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Antitumor effects of L6, an IgG2a antibody that reacts with most human carcinomas

(tumor antigen/complement activation/antibody-dependent cellular cytotoxicity)

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ABSTRACT **Mouse monoclonal antibody L6 (IgG2a subtype) recognizes a ganglioside antigen expressed at the surface of cells from human non-small-cell lung carcinomas, breast carcinomas, and colon carcinomas. We now show that this antibody can lyse L6 antigen-positive human tumor cells in the presence of Leu-11b-positive human lymphocytes (i.e., mediate antibody-dependent cellular cytotoxicity) or human serum (mediate complement-dependent cytotoxicity) and that it can inhibit the outgrowth of an L6 antigen-positive human tumor transplanted onto nude mice.**

Most neoplasms have tumor-associated antigens, many of which are differentiation antigens that are expressed more strongly in tumor cells than in normal cells from the adult host. Although none of these antigens are exclusively present on neoplastic cells, cell surface antigens that are expressed at significantly higher levels in neoplastic cells than in normal cells may be suitable targets for therapy. Monoclonal antibodies (mAbs) are logical vehicles for such therapy (1–3).

Some antitumor mouse mAbs of IgG2a and IgG3 isotypes can mediate antibody-dependent cellular cytotoxicity (ADCC) in the presence of human effector cells (4–8) and/or activate human complement (5–7), and there is evidence that a few of these mAbs have an antitumor activity in nude mice carrying human tumor xenografts (6–8) and even in human cancer patients (5, 9). Further characterization of the antibodies capable of participating in ADCC and/or the activation of human complement, and characterization of their target antigens, is much needed.

Mouse IgG2a mAb L6 identifies a ganglioside that is strongly expressed in most human carcinomas tested, including carcinomas of the lung, breast, colon, and ovary (10). Iodinated mAb L6 and its $F(ab')_2$ and Fab fragments target L-2981 xenografts in nude mice (P.L.B., unpublished data). In this paper we show that mAb L6 can kill antigen-positive tumor cells in conjunction with either human lymphocytes (as effector cells) or human serum (as complement), and that it can inhibit the outgrowth of L6 antigen-positive human tumor in nude mice. In view of the wide distribution of the L6 antigen in human cancer, this finding may have great clinical relevance.

MATERIALS AND METHODS

Monoclonal Antibodies. L6 is an IgG2a mAb that binds to its ganglioside antigen with an affinity constant of 4×10^8 M^{-1} (10). It is made by a hybridoma that was derived by fusing spleen cells from a BALB/c mouse immunized with human non-small-cell lung carcinoma, using NS-1 mouse myeloma cells as the fusion partner (10). According to immunohistology on frozen sections, performed as previously described for studies on melanomas (11), the L6 mAb binds strongly to cells from many human carcinomas, whereas it reacts very weakly and infrequently with cells from normal adult tissues. Table 1 summarizes specificity data on mAb L6, which is presented in more detail elsewhere (10).

Table 1. Specificity of antibody L6

Tumor type	No. positive/ no. tested
Lung tumors	
Adenocarcinoma	21/22
Squamous-cell carcinoma	8/10
Small-cell carcinoma	2/6
Large-cell carcinoma	2/2
Breast carcinoma	15/18
Colon carcinoma	11/11
Ovarian carcinoma	4/5
Melanoma	1/4
Lymphoma	0/5

mAb L6 was tested against various tumors by immunohistology on frozen sections (11). Reactivity is defined as staining of at least 75% of the cells in a tumor preparation. No binding was seen to normal colon, breast, liver, kidney, heart, brain, skin, thyroid, testis, vagina, ovary, retina, tonsils, or lymphocyte pellet. A few scattered cells were weakly positive in 2 of 19 normal lung samples and in 1 of 4 samples of normal spleen.

mAb MG-21 was used as a control. It is an IgG3 that binds to a GD3 ganglioside antigen most strongly expressed at the surface of human melanoma, and it can lyse melanoma cells in the presence of human complement or human leukocytes (6). MG-21 can also inhibit the outgrowth of human melanoma xenografts in nude mice (6).

mAb 96.5, used as another control, is an IgG2a specific for p97, a melanoma-associated cell surface glycoprotein (1). mAb 35.1 is an IgG2a specific for the Tp 50 epitope of a human T-lymphocyte surface protein associated with the erythrocyte rosette receptor (12). It was kindly provided by J. Ledbetter (Oncogen) and used as a control for the *in vivo* studies in nude mice. P1.17 is an IgG2a mouse myeloma protein obtained from the American Type Culture Collection. Neither P1.17 nor 35.1 binds to human tumor cells such as cells from lung carcinoma or melanoma (data not shown).

mAbs L6, 96.5, 35.1, and P1.17 were purified by affinity chromatography using protein A (13). The purification of mAb MG-21 has been described (6). This mAb, like most IgG3 antibodies, easily precipitates, unless the salt concentration is kept high prior to use or a stabilizing agent such as human serum albumin is added. Antibodies used for *in vivo* studies were prepared using buffers made from sterile, pyrogen-free water.

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Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; mAb, monoclonal antibody; NK cells, natural killer cells.

Table 2. ADCC as determined by a 4-hr ^{51}Cr-release assay

Target cells	Lymphocytes per tumor cell	mAb		% cytolysis*	
		Name	Conc., μg/ml	Exp. 1	Exp. 2
L-2981 lung adenocarcinoma (binds L6 but not MG-21)	100	L6	20	48	58
	100	L6	10	33	42
	100	L6	1	24	44
	100	None	—	7	10
	10	L6	20	37	39
	10	L6	10	41	35
	10	L6	1	40	26
	10	None	—	10	12
	1	L6	20	13	11
	1	L6	10	13	9
	1	L6	1	8	10
	1	None	—	4	5
	0	L6	20	0	0
	100	MG-21	20	9	NT
	100	96.5	20	7	NT
LX-1 lung small-cell carcinoma (binds L6)	100	L6	20	39	NT
	100	L6	10	33	NT
	100	L6	5	28	NT
	100	None	—	12	NT
	10	L6	20	29	NT
	10	L6	10	25	NT
	10	L6	5	20	NT
M-2669 cl 13 melanoma (binds MG-21 but not L6)	100	L6	10	10	NT
	10	L6	10	10	NT
	100	MG-21	20	NT	79
	100	MG-21	10	31	NT
	100	MG-21	5	NT	91
	10	MG-21	10	24	NT
	100	None	—	7	17
	10	None	—	5	17
	0	MG-21	20	0	0

NT, not tested.
*See *Materials and Methods* and ref. 6.

Target Cells. Lung carcinoma line L-2981 was established from a primary human adenocarcinoma of the lung (10). Colon carcinoma line C-3347 was derived from cells in the abdominal effusion of a patient with metastatic colon carcinoma. Melanoma line M-2669 clone 13 came from a metastatic human melanoma (14). Lung carcinoma line LX-1 was kindly provided by E. S. Neuwelt, Health Sciences University, Portland, OR. It was established from a human oat-cell carcinoma. The tumors were grown in Iscove's modified Dulbecco's medium (IMDM, from GIBCO) containing 10–15% heat-inactivated fetal bovine serum. Lung carcinoma line L-2981 was also propagated by serial transplantation in nude mice and used for the *in vivo* experiment described below. The lung carcinoma L-2981 and colon carcinoma C-3347 cells strongly express the ganglioside antigen defined by mAb L6 (≈100,000 sites per cell) but not the GD3 antigen defined by MG-21 according to tests with a binding assay (14), whereas the converse holds for the melanoma M-2669 cl 13 cells (14).

Tests for ADCC. Tests were carried out as previously described for mAb MG-21 (6). In brief, the target cells were labeled with ^{51}Cr and then exposed for 4 hr to effector cells [human lymphocytes purified by use of a lymphocyte-separation medium that has been described (6)] and mAb, which was added in concentrations varying between 1 μg/ml and 100 μg/ml, after which the release of ^{51}Cr from the target cells was measured as evidence of tumor-cell lysis (cytotoxicity). Controls included the incubation of target cells alone or with either lymphocytes or mAb; the total amount of ^{51}Cr that could be released also was measured. Several "criss-cross" experiments were done, in which lung (or colon) carcinoma and melanoma targets were tested in parallel with mAbs L6 and MG-21.

Effector cells were characterized by assessing their sensitivity to incubation with antiserum to the Leu-11b surface marker and guinea pig complement, using procedures that have been described (15). This was done to measure the expression of the Leu-11b marker, which characterizes natural killer (NK) cells (16) and is expressed by lymphocytes mediating ADCC against human melanoma cells in the presence of mAb MG-21.

Tests for Complement-Mediated Target-Cell Lysis. Our procedures, which have been published (6), were identical to those used for the ADCC assays except that human serum (as a source of complement) diluted 1:3 to 1:6 was added in place of the effector cells.

Tests for Antitumor Activity in Nude Mice. Female 8-week-old nude (*nu/nu*) BALB/c mice were bought from the breeding facilities of the Fred Hutchinson Cancer Research Center. The mice were placed in filter-top cages (five mice per cage) and maintained in condominium units in a specific pathogen-free room.

Mice were grafted on both sides of the back with approximately 1 × 1-mm pieces of the L-2981 tumor. One day before, and on days 1, 5, and 9 after tumor implantation, mice were randomized into three groups; two groups of 10 mice

Table 3. Leu-11b-positive human peripheral blood lymphocytes lyse L-2981 lung carcinoma cells in the presence of mAb (L6) that binds the target cells

Exp. group	Pretreatment of effector cells*	mAb added (10 μg/ml) to target cells	Cytolysis† ^{51}Cr release, cpm	%
1	Anti-Leu-11b + complement	None	37,972	7
2	Anti-Leu-11b + complement	L6	41,498	9
3	Anti-Leu-11b	None	49,468	13
4	Anti-Leu-11b	L6	72,805	26
5	Complement	None	44,685	11
6	Complement	L6	85,443	33
7	None (medium)	None	70,007	24
8	None (medium)	L6	135,444	60
9	No effector cells added	L6	24,584	0

*Where indicated, preincubation of lymphocytes for 30 min at room temperature with anti-Leu-11b was followed by incubation for 1 hr at 37°C with guinea pig complement.
†Measured in a 4-hr ^{51}Cr-release assay, using an effector/target ratio of 100:1. Calculation of % cytolysis was as in ref. 6. Spontaneous release was 24,880 cpm (0%), and total release was 210,198 cpm (100%).

were intravenously injected via the tail vein with either mAb L6 or mAb 35.1 (1 mg per mouse), and a third group of 20 mice was grafted with tumors but not injected with antibody and served as an untreated control. The cages in which the mice were kept were coded and the code was not known to the persons observing the mice and measuring the tumors.

The mice were inspected every 2–3 days starting 9 days after tumor implantation. Two perpendicular diameters were measured for each palpable tumor and averaged to give a mean dimension; since each mouse was transplanted on both sides of the back, data for two tumor "sites" per mouse were recorded. Three implant sites which measured 0 mm at all determinations were excluded (1 out of 20 in the L6 group, 0 out of 20 in the 35.1 group, and 2 out of 40 in the untreated group), since they represented failures of grafts to "take." Graft sites with originally palpable tumors that disappeared over the course of treatment were recorded; such disappearance may have resulted from tumor regression, but we cannot exclude that a failure of a small tumor implant to "take" may also have played a role. Student's t test was performed to statistically compare mean tumor dimensions among the three treatment groups, and χ^2 analysis was done to estimate the significance of differences in disappearance of palpable tumor implants between the L6-treated group and the two controls, using a computer with a statistical "package" (Minitab 82, Pennsylvania State University).

Table 4. Lymphocytes pretreated with mAb L6 are cytolytic to ^{51}Cr-labeled L-2981 lung carcinoma cells

Pretreatment of lymphocytes	mAb added (20 μg/ml) to target cells	% cytolysis
None	None	19
	L6	55
A	None	64
	L6	96
B	None	65
	L6	94

Human peripheral blood lymphocytes were incubated for 1 hr with L6 (20 μg/ml) at 37°C, followed by washing and further incubation (without L6) for 1 hr at either 4°C (pretreatment A) or 37°C (pretreatment B). They were tested in the ^{51}Cr-release assay at a ratio of 100 lymphocytes per target cell, either in the presence (20 μg/ml) or absence of L6.

Table 5. Complement-mediated cytolysis by mAbs L6 and MG-21

Target cells	mAb Name	mAb Conc., μg/ml	Complement added*	% cytolysis†
L-2981 lung carcinoma (binds L6 but not MG-21)	L6	50	Active	39
	L6	10	Active	34
	L6	1	Active	7
	L6	50	Inactive	4
	MG-21	50	Active	0
C-3347 colon carcinoma (binds L6 but not MG-21)	L6	10	Active	97
	L6	5	Active	83
	L6	2.5	Active	70
	L6	1.25	Active	70
	L6	10	Inactive	0
M-2669 cl 13 melanoma (binds MG-21 but not L6)	L6	50	Active	0
	MG-21	50	Active	79
	MG-21	10	Active	67
	MG-21	50	Inactive	0

*Human serum (diluted 1:5) was used as complement (active) or was heated to 56°C for 30 min and then used (inactive).
†Determined in a 4-hr ^{51}Cr-release assay.

RESULTS

Antibody L6 Mediates ADCC with Human Effector Cells. Table 2 presents two of several similar experiments in which cells from lung carcinoma line L-2981 were labeled with ^{51}Cr and incubated with a combination of human peripheral blood lymphocytes and mAb L6. The combination of mAb L6 and effector cells lysed a substantial fraction (24–58%) of the L-2981 cells (which express the L6 antigen), as long as 10 or more lymphocytes were added per target cell. Lysis was seen even at the lowest concentration of mAb tested (1 μg/ml). Lymphocytes alone gave 7–12% cytotoxicity when similarily tested, and mAb L6 alone had no measurable effect. When the ratio of lymphocytes was decreased to one per target cell, 8–13% cytolysis was observed with mAb L6, as compared to 4–5% with the same dose of lymphocytes alone; this difference was statistically significant ($P < 0.01$). Cells from an oat-cell carcinoma, LX-1 (which expresses the L6 antigen), were also killed by the combination of effector cells and mAb L6. Two antibodies to melanoma-associated antigens, mAbs MG-21 and 96.5, did not induce ADCC on L-2981 cells.

M-2669 cl 13 melanoma cells, which do not express significant amounts of the L6 antigen, were not lysed by mAb L6 and effector cells but were lysed when the effector cells were combined with mAb MG-21, which binds strongly to the M-2669 cl 13 cells; this is consistent with published results for MG-21 (6).

Cytotoxicity was completely abrogated when the lymphocytes were preincubated with anti-Leu-11 antibody and guinea pig complement; incubation with anti-Leu-11b antibody alone slightly depressed the cytolytic effect (Table 3). This indicates that the effector cells express Fc receptors and are related to NK cells (15, 16). Isolation of large granular leukocytes and T cells, respectively, by use of a fluorescence-activated cell sorter, further demonstrated that the effector function was entirely mediated by the large granular leukocyte fraction (data not shown).

Incubation of lymphocytes with mAb L6, followed by washing, made them capable of effectively lysing L6 antigen-

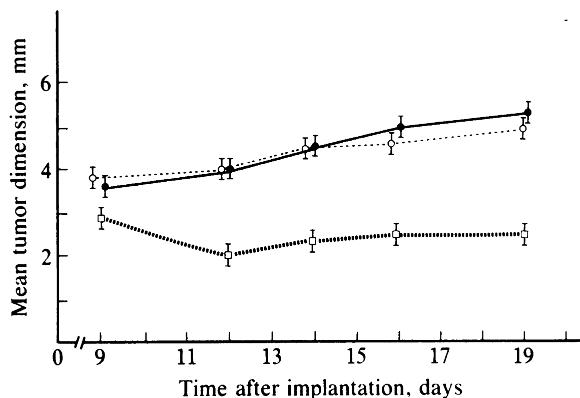

FIG. 1. Mean tumor dimension for groups of nude mice both implanted with two human lung carcinoma L-2981 xenografts and treated with mAb L6 (□; 10 mice, 19 sites) or 35.1 (●; 10 mice, 20 sites) or not treated with mAb (○; 20 mice, 38 sites). Mice in the L6 and 35.1 treatment groups received 1 mg of mAb the day before and on days 1, 5, and 9 after tumor implantation. Error bars show ±SEM.

positive target cells (up to 65% cytotoxicity, as shown in Table 4), whereas incubation with P1.17 myeloma protein (used as a control) had no such effect (data not shown). The cytolytic effect was further increased when mAb L6 was added to the target cells together with the "armed" effector cells (94–96% cytotoxicity; Table 4). Incubation of the pretreated lymphocytes for 1 hr at 37°C did not affect their cytotoxicity compared to lymphocytes kept at 4°C.

Antibody L6 Lyses Human Tumor Cells in the Presence of Human Complement. mAb L6, in the presence of human complement, lysed L6 antigen-positive L-2981 and C-3347 cells (Table 5). mAb MG-21, which does not bind to the L-2981 cells, did not lyse these cells. The L6 antigen-negative M-2669 cl 13 melanoma cells were not lysed by mAb L6, but they were killed (in the presence of complement) by mAb MG-21, which binds strongly to the melanoma cells. Inactivation of the complement at 56°C for 30 min abolished the antibody-dependent cytotoxicity.

Antibody L6 Suppresses the Outgrowth of L6 Antigen-Positive Tumor Cells in Nude Mice. Fig. 1 compares the outgrowth of L-2981 tumor grafts in groups of nude mice that received the specific mAb (L6), a nonspecific mAb (35.1), or no antibody. Tumor outgrowth was suppressed in the L6 group compared to either the 35.1 group or the no-treatment group. At 9 days after implantation, when the first evidence of tumor growth was observed, the mean tumor dimension of the L6 group was significantly different from that of the 35.1 group ($P < 0.04$) and the no-treatment group ($P < 0.09$). At all subsequent measurement times, including the last one (day 19), the difference between tumor dimension of the L6 group and either of the two control groups was significant at the $P < 0.001$ level; there were never any significant differences between the two control groups. Furthermore, 6 of the 19 (32%) graft sites in the L6 group displayed total disappearance of implanted tumor (regression?) as compared to 1 of 20 (5%) sites in the mAb 35.1 group and 3 of 38 (8%) in the untreated group; the differences between the L6 group as compared to the combined two control groups were significant at $\alpha < 0.001$ according to χ^2 analysis. There was no evidence that treatment of mice with mAb L6 had any adverse effects on their health over an observation period of 36 days. Neither has treatment of monkeys (*Macaca nemestrina*) with mAb L6 had any adverse effects, such as weight loss, induction of tissue damage detectable on gross pathological or histopathological examination, etc. (unpublished studies performed in collaboration with the University of Washington Primate Center).

DISCUSSION

Mouse mAb L6 recognizes a ganglioside antigen that is of particular interest because it is expressed at the surface of cells from most human carcinomas of the lung, breast, colon, and ovary, while it is present in only trace amounts at the surface of normal cells (ref. 10; also see data summarized in Table 1).

We now have shown that this mAb can mediate ADCC and complement-dependent cytotoxicity so as to lyse L6 antigen-positive tumor cells in the presence of human lymphocytes or human serum, respectively. The effector cells for the ADCC have the Leu-11b marker of NK cells. A high degree of target cytolysis (95%) was observed when the lymphocytes were first "armed" by *in vitro* incubation with mAb L6 and then added to the target cells in the presence of additional L6 antibody.

Encouraged by these findings, we studied whether mAb had any activity against an L6 antigen-positive tumor transplanted onto nude mice. Significantly smaller tumors were observed in mice injected repeatedly with a 1-mg dose of mAb L6, and a significantly greater number of tumors in this group disappeared than in control groups. Thus, the L6 antibody appears to seek out and destroy tumor cells *in vivo*.

We did not extend our studies in nude mice further, since we feel that data obtained in nude mice may as well underestimate as overestimate what may be therapeutically achieved in humans. Human complement and effector cells are more effective than their mouse counterparts in lysing human tumor cells. Furthermore, antibodies injected into a human patient might induce an immune response to the tumor, since human patients, unlike nude mice, do have a functioning T-cell system. Antitumor activity, when detected in humans and, so far, observed only with antibodies that, like L6, can kill tumor cells, is seen too late after antibody infusion to make it likely that it is only a direct result of tumor destruction by the antibody (5, 9, 17, §). One must also realize, of course, that an established tumor in a human patient is probably more resistant to an antibody-dependent effect than a tumor grafted onto a nude mouse, and the crossreactivity an antibody may have with normal cells that express the target antigen (albeit in a small amount) is a further complication.

In view of the specificity of mAb L6 for an antigen primarily expressed in human carcinomas and the interesting biological activities of this mAb, it should be an appropriate candidate for clinical trials in humans, as long as these are conducted with the proper caution.

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