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The majority of human CD3 epitopes are conferred by the epsilon chain

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Abstract

Transgenic mouse T cells expressing the human CD3ε chain bind the majority (29/36) of monoclonal antibodies (mAbs) specific for human CD3. A proportion of these mAbs are also able to recognize isolated CD3ε in a soluble, recombinant form. Thus, CD3ε can confer most CD3 epitopes on the TCR – CD3 complex, but many determinants may require assembly of the complex for their formation. A number of mAbs did not recognize ε-transgenic T cells and probably need other CD3 subunits for binding. CD3-specific mAbs from each of the three groups defined here, as well as mAbs directed against the TCRαβ heterodimer, are all able to activate T cells. Therefore mAb attachment at several different sites on the TCR – CD3 complex can give rise to activation signals. This suggests that the cross-linking function of mitogenic antibodies may be their most significant property, rather than the perturbation of a particular 'functional epitope'.

CD3 is a cluster of transmembrane proteins (termed γ, δ, and ε) associated with antigen receptor on the T cell surface (TCR – CD3 complex) (1,2). Most CD3-specific monoclonal antibodies (mAbs) have mitogenic properties (3) and, largely as a result of this finding, CD3 is thought to trigger T cell activation subsequent to antigen recognition. However, the large number of CD3-specific mAbs that have now been characterized can show marked variation in their ability to elicit certain T cell activation phenomena (4). Some of these effects may be attributable to the avidity of the antibody for its target (5,6) or to antibody isotype (reviewed in ref. 7), but in other cases it has been suggested that the site of mAb binding may be important (4), giving rise to the concept of 'functional' CD3 epitopes. It is expected that a functional epitope corresponds to one or more 'physical' epitopes. Therefore, mapping antigenic determinants on CD3 may define functionally important regions of the complex. Although several studies implicate multiple epitopes on the TCR – CD3 complex (8,9), little information on their localization has so far been obtained. Standard cross-blocking techniques on T cells have been largely uninformative since most CD3-specific mAbs tested seem to block each other at least partially (10 – 12). We have therefore taken two new approaches to mapping mAb binding sites within the CD3 complex, using (i) transgenic mice whose T cells express the human CD3ε gene, and (ii) recombinant soluble forms of CD3γ, -δ, and -ε.

Transgenic mice which carry the human CD3ε gene were found to express the transgene in thymocytes and peripheral T cells, and to deliver the human CD3ε protein to the cell surface as part of the mouse TCR – CD3 complex (A. Tunnacliffe *et al.*, in preparation). When cultured spleen T cells from the transgenic mice were stained with a panel of CD3-specific mAbs, it was found that most (29/36) antibodies were positive, although of varying intensity (Fig 1, Table 1) TCRαβ- or TCRγδ-specific mAbs were negative, as expected. All CD3-specific mAbs stained human peripheral blood T cells equally well and were negative on non-transgenic, littermate mouse T cells The 145-2C11 mAb, specific for mouse CD3ε (13), showed the converse staining pattern (Fig. 1). These results demonstrate that the majority of CD3 epitopes recognized by available CD3-specific mAbs are conferred by the epsilon chain. To test whether some epitopes rely for their formation on assembly of human CD3ε into the mouse TCR-CD3 complex, we assayed for binding of mAbs to isolated CD3ε in the form of a soluble recombinant molecule

Recombinant proteins have been generated which consist of the extracellular domain of either human CD3γ, -δ, or -ε linked to mouse C_κ. These soluble molecules—termed γ-κ, δ-κ, and ε-κ—are produced and secreted by myeloma-derived transfectant clones, and are immunoprecipitable by rat antisera raised against purified CD3 subunits (14). Among the mAbs which stained ε-transgenic T cells, 18 were able to immunoprecipitate surface-labelled, native CD3 (Figs 1 and 2a; summarized in Table 1). These mAbs were tested against metabolically labelled ε-κ, and as controls, γ-κ and δ-κ: a typical experiment is shown in Fig. 2(b – d). Six mAbs, including the widely used UCHT1 and Leu4 antibodies, bound ε-κ (Fig. 2d, Table 1) and therefore recognize CD3ε alone in the native TCR – CD3 complex.

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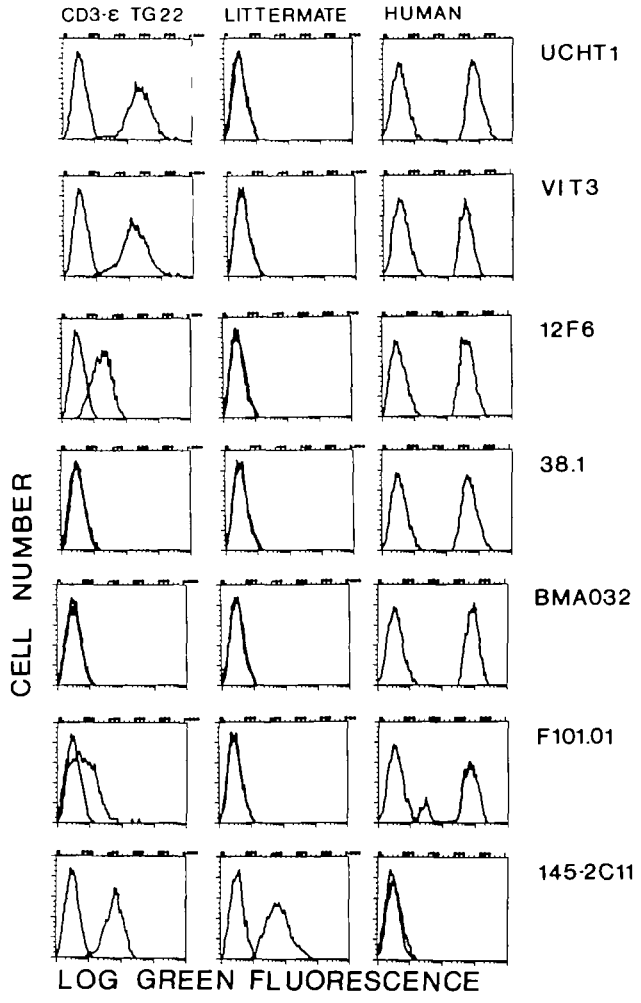

Fig. 1. Staining of CD3ε transgenic mouse, control littermate, and human T cells by CD3-specific monoclonal antibodies Results are depicted as curves of fluorescence distribution on a logarithmic scale The left histogram in each panel represents the binding of normal mouse serum as background control Details of the construction of transgenic mice carrying the human CD3ε gene will be published elsewhere (A. Tunnacliffe *et al.*, in preparation). Mice (of the TG22 line) carrying multiple copies (> 10) of the transgene were analysed here. Spleens were obtained from either TG22 mice or control littermates that did not carry the transgene and single cell suspensions were prepared from these organs Human peripheral blood was from Kantonsspital (Basel) blood bank donors, and mononuclear cells were isolated using Ficoll – Hypaque (Pharmacia, Uppsala, Sweden). Cells were maintained in 'complete medium', consisting of RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM HEPES, 50 mM 2-mercaptoethanol, and 10% heat-inactivated foetal calf serum (GIBCO, Paisley, UK). To prepare T cell lines, cells (2×10^5/ml) were cultured in the presence of anti-CD3 mAb and 50 U/ml recombinant interleukin 2 (rIL2) (Hoffmann-La Roche, Basel) for 4 days. Then the cells were washed to remove unbound antibody and expanded in complete medium containing 50 U/ml rIL2 by splitting the cultures (10^5 cells/ml) every second day. T cell lines used in the experiments reported here were cultured for 12 days The procedure for immunofluorescence surface staining of cells has been described (27). Fluorescein-conjugated goat anti-mouse or anti-rat Ig (Southern Biotechnology) was used as a second step reagent for human CD3-specific mAbs. 145-2C11 was directly conjugated with fluorescein. Quantitation of the surface immunofluorescence on 10^4 viable cells was performed with a FACScan instrument (Becton-Dickinson, Mountain View, CA, USA) equipped with a four decade logarithmic amplifier.

Table 1. Summary of CD3 epitope mapping data

CD3 mAb name	Workshop no. or reference	Isotype	Human PBL	ε transgenic	ε-x
Group I					
UCHT1	471	G1	+ +	+ +	+
VIT3b	478	G1	+ +	+ +	+
G19-4 1	484	G1	+ +	+ +	+
SK7/Leu4	492	G1	+ +	+ +	+
MEM-57	T096	G2a	+ +	+ +	+
CD3-4B5	T103	G1	+ +	+ +	+
Group II					
X35-3	472	G2a	+ +	+ +	–
VIT3	477	M	+ +	+ +	NP
BMA030 (BW264/56)	482	G2b	+ +	+ +	–
CLB-T3/3	489	G2a	+ +	+ +	–
CRIS7	490	G2a	+ +	+ +	ND
YTH12.5	491, T100	G2b (rat)	+ +	+ +	–
F111-409	T099	G1	+ +	+ +	–
CLB-T3 4/2a	ref 7	G2a	+ +	+ +	–
TR-66	ref. 20	G1	+ +	+ +	–
WT32	ref. 21	G2a	+ +	+ +	–
OKT3	ref. 22	G2a	+ +	+ +	–
SPV-T3b	ref 23	G2a	+ +	+ +	ND
11D8	ref. 23	G1	+ +	+ +	ND
XIII-141	473	G2a	+ +	+	NP
XIII-46	474	G2a	+ +	+	NP
XIII-87	475	G2a	+ +	+	NP
12F6	483, T094	G2a	+ +	+	–
T3/RW2-8C8	487	G2	+ +	+	NP
T3/RW2-4B6	488	G2b	+ +	+	–
OKT3D	T098	G1	+ +	+	–
M-T301	T101	G1	+ +	+	NP
SMC2	T104	M	+ +	±	NP
F101.01	ref. 16	G1	+ +	+	ND
Group III					
38 1	476	M	+ +	–	NP
T3/2T8-2F4	486	G1	+ +	–	NP
BL-A8	T093	G1	+ +	–	NP
T10/B9	T095	M	+ +	–	NP
MEM-92	T097	M	+ +	–	NP
M-T302	T102	G1	+ +	–	NP
anti-T3	T199	G2a	+ +	–	NP
TCR					
BMA031	479, T110	G2b	+ +	–	NP
BMA032	480	G2b	+ +	–	NP
WT31	ref. 21	G1	+ +	–	ND
TCRδ1	ref 24	G1	+ +[a]	–	ND
TCRγ/δ1	ref 25	G1	+ +[a]	–	ND
Clonotypes	T105-109	G2a	±	–	ND

The indicated TCR – CD3-specific mAb panel was assayed for surface immunofluorescence in CD3ε transgenic mice or human peripheral blood T cells (PBL) (+ +, bright; +, dull; and –, negative staining) and immunoprecipitation of recombinant soluble CD3ε molecules (ε-x) (+, precipitates; –, does not precipitate ε-x but precipitates membrane CD3, NP, fails to precipitate membrane CD3; ND, not tested).

[a]TCRγδ-specific antibodies stain brightly only a minor subset of PBL.

However, none of the 12 remaining mAbs, which stained ε-transgenic T cells, precipitated γ-x, δ-x, or ε-x, despite their ability to precipitate CD3 from T cells (e.g. Fig. 2, track 8; Table 1).

The data from the transgenic mouse and recombinant CD3 studies define a minimum of three groups of CD3-specific mAbs,

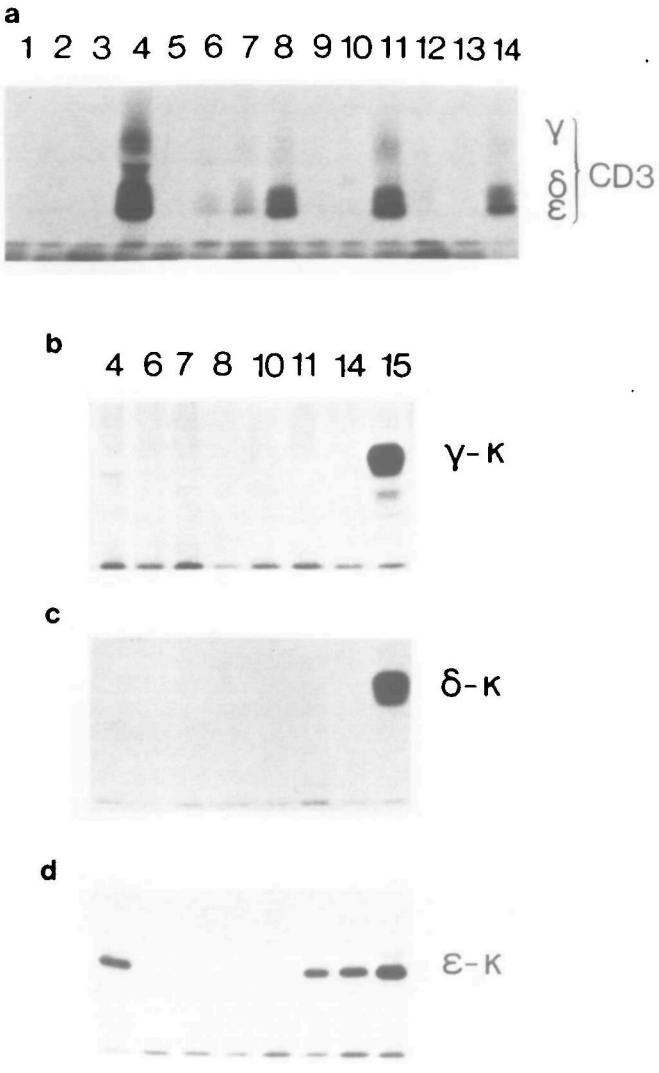

Fig. 2. Immunoprecipitation of native CD3 and recombinant CD3κ molecules by monoclonal CD3-specific antibodies. (a) Iodinated CD3 precipitated from surface-labelled human T cells by monoclonal antibodies. Lanes 1, BL-A8; 2, 12F6; 3, T10/B9; 4, MEM-57, 5, MEM-92; 6, OKT3D; 7, F111-409; 8, YTH12.5, 9, M-T301, 10, M-T302; 11, CD3-4B5, 12, SMC2; 13, 'anti-T3', 14, UCHT1. (b–d) Metabolically labelled γ-κ, δ-κ, and ϵ-κ respectively, precipitated by monoclonal antibodies functional in Fig 2a, numbered identically. Lane 15 is a polyclonal rabbit anti-mouse κ serum. Iodination of T cells (either Jurkat or a CD3$^+$ T cell clone) by the lactoperoxidase method and subsequent immunoprecipitation were adapted from Kaufman *et al.* (28). Cells were metabolically labelled for 4 h with 1 mCi [^{35}S]methionine after 1 h preincubation in methionine-free medium (Amimed, Basel, Switzerland). Immune complexes were first resuspended in RIPA (1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4) and washed in NET-NON (0 5% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, 50 mM Tris–HCl, pH 8, 0 05% sodium azide), followed by a PBS wash before electrophoresis. Antibody 12F6 gave variable results: while in the experiment shown it failed to precipitate native CD3, other experiments were positive

and therefore of corresponding epitopes (Table 1): group I, which recognize CD3ϵ, since they immunoprecipitate ϵ-κ and stain ϵ-transgenic T cells brightly; group II, which stain ϵ-transgenic T cells but do not recognize ϵ-κ; and group III, which do not bind

Table 2. Effect of TCR-CD3-specific mAbs on the proliferation of human T cells

mAb coated	[^{3}H]thymidine uptake (c p m. $\times 10^{-3}$)	
	PBM	PBT
Group I		
UCHT1	47	35
Leu4	68	46
Group II		
BMA030	31	ND
TR-66	44	ND
OKT3	70	58
T3/RW2-8C8	61	50
XIII-141	39	34
Group III		
T3/2T8-2F4	24	20
BL-A8	30	ND
M-T302	53	47
TCR		
BMA032	67	65
WT31	32	25
Control		
CD2 (OKT11)	0.5	0 7
CD5 (Leu-1)	1	0 8
PBS	0 7	0.5

For proliferation assays, human peripheral blood mononuclear (PBM) cells or T cells (PBT) purified using nylon wool columns (26) (2×10^5 cells/well) were cultured in flat-bottomed 96-well microtitre plates in complete medium supplemented with antibodies in the indicated combinations The mAbs (100 ng/well) were directly bound to the plastic surface of the wells via a second step goat anti-mouse Ig antiserum (Southern Biotechnology, Birmingham, AL, USA), as described elsewhere (26). Wells were pulsed with 1 μCi [^{3}H]methylthymidine for the last 9 h of a 4-day culture period and the radioactivity incorporated into the cells was estimated using a β-counter ND, not tested

ϵ-transgenic T cells or recombinant CD3 polypeptides but can bind well to human T cells

Solid-phase mAbs from all 3 groups, as well as TCR$\alpha\beta$-specific mAbs, were able to trigger human peripheral blood mononuclear or purified T cells in a proliferation assay (Table 2). It is therefore clear that a mitogenic stimulus can be delivered through a number of different epitopes on the TCR–CD3 complex.

In summary, it has been demonstrated that most CD3 epitopes (>80%) can be conferred by the human CD3ϵ chain when incorporated into a mouse TCR–CD3 complex. One-third of these epitopes (defined by group I mAbs) could also be seen on recombinant ϵ-κ, which unambiguously maps their position within the native receptor. The group II mAbs, which do not bind ϵ-κ but stain positively ϵ-transgenic T cells, may be recognizing conformational determinants which rely on association of CD3ϵ with other subunits in the complex for their formation. In this regard, the variable intensity of staining of ϵ-transgenic T cells by group II mAbs may be explained by how well assembly-dependent epitopes conferred by CD3ϵ are formed in the chimaeric mouse/human TCR–CD3 complex. A proportion (7/36) of the antibody panel (group III) did not appear to recognize epitopes conferred by CD3ϵ, at least in association with mouse TCR–CD3 subunits. These mAbs may require the presence of other human polypeptides for binding.

Other workers have previously shown by Western blotting that some of the group I mAbs identify CD3ϵ (15). They also demonstrated that these mAbs function in cytoplasmic immunofluorescent staining experiments, whereas group II and (group III) mAbs show no CD3-specific reactivity with either technique (15). Such epitopes are apparently not found either on individual CD3 chains or intracellular CD3 complexes. This further suggests that many group II mAbs may be recognizing conformational epitopes, dependent on the assembly of the whole TCR – CD3 complex. In the case of the F101.01 mAb, evidence has been presented in support of this (16).

That the majority of CD3-specific mAbs recognize epitopes involving CD3ϵ (directly or indirectly) is probably a reflection of the immunodominance of this subunit Strikingly, the differences between the N-terminal sequences of human and mouse CD3ϵ seem to be clustered, a phenomenon not seen for the CD3-γ or -δ chains, where sequence differences are more randomly distributed (17). These patches of sequence disparity may account for the strong mouse anti-human CD3ϵ immune response. Interestingly, two hamster anti-mouse CD3 mAbs also recognize the CD3ϵ chain (13,18). The bias of CD3-specific mAbs to CD3ϵ should temper the conclusions reached using such mAbs in tests for the presence of the CD3 complex as a whole, for example, in developmental studies. New mAbs are currently being developed using soluble CD3-γ and -δ as immunogens which will allow a more accurate assessment of the expression and function of the individual CD3 components in a given cell type.

Functional studies showed that CD3-specific mAbs in each group, as well as TCR$\alpha\beta$-specific mAbs, can promote proliferation of human T cells and therefore that T cell activation by receptor-binding mAbs can proceed through several, and possibly multiple, epitopes. This raises doubts about the existence of particular 'functional' epitopes on the CD3 complex, perturbation of which is responsible for activation. Rather, it suggests that the cross-linking function of mitogenic mAbs may be their most significant property. Indeed, monovalent CD3-specific mAbs do not seem to trigger T cells unless they are cross-linked or bridged to a second cell (19). Furthermore, despite several attempts to define functional epitopes on the TCR – CD3 complex (4), we have been unable to correlate the functional mAb groupings of other authors with those defined here. This suggests that the properties of individual antibodies, such as avidity and isotype, may have to be taken into account in such studies.

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Abbreviations

FACS	fluorescence-activated cell sorter
mAb	monoclonal antibody
PBS	phosphate-buffered saline
TCR	T cell receptor for antigen

References

1 Chang, T. W , Kung, P.C., Gingras, S.P , and Goldstein, G 1981 Does OKT3 monoclonal antibody react with an antigen-recognition structure on human T cells? *Proc. Natl Acad. Sci USA* 78:1805.
2 Reinherz, E L., Meuer, S., Fitzgerald, K A , Hussey, R E., Levine, H , and Schlossman, S. F. 1982. Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. *Cell* 30:735
3 van Wauwe, J. P., De Mey, J R , and Goossens, J. G. 1980 OKT3. a monoclonal anti-human T lymphocyte antibody with potent mitogenic properties. *J. Immunol* 124:2708.
4 McMichael, A J. *et al.*, eds. 1987 *Leucocyte Typing III* Oxford University Press, Oxford.
5 van Lier, R A W., Boot, J H A., Verhoeven, A. J., de Groot, E. R , Brouwer, M. and Aarden, L A. 1987. Functional studies with anti-CD3 heavy chain isotype switch variant monoclonal antibodies Accessory cell-independent induction of interleukin 2 responsiveness in T cells by ϵ-anti-CD3. *J. Immunol.* 139 2873
6 Roosnek, E., van Lier, R. A., and Aarden, L. A. 1987. Two monoclonal anti-CD3 antibodies can induce different events in human T lymphocyte activation *Eur J Immunol.* 17:1507
7 van Lier, R. A. W , Boot, J. H A., de Groot, E. R., and Aarden, L. A. 1987 Induction of T cell proliferation with anti-CD3 switch-variant monoclonal antibodies. effects of heavy chain isotype in monocyte-dependent systems. *Eur J. Immunol* 17:1599.
8 Clark, E A , Martin, P. J., Hansen, J. A., and Ledbetter, J A 1983. Evolution of epitopes on human and non-human primate lymphocyte cell surface antigens. *Immunogenetics* 18 599
9 Brochier, J. and Schmitt, D. 1984 Cross-reactivity of anti-lymphocyte monoclonal antibodies on human skin components. In Reinherz, E. L *et al.*, eds *Leucocyte Typing II*, Vol. 1, p. 407 Springer Verlag, New York.
10 Hansen, J. A , Martin, P J , Beatty, P. G , Clark, E. A., and Ledbetter, J A. 1984. Human T lymphocyte cell surface molecules defined by the workshop monoclonal antibodies ('T cell protocol'). In Bernard, A., Boumsell, L , Dausset, J., Milstein, C., and Schlossman, S. F , eds, *Leucocyte Typing*, p. 195 Springer Verlag, Berlin.
11 Kurrle, R , Seyfert, W , Trautwein, A., and Seiler, F R. 1985. Cellular mechanisms of T cell activation by modulation of the T3 antigen complex. *Transplant Proc.* 17 880
12 Wallace, D. L., Macintyre, E A., Linch, D. C., and Beverley, P. C L. 1987. Analysis of the activation signals induced by CD3 antibodies and their role in T-cell proliferation. In McMichael, A J *et al.*, eds, *Leucocye Typing III*, p. 167. Oxford University Press, Oxford
13 Leo, O., Foo, M., Sachs, D H., Samelson, L E , and Bluestone, J. A. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA* 84:1374.
14 Tunnacliffe, A., Olsson, C , Traunecker, A., Krissansen, G W., and Karjalainen, K. 1989. Production and secretion of recombinant soluble CD3 polypeptides by myeloma-derived transfectant clones *Immunol. Lett.* 21 243
15 van Dongen, J. J. M., Krissansen, G. W , Wolvers-Tettero, I. L. M., Comans-Bitter, W.M., Adriaansen, H. J., Hooijkaas, H , van Wering, E R., and Terhorst, C. 1988 Cytoplasmic expression of the CD3 antigen as a diagnostic marker for immature T-cell malignancies. *Blood* 71.603
16 Geisler, C., Plesner, T , Pallesen, G., Skødt, K., Ødum, N., and Larsen, J. K. 1988. Characterisation and expression of the human T cell receptor – T3 complex by monoclonal antibody F101.01. *Scand J. Immunol.* 27:685.
17 Clevers, H., Alarcon, B., Wileman, T , and Terhorst, C. 1988. The T cell receptor/CD3 complex. a dynamic protein ensemble. *Annu. Rev. Immunol.* 6:629.
18 Havran, W. L., Poenie, M., Kimura, J., Tsien, R., Weiss, A., and Allison, J. P. 1987. Expression and function of the CD3-antigen receptor on murine CD4+8+ thymocytes *Nature* 330:170.
19 Roosnek, E. and Lanzavecchia, A. 1989. Triggering T cells by otherwise inert hybrid anti-CD3/anti-tumor antibodies requires encounter with the specific target cell. *J. Exp. Med.* 170:297.
20 Lanzavecchia, A. and Scheidegger, D. 1987. The use of hybrid hybridomas to target human cytotoxic T lymphocytes. *Eur. J. Immunol.* 17.105.

21 Tax, W. J. M., Willems, H W., Reekers, P. P M , Capel, P. J A , and Koene, R. A. P. 1983. Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells *Nature* 304·445.
22 Kung, P.C , Goldstein, G., Reinherz, E L., and Schlossman, S. F 1979. Monoclonal antibodies defining distinctive human T cell surface antigens *Science* 206.347.
23 Spits, H , Keizer, G., Borst, J., Terhorst, C., Hekman, A , and de Vries, J E. 1983. Characterization of monoclonal antibodies against cell surface molecules associated with cytotoxic activity of natural and activated killer cells and cloned CTL lines. *Hybridoma* 2:423
24 Band, H., Hostenbach, F., McLean, J , Hata, S., Krangel, M. S , and Brenner, M. B. 1987 Immunochemical proof that a novel rearranging gene encodes the T cell receptor δ subunit. *Science* 238·682.
25 Borst, J., Van Dongen, J. J. M., Bolhuis, R L.H., Peters, P J , and van de Griend, R. J. 1988. Distinct molecular forms of human T cell receptor γδ detected on viable T cells by a monoclonal antibody *J Exp. Med* 167·1625
26 Carrera, A. C., Rincon, M., Sanchez-Madrid, F., Lopez-Botet, M , and O. de Landazuri, M. 1988 Triggering of co-mitogenic signals in T cell proliferation by anti-LFA-1 (CD18, CD11a), LFA-3 and CD7 monoclonal antibodies. *J. Immunol.* 141.1919.
27 Tonbio, M.L., de la Hera, A., Borst, J , Marcos, M. A R., Marquez, C., Alonso, J M , Barcena, A., and Martinez-A., C 1988. Involvement of the interleukin 2 pathway in the rearrangement and expression of both αβ and γδ cell receptor genes in human T cell precursors *J Exp. Med.* 168.2231
28 Kaufman, J F , Flajnik, M. F., du Pasquier, L., and Tiegert, P 1985 *Xenopus* MHC class II molecules. I. Identification and structural characterisation *J Immunol* 134·3248

Note added in proof

Some similar data to those described in this article have been presented by Transy *et al* 1989 *Eur. J. Immunol* 19·947