Histocompatibility antigens on astrocytoma cells

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SUMMARY Biopsied tumour cells from astrocytoma-bearing patients were grown in primary culture for 3-5 days. Both low and high grade tumours were represented in the study. The cultured cells could be shown to express the HLA-A and -B antigens using a multispecific allo-antiserum and a rabbit anti- β -2 microglobulin antibody. The tumour cells were negative for the HLA-DR determinants when tested with either rabbit anti-Ia-like antisera or specific anti-HLA-DR allo-antisera. They also failed to stimulate allogeneic lymphocytes in primary mixed lymphocyte-tumour cell cultures but stimulated lymphocytes primed to tumour cells in vitro. The tumour cells were also capable of stimulating autologous lymphocytes from the tumour-bearing patient in most of the combinations tested.

The expression of histocompatibility antigens (HLA) on malignant cells is of importance for a variety of reasons. These include the demonstration of alterations in the expression of histocompatibility antigens by cells which have undergone malignant transformation;¹ the increased susceptibility to particular malignant diseases in association with certain HLA antigens² and the importance of matching for histocompatibility antigens between T cytotoxic cells and transformed target cells in certain experimental situations.^{3,4}

Tumours of the central nervous system are unique in that they arise in an area considered immunologically privileged⁵ and that they very seldom metastasise. The presence of glioma-associated antigens has been suggested by various investigators^{6,7} and both cellular and hormonal responses have been demonstrated in patients with malignant gliomas⁸⁻¹¹ although many of the reported studies are still controversial.

We have studied the expression of the HLA-A, -B, -D and -DR antigens on astrocytoma cells in short term cultures. Our results indicate that although astrocytoma cells carry HLA-A and -B antigens, they do not react with anti HLA-DR allo-antisera or anti-Ia-like xenoantisera. Although

the tumour cells were capable of stimulating autologous lymphocytes from the tumour-bearing patient, they did not stimulate allogeneic cells from healthy donors.

Materials and methods

Patients Twenty-two patients with various grades of astrocytomas (I-IV) were used in this study. The grading system described in the Kernohan classification has been used throughout this paper.^{12,13} Tumour biopsies were obtained at the time of operative resection and peripheral blood samples were obtained during the first 3 days after operation. All of the patients were receiving steroid treatment (Dekadron) at various doses pre-operatively, but none after operation.

Peripheral blood mononuclear cells (PBM) Blood samples were obtained by venous puncture from either healthy donors or the glioma patients. The blood was immediately defibrinated and the PBM cells were separated using Ficoll-Isopaque flotation. All the cell donors were HLA-A, -B and -DR typed using standard methods.

Tumour cell preparation Tumour specimens, obtained at operation, were divided into two portions. The smaller portion was processed for conventional histopathological evaluation. The larger specimen was immediately minced into approximately 1-3 mm pieces and stored in culture medium (RPMI 1640 containing antibiotics and 5% human serum) on ice and transported to the laboratory. The transport time never exceeded 60 minutes. Clearly non-malignant, dead or vascular elements were discarded. The remaining tissue was carefully aspirated through a No 20 gauge syringe in 5 ml of culture medium 5-6 times. Large aggregates were

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allowed to sediment for 5 min and most of the supernatant was carefully collected. The cells in the supernatant were either in small cell clusters or appeared as individual cells. The cell suspension was carefully aspirated 4-5 times and then treated with 0.25% DNase (Sigma Chemical) for 10 min at 20°C. The cells were washed twice in culture medium containing 5% human serum, resuspended in 10 ml of medium and plated out in two tissue culture flasks, T-25 (Falcon plastics). Each flask contained approximately 2×10^6 cells. Following overnight incubation, all non-adherent erythrocytes, lymphocytes and non-viable tumour cells were removed by a triple wash with warm culture medium. The remaining cells were incubated at 37°C until 3-5 days.

Radio immunoassay Details of the method are given in the *Results* section. A microplate modification of the ^{125}I -labelled protein A microassay first published by Brown *et al* was used.^{14,15} Isotope levels in the supernatants were evaluated using the Skatron Titertek disposable supernatant harvester.¹⁶

Mixed lymphocyte tumour culture Tumour cells were freed from the monolayer cultures by a short collagenase treatment. A 0.2% collagenase solution was used with a 10 min. incubation at 37°C. This treatment does not free adherent monocytes. 5×10^4 cells were, after a triple wash, seeded into the wells of flat-bottomed 96 well microtitre plates (Titertek, Flow) and incubated overnight to allow them to adhere and possibly resynthesise putative antigens destroyed after enzyme treatment. The entire plate was irradiated (2000 rad) and autologous or allogeneic lymphocytes were added to each well. The plates were incubated for 6 days in a humidified atmosphere at 37°C with 5% CO_2 atmosphere, labelled with 1 μCi ^3H -thymidine (New England Nuclear NET-27A) and harvested 18 hr later on a Skatron multiple cell culture harvester (Skatron A/S, Lierbyen, Norway). The results are shown as the mean of triplicate cultures $\pm$ SE.

Priming of allogeneic lymphocytes to tumour antigens 5×10^6 PBM were co-cultured with 2.5×10^6 irradiated tumour cells in 10 ml of culture medium containing 10% human inactivated serum and antibiotics for 12 days in upright T-25 flasks. After this priming incubation, the cells were re-separated on Ficoll-Isopaque to remove dead cells and washed twice. 5×10^4 primed cells were co-cultured with an equal amount of irradiated tumour cells, which had been stored in liquid nitrogen and thawed 24 hrs before used. The secondary cultures were incubated for 5 days, labelled and harvested as previously described.

Results

TUMOUR CELL CULTURES

Twenty-two biopsied tumours were available for culturing. Four biopsies were grades I-II, eight grades III-IV (glioblastoma multiforme) and the remaining four as "not certain". Of 18 cultures started, 14 showed sufficient growth potential to cover most of the area in the culture flask. The cells generally grew out from small aggregates (fig 1),

Fig 1 Phase contrast micrographs at typical astrogloma culture showing typical growth from central region.

Fig 2 Close up phase contrast micrographs at tumour cells after 4 days of culture magnification $\times 600$.

forming colonies. After approximately 3-5 days, an interconnecting monolayer was generated. Typical atrogliia-like cell morphology was observed (fig 2) in all the cultures during the first 1-2 weeks, after which time degeneration of the cells occurred if they were not sub-cultured. Tumour cells which had been in culture longer than 5 days or did not display typical morphology were not used.

DETECTION OF HLA ANTIGENS ON TUMOUR CELLS

Five different cell donors were used in these experiments, two classified grade I-II and three grade III-IV. The tumour cells were first cultured for between 3-5 days. $0.5-1 \times 10^4$ cells were sub-cultured in the wells of flat-bottomed microplates overnight allowing them to adhere. $50 \mu\text{l}$ of diluted antiserum was added to each well. The antisera used were either allo-multispecific anti-HLA-A, -B, specific allo-anti-DR, zeno-anti-"Ia" or anti- β -2 microglobulin. The antisera were allowed to react with the cells for 30 min at 20°C followed by a triple wash.

Table 1 Binding of 125 I-protein A to antibody-coated tumour cells

Tumour grade	Antibody specificity				Normal serum
	HLA-A, -B*	anti-Ia†	anti-DR‡	anti-B2§	
III-IV	8751 $\pm$ 615	1054 $\pm$ 98	514 $\pm$ 100	11675 $\pm$ 1783	673 $\pm$ 75
III-IV	5373 $\pm$ 318	925 $\pm$ 101	491 $\pm$ 94	9747 $\pm$ 149	580 $\pm$ 81
III-IV	11725 $\pm$ 1922	1427 $\pm$ 163	527 $\pm$ 87	15515 $\pm$ 1615	544 $\pm$ 58
I-II	7967 $\pm$ 813	1003 $\pm$ 75	627 $\pm$ 69	9875 $\pm$ 1342	315 $\pm$ 49
I-II	8175 $\pm$ 1053	625 $\pm$ 79	515 $\pm$ 121	10613 $\pm$ 2111	611 $\pm$ 83

Anti HLA-A, -B, Ia and β -2-microglobulin antisera diluted 1:32

Anti HLA-Dr diluted 1:8

*multispecific anti HLA-A, -B alloantisera

†rabbit antihuman "Ia"

‡specific allo-anti DR

§rabbit antihuman anti β -2-microglobulin

Table 2 Allogeneic mixed lymphocyte/tumour cell cultures

Exp no	Patient	Tumour grade	3 H-thymidine incorporation cpm $\pm$ SE* stimulator cells			
			Ax	Cx	Tumour cells	SI†
1	AK	III-IV	320 $\pm$ 29	4821 $\pm$ 397	538 $\pm$ 43	1.7
2	AO	III-IV	930 $\pm$ 75	17920 $\pm$ 1630	1262 $\pm$ 121	1.4
3	AS	III-IV	201 $\pm$ 31	4344 $\pm$ 298	325 $\pm$ 27	1.6
4	JJ	III-IV	596 $\pm$ 51	2122 $\pm$ 301	708 $\pm$ 61	1.2
5	HO	III-IV	297 $\pm$ 18	10140 $\pm$ 937	562 $\pm$ 51	1.9
6	LD	I-II	171 $\pm$ 15	5379 $\pm$ 421	364 $\pm$ 31	2.1
7	BH	I-II	216 $\pm$ 18	6374 $\pm$ 537	281 $\pm$ 26	1.3

*Responding PBM in each experiment derived from different donors designated A.

†SI—Stimulation Index = $\frac{\text{cpm A} + \text{irradiated tumour cell}}{\text{cpm A} + \text{irradiated PBM A}}$

125 I-labelled protein A,¹¹ equivalent to 2×10^4 cpm was added to each well and the plates were incubated for 30 min at 20°C, followed by a triple wash. 200 μ l of 6 normal NaOH were added to each well and the plates incubated for an additional 60 min. This procedure dissolved the cells causing the bound radioactivity to be released into the supernatant. The supernatants were harvested using a modified Skatron-Titertek disposable supernatant harvester,¹⁶ from which the filters had been removed.

The results of five experiments are shown in table 1. Multispecific anti-HLA-A, -B and anti- β -2-microglobulin was bound to a significant degree compared to normal serum; cpm 10-15 times background. In contrast, both alloanti-DR and the zeno-anti-"Ia"-like antisera did not bind to any significant degree to the cells. Diluting the antisera gave comparable results.

ALLOGENEIC MIXED LYMPHOCYTE-TUMOUR CELL CULTURES

Cultured astrocytoma cells were obtained from seven different patients. Different responding and stimulating cells (designated A and C, table 2) were used in each experiment. All of the tumour cells used were from the first passage. In none of the allogeneic cultures was significant stimulation observed (table

2) although the responding cells responded well to stimulation by allogeneic lymphocytes (ACx combinations). Increasing the number of tumour cells in the culture over 5×10^4 had no effect.

One interpretation of the results shown in table 2 is that tumour cells are inhibitory to lymphocyte proliferation. Experiments were therefore designed where allogeneic irradiated tumour cells were added to mixed lymphocyte cultures to determine if they were inhibitory. Two typical experiments are shown in table 3 for two different tumour grades. No differences were observed in the response towards allogeneic lymphocytes in the presence of tumour cells for all of the combinations tested.

AUTOLOGOUS MIXED LYMPHOCYTE TUMOUR CELL CULTURES

PBM was isolated from the tumour-bearing patients. Mixed cultures were established employing tumour and lymphocyte combinations from five patients; three with glioblastoma multiforme and two with low grade astrocytoma (table 4). In all of the combinations tested a stimulation index over two was observed. Titration of the number of tumour cells gave decreasing cpm with decreasing numbers of stimulating cells. On the other hand, the tumour cells were inferior to lymphocytes as stimulator

Table 3 Mixed lymphocyte culture response in the presence of tumour cells

Tumour cells Grade	³ H-Thymidine incorporation cpm—SE					
I-II						
—	2049 ± 163	21841 ± 1810	16100 ± 1108	690 ± 37	7532 ± 611	6003 ± 518
+	1384 ± 79	22964 ± 1936	10166 ± 431	1325 ± 101	11982 ± 821	4388 ± 211
III-IV						
—	474 ± 38	3115 ± 305	7115 ± 510	321 ± 17	4344 ± 216	2437 ± 93
+	518 ± 41	2758 ± 241	8243 ± 631	429 ± 36	5230 ± 506	2426 ± 136

Table 4 Mixed lymphocyte autologous tumour cell culture

Patient	Tumour grade	³ H-Thymidine incorporation cpm ± SE stimulating cells			
		None	Cx	Tumour cells	SI*
AK	III-IV	474 ± 31	12319 ± 1125	1943 ± 162	4.1
SO	III-IV	92 ± 8	8417 ± 737	579 ± 64	6.3
JJ	III-IV	197 ± 12	3815 ± 286	768 ± 38	3.9
LD	I-II	321 ± 23	3703 ± 259	2664 ± 168	8.3
BH	I-II	215 ± 18	6381 ± 561	604 ± 37	2.8

*SI cpm: PBM patient
cpm: PBM from patient

Table 5

Tumour grade	Culture	Control*	MLC†	MLTC‡
I-II	primary	318 ± 21	6875 ± 632	494 ± 41
	secondary	527 ± 36	11413 ± 918	1838 ± 97
III-IV	primary	705 ± 37	5431 ± 318	872 ± 68
	secondary	372 ± 28	6039 ± 293	3276 ± 218

*Unstimulated responding lymphocytes.

†Response to allogeneic irradiated lymphocytes; secondary cultures primed with lymphocytes.

‡Response to allogeneic irradiated tumour cells; secondary cultures primed with tumour cells.

cells (table 4).

PRIMING OF ALLOGENEIC LYMPHOCYTES TO TUMOUR CELLS

Primary cultures of allogeneic PBM from healthy donors and irradiated tumour cells were established and incubated for 12 days. Cell aliquots taken out of the cultures on days 6 and 8 of culture showed no proliferation compared to controls. After 12 days of culture, the primed cells were rechallenged with fresh/frozen tumour cells. Control cultures were either non-stimulated or primed with allogeneic lymphocytes and rechallenged with lymphocytes from the same donor.

The results of one experiment are shown in table 5 for day 6 of primary culture and day 4 of secondary culture. Lymphocytes primed to allogeneic lymphocytes responded approximately equally in both primary and secondary cultures. In contrast, lymphocytes primed to tumour cells and showing no primary response demonstrated proliferation in secondary cultures towards tumour cell stimulation.

PBM cultured in the absence of stimulation for 12 days did not react to stimulation by tumour cells.

Discussion

The results of the experiments presented here and summarised in table 6 indicate that dissociated cells from astrocytomas of various degrees of malignancy do not express HLA-D/DR determinants. The evidence for this is two-fold; the tumour cells do not stimulate allogeneic lymphocytes and anti-HLA-DR antibodies do not bind to the tumour cells. On the

Table 6 Summary

	Tumour grade	
	III-IV	I-II
Binds HLA-A, -B antisera	+	+
Binds anti -2- antisera	++	++
Binds HLA-DR antisera	—	—
Stimulates in primary MLTC	—	—
Stimulates autologous lymphocytes	++	++
Stimulates in secondary MLTC	++	++

other hand, they do express the HLA-A,-B antigens as determined by binding of multispecific anti-HLA-A, -B antibodies as well as anti- β -2-microglobulin antibodies. Our data agree well with Howe and co-workers who examined three glioma cell lines using monoclonal anti-"Ia"-like antibodies and did not find bound antibody using a similar radio immunoassay system.¹⁷ In contrast, both our results and the results of others demonstrate that astrocytoma cells could stimulate autologous lymphocytes,^{9,18} albeit weakly. The lack of allogeneic stimulation combined with a positive autologous response might indicate that the patients developed an immunological response against their own tumour cells. Several other studies previously reported in the literature, for both humoral and cellular immunological response, support this concept.⁸⁻¹¹ Additionally, it proved possible to *in vitro* "immunise" allogeneic lymphocytes towards astrocytoma cells (table 5). Taken together, these results suggest antigenic system(s) on the tumour cells which are not stimulatory histocompatibility antigens (D/DR), but which can nevertheless lead to lymphocyte proliferation *in vitro* following either *in vivo* or *in vitro* immunisation.

It is now well established that T cells only respond *in vitro* when antigen is "presented" in an immunogenic form by some type of accessory cell (for review see ref. 19). This T cell to accessory cell cooperation is restricted by major histocompatibility complex products in all of the species tested including humans, and the only exception to MHC restriction appears to be allogeneic stimulation by D/DR antigens on certain types of stimulating cells. Stimulation by D/DR antigens also does not require pre-sensitisation of the responding T cells for a response to occur *in vitro*. The putative antigens on the glioma tumour cells which we have studied appear, therefore, to behave like "classical" soluble antigen requiring T cell pre-sensitisation and most probably accessory cell processing; although this latter point is presently unknown. Purified populations of T cells though have been reported to respond to autologous tumour cells,⁹ but even relatively small numbers of peripheral blood monocytes (less than 1%) are capable of restoring the antigen response of purified T cells *in vitro*, so the degree of monocyte depletion in the T cell fraction is a critical point.

The activation of T cells by antigen requires in principle two signals; the binding of antigen to the T cell receptor, probably in complex form together with MHC products, and the presence of a co-stimulating soluble fraction produced by the accessory cell (II-I).²⁰ Whether tumour cells are capable of supplying both signals is at present unknown,

but is most likely an important point, since the immunological rejection of both allografts and possibly tumour cells probably also requires two signals for its initiation.

The tumour cells used in this study were relatively fresh (3-5 days of incubation) and were all passaged *in vitro* only once. Detailed genetic studies of cloned glioma lines have shown a great deal of heterogeneity of the tumour cells in each solid tumour and that the cells which develop into lines are generally not representative of the cell populations in the tumour *in vivo*.²¹

Cell lines derived from solid tumours, therefore, are probably not as suitable as primary tumour cell cultures in the study of representative antigenic determinants on the cell surface.

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