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The effect of anti-CD4 on helper function of CD4,45RA$^+$ *versus* CD4,45RO$^+$ T cells

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SUMMARY

Here we have investigated and compared the effects of anti-CD4 on helper function of CD4,45RA$^+$ *versus* CD4,45RO$^+$ T cells. Only CD4,45RO$^+$ cells, but not CD4,45RA$^+$ cells were able to promote B cell differentiation resulting in immunoglobulin production *in vitro* (IgM as well as IgG) which could be inhibited by anti-CD4 MoAbs (MAX.16H5 and T151). In pokeweed mitogen (PWM)-induced B cell proliferation a similar pattern of responsiveness was obtained. When we studied the anti-CD4 effects on cytokine production in T cells stimulated in mixed lymphocyte reaction (MLR) or by mitogens, we found that neither IL-2 nor IL-4 production was dramatically influenced by anti-CD4 in CD4,45RO$^+$ cells. This led us to the conclusion that the inhibitory effect of anti-CD4 on B cell proliferation and immunoglobulin secretion was not due to inhibition of cytokine production. To clarify this point, we investigated the ability of anti-CD4 to inhibit conjugate formation between B and T cells. It was found that CD4,45RO$^+$ T cells formed more conjugates than CD4,45RA$^+$ cells, and that only the conjugate formation by CD4,45RO$^+$ T cells was inhibited by anti-CD4. These results suggest that (i) anti-CD4 inhibits T helper functions primarily by affecting CD4,45RO$^+$ cells, and (ii) this effect is probably mediated by contact inhibition in the early phase of T–B collaboration.

Keywords anti-CD4 CD45 isoforms T helper cells cytokines T–B cell conjugate

INTRODUCTION

Anti-CD4 antibodies have been extensively used in animal models for treatment of several autoimmune diseases and for the prevention of graft rejection [1–4]. Recently, a number of pilot studies were initiated to treat patients with autoimmune diseases, including multiple sclerosis and rheumatoid arthritis, by anti-CD4 [5–8].

Anti-CD4 MoAbs have been shown to block *in vitro* several T lymphocyte functions including helper activity for immunoglobulin synthesis [9]. In addition, the *in vivo* administration of anti-CD4 MoAb resulted in suppression of T cell-dependent antibody responses, as demonstrated recently in systemic lupus erythematosus [10].

T lymphocytes may be divided into subpopulations according to the expression of CD45 isoforms which are generated by alternative splicing of mRNA [11], and are designated as RA (205–220 kD) and RO (180 kD). Expression of CD45RA and CD45RO is exclusive in mature human T cells. The CD4,45RA$^+$ population contains naive, unprimed T cells that do not react to recall antigens and only provide minimal help for B cell differentiation, but induce CD8$^+$ T cells to exert suppressor function [9]. In contrast, the CD4,45RO$^+$ population contains memory T cells that provide help for immunoglobulin synthesis by B cells [12], although other evidence suggests that CD45RO$^+$ cells also represent recently primed or effector T cells [13].

Here we present data on the *in vitro* effects of anti-CD4 MoAbs on the induction of human B cell proliferation and differentiation mediated by T cell subpopulations. Both antibodies (MAX.16H5, T151) have already been used for therapeutic intervention in chronic inflammatory diseases [6,8,10]. We demonstrate that CD4,45RO$^+$ T cells are not only the most effective helper cells for the induction of B cell proliferation and differentiation, but also more susceptible to inhibition by anti-CD4. Interestingly, we found that neither IL-2 nor IL-4 production was dramatically reduced by anti-CD4 in CD4,45RO$^+$ cells. However, the formation of conjugates between CD4,45RO$^+$ T cells and B cells was significantly blocked by anti-CD4, while conjugates with CD4,45RA$^+$ cells were much less susceptible to anti-CD4. This may argue for interference at an early phase of physical T–B interaction as the most likely reason for the differential inhibition of B cell functions.

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MATERIALS AND METHODS

Reagents and cell line

The following reagents were used: anti-CD4 MoAbs T151 [14] (IgG2a), MAX.16H5 (IgG1) and $F(ab')_2$ fragments of MAX.16H5 [15] (inhibitor concentrations of anti-CD4 MoAbs are indicated in the figure legends; if not otherwise stated the antibody was present throughout the whole culture period). Isotype-matched control antibodies were BMA 25F9 (IgG1) directed to mature human macrophages not present in peripheral blood, and H 3-5/47 (IgG2a) directed to human endothelial cells (both from Dianova, Hamburg, Germany; Art. no. 0800 and Art. no. 0810), anti-CD45RA (MEM 56, kindly provided by Dr V. Horejsi, Prague, CR), anti-CD45RO (UCHL1, kindly provided by Dr P. Beverley, London, UK), anti-CD8 (OKT8, no. CRL 9014; American Type Culture Collection (ATCC), Rockville, MD), anti-CD3 (UCHT 1, kind gift from Dr P. Beverley) phytohaemagglutinin (PHA, Wellcome, Dartford, UK), heat-killed type A Streptococci preparation (Strep. A)[16], phorbol myristate acetate (PMA; Sigma, Deisenhofen, Germany), ionomycin (Calbiochem, Frankfurt, Germany), pokeweed mitogen (PWM; GIBCO, Eggenstein, Germany), rIL-2 (Eurocetus, Amsterdam, The Netherlands), rIL-4 (Genzyme, München, Germany), and Epstein–Barr virus (EBV)-transformed B cell line (kindly provided by Dr Barbara Bröker, Erlangen, Germany).

Isolation of lymphocyte populations

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers by Ficoll–Hypaque density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway). The lymphocytes were further fractionated by using 2-aminoethylisothiouronium bromide (AET; Sigma)-treated sheep erythrocytes [17] into rosette-positive (E^+) and negative (E^-) populations. The E^+ population obtained was >95% reactive with an anti-CD3 MoAb (UCHT1), and the E^- population was consistently <5% reactive with UCHT1. E^+ cells were further isolated into subpopulations by magnetic bead rosetting (Dynabeads; Dynal, Oslo, Norway) [18]. In brief, E^+ cells were incubated with the combinations of either OKT8 (anti-CD8) and MEM 56 (anti-CD45RA) or OKT8 and UCHL 1(anti-CD45RO) on ice for 30 min. After washing, the cells were incubated with M-450 Dynabeads coated with goat anti-mouse IgG at a ratio of 1:3 on ice for 30 min with occasional shaking. Ice-cold culture medium was added to the mixture and the negatively selected $CD4,45RA^+$ and $CD4,45RO^+$ subpopulations were then separated from rosetted cells by placing the tube on a flat cobalt samarium magnet. In cytofluorometric analysis, the subpopulations prepared in this way were regularly between 92% and 97% positive for CD4,45RA or CD4,45RO phenotype and 93–98% CD4 positive.

B lymphocyte proliferation

E^- cells (5×10^4/well) were cultured in triplicates with either irradiated (15 Gy) $CD4,45RA^+$ cells or $CD4,45RO^+$ cells (5×10^4/well) in 0·2 ml of RPMI 1640 medium (GIBCO) containing 2% L-glutamine (GIBCO), 25 mM HEPES (GIBCO),100 U/ml penicillin G, 100 μg/ml streptomycin (GIBCO) and 10% fetal calf serum (FCS; GIBCO) in the presence of PWM (1:200). After 7 days the cultures were pulsed with 1 μCi ^{3}H-TdR/well and harvested 18 h later. ^{3}H-TdR incorporation was measured by direct β-counting.

B cell differentiation

B cells were induced to differentiate into antibody-secreting cells by addition of $CD4^+$ T cells in autologous mixed lymphocyte reaction (AMLR [19]). E^- cells (1×10^6) were cultured in 24-well culture plates (Nunc, Roskilde, Denmark) with 1×10^6 irradiated (15 Gy) autologous $CD4,45RA^+$ or $CD4,45RO^+$ cells in a final volume of 1·0 ml medium. After 7 days the cultured cells were washed three times in order to prevent cross-reactivity of mouse MoAb added to the cultures with human immunoglobulin. Human immunoglobulin (IgG or IgM) in supernatants was measured on day 12 (i.e. after an additional 5 days) by ELISA [20]. Additional experiments were performed in the presence of PWM (1:200 of the stock solution).

Detection of immunoglobulin secreting cells

Immunoglobulin-secreting cells (ISC) were detected by using a solid-phase immunoenzymatic technique (SPOT-ELISA) [21]. In brief, flat-bottomed microtitre plates were coated with goat anti-human immunoglobulin (5 μg/ml, Sigma) overnight. The plates were blocked with 1% bovine serum albumin (BSA) for 1 h and 1×10^5/100 μl AMLR-activated E^- cells were added and incubated for 20 h. After thorough washing, goat anti-human immunoglobulin conjugated with alkaline phosphatase was added and incubated for 2 h at 37°C. Buffered substrate solution (5-bromo-4-chloro-3-indolylphosphate in 2-amino-2-methyl-1-propanol buffer, pH 10·25; Sigma) containing low gelling agarose was added. Blue spots developing after 12 h were counted.

Cytokine production

$CD4,45RA^+$ or $CD4,45RO^+$ T cells (1×10^6/ml) were stimulated with PHA (1 μg/ml), Strep. A (5×10^6/ml), PMA (10 ng/ml), ionomycin (0·5 μM/ml), anti-CD3 (4 μg/ml) or a combination of PMA and ionomycin in the presence of irradiated (30 Gy) autologous E^- cells (1×10^6/ml) . Supernatants were harvested at 48 h and were analysed for the presence of IL-2 and IL-4. In MLR the T cells were cultured with either irradiated (30 Gy) allogeneic (5×10^5/ml, allo-MLR) or irradiated autologous (1×10^6/ml) E^- cells for 48 h. Cytokine production was measured as described below.

Cytokine measurements

The IL-2 concentration was determined using the IL-2-dependent murine CTLL cell line as described [22] with a sensitivity threshold of 33 pg/ml. CTLL cells (5×10^4) were incubated in flat-bottomed microtitre plates in triplicates together with the diluted lymphokine-containing culture supernatants for 14 h. Proliferation was assessed by ^{3}H-TdR incorporation after pulsing of the culture (1 μCi/well) for the final 10 h of the 24-h culture period. IL-4 was quantified with an immunoenzymatic assay [23]. The IL-4 ELISA kit (sensitivity 45 pg/ml) was purchased from Genzyme. Initial time course experiments indicated an incubation time of 48 h being appropriate for both cytokines.

Conjugate formation

Antigen-independent T cell–B cell conjugate formation was studied according to the method described by Lecomte & Fischer [24]. Briefly, $CD4,45RA^+$ or $CD4,45RO^+$ T cells were

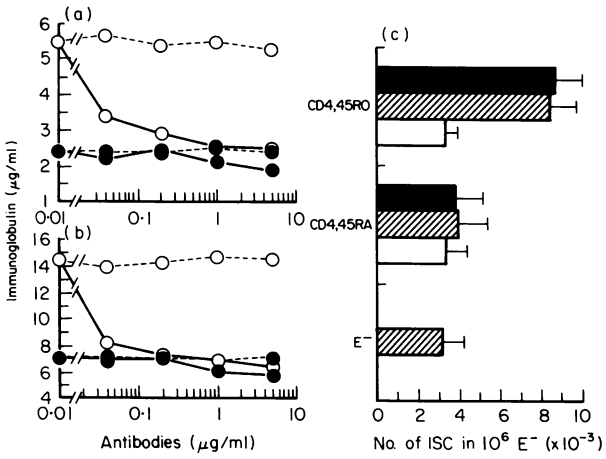

Fig. 1 Effects of anti-CD4 MoAb on induction of T-cell mediated immunoglobulin-secreting cells (ISC) and immunoglobulin production in autologous mixed lymphocyte reaction (AMLR). In a and b, E^- cells (1×10^6/ml) were cultured in triplicates with irradiated (15 Gy) autologous CD4,45RA$^+$ (●) or CD4, 45RO$^+$ (○) T cells (1×10^6/ml) without (a) or with (b) additional stimulation by pokeweed mitogen (PWM) in the presence or absence of varying amounts of MAX.16H5 (——) or isotype-matched IgG1 control MoAb (----). For assessment of immunoglobulin production, cultured cells were washed three times on day 7 and were then incubated for an additional 5 days before immunoglobulin was measured by an ELISA ($n=3$, one representative experiment is shown). In c, E^- cells (1×10^6/ml) were cultured in triplicates with irradiated (15 Gy) autologous T cell subpopulations (1×10^6/ml) in the presence of 5 μg/ml MAX.16H5 (□), 5 μg/ml isotype-matched IgG1 control MoAb (▩) or PBS (■). After 7 days ISC generation was assessed. Results are the mean of three independent experiments (±s.d.).

kept with or without anti-CD4 MoAbs (5 μg/ml) for 30 min at 37°C and the cells (2×10^6/ml) were then incubated with hydroethydine (40 μg/ml, gives red fluorescence; Polysciences, Warrigton, PA) for 15 min at room temperature. E^- cells or EBV-transformed cells (2×10^6/ml) were incubated with fluorescein diacetate (100 μg/ml, gives green fluorescence; Sigma) for 15 min at 37°C. After washing, 5×10^5 T cells were mixed with 5×10^5 E^- cells or EBV-transformed cells in 500 μl cell culture medium and incubated at 37°C for 20 min. Conjugates were identified as red/green pairs of cells under the fluorescence microscope. Large aggregates (>4 cells) were not counted.

RESULTS

Inhibition of B cell differentiation and proliferation

The effect of anti-CD4 MoAb on CD4,45RA$^+$ *versus* CD4,45RO$^+$ T cells with regard to their potential to provide help for B cell differentiation was analysed by culturing each subpopulation with B cells in the presence or absence of PWM. In these experiments T cells were irradiated before incubation with B cells and varying amounts of anti-CD4. As can be seen in Fig. 1, both T cell subpopulations retained their functional differences after being irradiated, in that the CD4,45RO$^+$ cells were able to induce substantial increase in immunoglobulin production in AMLR (Fig. 1a) and PWM-stimulated assays (Fig. 1b), while the CD4,45RA$^+$ cells lacked the capability to significantly induce B cell differentiation. The anti-CD4 MoAb

Table 1. Effects of anti-CD4 MoAb on pokeweed mitogen (PWM)-induced immunoglobulin isotype production

Cells	Secreted immunoglobulin (μg/ml)* IgG	IgM
E^- alone	4·12±1·21	1·53±0·72
E^- + CD4,CD45RA$^+$	5·42±1·00	1·69±0·82
E^- + CD4,CD45RA$^+$ + anti-CD4	4·23±1·21	1·58±0·62
E^- + CD4,CD45RO$^+$	9·59±2·32	2·96±1·2
E^- + CD4,CD45RO$^+$ + anti-CD4	4·47±1·52	2·00±0·71

* Mean of three independent experiments performed in triplicate cultures (±s.d.).

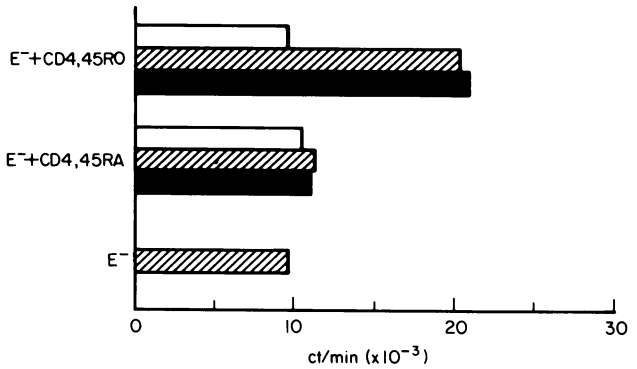

Fig. 2. Effects of anti-CD4 MoAb on pokeweed mitogen (PWM)-induced B cell proliferation. E^- cells (5×10^4/well) were cultured in triplicates with irradiated (30 Gy) CD4,45RA$^+$ or CD4,45RO$^+$ T cells (5×10^4/well) preincubated with anti-CD4 (5 μg/ml, □), isotype-matched IgG1 control MoAb (5 μg/ml, ▩) or PBS (■) in the presence of PWM. After 7 days of culture, ^{3}H-TdR incorporation was assessed ($n=3$, one representative experiment is shown).

MAX.16H5 clearly inhibited the immunoglobulin production induced by CD4,45RO$^+$ T cells. Production of both immunoglobulin isotypes was fully inhibited by MAX.16H5 (Table 1) as well as by T151 (data not shown). In the presence of CD4,45RA$^+$ cells no significant increase of immunoglobulin production was observed, and the anti-CD4 MoAb was not able to reduce immunoglobulin production of E^- cells below the control level. When the number of immunoglobulin-secreting B cells was assessed in AMLR, again CD4,45RO$^+$ cells were primarily affected (Fig. 1c).

The next experiments were undertaken to determine how anti-CD4 influences helper function of irradiated CD4,45RA$^+$ *versus* CD4,45RO$^+$ T cells in PWM-induced B cell proliferation (Fig. 2). Only CD4,45RO$^+$ cells provided help for B cell proliferation, and the help was reduced to background level by anti-CD4.

Inhibition of immunoglobulin secretion under influence of lymphokines

We then asked whether anti-CD4 would inhibit immunoglobulin secretion in the presence of lymphokines relevant for B cell

Table 2. Anti-CD4 MoAb on pokeweed mitogen (PWM)-induced immunoglobulin synthesis in the presence of exogenous lymphokines*

	Production of IgG (μg/ml)			Production of IgM (μg/ml)		
Cells	Medium	IL-2†	IL-4‡	Medium	IL-2	IL-4
E^- cells alone	3·86	4·13	5·72	1·20	1·32	1·58
E^- + $CD4,45RA^+$	3·98	4·21	5·43	1·30	1·39	1·67
E^- + $CD4,45RA^+$ + anti-CD4	3·34	3·97	4·92	1·24	1·36	1·69
E^- + $CD4,45RO^+$	7·90	12·00	9·20	2·42	2·96	1·98
E^- + $CD4,45RO^+$ + anti-CD4	3·64	10·20	7·20	1·94	2·10	1·84

* Mean of triplicate cultures.
† 100 U/ml IL-2.
‡ 0·1μg/ml IL-4.

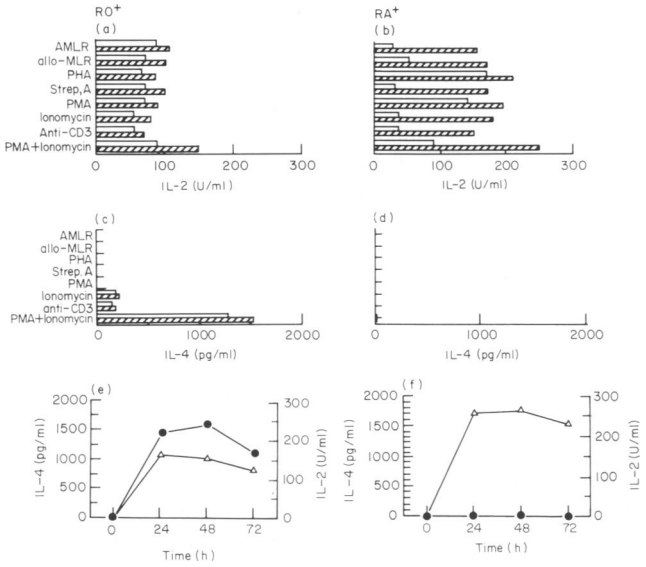

Fig. 3. Effects of anti-CD4 MoAb on cytokine production of $CD4,45RA^+$ *versus* $CD4,45RO^+$ T cells in response to mitogens and mitogenic MoAb. $CD4,45RO^+$ (a and c) or $CD4,45RA^+$ (b and d) T cells (1×10^6) were stimulated as indicated by either mitogen or mitogenic MoAb in the presence of irradiated (30 Gy) autologous E^- cells (1×10^6) with (□) or without (▨) anti-CD4 MoAb. Supernatants were harvested at 48 h and analysed for the presence of IL-2 and IL-4 ($n = 3$, one representative experiment is shown). In e and f, $CD4,45RA^+$ *versus* $CD4,45RO^+$ T cells were stimulated with a combination of phorbol myristate acetate (PMA; 10 ng/ml) and ionomycin (0·5 μM/ml). Supernatants were harvested at the time point indicated and were tested for IL-2 (△) and IL-4 (●) content.

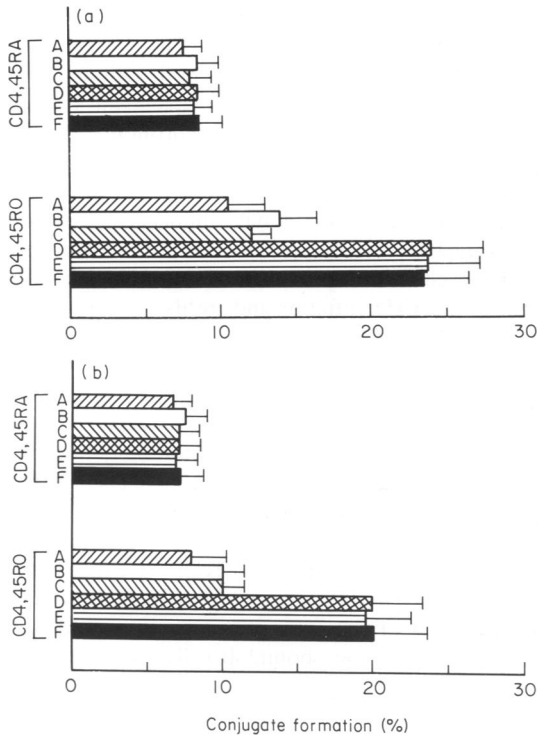

Fig. 4. Effects of anti-CD4 MoAb on conjugate formation by $CD4,45RA^+$ *versus* $CD4,45RO^+$ T cells with B cells. $CD4,45RA^+$ and $CD4,45RO^+$ T cells were incubated with $F(ab')_2$ fragments of MAX.16H5 (A), T151 (B), MAX.16H5 (C), isotype-matched IgG1 (D) and IgG2a (E) MoAbs or without anti-CD4 (F) for 30 min and the cells were then incubated with hydroethydine (40 μg/ml). E^- cells (a) and Epstein–Barr virus (EBV)-transformed B cells (b) were incubated with fluorescein diacetate (100 μg/ml) for 15 min respectively. The cells were then mixed and incubated. Only conjugates of less than four cells were counted after 20 min. Results are the mean of three independent experiments (±s.d.).

proliferation and differentiation. Based on cytokine titrations (data not shown), saturating concentrations were chosen for rIL-2 (100 U/ml) and rIL-4 (0·1 μg/ml). We tested whether the addition of IL-2 or IL-4 was able to compensate for the inhibitory effect of anti-CD4. As shown in Table 2, exogenous IL-4, but only marginally IL-2, supported PWM-induced B cell differentiation directly, as can be seen in the E^- cell cultures without addition of T cells. In contrast, addition of exogenous IL-2 seemed to stimulate primarily IgG secretion in the presence of $CD4,45RO^+$ cells. The data in Table 2 illustrate the capability of exogenous lymphokines, especially of IL-2, to further enhance IgG secretion promoted by $CD4,45RO^+$ T cells even in the presence of a strong polyclonal activator like PWM. Most importantly, despite of the exogenously added lymphokines, anti-CD4 was still able to inhibit T cell-mediated immunoglobulin secretion. However, the control levels measured without addition of cytokines were not reached, indicating a direct effect of high concentration of cytokines on B cells.

Inhibition of lymphokine production in T cell subpopulations

We next investigated whether the functional dichotomy between CD4,45RA$^+$ and CD4,45RO$^+$cells to induce B cell differentiation and proliferation resulted from different patterns of cytokine production, and whether anti-CD4 would differentially regulate cytokine secretion in the T cell subpopulations. Secretion of IL-2 and IL-4 was analysed in AMLR and allo-MLR and throughout stimulation of the subpopulations with various mitogens, mitogenic MoAb and a calcium ionophore (Fig. 3). Interestingly, the main differences between the two subpopulations were consistently observed with quite distinct stimuli. CD4,45RA$^+$ cells produced more IL-2 than CD4,45RO$^+$ cells with all protocols used. Allo-MLR, AMLR as well as PHA, Strep. A, and PMA, if used as single stimulants, did not induce detectable IL-4 production in either CD4,45RO$^+$ or CD4,45RA$^+$ T cells. However, production of IL-4 was seen in CD4,45RO$^+$ T cells following stimulation with ionomycin or anti-CD3 and at best with a combination of PMA plus ionomycin. Time course experiments (Fig. 3e, f) revealed a similar kinetics for IL-2 production in both subpopulations.

When we studied the anti-CD4 effects on lymphokine production we found with most stimuli a pronounced inhibition of IL-2 production in CD4,45RA$^+$ cells, and only a very modest inhibition in CD4,45RO$^+$ cells. Likewise, IL-4 production by CD4,45RO$^+$ cells was only minimally influenced. Therefore, the lack of anti-CD4-mediated inhibition of cytokine production in CD4,45RO$^+$ cells, which provide more effective help for B cell proliferation and differentiation and are also most susceptible to inhibition by anti-CD4, argues against effects on cytokines being important in the system.

Contact inhibition of T–B cell conjugate formation

We then investigated whether direct membrane contact of T and B cells might be involved by comparing CD4,45RO$^+$ with CD4,45RA$^+$ T cells in prevention of conjugate formation by anti-CD4. T and B cells were stained with hydroethydine and fluorescein diacetate respectively. The cells were then mixed and incubated. After 20 min the conjugates were identified (Fig. 4). When CD4,45RO$^+$ T cells were preincubated with MAX.16H5 or T151 for 30 min, they bound B cells less efficiently than without preincubation, an observation not made by using CD4,45RA$^+$ T cells. When F(ab')$_2$ fragments of MAX.16H5 were used, again CD4,45RO$^+$ cells were preferentially affected. There was no striking difference between freshly isolated B cells (Fig. 4a) and EBV-transformed B cells (Fig. 4b) in conjugate formation and inhibition. These results suggest (i) that anti-CD4 inhibits T–B interaction by primarily affecting CD4,45RO$^+$ cells, and (ii) that this effect is mediated by contact inhibition.

DISCUSSION

In this study we have demonstrated that the helper function for B cell proliferation and differentiation is mainly exerted by CD4,45RO$^+$ T cells and is sensitive to anti-CD4 treatment. It has been shown before that CD4,45RO$^+$ T cells are generally more effective helper cells than CD4,45RA$^+$ T cells (see for review [25]). The reasons are not clear. One possibility would be that CD4,45RO$^+$ T cells produced more lymphokines stimulating B cell functions like IL-2 [26,27]. Most studies including this one have shown that CD4,45RO$^+$ T cells in general secrete less IL-2 than CD4,45RA$^+$ T cells when stimulated by various stimuli [25,28]. However, there is controversy about anti-CD3-induced IL-2 stimulation in both populations [29,30]. Our findings support the data of Bettens *et al.* [31] which show—as with all other stimuli—more IL-2 being produced in CD4,45RA$^+$ human T cells after stimulation by anti-CD3. However, it is important to note that CD4,45RA$^+$ T cells are not effective in promoting B cell proliferation or differentiation [25,29], suggesting that IL-2 alone is not sufficient for optimal help, at least in non-preactivated B cells [32].

Previous studies have revealed the function of IL-4 as another important B cell helper factor [33]. It could be shown that most IL-4-producing T cells had a CD4,45RO$^+$ phenotype [34]. This was confirmed by our finding that only in this population but not in CD4,45RA$^+$ cells was IL-4 production inducible. However, significant amounts of IL-4 could be induced only by a strong stimulus like PMA plus calcium-ionophore. More physiological stimuli like allogeneic or autologous MLR did not induce measurable amounts of IL-4 in isolated naive or memory T cells. Also anti-CD3, which mimics the physiological stimulus by acting via the T cell receptor complex, could only induce small amounts of IL-4. This might be due to a very low cell number according to the observation published recently that only 1–3% of the lymphocytes from adult blood donors synthesized IL-4 even when stimulated by the strong stimulus PMA plus ionomycin [35]. Therefore, it seems not very likely that IL-4 production by CD4,45RO$^+$ T cells—under physiological conditions—was the only explanation for their excellent helper functions.

Another argument against a major contribution of IL-4 is provided by our finding that anti-CD4 treatment could hardly influence production of IL-2 or IL-4 in CD4,45RO$^+$ cells, though the capacity of this subpopulation to promote B cell differentiation was profoundly inhibited by anti-CD4. Even in the presence of a strong polyclonal activator like PWM and further addition of exogenous recombinant IL-2 or IL-4 we could still see a significant inhibition of immunoglobulin production by anti-CD4. However, under these conditions the immunoglobulin production was not reduced to the control level seen in cultures without exogenously added cytokines (see Table 2), thereby illustrating that cytokines once they are in the culture do promote a certain amount of immunoglobulin secretion which is not inhibitable by anti-CD4. It was interesting to note that anti-CD4 inhibited IL-2 production much more effectively in CD4,45RA$^+$ T cells, which were shown to be not very good helper cells.

The differential effect of anti-CD4 on both T cell subpopulations deserves an explanation. We have excluded distinct densities of surface CD4 by cytofluorometric analyses (data not shown), and there is no indication of different membrane charges in both subpopulations. However, an attractive alternative explanation may come from recent findings that have demonstrated an important role for direct membrane contact between B cells and activated T cells in T cell help [36,37]. In this study we have, therefore, investigated conjugate formation with different T cell subsets and the B cell preparation (E$^-$ cells) that was used throughout our experiments. This population included only a small portion of activated (large) B cells (data not shown). We could demonstrate by a double staining technique that even these freshly isolated B cells were able to form twice as many T–B conjugates with CD4,45RO$^+$ T

cells compared with the corresponding subpopulation. Since CD4,45RA$^+$ T cells express a smaller number of adhesion molecules (CD2, LFA-1, LFA-3) than CD4,45RO$^+$ T cells [38], it is likely that they will bind less efficiently to B cells.

We have shown that conjugate formation by CD4,45RO$^+$ T cells is much better inhibitable by anti-CD4 than conjugates with the corresponding subpopulation. This holds true not only for resting B cells, but has been demonstrated with EBV-transformed cells representing an activated B cell phenotype (see Fig. 4b). Since F(ab')$_2$ fragments were as effective as complete antibodies, binding to Fc receptors did not contribute to prevention of conjugate formation.

There remain other possibilities to explain the preferential effect of anti-CD4 on CD4,45RO$^+$ T cells which were not proven in this study. It was recently shown that activated helper T cells express a novel membrane molecule (gp39,CD40L) that triggers B cell growth and differentiation (see for review [39]). Since CD4,45RA$^+$ T cells contain resting, unprimed T cells [12] they could be assumed to have a lower rate of CD40L expression than most CD4,45RO$^+$ cells. This assumption can only be proven when antibodies to the human CD40L molecule become available.

Another possible explanation relates to the nature of the CD45 molecule, which is a tyrosine phosphatase in its cytoplasmic region involved in activation of the CD4-associated tyrosine kinase p56lck [40]. CD4,45RA$^+$ and CD4,45RO$^+$ T cells may differ (i) in their p561ck tyrosine kinase activities, or (ii) in substrates of phosphorylation that could modulate interaction with the signal–transduction complex in a different way [41–43]. Co-capping experiments have indicated that the CD4,45RO molecules but not the CD4,45RA molecules are able to associate with CD4 [41]. This could be an advantage for signal transduction in memory T cells, but, on the other hand, could also make them more susceptible to anti-CD4-mediated inhibition.

The advantage of CD4,45RO$^+$ T cells in promoting B cell proliferation as well as differentiation may result from a higher rate of antigen-independent conjugates formed beween T and B cells. Moreover, the primary adhesion of CD4,45RO$^+$ T cells with B cells seems to be more susceptible to interruption by anti-CD4 compared with CD4,45RA$^+$ T cells, which we suggest is at present the most likely explanation for a more profound inhibition of T cell-dependent B cell proliferation and differentiation.

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REFERENCES

1 Shizuru JA, Gregory AK, Chao CT, Fathman CG. Islet allograft survival after a single course of treatment of recipient with antibody to L3T4. Science 1987; **237**:278–80.

2 Wofsy D, Seaman WE. Reversal of advanced murine lupus in NZB/NZW F$_1$ mice by treatment with monoclonal antibody to L3T4. J Immunol 1987; **138**:3247–53.

3 Ranges GE, Sriram S, Cooper SM. Prevention of type II collagen-induced arthritis by *in vivo* treatment with anti-L3T4. J Exp Med 1985; **162**:1105–10.

4 Shizuru JA, Taylor-Edwards C, Banks BA, Gregory AK, Fathman CG. Immunotherapy of the nonobese diabetic mouse: treatment with an antibody to T-helper lymphocytes. Science 1988; **240**:659–62.

5 Hafler DA, Weiner HL. Immunosuppression with monoclonal antibodies in multiple sclerosis. Neurology 1988; **38**:42–7.

6 Herzog C, Walker C, Pichler W *et al.* Monoclonal anti-CD4 in arthritis. Lancet 1987; **2**:1461–2.

7 Wendling D, Wijdenes J, Racadot E, Morel-Fourrier B. Therapeutic use of monoclonal anti-CD4 antibody in rheumatoid arthritis. J Rheumatol 1991; **18**:325–7.

8 Horneff G, Burmester GR, Emmrich F, Kalden JR. Treatment of rheumatoid arthritis with an anti-CD4 monoclonal antibody. Arthritis Rheum 1991; **34**:129–40.

9 Takeuchi T, Schlossman SF, Morimoto C. The T4 molecule differentially regulating the activation of subpopulation of T4$^+$ cells. J Immunol 1987; **139**:665–71.

10 Hiepe F, Volk HD, Apostoloff E, von-Baehr R, Emmrich F. Treatment of severe systemic lupus erythematosus with anti-CD4 monoclonal antibody. Lancet 1991; **338**:1529–30.

11 Streuli M, Hall LR, Sage Y, Schlossman SF, Saito H. Differential usage of three exons generates at least five different mRNAs encoding human leukocyte common antigens. J Exp Med 1987; **165**:1548–66.

12 Koulova L, Yang SY, Dupont B. Identification of the anti-CD3-unresponsive subpopulation of CD4$^+$,CD45RA$^+$ peripheral T lymphocytes. J Immunol 1990; **145**:2035–43.

13 Eric BB, Sheila MS. Interconversion of CD45R subsets of CD4 T cells *in vivo*. Nature 1990; **348**:163–6.

14 Reiter C, Kakavand B, Rieber E, Schattenkirchner M, Riethmüller G, Krüger K. Treatment of rheumatoid arthritis with monoclonal CD4 antibody M-T151. Clinical results and immunopharmacological effects in an open study, including repeated administration. Arthritis Rheum 1991; **34**:525–36.

15 Emmrich F, Horneff G, Becker W *et al.* An anti-CD4 antibody for treatment of chronic inflammatory arthritis. Agents Actions Suppl 1991; **32**:165–70.

16 Emmrich F, Schilling B, Eichmann K. Human immune response to group A streptococcal carbohydrate(A-CHO). I. Quantitative and qualitative analysis of the A-CHO-specific B cell population responding *in vitro* to polyclonal and specific activation. J Exp Med 1985; **161**:547–62.

17 Saxon A, Feldhaus J, Robins A. Single step separation of human T and B cells using AET treated SRBC rosettes. J Immunol Methods 1976; **12**:285–8.

18 Fujii Y, Okumura M, Inada K, Nakahara K, Matsuda H. CD45 isoform expression during T cell development in the thymus. Eur J Immunol 1992; **22**:1843–50.

19 Rogozinski L, Bass A, Glickman E *et al.* The T4 surface antigen is involved in the induction of helper function. J Immunol 1984; **132**:735–9.

20 Noelle RJ, Daum J, Bartlett WC, McCann J, Shepherd DM. Cognate interactions between helper T cells and B cells. V. Reconstitution of T helper cell function using purified plasma membranes from activated Th1 and Th2 T helper cells and lymphokines. J Immunol 1991; **146**:1118–24.

21 Sedgwick JD, Holt PG. A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. J Immunol Methods 1983; **57**:301–9.

22 Hirohata S, Lipsky PE. T cell regulation of human B cell proliferation and differentiation. J Immunol 1989; **142**:2597–607.

23 Paliard X, de-Waal-Malefijt R, Yssel H *et al.* Simultaneous production of IL-2,IL-4 and IFN-γ by activated human CD4$^+$ and CD8$^+$ T cell clones. J Immunol 1988; **141**:849–55.

24 Lecomte O, Fischer A. Antigen-independent adhesion of CD45RA (naive) and CD45RO (memory) CD4 T cells to B cells. Inter Immunol 1992; **4**:191–6.
25 Akbar AN, Salmon M, Janossy G. The synergy between naive and memory T cells during activation. Immunol Today 1991; **12**:184–8.
26 Emmrich F, Moll H, Simon MM. Recombinant human interleukin 2 acts as a B cell growth and differentiation promoting factor. Immunobiol 1985; **169**:97–102.
27 Jelinek DF, Lipsky PE. Regulation of human B lymphocyte activation, proliferation, and differentiation. Adv Immunol 1987; **40**:1–59.
28 Salmon M, Kitas GD, Gaston JS. Interleukin-2 production and response by helper T-cell subsets in man. Immunology 1988; **65**:81–5.
29 Sleasman JW, Morimoto C, Schlossman SF, Tedder TF. The role of functionally distinct helper T lymphocyte subpopulations in the induction of human B cell differentiation. Eur J Immunol 1990; **20**:1357–66.
30 Byrne JA, Butler JL, Reinherz EL, Cooper MD. Virgin and memory T cells have different requirements for activation via the CD2 molecule. Int Immunol 1989; **1**:29–35.
31 Bettens F, Walker C, Gauchat JF, Gauchat D, Wyss T, Pichler WJ. Lymphokine gene expression related to CD4 T cell subset (CD45R/CDw29) phenotype conversion. Eur J Immunol 1989; **19**:1569–74.
32 Mond JJ, Thompson C, Finkelman FD, Farrar J, Schaefer M, Robb RJ. Affinity-purified interleukin 2 induces proliferation of large but not small B cells. Proc Natl Acad Sci USA 1985; **82**:1518–21.
33 Paul WE, Ohara J. B-cell stimulatory factor-1/ interleukin 4. Annu Rev Immunol 1987; **5**:429–59.
34 Holter W, Majdic O, Kalthoff FS, Knapp W. Regulation of interleukin-4 production in human mononuclear cells. Eur J Immunol 1992; **22**:2765–7.
35 Andersson U, Andersson J, Lindfors A, Wagner K, Möller G, Heusser CH. Simultaneous production of interleukin 2, interleukin 4 and interferon-γ by activated human blood lymphocytes. Eur J Immunol 1990; **20**:1591–6.
36 Hirohata S, Jelinek DF, Lipsky PE. T cell dependent activation of B cell proliferation and differentiation by immobilized monoclonal antibodies to CD3. J Immunol 1988; **140**:3736–44.
37 Suzuki N, Sakane T, Ueda Y, Murakawa Y, Tsunematsu T. Implications for the role of cognate interactions in *in vitro* human B cell activation by *Staphylococcus aureus* Cowan I and pokeweed mitogen. J Clin Invest 1986; **77**:294–300.
38 Sanders ME, Makgoba MW, Sharrow SO *et al.* Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3,CD2 and LFA-1) and three other molecules (UCHL-1, CDw29 and Pgp-1) and have enhanced IFN-γ production. J Immunol 1988; **140**:1401–7.
39 Noelle RJ, Ledbetter JA, Aruffo A. CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B-cell activation. Immunol Today 1992; **13**:431–3.
40 Koretzky GA, Picus J, Thomas ML, Weiss A. Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. Nature 1990; **346**:66–68.
41 Ostergaard HL, Shackelford DA, Hurley TR, Jahnson P, Hyman R, Sefton BM, Trowbridge IS. Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines. Proc Natl Acad Sci USA 1989; **86**:8959–63.
42 Mustelin T, Coggeshall KM, Altman A. Rapid activation of the T-cell tyrosine protein kinase pp56-lck by the CD45 phosphotyrosine phosphatase. Proc Natl Acad Sci USA 1989; **86**:6302–6.
43 Dianzani U, Luqman M, Rojo J *et al.* Molecular associations on the T cell surface correlate with immunological memory. Eur J Immunol 1990; **20**:2249–57.