DOI: 10.1002/cmdc.200700116

# Light Activation of Anti-CD3 in vivo Reduces the Growth of an Aggressive Ovarian Carcinoma

Stephen Thompson,* Robert Stewart, Jacqueline A. Smith, and Colin H. Self*[a]

In the preceding article we describe the controlled photoactivation of human T-cells by their illumination in the presence of the previously inactivated anti-human CD3 antibodies, OKT3 or UCHT1.[1] The photoreversible inhibition of the anti-human CD3 antibodies uses a coating of photocleavable 2-nitrophenylethanol (NPE) groups to block the antibody's activity.[2] The NPE groups could be removed by irradiation with UVA light, leaving the antibodies free to bind to, and activate, human T-cells at any given time and place. This provided a means to target human T-cells with a much higher degree of specificity to tumours, whilst minimising side effects in other non-illuminated areas, and was the first key step in our ultimate goal: the preparation of highly specific photoactivatable bispecific tumour-targeting antibody conjugates.

The next steps prior to the construction of the cancer-targeting conjugate were to examine 1) if an NPE-inactivated anti-T-cell antibody could be reactivated in vivo and in vitro, and 2) whether such an activation would have any effect on the growth of a tumour. We decided to switch to a C57BL6 murine system to carry out this work. Low levels of the hamster anti-murine CD3 monoclonal antibody 145-2C11 (a hamster equivalent of OKT3[3]), had been shown to prevent malignant tumour progression,[4] and we had frozen stocks of several virulent tumours which grew in C57BL6 mice. As 145-2C11 targets C57BL6 T-cells, we could obtain all the components to examine tumour growth in a syngeneic C57BL6 model system. The 145-2C11 hybridoma was obtained, and the antibody was coated with NPE in a similar manner to that used to inhibit OKT3 and UCHT1.[1] A progressive ovarian tumour, M5076, was selected for study, and the effects of the addition of 145-2C11, NPE-coated 145-2C11, in vitro UV-irradiated NPE–145-2C11, and in vivo UV-irradiated NPE–145-2C11 antibody on the growth of the tumour was then examined.

The 145-2C11 monoclonal antibody was quite susceptible to coating, with less NPE–COCl required to fully inhibit its activity (relative to the anti-human CD3 antibodies[1]). This caused the antibody to be prone to precipitation during dialysis to remove the excess NPE, and the large majority (>95%) spun out on centrifugation at 13000 rpm. When resuspended in isotonic saline, this NPE-coated 145-2C11 antibody could not bind to the CD3-expressing murine lymphoma cell line, EL4. However, after illumination of this NPE-coated antibody with UV light for 10 min in the presence of the EL4 cells, considerable binding of the antibody (70% of that given by uncoated antibody) could be detected (Table 1). This was an extremely important result demonstrating that we could regulate the anti-murine CD3 antibody in a similar manner to the anti-human CD3 antibodies, OKT3 and UCHT1.

**Table 1.** The binding of 145-2C11 and various NPE-coated 145-2C11 conjugates to EL4 cells.

| Conjugate | Fluorescence[a] |
|---|---|
| None | 12 |
| 145-2C11 (stock) | 59 |
| 145-2C11[b] | 56 |
| NPE–145-2C11 | 13 |
| NPE–145-2C11 + UV | 42 |

[a] Values given are the mean fluorescence of the single flow cytometry peak. [b] Control after going through the entire coating procedure, but with no NPE added.

Initial toxicology studies involved groups of six mice that were injected subcutaneously with 145-2C11 or NPE-coated 145-2C11 at two concentrations (30 and 5 μg per mouse). There were no observable deleterious short- or long-term effects on the mice. NPE–145-2C11 irradiated in vitro was then injected (at 30 $\mu g\,mL^{-1}$ only) to examine if the photolysed NPE products had any toxic effects. A second group was also injected with NPE-coated 145-2C11 and irradiated in vivo for 5 min through a shaved area of skin. (This allowed the possible formation of short-lived highly toxic products on irradiation). Again, no detrimental toxic side effects were observed in any animals over a 3-week time span. These studies demonstrated that neither NPE groups nor their photolysed products were cytotoxic in vivo, even at a sixfold excess over the dose of 5 μg per mouse that we had selected to use in our experiments to study tumour growth (4 μg per mouse had been used by Ellenhorn et al.[4]).

The M5076 ovarian tumour was grown in C57BL6 mice, finely diced, and 50 μL of the resulting tumour suspension was passaged into groups of six mice along with 50 μL of medium containing 5 μg of various 145-2C11 conjugates. After approximately 3 weeks the mice were humanely killed, and the final tumour weights were measured. The results obtained from two separate experiments are given in Table 2. In the first experiment the control tumours had an average weight of 200 mg in the mice that only received media with the transplanted diced tumour. When control uncoated 145-2C11 was injected with the diced tumour, final tumour weights markedly decreased to around 20 mg. This was an interesting finding in itself, as it demonstrated that if an anti-CD3 antibody was present in the area next to a tumour, it would stimulate the immune response, even though it was not being specifically targeted to the tumour by a bispecific antibody. When the

[a] *Dr. S. Thompson, R. Stewart, J. A. Smith, Prof. Dr. C. H. Self*
*Diagnostic and Therapeutic Technologies*
*School of Clinical and Laboratory Sciences*
*University of Newcastle upon Tyne, The Medical School*
*Framlington Place, Newcastle upon Tyne NE2 4HH (UK)*
*Fax: (+44) 191-222-6227 (C.H.S.)*
*E-mail: stephen.thompson@ncl.ac.uk*
*c.h.self@ncl.ac.uk*

  

**Table 2.** M5076 tumour weights obtained in mice treated with various 145-2C11 conjugates.[a]

| Medium Control | | Uncoated Ab Control | | NPE + UV in vitro | | NPE, no UV | | NPE + UV in vivo | |
|---|---|---|---|---|---|---|---|---|---|
| wt [g] | Avg. wt [mg] | wt [g] | Avg. wt [mg] | wt [g] | Avg. wt [mg] | wt [g] | Avg. wt [mg] | wt [g] | Avg. wt [mg] |
| | | | | Experiment 1 | | | | | |
| 0.06 | | nd | | 0.05 | | 0.08 | | 0.07 | |
| 0.27 | | < 0.01 | | 0.12 | | 0.19 | | nd | |
| 0.18 | 200 | < 0.01 | 20 | 0.18 | 110 | 0.24 | 160 | nd | 10[b] |
| 0.08 | | 0.02 | | 0.18 | | 0.19 | | nd | |
| 0.40 | | 0.13 | | 0.03 | | 0.13 | | nd | |
| 0.18 | | < 0.01 | | 0.08 | | 0.13 | | nd | |
| | | | | Experiment 2 | | | | | |
| 0.03 | | 0.09 | | 0.14 | | 0.53 | | 0.04 | |
| 0.47 | | 0.12 | | 0.46 | | 0.37 | | 0.03 | |
| 0.46 | 380 | 0.20 | 110 | 0.14 | 270 | 0.25 | 400 | 0.05 | 30 |
| 0.55 | | 0.10 | | 0.29 | | 0.32 | | < 0.01 | |
| 0.31 | | 0.12 | | 0.24 | | 0.56 | | nd | |
| 0.46 | | 0.05 | | 0.34 | | 0.37 | | 0.07 | |

[a] Groups of six mice were simultaneously injected (s.c.) with 50 μL diced M5076 tumour and 50 μL medium containing 145-2C11 conjugates. After approximately 3 weeks the resulting subcutaneous tumours were excised and weighed. Values are given in grams for each animal. Statistical analysis is not required, as the differences in tumour weights are so large in the various groups. Data are given for two separate sets of experiments; nd = not detectable. [b] No trace in five mice.

NPE-coated inactivated antibody was injected with the diced tumour the average weight of the final tumours increased in size almost back to the level of control tumours. If the NPE-coated 145-2C11 was irradiated in vitro for 5 min (in a cuvette) and injected with the transplanted tumour, then the final tumour weight decreased significantly, but not to the levels found with uncoated antibody. The most significant effect was clearly observed when the tumour and NPE-coated 145-2C11 was irradiated in vivo after the two components had been injected. There was a massive decrease in subsequent tumour growth to the extent that no trace of tumour was detectable in five of the six mice after 25 days. This was even more pronounced than the effect of uncoated antibody. This implies that irradiation of the NPE-coated 145-2C11 in situ with the tumour confers some additional potency.

To confirm these results, the experiment was repeated. Tumour growth was more vigorous on this passage of tumour, control tumours, and tumours treated with non-irradiated inactivated 145-2C11, reaching 400 mg in size after only 22 days of growth. Treatment with uncoated antibody again markedly decreased tumour growth to around 25% of control values, whereas in vitro irradiated NPE-coated antibody again reduced final tumour sizes to just over half control values. Despite the more vigorous growth of the tumour in this experiment, very little tumour was again obtained in the animals that were irradiated in vivo to reactivate the 145-2C11.

A third experiment was then carried out, primarily to gauge the effect of UV illumination alone on growth of the tumour. Some control animals were irradiated through a patch of shaved skin in addition to the animals treated with NPE-inactivated 145-2C11 (Table 3). Here the tumours again grew more vigorously to a very similar size (365 mg) obtained in the previous experiment. The irradiated control tumours were no smaller than non-irradiated control tumours, confirming that reductions in tumour growth were caused by the reactivated 145-2C11 antibody. The tumours in the latter group, the transplants of which had been irradiated in the presence of NPE-coated antibody, were again very small. Re-activation of the NPE-coated 145-2C11 antibody in vivo had therefore markedly inhibited the growth of transplanted M5076 tumour in C57BL6 mice on three separate occasions, even though it was not being specifically targeted to the tumour as part of a bispecific conjugate. The antitumour effect could be locally induced at the tumour site, or alternatively, reactivated 145-2C11 and tumour antigens could drain to the lymph nodes and activate T-cells there. Irrespective of the mechanisms involved in the reduction of tumour growth, these results demonstrate that enough light can penetrate the skin to reactivate antibodies in vivo, the main purpose of this study.

**Table 3.** M5076 tumour weights obtained in control mice relative to mice treated with NPE-coated 145-2C11.[a]

| Medium Control no UV | | Medium Control UV in vivo | | NPE-coated 145-2C11 UV in vivo | |
|---|---|---|---|---|---|
| 0.17 | | 0.37 | | < 0.04 | |
| 0.11 | | 0.50 | | < 0.03 | |
| 0.30 | 210 | 0.29 | 365 | < 0.07 | < 55 |
| 0.26 | | 0.30 | | < 0.08 | |

[a] Three groups of four mice were simultaneously injected with 50 μL diced M5076 tumour and 50 μL medium (two groups), or NPE-coated 145-2C11 (one group). One of the control groups and the NPE-coated antibody group were irradiated for 5 min through a shaved patch of skin.

The extent to which the UV light from the handheld lamp penetrates the skin is not known. This could important in determining as to which tumours could be treated by this procedure. Much more powerful light sources are available, but these would often harm tissues.[5] We have been able to reactivate antibodies using the light from a Caulk Nuva-light dental lamp. This transmits light at 365 nm along a 10-cm long, 1-cm diameter optical glass probe.[6] In clinical practice it is more likely that laser- or LED-[7] generated UV light would be delivered down optical fibres to deep-seated tumours. Given this demonstration that untargeted anti-T-cell antibody (albeit co-injected with the tumour transplant) can reduce tumour growth, this suggests a very promising future for the treatment of human cancer with bispecific conjugates[8] and partic-

  

ularly so when T-cell retargeting is involved.[9] Further improvements in patient therapy can be envisaged if light-reactivatable bispecific conjugates were used. If only the anti-T-cell portion of a tumour-targeting bispecific antibody conjugate was inactivated, then the antitumour marker portion of the bispecific antibody would be free to bind tumour. T-cell activity would then be upregulated with UV light only at specific sites where it is required, greatly increasing specificity. Thus the tumour-targeting antibody would not have to be exquisitely specific, as is currently required and in reality, seldom found. T-cell activity would be limited to illuminated areas. Higher doses of conjugate could be administered to allow more conjugate to target the tumour without risking damage to normal tissues targeted by both nonspecific cross-reactions and specific unwanted binding. We are therefore currently synthesising and analysing the effectiveness of photoactivatable bispecific anti-human conjugates.

## Experimental Section

The CD3+ murine cell line EL4 was grown in RPMI 1640 media containing 10% fetal calf serum. The 145-2C11 monoclonal antibody secreting hybridoma was obtained from ECACC. The antibody was obtained from serum-free media by $(NH_4)_2SO_4$ precipitation.

**NPE-coating**: 145-2C11 (0.5 mg mL$^{-1}$) was rendered inactive by the addition of 10 μL of NPE–COCl to 1.5 mL of antibody for 4 h at 20 °C followed by repeated dialysis to remove uncoupled NPE groups.[1,2,10]

**The binding of 145-2C11 antibody to EL4 cells by flow cytometry**: 100-μL aliquots of RPMI 1640 medium containing 200000 EL4 cells were added to individual wells of a 96-well plate. 20 μL of NPE-coated 145-2C11 (0.2 mg mL$^{-1}$) was added to the wells, and the cells were irradiated in the presence of the antibody for 10 min through the lid of the plate. Non-irradiated NPE-coated 145-2C11 and uncoated 145-2C11 were then added to relevant wells, and the cells were left for 1 h at 4 °C before they were washed. After washing, the cells were resuspended in 100 μL of a 1:100 dilution of biotinylated anti-hamster IgG (Vector labs, 10 μL diluted to 1 mL in phosphate buffered saline (PBS), pH 7.4) for 30 min, washed, and 100 μL of a 1:1000 dilution (again in PBS) of streptavidin–FITC (Sigma) was added for 30 min at 4 °C. After three further washes the cells were resuspended in PBS and analysed immediately with flow cytometry. All fluorescent peaks were single peaks with a good normal distribution similar to those shown in reference [1]. The values given in Table 1 are the mean fluorescence values of 5000 events.

**Tumour growth**: C57BL6 mice were purchased at 8 weeks old and were injected with tumour after they had been left for at least one week to acclimatise to their new surroundings. Frozen M5076 tumour pieces were thawed from liquid nitrogen storage. These were diced as finely as possible in 199 medium, and 50 μL of diced tumour was injected subcutaneously into each animal using a fine-gauge needle. After approximately 3 weeks the tumours were excised, and freshly diced tumour (50 μL) was simultaneously injected with 50 μL medium containing 5 μg of 145-2C11 conjugates. Controls contained medium alone. Control tumours excised from each experiment were diced and used for each subsequent experiment to minimise the number of mice required. All relevant local ethical committee and government licenses were obtained to carry out the animal procedures.

For in vivo photolysis a small area on the flank of the mice was shaved using hair clippers, the tumour and antibody were injected under the shaven area, and the shaven area was irradiated for 5 min with a handheld lamp (see below) from a distance of 2–3 cm above the mouse.

**Photolysis of conjugates**: The NPE-coated antibody samples were irradiated with a VL-206BL UVA lamp (2×6 W tubes) which has a total UVA irradiance of approximately 16 mW cm$^{-2}$ at a distance of 1 cm.

## *Acknowledgements*

*We thank Mr. J. Dessi for preparing the 145-2C11 antibody. Flow cytometry was carried out in the Department of Surgery with the kind assistance of Dr. B. K. Shenton. Funds were made available by BioTransformations Ltd. from a DTI SMART Award. C.H.S. is a founder of BioTransformations Ltd.*

**Keywords:** antibodies · caging · photoactivation · T-cells · tumour regression · UV light

[1] C. H. Self, A. C. Self, J. A. Smith, D. J. Self, S. Thompson, *ChemMedChem* **2007**, *2*, 1587 – 1590.
[2] a) C. H. Self, S. Thompson, *Nat. Med.* **1996**, *2*, 817 – 820; b) C. H. Self, S. Thompson, *Lancet* **2006**, *367*, 1038 – 1039.
[3] M. L. Alegre, P. Vandenabeele, M. Depierreux, S. Florquin, M. Deschodt-Lanckman, V. Flamand, M. Moser, O. Leo, J. Urbain, W. Fiers, *J. Immunol.* **1991**, *146*, 1184 – 1191.
[4] J. D. I. Ellenhorn, R. Hirsch, H. Schreiber, J. A. Bluestone, *Science* **1988**, *242*, 569 – 571.
[5] *Dynamic Studies in Biology: Phototriggers, Photoswitches and Caged Biomolecules* (Eds.: M. Goeldner, R. Givens), Wiley-VCH, Weinheim, **2005**.
[6] C. H. Self, M.-C. Fawcett, J. A. Spoors, L. B. Pulman, S. Thompson, *Biochem. Soc. Trans.* **1995**, *23*, 156S.
[7] Y. Bernardinelli, C. Haeberli, J.-Y. Chatton, *Cell Calcium* **2005**, *37*, 565 – 572.
[8] D. Schrama, R. A. Reisfeld, J. C. Becker, *Nat. Rev. Drug Discovery* **2006**, *5*, 147 – 159.
[9] a) P. A. Baeuerle, P. Kufer, R. Lutterbüse, *Curr. Opin. Mol. Ther.* **2003**, *5*, 413 – 419; b) L. G. Lum, P. Davol, *Cancer Chemother. Biol. Response Modif.* **2005**, *22*, 273 – 291.
[10] S. Thompson, M.-C. Fawcett, J. A. Spoors, C. H. Self, *Biochem. Soc. Trans.* **1995**, *23*, 155S.

Received: May 16, 2007
Revised: August 7, 2007

  