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Immunorecognition of Ganglioside Epitopes: Correlation between Affinity and Cytotoxicity of Ganglioside Antibodies

Wolfgang Dippold and Helga Bernhard

Cell-surface gangliosides have immunomodulatory effects that are presumed to play a role in tumour growth, progression, metastasis and therapy. To study the epitopes of gangliosides on human malignant melanomas and to search for monoclonal antibodies (Mabs) with superior immunological effector functions, 19 ganglioside antibodies were established. Specificity and affinity of nine antibodies of IgG3 isotype were evaluated by enzyme linked immunosorbent assay and thin layer chromatography with a panel of purified gangliosides. All antibodies recognised the ganglioside GD3, but their epitope specificity divided them into five groups. Their affinity constants for ganglioside GD3 ranged from 4.7×10^6 to 2.3×10^8, with 2×10^7 for Mab R-24. Two antibodies possessed a higher affinity for GD2 than for GD3. The functional properties of the antibodies were investigated *in vitro*. Differences in the degree of tumour lysis by complement fixation correlated with the affinity constants. Every ganglioside antibody differed in epitope recognition, affinity and cytotoxicity. Therefore some of these antibodies might even be more useful in the immunotherapy of malignant melanoma than Mab R-24.
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INTRODUCTION

GANGLIOSIDES, sialic acid-containing glycosphingolipids, are greatly increased in the membranes of neuroectodermal tumours. Gangliosides like GM2, GD2 and GD3 in particular have been associated to a high degree with tumour cells [1]. Every aspect of the study concerning gangliosides as surface antigens on tumour cells, their tissue distribution, biochemical characterisation and functional properties *in vitro* and *in vivo* has been greatly facilitated by Mabs directed to gangliosides [2–5]. First of all, mouse monoclonal ganglioside antibodies were found by chance after immunisation with whole tumour cells [6–8]. Then gangliosides were identified as important target molecules in the immune system in patients with malignant melanoma. Therefore, great efforts were made to generate additional ganglioside antibodies. However, purified gangliosides are known to be poorly immunogenic [9]. Possible explanations may be the immunosuppressive properties of gangliosides, their low molecular weight and their possible role as self-antigens [10]. To enhance immunogenicity various carrier molecules such as methylated bovine albumin, bacteria, liposomes and aminobeads were used. But even under these conditions monoclonal ganglioside antibodies were rarely obtained. In the present study, we have described a method of raising ganglioside antibodies of predefined specificity. Nine different GD3-ganglioside antibodies of IgG3 subclass were established and examined according to epitope specificity, affinity and functional properties.

MATERIALS AND METHODS

Cell lines

The following cell lines were used for immunisation, extraction and functional tests: SK-Mel-19, SK-Mel-28 and SK-Mel-31 melanoma cells. SK-Mel-19, -28, -29, -31, SK-RC-6 were obtained from Dr L. Old (Sloan-Kettering Memorial Cancer Center, New York). The ganglioside profile of these melanoma cell lines differs from each other, in particular concerning the expression of GD2 and GD3 [11]. B-16 mouse melanoma cell line (no detection of GD3) derived from C57BL/6 mice was kindly provided by Dr Burger (Basel Institute of Immunology). The liver cell carcinoma (MZ-Hep-1) and the gastric carcinoma (MZ-Sto-1) were established in our laboratory.

Generation of ganglioside antibodies

Three different immunisation schedules and fusions were performed. For each schedule a different melanoma cell line was used (SK-Mel-28, -19, -31). The gangliosides were presented as immunogen in the following way: 1×10^8 melanoma cells were mixed with the ganglioside extract from 1×10^8 melanoma cells of the same cell line and incubated for 1 h at room temperature. NZB-mice were immunised with this mixture twice weekly intraperitonally and in addition intravenously the last time. Freund's adjuvant (0.1 ml) was added for the first immunisation only. Mice were immunised three times with SK-Mel 28 cells or SK-Mel 31 cells and ganglioside extract before spleen cells were fused. In contrast, seven immunisations were carried out with SK-Mel 19 cells. Monoclonal antibodies were generated as described [6]. Culture supernatants of hybridomas were screened initially on ganglioside extract of the same melanoma which was used for immunisation. First supernatants were tested by enzyme linked immunosorbant assay and immunostaining (see below).

Positive clones were then tested on a panel of cultured human tumour cell lines consisting of five melanomas (SK-Mel-19, -28, -29, -31, B-16-Mel), 1 renal cell carcinoma (SK-RC-6), 1 pancreas carcinoma (Capan-1), 1 colon cancer (HT-29), 1 gastric cancer (MZ-Sto-1) and 1 liver cell carcinoma (Mz-Hep-1). Only antibodies which reacted with the melanoma cells were cloned and the isotypes were determined. Supernatants of hybridomas

Correspondence to W. Dippold.
W. Dippold and H. Bernhard are at the Medizinische Klinik, Johannes Gutenberg-Universität, D-6500 Mainz, Langenbeckstraße 1, Germany.
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were collected and centrifuged at 45000 rpm for 20 min to remove particulate material. Purification of Mab was carried out with protein A affinity chromatography. IgG3 Mab was eluted with 0.1 mol/l sodium citrate, pH 4–6. The purified IgG3 were dialysed extensively in PBS at 4°C and pH 7.4, concentrated using dextran, filtered through a 0.22-μm millipore filter and frozen at −70°C. Purity of the antibodies was tested by SDS/polyacrylamide gel electrophoresis and protein staining (Biorad). Antibody concentration was determined by colorimetric methods using a protein standard.

Isolation, thin layer chromatography and immunostaining of gangliosides

Gangliosides were isolated from cell pellets by a modification of Saito and Hakomori as described [11]. Purified gangliosides from bovine brain were obtained from Biocarb Chemicals (Lund, Sweden). The chemical structure of these gangliosides has been verified for purity by NMR spectrometry. Immunostaining of gangliosides was performed as previously described [11]. Briefly, gangliosides (1 μg/lane) were chromatographed on thin-layer plates and 100 μl/cm^2 of Mab R-24 at a concentration of 50 μg/ml were applied. Peroxidase-labelled rabbit anti-mouse serum (1:40; Dakopatts, Copenhagen, Denmark) was used as second antibody, 4-Chloronaphthol (Sigma) served as substrate for the enzyme reaction.

Ganglioside ELISA

Ganglioside mixture or purified gangliosides were dissolved in ethanol and adsorbed to microfluor plates (Dynatech) by exsiccation. In order to reduce non-specific binding, plates were rehydrated with 200 μl phosphate buffered saline (PBS) plus 5% bovine serum albumin (BSA) overnight; then 50 μl of antibody were incubated for 1 h at 4°C. Following washes of 30 min each with PBS plus 0.1% BSA, 100 μl of β-galactosidase-labelled F(ab)'$_2$ rabbit anti-mouse antibody (Zymed), appropriately diluted (1:500) in RPMI, plus 5% human serum albumin were incubated for 45 min; 200 μl 4-methylumbelliferyl-β-D-galactoside (1 mg dissolved in 60 ml PBS, pH 6,9, Sigma) were added to the washed plates and incubated for another 30 min at 37°C. Afterwards the samples were evaluated in a Dynatec microfluor reader.

Determination of affinity constants

Affinity constants were determined by ELISA, described above. Two methods were used. First, titration of antibodies (0.1–100 μg/ml) with a constant concentration of purified gangliosides (200 μ/ml) as antigen and second, titration of gangliosides (2–800 ng/ml) in the presence of a constant concentration of antibody (20 μg/ml). Calculation of affinity constant (K) was done using the following formula: K = (AbAg)/(Ab)(Ag) as described in Ref. [12]. AbAg stands for the molar concentration of the antibody–antigen complex, Ab for the molar concentration of the unoccupied antibody binding sites, and Ag for the molar concentration of the unoccupied antigen binding sites. The affinity constant K was calculated using the concentration of ganglioside or ganglioside antibody at the turning point, where the antibody–antigen complex was found at equilibrium. The molar concentrations were calculated assuming molecular weight of 180000 for IgG3 and 1500 for ganglioside GD2 or GD3. This indirect method was applied, because direct labelling of IgG3 ganglioside antibodies has proven to be impossible as experienced by collaborating laboratories and by ourselves. In general, all antibodies loose their binding properties to the ganglioside antigens.

Cell reactivity and complement-mediated cytotoxicity

Cells were cultured in microtest-plates (Nunc) for 3 days, Mabs added and stained by indirect immunoperoxidase as previously described [3]. FACS analysis of peripheral blood lymphocytes was performed according to standard procedures. Fluorescent, rabbit anti-mouse Mab were purchased from Coulter and diluted 1:100. Reactivity of cells was determined in a Coulter sorter. Cytotoxicity-assays were performed by a modification of Vogel and Cheresh [13, 14], using 10^4 ^{51}Cr-labelled target cells. Fifty μl human serum as a source of complement were added at concentrations pretested for lowest background cytolysis. Generally a dilution 1:4 was sufficient. After 4 h cytoxicity was calculated as follows: A−B/C−B×100; A:cpm in supernatant of tested samples; B: cpm in supernatant of target cells and medium; C: cpm in Nonidet NP40- treated targets for maximum ^{51}Cr-release.

RESULTS

A variety of ganglioside antibodies was established using melanoma cells and ganglioside extract as an immunogen. Ganglioside antibodies were screened by ELISA and also by immunostaining using melanoma gangliosides as the antigen source. Hybrids found to be positive by immunostaining were cloned. The various immunoglobulin classes obtained depended on the immunisation frequency. By immunising mice three times with SK-Mel 28 melanoma cells, the seven hybridomas selected produced ganglioside antibodies of the IgM subclass. With SK-Mel 31 melanoma cells as immunogen three ganglioside antibodies of the IgM subclass and one of the IgG3 subclass (O-17) were obtained. In contrast, all eight hybridomas established after seven immunisations with SK-Mel 19 melanoma cells produced antibodies of the IgG3 isotype (Z-21, A-4, Q-4, N-4, P-19, U-5, E-11, L-12). All the IgG3 producing hybrids were selected and the antibodies were comprehensively characterised. The GD3 antibody R-24 (IgG3) was used for comparison.

The epitope specificity of the nine Mabs was determined according to their reactivity with nine purified gangliosides from bovine brain (Fig. 1). Five groups were defined. The antibodies in the first group (Mab A-4 and Z-21) showed the same pattern as Mab R-24 reacting only with GD3 and less with GQ1b. Disialogangliosides GD2, GD1a and GD1b were not detected and none of the mono- or trisialogangliosides was recognised. Therefore binding appears to be restricted to the outer disialyl structure. In contrast to the antibodies belonging to the first group, Mab Q-4 (group II) recognises not only GD3 and GQ1b, but also GT1b, GD1b and GD2 indicating that this antibody detects all gangliosides having two connected sialic acids. Mab O-17, representing group III, also recognises two connected sialic acids also, but does not react with GT1b. Antibodies which are classified as group IV (U-5, E-11, L-12) react like Q-4 except that they do not detect GD2. Mabs N-4 and P-19 form group V, which resembles group I (R-24) in epitope specificity. However, these antibodies recognise GD1b in addition to GD3 and GQ1b. The ganglioside pattern of the antibodies described was confirmed by ELISA. Figure 2 demonstrates this for Mabs Z-21 (group I) and Mab Q-4 (group II). The strength of binding varies remarkably in the different reacting ganglisodes. In addition to purified bovine gangliosides, extracted gangliosides from melanoma cells were tested. All antibodies reacted equally well with GD3 derived from melanoma and bovine brain demonstrating that the different ceramid residues have no effect on the binding site.

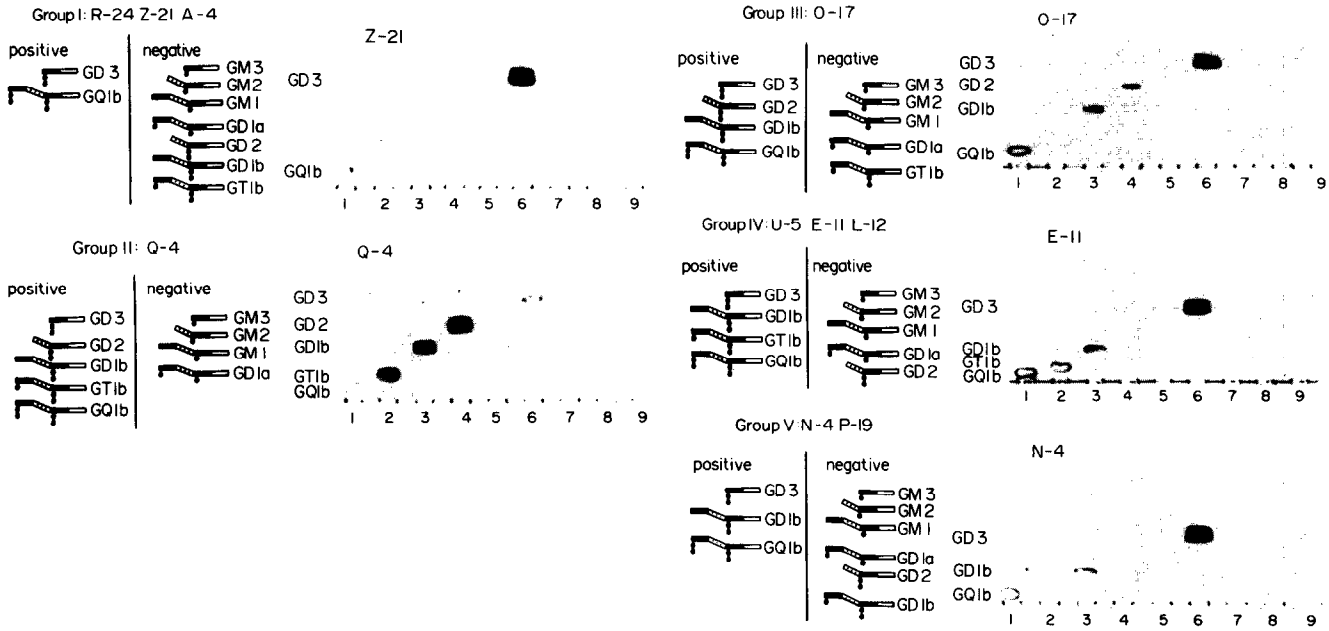

Fig. 1. Determination of epitope specificity of ganglioside antibodies tested by ELISA and thin layer chromatography. 1:GQ1b, 2:GT1b, 3:GD1b, 4:GD2, 5:GD1a, 6:GD3, 7:GM1, 8:GM2, 9:GM3;1 μg/lane. Schematic representation of the ganglioside structures. ▭ Glucose, ▬ Galactose, ▭ N-acetylgalactosamine, ♦ Neuraminic acid.

Affinity constants for all antibodies were determined using purified GD3 as antigen. Moreover, for Mabs Q-4 and O-17 detecting GD3 and GD2 affinity constants for both gangliosides were investigated. Constants calculated from the titration curves ranged from 4.7×10^6 to 2.3×10^8 mol/l^{-1} (Table 1). The results were independently obtained by both described ELISA methods. In comparison with Mab R-24 showing an affinity constant of 2.0×10^7 mol/l^{-1}, several antibodies possess a higher affinity to GD3. Mab Z-21 achieves the highest affinity constant with 2.3×10^8 mol/l^{-1}. This demonstrates that antibodies classified into the same group in terms of ganglioside epitope specificity have different affinities to GD3. Two Mabs (Q-4 and O-17) show a higher affinity to GD2 than to GD3.

Purified antibodies were tested on three melanoma cell lines with known ganglioside pattern, in particular concerning the gangliosides GD2 and GD3. Cell line SK-Mel 19 contains high amounts of GD2 and GD3. In SK-Mel 28 cells GD3 is highly but GD2 lowly expressed. In contrast SK-Mel 31 has low levels of GD3, but high levels of GD2. Positive reactivities demonstrate the ganglioside specificity of the antibodies (Table 2). All antibodies react with the three melanoma cell lines. Besides antigen specificity the titration end point of the antibodies reflects their affinity. For instance, Mab Z-21 reacts with SK-Mel 28 cells at a concentration lower than 1 μg/ml, whereas antibodies with lower affinity constants for GD3 (Q-4, O-17, A-4) reach their titration end point early (25–100 μg/ml). In contrast Mab Q-4 having a 5-fold higher affinity to GD2 than to GD3 was 100 times more reactive with SK-Mel 31 than with SK-Mel 28 melanoma cells.

All ganglioside antibodies were effective in complement-mediated lysis of melanoma target cells (Table 3). The proportion of lysed cells varied with regard to specificity and affinity

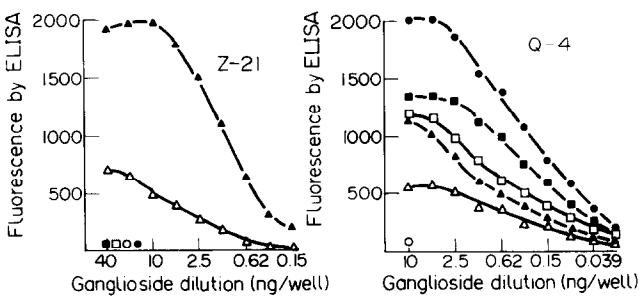

Fig. 2. Binding of ganglioside antibodies Z-21 and Q-4 tested by ELISA. ▲ GD3, △ GQ1b, ● GD2, ■ GD1b, □ GT1b, ○ GD1a, GM1,GM2,GM3.

Table 1. Affinity constants of ganglioside antibodies determined by ELISA

Antibody	Affinity constant (l/mol) GD3*	GD2*
Group I		
R-24†	2.0×10^7	
Z-21	2.3×10^8	
A-4	1.6×10^7	
Group II		
Q-4	2.7×10^7	1.3×10^8
Group III		
O-17	4.7×10^6	3.8×10^7
Group IV		
U-5	1.6×10^7	
E-11	1.0×10^7	
L-12	1.4×10^8	
Group V		
N-4	1.8×10^8	
P-19	2.0×10^8	

*Purified GD2 and GD3 from bovine brain, †purified ganglioside antibodies.

Table 2. Reactivity of ganglioside antibodies with in vitro *cultured melanoma cell lines*

Antibody	Mab - Concentration (μg/ml) End – point of positive reaction SK-Mel-28*	-19†	-31‡
Group I			
R - 24	3	3	25
Z - 21	< 1	<1	25
A - 4	25	25	100
Group II			
Q - 4	100	1	1
Group III			
O - 17	25	6	6
Group IV			
U - 5	3	3	70
E - 11	12	12	80
L - 12	< 1	<1	55
Group V			
N - 4	< 1	< 1	25
P - 19	< 1	< 1	10

*SK-Mel-28: GD3 level high, GD2 low,
†SK-Mel-19: GD2 and GD3 level high,
‡SK-Mel-31: GD2 level high, GD3 low.

of the ganglioside antibodies. For example Mab Z-21 with high specificity and affinity to GD3 mediated a high rate of complement dependent lysis on SK-Mel 28 cells. This antibody was effective up to a dilution of 1 μg/ml in comparison to Mabs A-4 or Q-4, where 32 μg/ml or 100 μg/ml were needed to obtain similar efficacy (Fig. 3). In contrast only minimal lysis was observed with 40 μg/ml Mab Z-21 on SK-Mel 31 cells, which contain only low levels of GD3 and no lysis was observed with lower affinity GD3 antibodies (R-24, A-4, U-5,E-11). On the

Table 3. Complement mediated cytotoxicity of ganglioside antibodies

Antibody	Mab - Concentration (μg/ml) End – point at 20% cytotoxicity SK-Mel-28	-19	-31
Group I			
R - 24	10	25	—
Z - 21	1	5	40
A - 4	32	25	—
Group II			
Q - 4	100	6	3
Group III			
O - 17	75	9	6
Group IV			
U - 5	9	52	—
E - 11	21	48	—
L - 12	2	3	25
Group V			
N - 4	< 1	2	37
P - 19	1	2	50

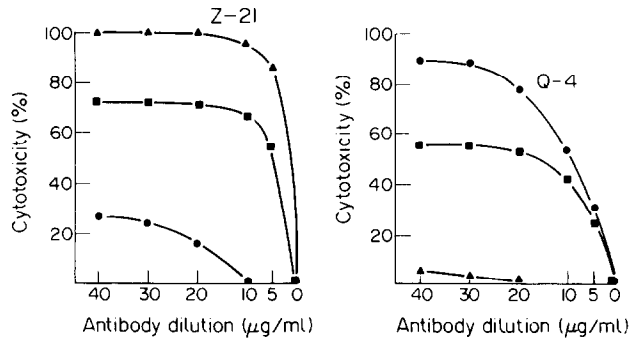

Fig. 3. Cytotoxicity of human melanoma cells by ganglioside antibodies in the presence of human complement (serum dilution 1:5). ▲ SK-Mel-28: GD3 level high, GD2 low; ■ SK-Mel-19: GD2 and GD3; ● Sk-Mel-31: GD2 level high, GD3 low.

other hand, Mab Q-4 with high affinity for GD2 lysed the highly GD2 expressing SK-Mel 31 cells up to low antibody concentrations (3 μg/ml) efficiently. Reactivity of all described ganglioside antibodies with peripheral blood lymphocytes (PBL) of 10 healthy donors was tested. Monoclonal antibodies O-17 and U-5 showed the highest reactivity with the different PBL. They stained 20–25% cells as determined by FACS analysis. Most of the other antibodies (R-24, A-4, L-12, N-4, P-19) reacted like Z-21 with 10% of the PBL only, Mab Q-4 and E-11 were negative.

DISCUSSION

Ganglioside antibodies with predefined specificity can be produced by immunisation with a combination of melanoma cells and ganglioside extracts. Hybridomas were generated in three separate experiments after immunisation with different melanoma cell lines each with a distinct distribution of gangliosides. All three fusions between spleen and myeloma cells resulted in a panel of ganglioside antibodies indicating that this approach is reliable in order to obtain ganglioside antibodies. Immunisation with a melanoma cell line containing a known ganglioside pattern induced mainly antibodies reacting primarily with the gangliosides present on the cells. Possibly the fatty acyl chain composition of melanoma gangliosides as compared to normal human brain gangliosides yields the enhancement of immunogenicity. Repeated immunisations induced more frequently antibodies of IgG3 isotype whereas fewer immunisations resulted in IgM antibodies. It appears remarkable that apart from IgG3 no other IgG isotype could be obtained. These findings agree with previous observations, showing that virtually all mouse IgG anticarbohydrate antibodies are of IgG3 isotype [15]. Epitope analysis of the monoclonal antibodies produced was performed using nine different gangliosides and melanoma cell extracts. According to their binding specificity the ganglioside antibodies are classified in five groups. The antibodies of the first group, R-24, A-4 and Z-21 react with the trisaccharide structure NeuAcα2-8NeuAcα2-3Gal, which must be in a terminal position. This reactivity has already been defined for Mab R-24 [16] and other antiganglioside antibodies [17]. In contast to these antibodies the epitope of Mab Q-4 comprises NeuAcα2-8NeuAc2-3Gal, in which the disialo-structure can be in a terminal position or within the carbohydrate chain. Mab O-17 also recognises two connected sialic acids, but does not react with GT1b indicating that the outer sialic acid probably affects the binding sites.Antibodies which are classified as belonging to

group IV or V recognise only gangliosides, which contain two connected sialic acid residues. However, the pattern of reactivity varies. Mabs from group IV (U-5, E-11, L-12) are very similar to Q-4. They recognise GD3, GD1b, GT1b and GQ1b, but not GD2, indicating that the binding specificity is affected by the adjacent free N-acetylgalactosamine. All antibodies recognise GD3 extracted from bovine brain as well as from melanoma cells, demonstrating that the ceramide moiety is not part of the determinant recognised by these antibodies.

With regard to the application of ganglioside antibodies for diagnosis and therapy in tumour patients, their epitope specificity and affinity are important factors as are variations in the amount of the respective ganglioside expressed by the tumour cells. It was found that reactivity to cultured malignant melanoma cells and the degree of complement-mediated cytotoxicity depended on the cell surface expression of gangliosides and antibody affinity. Application of two ganglioside antibodies with different specificity did not result in enhanced complement mediated lysis (data not shown). The extent of lysis was rather determined by the antibody which had the highest affinity. This correlated with the competence for complement mediated lysis. These observations are in agreement with results from Thurin *et al.* and Mujoo *et al.* [18, 19], who found that the cytotoxicity of ganglioside antibodies correlates with the degree of antibody binding to the tumour cells. The affinity constants of the isolated Mabs range from 4.7×10^6 to 2.3×10^8 l/mol. In some instances this difference may be due to the presence of light chain variants, as recently shown for Mab R-24 [20]. However, Mab Z-21 still shows a higher affinity than the highest affinity Mab R-24 preparations (data not shown). These data suggest that each ganglioside Mab has its own individual characteristics in terms of binding specificity, affinity and functional properties. In view of these findings, the simple term "GD3 ganglioside antibody" appears no longer appropriate [6, 14]. In this regard it will be very interesting and important to see how these different GD3 ganglioside antibodies differ in their immunocytochemical staining of human tissue specimen. This may be of practical relevance, because several clinical studies have revealed that intravenous or intralesional application of ganglioside antibodies induce inflammation and regression of tumours in a variable percentage of patients [21–23].

In order to compare the different clinical trials and above all to understand how the antibodies work and in which way the clinical success can be improved in the future, a detailed analysis of monoclonal ganglioside antibodies appears imperative. Furthermore, in view of the previous finding that Mab R-24 can induce lymphocyte proliferation *in vitro* [4, 24] and *in vivo* [23], the stimulation of immunocompetent cells by the different ganglioside antibodies should be analysed. Therefore, we have tested the binding behaviour of these antibodies to peripheral blood lymphocytes. There were striking differences concerning the percentage of cells stained by FACS analysis. A complementary approach to increase the efficacy of antibody treatment to enhance the ganglioside expression on human melanoma cells. Recently Gross *et al.* [25] reported that IFN gamma increases the surface expression of GD2 on human neuroblastoma cells *in vitro*. Cytokines, such as interleukin 2 or interferon alfa, may also upregulate effector mechanisms mediated by ganglioside antibodies, as recently demonstated [26, 27]. Humanised antibodies have been produced to circumvent the problem of the formation of human anti-mouse antibodies, with very promising results in a first clinical trial [28]. Mueller *et al.* recently developed a mouse/human chimeric GD2-ganglioside antibody, which showed an enhanced antibody-dependent cytotoxicity compared to the original mouse anti-GD2 antibody [29]. Therefore, we have started to humanise the highest affinity murine antibodies described here in order to compare them in clinical trials.

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Differences in the Level of DNA Double-strand Breaks in Human Tumour Cell Lines Following Low Dose-rate Irradiation

Anna M. Cassoni, Trevor J. McMillan, John H. Peacock and G. Gordon Steel

It is now well accepted that differences exist in the intrinsic radiosensitivity of human tumour cells although the molecular basis of this is still unclear. Current evidence suggests that of the lesions induced in DNA by ionising radiation, double-strand breaks (DSB) are the most closely linked to cell death. In this study, levels of DSB were measured by neutral filter elution under conditions of both repair inhibition and maximum recovery and compared with clonogenic survival curves for high (HDR) and low dose-rate (LDR) irradiation in human carcinoma lines of differing radiosensitivity. Four human lung carcinoma lines were used, two small-cell (SCLC; HC12 and HX149) and two non-small cell lines (NSCLC; HX147A7 and HX148G7). Cell survival was measured by soft agar and monolayer colony-forming assays as appropriate and a large variation in sensitivity of the cell lines was seen (α values of 0.06 to 0.56 Gy^{-1}). We have previously reported that the damage induced at high dose rate does vary in these cell lines but not in a way which correlates with their cell survival response [5]. Following irradiation to 15 Gy at low dose rate essentially no DSBs were detected in any of the four lines but at 70 Gy the more sensitive SCLC showed more residual damage than in the more radioresistant NSCLC lines. The prime determinant of the difference between the LDR and HDR damage curves is likely to be repair occurring during irradiation. These data suggest that whatever the determinant, whether the degree of damage induction or repair, the level of DSB after LDR correlates well with cellular sensitivity in these four cell lines. Thus, DNA damage studies after low dose-rate irradiation may not only enable the examination of irrepairable lesions which are important in cell killing but they may also provide a useful predictive test of cellular radiosensitivity.
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INTRODUCTION

THE ANALYSES of Fertil and Malaise [1] and Deacon *et al.* [2] have shown that if the initial portion of survival curves are examined, there is considerable variation in radiosensitivity of human tumour cell lines and this can be related to clinical curability. The factors underlying these differences in radiosensitivity have been considered in terms of recovery [3] and, by implication, repair ability and more recently in terms of damage induction [4, 5].

Of the many lesions induced in DNA, that which appears most closely linked to cell damage is the double-strand break (DSB). In yeast it has been shown that one unrepaired double-strand break is lethal [6]. Unfortunately, methods available for measuring DSB in human DNA are not sufficiently sensitive to be able to relate one unrepaired break directly to a lethal lesion. However, Radford [4, 7] has shown different levels of sensitivity to DSB induction in various cell lines. His finding of a direct and constant relationship between initial DSB and cell lethality

Correspondence to T.J. McMillan.
The authors are at the Radiotherapy Research Unit, Cotswold Road, The Institute of Cancer Research, Sutton, Surrey, U.K.
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