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Attempted Targeting of a Monoclonal Antibody in a Human Tumour Xenograft System

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Abstract—*The selective concentration of an internally labelled monoclonal anti-HLA antibody in a human tumour xenograft growing in a mouse has been attempted. Label was found to concentrate in the liver rather than the tumour at times from one to thirty two hours after intravenous injection of labelled antibody. Evidence is presented to suggest that the presence of circulating antigen may account for the failure of the antibody to concentrate in the tumour.*

INTRODUCTION

THE CONCEPT of attaching cell killing agents to tumour specific antibodies has been considered to have great therapeutic appeal [1]. A number of cell killing agents have been suggested for attachment to such antibodies. These include radioisotopes [2], diptheria toxin [3] and cytotoxic drugs [4–6].

One difficulty with this approach is that of unwanted antibody activity in the antiserum which has been prepared against the tumour, because tumour associated antigens only represent a proportion of the total number of potential antigenic determinants on the cell surface. Thus xenogeneic antisera prepared against tumour cells contain a large number of antibodies directed at antigens common to both the tumour cells and normal cells. Absorption of such unwanted antibodies may present problems [7]. The production of monoclonal antibodies from hybrid myelomas is a means of producing single pure antibodies against minor cell surface components and so provides an answer to the problem of the complexity of the antibody response evoked by a xenogeneic immunisation [8, 9]. The application of this approach to the specific targeting of killing agents may, therefore, overcome these difficulties.

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A necessary preliminary requirement before attempting to concentrate a cell killing agent selectively in a tumour by means of a carrier antibody is that the antibody itself will selectively concentrate in the tumour. A system in which a human xenograft is growing in a mouse provides a convenient model for testing this. A labelled mouse anti-human monoclonal antibody injected intravenously might be expected to concentrate selectively in the human tumour. In this paper we have investigated the distribution of W6/32, an anti-HLA monoclonal antibody[10] in a mouse bearing the HT29R human colonic adenocarcinoma.

MATERIALS AND METHODS

Monoclonal antibodies

Internally labelled monoclonal antibodies were prepared as follows: about 10^7 hybridoma cells from the appropriate exponentially growing culture were centrifuged, resuspended in Dulbecco's Modified Eagle's Medium without L-Lysine (−Lys DMM) and pelletted by centrifugation. These were then resuspended in 5 ml of incorporation medium (9 ml of −Lys DMM, 1 ml of L(4,5-^{3}H)Lysine monohydrochloride 5 mCi/5 ml, 0.5 ml of dialysed foetal calf serum) and incubated in a 37°C water saturated 10% CO_2 incubator. After 8 hr 10^7 cells prepared as above were added to the culture. Radioactive supernatant was collected after 16 hr and dialysed 3 times against 2 l. of phosphate-buffered saline before use. The particular antibodies

used were internally labelled W6/32HL anti-HLA (^{3}HLys W6/32) [10], the W3/15.2.8.g mouse anti-rat lymphocyte antibody (^{3}HLys W3/15) [8] and the NA1/34.HLK mouse anti-human T-cell antibody (^{3}HLysNA1/34).

Tumours

(1) HT29R human colonic adenocarcinoma, a once recloned variant of HT29, a gift from Dr. Jørgen Fogh [11]. This tumour has been shown to grow as a locally invasive, moderately poorly-differentiated mucin secreting adenocarcinoma in immuno-suppressed mice [12].

(2) EMT6/VJ/AC subline of the EMT6 mouse tumour described originally by Rockwell *et al.* [13, 14].

(3) MOLT4, a human T-cell lymphoma line capable of growth as a xenograft in a small percentage of immuno-suppressed mice.

Immuno-suppression of mice

Mice were immuno-suppressed by modification of the method of Kopper and Steel [15] as previously described [12]. Briefly, male CBA mice were thymectomised at four weeks of age and two weeks later subjected to whole body irradiation to a dose of 920 rads from a 60 Cobalt unit, followed by reconstitution with 3×10^5 syngeneic bone marrow cells. Tumours were initiated by subcutaneous injection of single cell suspensions of 10^6 cells the day after total body irradiation.

In-vivo *localisation of* ^{3}HLys W6/32 *in tumour bearing mice*

In order to determine where the labelled monoclonal anti-HLA antibody localised after intravenous injection, groups of four tumour bearing recipients each received 200 μl of supernatant containing labelled W6/32 antibody. This was injected intravenously into the tail vein of mice bearing tumours of between 100 and 200 mm^3.

At varying time periods after antibody injection the recipient mice were sacrificed and tumours, liver and kidneys were excised, weighed and digested in 4 ml of solium hydroxide (1.0 M). The total volume of each sample was adjusted to 10 ml with distilled water. A 400 μl aliquot from each digest was added to 3.6 ml of aquasol (New England Nuclear NEF 952). The samples were kept overnight in the dark at 4°C and the activity then counted in a liquid scintillation counter (Nuclear Chicago Model 724).

A correction for some of the quenching of scintillation counts by tissues was made for liver and tumour. Four uninjected animals from the same group as those which had received intravenous internally labelled monoclonal antibodies were sacrificed and livers and tumours subjected to sodium hydroxide digestion in identical manner to experimental mice. Samples of previously counted ^{3}HLys W6/32 from 5000 cpm to 40,000 count/min were added to these digests and the quenching of counts by the respective tissues measured.

In-vitro *blocking assay*

The serum of animals bearing tumours (TBMS) was tested for its ability to inhibit the direct binding of labelled monoclonal anti-HLA antibody to HT29R cells *in vitro*. 55 μl aliquots of HT29R TBMS at varying dilutions were incubated with 55 μl of ^{3}HLys W6/32 for 30 min at 4°C. Freshly trypsinised HT29R cells were washed three times by sequential centrifugation at 600 ***g*** and resuspension in PBS + heat inactivated FCS.

The cell pellet from the third wash was then gently resuspended in 100 μl of the preincubated mixture of ^{3}HLys W6/32 and TBMS and incubated at 4°C for 30 min. The cells were then washed a further three times, resuspended in 200 μl of distilled water and transferred to 1.8 ml of aquasol. Samples were counted for one minute each in a liquid scintillation counter. Control non-tumour bearing mouse sera (NTBMS) or EMT6 tumour bearing mouse serum (EMT6 TBMS) were treated identically to HT29R TBMS. The blocking activity was expressed as:–

$$\frac{\text{cpm with NTBMS}}{\text{cpm with TBMS}} \times 100$$

In-vitro *binding assay*

The serum of tumour bearing mice was examined for antibody directed against HT29R. After incubating HT29R cells with serum, rabbit anti-mouse immunoglobulin labelled with ^{125}Iodine (^{125}I) was used to detect any mouse antibody which had bound to the HT29R cells.

Freshly trypsinised *in-vitro* HT29R cells were washed three times by centrifugation at 600 ***g*** for five min at 4°C and resuspension of the cell pellet was carried out in Earle's balanced salt solution supplemented with HEPES (10 mM), bovine serum albumen (8% w/v) and sodium azide (0.1% w/v). All cell suspensions and reagents were kept on ice throughout the experiments. Serial dilutions of heat inactivated mouse sera were made using the same medium. The HT29R cells were then counted and adjusted to a concentration of 5×10^6 ml^{-1}.

100 μl of this cell suspension was introduced into a number of LP3 Luckham tubes spun at 600 **g** for five min at 4°C and the supernatant carefully removed from the cell pellet. 100 μl aliquots of serial dilutions of TBMS and non-tumour bearing mouse serum (NTBMS) were added in duplicate to the cell pellets which were gently resuspended in these sera. After incubation for 30 min at 4°C the HT29R cells were washed three times by centrifugation and resuspension as described above, and the cell pellets resuspended in 100 μl of ^{125}Iodine labelled rabbit anti-mouse IgG FAB_2 (^{125}I-RAMIG$(FAB)_2$). They were then incubated for 30 min at 4°C followed by three washes in fresh medium. The cell pellet was finally resuspended in 1 ml of medium and transferred to fresh LP3 tubes for counting on a gamma counter.

RESULTS

In-vivo *localisation of labelled antibody*

After *in-vivo* injection of labelled antibody into HT29R tumour bearing mice, ^{3}HLys W6/32 monoclonal anti-HLA antibody did not selectively concentrate in the tumour as compared to host mouse liver or kidney. It can be seen in Fig. 1 that from 30 min to 8 hr after intravenous injection the counts per minute were greater in liver and kidney than in HT29R. The greater number of counts in liver as compared to tumour was only observed when the labelled anti-HLa antibody was injected into a mouse bearing the human HT29R tumour. Figure 2 shows that at 8 hr after intravenous injections the greater number of counts in liver as compared to tumour was not observed when ^{3}HLys W6/32 was injected into animals bearing the EMT6 mouse tumour. When as a further control the tritium labelled monoclonal mouse anti-rat antibody (^{3}HLys W3/15) was injected into mice bearing HT29R tumours, the higher level of label in liver as compared to tumour was also not apparent in the counts. Time points taken at 16 and 32 hr did not show any later selective concentration of ^{3}HLys W6/32 in *in vivo* tumours. At eight hours after injection approximately 5–10% of the injected label could be detected as counts in sodium hydroxide digests of liver. The activity detected in HT29R tumours at 8 hr was between 2–4%.

Serum blocking of labelled antibody

Because labelled W6/32 did not appear to concentrate selectively in HT29R tumours *in vivo*, the serum of HT29R tumour bearing mice was examined for possible blocking activity. ^{3}HLys W6/32 antibody was preincubated with tumour bearing mouse serum prior to incubating with HT29R target cells. This resulted in a marked fall in the number of counts binding to HT29R *in-vitro* cells (Fig. 3). Serial dilution of HT29R TBMS progressively weakened the blocking activity which had almost disappeared at dilutions of 1:64. EMT6 TBMS produced only slight blocking of ^{3}HLys W6/32 binding, as did serum from non-tumour bearing immuno-suppressed CBA mice.

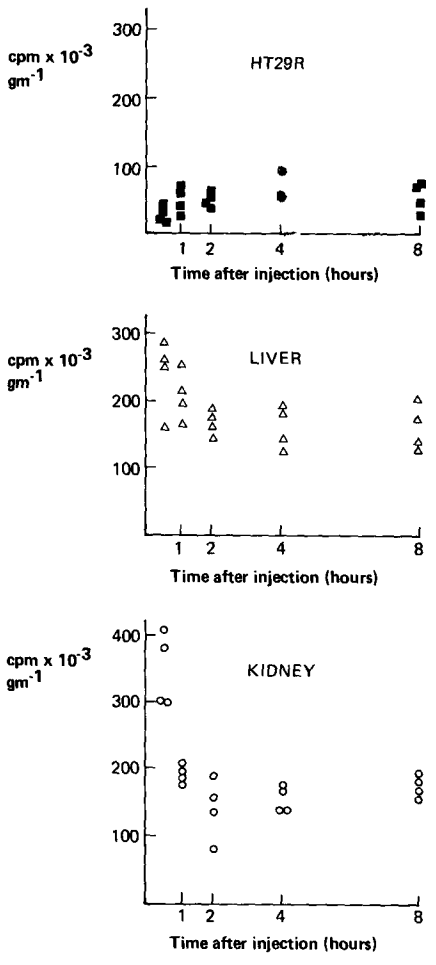

Fig. 1. The fate of ^{3}HLys W6/32 monoclonal antibody after its injection into HT29R tumour bearing mice. (4 mice each bearing one tumour were used for each time point. Counts are expressed per g of tissue.)

It was considered possible that the blocking of ^{3}HLys W6/32 binding to HT29R cells *in vitro* might be due to components in HT29R TBMS competing for the same binding sites on the cell.

For this reason an experiment was performed in which tumour bearing mouse serum was preincubated with HT29R cells which were then washed thoroughly and used as targets for ^{3}HLys W6/32. Preincubation of HT29R cells and tumour bearing mouse serum did not produce any blocking of ^{3}HLys W6/32 binding (Table 1). As a further check that serum block-

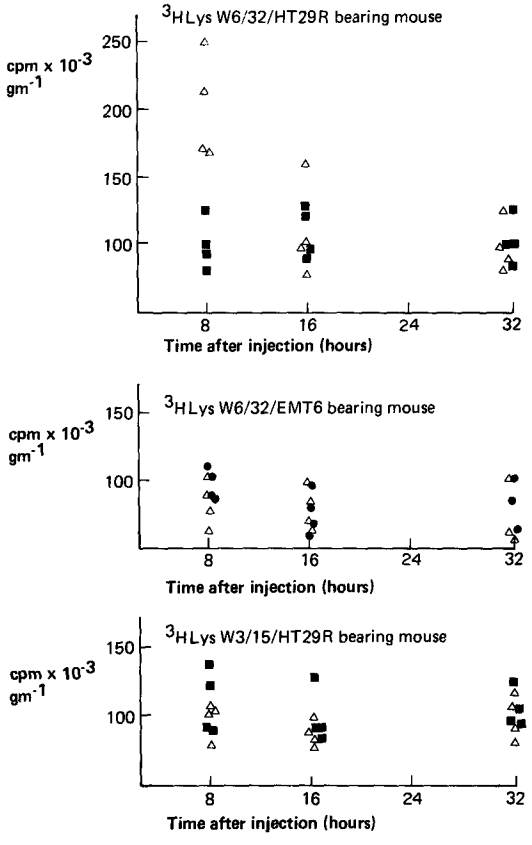

Fig. 2. The fate of ^{3}HLys W6/32 monoclonal antibody after injection into HT29R vs EMT6 tumour bearing mice and the fate of ^{3}HLys W3/15 monoclonal antibody after injection into HT29R tumour bearing mice. (4 mice bearing one tumour each were used for each time point. Counts are expressed per g of tissue). △, counts in liver; ■, counts in HT29R tumour; and ●, counts in EMT6 tumour.

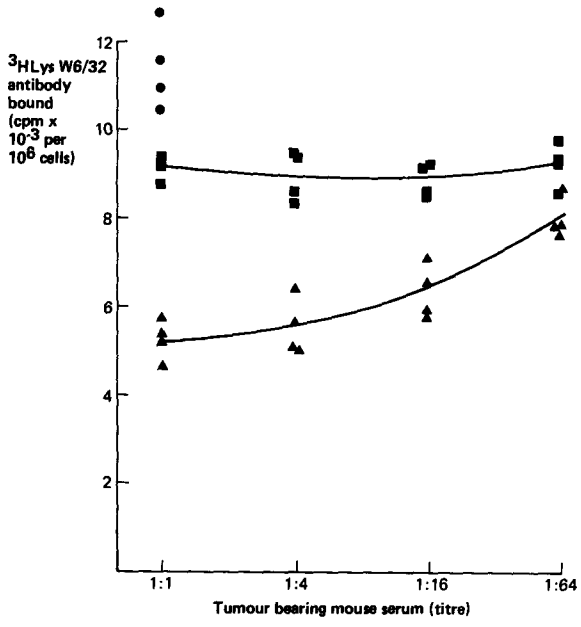

Fig. 3. The blocking of in-vitro *binding of ^{3}HLys W6/32 monoclonal antibody to HT29R* in-vitro *cells by tumour bearing mouse serum (TBMS). (Each point represents one serum sample from one mouse bearing one tumour. The labelled monoclonal antibody was pre-incubated with relevant serum for* 30 min *at* 40°C *prior to testing binding to HT29R).* ●, *^{3}HLys W6/32* 50 μl + *medium* 50 μl. ■, *^{3}HLys W6/32* 50 μl + *EMT* 6 *TBMS* 50 μl. ▲, *^{3}HLys W6/32* 50 μl + *HT29R TBMS* 50 μl.

ing activity was not the result of mouse antibody competing for the same antigenic sites on HT20R as W6/32, the tumour bearing mouse serum was sequentially absorbed three times against an equal volume of HT29R *in-vitro* packed cells. The duration of each absorption at 4°C was one hour. Table 2 shows that following absorption the binding activity of HT29R TBMS fell to the range for NTBMS. Absorption of this binding activity, however, did not reduce the blocking activity of HT29R TBMS on the direct binding of ^{3}HLys W6/32 to *in-vitro* HT29R cells.

Finally it was of interest to determine whether the serum blocking activity noted against W6/32 could also be demonstrated against other monoclonal antibodies. For this experiment the serum blocking of labelled W6/32 binding to the MOLT4 human T lymphoma line was compared to the serum blocking of another labelled monoclonal antibody (NAl/34) which recognised a different antigenic site on the same cell line.

Table 1. Lack of blocking of in-vitro *binding of ^{3}HLys W6/32 monoclonal antibody to HT29R* in-vitro *cells by pre-incubation of cells with HT29R TBMS*

	^{3}H CPM per 10^6 cells	
	Gross	Mean
Preincubation of HT29R with TBMS	58667 50077 52801 47928	52368
Preincubation of HT29R with NTBMS	50615 47402 52905 54854	51444
Preincubation of ^{3}HLys W6/32 with TBMS	9565 8436	9000

HT29R TBMS, HT29R Tumour Bearing Mouse Serum; NTBMS, Non Tumour Bearing Mouse Serum.

The MOLT4 human T cell line did not grow well as a xenograft. 10^7 cells were injected subcutaneously into 15 immuno-suppressed mice and four weeks later, only three of these bore tumours with volumes in excess of 100 mm^3. Sera from these three mice were pooled and

Table 2. The blocking of in-vitro *binding of* ^{3}*HLys W6/32 monoclonal antibody to HT29R* in-vitro *cells by HT29R TBMS before and after absorption against HT29R*

	Blocking assay ^{3}H Count/min 10^{6} cells		Binding assay ^{125}I count/min 10^{6} cells	
	Gross	Mean	Gross	Mean
HT29R TBMS Before absorption	6762	6957	2864	2695
	6584		2261	
	7280		2978	
	7201		2677	
HT29R TBMS After absorption	4660	4566	316	462
	4855		711	
	4332		523	
	4418		298	
NTBMS	36826	39537	305	490
	41295		544	
	38415		688	
	41613		423	

HT29R TBMS, HT29R Tumour Bearing Mouse Serum; NTBMS, Non Tumour Bearing Mouse Serum.

Table 3. Comparison of blocking of binding of ^{3}*HLys W6/32 and* ^{3}*HLys NA1/34 to MOLT4* in vitro *cells*

	^{3}H counts/min 10^{6} cells	
	Gross	Mean
W6/32 + NTBMS	8343	8324
	7536	
	7622	
	9795	
W6/32 + MOLT4 TBMS	6307	5350
	6112	
	4737	
	4244	
NAl/34 + MOLT4 TBMS	4179	4026
	4009	
	4083	
	3833	
NA 1/34 + NTBMS	3642	3934
	3211	
	4394	
	4485	

MOLT4 TBMS, Pooled Sera from 3 MOLT4 Tumour Bearing Mice; NTBMS, Non Tumour Bearing Mice.

examined for their ability to block the binding of ^{3}HLys W6/32 and ^{3}HLys NA1/34 to MOLT cells *in vitro*. Table 3 shows that serum from MOLT4 tumour bearing mice blocked W6/32 binding to *in-vitro* MOLT4 cells but did not block NA1/34 binding.

DISCUSSION

A number of problems may be encountered whilst attempting to investigate the feasibility of targeting labelled antibodies *in vivo*. Internally labelled monoclonal antibodies produced by the use of tritiated lysine in lysine-free tissue culture provide one possible test system.

The W6/32 preparation used in these experiments showed an 8–10 fold greater binding to HT29R *in-vitro* cells than to CBA mouse lymph node cells. It has also been demonstrated to selectively stain the human tumour component of frozen sections of HT29R xenografts [16]. However, in this paper we have found no evidence of selective concentration of the antibody in an HT29R tumour in the intact mouse. Between 30 min and 8 hr counts were higher in the liver and kidneys than in the tumour.

The serum of HT29R tumour bearing mice was found to inhibit the *in-vitro* binding of ^{3}HLys W6/32 to HT29R cells. Although it has been shown that the immuno-suppressed mice used in these experiments are capable of producing an antibody response against HT29R *in vivo* two pieces of evidence suggest that the blocking activity detected in these experiments is due to circulating antigen rather than to antibody competing for the same antigenic sites on HT29R as W6/32. Firstly, absorption of tumour bearing mouse serum with HT29R cells did not diminish the blocking activity and secondly blocking was not demonstrated when HT29R cells were incubated with HT29R tumour bearing mouse serum before testing binding of ^{3}HLys W6/32. The presence of shed antigen from *in-vivo* HT29R tumour cells may prevent effective targeting of specific antibody such as W6/32 *in-vivo*. Antibody and antigen may interact proximal to the tumour in the circulation and the high concentration of W6/32 in the liver and kidney of HT29R tumour bearing animals may reflect removal of labelled antigen antibody complexes.

The presence of specific antigen in the serum of animals bearing tumours induced by chemical carcinogenesis [17, 18] and antigen shedding by a human tumour growing *in-vitro*, or as a heterotransplant in nude mice [19], have been described. Evidence has been provided in animal systems that serum blocking activity of cell-mediated immunity may be due to antigen–antibody complexes [20]. How much antigen is in the form of antigen–antibody complexes in the serum of HT29R tumour bearing mice is uncertain. We have, however, subsequently confirmed that these immuno-suppressed animals are capable of producing antibodies

and can be used to produce tumour specific monoclonal antibodies to HT29R (data to be published). An assay of the blocking activity of the serum before and after precipitating immuno-complexes could provide further information on this point.

The attempts to target the ^{3}HLys W6/32 antibody *in vivo* to HT29R tumours were disappointing. However, the experiment with the MOLT4 tumour suggests that blocking activity in tumour bearing mouse serum may not occur in the case of all antibodies directed against the cell surface. The serum of MOLT4 tumour bearing mice did not show blocking activity against the NAl/34 monoclonal antibody, but did block W6/32 binding. This could be due to preferential shedding of cell surface antigens. Koch [21] has shown that the microvilli shed from the cell surface of an *in-vitro* mastocytoma are very rich in the major transplantation antigens (H2). If shedding of membrane fragments preferentially releases HLA, then W6/32 but not NAl/34 would be blocked by reacting with large amounts of circulating antigens. It is also possible that the antigen recognised by NAl/34 is unstable when no longer part of the cell surface. Unfortunately, the low take rate of MOLT4 did not provide sufficient animals to test whether ^{3}HLys NAl/34 would selectively target in this tumour as compared with ^{3}HLys W6/32.

Thus, although the shedding of antigen by tumours may interfere with the targeting of antibodies directed towards these antigens on the cell surface *in vivo*, it is possible that antibodies which are not blocked by antigen in the serum may be found. The results of this paper do not provide a good targeting monoclonal antibody model, but they establish a simple way in which a variety of new monoclonal antibodies can be tested before targeting experiments can be meaningfully performed.

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