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Functional analysis of the effects of a fully humanized anti-CD4 antibody on resting and activated human T cells

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SUMMARY

A fully humanized anti-CD4 antibody was studied for its effects on resting and activated CD4 T cells. Whereas the antibody was poorly lytic, it induced dramatic down-modulation of CD4 expression on both types of cell. In order to down-modulate CD4 on resting, normal CD4 T cells there was an absolute requirement for FcR-mediated cross-linking of the anti-CD4 antibody, and only CD4 levels were affected. When activated cloned T-cell lines were studied there was no requirement for cross-linking and several other cell surface markers were also affected. Although the total cellular CD4 was reduced in the down-modulated cells, as judged by Western blot analysis, that CD4 which remained was associated with p56lck. The results are discussed in relation to the potential use of humanized anti-CD4 antibodies in the therapy of autoimmune disease and the choice of antibody isotype for such a therapeutic antibody.

INTRODUCTION

Autoimmune syndromes represent some of the most debilitating chronic diseases suffered by humans, including rheumatoid arthritis (RA), myasthenia gravis, inflammatory bowel disease, psoriasis, thyroiditis and diabetes. Evidence for the autoimmune aetiology of these conditions comes from the observation of activated T cells at the inflammatory site, e.g. in the synovium of RA patients, from clinical observations of the effect of immunosuppressive agents on these conditions, and from the study of animal models of autoimmunity.[1] The animal models provide the most compelling evidence for the role of T cells, particularly $CD4^+$ T cells, in the aetiology of both induced and spontaneous autoimmune syndromes. The antigen-induced models of autoimmunity include myelin basic proteins-induced autoimmune allergic encephalomyelitis,[2] thyroglobulin-induced thyroiditis[3] and collagen-induced arthritis.[4] Spontaneous disease is seen in genetically predisposed strains such as the diabetic NOD mouse[5] and the New Zealand mouse strains—which exhibit a variety of autoimmune conditions including haemolytic anaemia and a lupus-like syndrome.[6] An important observation arising from the study of the animal models is that these diseases can be ameliorated by treatment with antibody directed against the CD4 antigen.[2–6]

Antibodies that either deplete all $CD4^+$ T cells or merely block the CD4 molecule have been shown to be effective in preventing and reversing the symptoms of autoimmunity in animal models.[7] The mechanism by which blocking the anti-CD4 antibody works is far from clear. Studies using soluble protein antigens and cellular alloantigens indicate that treatment with anti-CD4 antibody around the time of immunization can lead to a long-standing state of non-responsiveness after the antibody has disappeared from the system.[8,9] This 'reprogramming' of the immune system by a short course of therapeutic antibody is clearly an attractive proposition if it could be applied to human autoimmune disease. Current therapy for human conditions such as RA, systemic lupus erythematosus (SLE), myasthenia gravis and inflammatory bowel disease ranges from treatment with immunosuppressive drugs to surgery. While many patients can be managed well with the currently available therapies, it is clear that for many patients these treatments are inadequate and their quality of life is significantly diminished.

One problem of translating the experimental promise of monoclonal antibody therapy into clinical practice is that of anti-globulin responses, which can neutralize the effects of the therapeutic antibody. Anti-globulin responses have been seen in a significant number of patients that have been treated with rodent antibodies in early studies.[10] A major advance in the reduction of the immunogenicity of therapeutic monoclonal antibodies is the process of humanization.[11] This involves the grafting of the complementarity determining regions from a rodent antibody onto the framework of a human antibody using genetic engineering techniques. The first example of such a humanized antibody was the anti-CDw52 antibody CAMPATH-1H.[11] This pan-lymphocyte antibody has been used successfully in the treatment of non-Hodgkin lymphoma[12] and some autoimmune disease—namely RA and systemic

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vasculitis.[13,14] In this study we investigate the functional properties of a fully humanized anti-CD4 monoclonal antibody,[15,16] which is intended for use as a therapeutic antibody in humans. It is important that before any therapeutic moiety is administered to humans, the effects of that entity are studied in detail *in vitro*. This is important because it gives an indication of the biological effects which can be expected *in vivo*, and measurement of such effects allows the monitoring of the biological efficacy of the treatment. The data presented here indicate that a major biological effect of this particular humanized anti-CD4 antibody is the profound down-modulation of CD4 expression levels. The down-modulation in normal resting $CD4^+$ T cells is dependent on the interaction of the Fc region of the antibody with Fc receptors on other accessory cell types. These data have implications with regard to the choice of Fc region for humanized antibodies and the parameters to be measured during a clinical trial using a humanized anti-CD4 antibody.

MATERIALS AND METHODS

Reagents, cells and antibodies

All plastic tissue culture vessels were obtained from Costar (Cambridge, MA). Phorbol myristate acetate (PMA) was obtained from Sigma (St Louis, MO). Antibodies directly conjugated with fluorescein isothiocyanate (FITC) were obtained from either Becton Dickinson (Cambridge, MA) or Sigma. Humanized antibodies (anti-CDw52/CAMPATH-1H[12] and anti-CD4[15,16] were expressed in either Chinese hamster ovary (CHO) cells of NSO myeloma cells, respectively, and purified on either protein A or protein G columns. In a previous study we have shown that humanized anti-CD4 antibodies produced in CHO and NSO cell lines are functionally identical in *in vitro* assays.[16] Anti-CD4 antibody incapable of binding to FcR was produced by site-directed mutagenesis in which residues 234 (Leu) and 237 (Gly) in the CH2 domain were both changed to alanine.[17] These changes have been shown to abolish Fc receptor binding. $F(ab')_2$ and Fab fragments of anti-CD4 were prepared by pepsin or papain digestion, respectively, using standard methods.[18] Digestion was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Goat and rabbit anti-human Fc region antibody were obtained from Sigma. 5F3 anti-idiotypic antibody specific for the idiotype expressed on the anti-CD4 antibody was produced in our laboratory. Peripheral blood mononuclear cells (PBMC) were prepared by separation on Ficoll–Hypaque density gradients. Depletion of adherent cells to generate peripheral blood lymphocytes (PBL) was achieved by adherence to plastic for 2 hr in RPMI and 10% autologous serum. Purified $CD4^+$ T cells were prepared by incubating PBL with a cocktail of anti-CD8, anti-CD19, anti-CD16 and anti-HLA-DR antibodies (a Becton Dickinson) for 30 min, followed by washing and passage through an anti-mouse immunoglobulin column.[19]

Cultures of primary PBMC, PBL or purified $CD4^+$ T cells were performed in RPMI plus 10% heat-inactivated fetal calf serum (FCS) supplemented with penicillin–streptomycin and L-glutamine. Human T-cell clones specific for a peptide representing amino acids 202–229 of influenza A nucleoprotein (NP) were prepared as described previously[20] and passaged routinely by stimulation with irradiated (3000 rads) syngeneic PBMC, peptide (10 μg/ml) and 5% Lymphocult-T (Biotext AG, Dreieich, Germany). These cultures were performed in RPMI and 10% autologous human serum supplemented with penicillin–streptomycin and L-glutamine. For experiments in which cloned T cells were used as either targets for antibody-dependent cell-mediated cellular cytotoxicity (ADCC) or for CD4 modulation, the medium used contained FCS rather than human serum. Wien-133 cells were obtained from Dr A. Karpas (University of Cambridge, Cambridge).

ADCC

^{51}Cr-labelled targets, either Wien-133 cells, cloned T cells or activated T cells, were incubated for 6 h with PBMC effector cells in the presence of sensitizing antibody, after which time supernatants were removed for gamma counting. Percentage specific release was calculated by the following formula: percentage specific release = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

Flow cytometry

All flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson). Results are plotted either as histograms or as levels of mean fluorescence intensity. CD4 levels are down-modulation were measured using a non-competing anti-CD4 antibody (Leu-3a–FITC).

Immunoprecipitations and Western blotting

PBMC were lysed in 3% Nonidet P-40 (NP-40) lysis buffer (containing 20 mM MOPS, 15 mM EGTA, 3% NP-40, 2 mM PNSF, 1 mM Na_3VO_4, 10 μg/ml aprotonin, 2 mM EDTA) for 15 min on ice, after which time the lysate was centrifuged at 10 000 *g* for 5 min at 4°. The clarified supernatant was precleared with Sepharose 4B for 30 min at 4° and the precleared lysate was then incubated with Q4120-coupled Sepharose for 1 h at 4° with end-over-end rotation. The beads were washed twice in lysis buffer, resuspended in non-reducing SDS–PAGE sample buffer and boiled for 3 min. The samples were run on 8% SDS–PAGE gels, blotted onto nitrocellulose and the blots blocked with 3% non-fat milk in phosphate-buffered saline (PBS). The blots were probed with either biotin-Q4120 anti-CD4 followed by steptavidin–horseradish peroxidase (HRP) or with rabbit anti-p56lck followed by anti-rabbit IgG–HRP, and developed using enhanced chemiluminescence (ECL).

RESULTS

A humanized IgG1 anti-CD4 antibody capable of interacting with Fc receptors is poorly lytic on normal $CD4^+$ T cells

We studied the functional properties of a fully humanized anti-CD4 antibody of the IgG1 isotype. This isotype is 'permissive' for both complement (CMC) and cell-mediated cytotoxicity and ADCC inasmuch as antibodies with such a constant region were capable of lysing target cells with the appropriate specific antigen. For example, the humanized CAMPATH-1H IgG1 antibody against CDw52 was extremely potent in terms of both CMC and ADCC. We tested the humanized anti-CD4 antibody in complement lysis assays using human serum as

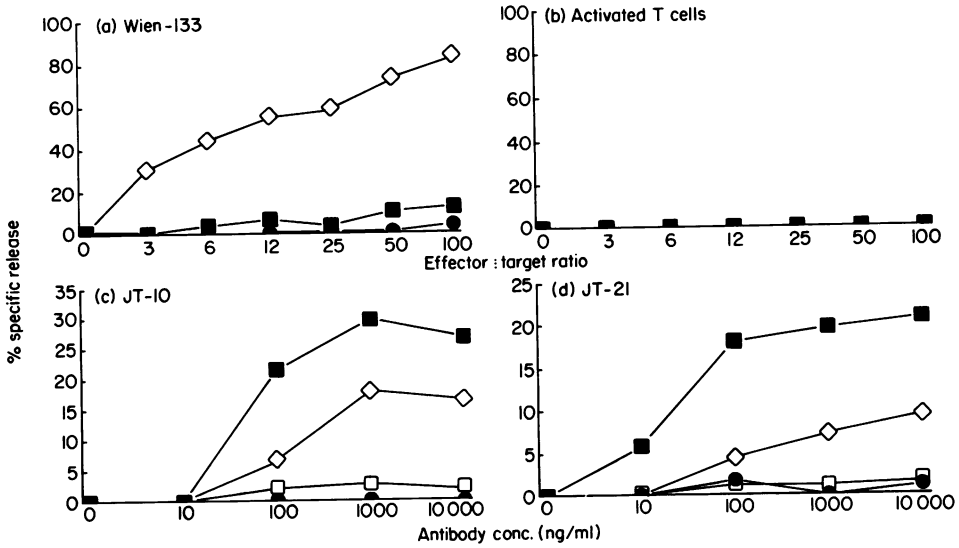

Figure 1. ADCC using anti-CD4 and anti-CDw52 antibodies. Target cells (Wien-133 cells, $CD4^+$ T cells activated with anti-CD3 antibody or cloned T-cell lines) were ^{51}C-labelled and co-cultured with normal PBMC effector cells at various effector : target ratios in the presence of 5 μg/ml of the indicated antibody (a, b) or at a fixed effector : target ratio of 100 : 1 in the presence of the indicated antibody concentration (c, d). Supernatants were harvested for calculation of specific ^{51}C-release after 6 hr. (a, b) (◆) CAMPATH-1H; (■) anti-CD4; (●) no antibody. (c, d) (■) effector cells + CAMPATH-1H; (□) CAMPATH-1H alone; (◆) effector cells + anti-CD4; (●) anti-CD4 alone.

the complement source and found no evidence of activity, whereas CAMPATH-1H was highly lytic (unpublished observations). We also tested both CAMPATH-1H and anti-CD4 for ADCC activity using normal PBMC as effectors, and either a long-term cloned T-cell or freshly activated $CD4^+$ T cells as targets (Fig. 1). Whereas it was possible to detect ADCC activity against the cloned T-cell line with both antibodies, the activity with anti-CD4 was much weaker than with CAMPATH-1H. On freshly activated $CD4^+$ T cells neither antibody was lytic, whereas in the same experiments the anti-CDw52 antibody was highly lytic against a tumour cell target (Wien-133) expressing CDw52. These data indicate that in exceptional circumstances the humanized anti-CD4 does give ADCC activity, evidence that the antibody possesses a functional Fc domain. However, against normal $CD4^+$ T cells as targets there was no activity with either anti-CD4 or CAMPATH-1H. Observations indicated that killing measured in short-term ^{51}Cr-release assays with non-activated PBMC effectors was entirely attributable to $CD56^+$ natural killer (NK) cells and was blocked by antibody to CD16 (FcγRIII) (data not shown). These data must cast some doubt on the relevance of redirected killing by NK cells as measured by ADCC assays to the clearance mechanism of CAMPATH-1H, as this antibody was extremely efficient in depleting $CD4^+$ T cells *in vivo*. Also, it was clear that factors other than expression of the relevant antigen were important for a target cell to be sensitive to ADCC.

Down-modulation of CD4 expression on human T cells by a humanized anti-CD4 antibody

Since it appeared that the humanized anti-CD4 antibody was not highly lytic, we looked at other functional effects of this antibody. PBMC from healthy volunteers were incubated *in vitro* with humanized anti-CD4 antibody and cell-surface expression of CD4 monitored with a non-competing anti-CD4 monoclonal antibody. Preliminary experiments (data not shown) indicated that after 24 hr the level of CD4 expression was reduced by approximately 80%, a greater level of down-modulation than observed with PMA after 2 hr. This modulation was specific as the levels of other cell-surface antigens was unaffected by the treatment with anti-CD4 antibody (data not shown). Immunoprecipitation of CD4 with a non-competing anti-CD4 followed by Western blotting

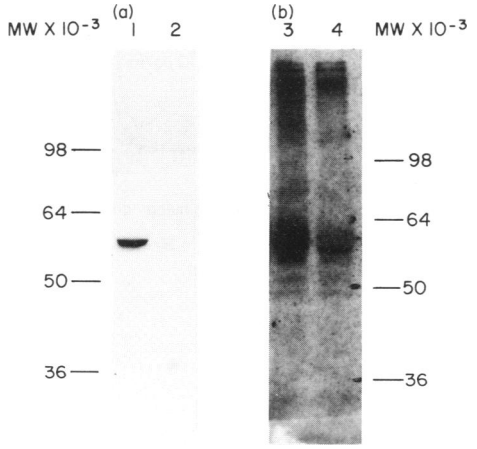

Figure 2. Western blot analysis of CD4 and p56lck expression. PBMC (10^7/ml) were incubated overnight with 10 μg/ml anti-CD4 or no antibody, after which time CD4 was immunoprecipitated as described in the Materials and Methods. The figure shows the levels of CD4 (lanes 1 and 2) and associated p56lck (lanes 3 and 4) in the two cell populations (lanes 1 and 3, control PBMC; lanes 2 and 4, down-modulated PBMC).

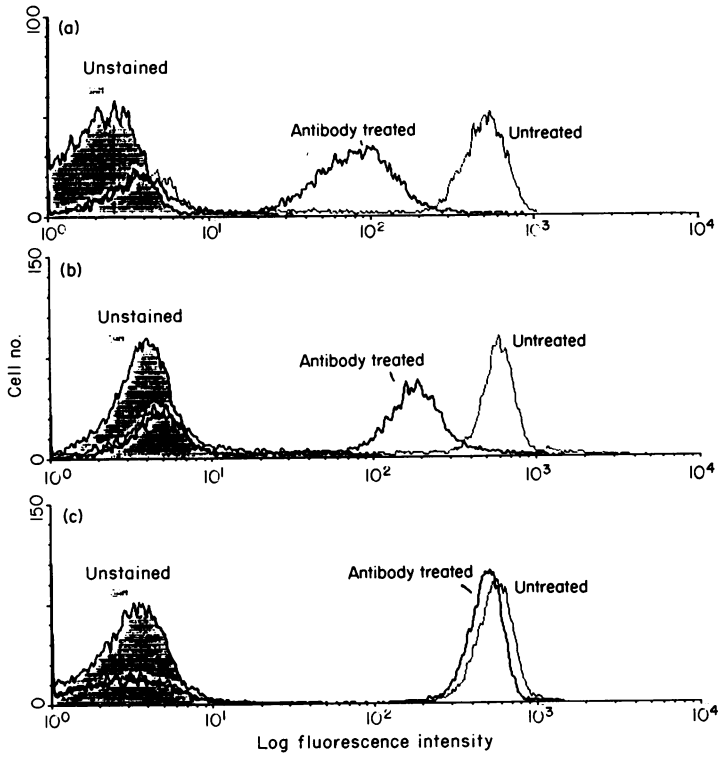

Figure 3. Requirement for cell–cell interaction in CD4 down-modulation. PBMC (a), adherent-cell depleted PBMC (PBL) (b) or purified $CD4^+$ T cells (c) (all at 10^7/ml) were incubated overnight with anti-CD4 at a final concentration of 10 μg/ml. After this time the cells were stained for CD4 expression and analysed by flow cytometry.

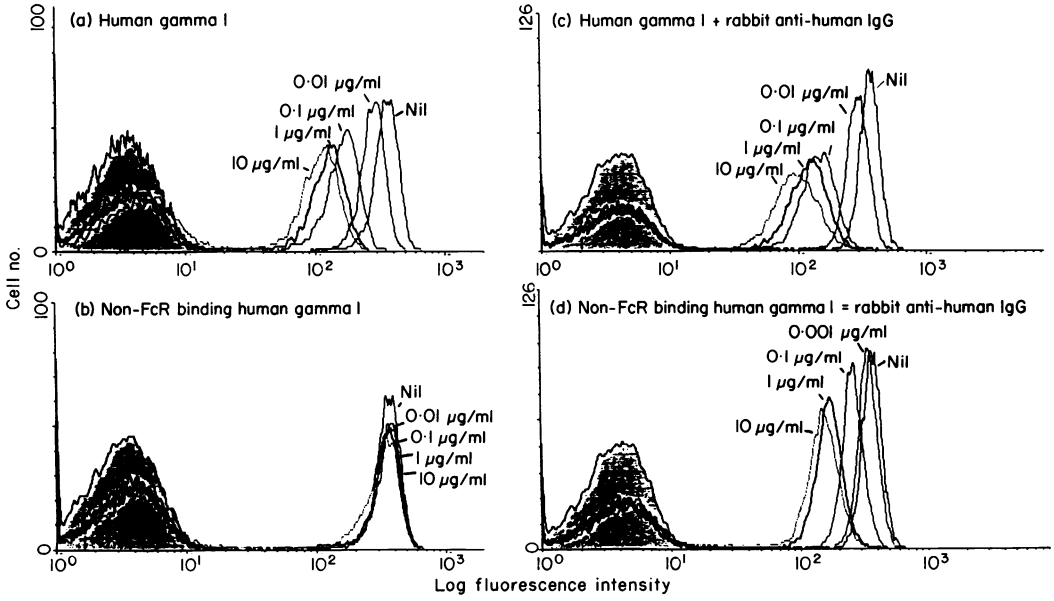

Figure 4. Antibody incapable of binding Fc receptors does not induce CD4 modulation in PBMC. PBMC were incubated at 10^7/ml for 60 mins with the indicated concentration of anti-CD4 antibody (a, c) or a genetically engineered version of the same antibody unable to bind to Fc receptors (b, d). After washing with PBS some cultures received rabbit anti-human IgFc region at antibodies at 10 μg/ml (c, d) or medium (a, b) and the cultures were incubated overnight at 37°. After 24 hr the level of CD4 expression was measured by staining with FITC-labelled Leu-3a anti-CD4 antibody.

indicated that the total cellular CD4 was also reduced in modulated PBMC (Fig. 2). Furthermore, the precipitated CD4 from both control and down-modulated T cells was associated with p56lck; however, much less was associated with CD4 in the down-modulated cells, consistent with the lower levels of CD4 expressed. Experiments were then performed to determine whether the down-modulation was the result of an interaction between the $CD4^+$ T cells and other cell types in PBMC. Figure 3 shows the effect of incubating PBMC, adherent-cell depleted PBMC (PBL) or purified $CD4^+$ T cells on the down-modulation induced by anti-CD4. The degree of reduction of CD4 expression was dependent on the presence of other cell types, as down-modulation in the absence of other cell types was minimal. Whereas unseparated PBMC showed a dramatic reduction in CD4 levels, the removal of adherent cells partially inhibited this effect, and removal of all accessory cells by purifing a $CD4^+$ population completely blocked down-modulation. This suggests that several different populations of FcR^+ accessory cells other than adherent immunocytes are capable of interacting with $CD4^+$ T cells to induce down-modulation. Possible candidates are B cells and NK cells, which also possess Fc receptors.

Down-modulation of CD4 expression on PBMC requires interaction of anti-CD4 with Fc receptors

In order to determine whether the observed cell–cell interaction was due to the cross-linking of the anti-CD4 antibody by Fc receptors on other cells, the ability of the antibody to bind to FcR was removed. This was achieved by either enzymatic digestion to produce a $F(ab')_2$ fragment or by site-directed mutagenesis of the anti-CD4 antibody heavy chain gene to produce an antibody incapable of FcR binding. Both antibodies retained full specific activity in terms of antigen binding (data not shown), but neither induced down-modulation of CD4 expression (Fig. 4). Interestingly, in the absence of down-modulation anti-CD4 antibody remained bound to surface CD4 for a considerable length of time, suggesting that the CD4 and anti-CD4 were either endocytosed or shed as a unit (Fig. 5). In the same culture the monocytes, as defined by light scattering properties, did not demonstrate any CD4 down-modulation and remained coated with anti-CD4 antibody (data not shown). The inability to down-modulate CD4 by the FcR binding-defective antibody could be overcome by cross-linking with a second antibody, either a polyclonal rabbit anti-human IgG (Fig. 6) or a murine monoclonal specific for the idiotype expressed on the humanized anti-CD4 antibody (Fig. 5). This anti-idiotypic antibody was able to bind to the humanized anti-CD4 when bound to CD4. The recognition of bound anti-CD4 by this anti-idiotype was not a consequence of univalent binding of the anti-CD4 antibody, as binding was also observed with the Fab fragment of anti-CD4 (data not shown). The effect of adding a secondary antibody was entirely due to the cross-linking by the secondary antibody, as the same effect could be obtained with a $F(ab')_2$ fragment of anti-human IgG, ruling out an interaction of the Fc region of the second antibody with Fc receptors (Fig. 5).

Activated $CD4^+$ T cells show a different response to treatment with humanized anti-CD4

The effect of anti-CD4 antibody on activated T cells was

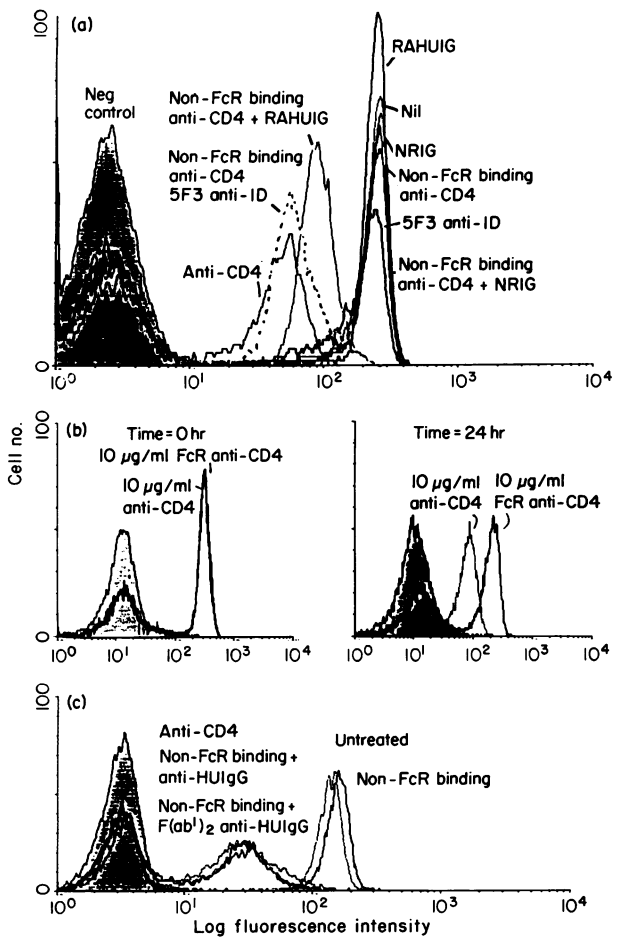

Figure 5. Requirement for cross-linking for both modulation of CD4 and bound anti-CD4 antibody. (a) PBMC (10^7/ml) were incubated with either anti-CD4 or non-FcR binding anti-CD4 (both at 10 ng/ml) for 60 min at 4°, washed twice in PBS and resuspended in medium containing either rabbit anti-human IgFc (RAHUIG), normal rabbit immunoglobulin (NRIG) (both at 10 ng/ml) or 5F3 anti-idiotypic antibody (neat hybridoma supernant). After 24 hr (10 ng/ml) CD4 levels were measured by flow cytometry. (b) PBMC were incubated with 10 μg/ml of anti-CD4 or non-FcR binding anti-CD4 antibody for 1 hr and washed three times in PBS before either staining with FITC-labelled anti-human immunoglobulin antibody or culturing overnight at 37°. The cultured cells were similarly stained for the presence of human immunoglobulin after 24 hr. The figures show equivalent binding of the two antibodies at time zero, however at 24 hr the level of detectable whole antibody was lower than that of the non-FcR binding antibody. (c) PBMC were incubated with anti-CD3 antibody (10 ng/ml) or non-FcR binding anti-CD4 followed by cross-linking with anti-human IgG (anti-IgG) or a $F(ab')_2$ fragment of the same antibody. The level of CD4 expression was measured by staining with FITC–Leu-3a after 24 hr.

investigated using a cloned $CD4^+$ T-cell line. The effects observed were quite different from those seen with the resting $CD4^+$ T cells. The cloned cells were used after antigen stimulation and feeding with interleukin-2 (IL-2)-containing medium. Incubation of these activated cells with anti-CD4 in the absence of accessory cells led to down-modulation of CD4 expression. Anti-CD4 incapable of binding to FcR, in this case

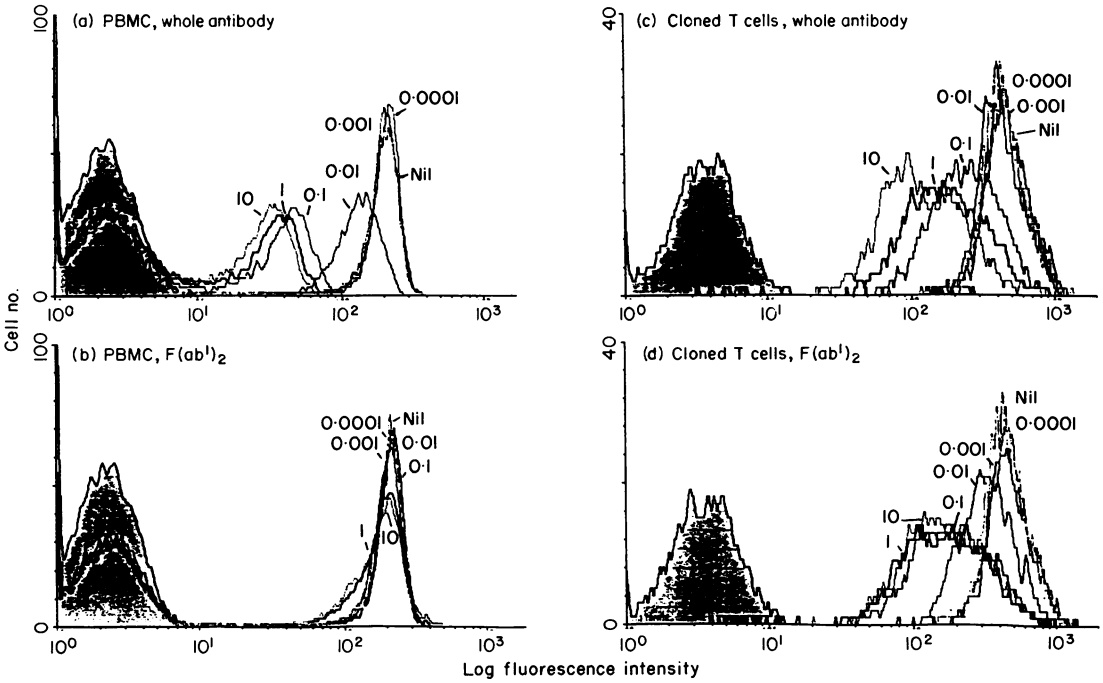

Figure 6. Requirement for FcR-mediated interactions for CD4 down-modulation in cloned, activated T cells. PBMC (10^7) (a, b) or cloned JT T cells (10^7) (c, d) were incubated overight with either anti-CD4 antibody (10 μg/ml) (a, c) or a $F(ab')_2$ fragment (10 ng/ml) of the same antibody (b, d). After 24 hr CD4 expression was measured by staining with FITC–Leu-3a.

a $F(ab')_2$ fragment, was equally effective in the down-regulation of CD4 on these cells, suggesting that cross-linking of the antibody was not necesary (Fig. 6). Furthermore, when the specificity of down-modulation was investigated it was found that not only CD4 expression was reduced but also expression of CD3, CD2, CD29 and CD18. Not all cell-surface molecules were down-regulated, however, as CD45 levels were unchanged. Figure 7 shows a dose–response curve for the down-modulation of CD4, CD3 and CD29 on a cloned T-cell line. Non-saturating levels of antibody showed this effect; the anti-CD4 antibody was saturating at around 100 ng/ml (data not shown) and down-modulation was observed down to 1 ng/ml.

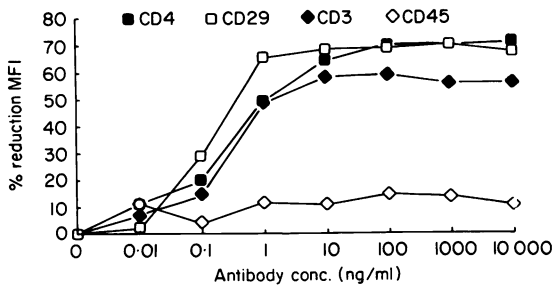

Figure 7. Dose–response of anti-CD4-mediated down-modulation. Cloned T cells (10^7/ml) were incubated with the indicated concentrations of anti-CD4 antibody. After 24 hr the cells were stained for the indicated CD marker with directly conjugated antibodies. Results are shown as percentage inhibition of the mean fluorescence intensity (MFI) of control cultures.

DISCUSSION

It is clear that CD4 has a major role to play in the activation of $CD4^+$ T cells, and since the role of CD4 is to enhance T-cell receptor (TCR) signalling it is possible that the level of CD4 expression may affect the efficiency of this co-receptor function. Antibodies specific for CD4 are extremely potent at inhibiting $CD4^+$ T-cell activation; early *in vitro* studies suggested that this occurred by preventing the recognition of and binding to class II MHC molecules.[21] It soon became apparent, however, that anti-CD4 antibodies could inhibit T-cell activation not involving MHC class II recognition.[22] This led to the initial concept that CD4 may play a role in signal transduction; the discovery of the CD4-associated p56lck tyrosine kinase revealed a potential mechanism for this signalling.[23,24] These observations could possibly be explained by interfering with the ability of CD4 to signal to the T cell, either by inappropriate signalling[25] or by sequestering p56lck away from the T-cell receptor.[26]

Later experiments showed that antibodies against CD4 and CD8 could be used successfully *in vivo* to suppress immune responsiveness.[9] The early studies used antibodies that depleted $CD4^+$ T cells, but subsequently anti-CD4 Fab_2 fragments that did not elicit effector mechanisms were also found to be effective.[27] This suggests that merely blocking the CD4 molecule may inhibit immune function *in vivo*. Other studies indicated that both antigen-induced and spontaneous autoimmune conditions in mice can be improved by treatmen with anti-CD4 antibody. This obviously has important implications for the treatment of human disease. However, realization of the promise of such an approach in humans has been slow. Early studies with anti-CD4 antibody in human autoimmune disease

have had muted success, giving some short-term benefit but no dramatic long-term remissions.[28] This may be a reflection of the necessarily cautious administration of an antibody capable of inducing immunosuppression. The doses administered on mg/kg basis have been orders of magnitude less in humans than in the animal models. In order to improve on such results in humans it will be necessary to be able to closely monitor the biological effect of anti-CD4 antibodies in the patient and fine tune the therapy accordingly. We have therefore investigated the *in vitro* effects of a humanized anti-CD4 antibody on human PBMC in order to establish what biological effects need to be monitored *in vivo*.

Our initial studies investigated the lytic potential of a fully humanized anti-CD4 antibody. It was found to be non-lytic in terms of complement-mediated lysis and only weakly lytic by ADCC. Indeed, preliminary studies in humans have shown that this antibody does not deplete circulating $CD4^+$ T cells, confirming the weak lytic potential. We therefore went on to investigate the physiological effects that this anti-CD4 antibody had on peripheral $CD4^+$ T cells. Preliminary experiments indicated that incubation of PBMC with anti-CD4 led to a dramatic decrease in CD4 expression, as measured by a non-competing anti-CD4 antibody. The ability to down-regulate CD4 expression varies considerably among anti-CD4 antibodies;[29] it is thererfore important to determine the properties of any potential therapeutic antibody before use in patients in order to anticipate rationally *in vivo* effects. On the resting $CD4^+$ T-cell population, the down-regulation was specific for CD4, as all other antigens studied remained at control levels. Using site-directed mutagenesis we changed the residues in the C_H2 domain responsible for FcR binding in order to determine the role of cell–cell interaction in this down-modulation. We found that binding of the anti-CD4 antibody alone was insufficient to induce down-modulation of CD4 on resting T cells and that cross-linking either by a FcR^+ accessory cell or by a secondary antibody was required. From the point of view of the choice of isotype for a therapeutic anti-CD4 antibody, it is therefore important to determine whether the decreased expression of CD4 has functional consequences. Whereas it might be considered desirable to use an antibody unable to interact with FcR in order to minimize the possibility of CD4 depletion and the side-effects often observed with therapeutic antibodies, this must be balanced against the potential therapeutic benefit of the ability of an antibody to reduce CD4 expression. In a subsequent paper we will discuss the functional consequences of CD4 down-modulation (Brett *et al.*, in preparation). In situations in which CD4 expression is not affected by binding of anti-CD4, e.g. when the antibody is unable to bind to FcR, the antibody remains on the surface of the T cell. When CD4 expression is down-modulated the antibody remaining on the T-cell surface is correspondingly decreased. How the modulation of CD4 expression is mediated at the molecular level is not clear.

The situation described above relates to resting $CD4^+$ T cells; activated $CD4^+$ T cells responded differently to anti-CD4 treatment in two important respects. Firstly, there was no requirement for accessory cells to obtain CD4 down-modulation, the $F(ab')_2$ fragment of anti-CD4 antibody induced responses equivalent to the whole molecule. Secondly, in several T-cell clones CD4 was not the only antigen that had reduced expression after CD4 down-modulation in such cells; the expression of other molecules important in T-cell activation, such as CD3, CD28 and CD29, was also dramatically reduced in parallel with CD4. There is some heterogeneity in this respect, in that we have observed that some cloned lines down-modulate CD4 only, but the lack of requirement for CD4 cross-linking is consistent. This suggests that the CD4 molecule is in a different state in activated T cells compared to resting $CD4^+$ T cells, and that in activated T cells CD4 may be complexed with other molecules involved in the activation of T cells. It is known that the activation cascade initiated by ligation of the TCR by processed antigen leads to the complexing of intracellular proteins through adapter proteins containing S_H2 and S_H3 domains. For example, after T-cell activation the tyrosine kinase ZAP-70 binds to the cytoplasmic region of members of the CD3 family,[30] and it has been shown that p56lck can bind to ZAP-70.[31] Likewise it has been shown tht p56lck can associate with CD2 and CD25,[32,33] whereas CD28 binds to Pl3 kinase in activated cells, and this has been shown to associate with p56lck as well.[34]

Although it is not possible to conclude whether all of these interactions occur physiologically, it is possible to envisage a large complex of signalling molecules becoming associated after T-cell activation. The differing behaviour of activated T cells after CD4 cross-linking may be important, as it is clear that many T cells at inflammatory sites are activated and may therefore respond differently to anti-CD4 therapy. In a subsequent report we will show that the ligation of CD4 on activated T cells without further cross-linking leads to rapid, strong phosphorylation of several substrates, whereas resting $CD4^+$ T cells response slowly and to a much lesser extent and require cross-linking to attain similar levels of phosphorylation (Brett *et al.*, in preparation). Again this may suggest that more signalling-associated molecules are associated with CD4 in activated T cells than in resting T cells. A corollary of this observation is that a non-FcR binding anti-CD4 may have a selective effect on activated T cells.

This study indicates that the interaction with FcR on accessory cells has important consequences as far as the biological effects of anti-CD4 antibody treatment are concerned. This suggests that the ability to bind to FcR may be desirable for anti-CD4 antibody, arguing against a non-FcR binding isotype or a genetically engineered non-FcR binding antibody. These studies also indicate an important parameter to measure during the treatment of patients with anti-CD4 antibody. The measurement of the modulation of CD4, especially at the site of inflammation, will indicate whether the antibody is mediating its biological effect. The potential impact of the modulation of CD4 upon T-cell function will be described in a subsequent paper (Brett *et al.*, in preparation).

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