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Light-Directed Activation of Human T-Cells

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Photocleavable "caged" biomolecules have been successfully used as photoswitches and phototriggers as reported in many biological studies,[1] but relatively few of these reports have described the effective caging and uncaging of proteins under physiological conditions.[2] We have developed a procedure that uses a coating of photocleavable 2-nitrophenylethanol (NPE) groups to reversibly inhibit antibodies.[3] The antibodies can be reactivated, when and where required, by irradiation with UVA light. Reactivation occurs even when the inactivated antibodies are irradiated through plastic in the presence of cells.[4] This led us to suggest that this procedure could be used to greatly increase the specificity of therapeutic antibodies,[4,5] and we recently described the construction of a photoactivatable cancer-targeting bispecific conjugate.[4] We proposed that if only the cytotoxic end of such a conjugate were to be reversibly inactivated, then the tumour-specific end would remain free to bind to its target tumour cells without damage to normal tissues also targeted through specific and nonspecific cross-reactions. Localised illumination of the tumour-targeted conjugate reactivates the cytotoxic end, maximising tumour destruction whilst minimising damage to healthy tissue.[4] Such a technique could also be used to improve the targeting of a patient's immune response to a tumour; this has been suggested as an elegant alternative to the use of toxins or enzymes.[6]

After early studies suggested that a low-level activation of T-cells in serum could prevent malignant tumour progression,[7] bispecific antibodies were designed to directly target cytotoxic T-cells to tumours.[6,8] In theory, one part of the antibody reacts with a specific tumour antigen and binds to the tumour cell surface whilst the other part of the antibody reacts with a T-cell marker (normally CD3), hence targeting the T-cell to the tumour cell (Figure 1). In practice this procedure suffers from two major drawbacks: Firstly, it has proved to be very challenging to obtain the exquisite specificity required to clinically differentiate between tumour and normal cells, perhaps not surprising when the large ratio of normal tissue to tumour tissue in most patients is taken into consideration. This limits the degree of specific localisation that can be achieved against certain tumours.[5] To date relatively few antibodies have been licensed for use against solid tumours.[9] Secondly, the introduction of anti-T-cell-based bispecific constructs results in them being bound by peripheral T-cells before the bispecific antibody reaches its tumour target. This both impedes the bispecific antibody and activates T-cells peripherally, leading to T-cell depletion and unwanted cytokine storms.[6a,9b,10] Both of these challenges could be effectively overcome if the anti-CD3 part of the bispecific antibody could be reversibly inactivated. The antitumour portion of the antibody would remain free to circulate and bind to tumour cells, but the anti-CD3 portion would not be able to bind, activate, and remove peripheral T-cells from the patient's circulation. Nonspecific cross-reactions or specific unwanted binding of the antitumour antibody would become irrelevant, as the anti-CD3 portion of the antibody would be inactive until deliberately reactivated in the area of the tumour (Figure 1). An added bonus would be that higher doses of conjugate could be administered, allowing more conjugate to target the tumour.

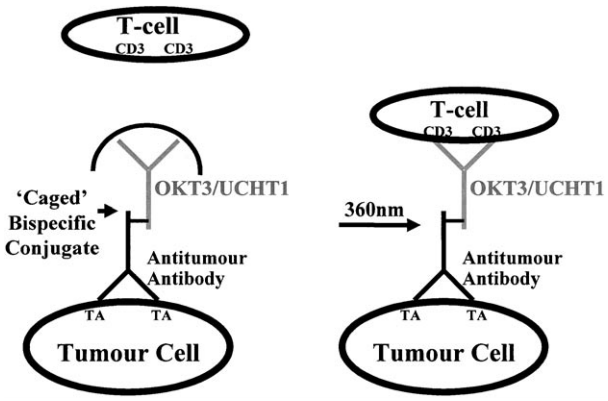

Figure 1. How a bispecific antibody conjugate links a T-cell to a tumour. This diagram shows the advantages of reversibly inactivating the anti-CD3 (OKT3 or UCHT1) portion of a cancer-targeting conjugate with NPE. The conjugate is reactivated only where it is bound to tumour by irradiation with UV light; TA = tumour antigen. Throughout these studies we used a human T-cell clone (H9), which expresses CD3 on its cell surface.

This report demonstrates the production of the most important component necessary to achieve this goal: photoreversibly inactivated anti-human CD3 antibodies. A coating of NPE groups[3] is used to block the activity of OKT3 or UCHT1 (two anti-human CD3 antibodies). On illumination with UVA light, the NPE groups cleave, leaving the antibodies free to bind to, and activate, the human H9 T-cell line. A coating of NPE groups was used to inhibit the biological activity of OKT3. 30 μL of NPE–COCl was used to coat 1 mL of antibody. The final yield of soluble inactivated OKT3 was routinely 20% (0.2 mg mL^{-1}) with approximately 50 NPE residues present on each antibody molecule (as determined by the increase in OD_{280} of the antibody). The inhibition and reactivation of OKT3 binding to the H9 T-cell line was then investigated by flow cytometry. As UV irradiation is needed to remove NPE from the coated OKT3 samples, and it is known to damage some biological molecules, it was first important to demonstrate that UV irradiation does not damage uncoated OKT3. Figure 2 shows

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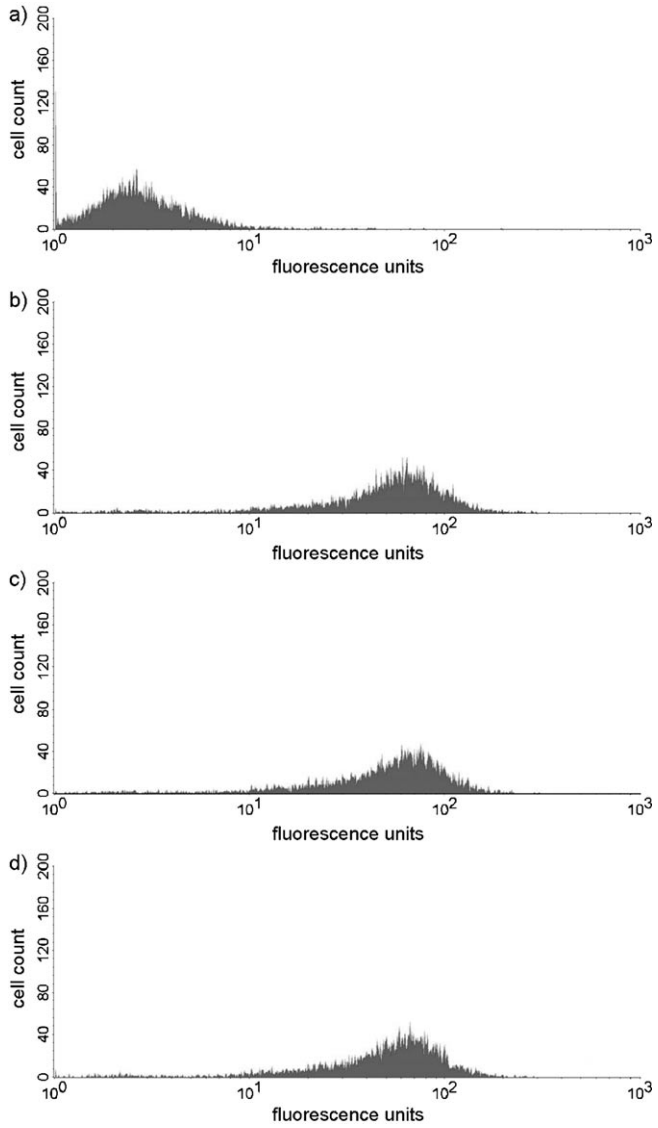

Figure 2. The binding of OKT3 antibody to H9 T-cells as measured by flow cytometry. a) Control nonspecific IgG or b–d) OKT3 (murine anti-human CD3) antibodies were added to H9 cells and UV irradiated for b) 0, c) 5, or d) 10 min. After a 1-h incubation, and washing, a fluorescein isothiocyanate (FITC)-labelled anti-murine antibody was added, and the amount of OKT3 bound to the cells was quantified by the amount the cells fluoresced.

that UVA irradiation has very little effect on the binding of OKT3 to H9 cells, the mean value of the fluorescence peak being 63, 63, and 61 fluorescence units after UV exposure for 0, 5, and 10 min. No fluorescence (mean 3 units) was detected when an irrelevant antibody was added to the cells as a control. The size (side and forward scatter) of the population of H9 cells measured in the assay is given in the Supporting Information. It is worth noting that the size and scatter of the cells did not change after they had been irradiated with UV light, demonstrating that little or no damage occurs to the cells. When NPE-coated OKT3 was added to the H9 cells it was not able to bind; fluorescence levels decreased to virtually nonspecific control levels (mean 4.6 units, Figure 3a). Irradiation with UVA light removed the NPE groups and reactivated the antibody, as

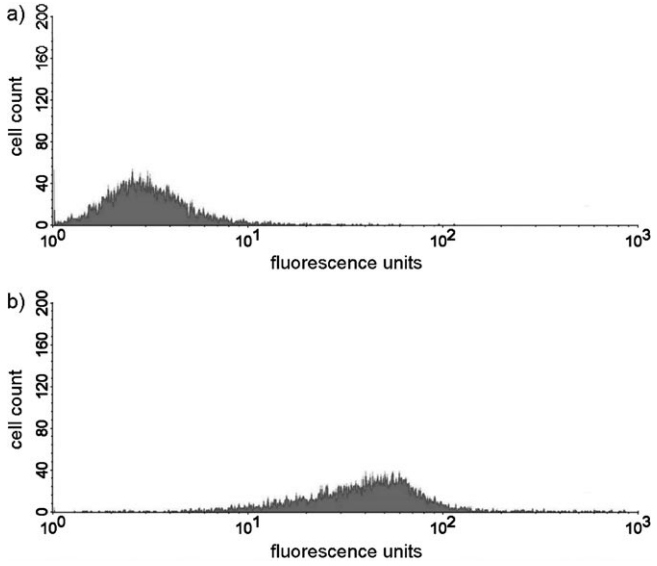

Figure 3. The binding of NPE-coated OKT3 antibody samples to H9 cells a) without UV exposure or b) UV irradiation for 6 min, as measured by flow cytometry.

shown by virtually all of the H9 cells fluorescing (mean 55 units, Figure 3b) at levels close to those given by untreated OKT3. Careful control of the degree of conjugation was found to be important. If less NPE–COCl (5–20 μL) was added (fewer NPE residues per antibody molecule), the OKT3 antibody activity was not fully inhibited, whereas the addition of too much NPE–COCl (>40 μL) caused the antibody to aggregate and precipitate.

After reversibly inactivating OKT3 on several occasions we decided to examine if we could also reversibly inhibit UCHT1, another anti-human CD3 antibody. Irradiation with UV light for 10 min had no effect on the binding of UCHT1 to H9 cells. Therefore 20- and 30-μL aliquots of NPE–COCl were added to two 1-mL aliquots of UCHT1. After centrifugation and dialysis, the supernatants contained 0.27 and 0.14 mg mL^{-1} of NPE-coated antibody. They had approximately 21 and 30 NPE residues coating each UCHT1 antibody molecule, respectively. Table 1 gives the values for the mean fluorescence given by each sample in a typical flow cytometry experiment. The addition of 20 μL NPE–COCl was not sufficient to fully inactivate the antibody; binding of UCHT1 to H9 cells was decreased to

Table 1. The binding of UCHT1 and two NPE-coated UCHT1 conjugates to H9 cells.

Conjugate	Fluorescence[a]
Control IgG	2
UCHT1 (stock)	86
NPE–UCHT1[b]	10
NPE–UCHT1[b] + UV	58
NPE–UCHT1[c]	3
NPE–UCHT1[c] + UV	56

[a] Values given are the mean fluorescence of the single flow cytometry peak. [b] 20 μL NPE. [c] 30 μL NPE.

approximately 12% of its initial activity. However, coating the antibody with 30 μL NPE–COCl resulted in virtually complete inhibition, reducing its activity to control background levels. On irradiation with UV light for 10 min, 65% of the initial antibody activity was regained in both samples. This further established the reproducibility of our coating procedure towards anti-T-cell antibodies.

The light-specific binding and subsequent activation of the H9 T-cell line was confirmed by the expression of early T-cell activation markers, CD69 and IL2, 3 h after the addition of the NPE–OKT3 complexes. In T-cells treated with non-illuminated NPE–OKT3, the levels of activation marker CD69 (Figure 4c) were only slightly increased above background fluorescence (Figure 4a). However, the CD69 levels were significantly increased on the T-cells illuminated in the presence of NPE–OKT3 (Figure 4d) to levels similar to those obtained with control antibody (Figure 4b). Medium from control H9 cells that

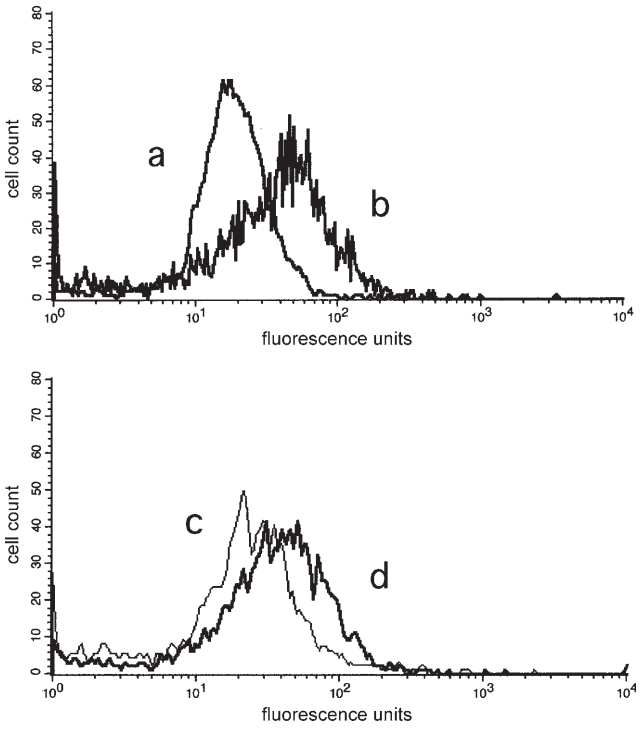

Figure 4. Activation of H9 T-cells as shown by the direct addition of an anti-human CD69–FITC conjugate and flow cytometry: a) irradiated T-cells alone; b) irradiated T-cells and OKT3; c) T-cells with NPE–OKT3 added after irradiation of the cells; d) irradiated T-cells and NPE–OKT3.

did not receive antibody contained 11 ± 3 pg mL^{-1} IL2. Positive control wells in which H9 cells received uncoated OKT3 contained 530 ± 40 pg mL^{-1}. In medium from cells treated with NPE–OKT3 the concentration of IL2 increased to 54 ± 13 pg mL^{-1} IL2 (possibly reflecting the presence of a small residual fraction of uncloaked antibody). However, illumination of the H9 cell NPE–OKT3 mixture increased the IL2 concentration to 211 ± 30 pg mL^{-1}. Values for H9 cells treated with UCHT1 samples were very similar. Control wells containing uncoated UCHT1 contained 615 pg mL^{-1}. Medium from wells treated with NPE–UCHT1 contained 30 pg mL^{-1}, but this increased markedly to 455 pg mL^{-1} on irradiation.

These data show that the biologically important anti-human CD3 monoclonal antibodies, OKT3 and UCHT1, can be reversibly inhibited by a simple nonspecific NPE coating procedure and that human T-cell activity can be upregulated in specific locations in vitro by illumination with UVA light in the presence of NPE-coated anti-CD3. This ability to regulate T-cell/CD3 activity in specific areas, without being part of a bispecific complex, could be used as a generally applicable way toward effecting immune modulation in the treatment of many other diseases as well as cancer. The extent of T-cell activation depends on which anti-CD3 antibody is used, and it is often reported that a second signal is required for maximal activation.[6a, 8b, 11] This is possibly why we needed to use ovarian cancer cell conditioned medium to obtain CD69 and IL2 expression by the H9 T-cell line after it had bound the reactivated anti-CD3 antibodies. In tumour targeting, the second signal may arise through cytokine release by the tumours, may be produced by the bispecific antibody itself,[12] or it is even possible that the cancer cells may express some of the B7 family of T-cell co-stimulatory molecules.[13] Irrespective of the mechanisms by which T-cell activation is obtained, antitumour/anti-CD3 bispecific complexes have been shown to kill human tumours in immune-deficient mice[14] and can also abolish tumour metastases.[12] This promising cancer-targeting procedure can only benefit from the increased specificity and decreases in peripheral binding obtained by using a photoactivatable conjugate. The next two critical steps are to 1) demonstrate that inactivated antibody can be reactivated both in vivo and in vitro and 2) to construct photoactivatable bispecific conjugates[4] for use in targeting tumours in vivo. We have already shown that the hamster anti-murine CD3 monoclonal antibody, NPE-145-2C11 (a murine equivalent of OKT3), can be reactivated in vivo, and that this alone can cause the regression of tumour growth in a C57L6 mouse system.[15] We are currently synthesising photoactivatable bispecific anti-human CD3 conjugates and analysing their effectiveness in vitro and in vivo.

Experimental Section

The CD3+ human T-cell line H9 and the OKT3-secreting hybridoma were obtained from ECACC and ATCC. UCHT1 (IgG2a subclass), was obtained from Cancer Research UK. OKT3 and UCHT1 were reversibly inhibited using 1-(2-nitrophenyl)ethoxycarbonyl chloride (NPE–COCl) as previously described.[3b,4] 250-μL aliquots of H9 T-cells had 30 μL (0.1 mg mL^{-1}) of control or NPE-coated OKT3/UCHT1 samples added to them. The amount of OKT3/UCHT1 bound was quantified by the addition of FITC-labelled goat anti-mouse and analysed by flow cytometry.

Photolysis of conjugates: The NPE-coated OKT3/UCHT1 samples were irradiated with a VL-206BL UVA lamp, which had a total UVA irradiance of ~16 mW cm^{-2} at a distance of 1 cm.

Expression of CD69 and IL2 by H9 cells: The expression of the activation marker CD69 was analysed by flow cytometry after the ad-

dition of FITC-labelled anti-CD69 and washing. IL2 concentrations were measured using a BD Biosciences human IL2 ELISA kit.

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[1] *Dynamic Studies in Biology: Phototriggers, Photoswitches and Caged Biomolecules* (Eds.: M. Goeldner, R. Givens), Wiley-VCH, Weinheim, **2005**.
[2] S. Loudwig, H. Bayley, *Dynamic Studies in Biology: Phototriggers, Photoswitches and Caged Biomolecules* (Eds.: M. Goeldner, R. Givens), Wiley-VCH, Weinheim, **2005**, pp. 253 – 304.
[3] a) S. Thompson, M.-C. Fawcett, J. A. Spoors, C. H. Self, *Biochem. Soc. Trans.* **1995**, *23*, 155S; b) C. H. Self, S. Thompson, *Nat. Med.* **1996**, *2*, 817 – 820.
[4] S. Thompson, M.-C. Fawcett, C. H. Self, *ChemMedChem* **2007**, *2*, 1162 – 1164.
[5] C. H. Self, S. Thompson, *Lancet* **2006**, *367*, 1038 – 1039.
[6] a) P. A. Baeuerle, P. Kufer, R. Lutterbüse, *Curr. Opin. Mol. Ther.* **2003**, *5*, 413 – 419; b) L. G. Lum, P. A. Davol, *Cancer Chemother. Biol. Response Modif.* **2005**, *22*, 273 – 291.
[7] J. D. I. Ellenhorn, R. Hirsch, H. Schreiber, J. A. Bluestone, *Science* **1988**, *242*, 569 – 571.
[8] a) A. B. van Spriel, H. H. van Oji, J. G. J. van de Winkel, *Immunol. Today* **2000**, *21*, 391 – 402; b) S. Withoff, W. Helfrich, L. F. de Leij, G. Molema, *Curr. Opin. Mol. Ther.* **2001**, *3*, 53 – 62.
[9] a) O. H. Brekke, I. Sandlie, *Nat. Rev. Drug Discovery* **2003**, *2*, 52 – 62; b) D. Schrama, R. A. Reisfeld, J. C. Becker, *Nat. Rev. Drug Discovery* **2006**, *5*, 147 – 159.
[10] G. Molema, J. W. Tervaert, B. J. Kroesen, W. Helfrich, D. K. Meijer, L. F. de Leij, *Br. J. Cancer* **2000**, *82*, 472 – 479.
[11] a) C. Renner, M. Pfreundschuh, *Immunol. Rev.* **1995**, *145*, 179 – 209; b) L. E. Porter, H. Nelson, G. I. Ethem, D. C. Rice, C. Thibault, A. I. Chapoval, *Cancer Immunol. Immunother.* **1997**, *45*, 180 – 183; c) A. Pfosser, M. Brandl, H. Salih, L. Grosse-Hovest, G. Jung, *Int. J. Cancer* **1999**, *80*, 612 – 616.
[12] a) A. I. Chapoval, H. Nelson, C. Thibault, *J. Immunol.* **1995**, *155*, 1296 – 1303; b) S. Riedle, M. Rosel, M. Zoller, *Int. J. Cancer* **1998**, *75*, 908 – 918.
[13] C. Rietz, L. Chen, *Am. J. Transplant.* **2004**, *4*, 8 – 14.
[14] a) L. da Costa, C. Renner, F. Hartmann, M. Pfreundschuh, *Cancer Chemother. Pharmacol.* **2000**, *46*, S33 – S36; b) A. Katzenwadel, H. Scheer, D. Gierschner, U. Wetterauer, U. Elsässer-Beile, *Anticancer Res.* **2000**, *20*, 1551 – 1555.
[15] S. Thompson, R. Stewart, J. A. Smith, C. H. Self, *ChemMedChem* **2007**, *2*, 1591 – 1593.

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