CD45 monoclonal antibody-mediated cytolysis of human NK and T lymphoma cells

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Background and Objectives. The CD45 rat monoclonal IgG2b antibodies YTH24.5 and YTH54.12 act synergistically to produce cytolysis of normal lymphocytes and have been safely given to patients in conditioning regimens for allogeneic stem cell transplantation. The antibodies are not lytic for hematopoietic stem cells, but the depletion of the lymphoid lineage cells is profound and sustained.

Design and Methods. We evaluated the YTH24.5 and YTH54.12 pair for complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and apoptotic and antiproliferative effects against a panel of non-Hodgkin's lymphoma (NHL) cell lines and against primary specimens.

Results. Significant CDC activity was observed against two of two NK and one of four T lymphoma cell lines; moderate activity was seen against two of four T, and four of eight B lymphoma cell lines. In the responding cell lines, the lytic activity of YTH24.5 and YTH54.12 was as least as strong as that of alemtuzumab or antithymocyte globulin. The combination of YTH24.5 and YTH54.12 also induced ADCC in one of two NK and two of four T lymphoma cell lines, as well as three primary specimens, but was ineffective in B-NHL. The antibodies decreased viability in two of two NK and one lymphoma cell line, measurable as apoptosis or direct cell death in the cell lines NK92 and CEM, respectively. In a tumor model of Jurkat lymphoma in SCID mice, administration of YTH24.5 and YTH54.12 impaired local tumor growth and delayed systemic disease progression.

Interpretation and Conclusions. CD45 antibodies YTH24.5 and YTH54.12 have lytic activity against NK and T lymphoma cells via CDC and ADCC, are effective in a preclinical tumor model, and may be candidates for immunotherapeutic approaches to the treatment of human NK and T cell lymphoma.

Key words: T-cell lymphoma, monoclonal antibody, CD45.

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Aggressive non-Hodgkin's lymphomas (NHL) are susceptible to chemo- and radiotherapy, leading to an initial complete remission in the majority of patients, and to long-term disease-free survival in about half of those treated with chemotherapy-based combined modality treatment protocols.[1,2] For B-cell NHL, addition of the chimeric anti-CD20 antibody rituximab to chemotherapy significantly improved disease-free survival,[3] an effect explained by complement fixation (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) as well as by reduction of bcl-2-associated chemotherapy resistance.[4-6] Patients with peripheral T-cell lymphoma, who are in general treated similarly to patients with B-cell NHL, have lower overall survival rates compared to these latter.[7] As in aggressive B-NHL, monoclonal antibody immunotherapy may prove a useful adjunct to chemotherapy in such patients. The humanized CD52 monoclonal antibody alemtuzumab, which has potent activity in prolymphocytic leukemia (PLL), has already shown promise in peripheral T-NHL.[8,9] The cell surface phosphatase CD45 is expressed on virtually all hematopoietic cells, with the highest expression occurring on T lymphocytes.[10] In rodent models as well as in humans, monoclonal antibodies against CD45 efficiently lysed normal T cells, mainly by complement fixation and ADCC.[11-13] In earlier studies, a pair of such monoclonal antibodies against CD45, YTH24.5 and YTH 54.12, had been selected for optimal lytic synergy in CDC and ADCC assays, and were successfully applied to deplete renal allografts of passenger leukocytes and to safely supplement conditioning regimens in allogeneic stem cell transplantation.[14,15] We have now explored whether YTH24.5 and YTH 54.12 have significant lytic activity against NK- and T-cell lymphomas *in vitro* and *in vivo*.

Design and Methods

Cells

The T-cell lymphoma/leukemia cell lines Jurkat, CCRF-CEM and HUT78 (DSMZ, Braunschweig, Germany) and the variant PM1 of HUT78,[16] as well as NK cell line YT (DSMZ, Braunschweig, Germany) were propagated in RPMI 1640 supplemented with 25mM HEPES, GlutaMAX I (Gibco-BRL), penicillin/streptomycin (Sigma, Biochrom) and 10% heat-inactivated fetal calf serum (FCS, Gibco-BRL). The NK cell line NK92 (kindly provided by T.Tonn, Frankfurt, Germany) was cultured in X-vivo medium (BioWhittaker) containing 5%

human AB plasma supplemented with 100U/mL interleukin (IL)-2 (R&D Systems). The aggressive B-cell lymphoma/leukemia cell lines Balm3, Raji, Karpas 422, Ramos, and SuDHL4 (DSMZ, Braunschweig, Germany), as well as the mantle cell lymphoma cell lines Granta 519, Jeko, Mino, and Sp53 were propagated in RPMI1640 supplemented as above. NK- and T-NHL cells from clinical specimens were obtained from diagnostic material, comprising the peripheral blood of a 64-year male patient with leukemia of NK-cell phenotype (patient 1), the peripheral blood of a 42-year old female patient with prolymphocytic leukemia of T-cell phenotype (T-PLL; patient 2), and the pleural effusion of a 16-year old female with acute T-lymphoblastic leukemia (patient 3) with malignant cell infiltrations of 100%, 82%, and 92.5%, respectively.

Antibodies and flow cytometry

The clinical grade rat IgG2b CD45 antibodies YTH24.5 and YTH54.12, originally developed by Waldmann`s group in Cambridge, UK,[11] were purified from cell culture supernatant by affinity chromatography and used at the concentrations indicated. Mouse fluorochrome-conjugated isotype control antibodies, fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-coupled anti-CD45 (clone OX-1, Coulter), PE-coupled anti-CD3 (clone UCHT1, DakoCytomation), FITC-coupled anti-CD52 (clone CF1D12, Caltag), and FITC-coupled anti-rat IgG2b (clone G15-337, Becton-Dickinson) were used following the manufacturers' instructions. Chimeric CD20 antibody (rituximab) was purchased from Roche Pharmaceutical (Basel, Switzerland), humanized CD52 antibody (alemtuzumab) from Medac-Schering (München, Germany), and rabbit anti-thymocyte globulin (ATG-F) from Fresenius Biotech (München, Germany). Flow cytometry phenotyping of tumor cells was performed on a FACScan (Becton Dickinson, Mountain View, CA, USA). Saturating amounts of antibodies were added to cells for 30 minutes at 4°C, before extensive washing and analysis. Mean fluorescence intensity (MFI) values were used as semiquantitative measures of CD45 expression. To assure comparability, peripheral blood lymphocytes from the same donor were run in parallel at the same instrument settings on the flow cytometer. For a direct comparison of CD45 and CD52 levels on the T and NK cell lines, the cells were incubated with the mouse monoclonal antibodies against CD45 and CD52, clones OX-1 and CF1D12, respectively, and antibody binding was detected with a secondary, PE-conjugated anti-mouse IgG F(ab')$_2$ fragment from sheep. MFI was measured on a flow cytometer and, after background fluorescence had been subtracted, the ratio of MFI_{CD45}/MFI_{CD52} was calculated. In some experiments investigating CDC and ADCC activity, we used a rat monoclonal antibody with irrelevant specificity (clone A110-2 against keyhole limpet hemocyanin, Becton Dickinson) as an isotype control.

Complement-mediated lysis, direct cytotoxicity and apoptosis

To measure direct and complement-dependent effects on tumor cell viability, antibodies were added at the concentrations indicated to lymphoma cells (1×10^5/100 µL) in complete medium supplemented with human serum (10%, 23% and 40% v/v, as indicated), obtained from volunteers at the University of Goettingen, Germany, with or without prior heat inactivation (56°C for 45 minutes). After 24 hours at 37°C, cell viability was determined in triplicate by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining as described before.[17] Briefly, the culture volume of 100 µL was supplemented with MTT in phosphate-buffered saline (PBS) to achieve a final concentration of 0.5 mg/mL. After incubation for 4 hours, the cells were spun, the supernatant discarded, and the pellet resuspended in 30% v/v dimethyl sulfoxide (DMSO), 5% v/v formic acid and 1% w/v Triton X100 (all from Sigma) dissolved in isopropanol. The light absorbance from formazan was measured at 540 nm on a Tecan SLT photometer (Tecan SLT Spectra). The effect on viability was expressed as the ratio of values from treated versus untreated samples, i.e. specific viability = 100×absorbance with antibody treatment/absorbance of untreated control. Differences between samples with and without antibody were tested using the two-tailed Student´s t-test and differences with a p value <0.05 were considered statistically significant. To assess the contribution of direct cytotoxicity and apoptosis to the reduction in viability, tumor cells (1×10^6/mL) were incubated in complete medium supplemented by 10% heat-inactivated FCS in the presence or absence of 1 µg/mL of the respective antibody. Apoptosis of cells was followed after 24 hours by combined staining with annexin V and propidium iodide according to the manufacturer's instructions (Becton-Dickinson) and by flow cytometric analysis with compensation for fluorescence from propidium iodide and annexin V set by controls after staining with either dye alone (FACScan, Becton Dickinson; *data not shown*).

Antibody-dependent cell-mediated cytotoxicity

Flow cytometric analysis of peripheral blood mononuclear cell (PBMC)-mediated cytotoxicity was performed as reported before.[18] Briefly, target lymphoma cells were washed and resuspended in phosphate buffered saline (PBS) to a concentration of 1×10^6/mL. Next, 2×10^6 cells were stained with 20 µL of 3 mM 3,3-dioctadecyloxacarbocyanine perchlorate (DiO) dissolved in DMSO (both from Sigma) for 20 min at 37°C. After washing twice with PBS, cells were resuspended in complete RPMI and PBMC from healthy volunteers were added at an effector to target ratio of 40:1 for 4 h at 37°C with YTH24.5 and YTH54.12 antibodies at a concentration of 0.1 µg/mL each. In a previous study, the optimal concentration of IgG2b antibody required for ADCC with YTH24.5 and YTH54.12 had been determined to be 0.1 µg/mL.[11] Samples without antibody and without effector cells served as the respective controls. The samples were stained with 2.5 µg/mL propidium iodide and analyzed by flow cytometry. DiO-stained viable target cells showed green fluorescence only, whereas dead cells showed both green and red fluorescence due to the propidium iodide counterstain. The specific lysis of DiO-labeled target cells induced by YTH24.5/YTH54.12 was calculated as: specific lysis (%) =[(lysis of DiO positive cells (%) - spontaneous lysis of DiO positive cells (%)]/[(100-spontaneous lysis of DiO positive cells (%)]. Specific and spontaneous lysis values were those with and without antibody addition,

respectively. The mean values of specific lysis from at least three independent experiments with and without YTH24.5/YTH54.12 for each cell line were analyzed by the two-tailed Student´s t-test and differences with p values <0.05 were considered statistically significant. In a previous study, Tighe *et al*. had tested several antibodies against CD45 for their ability to block lytic activity of human effector cells against mouse A20 lymphoma cells. Over a concentration range of 1 to 650 μg/mL, there was no inhibition of lysis by YTH24.5 or YTH54.12.[19] To exclude significant inhibition of effectors by the CD45 antibodies, we tested the effect of YTH24.5/YTH54.12 on the viability of the effector cells under the conditions of our ADCC assay (4 h at 37°C, 0.1 μg/mL) and found a loss of viability of 5.3% + 0.8% (*data not shown*). In additional experiments, the 10% heat inactivated FCS was substituted by either 10% heat inactivated or non-inactivated normal human serum to test the effects of complement on ADCC activity.

Human Jurkat T-cell lymphoma model in SCID mice

Six- to 8-week-old C.B-17 scid/scid (SCID) mice, bred and maintained under defined flora conditions in our own breeding facility, were injected with Jurkat T-cell lymphoma cells according to a protocol approved by the local and regional Animal Protocol Review Committees. On day 0, 2×10^7 washed Jurkat cells in 200 μL PBS were injected subcutaneously. The mice developed visible local tumor growth from day 10 and were all sacrificed on day 20 whether or not they had signs of systemic disease. The animals were dissected, macroscopically visible tumor lesions documented and the spleens weighed. The tumor volume was calculated using the equation: $tumor\ volume = a \times b \times c\ \pi/6$, where a, b and c are the three orthogonal tumor diameters. For the assessment of bone marrow infiltration, single-cell preparations from hind-limb bones were analyzed by flow cytometry for $CD45^+$ and $CD3^+$ cells. Estimation of bone marrow infiltration was based on 1×10^5 viable cells per measurement, and the differences in bone marrow infiltration in untreated and treated animals were compared by the two-tailed Student's t-test. Tissues from tumor, spleen and liver were processed by formalin fixation and standard paraffin embedding for immunohistochemical studies.

Results

Targeting lymphoma cells with CD45 monoclonal antibody

A prerequisite for the lytic activity of the antibodies YTH24.5 and YTH54.12 against different lymphoma cell populations is the expression and accessibility of the CD45 protein on the tumor cell surface. We, therefore, analyzed CD45 antibody binding with YTH24.5 and YTH54.12 by flow cytometry. CD45 expression levels differed widely, but in general lymphoma cells showed levels of CD45 expression equivalent to those on peripheral blood lymphocytes (Figures 1 A,B). Very low or absent CD45 expression was seen only on one B-cell lymphoma (SuDHL4, Figures 1A,D). In most NHL a unimodal distribution of CD45 expression levels was found, except in cell lines HUT78, SP53 and KMH2, which exhibited population heterogeneity of antigen expression (Figure 1C). Furthermore, the expression of CD45 was compared to that of CD52 and the epitopes recognized by antithymocyte globulin on the NK and T NHL cell lines, as well as CD52 on the cells of three primary samples (patient 1 with leukemic NK cell lymphoma, patient 2 with T-PLL, and patient 3 with T-lymphoblastic leukemia). The expression of CD52 was consistently lower on the cell lines and primary cells, with some cells lines such as CEM and YT exhibiting no binding of CD52 antibody (Figure 1E, Figure 2). By indirect immunofluorescence with flow cytometric quantification, the ratios (MFI_{CD45}/MFI_{CD52}, n=3) of anti-CD45 versus anti CD52 antibody binding were: 2.3±0.1 for peripheral blood, 4.0±1.6 for YT, 15.4±8.7 for NK92, 22.6±0.6 for Jurkat, 21.4±7.9 for CEM, 20.3±2.1 for PM1 and 1.6±0.2 for HUT78. Furthermore, antithymocyte globulin bound to all cell lines, although at consistently lower levels than an antibody against CD45 (Figure 2). Thus, the CD45-specific monoclonal antibodies, YTH24.5 and YTH54.12, can target T and B NHL with variable binding levels, and with higher efficiency than antibodies against CD52 and antithymocyte globulin.

Complement-dependent cytolysis of NK- and T-cell lymphoma cell lines

The monoclonal antibodies YTH24.5 and YTH54.12 effectively lyse T lymphocytes by complement fixation, and because they bind to proximate but non-competing epitopes, they are most effective when used at equimolar concentrations.[11] We, therefore, measured the viability of lymphoma cells after 24 hours of exposure to antibody in the presence of complement. A substantial reduction in cell viability was observed in two of two NK cell lines and one of four T-cell lines; minor effects were seen in two of four T-cell lines (Figure 3A). The lysis induced by YTH24.5 and YTH54.12 correlated weakly with the binding levels of the antibody combination as determined by flow cytometry (correlation coefficient 0.71). To compare the lytic activity of YTH24.5/YTH54.12 with that of other antibodies, we measured the CDC of YTH24.5/YTH54.12, of the CD52 monoclonal antibody (alemtuzumab), and of antithymocyte globulin. The CD45 antibodies YTH24.5 and YTH54.12 were at least as effective as alemtuzumab and antithymocyte globulin. YTH24.5/YTH54.12 also showed efficient CDC activity against Jurkat cells, which were resistant to alemtuzumab and antithymocyte globulin (Figure 3A). The relevance of CDC was further addressed by exposing cell lines to increasing amounts of complement.

In the NK cell line YT and the T-cell line Jurkat the lytic activity of YTH24.5 and YTH54.12 increased with the amount of complement, while in the cell lines CEM and HUT78 these effects were not observed (Figure 4). It was noteworthy that addition of human serum had a stimulatory effect on YT and Jurkat cell proliferation, which was more than counterbalanced by YTH24.5 and YTH54.12 CDC activity (Figure 4). Thus the combination of anti-CD45 antibodies YTH24.5 and YTH54.12 had CDC activity against NK and T lymphoma cell lines, with a potency that was as least as strong as that of alemtuzumab or antithymocyte globulin.

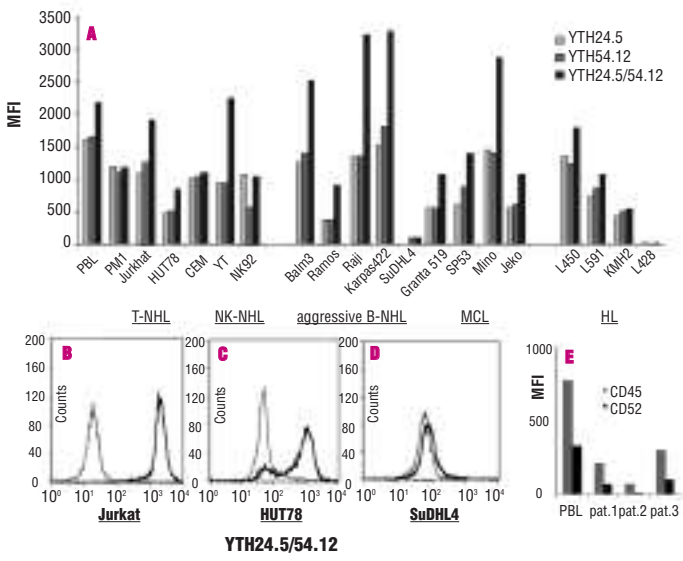

Figure 1. Binding patterns of the YTH24.5 and YTH54.12 monoclonal antibodies on lymphoma cell lines. Binding of YTH24.5, YTH54.12 or the combination (YTH24.5/54.12) on peripheral blood or the lymphoma cell lines was detected with an FITC-labeled antibody against rat immunoglobulin, and the mean fluorescence intensity (MFI) measured by flow cytometry (A). All cell lines, representing T-NHL, NK-NHL, aggressive B-NHL, mantle cell lymphoma (MCL) and Hodgkin's lymphoma (HL) were run in parallel at the same instrument settings, and the MFI of the respective isotype control was subtracted for each sample. In most cell lines a unimodal staining pattern was found, as represented by the Jurkat cell line (B; binding of YTH25.5/YTH54.12 represented by the bold line, binding of the isotype control by the fine line). In the HUT78, SP53 and KMH2 cell lines, subpopulations with low antibody binding were found (HUT78 in C), whereas the cell line SuDHL4 showed only marginal binding (SuDHL4 in D). The expression of CD45 and CD52 on three primary NK- and T-NHL samples was tested by flow cytometry with an FITC-labeled antibody against CD45 (clone OX-1), and CD52 (clone CF1D12), respectively, and the results shown in comparison to expression on peripheral blood lymphocytes (PBL) after subtraction of background fluorescence values from the isotype controls (E).

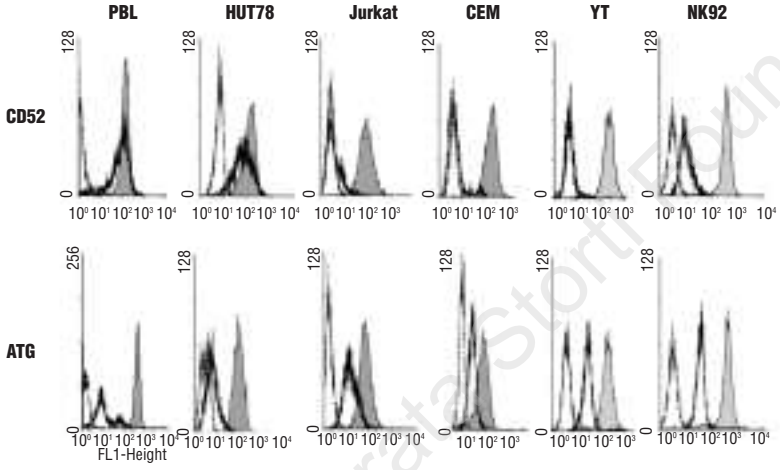

Figure 2. Expression of CD45, CD52 and antithymocyte globulin epitopes on NK- and T-NHL cell lines. Peripheral blood lymphocytes (PBL), NK cell lines (YT, NK92) and T cell lines were stained with FITC-labeled antibodies against CD45 (clone OX-1, shaded curves), CD52 (clone CF1D12, bold line in the upper row), as well as with antithymocyte globulin (ATG) and FITC-labeled anti-rabbit immunoglobulin (bold line in the lower row). The respective isotype controls are given as dotted curves in the histogram overlays.

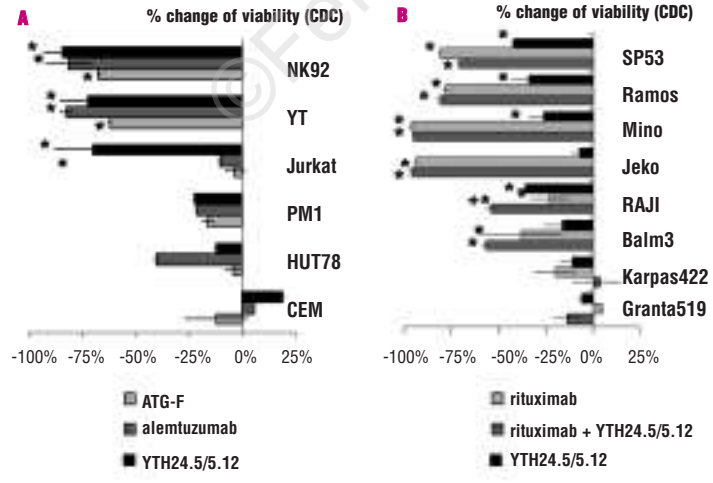

Figure 3. Complement-dependent cytolytic activity of YTH24.5, YTH54.12, alemtuzumab and antithymocyte globulin (ATG-F) against lymphoma cell lines. The viability of cells was measured by the MTT test after incubation for 24 hours with the combined antibodies YTH24.5/54.12, or alemtuzumab, rituximab, and ATG-F at concentrations of 1 μg/mL each. The antibodies were added to cell samples containing 23% non-heat-inactivated normal human serum. Reductions in viability compared to the controls without antibody addition are reported as mean values of triplicate experiments. The differences in the samples with and without the respective antibodies were tested by a two-tailed Student´s t-test, and results with $p<0.05$ are marked (*). In the Raji cell line, the combination of YTH24.5/YTH54.12 with rituximab led to a significantly greater reduction in viability than that produced by rituximab alone (+). The results for NK and T lymphoma cell lines are summarized in (A), the results for B lymphoma cell lines in (B).

Complement-dependent cytolysis of B-NHL cell lines

Since B-cell lymphomas are susceptible to CDC activity, as demonstrated by the strong CDC activity of the chimeric CD20 antibody rituximab,[20] we tested YTH24.5 and YTH54.12 for CDC activity against B-NHL cell lines. Our results confirmed the strong CDC activity of rituximab against four of the eight B-cell lymphoma cell lines tested (Figure 3B). We also observed that YTH24.5/54.12

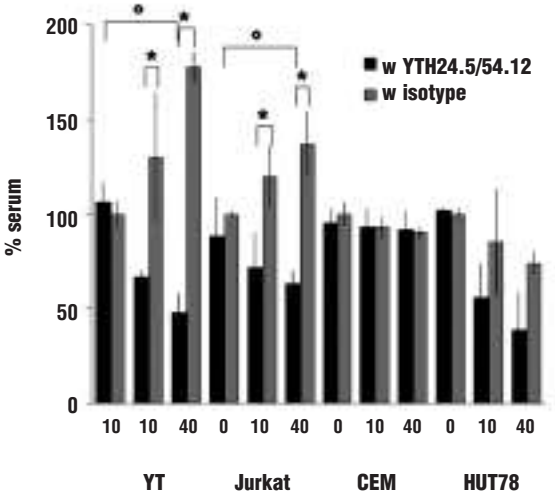

Figure 4. Complement-depending cytolytic activity of YTH24.5 and YTH54.12 against NK and T lymphoma cell lines. The viability of cells was measured by the MTT test after incubation for 24 hours with the combined antibodies YTH24.5/54.12 (black columns) or an isotype control (clone A110-2, gray columns) at concentrations of 1 μg/mL each. The antibodies were added to cell samples containing increasing amounts of non-heat-inactivated normal human serum, i.e. 0%, 10% and 40%, as indicated. Viabilities, represented by mean values of triplicate experiments, are reported in percent of the sample without human serum and with isotype addition for each cell line. The differences in the samples with YTH24.5/54.12 and isotype control were tested by a two-tailed Student´s t-test, and results with $p<0.05$ are marked (*). The viabilities observed with 0% and 40% were also compared by a two-tailed Student´s t-test, and results with $p<0.05$ are, as for the cell lines YT and Jurkat, marked (°). It shoud be noted that adding human serum increased the viability in the YT and Jurkat cell lines.

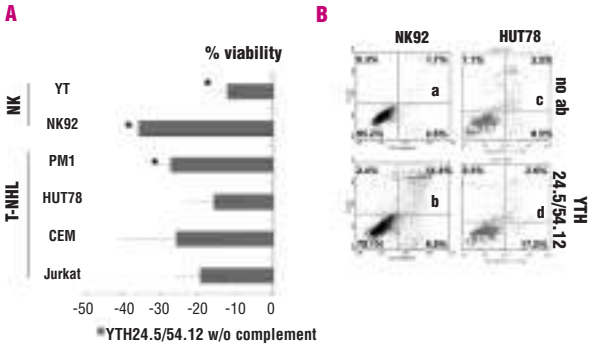

Figure 5. Direct effects of YTH24.5 and YTH54.12 against NK and T lymphoma cell lines. The viability of cells was measured by the MTT test after incubation for 24 hours with the combination of YTH24.5 and YTH54.12 monoclonal antibodies in the absence of complement. A statistically significant reduction of viability was seen in the NK cell lines YT and NK92, as well in the T lymphoma cell line PM1 (B). Using staining with propidium iodine (y-axis) and annexinV (x-axis) as markers for dead and apoptotic cells, respectively, flow cytometric analysis revealed induction of apoptotic death in NK92 cells by combined YTH24.5/YTH54.12 (a,b). In the HUT78 cell line, neither antibody nor the combination induced measurable effects (e,f).

produced a statistically significant reduction of viability in four of eight B-cell lines, albeit at lesser degrees in the range of 25-50% (Figure 3B). The lysis induced by YTH24.5 and YTH54.12 did not correlate with the binding levels of the antibody combination (correlation coefficient 0.074). The combination of rituximab and YTH24.5/54.12 had no effect over rituximab or YTH monoclonal antibody alone, except for the cell line Raji, in which YTH24.5/YTH54.12 moderately enhanced the cytolytic effects of rituximab (Figure 3B). The non-complement lytic activity of YTH24.5/54.12 was below 30% in all B-cell lymphoma cell lines tested (*data not shown*). Thus the anti-CD45 antibodies YTH24.5 and YTH54.12 had limited CDC activity against B-cell lymphoma cells, but this was less than that produced by rituximab and added to that drug's effects in only one of eight cell lines.

Antiproliferative and apoptotic effects of YTH24.5 and YTH54.12 in NK and T lymphoma cell lines

Monoclonal antibodies may also exert effects against tumor cells by direct mechanisms such as receptor-mediated growth arrest and apoptosis. CD45 is a tyrosine phosphatase and since antibodies may impede its function, we determined the viability of lymphoma cells after 24 hours of exposure to YTH24.5 and YTH54.12 in the absence of complement. A significant reduction in cell viability, in the range of 12% to 35%, was observed in the NK cell lines YT and NK92, as well as in the T cell lymphoma PM1, whereas the effect in the other cell lines did not reach statistical significance (Figure 5). The reduction in viability did not correlate with the binding levels of YTH24.5/YTH54.12 on the lymphoma cells (correlation coefficient 0.034). To investigate the reduction of viability summarized by the MTT test, we incubated the cell lines with YTH24.5 and YTH54.12, and analyzed annexinV and propidium iodide staining as markers for apoptotic and dead cells, respectively. In NK92 cells, annexin V staining showed that incubation with the antibodies led to cell death via apoptosis with annexin V/propidium iodide double positive cells (Figures 5 A,B). In the T-lymphoblastic cell line HUT78, however, we observed no effects of YTH24.5 and YTH54.12 on either the proportion of dead or apoptotic cells in this assay (Figures 5 E,F). Thus ligating the CD45 cell surface protein with YTH24.5 and YTH54.12 antibodies induced direct effects in some NK and T lymphoma cell lines, associated with apoptosis in the NK92 cell line. The precise mechanisms of the antiproliferative and cytotoxic effects of the antibodies in the absence of complement or effector cells do, however, remain to be elucidated.

ADCC of NK and T-cell lymphoma cell lines and primary samples

Besides complement fixation, ADCC via recruitment of effector cells bearing Fc-receptors is a major mechanism of lytic activity by many monoclonal antibodies including YTH24.5/YTH54.12.[21] The YTH24.5/54.12 antibodies enhanced the efficacy of ADCC in the NK cell line NK92 and the T lymphoma cell lines Jurkat and CEM, but had little effect on ADCC of YT, PM1 and HUT78 (Figure 6 A,B). Similarly, in three primary T-cell lymphoma specimens the antibodies induced specific lysis of 11.1%, 4.3% and 9.7%, respectively (Figure 6 A,B). Furthermore, we

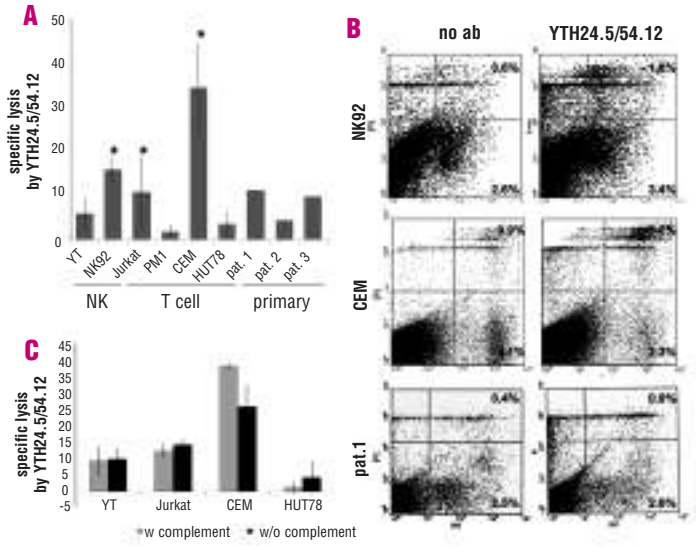

Figure 6. Antibody-dependent cell-mediated cytotoxicity against NK and T-cell lines. The cell lines as well as PBMC of three patients with NK (pat. 1) and T cell lymphoma (pat. 2 and 3) were labeled green fluorescent with DiO, exposed to PBMC for 4 hours with or without 0.1 μg/mL YTH24.5 and YTH54.12, and the fraction of propidium iodine (PI)-positive, i.e. dead, target cells determined by flow cytometry. The fluorescence of DiO-labeled target cells is shown on the x-axis, the fluorescence of PI positive cells on the y-axis, exemplified for a NK-cell lymphoma (NK92), a T-cell lymphoma (CEM), and a primary NK-cell lymphoma sample (B). Thus unlabeled effector cells are found in the upper and lower left quadrants, DiO-labeled target cells in the upper and lower right quadrants. The specific lysis of DiO-labeled target cells induced by YTH 24.5/YTH 54.12 was calculated as: specific lysis (%) = (lysis of DIO positive cells (%) - spontaneous lysis of DiO-positive cells (%))/(100 - spontaneous lysis of DiO positive cells (%)). Specific and spontaneous lysis were lysis in the presence and absence of antibody, respectively. Specific lysis caused by antibody addition, represented as the mean values of three independent experiments per cell line, is summarized in (A). The differences between the samples with and without YTH24.5/YTH54.12 were analyzed by a two-tailed Student´s t-test. Differences <0.05 were considered statistically significant and are marked (*). The increase in ADCC activity caused by the addition of YTH24.5/YTH54.12 reached statistical significance in the NK92, Jurkat, and CEM cell lines. The addition of 10% heat inactivated or non-heat-inactivated normal human serum instead of heat-inactivatd FCS in the ADCC assay was tested for the cell lines YT, Jurkat, CEM and HUT78 (C), and no significant differences with and without heat inactivation were observed.

analysed the effects of complement addition to ADCC activity. In the presence of 0.1 mg/mL YTH24.12/YTH54.12, complement addition did not significantly change ADCC activity against the cell lines YT, Jurkat, CEM and HUT78 (Figure 6C). Thus we observed ADCC activity against one of two NK cell lines, two of four T lymphoma cell lines and, to a moderate degree, three primary specimens.

Activity of YTH24.5/54.12 against Jurkat lymphoma cells in vivo

We next addressed the value of YTH24.5 and YTH54.12 in an *in vivo* model of T-cell lymphoma. We xenotransplanted human Jurkat T-cell lymphoma cells into SCID mice, and found 2×10^7 tumor cells sufficient to establish measurable local tumor growth within 10 days (Table 1). Moreover, Jurkat cells also showed an early systemic spread to other organs, such as spleen, liver and bone marrow (Table 1, Figure 7). We then administered YTH24.5/54.12 antibodies on day 6 to 9 after lymphoma cell inoculation at a daily dose of 400 μg/kg body weight, equivalent to the MTD established for humans in a phase I clinical trial.[15] Analyzing the animals after sacrifice on day 20, we found a significantly smaller tumor volume at the site of inoculation in the treated animals (Table 1, p=0.016, unpaired Student´s t-test). Furthermore, we observed that macroscopic metastatic liver involvement occurred in all of five control animals, but in only one of five antibody treated animals, while ascites was observed in three of five controls and none of five antibody-treated animals (Table 1). Microscopically, lymphoma infiltration of the liver was confined to the periportal region after antibody treatment, but extended into the liver plate with areas of liver parenchyma necrosis in the untreated control animals (Figures 7 D-F). In the spleen, which normally has low cellularity in SCID mice (Figure 7G), there was diffuse, dense

Table 1. Effects of YTH24.5/YTH54.12 treatment on Jurkat lymphoma disease in SCID mice.

Group	*n*	*YTH24.5/54.12*	*Primary tumor pos/all*	*Tumor volume [cm³]*	*Spleen weight [g]*	*Liver nodules pos/all*	*Ascites pos/all*	*BM infiltration pos/all*	*BM infiltration [%]*
1	5	y	5/5	0.14*[1]±0,10	0.128±0.047	1/5	0/5	3/5	0.05%*[2]±0.05%
2	5	n	5/5	0.86*[1]±0,45	0.182±0.037	5/5	3/5	5/5	1.07%*[2]±0.09%

**[1]p=0.016; *[2]p=0.020. SCID mice were implanted subcutaneously with 2x107 Jurkat cells and developed systemic lymphoma disease. The animals were sacrificed on day 20, dissected and macroscopic extension of the disease estimated by measurement of primary tumor incidence and volume, spleen weight and occurrence of liver nodules. Liver and spleen were also analyzed by light microscopy, and bone marrow infiltration by flow cytometry (Figure 7). Tumor volumes, spleen weights and bone marrow infiltration of the treatment groups were compared by two-tailed Student's t-tests for unpaired samples.*

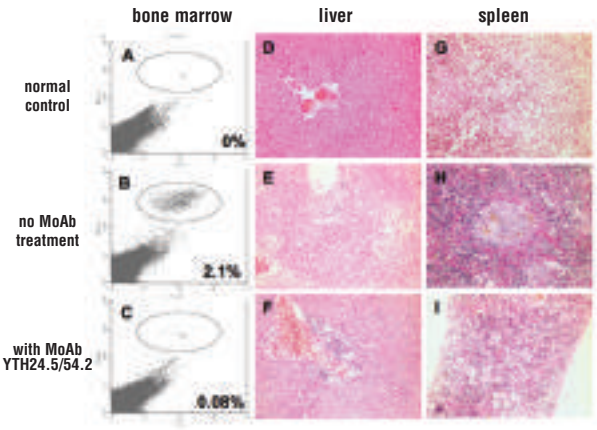

Figure 7. Effects of YTH24.5/YTH54.12 treatment on Jurkat lymphoma disease in SCID mice. SCID mice implanted with Jurkat cells developed systemic lymphoma disease within 20 days. Bone marrow infiltration was detected and quantified by flow cytometry of 100,000 events after staining with anti-human CD45 (x-axis) (A-C) and anti-human CD3 (y-axis) (A-C). Compared to a normal SCID mouse without human cells in the bone marrow (A), infiltration was found in all five untreated animals (example in B) and with lower percentages of infiltration in three of five animals treated with YTH24.5/YTH54.12 (C). Likewise, lymphoma infiltration of liver (D-F) and spleen (G-I) as documented by hematoxylin-eosin stain (40×) was less prominent after antibody treatment (F,I) than in the untreated controls (E,H).

tumor cell infiltration in untreated animals while the treatment group retained the appearance of low cellularity (Figure 7I). Splenic weight was lower in the antibody-treated animals than in the untreated controls, although the difference did not reach statistical significance (0.128±0.047g versus 0.182±0.037g, p=0.07, unpaired Student´s t-test, Table 1). Finally in the bone marrow, we found a mean tumor cell infiltration of 0.05%, range 0% - 0.11%, after antibody treatment, compared to 1.07 %, range 0.52% to 2.37%, in the untreated control group (p=0.04, unpaired Student´s t-test, Figure 7 B and C). Thus in this model, systemic application of YTH24.5 and YTH54.12 reduced tumor growth both at the inoculation site and at sites of metastatic disease.

Discussion

We have shown that the rat monoclonal CD45 antibodies YTH24.5 and YTH54.12 have *in vitro* and *in vivo* lytic activity against primary cells and cell lines derived from NK and T-cell lymphomas. The lytic effects against NK and T-NHL cell lines were strongest in the CDC assay, supplemented by modest cytolytic activity mediated by ADCC and by direct cytotoxic effects. Cell lines with a NK-cell phenotype (NK92 and YT) and the Jurkat T lymphoma cell line were more susceptible than B-cell lymphomas. *In vivo* implantation of Jurkat cells in SCID mice also confirmed responsiveness of T-cell lymphomas to these monoclonal antibodies, used at clinically tolerable doses. The reasons for the differential sensitivity of NK, T and B-cell NHL are not yet clear, since levels of CD45 expression were high. Of note, administration of CD45 monoclonal antibodies to patients prior to stem cell transplantation produced a similar pattern of depletion of normal lymphocytes, with T cells being the most susceptible, and B cells the least.[15] Since CDC is the main mechanism of cell death mediated by YTH24.5 and YTH54.12 *in vitro*, differences in susceptibility to complement-mediated lysis may be responsible for the differences we observed. Manches *et al.* found complement-dependent cytolysis of B-NHL by the CD20 antibody rituximab was reversibly associated with the ratio of CD20 expression levels to the expression levels of the complement resistance proteins CD46, CD55 and CD59. The Raji and Karpas cell lines are certainly resistant to both anti-CD45 and anti-CD20 mediated lysis (Manches *et al.* 2003, Figure 3B), but the Mino and Balm3 cell lines were highly susceptible to CD20 mediated CDC and less susceptible to CD45 CDC, even though high levels of antibodies were bound. These results suggest different resistance mechanisms for anti-CD52 and anti-CD45 mediated CDC, a notion supported by the higher efficacy of CD45 treatment in T-cell-derived compared to B-cell-derived malignancies (Figure 3), even when the levels of antibody binding were equivalent. The distinct consequences of CD45 antibody binding in these two populations may reflect differences in the consequences of ligating CD45 surface protein. CD45 phosphatase activity both positively and negatively regulates signaling through the activation of the Src family tyrosine kinases,[22] and the effect of CD45 monoclonal antibodies on these functions remains to be elucidated. Ligation of CD45 may inhibit B-cell proliferation after anti-IgM or anti-CD40 stimulation, interfere with leukocyte adhesion and migration, and modulate the lytic activity of natural killer cells.[23-25] If ligation with YTH24.5 and YTH54.12 antibodies interferes with the specific function of CD45 in T-lymphocytes, this interference may render such cells more prone to CDC lysis, and may contribute to the heterogeneous picture of direct cytotoxic effects observed (Figures 3,4). Certainly, the induction of apoptosis and the loss of viability that may follow CD45 monoclonal antibody binding suggest that this event has substantial downstream consequences for the NK or T cell. Of note, the insensitivity of CD45$^+$ B-NHL cells to the CD45 monoclonal antibodies extends also to Hodgkin's lymphoma. Consistent with their presumed B-cell origin, we found that the CD45$^+$ Hodgkin's cell lines L-540, L-549, L-428 and KMH2 were entirely insensitive to ADCC, CDC or direct killing mediated by the YTH24.5 and YTH54.12 antibodies. The YTH24.5 and YTH54.12 antibodies had shown synergistic lytic activity on normal T cells, binding non-competingly to the distinct epitopes P2 (YTH 24.5) and Q2 (YTH54.12) on purified CD45 antigen.[11,19] The sub-additive fluorescence intensity of combined YTH24.5 and YTH54.12 staining in some lymphoma cell lines observed here is unexplained. As the original competition assays were performed on the purified protein and not on viable cells, the differences in fluorescence intensities of the antibody combination are presumably due to steric inhibition between the antibodies in the

context of CD45 antigen insertion in the plasma membrane.

What are the therapeutic prospects for these CD45 antibodies?

Based on their *ex vivo* activity, it appears that the CD45 monoclonal antibody pair may be most likely to contribute to the therapy of NK and T-NHL. CD45 antibodies may most readily be envisioned as an adjunct to chemotherapy, comparable to the application of anti-CD20 and anti-CD52 in B-cell lymphomas.[3,26] A possible limitation of this approach, however, may be hematopoietic toxicity, and indeed, Dahlke *et al*. have recently reported fatal aplasia induced by a different rat monoclonal IgG2b anti-CD45 antibody in a rat model.[12] Our own data in a murine model with a similar CD45 antibody (30F11)[27] showed no evidence of long-term hematopoietic toxicity from CD45-mediated cytoreduction, and in clinical studies with YTH24.5 and YTH54.12, no impairment of stem cell activity has been observed.[15] The low level of CD45 on the most immature progenitor cells[28] evidently spares the cells from destruction. As a consequence of stem cell sparing and of their short half life,[15] the effect of the antibodies on neutrophils is transient, with neutropenia lasting only 2-4 days after administration of the monoclonal antibody.[15] While even short-lasting neutropenia is an undesirable consequence of therapy, the brevity of this effect will likely compare well with the more prolonged neutropenia observed with many of the other cytotoxic agents currently used to treat T and NK-cell lymphomas. Although there are no data yet on the efficacy of YTH24.5 and YTH54.12 in combination with chemotherapy against leukemias and lymphomas, our assay systems might serve as a platform to test such combinations.

Second, CD45-mediated cytoreduction may be used as an adjunct to the conditioning regime for allogeneic stem cell transplantation in CD45-positive NK and T-cell lymphomas. Anti-CD45 treatment may serve two purposes: targeting the neoplasia and targeting host T cells and dendritic cells. For the former purpose, antibodies against CD45 appear promising as NK and T cell lymphomas retain ample CD45 on their cell surface. Anti-CD45 treatment may also serve a similar role as antithymocyte globulin, targeting host T cells and dendritic cells relevant for engraftment and development, and our previous data in a murine model showed the beneficial effect of anti-CD45 conditioning for allogeneic engraftment.[13,27]

In summary, we have shown that anti-CD45 treatment for NK and T-cell lymphomas using the clinically applicable antibodies YTH24.5 and YTH54.12 is feasible and may be worthy of clinical testing.

GGW: design of the study, experimental work, wrote the manuscript; AB: in vitro experimental work; BG: design of the study; BH: pathological evaluation of in vivo experiments; RS: design of the study; MKB: design of the study, wrote the manuscript; LT: design of the study. The authors reported no potential conflicts of interest.

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