγ9-Vδ2 clones; 1042, N-4, KOBA70, and PG60, five Vδ1 clones; ER22, ER25, ER44, WiK, AK119, one Vγ9-Vδ2-, Vδ1 negative clone; PJ4, and two Vγ9-Vδ1 clones; ThyCl5 and ThyCl10.
Data presented as the mean percentage lysis ± SD of five experiments.
*: Vγ9-Vδ2,Vδ1 negative clone PJ4, data presented as the mean percentage lysis ± SD of two experiments at an effector/target cell ratio of 10/1.
▼: Vγ9-Vδ1 clones ThyCl5 and ThyCl10, data presented as the mean percentage lysis ± SD of two experiments.

Nucleotide diversity at the junctional region of the TCR-Vδ2 gene-segment does not contribute to Daudi cell specificity.

TCR diversity arises from both combinatorial and junctional diversity. As shown, all TCRγδ lymphocytes expressing the Vγ9-Vδ2 encoded TCR specifically respond to Daudi cells. TCRγδ clones generated from PBL from different donors have not descended from a single precursor cell. However, all Vγ9-Vδ2 clones may express identical TCRγδ chains. Therefore, the nucleotide sequences at the V-D-J junction of the TCR-δ chain of four Vγ9-Vδ2 clones were determined (Table II). The nucleotide sequences of these four Vγ9-Vδ2 clones were found to be diverse. Furthermore, three of the Vγ9-Vδ2 clones used the Jδ1 gene-segment, whereas the fourth Vγ9-Vδ2 clone used the Jδ4 gene-segment. Extrapolating from the results obtained with these four TCRγδ clones, we conclude that most if not all Vγ9-Vδ2 clones are derived from distinct precursor cells; express a distinct TCR, and all have Daudi cell specificity.

TCRγδ clone	Seq.	Vδ2	N D N	Jδ
1004	Vδ2\Jδ1	TACTGTGCCTGTGAC	ACCCTGGGAGGT	AACACCGATAAACT
1018	Vδ2\Jδ1	TACTGTGCCTGTGAC	AACATGGGGGAT	AACACCGATAAACT
1019	Vδ2\Jδ1	TACTGTGCCTGTGAC	ACTGTAACTGCC	ACCGATAAACT
N-4	Vδ2\Jδ4	TACTGTGCCTGTGAC	ACGCTGGGGGATAAAGGGTCTTTGACAGCACAA	CTCTTCTTTGGA

LEGEND table II. Organization and nucleotide sequence of the V-D-J junction of the TCR-δ gene. Four Vγ9-Vδ2 clones were analysed, the V, and J elements are shown, as are the nucleotides representing the N-D-N sequences.

Daudi cells are not intrinsically resistant to lysis by Vδ1 clones

The inability of Vδ1 clones to lyse Daudi cells could be due to an intrinsic resistance of Daudi cells to lysis by Vδ1 clones. Therefore we used TCR/CD3 specific mAb to provide optimal crosslinking of TCR/CD3 complexes via the FcγR expressed on Daudi cells. This crosslinking resulted in efficient lysis of the Daudi cells by Vδ1 clones [41-42] (Fig. 4). These results prove that Daudi cells can be lysed by the Vδ1 clones, when these clones are activated via the TCRγδ/CD3 complex. Thus Daudi cells are not intrinsically resistant to lysis by Vδ1 clones (Fig.4). Identical to the results obtained with peripheral blood TCRγδ lymphocytes, also cloned TCRγδ lymphocytes, with Daudi cell specificity belong exclusively to the Vγ9-Vδ2 lymphocyte subset.

Vγ9-Vδ2 and Vδ1 clones conjugate equally well to Daudi cells

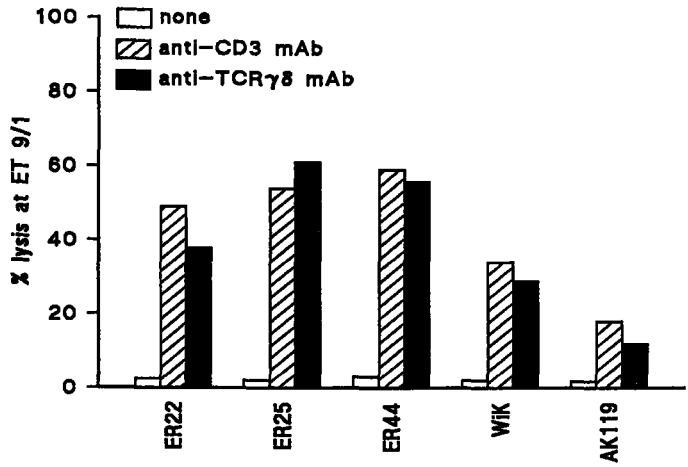
Conjugate studies were performed to investigate whether the resistance to lysis of Daudi cells by Vδ1 clones resulted from a lower binding capacity to Daudi cells as compared to the binding capacities of Vγ9-Vδ2 clones.Table III shows the percentage spontaneous and CD3 specific mAb induced conjugates between various TCRγδ clones and Daudi cells. The Vδ1 and Vγ9-Vδ2 clones form equally efficient spontaneous effector-/target cell conjugates with Daudi cells. There was no significant enhancement in the percentage of conjugates at a concentration of CD3 specific mAb sufficient to induce Daudi cell lysis by Vδ1 clones.

Thus the resistance to lysis of Daudi cells by Vδ1 clones was not due to a diminished capacity to form effector-target conjugates with Daudi cells.

Table III: Conjugate formation between TCRγδ clones and Daudi cells

	% conjugates between TCRγδ clones and Daudi cells	
Clone Vδ1	Daudi*	Daudi + anti-CD3 #
ER22 +	19	28
ER25 +	30	44
ER44 +	39	40
WiK +	26	26
AK119 +	28	35
Clone Vγ9-Vδ2		
N-4 +	40	33
PG60 +	23	30

LEGEND table III: - % conjugates was calculated as described in materials and methods.
* % spontaneously formed conjugates
\# % anti-CD3 mAb induced conjugates.



LEGEND fig.4: Induction of lysis by anti-CD3 and anti-TCRγδ mAb of Daudi target cells by Vδ1+ clones. Anti-CD3 mAb (CLB-T3/4.1) saturating concentration 1:10.000 as determined by fluorescence analysis) or anti-TCRγδ mAb TCRδ-1) used at a 0.22 μg/ml concentration were added at the onset of the ^{51}Cr release assay.

DISCUSSION

Despite the large potential combinatorial diversity of the TCRγδ gene-segments, the variety of functional gene combinations utilized by human TCRγδ lymphocytes in the periphery appears limited. We have investigated whether the use of a particular Vγ or Vδ gene rearrangement corresponds with specific proliferative and/or cytolytic capacities. Our study details four major findings: First, stimulation of PBL with Daudi cells results in a selective outgrowth of TCRγδ lymphocytes, restricted to the Vγ9-Vδ2 subpopulation only. Second, cloned Vγ9-Vδ2 but not Vδ1 lymphocytes specifically lyse Daudi cells. Third, nucleotide diversity at the junctional region of the TCR-Vδ2 gene does not contribute to this Daudi cell specificity. Fourth, all TCRγδ clones exhibit MHC unrestricted cytolytic activity, but on average the Vγ9-Vδ2 clones exert higher levels of MHC unrestricted lysis than the Vδ1 clones.

Proliferation of purified TCRαβ and TCRγδ populations can be sustained by stimulation with certain B-LCL [15,23]. We observed that stimulation of PBL with Daudi cells results in a selective expansion of Vγ9-Vδ2 TCRγδ lymphocytes. Stimulation of PBL with a mixture of two other B-LCL, i.e. APD and BSM, does not result in a selective expansion of TCRγδ lymphocytes. Moreover, Vγ9-Vδ2 clones obtained from PBL cultures stimulated with APD and BSM do not lyse the APD or BSM cell lines, whereas all of them specifically lyse Daudi cells. These results show that the Daudi cell specificity of Vγ9-Vδ2 clones is independent of the stimulator cell. The data also suggest that the antigenic structure on Daudi cells, which is specifically recognized by Vγ9-Vδ2 lymphocytes, is not expressed by the APD and BSM cell lines.

The Daudi cell specificity of fresh Vγ9-Vδ2 lymphocytes may result from an antigen driven proliferation of a single precursor cell. However, our TCRγδ clones were derived from nine individuals, excluding a single precursor cell origin of the Vγ9-Vδ2 clones. Nevertheless, the Vγ9-Vδ2 clones obtained from these nine individuals may express identical TCRγδ chains. Human TCRγδ lymphocytes in the periphery express extensive junctional diversity at the V-(D)-J joints of functionally rearranged TCR genes [43-44]. Four of our Vγ9-Vδ2 clones were analysed for junctional diversity. The N-nucleotides used in the junctional sequences of the Vδ2 gene-segment of the four clones were all different. Moreover Vγ9-Vδ2 clones derived from multiple other individuals all show distinct Vγ junctional sequences (Dr. M.-P. Lefranc, personal communication). Therefore, we conclude that 1:) most if not all Vγ9-Vδ2 clones express a distinct TCR but all specifically lyse Daudi cells and 2:) the nucleotide diversity at the junctional regions of TCRγ and TCRδ chains is not involved in Daudi cell specificity.

In vitro activated TCRγδ lymphocytes possess two mechanisms by which they can exert cell mediated cytotoxicity. Firstly activated TCRγδ lymphocytes display MHC unrestricted cytolysis [3,8-15], that supposedly does not involve the TCR as a recognition structure [16]. Secondly, recognition of target may be directly mediated via the TCR [4,20]. Because, it is well established that Daudi cells are susceptible to MHC unrestricted lysis by activated CD3- lymphocytes [27,45], it remains to be ascertained whether the TCR of Vγ9-Vδ2 clones or other membrane structures account for the specific recognition of Daudi cells. Adhesion cell surface molecules such as CD2, CD11a/18 and LFA-3 are equally expressed on Vγ9-Vδ2 and Vδ1 clones [45]. It is therefore unlikely that these adhesion molecules give rise to the differences in Daudi cell lysis by Vγ9-Vδ2 and Vδ1 clones. Vγ9-Vδ2 and Vδ1 lymphocytes have been reported to differ in their cell surface matrix composition, which is reflected by differences in their respective adherence and motility capacities [46]. Different capacities in their respective recognition mechanisms could represent a further reflection of these matrix composition differences resulting in different target cell repertoires [47]. However, our finding that freshly isolated Vγ9-Vδ2 PBL, that do not display MHC unrestricted cytolysis [10], specifically proliferate to Daudi cells but not to other B-LCL or K562 cells supports the concept of direct TCRγδ involvement in the recognition of Daudi cells.

Monoclonal antibody mediated blocking or modulation studies of the TCR do not allow to study this involvement of the TCR. Fab_2 fragments of TCRγδ or CD3 specific mAb do not only inhibit TCR mediated lysis but also MHC unrestricted cytolysis, possibly through the delivery of an overall negative signal to the lymphocyte resulting in inhibition of cytolysis [11]. Indeed, we observed that Fab_2 fragments of TCRγδ or CD3 specific mAb not only inhibited the cytolysis of Daudi cells but also that of other target cells such as K562 by Vγ9-Vδ2 clones (data not shown) [11].

What is the nature of the antigen expressed on Daudi cells that is specifically recognized by Vγ9-Vδ2 lymphocytes? The CD10 (CALLA) antigen, is unlikely to serve as the target antigen because, although Raji and K562 cell lines express CD10 [48], they do not specifically activate Vγ9-Vδ2 lymphocytes. Our preliminary blocking studies with anti-MHC-class II mAb do not suggest a role for the MHC-class II antigens in Daudi cell recognition. MHC class I and CD1 molecules, reported to serve as specific TCRγδ ligands [19-20,49], are not expressed on Daudi cells [40,50].

The specific proliferation to and cytolysis of Daudi cells of the entire subpopulation of Vγ9-Vδ2 lymphocytes is reminiscent of a superantigen response, as described for Vβ6 and Vβ8.1 lymphocytes to Mls and for Vβ8 or Vγ9 lymphocytes to Staphylococcal enterotoxins [51-53]. In superantigen stimulation, the Vβ8 or the Vγ9 encoded TCRγ chain in itself is

sufficient to impose superantigen specificity, i.e. independent of the Vα or Vδ usage in the associated TCRα or TCRδ chain [51-53]. When analogy would exist then the Vγ9 chain in itself is expected to dictate Daudi cell specificity. However, we found that Vγ9-Vδ1 clones of thymic origin do not lyse Daudi cells, implying that the Vγ9 encoded TCRγ chain in itself is not sufficient to dictate Daudi cell specificity. Therefore either the expression of a Vδ2 encoded TCRδ chain alone is sufficient to impart Daudi cell specificity or alternatively, the combination of Vγ9 and Vδ2 encoded TCR chains.

Recently evidence has accumulated to indicate that TCRγδ lymphocytes recognize ligand(s) expressed by mycobacteria (our data not shown) and [17,21,54-55]. This reactivity is widely thought to be TCRγδ mediated. In view of the conserved nature of mycobacterial and/or autologous heat shock proteins (HSP) and the reported expression of the latter on the membrane of certain B-LCL [56], makes HSP or related molecules likely candidates for the Daudi cell structure recognized by Vγ9-Vδ2 lymphocytes. Specificity of the Vγ9-Vδ2 lymphocyte subset in the periphery for autologous HSP would then explain the overrepresentation of these lymphocytes at sites of autoimmune reactions, where HSP are abundantly present [21]. Vγ9-Vδ2 lymphocytes may then function in the elimination of stressed cells, either by lymphokine production to elicit a cytolytic reponse or by directly killing them.

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CHAPTER IV

RECOGNITION BY HUMAN Vγ9/Vδ2 T CELLS OF A GROEL HOMOLOG ON DAUDI BURKITT'S LYMPHOMA CELLS

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SUMMARY

All human γδ T cells coexpressing the products of the variable (V) region T cell receptor (TCR) gene segments Vγ9 and Vδ2 recognize antigens from some mycobacterial extracts and Daudi cells. Exogenous and endogenous ligands on the cell surface, homologous to the groEL heat shock family, induced reactivities that resembled superantigen responses in this major subset of human peripheral blood γδ T cells. Stimulation of human Vγ9/Vδ2 T cells is not restricted by human leucocyte antigens (HLA), including non-polymorphic β_2-microglobulin (β_2m) associated class Ib molecules. These data may be important for understanding the role of γδ T cells in autoimmunity and in responses to microorganisms and tumors.

INTRODUCTION

A subset of T cells expresses the γδ T cell receptor (TCR) [1] and has limited variable (V) region repertoire [2]. Antigens that stimulate γδ T cells are only now being identified [3], and it is not clear whether they need to be processed and presented as is the case for αβ T cells [4]. The non-classical major histocompatibility complex (MHC) class Ib molecules also have limited polymorphism and have been proposed as antigen presenting molecules for γδ T cells [5]. Both murine [6,7] and human [8] γδ T cells can respond to mycobacterial extracts. In the mouse, the 65-kD mycobacterial heat shock protein (hsp65), and peptides from it can stimulate γδ T cells [6]. Hsp65 is homologous to the groEL protein of E.coli and similar stress proteins in prokaryotic and eukaryotic cells [9,10]. Examination of over 2000 human γδ T cells clones revealed that all of those expressing both Vγ9 and Vδ2 [11,12] are particularly efficient killers of the Daudi Burkitt's lymphoma cell line [13]. In this study, we examined the proliferative responses of human γδ T cells to endogenous and exogenous antigens. The proliferative response of human Vγ9/Vδ2 T cells to Daudi cells and bacterial extracts could be inhibited by antiserum to the mammalian groEL related heat shock proteins (hsp58 or hsp60) [14]. This serum immunoprecipitated a molecule of corresponding molecular size from the surface of Daudi cells. We found that, unlike previous results, β_2m binding MHC proteins are unlikely to be involved in the presentation of antigen to human Vγ9/Vδ2 T cells.

RESULTS & DISCUSSION

Initially, we tested our clones for their ability to proliferate in response to various cell lines and to mycobacteria (Table 1). The Vγ9/Vδ2 clones, but none of the other, were stimulated by the Daudi cell line and by some preparations of sonicated mycobacteria [15], when presented by appropriate antigen presenting cells (APCs). This was the first exposure of the clones to Daudi cells or mycobacterial antigens in vitro [16]. Lymphoblastoid B cell lines (LCLs) other than Daudi cells stimulated the Vγ9/Vδ2 clones only in the presence of the mycobacterial extracts (Table 1). However, intracellular processing of the mycobacterial extracts was not necessary; because paraformaldehyde fixation of the APC did not eliminate stimulation by the mycobacterial antigens (Fig.1). Allogenic mutant LCLs that do not express polymorphic human leucocyte antigens (HLA) class I (LCL 721.221) or class II (LCL 721.180) determinants [17], the parent nonmutated LCL 721, and Daudi cells [18] presented the mycobacterial antigens to the Vγ9/Vδ2 clones (Fig.1). Daudi cells do not express β_2m-associated HLA class I proteins because the translated class I α-chains in Daudi are not transported to the cell surface due to a mutation of the β_2m mRNA initiation codon [18]. A Daudi variant that expresses cell surface HLA class I [18] was as stimulatory for the human Vγ9/Vδ2 T cells as the parent Daudi cell line (Table 1). Thus, the response to Daudi was not associated with the absence of HLA class I antigens on the cell surface. However, other APCs (such as K562, Molt4, or Raji cells), independent of their surface class I or class II expression did not present mycobacterial antigens to the γδ T cells (Fig.1). Daudi cells presented mycobacterial antigens, even after adaptation to serum-free medium and fixation (Fig.1). This excludes the possibility that serum β_2m allowed the transport of HLA class I molecules to the Daudi cell surface. Thus, the antigens for the Vγ9/Vδ2 T cells do not require presentation by conventional polymorphic or β_2m-associated MHC molecules. This would be consistent with the absence of CD4 or CD8 accessory molecules on most γδ T cells, and the normal development of γδ T cells in β_2m-deficient mice [19].

We then examined the reactivity of fresh peripheral blood lymphocytes (PBL) to the antigens that stimulate the Vγ9/Vδ2 clones. As determined by flow cytometry, both the mycobacterial preparation and Daudi cells, but not Candida antigens or Raji cells, induced strong proliferation of human peripheral blood Vγ9/Vδ2 T cells (Fig. 2). This indicates that the immunological reactivity of Vγ9/Vδ2 T cells was not a phenomenon detected only after in vitro culture of γδ T cell clones with interleukin-2 (IL-2).

Human γδ T cells also expanded and Vγ9/Vδ2 clones were stimulated by some Escherichia coli extracts [20]. The reactivity of Vγ9/Vδ2 T cells to Daudi and mycobacterial antigens was detected in both tuberculin skin test-negative and positive individuals. Daudi stimulation

Simulator	Responder clone						
	BT60	FW1	GPE5	GPC4	PJ4	TS4B	AD4
Medium	537 ± 71	328 ± 91	468 ± 171	544 ± 77	285 ± 14	779 ± 26	644 ± 17
IL-2	NT	7,476 ± 136	13,350 ± 161	11,897 ± 156	62,802 ± 881	8,276 ± 257	18,795 ± 271
Anti-CD3 MAb	19,874 ± 1,973	13,103 ± 1,791	12,602 ± 508	10,951 ± 252	14,484 ± 580	17,593 ± 417	9,252 ± 103
Daudi	16,330 ± 551	6,765 ± 198	12,784 ± 231	8,968 ± 138	1,197 ± 37	792 ± 206	942 ± 103
β_2M-Daudi	NT	7,017 ± 200	14,253 ± 783	9,397 ± 242	1,500 ± 156	NT	328 ± 32
Raji	585 ± 275	300 ± 115	812 ± 353	423 ± 35	1,281 ± 105	772 ± 186	233 ± 92
LCL 68	903 ± 119	280 ± 127	521 ± 196	323 ± 26	5,291 ± 316	872 ± 76	1,421 ± 23
LCL 68 + C	NT	261 ± 238	123 ± 37	78 ± 31	NT	923 ± 75	1,041 ± 49
LCL 68 + H	8,930 ± 1,866	6,160 ± 149	16,752 ± 566	12,427 ± 513	3,122 ± 177	624 ± 85	1,166 ± 63

*C, Candida; H, H37Rv

Table I. Specific proliferation of Vγ9-Vδ2 clones to Daudi cells and mycobacterial extracts. Numbers represent [^{3}H] thymidine incorporation (mean count per minute + standard error of the mean of quadruplicates). The proliferative assays are described [30]. The expression of the V regions was determined with mAbs to Vγ9, Vδ2, and Vδ1 [11]. Representative are the Vγ9/Vδ2 T cell clones BT60, FW1, GPE5, and GPC4, other γδ T cell clones TS4B and PJ4 and αβ TCR clone AD4. Proliferation was measured in medium alone, in the presence of IL-2 (500 units/ml), surface immobilized mAb CD3 (OKT3), or diverse target cell lines (Daudi, β2m-Daudi is the HLA class I+ Daudi variant after transfection with the mouse β2m gene [18]; LCL68 is representative for other LCL lines studied. Stimulation by LCL68 was measured in the absence or presence of the H37Rv mycobacterial antigens [15] or Candida antigens at concentrations determined as optimal in preliminary experiments. Control PBL cultures primed to H37Rv and Candida showed specificity for these antigens. NT= not tested, The Vγ9-Vδ2 clones but not the other clones lysed Daudi cells in cytotoxicity assays although all clones could lyse susceptible targets [13]

of umbilical cord blood mononuclear cells (less than 0.1% γδ T cells, predominantly Vδ1+ [12,21]), induced expansion of γδ T cells that coexpressed Vγ9/Vδ2 genes (50 to 80 percent Vγ9/Vδ2 T cells three weeks after two sequential exposures to Daudi cells in vitro). In addition, γδ T cells from Rhesus monkeys (reactive with the monoclonal antibody (MAb) TCRδ1 to human γδ TCR) proliferate in response to Daudi (but not Raji) cells [22]. These observations suggest that the reactivity of human γδ T cells to Daudi is displayed early during ontogeny and is conserved during phylogeny.

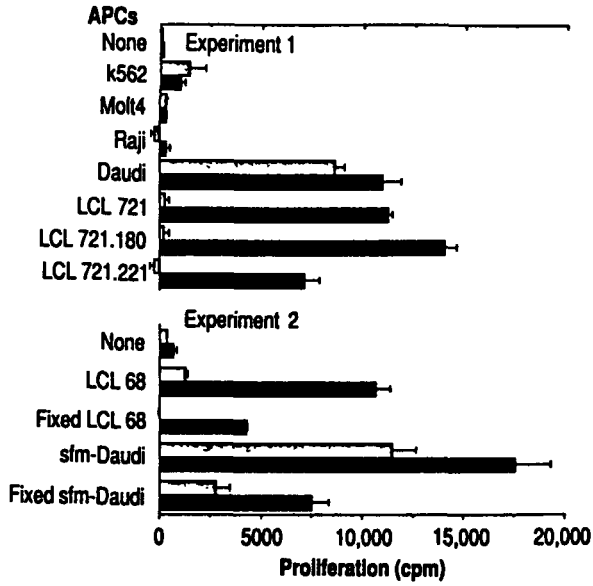

Fig. 1. Presentation of mycobacterial antigen to Vγ9/Vδ2 T cells by different APCs
Proliferation ([^{3}H] thymidine incorporation as mean counts per minute ± standard error of the mean of quadruplicates) of the clone GPC4 to diverse APCs in the absence (dotted bars) or presence (filled bars) of sonicated H37Rv mycobacteria [15]. The proliferative assays were performed as described [30]. APCs were K652, Molt4, Raji, Daudi cells, LCL 68 (The autologous LCL for clone GPC4), LCL 721, and variants from LCL721 that have partial deletions of the chromosome 6, LCLs 721.180 and 721.221. LCL 721.180 has reduced levels of HLA class I on the surface and does not express class II encoded by the DR, DP, and DQ loci, whereas LCL 721.221 does not express HLA class I antigens A, B of C [17]. Daudi cells that had been adapted to serum-free medium (HL-I, Ventrex Laboratories) before the assay are designated sfm-Daudi. All proliferative assays were performed in the presence of 10% human serum. LCL 68 and sfm-Daudi cells were fixed by 5 min incubations in 0.15% paraformaldehyde [23]. Similar results with these APCs were obtained with other Vγ9/Vδ2 clones in six independent experiments. The antigen presenting capacity of LCL 721, LCL 721.180, and LCL 721.221 were comparable, whereas some other LCL lines were less effective as APCs. The sfm-Daudi cells were lysed by the Vγ9/Vδ2 clones equally well as the Daudi cells, grown in serum containing medium, even when the cytotoxicity assays were performed in serum-free medium.

Because hsps are a major antigenic component of mycobacterial extracts, we tested polyclonal rabbit antisera specific for the hsp70 and groEL hsp families for inhibition of the proliferative response of the Vγ9/Vδ2 T cells to Daudi. The proliferation of the peripheral blood γδ T cells and the Vγ9/Vδ2 clones to Daudi was suppressed by the hsp58-specific antiserum [14], but not by hsp70-antisera [14,23] or nonimmunune sera (Fig.3). Thus, Vγ9/Vδ2 T cells recognize a ligand on the cell surface of Daudi cells related to the groEL hsp family.

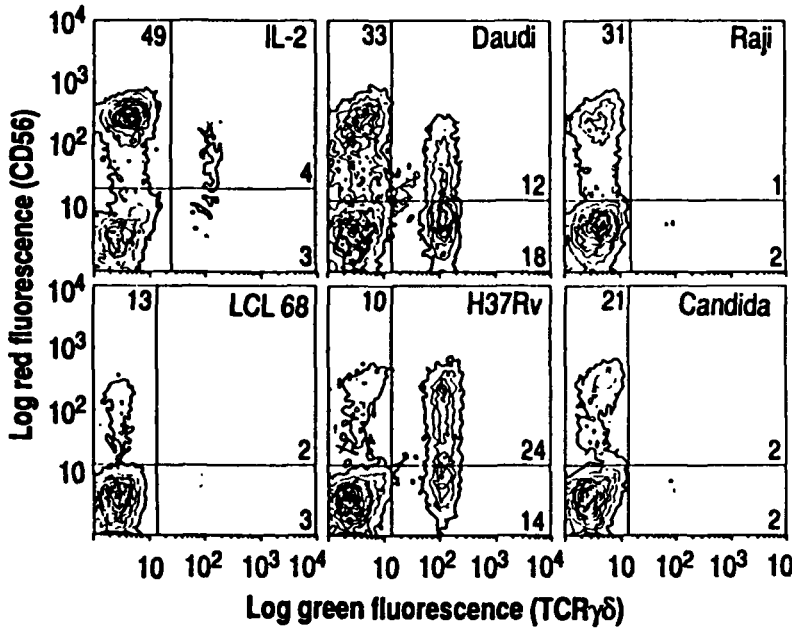

Fig.2 Flow cytometric analysis of the proliferation of human peripheral blood Vγ9/Vδ2 T cells after stimulation with Daudi cells or mycobacterial antigens.
PBLs (10^6/ml) were stimulated with IL-2 (100 units/ml), irradiated Daudi, Raji, or LCL 68 cells (all at $2x10^5$/ml), extracts from H37Rv mycobacteria [15] or Candida. On day 7, cells from all cultures were labeled with mAbs and 10,000 events per sample were analyzed on a FACStar^{plus} (Becton-Dickinson) using four decade log signal amplification. All viable cells were gated on forward scatter and by exclusion of propidium iodide (1 μg/ml). Two color analysis with mAbs to the γδ TCR (TCRδ1), conjugated to fluorescein isothiocyanate (green fluorescence: x-axis), and mAbs to CD56 (Leu19), conjugated to phycoerythrin (red fluorescence: y-axis), is shown. A marked expansion of human peripheral blood γδ T cells (right upper and right lower quadrants) was seen after stimulation with Daudi and the H37Rv preparation but by none of the other antigens. The cells from the left upper quadrants were identified as NK cells and in the left lower quadrants as αβ T cells, by parallel staining with other mAbs. The percentages of cells in the quadrants are indicated. The γδ T cells expanded by Daudi and mycobacteria coexpressed Vγ9 and Vδ2, but they did not express Vδ1, as determined by the Mabs described [11].

This is consistent with the specific recognition of Daudi cells by Vγ9/Vδ2 clones in cytotoxicity assays [13] and with the lack of proliferation-inducing activity in cell-free Daudi culture supernatants. Also, the expansion of human γδ T cells from PBL induced by bacterial antigens could be markedly inhibited by the hsp58 antiserum (Fig.3), suggesting that the Vγ9/Vδ2 T cells recognize related endogenous ligands on Daudi and exogenous ligands from some bacterial preparations [14].

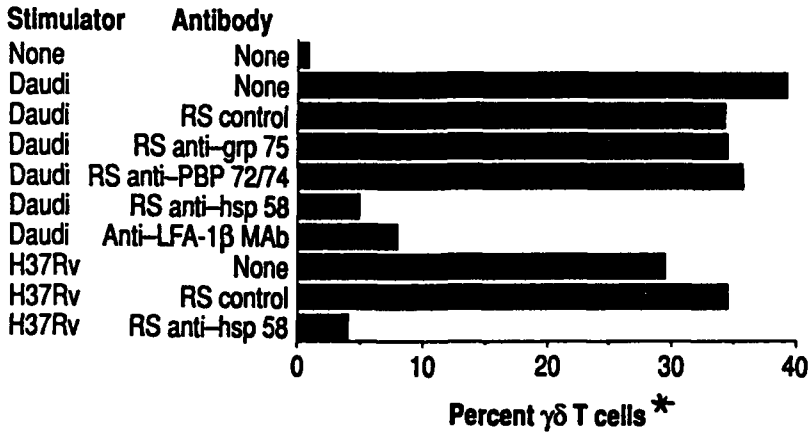

Fig.3 The proliferation of peripheral blood γδ T cells, induced by Daudi and mycobacteria, can be inhibited by antiserum against the mitochondrial hsp58.
*: % TCRγδ lymphocytes within the total lymphocyte yield.
PBL were activated with irradiated Daudi cells and sonicated mycobacteria (H37Rv) in the absence or presence of hsp58 antiserum and control antibodies. The antibodies were control non-immunune rabbit sera, rabbit antisera to the hsp70 family (PBP 72/74 [23] and grp75 [14]), rabbit antiserum specific to hsp58[14], and mouse mAb to LFA-1β (mAb TS1/18)[31]. The cultures were analyzed by flow cytometry. Only the hsp58 antiserum and the mAb to LFA-1β inhibited the expansion of γδ T cells. The hsp58 antiserum could also inhibit the proliferation of peripheral blood γδ T cells induced by E.coli extracts [14], but did not block the stimulation of γδ T cells by IL-2 or mAbs to CD3, the expansion of NK cells by Daudi, or the proliferation of Daudi cells. All rabbit sera were tested at a 1:40 dilution. The data were confirmed in three independent experiments.

We used the hsp58-specific antiserum to immunoprecipitate groEL related molecules from surface iodinated Daudi cell lysates. Two-dimensional gel-electrophoresis of the immunoprecipitate revealed the presence of 58-kD and 66-kD molecules (Fig.4). A similar complex of hsp58 and an hsp70 molecule is immunoprecipitated from the mitochondria of

internally labeled HeLa cells, using the same hsp58 antiserum [24]. To control for the surface-specificity of the iodination, we immunoprecipitated with a mAb to class I α-chain. We did not detect any iodinated class I α-chains (Fig.4C) in the same Daudi lysate, whereas the HLA class I α-chains were precipitated from the cytoplasm of biosynthetically labeled Daudi cells (Fig.4D) and from iodinated cells expressing HLA class I on their surface. Moreover, no ^{125}I-labeled actin was detected in the cell lysate from surface labeled Daudi cells (Fig.4, A to C) (actin binds non-specifically to the immunoglobulin coated beads, used for the immunoprecipitation), confirming the specificity of the surface iodination procedure.

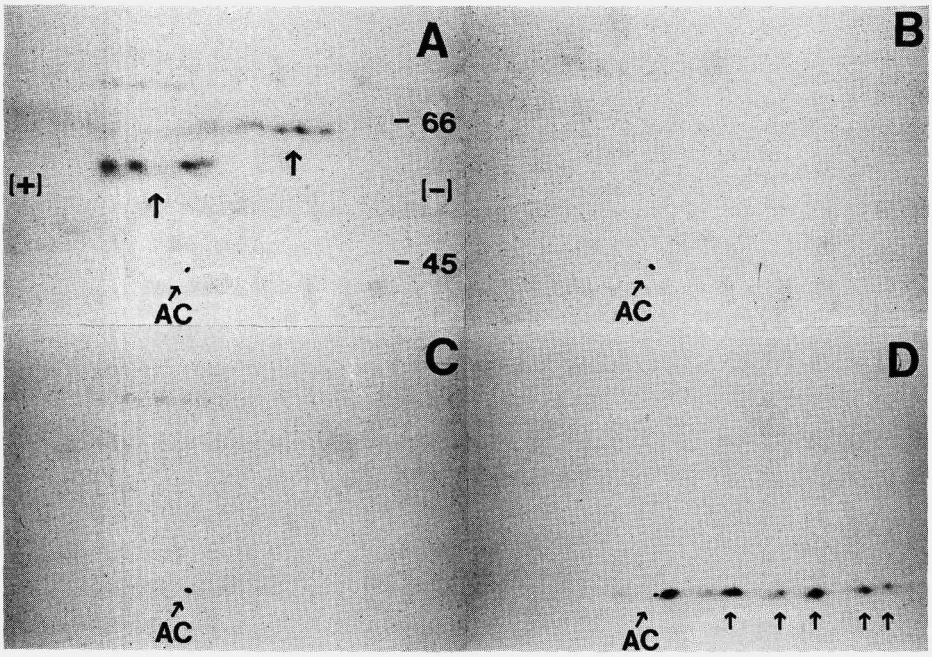

Fig. 4. Hsp58 can be detected on the cell surface of Daudi whereas HLA class Iα -chains are only detectable in the cytoplasm.
(A) surface labeled Daudi cells, immunoprecipitated with the hsp58 antiserum [14]; (B) surface labeled Daudi cells, immunoprecipitated with control, nonimmune rabbit serum; (C) surface labeled Daudi cells, immunoprecipitated with Mab Q1/28 that binds to the HLA class I α-chains in the absence of β_2m [32]; (D) metabolically labeled Daudi cells, immunoprecipitated with mAb Q1/28. AC is the position of actin; purified actin was added to the samples to aid in orientation of the spots in the second dimension. The gels are oriented from acidic (+) to basic (-). The arrows point to the immunoprecipitated proteins at 58 and 66 kD in (A) and to the HLA class I isoforms at 44kD in (D). For surface labeling, 2,5 x 10^7 Daudi cells were surface iodinated with ^{125}I (1.0 mCi) with Iodogen (Pierce Chemicals) [33]. The metabolic labeling with (^{35}S) methionine, immunoprecipitations, and 2-D gel analysis were done as described [34].

Although groEL hsps are intracellular proteins and as yet have not been characterized on the cell surface [9,10], the reactivity of T cells may provide a very sensitive method of their surface detection [25,26]. The groEL- related protein we detected at the cell surface could be a novel member of the groEL family or the known form of groEL could be transported to the cell surface with another molecule. An MHC class I-like molecule not binding β_2m [27] may function as a transport mechanism for groEL. It is also possible that the 66-kD molecule associated with hsp58 on the cell surface (Fig.4A) might be involved in the transport of groEL to the cell membrane. Molecules that bind and present endogenous antigens might also present homologous exogenous antigens to γδ T cells. Such determinants could be expressed at low levels [26] on APCs that stimulated γδ T cells in the presence, but not in the absence, of exogenous antigens.

Thus, all human T cells coexpressing Vγ9 and Vδ2 genes proliferate in response to Daudi cells and to certain mycobacterial extracts, whereas the Daudi non-reactive γδ T cells (that is, the Vδ1 subset [12]) does not recognize mycobacteria. The ligands recognized by the Vγ9/Vδ2 T cells appear to be homologs of the groEL hsp family, either as endogenous ligands, expressed on the cell surface of Daudi, or as exogenous bacterial antigens that can be presented by APCs without antigen processing. Recognition by human Vγ9/Vδ2 T cells of these antigens does not require antigen presentation by β_2m-associated HLA class I or polymorphic HLA class II molecules. Most crude mycobacterial antigens do not stimulate the human γδ T cells effectively, and only a few preparations are markedly stimulatory [15]. Purified hsp65 antigens [15], including the groEL peptides that stimulated murine γδ T cells hybridomas [6], did not stimulate our human γδ T cells. Our results suggest that the stimulatory epitopes for human Vγ9/Vδ2 T cells may depend on the conformation of groEL molecules or their peptide fragments and on suitable presentation on the cell surface. Ineffective antigen presentation to γδ T cells [6] could explain why only few investigators found purified hsp65 preparations stimulatory for human mycobacterial reactive γδ T cells [8]. The proliferative responses of all Vγ9/Vδ2 T cells, but not other γδ T cells, to Daudi cells and mycobacterial antigens support the idea of TCR mediated antigen recognition. Because human peripheral blood γδ T cells have extensive junctional diversity of both the γ and δ chains [2], it is unlikely that this recognition is determined by the junctional diversity. Coexpression of Vγ9 and Vδ2 genes seems to be required for antigen responsiveness to Daudi and mycobacteria [28]. The requirement for specific V regions, irrespective of the diversity of junctional segments and the proliferation of the whole T cell subset in response to the same exogenous and endogenous antigens resemble superantigen responses of murine Vβ8 T cells to Staphylococcal enterotoxins and Mls [29]. Peripheral human Vγ9/Vδ2 cells are selectively expanded in the first ten years after birth, but thymic Vγ9/Vδ2 T cells remain

only a minor fraction of γδ T cells [12,21]. Our results indicate that endogenous and exogenous ligands, homologous to groEL hsps, could be responsible for this in vivo expansion [21]. Recognition of groEL-related proteins by T cells may play a role in the pathogenesis of infectious and autoimmune diseases [9,10,25] and could explain the reactivity to the Daudi lymphoma. Therefore, the Vγ9/Vδ2 T cell subset may participate in diseases of infectious, autoimmune, and neoplastic origins.

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16. The clones were derived by sorting human γδ T cells from peripheral blood with various mAbs [11] to the γδ TCR with a FACStar plus (Becton Dickinson). The sorted cells were cloned and expanded with LCL (such as LCL 68) and PBL feeder cells as described [13], but never with Daudi cells or bacterial antigens.

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28. All Vγ9/Vδ2 T cells react strongly to these antigens, whereas the majority of Vδ1+ (including Vγ9/Vδ1) T cells do not. Preliminary results suggest that very rare Vγ9+/Vδ1+ T cell clones [as determined by mAbs (11)] may react with lower affinity.

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CHAPTER V

EXPRESSION OF CD45-ISOFORMS BY FRESH AND ACTIVATED HUMAN γδ T LYMPHOCYTES AND NATURAL KILLER CELLS

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SUMMARY

Naive and primed αβ T cells can be distinguished on the basis of their differential expression of CD45RA and CD45RO, respectively. The present study indicates that these CD45-isoforms also identify naive and primed maturational stages of γδ T cells and natural killer cells. In peripheral blood, all Vγ9-Vδ2 T cells reportedly express CD45RO whereas all Vδ1 T cells lack CD45RO. Here, we show that these CD45RO- Vδ1 T cells all express CD45RA and the CD45RO+ Vγ9-Vδ2 T cells lack the expression of CD45RA. The Vδ1 T cells acquired CD45RO expression and lost part of their surface CD45RA, following in vitro activation with PHA or IL-2. Also the CD3-, CD16+ NK cells in peripheral blood, that are uniformly CD45RA+, CD45RO- completely converted to the CD45RA-, CD45RO+ phenotype upon in vitro activation. Moreover, all cloned Vγ9-Vδ2 and Vδ1 T cells and NK cells express CD45RO and lack the expression of CD45RA. Our results strongly suggest that CD45RA and CD45RO are genuine markers for naive and primed lymphocytes that represent distinct differentiation lineages.

INTRODUCTION

CD45 is a family of high molecular weight glycoproteins expressed by all haemopoietic cells except mature erythrocytes [1]. The CD45 family comprises four isoforms of different molecular weight (180-220 kDa) [2,3]. The 180 kDa isoform, designated CD45RO, can be identified with the monoclonal antibody (mAb) UCHL-1 [4]. The 220 kDa isoform, defined as CD45RA, is recognized by mAb such as 2H4 and Leu-18 [5]. Human CD4+ or CD8+ αβ T lymphocytes can be divided into two reciprocal subsets on the basis of a differential expression of these two isoforms of CD45 [6,7]. This phenotypic division correlates with functional diversity, e.g. the frequency of T lymphocytes responding to recall antigens is much lower in the CD45RA+ than in the CD45RO+ lymphocyte subset, both among CD4+ and CD8+ T cells [8,9]. CD45RA+ T lymphocytes are also hyporesponsive to anti-CD3 or anti-CD2 monoclonal antibody (mAb) stimulation as compared to CD45RO+ T lymphocytes [10,11]. Following polyclonal activation, CD45RA+ T lymphocytes gradually lose CD45RA and acquire CD45RO expression, indicating that CD45RA and CD45RO represent different stages of maturation [6,10,12]. These data led to the conclusion that naive αβ T lymphocytes express CD45RA and lack the expression of CD45RO whereas previously activated (primed) αβ T lymphocytes are instead CD45RA-, CD45RO+.

A minor population of T lymphocytes (0-5%) expresses a T cell receptor (TCR) composed of a γ and a δ chain [13,14]. γδ T lymphocytes are present in all lymphoid organs, epithelial

cell layers and blood [15]. γδ T lymphocytes in peripheral blood can be dissected into two major non-overlapping subsets on the basis of their use of Vγ and Vδ gene segments. The major subset of γδ T lymphocytes expresses the Vγ9-Vδ2 encoded TCR, whereas the minor subset expresses a δ chain encoded by the Vδ1 gene segment in combination with a γ chain encoded by a Vγ gene segment from the VγI subgroup [16,17]. Natural killer (NK) cells are a heterogeneous population of large granular lymphocytes defined by their ability to lyse NK-susceptible target cells, such as K562, in a non-MHC restricted way without prior sensitization [18]. NK cells do not rearrange or productively transcribe TCR genes nor do they express the CD3γ and δ transcripts [19,20]. In the present study, we analyzed the expression of CD45RA and CD45RO by γδ T lymphocytes subsets and by CD3$^-$, CD16$^+$ NK cells, prior to and after their activation in vitro. Our results suggest that also for γδ T lymphocytes and NK cells, CD45RA and CD45RO are genuine markers for naive and primed cells respectively.

MATERIALS AND METHODS

Cells and media

Peripheral blood mononuclear cells were isolated from healthy donors by centrifugation of whole blood on Ficoll-Isopaque (Pharmacia Fine Chemicals, Uppsala, Sweden). Human thymus was obtained from children undergoing cardiac surgery at the Thorax Center, Academic Hospital Dijkzigt, Rotterdam. Thymus fragments were minced and squeezed over a nylon mesh to obtain single cell suspensions. αβ and γδ T lymphocyte and NK cell clones were generated by limiting dilution from PBL from healthy individuals and patients, as described [21-23]. All clones were expanded in culture medium: RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (4.8 g/l) (Gibco, BRL, Paisly, Scotland) supplemented with 25 U/ml recombinant human IL-2 (rIL-2) (Eurocetus, Amsterdam, The Netherlands), 4mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin and 1 μg/ml indomethacine in the presence of irradiated (2500 Rad) allogeneic PBL and lymphoblastoid B cell lines as feeder cells and 1 μg/ml leucoagglutinin (Pharmacia, Uppsala, Sweden), as described earlier [21].

Monoclonal antibodies (mAb)

Antibody reagents used for immunomagnetic depletions and immunofluorescence stainings were: anti-TCRαβ framework mAb, BMA031 [24]; anti-CD20 mAb 1F5; anti-TCRγδ framework mAb, TCR γδ-1 [25]; Vδ2 specific mAb, BB3 [26]; Vδ1-specific mAb, δTCS-1 (T-Cell Sciences, Cambridge, MA) [27]; FITC-conjugated CD45RO-specific mAb UCHL-1

(Dakopatts, Glostrup, Denmark) [4]; FITC-conjugated anti-CD3 mAb, anti-Leu-4, FITC-conjugated anti-CD45RA mAb, anti-Leu-18 [5] PE-conjugated anti-CD16 mAb, anti-Leu-11c (Becton and Dickinson, Mountain View, CA) and PE- conjugated goat anti-mouse Ab specific for IgG1 (GAM/IgG1/PE) (Southern Biotech., Birmingham, AL).

Lymphocyte activation

PBL were cultured in 24 well plates (Costar, Cambridge, MA) at a concentration of 1 x 10^6 cells/ml in a volume of 1 ml culture medium, for three days in case of stimulation with 1 μg/ml phytohemaglutinin (PHA) (HA16, Willcome, Dartford, England) or for three and seven days in case of stimulation with 1000 U/ml rIL-2. Three x 10^5 PBL/ml were also stimulated with 2 x 10^5 irradiated (2500 Rad) Daudi Burkitt lymphoma cells and 25 U/ml rIL-2 for seven days , as described [22].

Immunomagnetic depletion of lymphocyte subsets

PBL of healthy donors were depleted for αβ T and B lymphocytes. Pelleted PBL were incubated with saturating concentrations of anti-TCRαβ mAb BMA031 and anti-CD20 mAb 1F5 for 30 min at 4 °C. Cells were washed twice, 50 x 10^6 cells were resuspended in 250 μl RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) and mixed with an equal volume of two times diluted GAM-coated chromium dioxide particles (Dupont, Wilmington, DE). The mixture was incubated on a rotator at 4 °C for 1 hr to ensure proper contact between cells and particles. Immediately before magnetic separation, the mixture was diluted in RPMI + 10 % FCS to a volume of 10 ml, to avoid trapping of non-magnetized cells. Cells bound to the particles were removed by two immunomagnetic separation steps of 15 min using a Magnetic Particle Concentrator (Dynal, Oslo, Norway). The remaining non-magnetized cells were washed with PBS +1% BSA, checked microscopically for the absence of residual particals and subsequently stained for immunofluorescence analysis.

Immunofluorescence analysis of lymphocytes

Fresh, depleted or activated PBL were washed with PBS/BSA and stained either with anti-Leu-11c PE or with TCRγδ-1, (IgG1), BB3, (IgG1) or δTCS-1, (IgG1) for 30 min followed by GAM/IgG1/PE. After washing once, the GAM /IgG1/PE labeled cells were incubated with 10 μg/ml purified mouse IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) to block free GAM/IgG1/PE binding sites. Subsequently, cells were stained with either UCHL-1/FITC or anti-Leu-18/FITC for 30 min. followed by two washes. One sample of anti-Leu-11c stained cells was stained with anti-Leu-4/FITC to

determine the % CD3+ cells within the CD16 fraction. All incubations and washes were performed at 4°C. Two colour flow cytometry was performed on a FACS IV fluorescence activated cell sorter (Becton and Dickinson, Mountain View, CA). Viable lymphocytes were gated on the basis of their FSC and SSC characteristics. Data of 10.000 cells were collected and analyzed by FACscan software. To allow a better analysis of the CD45RO (UCHL-1) and CD45RA (anti-Leu-18) profiles of the relatively small NK or γδ T cell subsets in total PBL, the live gate was extended with FL2, (TCRγδ-1, BB3, δTCS-1, anti-LEU-11c) in such a way that only positive cells were included in the gate. Thereafter data on 2-10x10^3 gated cells were collected. Green fluorescence histograms, showing the UCHL-1 or anti-Leu-18 profiles of the gated lymphocyte subsets were made, using the FACScan software.

RESULTS

The Vδ1 and Vδ2 T cell subsets in peripheral blood selectively express CD45-isoforms

To analyze the expression of CD45-isoforms by human γδ T cells in peripheral blood, we enriched peripheral blood lymphocytes (PBL) from healthy donors for γδ T cells by depleting αβ T and B lymphocytes, using immunomagnetic negative selection. The remaining cells were 6-12 fold enriched for γδ T cells, sufficient to allow an accurate phenotypic analysis of the γδ T cell subsets. Cells were analyzed by 2 colour flow cytometry after staining with TCR-γδ – and CD45 isoform-specific mAb. In the majority of healthy individuals, Vγ9/Vδ2 T cells comprise 70-95% of all γδ T cells in peripheral blood [16]. Fig.1a shows the profiles representative for these donors. Analysis of the total peripheral γδ T lymphocyte subset showed that most γδ T cells express CD45RO, while the expression of CD45RA, was dull to absent. Analysis of the two peripheral γδ T cell subsets separately confirmed the observation by Parker et al. [28] that in PBL virtually all Vδ2 T cells express CD45RO whereas the entire Vδ1 T cell subset lacks the expression of CD45RO. Our observation that the expression of CD45RA is inversely proportional to the expression of CD45RO in these γδ T cell subsets extends their findings. To ascertain the expression of CD45 isoforms on Vδ1 T cells in peripheral blood, PBL of two selected donors, which comprised mainly Vδ1 T cells within the γδ T cell population, were analyzed (Fig.1b). Again, Vδ1 T cells were CD45RA+ CD45RO-, whereas the reciprocal staining pattern was observed for the Vδ2 T cell subset.

To exclude that the differential expression of CD45-isoforms on γδ T cell subsets was due to selection introduced by the magnetic particle separation, total PBL were also directly

double stained and analyzed by flow cytometry. To accurately analyze CD45-isoform expression on the small fraction of γδ T cells (<5%), only data of the γδ T cell subset of interest were acquired by setting a gate on either all γδ T cells or on Vδ2 or Vδ1 T cells only. The histograms displaying CD45RO expression of the gated γδ T cell subsets (Fig.2), confirmed the results obtained with the γδ T cell enriched fractions. The selective expression of CD45 isoforms on γδ T cells in peripheral blood was observed in all 8 PBL samples analyzed, irrespective of the PBL processing.

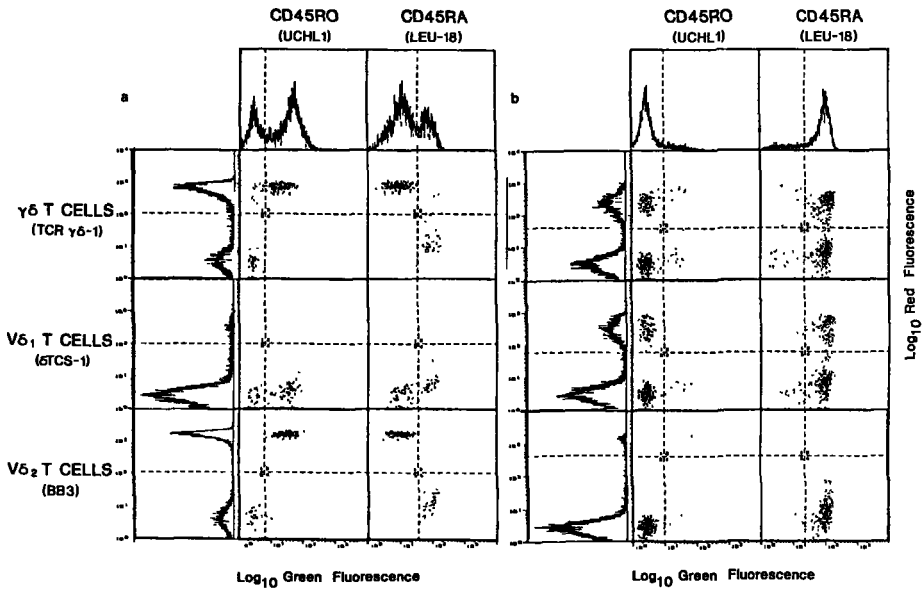

Fig.1. Expression of CD45RA and CD45RO on human γδ T lymphocytes in peripheral blood;
a, γδ T lymphocyte enriched PBL containing mainly Vγ9/Vδ2+ T lymphocytes;
b, γδ T lymphocyte enriched PBL containing mainly Vδ1+ T lymphocytes.
PBL were enriched for γδ T lymphocytes by depleting αβ T and B lymphocytes using mAbs BMA031 and IF5 and immunomagnetic separation. The remaining cells were stained with TCRγδ-1 (upper panel), BB3 (middle panel) or δTCS-1 (lower panel) followed by GAM/IgG1/PE and either UCHL-1/FITC or anti-Leu-18/FITC. Lymphocytes were distinguished from monocytes on the basis of their characteristic FSC and SSC profiles. Based on the Ig control samples and the single FL-1 and FL-2 histograms, the dot plots were divided into quadrants.

Vδ1 T cells acquire CD45RO following activation

The fact that CD45RA and CD45RO identify naive and primed lymphocytes of the αβ T cell population does not necessarily apply to other lymphocyte populations. PHA activation of αβ T cells results in a conversion of a CD45RA to a CD45RO phenotype [6,12]. When PHA activation of γδ T cells results in a similar conversion, then this would support the notion that CD45-isotypes represent primed or naive markers for the entire T cell population. Passively enriched γδ T cell fractions of PBL did not respond to PHA activation (data not shown), possibly because proliferation of γδ T cell subsets requires lymphokines produced by accessory cells. Therefore, total PBL were activated with PHA for 3 days, CD45RA and CD45RO expression of the gated γδ T cell fractions were compared before and after stimulation (Fig.2). PHA stimulation of total PBL resulted in a small, but reproducible, increase in the level of CD45RO expression on the naive, i.e. Vδ1 T lymphocytes. Because the acquisition of CD45RO on Vδ1 T lymphocytes is accompanied by a simultaneous decrease in CD45RA expression, the appearance of CD45RO is not due to the PHA-induced increase in cell size, but reflects an increased density of CD45RO on Vδ1 T lymphocytes. CD45RO expression on Vδ2 T lymphocytes remained uniformly positive before and after PHA stimulation.

Table I. CD45RA and CD45RO expression on cloned T lymphocytes and NK cells

Phenotype	number of clones tested	expression of CD45RA	CD45RO
TCRαβ /CD3+,4-,8+	2	-	++
TCRαβ /CD3+,4+,8-	3	-	++
TCRαβ /CD3+,4+,8+	2	-	++
TCRγδ /CD3+,Vγ9/Vδ2+	4	-	++
TCRγδ /CD3+,Vδ1+	10	-	++
TCR /CD3-,16+	3	-	++

Table 1. Cloned lymphocytes were stained with UCHL-1/FITC or anti-Leu-18/FITC

Because γδ T lymphocytes acquire MHC-unrestricted cytotoxic activity upon activation with IL-2 [13,29], we examined whether IL-2 alone could also induce a conversion of the CD45RA to the CD45RO phenotype on Vδ1+ T lymphocytes. Following a seven day

activation with 1000 U/ml rIL-2, part of the Vδ1+ T lymphocytes acquired CD45RO and lost CD45RA (Fig.3). These results, combined with the fact that all our Vδ1 and Vδ2 T cell clones express CD45RO and lack CD45RA (Table 1) suggest that CD45RA and CD45RO represent genuine markers for naive and primed γδ T cells, respectively.

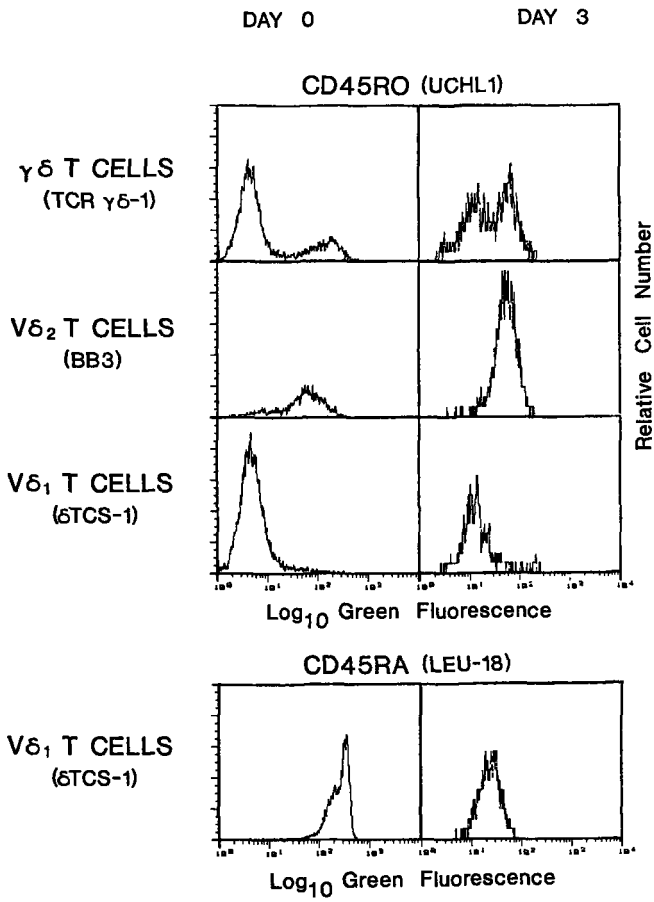

Fig. 2: CD45RO and CD45RA expression by γδ T lymphocyte subsets before and after activation of PBL with phytohemaglutinin (PHA).
One x 10^6 PBL/ml were stimulated with 1 μg/ml PHA for three days. Fresh and PHA-activated PBL were stained for two colour flow cytometry as described in the legend to figure 1. Gates were set on FSC, SSC and FL-2 before data collection on a FACScan. FCS and SSC settings were used to gate for living lymphocytes. The FL-2 gate was set in such a way that only red fluorescent lymphocytes (TCRγδ-1+, BB3+ or δTCS-1+) were included in the gate. Thereafter data on 2-10 x 10^6 lymphocytes were collected. Green fluorescence histograms, showing the UCHL-1 or anti-Leu-18 profiles of the gated T lymphocyte subsets were made.

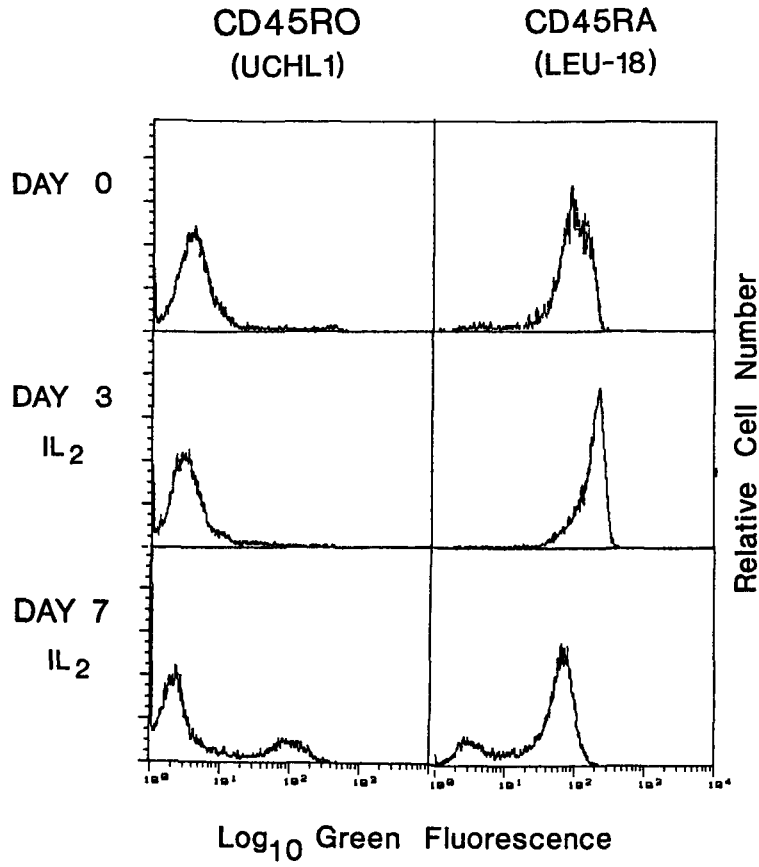

Fig.3. CD45RO and CD45RA expression by fresh and IL-2 stimulated Vδ1 lymphocytes. One x 10^6 PBL/ml were stimulated with 1000 U/ml rIL-2 for three or seven days. Cells were stained with δTCS-1, GAM/IgG1/PE and either UCHL-1/FITC or Leu-18/FITC. Two colour flow cytometric analysis was performed as described in the legend to figure 2.

Subdivision of Vδ1 thymocytes into a CD45RA and CD45RO expressing subset

γδ Thymocytes constitute no more than 2% of the total thymocyte population. In contrast to peripheral blood, the majority of γδ thymocytes expresses Vδ1 whereas Vγ9-Vδ2 thymocytes are rare, [30, data not shown]. The Vδ2 thymocyte subset was too small to allow analysis of CD45-isoform expression. As opposed to the uniform CD45RA+, CD45RO- phenotype of Vδ1 T cells, in peripheral blood, the Vδ1 thymocyte subset comprised two more or less equally large CD45RA+ and CD45RO+ subsets (Fig.4).

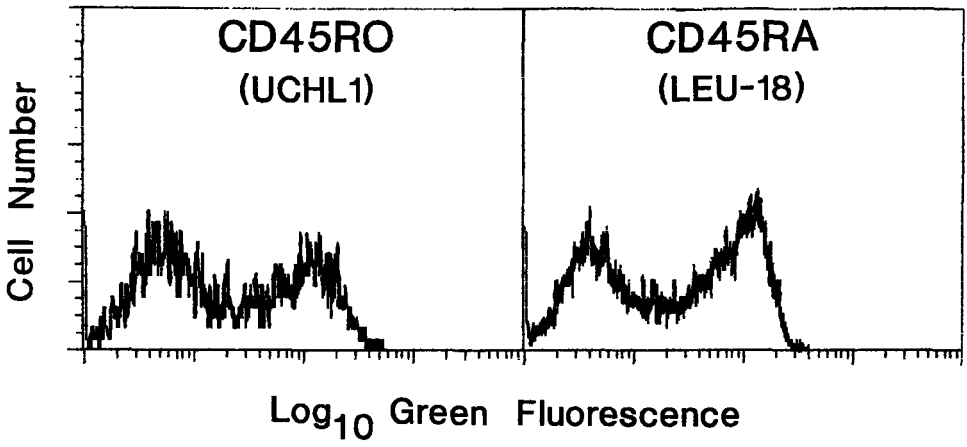

Fig.4. CD45RA and CD45RO expression on Vδ1 thymocytes.
Single cell suspensions of thymocytes were stained with δTCS-1, GAM/IgG1/PE and either UCHL-1/FITC or anti-Leu-18/FITC as described in figure 1. UCHL-1 and anti-Leu-18 histograms of the gated Vδ1 thymocytes were made and presented as described in figure 2.

Transition of CD45RA to CD45RO phenotype by CD3-, CD16+ NK cells following activation

CD45-isoform expression by NK cells was analyzed after staining with a combination of CD16, CD45-isoform and/or CD3-specific mAb. Gated CD16+ lymphocytes contained less than 5 percent CD3+ T lymphocytes (data not shown). All CD16+, CD3- NK cells in peripheral blood had the CD45RA+, CD45RO- phenotype (Fig.5). Following activation of PBL with PHA, the percentage CD16+, CD3- NK cells rapidly declined as a result of massive T cell proliferation. In order to analyze CD45-isoform expression on in vitro activated NK cells, we therefore stimulated PBL with 1000 U/ml rIL-2. After 7 days, a fraction of CD16+, CD3- NK cells converted from the naive CD45RA to the primed CD45RO phenotype (Fig.5).

Following 7 day activation by irradiated Daudi cells and 25 U/ml rIL-2, all CD16+, CD3- NK cells converted to the CD45RO phenotype (Fig.5). Finally, like αβ and γδ T cell clones, also all CD16+, CD3- NK cell clones were CD45RA-, CD45RO+ (Table 1).

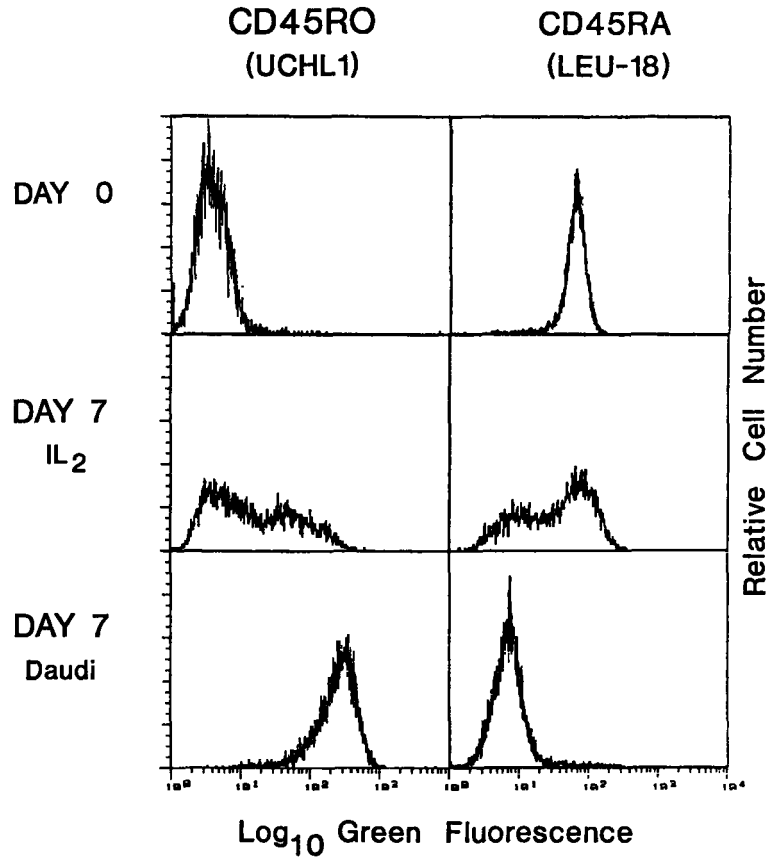

Fig.5. CD45RA and CD45RO expression of fresh and activated CD3-, CD16+ NK cells. PBL were stimulated with 1000 U/ml rIL-2 or with 25 Gy irradiated Daudi cells and 25 U/ml rIL-2 for 7 days. Fresh and activated PBL were stained with anti-Leu-11c/PE and either UCHL-1/FITC or anti-Leu-18/FITC. In the anti-Leu-11c/PE, anti-Leu-4/FITC control sample (not shown) more than 95% or the CD16+ lymphocytes were CD3 negative. UCHL-1 and anti-Leu-18 histograms of the gated CD16+, CD3- NK cells were made and presented as described in figure 2.

DISCUSSION

The present study describes the expression of isoforms of CD45 by γδ T lymphocyte subsets and NK cells prior to and after their activation. Our results confirm the recently reported differential expression of CD45RO on the two major γδ T lymphocyte subsets in PBL [28,31]. Here, we report that, in peripheral blood, the expression of CD45RA and CD45RO on both subsets of γδ T cells is reciprocal. Moreover, fresh CD3-, CD16+ NK cells in peripheral blood, like the Vδ1 T cell subset, are all CD45RA+, CD45RO-. In vitro activation of CD45RA+ Vδ1 T lymphocytes and NK cells resulted in the acquisition of CD45RO expression and a gradual loss of CD45RA . These results, combined with the fact that all our Vδ1 and Vδ2 T cell and NK cell clones express CD45RO and lack CD45RA suggest that, like in αβ T cells, CD45-isoforms can identify naive and primed maturational stages of γδ T lymphocytes and NK cells. Whether CD45RO identifies memory lymphocytes is debatable in view of the lack of immunological memory by TCR negative NK cells (see below).

The expression of CD45RO on the entire Vγ9/Vδ2 T lymphocyte subset in peripheral blood is difficult to reconcile with in vivo stimulation of these lymphocytes by an extensive repertoire of immunogenic antigens. Rather, it favors the activation by a single antigen or by a closely related family of antigens. The uniform CD45RA-, CD45RO+ phenotype of Vγ9/Vδ2 T lymphocytes in peripheral blood can not be explained by clonal expansion of a few antigen-reactive Vγ9/Vδ2 T lymphocytes because human γδ T lymphocytes in blood reportedly express extensive junctional diversity at the Variable-Diversity-Joining (V-D-J) region of functionally rearranged TCR-δ genes [32]. In analogy to subsets of αβ T cells, which share a particular Vβ region and which can be activated by superantigens [33], Vγ9/Vδ2 T lymphocytes in peripheral blood may be in vivo activated by a superantigen [34]. If this hypothesis is correct it would imply that this superantigen is ubiquitous in man. Attractive candidates for such superantigens are autologous and mycobacterial heat shock proteins which indeed are ubiquitous in man and mice and against which human and mouse T lymphocytes can respond [35,36]. The Vδ1 T lymphocytes in peripheral blood have the naive immunophenotype and consequently these cells have not been activated in vivo or alternatively, once activated, may not recirculate in peripheral blood.

Most human cortical thymocytes express CD45RO and lack CD45RA [37]. It is believed that these CD45RA-, CD45RO+ thymocytes are committed to intrathymic death because the generative potential is found almost exclusively in the CD45RA+, CD45RO- thymocyte subset [38]. This probably also holds true for γδ thymocytes since self-reactive γδ T cells are eliminated in the thymus [39]. Our observation that Vδ1 thymocytes could be divided in

a CD45RA+, CD45RO- and a more or less equally large CD45RA-, CD45RO+ subset is consistent with the concept that Vδ1 T cells in the periphery descend from thymic precursors and that Vδ1, CD45RO+ thymocytes are destined to die in the thymus.

Circulating CD3-, CD16+ NK cells uniformly express CD45RA and lack CD45RO expression, in accordance to previous findings based on a CD3-, CD4-, CD8- [40] or a CD56+ (anti-Leu-19) [4] phenotype of NK cells. The naive phenotype of circulating NK cells appears to contrast their ability to spontaneously lyse NK-susceptible target cells. However, activation of NK cells with a high dose IL-2 results not only in the acquisition of CD45RO expression but also in the acquisition of LAK activity [41]. Thus, like αβ T lymphocytes, primed CD45RO+ NK cells are functionally hyperreactive compared to naive CD45RA+ NK cells. The absence of primed NK cells in peripheral blood of healthy individuals suggests that in vivo NK cells, once activated, do not recirculate, as suggested above for Vδ1 lymphocytes. The maturational stage identified by CD45RO expression has been termed "memory" [5,8-10] and "primed" [12]. The fact that CD45-isoforms identify different maturational stages of NK cells argues against the term "memory", because NK cells lack a TCR and thus by definition do not possess classic immunological memory. We therefore favour the terminology "primed" for CD45RO+ lymphocytes.

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CHAPTER VI

COMPARISON OF T-CELL-RECEPTOR GAMMA/DELTA GENE-SEGMENT REARRANGEMENTS IN HUMANS AND CHIMPANZEES

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SUMMARY

In analogy to human peripheral blood lymphocytes (PBL), the TCRγδ lymphocyte population in chimpanzee PBL represents only a minor subset (3-8%) of T lymphocytes. In the periphery, the TCRγδ population, in human as well as in chimpanzee, shows a restricted combinatorial receptor repertoire. The Vδ1 and Vδ2 gene products are expressed in a mutually exclusive fashion and in general the Vδ2 and Vγ9 encoded proteins show a coordinated expression. Restriction fragment length polymorphism (RFLP) analysis showed the conservation of the restriction sites which identify the Vγ9 and Vδ2 rearrangements. The human TCR-Vγ9 gene has two alleles, TCR-Vγ9A1 and TCR-Vγ9A2 which differ at postition 42 in a Threonine (Thr) or Lysine (Lys) residue respectively. Comparison of the human TCR-Vγ9 alleles and deduced amino-acid sequence of the chimpanzee TCR-Vγ9 gene revealed that the chimpanzee TCR-Vγ9 gene contains two amino-acid substitutions when compared with the human TCR-Vγ9A1 allele and three amino-acid substitutions when compared with the TCR-Vγ9A2 allele. In man the Vγ9-Vδ2 lymphocytes show a specific proliferative and cytolytic response to Daudi Burkitt's lymphoma cells. The chimpanzee Vγ9-Vδ2 lymphocytes appear to exert an identical immune reactivity and therefore, the amino-acid substitutions found in the Vγ9 gene-segment do not affect recognition in a trans-species fashion.

INTRODUCTION

Until recently, T lymphocytes were considered to express an unique form of T cell receptor (TCR), composed of a disulphide linked α and β protein. This TCR αβ complex recognizes antigen in the context of major histocompatibility complex (MHC) molecules and is expressed by the majority of peripheral blood lymphocytes (PBL) (1,2). However, a minor subset of T lymphocytes were found to express a distinct type of TCR, composed of a γ and δ chain (3-5). Like the TCRαβ, the TCRγδ is non-covalently associated with the CD3 molecular complex. The TCRγδ is either a disulphide or a non-disulphide linked receptor (6). TCRγδ lymphocytes generally lack the expression of CD4 and CD8 and have a limited variable (V) gene repertoire. The human TCRγ locus comprises of two constant (TCR-Cγ), five joining (TCR-Jγ) and fourteen variable gene segments (TCR-Vγ) belonging to four different subgroups. The human TCRδ locus is embedded between the Vα and Jα gene segments. This locus comprises eight identified variable (TCR-Vδ), three joining (TCR-Jδ), two diversity (TCR-Dδ) and an unique constant gene-segment (TCR-Cδ) (7-10). In human peripheral blood, the major population of TCRγδ lymphocytes expresses the protein

products encoded by the Vγ9 and the Vδ2 gene-segments, whereas a minor population uses the protein products encoded by the Vδ1 gene-segment (11-13). Although a number of investigators have tried to unravel the function of TCRγδ lymphocytes (14-16), it is still enigmatic which biological functions the TCRγδ lymphocytes exert. TCRγδ lymphocytes have been studied in a wide variety of vertebrates: birds, ruminants, rodents and humans but never in non-human primates. Human and chimpanzee *(Pan troglodytes)* lineages separated about 5-7 million years ago, and are each others closest living relatives (17). This phylogenetic relationship provides the reason, why the chimpanzee represents such a suitable experimental model for a; the analysis of the TCRγδ gene repertoire, which depends on the number of variable genes and their polymorphism and for b; the specificity of the TCRγδ lymphocyte population.

To gain further insight into the biological significance of the TCRγδ population, we investigated the evolutionary conservation of the TCR-γ and TCR-δ gene rearrangements. To this end we performed phenotypic, biochemical, molecular biological and functional analyses, and demonstrate that the generation of the TCR-Vγ9 and TCR-Vδ2 gene rearrangements and the specificity of the Vγ9-Vδ2 encoded TCRγδ receptor predates the divergence of chimpanzee and man.

MATERIALS & METHODS

Cells

Cloned TCRγδ lymphocytes were generated from PBL obtained from different chimpanzees. Clones A8, A16, S17 and S28 were obtained directly after limiting dilution procedures. Clones GD26, GD19, GD7, SD1 and SD18 were obtained after stimulation with irradiated Daudi, Burkitt's lymphoma cells. Human PBL and TCRγδ clones N-4 (Vγ9-Vδ2), AK 119 (Vδ1) were analyzed by Southern blotting whereas the human clones, 1012 (Vγ9-Vδ2) and ER25 (Vδ1) were analyzed by SDS-Page. All clones were cultured in RPMI 1640; 10% human serum; 4 mM L-glutamin; 100 IU/ml penicillin/ streptomycin; 1 ng/ml indomethacine; 1 μgr/ml leuco-agglutinin (Purified PHA, Pharmacia, Upssala, Sweden) and 25 U/ml rIL-2. Irradiated (25Gy) feeder cells were added weekly (18).

Monoclonal antibodies

Five mAb reactive with the particular human TCRγδ complexes were used; TCRγδ-1 (19) and TCRδ1 (20), both reactive with all human TCRγδ lymphocytes, TiγA, specific for the Vγ9 encoded protein (21), BB3, specific for the Vδ2 encoded protein (22) and δTCS-1, specific for the Vδ1 encoded protein (23). Anti-CD3 (OKT3 hybridoma culture

supernatant), anti-CD4 (OKT4 hybridoma culture supernatant), anti-CD8 (B116.1) and anti-TCRαβ (BMA031) mAb were used in immunofluorescence studies. Cell surface expression was analyzed by flow cytometry on a FACSscan (Becton & Dickinson, Mountain View, CA), after indirect labeling, using 1:40 dilution of fluorescein isothiocyanate conjugated (FITC goat anti-mouse Ig (GAMIg) (Nordic, Tilburg, The Netherlands). For the immunoprecipitation studies anti-CD3 mAb CLBT3/4.2a was used (24).

Cytotoxicity assay

Cytotoxic activity was measured in a standard three hour ^{51} Cr release assay. Various numbers of effector cells were added to a fixed amount (n=2000) of ^{51} Cr labeled target cells. After three hours of incubation at 37°C, the supernatants were harvested and counted in a gamma counter (25). The following human tumor cell lines were used as target cells in the cytotoxicity assays; K562, an erythromyeloid derived cell line and Daudi, a Burkitt lymphoma derived cell line.

Radiolabeling and immunoprecipitation

Approximately 10x 10^6 TCRγδ cells were surface iodinated with ^{125} Iodine (Amersham Corp. Buckinghamshire, UK) using the lactoperoxidase method and immunoprecipitation was performed according to ref. 11.

Probes

TCR-Jγ1 probe PH60, a 700 bp HindIII-EcoRI fragment, isolated from M13H60 and containing the Jγ1 fragment cloned in the PUC vector. This probe cross-hybridizes to the Jγ2 containing fragment (9). TCR-Vγ9 probe pV9PH, a 700 bp PstI-HindIII fragment from λSHV7 containing TCRVγ9, cloned in PUC vector (10). TCR-Jδ1 probe, clone JδS16, a 1.5 kb SacI fragment containing the 3' region of the Jδ1 segment, cloned in PUC vector (26). TCR-Vδ2 probe, clone PVDSP0.5, a 500 bp SmaI-PstI fragment, containing the germline TCR-Vδ2 subcloned in PUC (27).

Southern blotting

Genomic DNA obtained from chimpanzee and human PBL or cloned TCR γδ lymphocytes, was digested with the restriction endonucleases EcoRI, HindIII, BamHI, KpnI and XbaI, size separated on a 0.8% agarose gel and transferred to Hybond N+ (Amersham, UK) nylon membranes. Filters were hybridized with the by random priming ^{32}P-αdCTP radiolabeled probes according to a standardized Southern blot protocol (28,29). In brief, hybridization

(12h) was performed at 42 °C in a mixture containing 5x saline-sodiumphosphate-EDTA (SSPE; 0.75 M NaCl, 0.05 M Na_2PO_4, 5 mM EDTA, pH 7.7), 50% formamide, 10% dextransulphate, 1 M NaCl and 1% SDS). Filters were washed, dried and exposed to KODAK X-AR-5 films.

Polymerase chain reaction

Genomic DNA (0.5μg) was heated to 95 °C for 10 min. and was amplified under paraffin in a 100 μl reactionmixture (0.5 mM of each deoxyribonucleosidetriphosphate, 50 mM KCl, 10 mM Tris/HCl pH 8.4, 4 mM $MgCl_2$, 0.06 mg/ml BSA, 5 U Taq polymerase and 20 pmol of each oligonucleotide) (30) for 3 cycli of 1.5 min, followed by 35 cycli of 1 min. Each PCR cycle consisted of a denaturation step at 94 °C, an annealing step of 55 °C and an extension step at 74 °C. The extension step following the last cycle was for 10 min. Five μl of each reaction was analyzed by 2% agarose gel-electrophoresis. The remainder was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and was ethanol precipitated. Pelleted DNA was dissolved and digested using the endonucleases XbaI and SalI, and purified using low melting agarose electrophoresis. Fragments were then cloned into appropriate digested vectors prepared from M13tg130 and M13tg131 (31), and transformed in E.Coli XL1-blue bacteria. Nucleotide sequence analysis of single strand DNA was carried out by dideoxy chain termination procedures using the M13 vectors and T7 polymerase (Promega Corporation, Madison, WI) (32). The reported nucleotide sequence represents the consensus of at least three clones.

Oligonucleotides used for the PCR were:

Vγ9; SalI : 5'CCGGTCGACGCTGGCAGTCCTTGGGGCTCT.

XbaI : 5'TGCTCTAGAGGGATGGCTCAAACTGTC.

RESULTS

Chimpanzee TCRγδ lymphocytes lack reactivity with the 'pan' TCRγδ specific mAb TCRγδ-1

Human TCRγδ lymphocytes can be divided into two mutually exclusive subsets on the basis of their reactivity with anti-TCRγδ specific mAb (11-13). The major subset expresses the Vγ9 encoded determinant as identified by mAb TiγA (21) which, in general, is expressed in a coordinated fashion with the Vδ2 encoded determinant as identified by mAb BB3 (22).

The minor subset expresses a Vδ1 encoded determinant as identified by mAb δTCS-1 (23). All human TCR γδ lymphocytes share the expression of epitopes recognized by the 'pan' TCRγδ mAb, TCRδ-1 (20) and TCRγδ-1 (19) respectively.

Cloned chimpanzee TCRγδ lymphocytes (n=9) showed an identical mutually exclusive expression of the Vγ9-Vδ2 and the Vδ1 epitopes, as demonstrated by immunophenotypic analysis (Table I) suggesting their conservation during phylogeny. The reactivity of both TCR-Vδ1 and TCR-Vδ2 gene product specific mAb suggests that the chimpanzee TCR-δ locus comprises at least two V gene-segments. All chimpanzee TCRγδ lymphocytes analyzed expressed the CD3 molecular complex. The CD8 antigen appeared to be differentially expressed by cloned TCRγδ lymphocytes whereas the CD4 antigen was not expressed (Table I). Chimpanzee peripheral blood TCRγδ lymphocyteslacked reactivity with the 'pan' TCRγδ mAb TCRγδ-1, which identifies all human TCRγδ lymphocytes (19). Therefore the expression of the epitope recognized by this specific antibody seems to be unique for human TCRγδ lymphocytes.

Table I. Immunophenotype of cloned chimpanzee TCRγδ lymphocytes

				anti-TCRγδ mAb				
Clone	CD3	CD4	CD8	TCRγδ-1	TCRδ1	TiγA (Vγ9)	BB3 (Vδ2)	δTCS-1 (Vδ1)
GD7	+	-	+/-	-	+	+	+	-
GD26	+	-	+	-	+	+	+	-
SD1	+	-	+/-	-	+	+	+	-
SD18	+	-	-	-	+	+	+	-
A8	+	nt	nt	-	+	-	-	+
A16	+	nt	nt	-	+	-	-	+
S17	+	nt	nt	-	+	-	-	+

Table I. Binding of the mAb was determined by immunofluorescence. T cell clones were incubated with OKT3 or OKT4 hybridoma culture supernatant, B116.1, 10 µg/ml, TCRγδ-1 hybridoma culture supernatant, TCRδ1 1/100 dilution of ascites, TiγA 1/500 dilution of ascites, BB3, 1/1000 dilution of ascites and δTCS-1, 3.75 µg/ml followed by incubation with FITC conjugated GAM-Ig. nt= not tested

Similar protein forms of the TCRγδ receptor complex expressed by human and chimpanzee TCRγδ lymphocytes

To assess the protein forms of the chimpanzee TCR γδ receptor complex, and to reveal the lack of reactivity with the TCRγδ-1 mAb, SDS-Page analysis was performed under, both, reducing and non-reducing conditions. Radiolabeled immunocomplexes were immunoprecipitated from digitonin cell lysates by an anti-CD3 mAb, or from NP40 cell lysates by an anti-TCRγδ mAb. Under non-reducing conditions using anti-CD3 mAb and digitonin cell lysates, obtained from Vγ9-Vδ2 chimpanzee clones (GD26 and SD18), a disulphide linked protein of approximately 75-80 kD was immunoprecipitated (Fig.1 lanes A and B), whereas from Vδ1 clones, A8 and S17, (lanes C, D) a non-disulphide linked protein structure with a 55kD TCR-γ and 37 kD TCR-δ chain was precipitated. In human the 55kD TCR-γ protein is a result of rearrangement of the TCR-Cγ2 gene-element with a triplication of exon-2. Lanes E and F contain disulphide (80kD) and non-disulphide linked (41kD TCR-γ and 40 kD TCR-δ protein) human TCRγδ proteins immunoprecipitated from a human Vγ9-Vδ2 and Vδ1 clone, respectively (SDS-Page analysis under reducing conditions, not shown). In human the disulphide linked TCRγδ receptors are a result of a TCR-Cγ1 rearrangement.

The anti-CD3 mAb mediated precipitations show that the chimpanzee TCRγδ proteins, disulphide or non-disulphide linked forms, are non-covalently associated with the CD3 complex. The anti-TCRγδ mAb, TCRδ1 (lanes G-I) precipitated a non-disulphide linked receptor (55kD TCR-γ and 37 kD TCR-δ protein) from NP40 cell lysates of chimpanzee Vδ1 clones A8, S17 and a 41 TCR-γ protein from the human Vδ1 clone, ER25, the TCR-δ protein is not detected. as found for man, the Vγ9-Vδ2 encoded receptor is expressed in a 75-80 kD disulphide linked form, and the Vδ1 encoded receptor in a non-disulphide linked form. It is concluded that not only the epitopes recognized by the anti-TCRγδ mAb (TiγA, BB3, δTCS-1 and TCRδ1) are conserved during phylogeny, but also the cysteine residues responsable for the disulphide binding of the TCRγ and TCRδ polypeptides

TCR-Vγ9 and Vδ2 gene rearrangements are indentical between human and chimpanzee

Because the TCR-Vγ9 and TCR-Vδ2 genes and products are preferentially expressed in PBL TCRγδ lymphoytes of both, man and chimpanzee, it was of interest to study the genomic organization using the available human probes to detect chimpanzee TCR-Vγ and Vδ gene rearrangements. Rearrangements in the TCRγ chain are detectable by the combined use of EcoRI, HindIII and BamHI digests and the PH60 probe which hybridizes with all Vγ rearrangements involving Jγ1 and Jγ2 (9,10). KpnI digests show rearrangements to JγP,

JγP1 or JγP2 (33). The Vγ rearrangements of the Vγ9-Vδ2 clone GD26, representing the major subset of TCRγδ lymphocytes, are shown in Fig.2. Assignments of the TCRγ rearrangements to given Vγ and Jγ segments on basis of relative mass markers detected in Southern blot analysis is according to ref. 10.

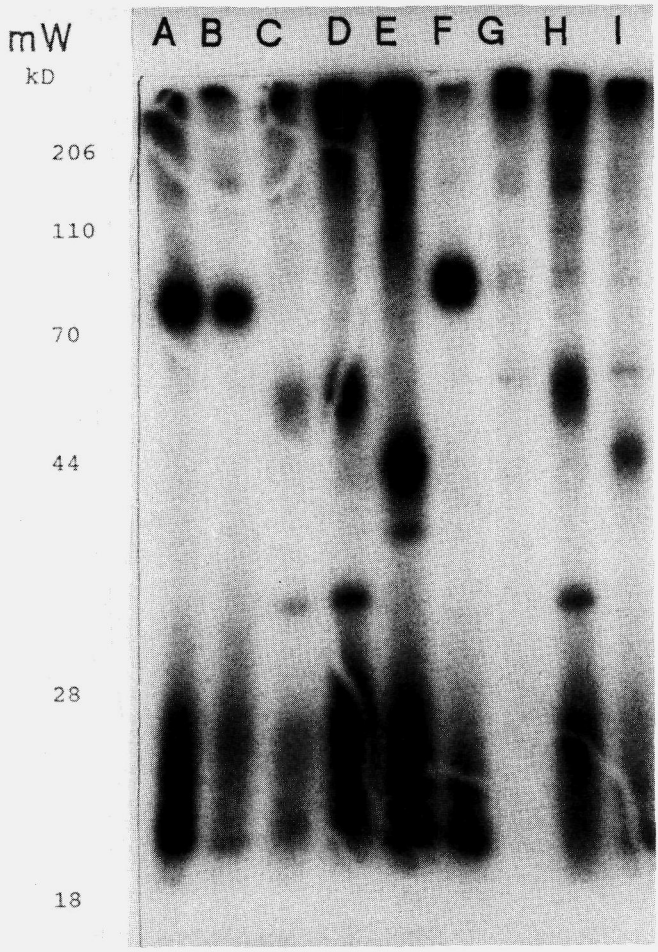

Figure 1: SDS-Page analysis of surface molecules immunoprecipitated under non-reducing conditions from chimpanzee and human TCRγδ clones. Ten x 10^6 cells were surface iodinated, fifty percent of the cells were lysed in buffer containing 1% digitonin and the remaining cells were lysed in buffer containing 1% NP40. Digitonin cell lysates were precipitated with anti-CD3 mAb CLB-T3/4.2a (lanes A-F), whereas NP40 lysates were precipitated with the anti-TCRγδ mAb; TCRδ1 (lanes G-I). Lanes A and B, GD26 and SD18 chimpanzee Vγ9-Vδ2 TCRγδ clones, lanes C, D, G and H, A8 and S17 chimpanzee Vδ1 TCRγδ clones, lane F the human Vγ9-Vδ2 clone 1012 and in lanes E and I the human Vδ1 clone ER25.

The EcoRI digest (E), showed a 3.2 kb signal corresponding to the Jγ2 germline fragment and a 2.4 kb hybridizing signal probably the Jγ1 germline fragment. The human Jγ1 germline segment is a 1.5 kb fragment. The HindIII digest (H) showed a 5.1 kb germline Jγ2 and a 2.1 kb germline Jγ1 fragment. The BamHI (B) digest showed two hybridizing signals, a 16 kb germline Jγ2 and a 22 kb germline Jγ1 gene segment (10). The KpnI (K) digest showed a 16 kb JγP2 germline fragment, a 14 kb JγP1 germline fragment and the 12 kb fragment corresponding to the VγJγP rearrangement (33). These results show that the chimpanzee GD26 clone indeed has the Vγ9-JγP rearrangement on one allele. As shown in Fig.2, this Vγ9-JγP rearrangement was confirmed by the 1.9 kb EcoRI, 2.0 kb HindIII, 19 kb BamHI and 12 kb KpnI hybridizing signals to the TCR-Vγ9 specific probe pV9PH.

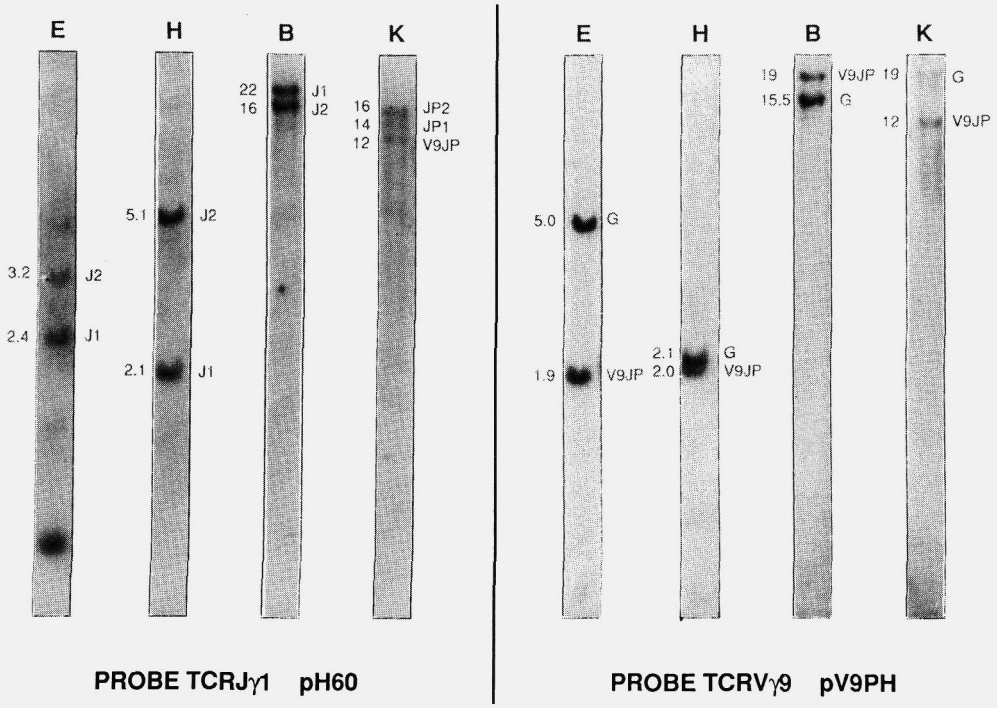

Figure 2. Southern hybridization to the TCRγ probe PH60 and the TCR-Vγ9 probe pV9PH. DNA of chimpanzee TCRγδ clone GD26 was digested with the endonucleases EcoRI (E), HindIII (H), BamHI (B) and KpnI (K). Assignments to given Vγ and Jγ segments is according to ref. 9 and 10. The relative mass markers are given in kb.

Other chimpanzee clones with the TiγA-BB3 phenotype showed identical hybridizing fragments which identify the Vγ9 rearrangement. These results indicate that not only the Vγ9 gene product but also the Vγ9 gene configuration and identifying restriction sites are identical between human and chimpanzee

Table II. Restriction fragment sizes of the TCR-Vδ and TCR-Jδ genes using the Vδ2 and Jδ1 specific probes

Clone	ECORI	HINDIII	XBAI	Rearrangement
SD18 (chimp)	5.5*	6.0	4.0	Vδ2-Jδ1
GD26 (chimp)	5.5	6.0	4.0	Vδ2-Jδ1
1012 (human)	5.5	6.0	4.0	Vδ2-Jδ1
N-4 (human)	5.5	6.0	4.0	Vδ2-Jδ1

Table II. The TCRJδ1 probe (JδS16) (26) detects all rearrangements to Jδ1, whereas the Vδ2 specific probe (pVDSP0.5) (27) only detects rearrangements to the corresponding Vδ gene.
* the size of restriction fragments is given in kb.

The Jδ and Vδ rearrangements of BB3+ TCRγδ clones were studied using the Jδ1 (JδS16)(26) and Vδ2 (pDVSPO.5)(27) probes. As shown in Table II, chimpanzee TCRγδ clones GD26 and SD18 displayed a 5.5 kb EcoRI, a 6.0 kb HindIII and a 4.0 kb XbaI fragment, as detected by both the Vδ2 and Jδ1 probes, demonstrating a Vδ2-Jδ1 rearrangement. The restriction fragment lengths of the hybridizing signals found for the chimpanzee rearrangements are identical to those found for human (11). Therefore, as for the Vγ9 gene, the Vδ2 gene rearrangement and some of its restriction sites predates the divergence of human and chimpanzee lineages. The data confirm that the TiγA and BB3 mAb also identify the Vγ9 and Vδ2 rearrangements in chimpanzee. Southern blot analysis can not discriminate between functional or non-functional rearrangements. The correlation between the reactivity of human Vγ or Vδ gene-segment specific mAb with the corresponding rearrangements can be used to determine functional rearrangements not only in human, but as shown in this study, also in chimpanzee TCRγδ lymphocytes.

Nucleotide sequence of the chimpanzee TCR-Vγ9 gene

The human TCR-Vγ9 gene has two allelic forms; TCR-VγA1 and TCR-VγA2, which have different amino-acid residues at position 42. The TCR-VγA1 allele has a Threonine residue whereas the TCR-VγA2 has a Lysine residue at that position (10). To further extend the similarity between human and chimpanzee genes as detected by Southern blot analysis, we sequenced the chimpanzee germline TCR-Vγ9 gene after PCR amplification.

Figure 3a: Sequence comparison of the human and chimpanzee TCR-Vγ9 genes

```
                                                                V
Hu Vγ9 :  0 TCA ACG CTG GCA GTC CTT GGG GCT CTG TGT GTA TAT  36
Ch Vγ9 :                                    --- --- --- ---

         37 GGT GCA GGT CAC CTA GAG CAA CCT CAA ATT TCC AGT  72
            --- --- --- --- --- --- --- --- --- --- --- ---

         73 ACT AAA ACG CTG TCA AAA ACA GCC CGC CTG GAA TGT 108
            --- --- --- --- --- --- --- --- --- --- --- ---

        109 GTG GTG TCT GGA ATA ACA ATT TCT GCA ACA TCT GTA 144
            --- --- --- --- G-- --- --- --- --- --- --- ---

        145 TAT TGG TAT CGA GAG AGA CCT CGT GAA GTC ATA CAG 180
            --- --- --- --- --a --- --- --- --- --- --- ---

        181 TTC CTG GTG TCC ATT TCA TAT GAC GGC ACT GTC AGA 216
            --- --- --- --- --- --- --- --- --- --- --- ---

        217 AAG GAA TCC GGC ATT CCG TCA GGC AAA TTT GAG GTG 252
            --- --- --- A-- --- --a --- --- --- --- --- ---

        253 GAT AGG ATA CCT GAA ACG TCT ACA TCC ACT CTC ACC 288
            --- --- --- --- --- --- --- --- --- --- --- ---

        289 ATT CAC AAT GTA GAG AAA CAG GAC ATA GCT ACC TAC 324
            --- --- --- --- --- --- --- --- --- --- --- ---

        325 TAC TGT GCC TTG TGG GAG GTG CAC AGC AGC AGA CAG 360
            --- --- --- --- --- --- --- --- --- --- --- ---

        361 TTT GAG CCA TCC
            --- --- --- ---
```

Figure 3a. Nucleotide sequence comparison of the human and chimpanzee TCR-Vγ9 gene.
Underlined is the conserved heptamer sequence.
The onset of the V region is marked by V.

Primers were chosen just between the conserved heptamer-nonamer sequence at the 3' end of the germline human TCR-Vγ9 gene, and in the leader sequence at the 5' end of this gene (34). This set of primers amplified the entire chimpanzee TCR-Vγ9 gene. Nucleotide and deduced aminoacid sequences of human and chimpanzee are shown in Fig.3a and 3b.

Figure 3b: Deduced aminoacid sequence comparison of human and chimpanzee Vγ genes

Hu Vγ9:	0										Cys	Val	Tyr	12
Ch Vγ9:											-	-	-	
	13	Gly	Ala	Gly	His	Leu	Glu	Gln	Pro	Gln	Ile	Ser	Ser	24
		-	-	-	-	-	-	-	-	-	-	-	-	
	25	Thr	Lys	Thr	Leu	Ser	Lys	Thr	Ala	Arg	Leu	Glu	Cys	36
		-	-	-	-	-	-	-	-	-	-	-	-	
	37	Val	Val	Ser	Gly	Ile	Thr	Ile	Ser	Ala	Thr	Ser	Val	48
		-	-	-	-	Val	-	-	-	-	-	-	-	
	49	Tyr	Trp	Tyr	Arg	Glu	Arg	Pro	Gly	Glu	Val	Ile	Gln	60
		-	-	-	-	-	-	-	-	-	-	-	-	
	61	Phe	Leu	Val	Ser	Ile	Ser	Tyr	Asp	Gly	Thr	Val	Arg	72
		-	-	-	-	-	-	-	-	-	-	-	-	
	73	Lys	Glu	Ser	Gly	Ile	Pro	Ser	Gly	Lys	Phe	Glu	Val	84
		-	-	-	Ser	-	-	-	-	-	-	-	-	
	85	Asp	Arg	Ile	Pro	Glu	Thr	Ser	Thr	Ser	Thr	Leu	Thr	96
		-	-	-	-	-	-	-	-	-	-	-	-	
	97	Ile	His	Asn	Val	Glu	Lys	Gln	Asp	Ile	Ala	Thr	Tyr	108
		-	-	-	-	-	-	-	-	-	-	-	-	
	109	Tyr	Cys	Ala	Leu	Trp	Glu	Val						120
		-	-	-	-	-	-	-						

Figure 3b. Deduced amino acid sequence comparison of the human and chimpanzee TCR-Vγ9 gene, predicted from the nucleotide sequence of the TCR-Vγ9 allele.

Comparative analysis revealed that the cysteine residue at position 110, which is probably involved in the intrachain disulphide bond (35), is conserved. The chimpanzee TCR-Vγ9 gene showed four nucleotide substitutions when compared to the human TCR-Vγ9A1 allele. At position 121 an adenine has been substituted for a guanine resulting in a amino-acid change of isoleucin to valine (position 41). At position 226 a guanine has been substituted for an adenine, resulting in an amino-acid substitution of glycine to serine (position 76), These amino-acid altering substitutions are non-synonomous. At positions 159 and 234 a guanine has been substituted for an adenine not resulting in an amino-acid change. These are socalled silent substitutions. The identified chimpanzee TCR-Vγ9 sequence resembles the human TCR-VγA1 allele in a Threonine residue at position 42.

The immune reactivity of Vγ9-Vδ2 lymphocytes to Daudi Burkitt's lymphoma cells is conserved during phylogeny

Human Vγ9-Vδ2 TCRγδ lymphocytes show immune specificity to Daudi Burkitt's lymphoma cells, which is related to the expression of a groEL homolog, HSP58 (36). To investigate whether this specificity is conserved during the phylogeny of great ape and homonoids, we cultured unfractionated PBL from different individual chimpanzees in the presence of various irradiated human B-LCL as described (16).

As shown in Fig. 4. stimulation with human Daudi cells, but not with other lines of human EBV transformed B-LCL (APD/BSM), resulted in the selective outgrowth of chimpanzee TCRγδ lymphocytes. This selective TCRγδ proliferation, as for human, was confined to the Vγ9-Vδ2 lymphocytes (data not shown). The data indicate that both chimpanzee and human Vγ9-Vδ2 lymphocytes may recognize identical antigenic structures expressed by Daudi cells. Proliferation inhibition studies using the polyclonal antiserum directed against HSP58 may provide evidence for this notion.

Human TCRγδ lymphocytes mediate MHC unrestricted cytolysis when activated in vitro (3,19). Using two human tumor target cell lines of distinct histologic origin, we found that all chimpanzee clones (Table III) exert MHC unrestricted lysis of the K562 cell line.The chimpanzee Vγ9-Vδ2 clones specifically lysed Daudi cells, whereas none of the Vδ1 clones could (16,36). These results conclusively demonstrate that the specificity of Vγ9-Vδ2 lymphocytes is identical between the non-human and human primate species demonstrating an interspecies maintenance of this specificity.

Figure 4: Daudi cells specifically stimulate proliferation of chimpanzee TCRγδ lymphocytes

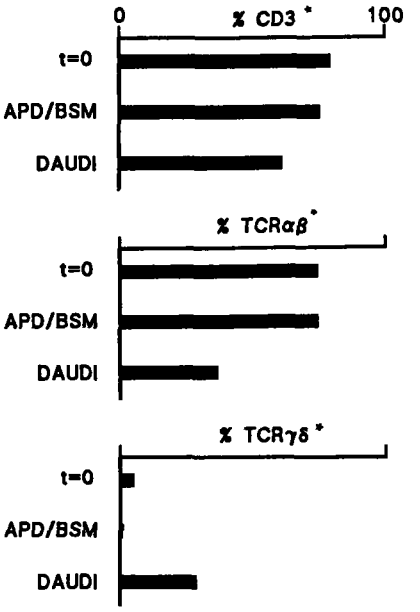

Figure 4. PBL were isolated and directly stained (t=0) with anti-CD3, anti-TCR αβ and anti-TCRγδ mAb followed by incubation with FITC conjugated GAM-Ig. These PBL were cultured for a seven day period with irradiated Daudi, Burkitt's lymphoma cells or a mixture of APD/BSM (EBV-BLCL) feeder cells, after which the lymphocytes were stained with identical mAb as used at t=0.
* % CD3, TCRαβ and TCRγδ lymphocytes in total lymphocyte yield.

Table III. Cytotoxic activity of chimpanzee TCRγδ clones

Effector		target cell lines K562	Daudi
SD18	(Vγ9-Vδ2)	66*	57
GD26	(Vγ9-Vδ2)	65	71
SD21	(Vγ9-Vδ2)	61	53
A8	(Vδ1)	48	0
A16	(Vδ1)	41	0

Table III. * data presented as percentage ^{51}Cr release in a standard 3h assay as described (25). Effector/target ratio 10:1

DISCUSSION

The dichotomy of TCRγδ lymphocytes into the Vγ9-Vδ2 and Vδ1 subsets and the functional features of these cells (11-13), raises the question how the TCRγδ lymphocyte population has developed through evolution. The species most closely related to humans are the African anthropoid apes, which comprises the chimpanzee and gorilla. Human and chimpanzee lineages have separated 5-7 million years ago. Therefore the analysis of chimpanzee TCRγδ polymorphism and receptor specificity may provide information for its immunological importance and for the evolutionary significance of the TCRγδ population in human and non-human primates. This study shows that the identifying restriction sites for particular TCR-γδ gene-segment rearrangements are identical between human and chimpanzee. It is also shown that the chimpanzee TCR-δ locus comprises at least two TCR-V gene-segments, TCR-Vδ1 and TCR-Vδ2 as identified by the human TCR-Vδ1 and Vδ2 specific mAbs. The TCR-γ locus comprises at least one V gene-element, TCR-Vγ9, identified by the human TCR-Vγ9 specific mAb and probably two TCR-Cγ gene-elements. These different TCR-Cγ elements could, like in human, encode for either a disulphide linked or a non-disulphide linked TCRγδ heterodimer. The diversity of both TCRγ and δ polypeptides was analyzed at the cell surface of chimpanzee TCRγδ clones. The immunofluorescence analysis revealed a coordinated expression of the Vγ9-Vδ2 epitopes which were mutually exclusive expressed to the Vδ1 epitope. This dichotomy in the TCRγδ population was earlier reported for the human TCRγδ population (11-13). Four out of five anti-human TCRγδ mAb also reacted with the relevant chimpanzee TCRγδ lymphocyte subsets. MAb TCRγδ-1 reacts with all human TCRγδ lymphocytes (19). The epitope recognized by this mAb has not as yet been defined. Remarkably, the TCRγδ-1 mAb did not react with the chimpanzee TCRγδ lymphocytes. Therefore, the TCRγδ-1 mAb, may specifically recognize a human determinant. Given the resemblance of the chimpanzee and human TCRγδ protein complexes, it may well be that the TCRγδ-1 mAb recognizes an unique conformational epitope created by the quarternary structure of the human TCRγδ complex.

The data showed that the immune reactivities of chimpanzee Vγ9-Vδ2 lymphocytes are identical to those reported for human Vγ9-Vδ2 lymphocytes (16,36). This by chimpanzee and man species mismatched expression of antigenic determinants, which are effectively recognized by Vγ9-Vδ2 lymphocytes of both species, indicates that an identical antigen is recognized. Similar observations have been made for the species mismatched presentation of PPD (purified protein derivate), restricted by MHC class II molecules (37). Daudi cells, reportedly express determinants homologous to the groEL heat shock family (HSP58) (36). These HSP are homologous to mycobacterial antigens, and thought to be widely expressed

between species. They show a conserved nature and are expressed by 'stressed' cells (38). It has been shown that TCRγδ lymphocytes recognize HSP without MHC restriction (15, 39-41). Because chimpanzees are susceptable to identical infectious diseases as man, the expression of heat shock proteins on 'stressed' cells is likely to occur. Consequently, the heat shock proteins also represent likely candidates for the antigen recognized by chimpanzee Vγ9-Vδ2 lymphocytes, as reported earlier for human Vγ9-Vδ2 lymphocytes (36). This superantigenic reactivity would explain the polyclonal expansion of the Vγ9-Vδ2 lymphocytes in vivo. Comparison of the deduced aminoacid sequences of the human and chimpanzee TCR-Vγ9 gene revealed that the chimpanzee TCR-Vγ9 gene resembles the human TCR-Vγ9A1 allelic form at position 42 with a Threonine residue. Two non-synonomous substitutions have been identified. These aminoacid changes are a result of point mutations contributing to the allelic diversity of the TCR-Vγ9 gene but do not affect the receptor specificity. These non-synonomous substitutions may results in an epitope change and therefore explain the lack of reactivity with the human 'pan' TCRγδ mAb, TCR γδ-1. These substitutions are then species-specific. The evolutionary rate of silent substitutions has been determined to be 1.1×10^{-9} substitutions per site per year for the human-chimpanzee lineage (42). When only the TCR-Vγ9 seqence at the third codon positions are compared, the evolutionary rate in terms of nucleotide substitutions for the human and chimpanzee TCR-Vγ9 gene-segment is about 1.7×10^{-9}. This rate is within the range of expectation of neutral drift of a gene in two species so recently separated. However, the antigen specificity of the Vγ9-Vδ2 lymphocytes has been conserved, suggesting that the TCR-Vγ9 gene is a functional gene. In summary, the finding that the functional gene-rearrangements such as the TCR-Vγ9, TCR-Vδ1 and TCR-Vδ2 are generated before the divergence of human and chimpanzee; the persistence of the dichotomy into the Vγ9-Vδ2 and Vδ1 subsets, and the conservation of the Vγ9-Vδ2 lymphocyte antigen specificity, support the idea that TCRγδ lymphocytes have an important function within the immune network of human and non-human primates

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CHAPTER VII

GENERAL DISCUSSION

DISCUSSION

Since the discovery of TCRγδ lymphocytes four years ago, TCRγδ lymphocytes have become a popular subject and are extensively studied. Despite the large amount of information on genetical, biochemical, and functional properties of TCRγδ lymphocytes in both mice and man, their biological role still remains enigmatic. In this chapter, the data presently available on TCRγδ lymphocytes and its subsets in peripheral organs and thymus will be discussed. Also their origin, functional properties in vitro, and their putative physiological role in vivo will be summarized and discussed.

The diversity of the gene repertoire of TCRγδ lymphocytes in the periphery is more limited than the germline V, D, J and C elements would allow. In fact, more than 95 % of the TCRγδ lymphocytes in the circulation belongs to one of two subsets; 1) the Vγ9-Vδ2 encoded receptors are used by the majority of TCRγδ lymphocytes in the periphery; 2) a minority however, expresses the Vδ1 gene element in association with a Vγ gene element from the VγI subgroup. In postnatal thymus, the Vδ1 encoded receptor is predominantly expressed by the TCRγδ thymocytes. This mutually exclusive expression of these two T cell receptor types raises intriguing questions regarding the thymic selection of the TCRγδ lymphocytes. Mechanisms that may govern the selection process of the TCRγδ lymphocyte repertoire are a; sequential gene rearrangements, in which the rearrangement of a particular gene element dictates the rearrangement of the other specific gene element, e.g. the functional Vγ9 gene rearrangement may subsequently dictate the functional rearrangement of the Vδ2 gene. b; the preferential pairing of Vγ9-Vδ2 protein products as a result of physiological constraints. c; the negative selection of those TCRγδ receptors which express protein products other than the Vγ9-Vδ2 encoded peptides, and d; the selective expansion of the Vγ9-Vδ2 expressing TCRγδ lymphocyte subset in the periphery. The following observations argue in favor of or against the above mentioned selection mechanisms for TCRγδ lymphocytes.

The finding that Vγ9 encoded protein products associate with Vδ1 encoded δ chains argues against sequential gene rearrangments as a selection mechanism (1).

Transfection experiments in a TCR-δ negative mutant cell line indicate that the Vγ9 encoded chains pair equally well with a Vδ2 as with a Vδ3 encoded δ chain (2). This implies that physiological constraints are not involved in the preferential pairing of Vγ9-Vδ2 encoded receptors.

The majority of TCRγδ lymphocytes in the circulation expresses the Vγ9-Vδ2 encoded TCRγδ heterodimer (3,4). However, other TCRγδ types have also been found e.g. Vγ9+-Vδ1+, VγI+-Vδ1+ and Vγ9-Vδ2-Vδ1- (1). Albeit rarely, in some donors, the majority of TCRγδ

lymphocytes in the circulation expresses the Vδ1 encoded receptor. Therefore, these observations suggest that a negative selection mechanism for TCRγδ lymphocytes which express receptor types other than the Vγ9-Vδ2 heterodimer is not the predominant one.
Are the Vγ9-Vδ2 TCRγδ lymphocytes in the periphery then selectively expanded? The relatively low frequency of Vγ9-Vδ2 lymphocytes in the thymus, together with the age-related selective expansion of previously activated Vγ9-Vδ2 lymphocytes in the periphery (5) suggests that Vγ9-Vδ2 lymphocytes are subject to extrathymic selection. In addition, this subset is the major responder to mycobacteria (6).

Are the Vδ1 and Vγ9-Vδ2 lymphocytes both derived from thymic precursors?
The Vδ1-Dδ1(Dδ2) gene rearrangement together with a VγI subgroup gene rearranged to Jγ2 is the dominating gene rearrangement in postnatal thymus (7). The analysis of CD45 isoform expression on the Vδ1 thymocyte population showed that Vδ1 thymocytes express either the CD45RA (naive) or the CD45RO (prior activated) antigens (8). For TCRαβ thymocytes, it is believed that the CD45RA-, CD45RO+ subset is destined to intrathymic death because the generative potential is almost exclusively found in the CD45RA+, CD45RO- thymocyte subset (9). This probably is also true for the TCRγδ thymocyte population, since self-reactive TCR γδ thymocytes are eliminated in the thymus (10). Taken together, these observations are consistent with the concept that Vδ1 lymphocytes in the periphery descend from CD45RA+, CD45RO- Vδ1 thymic precursors.
Do Vγ9-Vδ2 lymphocytes in the periphery also descend from thymic Vγ9-Vδ2 precursors? In contrast to human postnatal thymus, the Vδ2 gene element rearranged to the Dδ3 and Jδ3 gene elements is the predominantly expressed TCRδ gene in fetal thymus (11). The initial TCRγ locus rearrangement events involve the Vγ8 and Vγ9 to the Jγ1 gene element. In contrast to the fetal Vγ9-Vδ2 thymocytes, which show limited N-nucleotide diversity, the Vγ9-Vδ2 lymphocytes in the periphery display an extensive N-linked diversity, and use the Jδ1 gene element in functional rearrangements (12). Therefore, the fetal Vγ9-Vδ2 thymocyte population is unlikely to be the precursor of the peripheral blood Vγ9-Vδ2 lymphocytes. Also the observations that in nude mice and athymic DiGeorge patients the Vγ9-Vδ2 peripheral blood population is present suggest a thymus independent selection mechanism (13). The fetal thymic Vγ9-Vδ2 population may be destined to die within the thymic environment. The analysis of CD45 isoform expression on this thymic Vγ9-Vδ2 population may provide evidence to support this hypothesis. In analogy with the murine Vγ3-Vδ1 population in the early murine fetal thymus (14), which are the first to leave the thymus and specifically localize in epithelial layers to become dendritic epidermal cells (DEC) (14), the human fetal Vγ9-Vδ2 population may preferentially localize in a yet unknown homing site.

The murine DEC lymphocytes and human fetal Vγ9-Vδ2 lymphocytes both show very limited N-nucleotide diversity (11,14).
This positive selection in the peripheral compartment of Vγ9-Vδ2 lymphocytes may result from the environmental challenge by superantigens. Such superantigens are capable of activating an entire T lymphocyte subset which expresses identical V gene products

Superantigen activation of Vγ9-Vδ2 lymphocytes in vivo

The expression of CD45RO on the entire Vδ2 lymphocyte subset in peripheral blood is difficult to reconcile with stimulation of these lymphocytes by an extensive repertoire of antigens, matching the heterogeneity of the Vγ9-Vδ2 lymphocyte population based on the extensive junctional diversity at the V-D-J joints of functionally rearranged TCR-δ genes. Rather it points at an activation by a single antigen or by a closely related family of antigens. In analogy to subsets of TCRαβ lymphocytes which share a particular Vβ region and which can be activated by so-called superantigens (15), we propose that Vγ9-Vδ2 lymphocytes in the periphery are in vivo activated by superantigens (see below). Vδ1 lymphocytes in peripheral blood have the naive CD45RA+ phenotype, and consequently these lymphocytes have not yet been activated in vivo or, alternatively, once activated do not remigrate to peripheral blood. If the hypothesis is correct that all Vδ2 but none of the Vδ1 lymphocytes are in vivo activated, this would imply that such a putative superantigen is ubiquitous in man. Autologous and mycobacterial heat shock (stress) proteins which are ubiquitous in man are attractive candidates for such superantigens. Indeed mouse and man TCRγδ lymphocytes have been reported to specifically respond to these antigens (16,17). This superantigen activation in vivo may either occur by mature Vγ9-Vδ2 lymphocytes in the periphery or operate in the positive selection during extrathymic differentiation.

How do superantigens activate T lymphocytes?

Exogenous superantigens stimulate the polyclonal expansion of an entire subpopulation of T lymphocytes with a particular Vβ or Vγ receptor element. The stimulation of T lymphocytes by bacterial toxins depends upon the presence of MHC class II expressing cells (18,19). These superantigens bind to the outsides of the peptide binding groove of the presenting MHC molecule, thereby bringing the TCR and MHC into close proximity of each other (20). Recently, it has been reported that both human and mouse MHC class II negative target cells could be recognized in a Staphylococcal Enterotoxin (SE) dependent fashion by mouse CD8+ CTL clones (21). This implicates the existence of a novel SE receptor distinct of MHC class II molecules. The CD4-/CD8- TCRγδ lymphocytes also recognize superantigens which are presented by MHC class II negative target cells (22). Therefore, the

TCRγδ lymphocytes may recognize a novel MHC class II molecule or such a putative SE receptor. Endogenous equivalents of the toxins are the Mls antigens in mice. These self superantigens cause the elimination of virtually all T lymphocytes bearing the target Vβ (18). The analysis of lymphokine production by a murine CD4+ clone which displayed antigen specific as well as superantigen recognition, showed that IFNγ gene expression was induced in the former but not in the latter recognition process (23). This indicates that superantigen recognition is distinct from T cell recognition of antigenic peptides and leads to a different pattern of lymphokine production.

A putative physiological role of Vγ9-Vδ2 lymphocytes

Heat shock (stress) proteins are constitutatively expressed intracellularly to sustain essential normal cell growth functions (24). Such HSP self proteins may not be functionally present on the membrane of normal cells. However, when these cells are stressed or damaged, they may express heat shock proteins in an immunogenic form at their surface. This may result in the activation of the Vγ9-Vδ2 lymphocytes in vivo. Their physiological function may be to produce lymphokines that are needed to elicit a specific immune response against the 'stressed' cell, or to directly lyse the cell expressing the HSP. In addition, the Vγ9-Vδ2 lymphocytes may have a specific role in the immuno-surveillance of bacterial infections. Indeed, Vγ9-Vδ2 lymphocytes have been shown to specifically respond to a variety of bacteria and bacterial enterotoxins (6,24). These reponses include lymphokine production and cytotoxic activity. Activation of cytolytic T lymphocytes may contribute to the protection against mycobacterial infections. The appearance of TCRγδ lymphocytes prior to the antigen specific TCRαβ lymphocytes during infection with Calmette Guerin Bacillus (26) suggests that the TCRγδ lymphocytes serve as a first line of defense in mycobacterial immunity. The proliferation and clonal expansion of antigen specific TCRαβ lymphocytes takes several days. Therefore in the early stages of infection the TCR γδ lymphocyte population can serve as an effective host defense mechanism through direct elimination of the infected cells.

Evolutionary importance of TCRγδ lymphocytes

The analysis of the TCRγδ lymphocyte characteristics among species shows an apparent heterogeneity. For instance, the absolute number of TCRγδ lymphocytes in the periphery varies considerably. In peripheral blood of sheep and cattle the TCRγδ lymphocyte population comprises about 20-30% of the total T lymphocytes (27), whereas in non-human and human primates the TCRγδ population comprises only 3-10% of the total T lymphocyte population in the periphery. These differences in numbers of circulating TCRγδ lymphocytes may relate to differences in pathogenic challenges, environmental situations

and the anatomy between species. Non-human and human primates show an identical restricted TCRγδ repertoire in peripheral blood and conservation of the Vγ9-Vδ2 lymphocyte antigen specificity. This may well be the result of preservation of functional diversity during evolution. The study of TCRγδ lymphocytes, their genomic repertoires and functions in non-human primates other than chimpanzee, which is the most closest living relative of man, could reveal the evolutionary timepoint where the TCR gene rearrangements and receptor specificity are generated. Also, the analysis of more primitive species, which may have an immune system that comprises mainly of TCRγδ lymphocytes could reveal the immunological functions of this TCRγδ population in that particular species host-defense mechanisms. A suitable model for the analysis of the functional capabilities of TCRγδ lymphocytes may be TCRαβ depleted mice. Although, secondary changes in other components of the immune system must be considered. Mice treated with anti-TCRαβ mAb were completely abrogated from TCRαβ lymphocytes but showed no significant changes in size of TCRγδ populations in thymus and peripheral lymphoid organs (28). Since the frequency of alloantigen-reactive TCRγδ lymphocytes is considerably lower than among TCRαβ lymphocytes, the TCRαβ lymphocyte depleted mice were stimulated with alloantigens. Alloreactivity was not detectable in bulk lymphocyte cultures. Thus, the alloreactivity mainly resides within the TCRαβ lymphocyte population. The notion that TCRγδ lymphocytes specifically recognize mycobacterial antigens is supported by the accumulation of these lymphocytes in the draining lymphnodes of TCRαβ suppressed mice immunized with purified protein derivate (28).
Selective depletion in vitro of the human Vγ9-Vδ2 TCRγδ population from a total PBL population, completely abrogated the proliferative response of all TCRγδ lymphocytes to mycobacterial antigens, while leaving the reponse of TCRαβ lymphocytes unaltered (29). Together with the model of TCR αβ lymphocytes depleted mice, these findings indicate that TCRγδ lymphocytes represent a separate lineage of T lymphocytes. In the T lymphocyte population, TCRαβ lymphocytes represent the antigen specific subset and the Vγ9-Vδ2 lymphocytes play a role in the early stages of mycobacterial immunity.

Resumé and conclusions

The purpose of the study, described in this thesis was to analyze the TCRγδ genomic gene repertoire, the actual receptor use in thymus and peripheral blood, and to determine some of the immunological reactivities of this T lymphocyte population within the immune network. In the periphery, the TCRαβ and TCRγδ receptors are expressed in a mutually exclusive fashion and thus represent two separate T lymphocyte lineages. The TCRγδ population in

peripheral blood can be subdivided into two subsets on the basis of their functional gene rearrangments, which can be identified by specific mAb. The majority of TCRγδ lymphocytes in peripheral blood expresses a Vγ9-Vδ2 encoded receptor, whereas a minority expresses a VγI-Vδ1 encoded receptor. The dichotomy in functional receptor usage is also reflected by the immune-reactivities of both subsets. The Vγ9-Vδ2 subset shows reactivitiy with HSP and mycobacterial antigens and expresses the CD45RO isoform which is associated with prior activation. This HSP and mycobacterial activation of an entire subset of lymphocytes with a particular V gene rearrangement reflects a superantigen driven polyclonal expansion of the Vγ9-Vδ2 lymphocyte subset. However, the Vδ1 subset expresses the CD45RA isoform which is associated with a 'naive' phenotype. After activation in vitro TCRαβ, Vδ1 lymphocytes and NK cells acquire CD45RO expression and lose their CD45RA phenotype. Therefore, the CD45 isoforms are genuine activation markers for T lymphocytes and NK cells. The Vδ1 lymphocytes exert antigen-specific functions. Therefore, the TCRγδ population can exert functions similar as those found for TCRαβ lymphocytes. The finding that particular TCRγδ gene configurations and specificities have been conserved during a 5-7 million year period of evolution. Together with the persistence of the dichotomy into the Vγ9-Vδ2 and Vδ1 subsets, and the conservation of immunereactivity of the Vγ9-Vδ2 lymphocytes against HSP suggests a selection and preservation of functional diversity within the TCRγδ lymphocyte population. Also, the sequence similarity found for the human and chimpanzee TCR-Vγ9 gene indicates that this gene already existed before the divergence of chimpanzee and man.

Future prospects

The search for and analysis of antigen specific TCRγδ lymphocytes will eventually reveal what specific role the TCRγδ lymphocytes play in the immune response. Limiting dilution analysis can determine the frequency of the Ag-specific Vδ1 lymphocytes.

TCRγδ lymphocytes generally lack expression of the CD4 and CD8 antigens. The CD8 antigen expressed, is an α-α homodimer, which serves as a less efficient coreceptor than the CD8 αβ heterodimer. Since the CD45 tyrosine phosphatase counteracts the CD4 or CD8 associated tyrosine protein kinase, the absence of these coreceptor molecules could have important implications for the way in which TCRγδ lymphocytes transduce activation signals received by their receptor. In contrast to TCRαβ lymphocytes, TCRγδ lymphocytes can be stimulated by phorbol myristate acetate (PMA) alone to produce a variety of lymphokines. The study of activation signal transduction pathways resulting in protein phosphorylations, such as the ζ chain could unravel the differences in activation requirements between TCRαβ and TCR γδ lymphocytes.

Comparison of human and various non-human primate species may elucidate the evolutionary timepoint where TCRγδ polymorphisms and specificities have evolved. Although the genomic organization and biochemical features of the human TCRγδ lymphocyte populations have been elucidated, the study of their biological function remains an intriguing challenge for further research.

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SUMMARY

SAMENVATTING

SUMMARY

The ultimate goal of the immune system is to recognize and destroy any foreign antigen invading the body. To obtain this goal, the immune system has several effector cells at its disposal. These lymphocytes can recognize the foreign antigen and subsequently remove the invader, either by cell mediated interactions or by eliciting an antibody response.
T lymphocytes and NK cells play a major role in the immune response. The activation of these lymphocytes is a multistep event, in which several of their membrane structures are involved. The structure that provides the T lymphocytes with their antigen specificity consists of a constant domain and a polymorphic variable domain. This structure is called the T cell receptor (TCR). Within the immune-network the T lymphocyte population can be divided into two mutually exclusive subpopulations. Most T lymphocytes express a TCR composed of a disulphide linked TCR-α and TCR-β chain. These TCR αβ lymphocytes recognize antigens which are presented by a major histocompatibility complex (MHC) molecule expressed by the target cell. A minor population of T lymphocytes, 3-10% of the total T lymphocyte population, expresses a TCR composed of either a disulphide linked or a non-disulphide linked TCR-γ and TCR-δ chain. In general, these TCRγδ lymphocytes recognize antigen without MHC restriction. Foreign antigens are recognized via the polymorphic variable domains of the TCR and the resulting activation signal is then transduced via the CD3 molecular complex. The CD3 complex comprises five non-polymorphic proteins and is non-covalently associated with the TCR. Activation of the effector lymphocyte can also be achieved via other signal transducing molecules, such as CD2 or CD16. In addition of the CD2, CD3/TCR and CD16 molecules, accessory molecules mediate the antigen non-specific adhesion, thereby bringing the effector cell and target cell into close proximity of each other. Thus, after recognition of the antigen, a whole cascade of events takes place, the signal transduction routes such as the inositol-lipid pathway and the tyrosine kinase pathway, become activated. Several enzymes are involved, e.g. stimulation via the inositol pathway results in the activation of protein kinase C, which in turn phosphorylates many protein substrates. This finally results in the activation of the effector lymphocyte to produce lymphokines or display cytolytic activities, thereby eliminating the target cell. Several membrane molecules possess enzymatic activities; CD45 is a tyrosine phosphatase; CD4 and CD8 represent two protein tyrosine kinases. The CD45 and the CD4, CD8 antigens act antagonistic.
In chapter I, the introduction, are the different types of cytotoxic lymphocytes, their activation pathways and surface molecules involved in the regulation of the immune response described.

The cytolytic effector functions of TCRαβ lymphocytes and NK cells against allogeneic, virus infected and tumor cells are well documented. The discovery of the TCRγδ lymphocytes raised new questions about their receptor structures, ligands and their immunological role within the immune network. The following chapters of this thesis deal with this particular lineage of T lymphocytes; the TCRγδ lymphocytes, their membrane structures and functions are detailed.

TCRγδ lymphocytes represent a minor population of T lymphocytes. The genomic organization of the TCRγδ gene elements resembles that of the immunoglobulin gene superfamily. Different V, D, J, and C elements rearrange and form a functional protein expressed on the cell membrane. TCRγδ lymphocytes are localized in lymphoid tissues and peripheral blood. Upon activation in vitro, TCRγδ lymphocytes exert MHC unrestricted cytolysis towards a large variety of tumor target cells of distinct histological origin, in which they resemble the NK cells. Also, like TCRαβ lymphocytes, TCRγδ lymphocytes exert antigen-specific cytolytic activity. In peripheral blood, the Vγ9-Vδ2 gene element encoded, disulphide linked TCRγδ heterodimer is the predominant one. Only, a minor population of TCRγδ lymphocytes in peripheral blood uses the Vδ1 gene element encoded, either as a disulphide or as a non-disulphide linked TCRγδ heterodimer, as described in chapter II. In postnatal thymus an inverse occurrence of TCRγδ receptors is found.

TCRγδ lymphocytes generally lack the expression of the CD4 and CD8 antigens, which serve as co-receptors in MHC restricted antigen recognition. Therefore, TCRγδ lymphocytes recognize antigens in an MHC unrestricted manner. In chapter III is shown, that the dichotomy in receptor structure is also reflected by a difference in functional capabilities of the two subsets of TCRγδ lymphocytes. Only the Vγ9-Vδ2 lymphocytes show a specific proliferative and cytolytic response to Daudi Burkitt's lymphoma cells, although the Vδ1 lymphocytes can form effector/target conjugates with Daudi cells. However, the Vδ1 lymphocytes are able to lyse Daudi cells when an anti-CD3 monoclonal antibody is used to provide optimal crosslinking between the Vδ1 lymphocyte, via the CD3 complex, and the Daudi cells, via the FcγR expressed by these cells. Chapter IV discloses the nature of the structure on Daudi cells that is recognized by the Vγ9-Vδ2 lymphocytes. Daudi cells express the HSP58 protein. Such HSP or stress proteins are produced by prokaryotic and eukaryotic cells in response to a variety of insults. The high sequence homology between HSP and mycobacterial antigens can explain the 'specificity' of the entire population of Vγ9-Vδ2 lymphocytes to Daudi cells and mycobacterial extracts. The stimulation of an entire subpopulation of lymphocytes, which all express a particular V gene element is called a superantigen response. The expression of the CD45RO antigen on the Vγ9-Vδ2 lymphocytes substantiates that these lymphocytes are already in vivo activated,

presumably by such a superantigen, as described in chapter V. The expression of the CD45RA antigen on freshly isolated Vδ1 lymphocytes and NK cells and the conversion to the CD45RO phenotype after activation argues in favor of these antigens as genuine markers for lymphocyte activation, rather than naive and memory respectively.

To gain further insight in the biological importance of the TCRγδ population, we investigated the evolutionary timepoint were the TCR-γ and TCR-δ gene gene rearrangements are generated. In chapter VI, the TCRγδ population in chimpanzee, which is the closest living relative of man, was investigated. The persistence of the dichotomy into Vγ9-Vδ2 and Vδ1 subsets and the conservation of the Vγ9-Vδ2 lymphocyte specificity show the preservation of functional diversity during 5 million years of evolution. The final chapter of the thesis discusses the functional properties of the TCRγδ lymphocytes in vitro and their putative role in the immune reponse against bacterial infections.

The study described in this thesis unravels the receptor structures used by TCRγδ lymphocytes in peripheral blood and gives an insight in the putative ligands recognized. It suggests a role for TCRγδ lymphocytes as an early defense system against 'stress' signals and bacterial infections. Together with the fact that TCRγδ lymphocytes have been found in all vertebrates analysed, this thesis shows that TCRγδ lymphocytes have a specific and unique function and play an important role within the immune-network.

SAMENVATTING

Het voornaamste doel van het immuunsysteem is de herkenning en de vernietiging van ieder lichaamsvreemd antigeen. Om dit doel te bereiken beschikt het immuunsysteem over verschillende uitvoerende cellen. De lymfocyten kunnen de 'vreemde' antigenen herkennen en vervolgens processen op gang brengen die deze antigenen elimineren. De eliminatie vindt plaats door middel van cellulaire interacties of door middel van het genereren van een antilichaam reactie.

T lymfocyten en NK cellen spelen een belangrijke rol in de cellulaire reactie van het immuunsysteem tegen 'vreemde' antigenen. De activering van de lymfocyten kent vele fasen, waarbij verschillende membraanstructuren betrokken zijn. De membraanstructuur, die de T lymfocyten hun antigeen specificiteit geeft bestaat uit een constant gedeelte en een polymorf variabel deel. Deze structuur wordt de T cel receptor genoemd. In het immuun-netwerk bestaat de T lymfocyten populatie uit twee subpopulaties. De meeste T lymfocyten brengen een T cel receptor tot expressie, die bestaat uit een TCR-α en TCR-β eiwitketen, die door een zwavelbrug met elkaar verbonden zijn. De andere subpopulatie bestaat uit T lymfocyten die een T cel receptor bestaande uit de TCR-γ en TCR-δ eiwitketens tot expressie brengen. Deze TCR-γ en TCR-δ eiwitketens zijn al dan niet door een zwavelbrug verbonden. Het T cel receptor-complex is direct betrokken bij de antigeen-herkenning. Nadat het antigeen herkend is, wordt het activatiesignaal doorgegeven via het CD3 eiwit complex. Dit CD3 molecuul bestaat uit vijf non-polymorfe eiwitketens, die non-covalent met de T cel receptor verbonden zijn. Activatie van de lymfocyt kan ook worden bereikt via andere non-polymorfe signaalgeleidende moleculen, zoals CD2 en CD16. Tevens spelen adhesiemoleculen die de niet specifieke antigeen-adhesie tot stand brengen, een belangrijke rol. Deze adhesiemoleculen brengen de effector en doelwitcel in nauw contact, waarna het antigeen door de TCR herkend wordt. Na de antigeen-herkenning vindt er een groot aantal gebeurtenissen plaats. De signaalgeleidings routes, zoals de inositol-lipide en tyrosinekinase route, worden geactiveerd, waarbij verschillende enzymen betrokken zijn. Stimulatie via de inositol-lipide route heeft tot gevolg dat in de cel het enzym protein-kinase C (PKC) wordt geactiveerd. PKC fosforyleert een groot aantal eiwitten. Dit leidt bijvoorbeeld tot de productie van lymfokines of tot activatie van het cytolytische mechanisme van de effector lymfocyt. Als gevolg hiervan wordt de doelwitcel geëlimineerd. Eliminatie van het 'vreemde' antigeen is het uiteindelijke resultaat. Verscheidene membraanmoleculen hebben enzymatische activiteiten zoals CD45, dat een tyrosinefosfatase is, of CD4 en CD8, die eiwit tyrosine kinases zijn. Het CD45 molecuul is de tegenpool van de CD4 en CD8 moleculen.

De verschillende typen van cytotoxische cellen, hun activatieroutes en membraanmoleculen, die betrokken zijn bij de regulatie van de immuunrespons worden beschreven in hoofdstuk I, de introductie.

De cytolytische effector functies van TCRαβ lymfocyten en NK cellen in de immuunrespons tegen allogene, virus geïnfecteerde en tumor cellen zijn de laatste jaren uitgebreid beschreven. De ontdekking van de TCRγδ lymfocyten heeft nieuwe vragen opgeworpen omtrent hun immunologische rol binnen het immuun-netwerk. Met name de receptor structuur en de respectievelijke liganden, alsmede de interacties met de TCRαβ en NK cellen binnen het immuun-netwerk zijn belangrijke vraagstukken. Dit proefschrift behandelt de TCRγδ lymfocyten; hun receptorstructuren en functies. De TCRγδ lymfocyten vormen een kleine populatie (3-10%), van T lymfocyten in het immuunsysteem. De genomische organisatie van de TCRγδ gen elementen is gelijk aan de immunoglobulinen. Verschillende V,D,J, en C elementen rangschikken en vormen zo een functioneel eiwit, dat tot expressie komt op de membraan. Zowel in het perifere bloed als in de thymus komen TCRγδ lymfocyten voor. TCRγδ lymfocyten kunnen in vitro geactiveerd worden door lymfokines en hebben dan een MHC niet-gerestricteerde cytolytische repons tegen een groot scala van allogene tumor target cellen. In dit opzicht zijn de TCRγδ lymfocyten vergelijkbaar met de NK cellen. In andere opzichten zijn de TCRγδ lymfocyten vergelijkbaar met de TCRαβ lymfocyten, nl. antigeen specificiteit en MHC restrictie.

De meerderheid van de TCRγδ lymfocyten in de periferie beschikt over een Vγ9-Vδ2 gen-rangschikking. De TCR-Vγ9 en TCR-Vδ2 eiwitketens zijn verbonden door een zwavel verbinding. Slechts een kleine populatie van TCRγδ lymfocyten in de periferie gebruikt het Vδ1 gen element, resulterend in de expressie van een receptor eiwit dat al dan niet een zwavelverbinding bevat, zoals beschreven is in hoofdstuk II. In de thymus is de verdeling van receptor typen andersom. Daar beschikt het grootste deel van de TCRγδ lymfocyten over een Vδ1 gen rangschikking.

Vrijwel geen TCRγδ lymfocyt brengt het CD4 of het CD8 molecuul tot expressie. Deze moleculen fungeren als co-receptoren in MHC-gerestricteerde herkenning van antigenen, zoals beschreven is voor TCRαβ lymfocyten. De antigeen herkenning van de CD4-CD8- TCR lymfocyten is derhalve niet MHC-gerestricteerd.

De dichotomie in receptorstructuur wordt weerspiegeld in een functioneel verschil tussen de Vγ9-Vδ2 en Vδ1 TCRγδ lymfocyten. De Vγ9-Vδ2 lymfocyten geven een specifieke proliferatieve en cytotoxische respons op stimulatie met Daudi Burkitt's lymfoma cellen. Hoewel de Vδ1 lymfocyten effector/doelwitcel conjugaten kunnen vormen met de Daudi doelwitcel, vindt er geen cytolytische reactie plaats, zoals beschreven is in hoofdstuk III. De Vδ1 lymfocyten zijn echter wel in staat om de Daudi cellijn te lyseren, wanneer er gebruik

gemaakt wordt van een anti-CD3 monoclonaal. Dit anti-CD3 monoclonaal fungeert als brug tussen de Daudi cel (via de Fcγ receptor) en de Vδ1 lymfocyt (via het CD3 complex), zodat deze geactiveerd wordt en de Daudi cel lyseert.

Hoofdstuk IV onthult het karakter van de structuur op Daudi cellen, dat herkend wordt door alle Vγ9-Vδ2 lymfocyten. Daudi cellen brengen het heat shock eiwit, HSP58, tot expressie. HSP of 'stress' eiwitten worden geproduceerd door prokaryotische en eukaryotische cellen als gevolg van invloeden van buiten. De grote mate van sequentie homologie tussen HSP en mycobacteriele antigenen vormt een logische verklaring voor de 'specificiteit' van alle Vγ9-Vδ2 lymfocyten voor Daudi cellen en mycobacteriele preparaten. De stimulatie van de totale subpopulatie van lymfocyten, die alle een identiek V gen element expresseren, heet een superantigeenstimulatie. De CD45RA en CD45RO moleculen discrimineren tussen niet-geactiveerde en 'ooit' geactiveerde lymfocyten. De expressie van het CD45RO molecuul op de membraan van alle Vγ9-Vδ2 lymfocyten geeft aan dat deze lymfocyten reeds in vivo zijn geactiveerd, waarschijnlijk door een superantigeen (Hoofdstuk V). De expressie van het CD45RA molecuul, hetgeen de 'naieve' lymfocyten identificeert, op vers geïsoleerde Vδ1 lymfocyten en NK cellen en de conversie naar het CD45RO fenotype door activatie geeft aan dat de CD45RA en CD45RO moleculen inderdaad kunnen discrimineren tussen niet-geactiveerde en 'ooit' geactiveerde lymfocyten.

Om een beter inzicht te krijgen in de biologische betekenis van de TCRγδ lymfocyten populatie, hebben we onderzocht in welke mate de TCRγδ structuur geconserveerd is in de evolutie. In hoofdstuk VI wordt het voorkomen en functioneren van de TCRγδ populatie in chimpanzees beschreven. Immers, de chimpanzee is het meest verwant aan de mens. Het voortbestaan van de dichotomie tussen de Vγ9-Vδ2 en Vδ1 receptortypen, alsmede de conservering van de antigene specificiteit van de Vγ9-Vδ2 lymfocyten, bewijzen dat de functionele diversiteit gedurende 5 miljoen jaren van evolutie bewaard gebleven zijn. Het laatste hoofdstuk van dit proefschrift, behandelt op grond van de gepresenteerde resultaten en de beschikbare literatuur gegevens, de functionele eigenschappen van de TCRγδ lymfocyten in vitro en hun mogelijke fysiologische rol in de respons tegen bacteriele infecties.

Het onderzoek zoals beschreven is in dit proefschrift levert een bijdrage aan de ontrafeling van de vraagstukken omtrent de receptor structuren en liganden van de TCRγδ lymfocyten populatie. Het plaatst de TCRγδ lymfocyten in de eerste stadia van de immuunrespons, voornamelijk bij de herkenning van 'stress' signalen en bacteriele infecties. Het feit dat in alle tot nu toe geanalyseerde vertebraten TCRγδ lymfocyten gevonden zijn, suggereert dat TCRγδ lymfocyten specifieke/unieke functies hebben en dus een noodzakelijke rol spelen in het immuun-netwerk.

ABBREVIATIONS

ADCC	: antibody dependent cellular cytotoxicity
Ag	: antigen
B-LCL	: lymphoblastoid B cell line
C	: constant
Ca^{++}/Mg^{++}	: calcium, magnesium
CD	: cluster of differentiation
CTL	: cytotoxic T lymphocyte
CSF	: colony stimulating factor
D	: diversity
DG	: diacylglycerol
EBV	: Epstein-Barr virus
EDTA	: ethylene diamine tetraacetic acid
FACS	: fluorescence activated cell sorter
Fc R	: Fc receptor for IgG
FITC	: fluorescein isothiocyanate
GAM	: goat anti-mouse
GM-CSF	: granulo-macrophage colony stimulating factor
HLA	: human leucocyte antigen
HSP	: heat shock protein
J	: joining
ICAM	: intercellular adhesion molecule
IFN	: interferon
Ig	: immunoglobulin
IL	: interleukin
IP3	: inositol triphosphate
kb	: kilobase
kD	: kilodalton
LFA	: leucocyte function associated antigen
mAb	: monoclonal antibody
MHC	: major histocompatibility complex
mW	: molecular weight
NP40	: Nonidet-P40 (detergens)
PBL	: peripheral blood lymphocytes

PCR : polymerase chain reaction
PHA : phytohaemagglutinin
PIP2 : phosphatidyl inositol 4,5 biphosphate
PKC : protein kinase C
PLC : phospholipase C
PMA : phorbol myristate acetate
RFLP : restriction fragment length polymorphism
SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA : staphylococcal enterotoxin A
SRBC : sheep red blood cell
TCR : T cell receptor
TNF : tumor necrosis factor
TPK : tyrosine protein kinase
V : variable

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CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 4 oktober 1962 te Haarlem. Na het behalen van het HAVO diploma in 1980, aan het Coornhert lyceum te Haarlem werd in 1981 het staatsexamen VWO, te 's Gravenhage behaald. In datzelfde jaar werd aan de Rijksuniversiteit Leiden een aanvang gemaakt met de studie Biologie. In december 1987 behaalde zij het doctoraal examen met als hoofdvak Immunologie (Dr. M.J. Giphart) en de bijvakken Moleculaire Immunologie (Drs. H. Spits en J.E. De Vries) en Gistgenetica (Dr. B. Zonneveld).
Vanaf januari 1988 is zij werkzaam op de afdeling Immunologie van de Dr. Daniel den Hoed Kliniek, vanaf 1989 op een door de Nederlandse Kankerbestrijding " Koningin Wilhelmina Fonds" gefinancierd project. Onder de inspirerende leiding van Dr. R.L.H. Bolhuis en Prof. Dr. D.W. Van Bekkum (Instituut Toegepaste Radiobiologie en Immunologie, ITRI) werd het in dit proefschrift beschreven onderzoek verricht.

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