# Combinatorial functions of two chimeric antibodies directed to human CD4 and one directed to the α-chain of the human interleukin-2 receptor

Winfried Weissenhorn [a,*], Werner Scheuer [b], Brigitte Kaluza [b], Marina Schwirzke [b], Christian Reiter [a], Dimitri Flieger [a], Helmut Lenz [c], Elisabeth H. Weiss [a], Ernst Peter Rieber [a], Gert Riethmüller [a] and Ulrich H. Weidle [b]

[a] Institut für Immunologie der Universität München, W-8000 Munich, Germany. Tel. (49-89)5996-689; [b] Boehringer Mannheim, GmbH, Department of Biotechnology, W-8122 Penzberg, Germany; [c] Biochemical Research Center, Boehringer Mannheim, GmbH, W-8132 Tutzing, Germany. Tel. (49-8158)22420

## SUMMARY

The general feasibility of chimerization of monoclonal antibodies (mAbs) has already been shown for a large number of them. In order to evaluate in vitro parameters relevant to immunosuppressive therapy, we have chimerized and synthesized two anti-CD4 mAbs recognizing two different epitopes on the human T-lymphocyte antigen, CD4. The chimerized mAbs are produced at levels corresponding to those of the original hybridoma cell lines. With respect to activation of human complement, the individual Abs are negative; however, when used in combination, complement activation was performed. When applied in combination, they were found to modulate the CD4 antigen, whereas the individual mAb do not display this property. Individually they mediate an up to 60% inhibition of the mixed lymphocyte reaction (MLR). However, by combination of an anti-CD4 mAb with one directed against the α-chain of the human IL2 receptor, nearly 100% inhibition of the MLR was achieved, even with reduced dosage of the mAbs. Our data suggest that the combination of an anti-CD4 mAb and an anti-IL2Rα chain mAb is more effective with respect to immunosuppression than each mAb by itself, indicating that this mAb cocktail could be a new strategy for immunosuppressive therapy.

Correspondence to: Dr. U.H. Weidle, Department of Biotechnology, Boehringer Mannheim, GmbH, Nonnenwald 2, Postfach 1152, W-8122 Penzberg, Germany. Tel. (49-8856)60-2801; Fax (49-8856)60-2659.
* Present address: Howard Hughes Medical Institute, Laboratory of Molecular Medicine, 300 Longwood Ave., Boston, MA, USA. Tel. (617)735-7373; Fax (617)730-0506.

Abbreviations: aa, amino acid(s); Ab, antibody; cDNA, DNA complementary to mRNA; CDR, complementarity-determining regions; CD4, T-cell surface antigen; D, diversity regions; Fab, antigen-binding fragments of Abs that are derived by papain digestion and contain the L chain and part of the H chain (variable region and first constant region); FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; H, heavy chain of Ab; Ig, immunoglobulin; IL2, interleukin 2; IL2R, IL2 receptor; IL2Rα, α chain of IL2R; J, joining region; kb, kilobase(s) or 1000 bp; L, light chain of Ab; mAb, monoclonal Ab; MES, morpholinoethanol-sulfonic acid; MLR, mixed lymphocyte reaction; nt, nucleotide(s); PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline (0.15 M NaCl/10 mM Na·phosphate pH 7.5); PCR, polymerase chain reaction; RPMI, Roswell Park Memorial Institute (medium); V, gene (DNA) encoding V; VDJ, variable region of rearranged H chain; $V_H$, variable region of heavy chain; VJ, variable region of rearranged L chain; $V_L$, variable region of light chain.

## INTRODUCTION

Antibodies that recognize antigens on the surface of human lymphocytes have been used as immunosuppressive agents for more than 20 years. Since the development of techniques to produce mAb (Köhler and Milstein, 1975)

that recognize a variety of antigens on the lymphocyte surface, mAbs are clinically applied for the diagnosis and ther-apy of an array of human disorders. Possible targets for immune intervention focus on two antigens expressed by T lymphocytes. CD4 is only expressed by a subset of T lymphocytes. This phenotype is usually, but not exclusively,

(a)
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1                    .         .         .         .         .         .
ATGATGTCCTCTGCTCAGTTCCTTGGTCTCCTGTTGCTCTGTTTTCAAGGTACCAGATGT
MetMetSerSerAlaGlnPheLeuGlyLeuLeuLeuLeuLeuCysPheGlnGlyThrArgCys
-20

61                   .         .         .         .         .         .
GATATCCAGATGACACAGACTATATCCTCCCTCTCTGCCTCTCTGGGAGACAGAGTCACC
AspIleGlnMetThrGlnThrIleSerSerLeuSerAlaSerLeuGlyAspArgValThr
1                                                            20

121                  .         .         .         .         .         .
ATCAGTTGCAGGGCAAGTCAGGACATTAACAATTATTTAAGCTGGTATCAGCAGAAACCA
IleSerCysArgAlaSerGlnAspIleAsnAsnTyrLeuSerTrpTyrGlnGlnLysPro
                        CDR1                                 40

181                  .         .         .         .         .         .
GATGGAACTGTTAAACTCCTGATCTACTACACATCAAGATTACATTCAGGAGTCCCATCA
AspGlyThrValLysLeuLeuIleTyrTyrThrSerArgLeuHisSerGlyValProSer
                                       CDR2                   60

241                  .         .         .         .         .         .
AGGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTACCAACCTGGAGCAA
ArgPheSerGlySerGlySerGlyThrAspTyrSerLeuThrIleThrAsnLeuGluGln
                                                             80

301                  .         .         .         .         .         .
GAAGATGTTGCCACTTACTTTTGCCAACAGGGTAATACGCTTCCGTACACGTTCGGAGGG
GluAspValAlaThrTyrPheCysGlnGlnGlyAsnThrLeuProTyrThrPheGlyGly
                        CDR3                                 100

361
GGGACCAAGCTGGAAATAAAA
GlyThrLysLeuGluIleLys
             107
```

(b)
```
1                    .         .         .         .         .         .
ATGGCTTGGGTGTGGACCTTGCTTTTCCTGATGGCAGCTGCCCAAAGTATCCAAGCACAG
MetAlaTrpValTrpThrLeuLeuPheLeuMetAlaAlaAlaGlnSerIleGlnAlaGln
-19                                                           1

61                   .         .         .         .         .         .
ATCCAGTTGGTGCAGTCTGGACCTGAGCTGAAGACGCCTGGAGAGACAGTCAAGATCTCC
IleGlnLeuValGlnSerGlyProGluLeuLysThrProGlyGluThrValLysIleSer
                                                             21

121                  .         .         .         .         .         .
TGCAAGGCTTCTGGTTATACCTTCACAGACTATTCAATACACTGGGTGAAGCAGGCTCCA
CysLysAlaSerGlyTyrThrPheThrAspTyrSerIleHisTrpValLysGlnAlaPro
                        CDR1                                 41

181                  .         .         .         .         .         .
GGGAAGGATTTAAAGTGGATGGGCTGGATAAACACTGAGACTGGTGAGCCAACATATGCA
GlyLysAspLeuLysTrpMetGlyTrpIleAsnThrGluThrGlyGluProThrTyrAla
                                       CDR2 61

241                  .         .         .         .         .         .
GATGACTTCACGGGACGGTTTGCCTTCTCTTTGGAAACCTCTGCCAGCACTGCCTATTTG
AspAspPheThrGlyArgPheAlaPheSerLeuGluThrSerAlaSerThrAlaTyrLeu
                                                             81

301                  .         .         .         .         .         .
CAGATCAACAACCTCAAAAATGAGGACACGTCTACATATTTCTGTGCTATTCATTACTAC
GlnIleAsnAsnLeuLysAsnGluAspThrSerThrTyrPheCysAlaIleHisTyrTyr
                                                            101

361                  .         .         .         .         .         .
GCCTACGGGGATCCTTTGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA
AlaTyrGlyAspProLeuAspTyrTrpGlyGlnGlyThrSerValThrValSerSer
   CDR3                                                 120
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(c)
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1                    .         .         .         .         .         .
ATGGAGACAGACACAATCCTGCTATGGGTGCTGCTGCTCTGGGTTCCAGGCTCCACTGGT
MetGluThrAspThrIleLeuLeuTrpValLeuLeuLeuTrpValProGlySerThrGly
-20

61                   .         .         .         .         .         .
GACATTGTGCTGACCCAATCTCCAGCTTCTTTGCCTATGTCTCTAGGGCAGAGGGCCACC
AspIleValLeuThrGlnSerProAlaSerLeuProMetSerLeuGlyGlnArgAlaThr
1                                                            20

121                  .         .         .         .         .         .
ATCTCCTGCAAGGCCAGCCAAAGTCTTGATTATGATGGTGATAGTTATATGAACTGGTAC
IleSerCysLysAlaSerGlnSerLeuAspTyrAspGlyAspSerTyrMetAsnTrpTyr
                        CDR1                                 40

181                  .         .         .         .         .         .
CAACAGAAACCAGGACAGCCACCCAAACTCCTCATCTATGCTGCATCCAATCTAGAATCT
GlnGlnLysProGlyGlnProProLysLeuLeuIleTyrAlaAlaSerAsnLeuGluSer
                                       CDR2 60

241                  .         .         .         .         .         .
GGGATCCCAGCCAGATTTAGTGGCAGTGGGTCCGGGACAGACTTCACCCTCAACATCCAT
GlyIleProAlaArgPheSerGlySerGlySerGlyThrAspPheThrLeuAsnIleHis
                                                             80

301                  .         .         .         .         .         .
CCTGTGGAGGAGGAGGATGCTGCAACCTATTACTGTCAGCAAAGTAGTGAGGATCCTCCG
ProValGluGluGluAspAlaAlaThrTyrTyrCysGlnGlnSerSerGluAspProPro
                                       CDR3 100

361
ACGTTCGGTGGAGGCACCAAGCTGGAAATCAAA
ThrPheGlyGlyGlyThrLysLeuGluIleLys
                              111
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(d)
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1                    .         .         .         .         .         .
ATGGAATGGAGGATCTTTCTCTTCATCCTGTCAGGAACTGCAGGTGTCCACTCCCAGGTT
MetGluTrpArgIlePheLeuPheIleLeuSerGlyThrAlaGlyValHisSerGlnVal
-18                                                           1

61                   .         .         .         .         .         .
CACCTGCAGCAGTCTGGACCTGAGCTGGTGAAGCCTGGGCCTTCAGTGAAGATGTCCTGC
HisLeuGlnGlnSerGlyProGluLeuValLysProGlyProSerValLysMetSerCys
                                                             22

121                  .         .         .         .         .         .
AAGGCTTCTGGATACACATTCACTGACTATGTTGTAAGTTGGATGCAACAGAGAACTGGA
LysAlaSerGlyTyrThrPheThrAspTyrValValSerTrpMetGlnGlnArgThrGly
                        CDR1                                 42

181                  .         .         .         .         .         .
CAGGTCCTTGAGTGGATTGGAGAGATTTATCCTGGAAGTGGTAGTGCTTATTACAATGAA
GlnValLeuGluTrpIleGlyGluIleTyrProGlySerGlySerAlaTyrTyrAsnGlu
                                       CDR2 62

241                  .         .         .         .         .         .
AAATTCAAGGGCAAGGCCATACTGACTGCAGACAAATCCTCCAGCACAGCCTACATGGAG
LysPheLysGlyLysAlaIleLeuThrAlaAspLysSerSerSerThrAlaTyrMetGlu
                                                             82

301                  .         .         .         .         .         .
TTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTTTTTCTGTGCAAGACGGGGGGATGGT
PheSerSerLeuThrSerGluAspSerAlaValPhePheCysAlaArgArgGlyAspGly
                                                            102

361                  .         .         .         .         .
TCCCTCGGCTTTGCTCACTGGGGCCAAGGGACTCTGGTCAGTGTCGCTGCA
SerLeuGlyPheAlaHisTrpGlyGlnGlyThrLeuValSerValAlaAla
CDR3                                             119
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Fig. 1. Sequences of (a) L and (b) H chains of mAb M-T151 and (c) L and (d) H chains of mAb M-T310. The CDR regions (Kabat et al., 1987) are underlined. The numbers below the sequence indicate aa positions; 1 indicates the first aa of the mature protein; upstream aa represent the signal sequence. The numbers on the left margin specify the nt positions, with nt = 1 corresponding to the A of the ATG start codon. In (a) the $J_2$ region of the L chain of mAb M-T151 is from Tyr[96] to Lys[107], in (b) the $J_4$ region of the H chain is from Asp[108] to Ser[120], in (c) the $J_1$ region of the L chain of mAb M-T310 is from Thr[101] to Lys[111], in (d) the $J_3$ region of the H chain is from His[107] to Ala[118]. **Methods.** RNA was isolated from $1 \times 10^7$ hybridoma cells secreting mAbs M-T151 and M-T310 and selected for poly(A)$^+$ RNA on oligo(dT) cellulose (Maniatis et al., 1982). cDNA was synthesized with the BRL cDNA synthesis kit according to the instructions of the supplier and the sequences determined by the dideoxy method (Sanger et al., 1977; Weissenhorn et al., 1991).

associated with helper/inducer T cell functions such as promoting immunoglobulin production and cytotoxicity. It has been shown that mAb targeting of CD4 T cells induced profound humoral and cellular immunological unrespon-siveness. The experimental findings in the mouse system (Cobbold et al., 1984; Miller et al., 1985) have led to clinical evaluation of anti-CD4 mAbs in the serotherapy of diseases (Herzog et al., 1987; Reiter et al., 1991; Reinke et al., 1991). The IL2R is a target of considerable interest, particularly as it is an activation antigen, composed of an inducible $\alpha$ chain (55 kDa) and a $\beta$ chain (75 kDa), which only appears on the surface of T lymphocytes when they are activated by antigen or mitogen. Successful therapeutical application of an IL2R mAb has been already shown by different groups (Kyle et al., 1989; Hervé et al., 1990; Waldmann, 1991a).

Since there are limitations to administering non-human proteins, efforts to engineer new generations of antibodies are underway and are directed towards making the antibodies more human-like, with the aim of lowering the immunogenicity of these proteins in man and optimizing their physiological effector functions and pharmacokinetic characteristics. One approach to decrease mAb immunogenicity (Morrison et al., 1984) is the synthesis of chimeric mAb. After the demonstration that lymphoid cells can express cloned, transfected Ig genes, mouse/human chimeric mAbs were generated with specificities directed towards a wide range of tumour antigens and T-cell surface molecules. The benefits of chimeric mAb for serotherapy in humans are widely accepted.

The aim of this work was to generate chimeric mouse/human antibodies directed against the T-cell antigen CD4. Furthermore, the chimeric mAbs M-T310 and M-T151 were analysed alone and in combination with MAK 179, an IL2Rα chimeric mAb (Kaluza et al., 1991), with respect to their physiological effector functions. With the aim of possible clinical application, we have evaluated in vitro possible synergistic effects between the two anti-CD4 mAb M-T310 and M-T151, recognizing two different epitopes on the first and second extracellular domains of the T-cell surface glycoprotein CD4 (Peterson and Seed, 1988; Sattentau et al., 1989; Ashkenazi et al., 1990), and combinations of the two anti-CD4-specific chimeric mAbs with the IL2Rα-specific chimeric mAb MAK 179.

## RESULTS AND DISCUSSION

### (a) Cloning and sequence analysis of V region sequences

The functionally rearranged $V_H$ and $V_L$ regions of mAbs M-T310 and M-T151 have been cloned and subsequently fused to human constant region gene segments encoding the human $\gamma 1$ chain (vector pUHWγ1) and human $\kappa$ chain (vector pUHWκ), as described (Weissenhorn et al., 1991). The nt and the deduced aa sequences of anti-CD4 mAb $V_H$ and $V_L$ regions are shown in Fig. 1. Both the $V_H$ and $V_L$ sequences of anti-CD4 mAbs M-T310 and M-T151 show no significant homology with respect to their nt and aa sequences. The $V_H$ region genes can be attributed to two different $V_H$ gene families according to previously defined

**A**

**B**
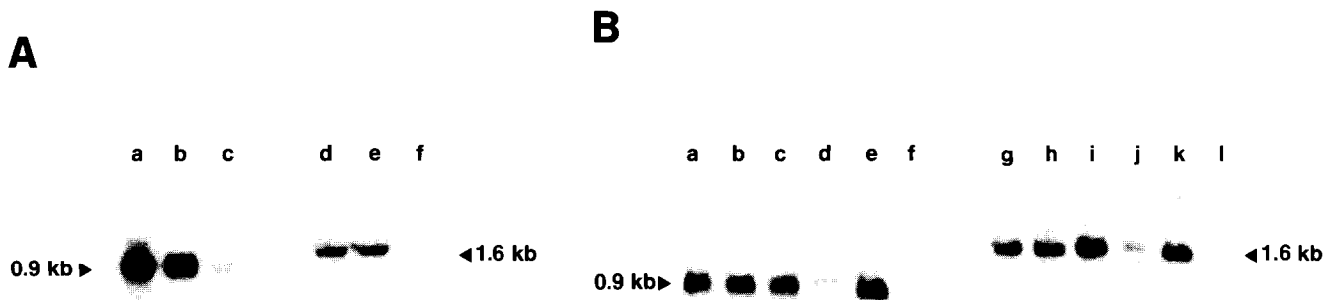


Fig. 2. Northern blot analysis of hybridomas and transfectomas secreting murine and chimeric mAb M-T151 and M-T310. Panel A displays the analysis of $\kappa$ and $\gamma$ transcripts for mAb M-T310: lanes a–c $\kappa$ transcripts of mAb M-T310; a, transfectoma; b, hybridoma; c, recipient cell line Sp2/0. Lanes d–f, $\gamma$ transcripts of mAb M-T310. Panel B shows the equivalent analysis for mAb M-T151. The sizes of the detected transcripts are shown in kb. $\kappa$ and $\gamma$ transcripts of mAb M-T151: a–f, $\kappa$ transcripts; g–l, $\gamma$ transcripts; a–d, four transfectomas; e, hybridoma; f, Sp2/0, g–j, four transfectomas corresponding to those in lanes a–d; k, hybridoma; l, Sp2/0. **Methods.** Total RNA was extracted from $1 \times 10^8$ cells according to the method described by Auffray and Rougeon (1980). Ten $\mu$g of total RNA was electrophoresed on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a nitrocellulose membrane (Amersham, Braunschweig, Germany) by standard procedures (Maniatis et al., 1982). The blots were hybridized with V-specific probes. For detection of $\kappa$ mRNA a 274-bp EcoRV-NotI fragment was used for M-T151; for M-T310 a 248-bp KpnI-NotI fragment was used as a probe. The $\gamma$ transcripts were detected with the following probes: a 767-bp StyI fragment for mAb M-T310 and a 367-bp BamHI-BglII fragment for M-T151. Radioactive probes were obtained by labelling the fragments with [$\alpha$-$^{32}$P]dCTP using the random primed DNA-labelling kit supplied by Boehringer Mannheim. Then $2 \times 10^6$ cpm per labelled fragment were used for hybridization, which was performed as described in Maniatis et al. (1982).

$V_H$ gene families. $V$ gene families comprise groups of related $V$ genes that in general share greater than 80% sequence similarity (Brodeur and Riblet, 1984; Winter et al., 1985; Kabat et al., 1987). The $V_H$ region gene segment of mAb M-T310 is a member of the J558 gene family, and the $V_H$ region of mAb M-T151 belongs to the $VGam$ gene family. The $V_H$ regions display different D region segments which have been rearranged either to J3 or to J4. The heterogeneous nature of the 'D' sequences makes the precise determination of $V_H$-D and D-$J_H$ boundaries impossible. Although none of these somatic D regions corresponds exactly to identified germline D segments (Kurosawa and Tonegawa, 1982), they all show regions of homology flanked by relatively G-rich sequences (N sequences) which have previously been proposed to be inserted during $V_H$-D and D-$J_H$ joining events (Alt and Baltimore, 1982). Similar results were obtained for the V$\kappa$ sequences. M-T310 V$\kappa$ is a member of the V$\kappa$21 subgroup and V$\kappa$ M-T151 of the V$\kappa$10 subgroup (Kabat et al., 1987; Kofler et al., 1989; Strohal et al., 1989). V$\kappa$ M-T310 is rearranged to J1 and V$\kappa$ M-T151, to J2. This diversity in sequence correlates with their different CD4 antigen binding pattern. Both mAbs recognize different epitopes on the CD4 molecule as indicated by cross-blocking experiments (data not shown), and they map to different antigenic regions, as shown by mutational analysis of the CD4 molecule (Peterson and Seed, 1988; Sattentau et al., 1989; Ashkenazi et al., 1990).

## (b) Synthesis of chimeric mAb M-T151 and M-T310 in transfectomas

Genomic VJ and VDJ regions of mAbs M-T151 and M-T310 were isolated by the PCR reaction, fused to human $\kappa$ and $\gamma$1 constant regions, transfected into Sp2/0 cells and stable transformants isolated by G418 selection. Details are described in Weissenhorn et al. (1991). Fig. 2 reveals that transfectants with steady state levels of $\kappa$- and $\gamma$1-specific transcripts corresponding in abundance to those of the original hybridoma cells could be isolated after screening about 200 transfectants for each chimeric mAb. This holds true with the exception of the transfectant shown in Fig. 2B, lanes d and j. With the $\kappa$-specific V region probe, slight cross-hybridisation to an endogeneous $\kappa$ message in the recipient cell line Sp2/0 was observed (Fig. 2A, lane c). This message contains a premature stop codon and does not mediate synthesis of a secreted, truncated protein (Cabilly and Riggs, 1985). Synthesis of murine and chimeric mAb corresponds to 15–20 $\mu$g/ml/$10^6$ cells/24 h. The isolated VJ and VDJ regions contain the authentic introns in the signal sequences of L and H chains. Expression of the chimeric genes is mediated by a tandem combination of a H chain promoter/enhancer element (Weissenhorn et al., 1991). The result indicates that transfectomas with expression levels of chimeric mAb corresponding to those of the original hybridoma can be established with this experimental design without the need to amplify transfected genes.
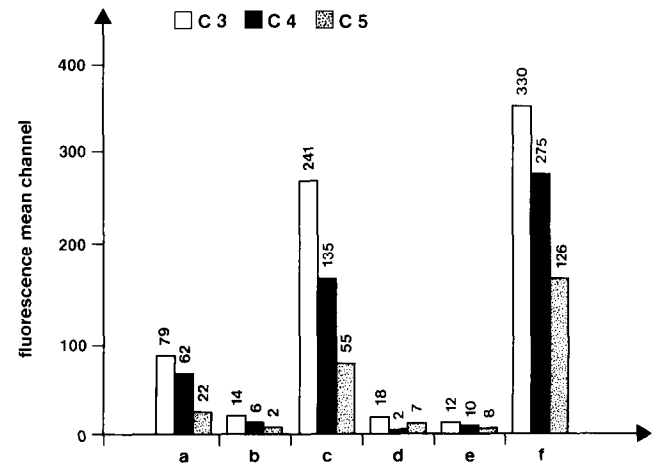


Fig. 3. Activation of human complement by anti-CD4 mAb. Deposition of complement components C3c, C4 and C5 is shown as total fluorescence mean channel after detection by C3-, C4- and C5-specific FITC-conjugated antisera in a FACscan analysis; a, m M-T151 (IgG$_{2a}$); b, m M-T310 (IgG1); c, m M-T151+m M-T310; d, chim M-T151 (IgG1); e, chim M-T310 (IgG1); f, chim M-T151+chim M-T310. Open bars represent C3c, filled bars C4 and stippled bars C5. **Methods.** Hybridomas and transfectomas were fermented in a 10-liter reactor in suspension culture with 5% FCS. After concentration of the eluate to 700 ml, the $\gamma$ globulin fraction was precipitated by adjusting the supernatant to 2 M $(NH_4)_2SO_4$ pH 7. After dialysis against 10 mM MES buffer pH 5.8, the fraction was applied to an S-Sepharose column (Pharmacia) equilibrated with the same buffer at 4°C, eluted with a linear NaCl gradient (0.1–0.25 M pH 5.8), and fractions containing mAbs were pooled. The material was adjusted to 1 M $(NH_4)_2SO_4$ pH 9, applied to a Protein A Sepharose column (Pharmacia) equilibrated with the same buffer and eluted with 0.1 M Na$_3$·citrate pH 3.4. After dialysis against PBS buffer pH 7.5 and ultrafiltration, contaminating bovine IgG was removed by adsorption to Spherosil conjugated with sheep anti-bovine IgG. For the complement activation experiments, PBL from EDTA-blood of healthy adults were isolated by Ficoll-Hypaque density centrifugation. Then 1 × $10^5$ PBL were incubated with 50 $\mu$l of purified mAb (10 $\mu$g/ml of each mAb) for 30 min on ice. After washing twice with ice-cold PBS, 50 $\mu$l of fresh human serum was added to the cells and incubated for 20 min at 37°C. For the negative control, mAb-targeted cells were incubated with heat-inactivated serum. The cells were washed twice with ice-cold PBS and subjected to complement component-specific antisera and incubated on ice for 20 min (for detection of C3c a rabbit anti-C3c, for C4 a rabbit anti-C4 FITC-conjugated serum and for C5 a rabbit anti-C5 serum was used; all from Dakopatts). After washing cells twice with PBS, the anti-C3c and anti-C5 targeted cells were further incubated with goat anti-rabbit Ig FITC-conjugated serum (Dianova) for 20 min on ice, washed twice with PBS and fixed with 1% paraformaldehyde. The fluorescence intensity was quantified by FACscan analysis as fluorescence mean channel of living CD4$^+$ cells on a log scale. The value of the mean channel was obtained by subtraction of the value of the mean channel of the negative control (incubation of CD4-mAb-targeted T cells with heat-inactivated serum and detection of cells with complement-specific antisera) from total mean channel of positive experiments.

## (c) Complement activation

As the aim of targeted mAb therapy is either modulation of the CD4 molecule (Lifson and Engleman, 1989) or elimination of reactive T cells, it was important to evaluate the complement activation properties of our mAbs. Compared with other human Ig subclasses, the greatest effectiveness with respect to physiological effector functions has been described for the IgG1 isotype (Brüggemann et al., 1987; Riechmann et al., 1988). We therefore selected the human IgG1 isotype for chimerization of the anti-CD4 mAbs. The activation of complement was assayed by determination of the ability of the mAbs to induce deposition of components of the complement cascade on the surface of CD4$^+$ PBL.

The results show that murine M-T151 (IgG$_{2a}$) activates human complement weakly (Fig. 3a), whereas murine M-T310 (IgG1) is negative with respect to this property (Fig. 3b). The combination of these murine anti-CD4 mAbs results in a dramatic enhancement of activation of human complement (Fig. 3c). The chimeric mAbs M-T151 (IgG1) and M-T310 (IgG1) do not display human complement-activating properties (Fig. 3d,e) by themselves; however, a combination of these mAbs results in complement activation stronger than the activation induced by a combination of the murine mAb (Fig. 3f). As a negative control, mAb-targeted cells have been incubated with heat-inactivated serum. Consistently, only background levels of fluorescence intensity have been observed (data not shown). Unfortunately, a lytic mAb pair was not available for our studies. Synergy with respect to complement activation for pairs of mAbs has also been shown by Bindon et al. (1985) and Hughes-Jones et al. (1983).

## (d) Immunomodulation by chimeric anti-CD4 mAbs

To determine the possible role of modulation of CD4$^+$ cells rather than elimination of these cells, the effect of anti-CD4 mAbs on whole blood cells was determined. Fig. 4 shows the result of these experiments. The blood of both donor A and donor B did not show any effect when coculturing the whole blood with only one chimeric anti-CD4 mAb alone. The amount of CD4$^+$ cells from time point 0 until 48 h, differentiating into total CD4, high-expressing and low-expressing cells, resulted in the same graphs in the control samples as well as in the samples containing either the anti-CD4 mAb M-T310 or M-T151. There is no significant decrease of CD4$^+$ cells induced by the chimeric anti-CD4 mAb. By adding both anti-CD4 mAbs, M-T310 and M-T151, the CD4 level changes from time point 0 to 48 h. Samples of both blood donors show a 90% (A) and 70% (B) reduction of high-expressing CD4 cells and an increase of low-expressing CD4 cells (by a factor of 5). The reduction of high-expressing cells correlates with the increase of low-expressing cells. Summing up the amounts of high- and low-expressing CD4 cells at time point 48 h leaves the total amount of all CD4$^+$ cells unchanged. Therefore, we conclude from these experiments that our chimeric anti-CD4 mAbs induce modulation of the CD4 antigen rather than elimination of CD4-expressing cells.
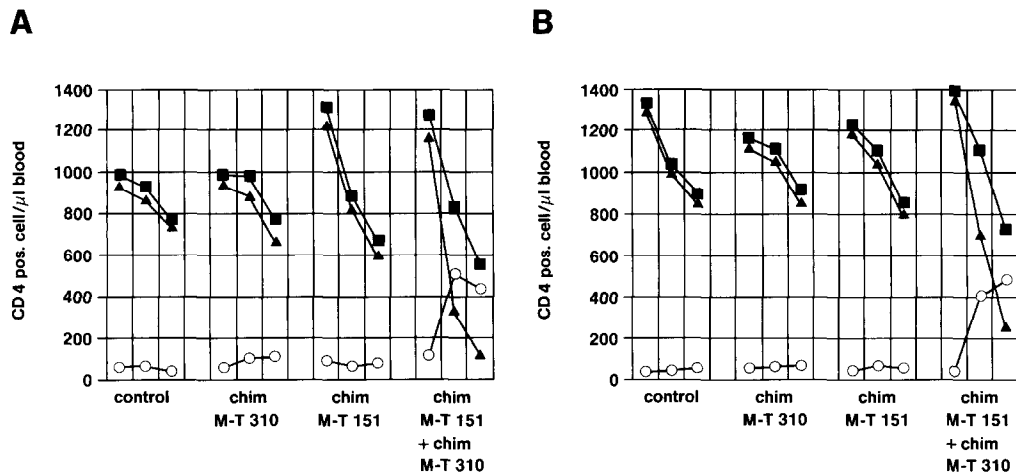


Fig. 4. Immunomodulation by chimeric CD4 mAbs. Reduction of total and high CD4-expressing cells as well as increase of low CD4-expressing cells are shown by the different graphs after incubation of whole blood cells with chimeric mAbs or combinations thereof. In graphs **A** and **B** blood of two different donors was analysed. Total, high and low CD4-expressing cells were determined at the beginning of the experiment and at 24 and 48 h after incubation with the mAbs, respectively. Filled squares represent total CD4 cells, filled triangles high CD4-expressing cells, and open circles display low CD4-expressing cells. **Methods.** 100 µl of whole heparin-treated blood was cocultured with chimeric anti-CD4 mAb (100 µg/ml) at 37°C in flat-bottom microtiter plates in duplicates. At time points 0, 24 and 48 h, the amount of CD4$^+$ cells was determined by FACscan analysis with the FITC-conjugated mAb OKT4 (Ortho). CD4$^+$ cells have been determined in total as well as grouped into low and high CD4-expressing cells according to their fluorescence intensity. For flow cytometry 10$^5$ FITC-labelled CLL cells (plus 10 µg/ml propidium bromide) were added to each sample as the standard cell population. Total CD4$^+$ cells were determined relative to the amount of detected standard cells. There is no cross-blocking of the chimeric Abs by OKT4.

TABLE I

Dose-dependent inhibition of MLR by individual chimeric mAbs

| mAb concentrations (ng/ml) | Inhibition of MLR [a] (%) | | |
|---|---|---|---|
| | MAK 179 | M-T310 | M-T151 |
| 1 | 4 | 9 | 5 |
| 3 | 11 | 18 | 2 |
| 10 | 28 | 26 | 24 |
| 30 | 48 | 57 | 52 |
| 100 | 58 | 58 | 54 |
| 300 | 61 | 67 | 67 |
| 1000 | 61 | 68 | 67 |
| 30000 | 61 | 70 | 70 |

[a] **Methods.** Human PBL were isolated from a single healthy donor by elutriation (Red Cross, Munich, FRG) followed by discontinuous gradient centrifugation with lymphocyte separation medium (Boehringer Mannheim, Germany). Cells at the interphase were collected and washed twice with RPMI 1640 (supplemented with 10% fetal calf serum/2 mM glutamine/1% BME vitamins/100 $\mu$g penicillin/100 $\mu$g streptomycin, all per ml, from Boehringer Mannheim, Germany. Then $10^7$ stimulator cells (human lymphocyte cell line RPMI 1788, ATCC CCL 156) were treated for 45 min at 37°C and 5% $CO_2$ in a volume of 1 ml with 50 $\mu$g mitomycin C/ml. After centrifugation, cells were washed twice with RPMI 1640 and adjusted to a cell titer of $10^6$ cells/ml. MLR was performed by co-culturing $10^5$ PBL/100 $\mu$l with the same amount of stimulator cells in flat-bottom microtiter plates (Nunc). The mAbs were added either alone or in combination in a 20-$\mu$l volume to give the final concentration as indicated in the table. The allogeneic response was assayed on day four by pulsing the cells with 0.5 $\mu$Ci [$^3$H]-thymidine (25 Ci/mM, Amersham Buchler). After 18 h the cultures were terminated by harvesting the cells on glassfiber filters, and the amount of radioactivity incorporated into the cellular DNA was determined with the filter counting system INB-384 (Inotech, Wohlen, Switzerland).

All assays were done in triplicates and the results expressed as:

$$1 - \left(\frac{cpm_{experiment} - cpm_{background}}{cpm_{positive\ control} - cpm_{background}}\right) \times 100 = \%\ \text{inhibition in MLR}$$

where cpm = counts per min; $cpm_{experiment}$ is the MLR response with mAbs; $cpm_{positive\ control}$ is the MLR response without mAbs; $cpm_{background}$ represents the spontaneous proliferation of responder cells.

Anti-CD4 mAb has the potential to modulate the function of CD4$^+$ cells either by delivering an inactivating signal (Rosoff et al., 1987) or by inhibiting the association of the CD4 molecule with the T cell receptor-CD3 complex or by blocking its interaction with MHC class II molecules on cooperating cells. In vivo experiments have shown that the immunosuppressive effect and the induction of tolerance by anti-CD4 mAbs does not entirely depend on cell depletion (Benjamin et al., 1988; Alters et al., 1990; Cobbold et al., 1990; Qin et al., 1990). Furthermore it has been shown that Fab fragments of an anti-CD4 mAb mediate immunosuppression (Carteron et al., 1988). Qin et al. (1987) have demonstrated an improved clearance of cells in vivo and tolerance induction, using synergistic pairs of anti-CD4 mAbs.

TABLE II

Inhibition of MLR by selected combinations of mAbs

| mAb concentrations (ng/ml) [a] | | | | Inhibition of MLR (%) |
|---|---|---|---|---|
| M-T310 | M-T151 | MAK 179 | MAK 215 | |
| 1 | | | | 6 |
| 10 | | | | 38 |
| 100 | | | | 59 |
| | 1 | | | 0 |
| | 10 | | | 29 |
| | 100 | | | 57 |
| | | 1 | | 0 |
| | | 10 | | 23 |
| | | 100 | | 53 |
| | | | 10000 | 5 |
| 1 | 100 | | | 65 |
| 10 | 100 | | | 64 |
| 100 | 100 | | | 60 |
| 100 | 1 | | | 58 |
| 100 | 10 | | | 58 |
| 100 | 100 | | | 60 |
| 100 | | 1 | | 59 |
| 100 | | 10 | | 69 |
| 100 | | 100 | | 92 |
| | 100 | 1 | | 58 |
| | 100 | 10 | | 64 |
| | 100 | 100 | | 96 |
| 100 | | | 10000 | 40 |
| | 100 | | 10000 | 45 |

[a] The mAbs used are chimeric (IgG1), with the exception of MAK215. This mAb (IgG$_{2b}$) is directed against the $\alpha$ chain of the IL2R and does not compete with IL2 binding (unpublished observations). For methods see Table I.

### (e) Inhibition of MLR

When lymphocytes from two genetically different individuals of a species are mixed in cell culture medium, the T cells of each individual respond to the MHC antigens of the other by differentiation and proliferation. This process is called mixed lymphocyte reaction (MLR). Allogeneic antigens presented on accessory cells stimulate precursor cells, and the effector cells secrete IL2 and other lymphokines that cause maturation of effector T cells. Since these mechanisms are responsible for allograft rejection, it is of great interest to suppress T-cell reactivity in allograft patients. In the present study we demonstrate that each chimeric anti-CD4 mAb by itself inhibits markedly MLR. This can also be achieved by the anti-IL2R$\alpha$ mAb MAK179. As shown in Table I, a dose-dependent inhibition of the MLR up to a concentration of 300 ng/ml can be obtained with these three mAbs. The maximum inhibition of MLR is

about 60–70%. An increase of the mAb concentration to 30 μg/ml did not result in any further increase in inhibition. Probably, the proliferation of lymphocytes is supported by additional signals, besides those mediated by CD4 and IL2R.

For clinical applications it is important to accomplish a high degree of suppression of T-cell proliferation. In Table II we show that a combination of the chimeric anti-CD4 mAbs M-T310 and M-T151 did not lead to a marked increase in MLR inhibition; however, either of the anti-CD4 mAbs (M-T310 or M-T151) in combination with the anti-IL2Rα mAb MAK 179 mediates an almost 100% inhibition of MLR, using each mAb in a concentration of 100 ng/ml. The combination of each anti-CD4 mAb and another anti-IL2Rα mAb M215, which does not compete with binding of IL2 to its receptor (unpublished observations), did not show any synergistic effect, even when used at a concentration of 10 μg/ml (data not shown). In addition, even at reduced concentrations (10 ng/ml of each mAb) the combination of either one of the two anti-CD4 mAbs and the anti-IL2Rα mAb MAK 179 resulted in almost 50% inhibition of the MLR.

These data show that an inhibition of the MLR can be achieved very effectively by the combined administration of one of the two anti-CD4 mAbs and the anti-IL2Rα mAb MAK 179. During treatment of autoimmune diseases and following organ transplantation, mAbs are administered in doses ranging from 20 to 50 mg per day. By using an anti-CD4/IL2Rα mAb cocktail, we assume that the same immunosuppressive effect might be obtained by a 5–10 times lower dose than when using either one alone.

### (f) Conclusions

(1) We were able to establish transfectomas with Ig-specific mRNA steady state levels comparable to those of the original hybridoma cell lines with our cloning/expression system. The mRNA steady state levels correlated with the secretion of reconstituted mAb into the culture supernatants.

(2) Selective T-cell-targeted therapy is an attractive alternative to non-specific immunosuppressive drugs in the treatment of graft rejections as well as autoimmune disorders. The chimerization of mAbs reduces their immunogenicity (Brüggemann et al., 1989) for serotherapy in humans, it enhances the serum half-life and improves physiological effector functions (reviewed by Waldmann, 1991b).

(3) Chimeric mAbs M-T151 and M-T310 seem to be suitable for the therapy of autoimmune diseases. The murine mAb M-T151 has been already used successfully in a limited trial (Herzog et al., 1987). Complement activation and antigen modulation are only performed by a combination of the chimeric anti-CD4 mAb; the individual mAbs do not display these properties. Therefore, we suggest that

a combination of both chimeric mAbs is more effective than the administration of a single mAb.

(4) For the treatment of graft rejections, a combination of an anti-IL2Rα-chain mAb and an anti-CD4 mAb seems to be a very effective combination, as T-cell proliferation can be almost completely suppressed.

(5) Our study shows that combinations of mAbs are more effective than single mAbs with respect to several properties (complement activation, CD4 modulation, inhibition of MLR). Cocktails of mAbs containing anti-CD4 mAbs and/or IL2Rα mAbs and also mAbs directed to other T-cell accessory molecules should provide new tools for immunotherapy.

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