The administration of anti-CD3 antibodies, either unmodified or in bispecific formats, has been shown to kill tumors. However, their activity needs to be carefully controlled. We have approached this problem by inhibiting their anti-CD3 activity until it is required. Folated anti-human CD3 antibody bispecific conjugates were therefore synthesised in which the folate portion of the conjugates remained free to bind to folate receptor (FR) expressing cancer cells, whilst their anti-CD3 activity was reversibly inhibited. On irradiation with UV-A light, the T-cell binding activity of the anti-CD3 antibody can be restored only when and where it is required, i.e., adjacent to a tumor. Conjugate bound to FR expressed on normal tissues in other parts of the body remains inactive. This report describes the preclinical in vivo testing of these conjugates in transgenic mice whose T-cells express human CD3 molecules. When the ‘cloaked’ conjugates were reactivated in the region of the primary tumor, both primary tumor growth and liver metastasis were markedly reduced. That the deliberate targeting of T-cell activity locally to the primary tumor also resulted in reduced distant metastatic growth was a key finding. Light-activatable bispecific antibody conjugates similar to those described here offer a means to control T-cell targeting with a much higher degree of specificity to tumors because they minimize potentially dangerous and unwanted side effects in non-illuminated areas. The addition of light-specific targeting to the inherent tumor specific targeting of therapeutic antibody conjugates could result in the development of safer treatments for patients.

Introduction

Seminal work by Ellenhorn et al. demonstrated that the in vivo administration of low levels of anti-CD3 antibodies could not only prevent tumor growth, but also induce immunity against further injections of tumor. The level of anti-CD3 administered was critically important because higher levels of anti-CD3 resulted in immuno-suppression and more rapid tumor growth. This work led to proposals that immune recruitment to specific areas of the body via targeting T-cells to tumors could be a major step forward in tumor therapy.2,3 This process is normally brought about by using antibodies that bind to specific CD antigens on the T-cell surface (usually CD3 and/or CD28), and cause T-cell activation. However, the administration of such anti-CD antibodies can cause very dangerous cytokine storms, as might have occurred in the human volunteers at Northwich Park.6,7 Whilst the approach is clearly very powerful, it requires close control of both the amount and the location of the active antibody.

Bispecific antibodies were developed to minimize these problems and focus T-cell activation to the site of the tumor.2,3 In these bispecific constructs, one binding site of the antibody reacts with a tumor specific antigen (TSA), whilst the other reacts with the T-cell CD antigen.2,3,8-10 This should result in T-cells being targeted directly to the tumor surface. In practice, the constructs contain active anti-T-cell antibodies when they are injected, so they can bind to, and activate, peripheral T-cells in areas of the body that are well away from the tumor. Perhaps more importantly, there are very few, if any, truly specific tumor antigens.11-13 Normal tissues that express low levels of the TSA are also targeted, leading to the possibility of damaging side effects. The problem is not so much the binding selectivity of the antibody, but more the distribution of TSA throughout the body.

If a construct was created in which the potentially toxic anti-CD antibody portion of the bispecific antibody were to be initially inactive, then this would provide a means to circumvent both of these inherent problems. Peripheral T-cells would not be activated, and all tissues which expressed the TSA would remain unharmed until the anti-CD3 (T-cell targeting) activity was restored. We had previously demonstrated a procedure to photo-reversibly deactivate antibodies in such a way that antibody activity could be restored.
by localized irradiation with UV-A light.\textsuperscript{14,15} This procedure was therefore used to construct photo-activatable, folated, anti-human CD3 conjugates in which the anti-CD3 portion was initially inactive.\textsuperscript{16}

The folate receptor (FR) has been suggested as a therapeutic target\textsuperscript{4,17,18} because many human carcinomas are known to express high levels of the receptor,\textsuperscript{18,19} and folic acid is both readily available and easily coupled to toxins and antibodies. Unfortunately, folate receptors are also expressed on a number of healthy tissue types, including the kidney and lung.\textsuperscript{19} Therapeutic antibodies that are directed to their targets by folate will also be directed to these healthy tissues, potentially leading to harmful side effects. These side effects will not occur when the therapeutic anti-CD3 antibody has been rendered reversibly inert prior to its administration.

Two anti-human CD3 antibodies, OKT3 and UCHT1, were first folated to enable them to bind to FR-expressing tumor cells.\textsuperscript{16} After this initial treatment, anti-CD3 T-cell targeting activity was inhibited by a coating of photocleavable 1-(2-nitrophenyl)ethanol groups.\textsuperscript{14} These photo-activatable, folated and NPE coated anti-CD3\textsuperscript{16} conjugates bind to the FR-expressing murine ovarian cell line M5076\textsuperscript{20} in vitro both before and after irradiation, whilst T-cell binding only occurs after irradiation.\textsuperscript{16} In clinical use, the conjugate will be administered and allowed to bind to the target, then local irradiation with UV light will reactivate the anti-T cell antibody only where it is required. The conjugate will then bind and activate killer T-cells, thereby killing the tumor. Two critical components were necessary for the preclinical assessment of the efficacy of this approach: transgenic mice that expressed human CD3 on their T-cells, thus allowing us to employ anti-human CD antibodies; and a tumor that expressed folate receptors on its surface and was capable of growing in the transgenic mice. Toward this end, C57BL6/human CD3\textsuperscript{ε} transgenic mice were obtained.\textsuperscript{21} As the FR-expressing ovarian carcinoma M5076 originated in C57BL6 mice, we could then directly study the effects of the anti-human CD3 targeting conjugates on the growth of this murine tumor in the transgenic mice.

\section*{Results}

\textbf{NPE-OKT3-folate and NPE-UCHT1-folate preparations.} Two anti-human CD3 antibodies UCHT1 and OKT3 were folated to enable them to bind to FR-expressing M5076 cancer cells.\textsuperscript{16} The folated antibodies were then coated with NPE residues to reversibly inactivate their activity.\textsuperscript{15,16} The folation had to be carried out prior to the anti-CD3 activity being inhibited, probably because both NPE and folate bind via the same amine residues on the antibody.\textsuperscript{16} In all conjugates, the folate portion of the conjugate remained free to bind to the FR-expressing cancer cells even after the anti-CD3 activity was rendered inert by the NPE coating.\textsuperscript{16} Indeed, the NPE coating appeared to increase the availability of the folate to bind to M5076 cells rather than decrease it.\textsuperscript{16} On irradiation with UV light, the NPE cleaves and the anti-CD3 activity was restored.

Four NPE-UCHT1-folate conjugates and one NPE-OKT3-folate conjugate were selected for this study. Two conjugates had higher levels of folate substitution, and three had low levels of folate substitution. All five conjugates had minimal levels of anti-CD3 activity. Their in vitro T-cell and M5076 tumor cell binding capabilities are listed in Table 1. The data demonstrates that folation irreversibly decreases the activity of the anti-CD3 antibodies by about 30%. When the folated-conjugates are coated with NPE their anti-CD3 activities reduce to nearly background levels, but after irradiation the conjugates regain 25–30% of their original uncloked binding capacities.\textsuperscript{15,16,22} This degree of reactivation is acceptable given that the most important parameter in the actual application of these conjugates is the degree of inactivation achieved.

\textbf{Tumor growth studies.} Tumor growth when anti-CD3-folate conjugates were added with the diced tumor. We had previously demonstrated that co-injection of 5 μg of an anti-murine CD3 antibody (145-2C11) with tumor pieces could reduce the growth of a M5706 ovarian tumor in wild type C57BL6 mice.\textsuperscript{22} It was not known whether folated human anti-CD3 conjugates would be

\begin{table}[h!]
\centering
\caption{The T-cell binding and M5076 binding characteristics of various folated-anti-human CD3 conjugates as measured by flow cytometry}
\begin{tabular}{|l|l|l|l|}
\hline
Antibody conjugate & Mean T-cell binding & Mean cancer binding \\
\hline
IgG Control & 2.3 & 2.2 & 20 \\
OKT3 & 139 & 153 & 32 \\
OKT3-Folate (5.3 res folate) & 121 & 129 & nd \\
NPE-OKT3-Folate (31 res NPE) & 7.1 & 8.8 & 94 \\
NPE-OKT3-Folate + UV & 77 & 71 & nd \\
IgG Control & 2 & 2.2 & 20 \\
UCHT1 (a) & 134 & 111 & 28 \\
UCHT1-Folate (9 res folate) & 93 & nd & nd \\
NPE-UCHT1-Folate (33 res NPE) & 8 & 6 & 68 \\
NPE-UCHT1-Folate + UV & 35 & 32 & 48 \\
IgG Control & 2 & 2 & 28 \\
UCHT1 (b) & 98 & 163 & 36 \\
UCHT1-Folate (2.4 res folate) & 64 & 110 & 57 \\
NPE-UCHT1-Folate (44 res NPE) & 4.7 & 6.8 & 76 \\
NPE-UCHT1-Folate + UV & 25 & 46 & nd \\
IgG Control & 3 & nd & 4 \\
UCHT1 (c) & 74 & nd & 4.8 \\
UCHT1-Folate (1.4 res folate) & 44 & nd & 6 \\
NPE-UCHT1-Folate (48 res NPE) & 4 & nd & 19 \\
NPE-UCHT1-Folate + UV & 34 & nd & 7 \\
IgG Control & 3 & nd & nd \\
UCHT1 (d) & 84 & nd & nd \\
UCHT1-Folate (2.1 res folate) & 57 & nd & nd \\
NPE-UCHT1-Folate (27 res NPE) & 5 & nd & nd \\
NPE-UCHT1-Folate + UV & 23 & nd & nd \\
\hline
\end{tabular}
\footnotesize{The values given are the mean fluorescence of the single fluorescent peak. UV irradiation of NPE coated conjugates was carried out for 10 min; res, no of residues per antibody molecule; nd, not determined.}
\end{table}
as effective. Two new factors had to be taken into consideration: firstly, that the folation procedure had deactivated approximately 30% of the antibody; and, secondly, that whilst all of the transgenic mice expressed human CD3 on their T-cells, they also expressed murine CD3. Our folated anti-human CD3 conjugates might not, therefore, be expected to be as effective in stimulating a T-cell response.

We carried out an initial experiment in which 5 μg of various folated-UCHT1 conjugates (batch a) were co-injected with transplanted tumor pieces. After four weeks the transgenic animals were killed and their primary subcutaneous tumors excised along with their livers. The weight of the primary tumor was measured, and the weight and appearance of the livers was noted as a measure of metastasis. The results are summarized in Figure 1 (See also Table 2, where the detailed results for each animal are listed). As can be seen from the data, final primary tumor weights in all treated animals were markedly reduced, with the in vitro irradiation group having by far the smallest tumors, even smaller than the tumors found in animals which had received the fully active uncoated folated-UCHT1. This implied an additive effect of the NPE coating, or its hydrolysis products, to the inherent anti-CD3 activity in reducing final tumor growth. The lowest reductions were, as expected, in animals that received NPE inactivated conjugate, although even this treatment had some effect, possibly through the presence of a very small amount of residually active conjugate. There was also an obvious difference in the condition of the livers in the groups treated with both fully active folated-anti-CD3 conjugates, and

Table 2 Results for final tumor growth when tumor pieces were co-injected with various UCHT1-Fol conjugates (batch a)

| Group             | Sex | Body (gm) | Tumor (mg) | Liver (mg) | Liver appearance |
|-------------------|-----|-----------|------------|------------|------------------|
| Control           | F   | 22.8      | 575        | 2200       | Riddled          |
|                   | F   | 25.3      | 200        | 1260       | Normal           |
|                   | F   | 25.1      | 362        | 1520       | Riddled          |
|                   | F+  | 26.6      | 428        | 1700       | Riddled          |
|                   | M   | 34.5      | 320        | 1700       | Riddled          |
|                   | M   | 29.2      | 338        | 1740       | Riddled          |
|                   | M   | 34.1      | >320       | >2000      | Riddled (large nodules) |
| Mean ± SD         |     | 363 ± 115 | 1731 ± 305 |            |                  |
| UCHT1-Fol         | F   | 24.5      | 63         | 1190       | Normal           |
|                   | F   | 24.9      | 171        | 1290       | Normal           |
|                   | F   | 22.9      | 154        | 1210       | Normal           |
|                   | M   | 34.8      | 129        | 1870       | Normal           |
|                   | M   | 33.2      | 220        | 1850       | Normal           |
|                   | M   | 29.7      | 245        | 1750       | Normal           |
| Mean ± SD         |     | 164 ± 65  | 1526 ± 329 |            |                  |
| UCHT1-Fol-NPE     | F   | 23.0      | 159        | 1170       | Normal           |
|                   | F   | 21.6      | 114        | 1130       | Normal           |
|                   | F   | 24.5      | 163        | 2250       | Riddled          |
|                   | M   | 34.6      | 415        | 2750       | Riddled          |
|                   | M   | 30.9      | 82         | 1760       | Normal           |
|                   | M   | 29.0      | 350        | 1760       | Normal           |
| Mean ± SD         |     | 214 ± 136 | 1803 ± 625 |            |                  |
| UCHT1-Fol-NPE     | F   | 22.8      | 124        | 1360       | Normal           |
| + UV in vivo      | F   | 25.3      | 185        | 1420       | Normal           |
|                   | F   | 25.1      | 346        | 1290       | Normal           |
|                   | M   | 34.5      | 72         | 1790       | Normal           |
|                   | M   | 29.2      | 280        | 1900       | Pale             |
|                   | M   | 34.1      | 75         | 2170       | Normal           |
| Mean ± SD         |     | 180 ± 113 | 1655 ± 352 |            |                  |
| UCHT1-Fol-NPE     | F   | 28.4      | 68         | 1420       | Normal           |
| + UV in vitro     | F   | 27.8      | 148        | 1350       | Normal           |
|                   | F   | 25.7      | 72         | 970        | Normal           |
|                   | M   | 32.1      | 44         | 1670       | Normal           |
|                   | M   | 33.1      | 128        | 1830       | Normal           |
|                   | M   | 31.6      | 96         | 1640       | Normal           |
| Mean ± SD         |     | 93 ± 39   | 1480 ± 304 |            |                  |

Individual animal data from each animal experiment. 5 μg conjugate per mouse. 5 min UV irradiation, both in vitro and in vivo. Riddled means that the number of small colonies were too numerous to count. Riddled livers were always very pale pink due to the amount of tumor. Healthy normal livers were a dark red/brown color.
in vitro irradiated NPE-UCHT-folate conjugates. The livers in both of these groups had the firm texture and dark reddish-brown appearance of normal livers, whilst livers from the control group were very soft and light pink in color, and included many obvious small white tumor colonies. This reduction in metastasis to the liver was confirmed by reductions in weights of the livers in these 2 groups. As the tumors in the in vivo irradiated group were not as small as those obtained with the in vitro activated group, this implied that not enough conjugate had been reactivated by 5 minutes in vivo UV irradiation. We therefore decided to both increase the amount of antibody injected to 10 μg instead of 5 μg per mouse, and the in vivo irradiation time to 15 minutes for all further experiments.

This experiment, and previous work, established that we could reduce the growth of the murine ovarian carcinoma when tumor and antibody were co-injected. In clinical applications, the tumor would be established before it was discovered. In all subsequent experiments mice were injected with diced tumor and left for tumors to establish before treatment was commenced. This situation more accurately represents the human experience.

Tumor growth when anti-CD3-folate conjugates were added four days after tumor growth had been initiated. In all further experiments, tumor pieces were transplanted by subcutaneous injection and left to grow for four days to small, established, palpable masses. After this tumor growth stage, 10 μg of each antibody conjugate was injected. In vivo irradiation, through a shaved patch of skin, was then carried out for 15 min. As in vitro irradiation produces a very similar control to the animals receiving uncoated folated-antibody, this group was discontinued to further minimize the number of animals needed.

In a first experiment, OKT3-folate conjugates were administered near to the tumor, under the same bald patch, 4 days after the tumor. These tumors grew much faster than expected, necessitating that the animals be killed after 22, rather than 28 days; however, the results were again promising (Fig. 2, Table 3) with the exception of the group treated with uncoated folated-OKT3 conjugate, which had no effect on this occasion. This surprising result was attributed to the sample of folated-OKT3 having somehow denatured, previous preliminary experiments in which only 5 μg of folated-OKT3 conjugates were co-injected with tumor had markedly reduced tumour growth in a similar fashion to that found with folated-UCHT1 as described above. The in vivo irradiated group had much smaller primary tumors and all their livers appeared normal in color, although two animals had 3–4 large (3 mm diameter) tumor colonies visible on their surface. The control animals had large primary tumors and three animals had livers riddled with tumor colonies. This reduction in liver metastasis was also reflected in the gross liver weights.

A second experiment was therefore performed using a NPE-UCHT1-folate conjugate (batch b). The results are summarized in Figure 3. (Results from individual animals are given in Table 4). As expected, the animals treated with unirradiated NPE-UCHT1-folate had tumors and livers very similar to the control untreated group. However, both primary tumors and liver metastasis were markedly reduced when a second group that had the same conjugated injected was irradiated in vivo for 15 min to reactivate the conjugate. The uncoated, folated-UCHT1 group was, as expected, very effective at reducing primary tumor growth, but did not have as great an effect on reducing liver metastasis as judged by both liver condition and weight. These results were repeated in a further experiment using the same NPE-UCHT1-folate conjugate (Table 5). Here the tumor grew much more vigorously, but both primary tumor growth and liver metastasis were again markedly reduced in the treated animals. Indeed, no metastatic growth was apparent in the liver.

The experiments were repeated using a further two batches (c and d) of NPE-UCHT1-folate to establish whether the above effect was reproducible from batch to batch (Tables 6–8). Primary tumor growth and liver metastatic growth were considerably reduced on all occasions. Data from all of the NPE-UCHT1-folate experiments (Tables 4–8) is summarized in Figure 4. The average value for the size of the primary tumors (29 animals) in untreated animals was 426 ± 46.7 mg (mean ± SE); this decreased to 141 ± 16.6 mg (mean ± SE) when the animals were treated with UCHT1-Fol-NPE conjugates and irradiated for 15 min with
In vivo photoactivation of bispecific antibodies

reported, then this result could be of great importance in human clinical use because the treatment could prevent recurrences of tumors. Of particular relevance is the very recent work of Kabingu et al. who reported that localized photodynamic therapy (PDT) directed to subcutaneous murine tumors reduced the growth of the same tumor at distant sites (the lungs). They attributed this result to the systemic immune response to the tumor being upregulated by the generation of CD8\(^+\) memory T-cells. This was thought to be a consequence of the PDT causing massive inflammation and cytokine release adjacent to the primary tumor. As our approach deliberately targets the immune response directly to the tumor surface, a much more efficient polyclonal T-cell response should be elicited, and it is probable that this upregulation of T-cell activity is involved in the killing of the liver metastatic deposits. This leads to the intriguing possibility that localized irradiation by light of accessible secondary metastatic deposits could be used to treat inaccessible primary tumors and other metastases. In clinical use, anti-CD3 conjugates could be reactivated through the skin as described here, or by irradiating the interior of patient following a de-bulking operation (and an injection of inactive conjugate). A further option would be to deliver the light to deep-seated tumors via light through an optical probe. We have previously demonstrated that 360 nm light delivered through a 10 cm long x 1 cm diameter dental probe efficiently removes NPE residues.

UV light (30 animals). The difference between these means is 285 mg and the SE between the means is 49.5 mg. As the ratio of these two values is 5.75 the probability of these results occurring by chance is much less than \( p < 0.001 \) using normal distribution tables. Similarly the liver weight in untreated animals was 1856 ± 138 mg and treated animals 1389 ± 54 mg (both mean ± SE). Here the ratio of the difference between the means (467 mg) and the standard error between the means (149 mg) is 3.14, the probability of this difference occurring by chance is \( 0.002 > p > 0.001 \) using normal distribution tables.

Discussion

Tumor-bearing, immunocompetent transgenic mice that express human CD3 on their leukocytes were treated with several different batches of NPE-coated, folated-anti-human CD3 antibodies. In all cases where the conjugate was administered four days after the tumor, irradiation of the conjugate in vivo next to the primary tumor reduced both primary tumor growth and metastatic liver growth. It is not yet known if the treatment prevents the tumor from metastasising or inhibits the growth of metastatic deposits. If reduction in metastasis occurs through an immunization effect similar to the protection against further injections of the same or different tumors that has been previously reported, then this result could be of great importance in human clinical use because the treatment could prevent recurrences of tumors. Of particular relevance is the very recent work of Kabingu et al. who reported that localized photodynamic therapy (PDT) directed to subcutaneous murine tumors reduced the growth of the same tumor at distant sites (the lungs). They attributed this result to the systemic immune response to the tumor being upregulated by the generation of CD8\(^+\) memory T-cells. This was thought to be a consequence of the PDT causing massive inflammation and cytokine release adjacent to the primary tumor. As our approach deliberately targets the immune response directly to the tumor surface, a much more efficient polyclonal T-cell response should be elicited, and it is probable that this upregulation of T-cell activity is involved in the killing of the liver metastatic deposits. This leads to the intriguing possibility that localized irradiation by light of accessible secondary metastatic deposits could be used to treat inaccessible primary tumors and other metastases. In clinical use, anti-CD3 conjugates could be reactivated through the skin as described here, or by irradiating the interior of patient following a de-bulking operation (and an injection of inactive conjugate). A further option would be to deliver the light to deep-seated tumors via light through an optical probe. We have previously demonstrated that 360 nm light delivered through a 10 cm long x 1 cm diameter dental probe efficiently removes NPE residues.

Figure 3. Final tumor weights given by animals to whom UCHT1-Fol (batch b) conjugates were administered four days after tumor initiation. Control animals received medium without antibody. UF animals received UCHT1-Folate. NUF animals received NPE-UCHT1-Folate conjugates and half of these animals were irradiated in vivo for 15 min. All mice received 10 μg of antibody conjugate.

Figure 4. A summary of final tumor sizes and liver weights in all of the animals treated with NPE-UCHT-Folate conjugates (batches b, c and d) followed by UV irradiation in vivo for 15 min, as compared to control untreated animals which only received an injection of media.
In vivo photoactivation of bispecific antibodies

We used a bispecific conjugate in which the antibody is folated to allow the conjugate to target cancer tissues that express elevated levels of FR. The conjugates are only active after irradiation, which prevents normal tissues and organs that also express FR from being damaged. Most bispecific therapeutic antibodies are composed of two antibody binding sites. One of the binding sites binds to a tumor antigen and the other to a T-cell. The two different binding sites may both be present in the same antibody molecule or a conjugate of more than one antibody can be made. In either case, the photo-activation technique described here could be used to greatly enhance the specificity of any therapeutic T-cell re-targeting conjugate. Many otherwise valuable antibodies, raised against different types of tumor cells, have been discarded in the past because they had unacceptable levels of both specific and non-specific cross reactions with normal tissue. The possibility of forming a bispecific conjugate in which the anti-CD3 portion is inactive until irradiated might allow these discarded antibodies to be reconsidered, thereby potentially increasing the range of tumors that can be treated. Higher doses of conjugate could also be administered. More conjugate would be available to target the tumor,

### Table 3 Final tumor weights from animals to whom OKT3-Folate conjugates were administered 4 days after tumor initiation

| Group | Sex | Body (gm) | Tumor (mg) | Liver (mg) | Liver appearance |
|-------|-----|-----------|------------|------------|-----------------|
| Control | F | 12.9 | 168 | 1100 | Riddled |
|       | F | 23.8 | 348 | 1420 | Very Pale |
|       | F | 21.0 | 387 | 1110 | Normal |
|       | F | 21.5 | 400 | 1170 | Normal |
|       | M | 31.9 | 126 | 1930 | Normal |
|       | M | 32.8 | 505 | 1880 | Normal |
|       | M | 31.6 | 686 | 2070 | Riddled |
|       | M | 30.7 | 606 | 3160 | Riddled |
| Mean ± SD | | 403 ± 195 | 1730 ± 698 |           |                 |
| OKT3-Fol | F | 24.9 | 356 | 2150 | Riddled |
|         | F | 24.2 | 303 | 1850 | Normal |
|         | F | 24.3 | 464 | 2240 | Riddled |
|         | M | 29.5 | 945 | 2120 | Riddled |
|         | M | 26.9 | 42 | 1690 | Normal |
|         | M | 28.5 | 154 | 1710 | Normal |
|         | M | 26.4 | 336 | 2010 | Riddled |
| Mean ± SD | | 371 ± 289 | 1969 ± 220 |           |                 |
| OKT3-Fol-NPE | F | 25.3 | 276 | 1170 | Normal |
|          | F | 27.9 | 775 | 2830 | Riddled |
|          | F | 25.4 | 215 | 1970 | Normal |
|          | M | 29.7 | 273 | 1820 | Normal |
|          | M | 28.8 | 138 | 2010 | Normal |
|          | M | 30.6 | 133 | 1610 | Normal |
| Mean ± SD | | 302 ± 240 | 1901 ± 549 |           |                 |
| OKT3-Fol-NPE + UV in vivo | F | 24.2 | 92 | 1360 | Normal |
|          | F | 24.9 | 129 | 1200 | Normal |
|          | F | 25.8 | 160 | 1440 | Few large colonies |
|          | M | 28.6 | 136 | 1590 | Normal |
|          | M | 29.7 | 123 | 1710 | Few large colonies |
|          | M | 29.2 | 104 | 1520 | Normal |
| Mean ± SD | | 124 ± 24 | 1470 ± 73 |           |                 |

IP signifies that the tumor had also spread intraperitoneally.

### Table 4 Final tumor weights from male animals to whom UCHT1-Folate (batch b) conjugates were administered 4 days after tumor initiation

| Group | Body (gm) | Tumor (mg) | Liver (mg) | Liver appearance |
|-------|-----------|------------|------------|-----------------|
| Control | 30.3 | 520 | 2110 | Riddled |
|         | 30.7 | 672 | 1840 | Riddled |
|         | 29.7 | 275 | 1950 | Riddled |
|         | 24.3 | 364 | 1440 | Few small nodules |
|         | 29.4 | 170 | 1600 | Riddled |
|         | 27.7 | 380 | 1550 | Riddled |
|         | 29.3 | 510 | 2230 | Riddled |
| Mean ± SD | | 413 ± 169 | 1818 ± 299 |           |                 |
| UCHT1-Folate | 31.1 | 32 | 1680 | 3 nodules good color |
|          | 31.3 | 265 | 1920 | Very pale |
|          | 31.2 | 34 | 1800 | 1 nodule |
|          | 28.4 | 23 | 1540 | Normal |
|          | 29.4 | 112 | 1590 | Normal |
|          | 28.5 | 274 | 2060 | Riddled |
|          | 30.8 | 100 | 1520 | Normal |
| Mean ± SD | | 120 ± 108 | 1730 ± 205 |           |                 |
| UCHT1-Folate-NPE | 25.8 | 420 | 1610 | Normal |
|             | 26.1 | 340 | 1600 | Many nodules |
|             | 25.0 | 139 | 1330 | 5 or 6 nodules |
|             | 28.3 | 280 | 1730 | Some nodules |
|             | 30.6 | 248 | 1860 | Unhealthy, very pale |
|             | 30.8 | 284 | 1980 | Riddled |
| Mean ± SD | | 285 ± 94 | 1685 ± 227 |           |                 |
| UCHT1-Fol-NPE + UV in vivo | 30.7 | 40 | 1600 | Normal |
|             | 26.4 | 41 | 1410 | 3 nodules |
|             | 27.8 | <5 | 1470 | Normal |
|             | 26.9 | 93 | 1420 | 3 nodules |
|             | 27.8 | 175 | 1690 | Normal |
|             | 28.5 | 217 | 1600 | Normal |
|             | 28.9 | 212 | 1570 | 3 nodules |
| Mean ± SD | | 112 ± 89 | 1537 ± 105 |           |                 |

IP signifies that the tumor had also spread intraperitoneally.
In vivo photoactivation of bispecific antibodies

Reactions that can occur, or the distribution of the targeted marker in the body. These advances in antibody technology may, however, be combined with the photo-activation technology described here. It could prove possible to build extremely effective, highly tumor-specific, cancer targeting conjugates in the near future using light-specific targeting in conjunction with the inherent tumor targeting of natural or recombinant antibody conjugates.

Table 5  Final tumor weights from a second group of male animals to whom UCHT1-Folate conjugates (batch b) were administered four days after tumor initiation

| Group               | Body (gm) | Tumor (mg) | Liver (mg) | Liver appearance |
|---------------------|-----------|------------|------------|------------------|
| Control             | 29.5      | 510        | 2730       | Riddled         |
|                     | 31.1      | 1380       | 3130       | Riddled         |
|                     | 31.5      | 890        | 3890       | Riddled         |
|                     | 28.0      | 385        | 3620       | Riddled         |
| Mean ± SD           | 3791 ± 450| 3342 ± 515 |            |                 |

UCHT1-Fol-NPE + UV in vivo

| Group               | Body (gm) | Tumor (mg) | Liver (mg) | Liver appearance |
|---------------------|-----------|------------|------------|------------------|
|                     | 31.0      | 258        | 1330       | Normal           |
|                     | 26.8      | 42         | 1270       | Normal           |
|                     | 29.2      | 155        | 1510       | Normal           |
| Mean ± SD           | 142 ± 90  | 1380 ± 104 |            |                 |

Table 6  Final tumor weights from female animals to whom UCHT1-Folate conjugates (batch c) were administered four days after tumor initiation

| Group               | Body (gm) | Tumor (mg) | Liver (mg) | Liver appearance |
|---------------------|-----------|------------|------------|------------------|
| Control             | 27.0      | 115        | 1410       | Normal           |
|                     | 30.3      | 120        | 1820       | Slight mottle    |
|                     | 32.1      | 260        | 1870       | Normal           |
|                     | 34.3      | 230        | 1930       | Slight mottle    |
| Mean ± SD           | 162 ± 84  | 1676 ± 218 |            |                 |

UCHT1-Fol-NPE + UV in vivo

| Group               | Body (gm) | Tumor (mg) | Liver (mg) | Liver appearance |
|---------------------|-----------|------------|------------|------------------|
|                     | 24.8      | 140        | 1330       | Normal           |
|                     | 26.0      | 180        | 1340       | Normal           |
|                     | 26.1      | 220        | 1320       | Normal           |
|                     | 25.8      | 420        | 1730       | Riddled          |
|                     | 24.7      | 90         | 1240       | Normal           |
| Mean ± SD           | 210 ± 127 | 1392 ± 193 |            |                 |

Table 7  Final tumor weights from male animals to whom UCHT1-Folate conjugates (batch d) were administered four days after tumor initiation

| Group               | Body (gm) | Tumor (mg) | Liver (mg) | Liver appearance |
|---------------------|-----------|------------|------------|------------------|
| Control             | 36.5      | 250        | 1850       | Bad pale color   |
|                     | 35.1      | 570        | 1680       | Bad color, mottled |
|                     | 33.0      | 340        | 2320       | Riddled          |
|                     | 37.2      | 330        | 1460       | Motiled          |
| Mean ± SD           | 394 ± 129 | 1874 ± 333 |            |                 |

UCHT1-Fol-NPE + UV in vivo

| Group               | Body (gm) | Tumor (mg) | Liver (mg) | Liver appearance |
|---------------------|-----------|------------|------------|------------------|
|                     | 25.5      | 210        | 900        | Pale             |
|                     | 22.7      | 260        | 800        | Pale             |
|                     | 25.5      | 110        | 1250       | Normal           |
|                     | 25.7      | 210        | 1200       | Normal           |
|                     | 24.8      | 150        | 1200       | Normal           |
|                     | 28.7      | 180        | 1310       | Normal           |
| Mean ± SD           | 216 ± 91  | 1164 ± 238 |            |                 |

UCHT1-Fol-NPE UV in vivo

| Group               | Body (gm) | Tumor (mg) | Liver (mg) | Liver appearance |
|---------------------|-----------|------------|------------|------------------|
|                     | 21.4      | 80         | 870        | Normal           |
|                     | 21.2      | 120        | 840        | Normal           |
|                     | 25.4      | 110        | 1330       | Pale             |
|                     | 22.0      | 70         | 910        | Normal           |
|                     | 26.7      | 70         | 1400       | Normal           |
|                     | 26.1      | 220        | 1210       | Normal           |
|                     | 20.9      | 50         | 680        | Normal           |
|                     | 24.1      | 80         | 1130       | Normal           |
| Mean ± SD           | 100 ± 53  | 1040 ± 258 |            |                 |

while normal tissues similarly targeted because they express lower levels of the same TSA would remain unaffected.

Given that the task of identifying truly tumor-specific markers has proved to be challenging, research has concentrated on alternative recombinant strategies to produce tumor-targeting Fv antibodies from phage display libraries8-10,27,28 with higher binding affinities. Antibodies have also been humanized to prevent first-generation, rodent-derived therapeutic antibodies from being scavenged by a patient’s immune defences.29 Unfortunately, neither of these approaches affects the levels of non-specific cross reactions that can occur, or the distribution of the targeted marker in the body. These advances in antibody technology may, however, be combined with the photo-activation technology described here. It could prove possible to build extremely effective, highly tumor-specific, cancer targeting conjugates in the near future using light-specific targeting in conjunction with the inherent tumor targeting of natural or recombinant antibody conjugates.
We also propose that use of the immunocompetent C57BL6 transgenic mouse line, in which the mice express human CD3 on their T-cells, is also a major advance on the models currently available to study bispecific anti-CD3 retargeting antibodies. In a recent article, Schlereth et al.9 grew human Ep-CAM transfected murine tumors in C57BL6 mice, and treated the mice with anti-human Ep-CAM/murine CD3 bispecific antibodies (BiTE or bispecific T-cell engager). They demonstrated that both primary subcutaneous growth and disseminated lung growth could be potently inhibited by repeated injections with small amounts of this BiTE without an obvious need for co-stimulation of T cells by secondary agents. They also discussed the concept that use of immunocompetent mice was far superior to use of NOD/SCID mice with human tumors and human T-cells. They described the use of immunocompetent mice as the ‘next important step’ in preclinical studies because tissue distribution of the BiTE could be better examined, and side effects related to tumor lysis and polyclonal T-cell activation could be predicted. However, they had to use murine CD3 antibodies in their conjugates. If the transgenic mice described in this study are utilized, then human CD3 bispecific therapeutic constructs could be directly studied prior to their use in clinical trials.

Materials and Methods

Antibodies and cell lines. The CD3+ T-cell line H9 was obtained from ECACC. The UCHT1 secreting hybridoma (IgG2a subclass) was obtained from Cancer Research UK. OKT3 was purchased from Ortho Biotech. The FR-expressing murine ovarian cell line M5076,20 was kindly supplied by Dr. GA Turner.

Coupling of folate residues to the UCHT1 and OKT3 anti-human CD3 antibodies. Folic acid (5 mg/ml) was converted to its NHS ester by the addition of N-hydroxsuccinimide and dicyclohexylcarbodiimide (DCC) in dimethylformamide as previously described.16 30 μl aliquots of this solution were added per ml to UCHT1 or OKT3 (1.0 mg/ml) dissolved in 0.1 M sodium bicarbonate. These reaction conditions reproducibly coupled around two folate residues per antibody molecule with a 75% antibody yield.16

Coating of the folated-UCHT1 with 2-nitrobenzyl groups. Folated-UCHT1 and folated-OKT3 (0.75 mg/ml) dissolved in 0.1 M sodium bicarbonate were coated with NPE by the addition of 10 μl and 20 μl (per ml) NPE-chloroformate, respectively. After an o/n incubation, dialysis and centrifugation, the final yield of NPE coated folated antibodies was approximately 0.2 mg/ml with around 40 NPE residues per antibody molecule.

T-cell binding assay. The human T-cell line, H9, was grown in RPMI-1640 media. 10 μl aliquots of the diluted antibody conjugates (all diluted to 0.05 mg/ml) were added to 250 μl aliquots of a H9 cell suspension (10⁶ cells/ml) and were left to bind for 30 min at 4°C. After washing the cells were resuspended in 200 μl of goat anti mouse FITC antibody (BD Pharmingen, 5 μl/ml in PBS) for a further 30 min. After further washing, the cells were resuspended in 500 μl PBS and their fluorescence (5,000 gated cells) was measured using a Becton Dickinson flow cytometer.

Cancer cell binding assay. This assay was carried out under identical conditions to those used in the T-cell binding assay above, except in these assays, the FR-expressing mouse cancer cell line M5076 was used as the target cell. The M5076 cells were maintained as a suspension in folate restricted media to upregulate their FR expression.16

 Provision of C57BL6/humanCD3+ transgenic mice. The mice transfected with the human CD3 gene originated at the Beth Israel Deaconess Hospital (BIDH), Harvard21 as an AJ8/C57BL6 colony, but had since been repeatedly back crossed with wild type C57BL6 to a completely C57BL6 phenotype by Novartis. These mice were kindly provided by Novartis with the permission of BIDH.

(1) Double positive stock. In order to carry out our efficacy studies, we required a mouse line whose T-cells could bind our anti-human T-cell conjugates. Six pairs of double positive homozygous C57BL6/humanCD3+ transgenic mice were obtained, and these were first inbred to establish a larger stock colony. Keeping the mice as a double positive colony negated the need for expensive phenotyping and waste of mice that a heterozygous colony would incur. However, these mice have very few T-cells because they carry two copies of the human T-cell gene. This also means that the stock colony should be maintained in an isolator; otherwise they are very susceptible to helicobacter infections.

(2) Heterozygous mice. For experimental work, normal wild type C57BL6 female mice were purchased and bred with our double positive males to generate heterozygote +/- mice with one copy of the human CD3 gene and one copy of the mouse gene. These mice had normal numbers of T-cells which all expressed many copies of the human CD3 marker.21

Tumor growth. C57BL6 mice were purchased at eight weeks old, and were injected with tumor after they had been left for at least one week to acclimatise to their new surroundings. Frozen M5076 tumor pieces were thawed from liquid nitrogen storage. These were diced as finely as possible in 199 medium and 50 μl of diced tumor was injected subcutaneously into each animal using a fine gauge needle. After 3–4 weeks, the tumors were excised and freshly diced tumor (50 μl) was injected into the transgenic mice. This procedure was found to give more reproducible final primary tumor growth than the injection of M5076 cells straight from tissue culture. In a preliminary experiment, the tumor was simultaneously injected with 50 μl of medium containing a UCHT1-folate conjugate (batch a). In all other experiments the tumor was injected and allowed to establish for four days before being treated with the conjugates. Controls had only medium injected and were irradiated for the same length of time as the in vivo treated group. Control tumors excised from each experiment were diced, and used to inject all groups in the next experiment to minimise the numbers of mice required. All relevant local ethical committee and government licences were obtained to carry out the animal procedures.

In order to enable in vivo photolysis a small area (15–20 mm diameter) on the flank of the mice was shaved using hair clipsers. The tumor and antibody were injected under this shaved area, either together or four days apart, and the shaved area was
irradiated for 5–15 min with a hand held lamp (see below) from a distance of 2–3 cm above the mouse. Irradiation was carried out approximately 15 minutes after injection of the conjugates. The mice were held under light anaesthesia during the irradiation to ensure that all the animals received the same reproducible dose of UV light. This also allowed the numbers of animals used in each group to be kept to a minimum.

**Photolysis of conjugates.** Antibody samples were irradiated in vitro in a quartz cuvette for 10 min with a VL-206BL UV-A hand held lamp (2 x 6 W tubes) that had a total UV-A irradiance of approximately 16 mW/cm² at a working distance of 1 cm.

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**References**

1. Ellenhoorn JDJ, Hirsch R, Schreiber H, Bluestone JA. In vivo administration of anti-CD3 prevents malignant progresor tumor growth. Science 1988; 242:569-71.
2. Baeuerle PA, Kufer P, Lutterbuse R. Bispecific antibodies for polyclonal T-cell engagement. Curr Opin Mol Therap 2003; 5:413-9.
3. Lum LG, Davol PA. Retargeting T cells and immune effector cells with bispecific antibodies. Cancer Chemother and Biological response modifiers 2005; 22:273-91.
4. Roy EJ, Gawlick U, Orr BA, Kranz DM. Folate mediated targeting of T-cells to tumors. Adv Drug Delivery Rev 2004; 56:1219-31.
5. Wilthner S, Helfrich W, de Leij LF, Molema G. Bispecific antibody therapy for the treatment of cancer. Curr Opin Mol Therap 2001; 3:53-62.
6. Hopkin M. Can super-antibody drugs be tamed? Nature 2006; 440:855-6.
7. Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, Hopkin M. Can super-antibody drugs be tamed? Nature 2006; 440:855-6.
8. Schlereth B, Fichtner I, Lorenczewski G, Kleindienst P, Brischwein K, da Silva A, et al. Eradication of tumors from a human colon cancer cell line and from ovarian cancer metastases in immunodeficient mice by a single-chain Ep-CAM/CD3-bispecific antibody construct. Cancer Res 2005; 65:2882-9.
9. Schlereth B, Fichtner I, Lorenczewski G, Kleindienst P, Brischwein K, da Silva A, et al. Eradication of tumors from a human colon cancer cell line and from ovarian cancer metastases in immunodeficient mice by a single-chain Ep-CAM/CD3-bispecific antibody construct. Cancer Res 2005; 65:2882-9.
10. Buhler P, Wolf P, Gierschner D, Schaber I, Katzenwadel A, Schultze-Seemann W, et al. A bispecific diabody directed against prostate-specific membrane antigen and CD3 induces T-cell mediated lysis of prostate cancer cells. Cancer Immunol Immunother 2008; 57:43-52.
11. Christiansen J, Rajasekaran AK. Biological impediments to monoclonal antibody-based cancer immunotherapy. Mol Cancer Ther 2004; 5:1403-501.
12. Nobis L, Buchegger F, Guerri R, Allemann E. Bispecific antibodies for the treatment of prostate cancer: strategies to design and validate bispecific antibodies. Mol Cancer Ther 2006; 6:429-51.
13. Self CH, Thompson S. How specific are monoclonal antibodies? Lancet 2006; 367:1038-9.
14. Self CH, Thompson S. Light activatable antibodies: Models for remotely activatable proteins. Nature Medicine 1996; 2:817-20.
15. Self CH, Self AC, Smith JA, Self DJ, Thompson S. Light directed activation of human CD3ε transgenic mice. Transplantation 2002; 73:1658-66.
16. Thompson S, Steward R, Smith JA, Self CH. Light activation of anti-CD3 in vivo reduces the growth of an aggressive ovarian carcinoma. Cancer Chemother Biochem 2004; 2:1591-3.
17. Porter LE, Nelson H, Ethem GL, Rice DG, Thibault C, Chapoval A. T cell activation and retargeting using staphyloccocal enterotoxin B and bispecific antibody: an effective in vivo antitumor strategy. Cancer Immunol Immunother 1997; 45:180-3.
18. Rau P, Lindhofer H. Induction of a long-lasting antitumor immunity by a bifunctional bispecific antibody. Blood 2001; 98:2526-34.
19. Kabinga E, Vaughan L, Owczarzak B, Ramsey KD, Gollnick SO. CD8+ T-cell-mediated control of distant tumors following local photodynamic therapy is independent of CD8+ T cells and dependent on natural killer cells. Br J Cancer 2007; 96:1839-48.
20. Self CH, Fawcett M-C, Spoors JA, Palman LB, Thompson S. Studies on photoactivatable nitrobenzyl-bovine serum albumin conjugates. Biochem Soc Trans 1995; 23:156.
21. Winter G, Harris WJ. Humanised antibodies. Immunol Today 1993; 14:243-6.