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Immunology

Study of antibodies against human melanoma produced by somatic cell hybrids

(human tumor/cell fusion/mouse myeloma/antitumor activity)

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ABSTRACT **We fused spleen lymphocytes obtained from mice immunized against a human melanoma cell line and melanoma-mouse hybrid cells with the P3 × 63 Ag8 mouse myeloma in order to produce hybrids secreting antibodies against a human melanoma. Antibodies secreted by individual hybrids were tested for their reaction with a panel of human melanoma, colorectal carcinoma, and normal cells in an indirect radioimmunoassay, and they displayed different specificities and crossreactivities. Some reacted only with melanomas, whereas others crossreacted with normal human or human colorectal carcinoma cells. By analysis of competitive binding of mixtures of monoclonal antibodies, it was possible to delineate different epitopes on melanomas. Hybrids growing in *nude* mice and producing antimelanoma antibody suppressed growth of melanoma tumors.**

We previously reported production of monoclonal antibodies specific for antigenic determinants of influenza and parainfluenza viruses (1, 2) by somatic cell hybrids (hybridomas) between spleen cells obtained from immunized mice and mouse myeloma cells (3). Also, hybridomas were subsequently produced that secrete antibodies specific for the simian virus 40 tumor (T) antigen (4).

Antigenic analyses of compounds expressing various undefined, distinct antigens have been difficult. This difficulty primarily reflects the fact that antisera raised *in vivo* against these compounds are likely to contain many antibody populations that exhibit distinct specificities. Thus, polyspecific antisera produced *in vivo* delineate an undefined part of the overall, rather than the individual, antigenic determinants. Furthermore, polyspecific antisera in general are unable to discriminate among small antigenic differences on an otherwise identical background or small antigenic similarities on a different background. Although the polyspecificity of antisera produced *in vivo* can be reduced by using an appropriate host for immunization or by absorption *in vitro* of unwanted antibody specificities, neither of these methods can rigorously establish the monospecificity of an antibody preparation. In addition, both methods require some prior knowledge of the antigenic makeup of the immunizing compound, the immunized host, or the compounds used for cross-absorption. Monoclonal antibodies, on the other hand, circumvent these problems because they represent a single population of antibody-combining sites. Because attempts to characterize antigenic determinants of human tumor cells by antisera raised *in vivo* were largely unsuccessful, we have decided to produce monoclonal antibodies in a hybridoma system against human tumors. Availability of several lines of human melanomas grown in culture (5, 6) led us to select this tumor for the experiments.

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MATERIALS AND METHODS

Human Cells. Human melanoma cell lines SW: 489, 690, 691, 843, 1614, and 1694 and human colorectal carcinoma cell lines SW: 403, 480, 948, 620, and 1116 were kindly supplied by A. Leibovitz (5, 6). Normal human cells used in this study were WI38 (7), HFF (8), and FS2 (9).

Mouse Cells. BALB/c myeloma cells (P3 × 63 Ag8), derived from the MOPC-21 line (10) and deficient in hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) (3, 11), were obtained from C. Milstein. They were maintained in Eagle's minimal essential medium containing 10% fetal calf serum, 10% horse serum, and 15 μg of 8-azaguanine per ml. Other mouse cell lines used were: IT22, fibroblasts deficient in thymidine kinase (EC 2.7.1.75) (12) and GMM, simian virus 40-transformed BALB/c macrophages (13).

Human–Mouse Hybrid Cells. We have produced several hybrids between human melanoma cells and IT22 mouse cells (14). One such hybrid, derived from human melanoma SW691 cells and IT22 cells (691–I-5), was found to be tumorigenic in *nude* mice; tumor cells of *nude* mouse origin established in culture (691–I-5Nu) were used for immunization. 691–I-5Nu tumor cells contained human chromosomes 14, 17, and 21 as established by karyotyping and isozyme analysis.

Immune Mouse Spleen Cells. BALB/c and ICR Swiss mice were primed by an intraperitoneal injection of 3×10^7 human tumor or human–mouse hybrid cells washed three times in phosphate-buffered saline. After 1 month, the mice received an intravenous booster dose of 10^6 cells. The mice were sacrificed 3 days later and a spleen cell suspension was prepared as described (1).

Production of Hybridomas. Fusion of spleen cells with P3 × 63 Ag8 cells was done as described (1). Hybrids were selected in a medium containing hypoxanthine/aminopterin/thymidine (15) by using established techniques (1, 2). Fused cells were seeded in wells of tissue culture plates (Linbro FB-16-24-TC).

Radioimmunoassay. Analysis and quantitation of antibodies were performed by using the radioimmunoassay described for influenza virus (1) except that cells were the immunoadsorbent. Briefly, trypsinized tumor or normal human cells were seeded in Falcon microtiter plates and incubated for 16–48 hr until about 95% confluency was established. After removal of growth medium, the cells were washed and fixed with 0.15% glutaraldehyde. The fixed cells were stored at 4° in phosphate buffered saline containing 10% agamma horse serum and 0.08% sodium azide.

Cells were washed with the storage buffer before being assayed as described (1). The anti-$F(ab')_2$ serum produced in rabbits was kindly supplied by W. Gerhard (The Wistar Institute).

In some cases, the radioimmunoassay was performed with live target cells instead of fixed cells by using the modified

procedure of Goldstein *et al.* (16, 17). Briefly, 5×10^5 target cells and dilutions of antibody in 0.5 ml of the storage buffer were added to Linbro U-bottom microtest plates and placed on a microshaker for 1 hr at room temperature. Plates were centrifuged at $200 \times g$ for 3 min and the supernatant was removed. The cells were washed three times with the storage buffer. To each well 0.05 ml of ^{125}I-labeled anti-F(ab')$_2$ was then added, and the remainder of the procedure was as described (1). All data are expressed as a mean cpm of triplicate test samples minus the mean cpm of triplicate samlpes of the P3 × 63 Ag8 control culture. For each assay the SEM of triplicate cpm was calculated and when SEM exceeded 10%, the test was repeated. Only counts that differed significantly (99% limit) from background counts were included in tables and figures.

***In Vivo* Experiments.** *Nude* mice (*nu/nu*) were used to study tumor growth. One group of mice was injected subcutaneously with P3 × 63 Ag8 mouse myeloma cells to serve as a control, and the other groups were injected with hybridoma cells. Two days later all animals were challenged by subcutaneous inoculation with 691-I-5Nu tumor cells.

The size of the 691-I-5Nu tumor was monitored. We arrived at the arbitrary units of tumor growth by adding three dimensions—width (W), length (L), depth (D)—in centimeters and dividing by 2: Tumor size = $W + L + D/2$.

RESULTS

Antibodies against Human Melanoma. Twenty-nine hybrid cultures were obtained by fusion of P3 × 63 Ag8 myeloma cells (referred to hereafter as P3 cells) with lymphocytes obtained from spleens of mice immunized with melanoma 691. Of these, nine showed the presence of antimelanoma antibody (Table 1). Antibodies in hybrid culture 691-13 reacted with three melanomas—690, 691, and 1694—but not with any other melanoma, colorectal carcinoma, or normal human cells. Antibodies of hybrid culture 691-19 were similarly restricted, reacting with three melanomas and possibly crossreacting slightly with WI38 and melanoma 489 cells. In contrast, antibodies of hybrid cultures 691-2, 691-5, and 691-12 reacted in radioimmunoassay with all melanomas (except for 691-5 with melanoma 1694), four out of five colorectal carcinomas, and with the three normal human cultures. Culture fluids from hybridomas 691-9 and 691-11 exhibited low cpm in radioimmunoassay for binding even with the homologous tumor (691). Thus, it is difficult to draw conclusions concerning the crossreactivity of these antibodies with other cell lines.

Antibodies secreted by hybridomas 691-4 and 691-6 interacted better with the cells used for immunization (691) than with other melanomas but differed from each other in their reaction with other cell lines, hybridoma 691-6 showing less crossreactivity than 691-4. None of the hybridomas secreted antibodies crossreacting with the mouse cell lines IT22 and GMM. Four of the hybridomas (691-9, -11, -12 and -19) reacted slightly with the hybrid cells 691-I-5Nu.

Of the six hybridomas produced by fusion of P3 cells with lymphocytes obtained from spleens of mice immunized with 691-I-5Nu hybrid cells, three secreted antibodies that reacted with melanoma cells (Table 1): 691-I-5Nu-6 showed the highest bindings with four melanomas (690, 691, 1614, and 1694), the melanoma hybrid 691-I-5Nu not shown in Table 1, and normal WI38 human cells and was crossreactive with other melanomas, normal human cells, mouse cells, and possibly two out of four colorectal carcinomas tested. Conversely, antibody produced by 691-I-5Nu-4 reacted best with melanoma 1694 followed by melanomas 691, 843, and 690 and hybrid 691-I-5Nu. It showed either little or no crossreactivity with other cells. Antibody secreted by hybridoma 691-I-5Nu-2 reacted with four melanomas, melanoma hybrid, and probably with no other cells.

Reactivity of Antibodies Produced by Clones of Hybridomas. Several hybridoma cultures were cloned at limiting dilution in conditioned medium. All but three clones of 691-4 and four clones of 691-13 produced detectable levels of antibody. With the exception of one clone of 691-6, all hybridoma clones produced antibodies with specificities similar to those of antibodies secreted by the parental hybrid culture (Table 2). The immunoglobulin isotypes expressed by the mass culture were, in general, also expressed by the clones. A rare exception was clone 42 of 691-I-5Nu-4 hybridoma (see below) which did not express IgG1 (produced by P3 cells) but continued to produce IgG2a.

Because most hybrid clones derived from the same culture exhibited identical crossreactions, only one or two clones of five hybridomas were selected for a more detailed analysis of

Table 1. Immunoreactivity of hybridomas produced by fusion of lymphocytes from mice (immunized with human melanoma) with P3 × Ag8 cells

Donor mice immunized with:	Ratio showing immunoreactivity	No.	Results of radioimmunoassay against cells of: Hybridomas: Melanoma 690	691	489	843	1614	1694	Colorectal carcinoma 480	948	403	1116	837	Normal human WI38	HFF	FS2
		2	1179	785	1035	914	852	1303	387	504	0	309	254	639	159	449
		4	1266	1611	458	125	803	467	51	145	0	0	0	215	241	112
		5	652	2409	676	495	779	0	367	440	0	185	274	244	301	730
691	9/29	6	948	1938	297	536	615	450	0	0	0	254	286	0	47	0
		9	258	503	99	204	320	0	192	335	78	77	175	231	221	183
		11	283	475	51	351	351	172	141	259	175	60	123	253	158	238
		12	1614	2279	1923	658	744	1031	596	292	89	151	252	289	438	221
		13	1053	1487	42	0	0	1101	0	0	0	0	0	0	0	0
		19	803	1030	136	68	301	67	0	86	0	0	0	143	73	0
691-I-5Nu hybrid*	3/6	2	70	402	508	599	65	220	0	107	0	125	ND	0	0	89
		4	317	520	103	377	88	1207	0	0	0	39	ND	0	0	65
		6	1474	1277	392	302	1572	1278	0	182	0	124	0	1041	533	415

* With IT-22 mouse cells.

Table 2. Immunoreactivity of clones of hybridomas

Hybridoma	Ratio of antibody-producing clones	Ratio of clones showing same reactivity as mass cultures
691-2	9/10	9/9
691-4	10/13	10/10
691-6	9/9	8/9
691-13	22/26	22/22
691-1-5Nu 4	12/12	12/12

crossreactivity. As shown in Fig. 1, antibodies secreted by clones 155 and 27 of hybridoma 691-4 exhibited the greatest extent of binding to homologous (691) and 690 melanomas and some crossreactivity with WI38, FS2, and melanoma 489. Binding of the antibody to melanoma 843 was probably not above the background level.

Antibodies produced by clone 4 of 691-2 hybridoma reacted with all melanomas except melanoma 1614 and with the three normal human cells. These antibodies did not seem to crossreact with the four colorectal carcinomas. Antibodies produced by clone 17 of 691-13 hybridoma reacted only with three melanomas—690, 691, and 1694—and not with other cells including human erythrocytes and lymphocytes (not shown in Fig. 1). The results obtained with clone 691-6-5 indicate that these antibodies reacted better with the four melanomas 691, 690, 1614, and 843 than with melanoma 489 and colorectal carcinoma 1116. No reactivity was detected in the test with either HFF or FS2 normal human cells, or human erythrocytes, or lymphocytes (not shown in Fig. 1). Culture fluid of clone 42 of 691-I-5Nu-4 hybridoma exhibited low cpm in radioimmunoassays with 691 and 690 melanomas (Fig. 1) and even lower binding with homologous 691-I-5Nu tumor (Fig. 2). When 5×10^5 live rather than 4×10^4 glutaraldehyde-fixed cells were used as immunoadsorbent (Fig. 2), much higher binding of clone 42 antibody to 691 and 691-I-5Nu tumor cells was observed. When live cells were substituted for glutaraldehyde-fixed cells in the radioimmunoassay, culture fluids of other 691-I-5Nu hybridomas also exhibited much higher binding to target cells. However, antibodies that did not bind to glutaraldehyde-fixed cells also did not interact with live cells.

In order to determine the topographical relation between antigenic determinants recognized by various hybridoma antibodies, we tested by radioimmunoassay mixtures of antibodies obtained from clones of different hybridomas for competitive binding to 691 melanoma. As shown in Fig. 3, each antibody was used under saturating conditions because the use of a double concentration of the antibody did not lead to a further increase of cpm (shown only for 691-6-5 in Fig. 3). It can be seen, however, that after saturation of target cells with antibody from either clone 691-13-17 or clone 691-19-19, 691-6-5 antibody can still bind to the target cells. Because the amount of labeled antibody used in the assay is certainly not limiting

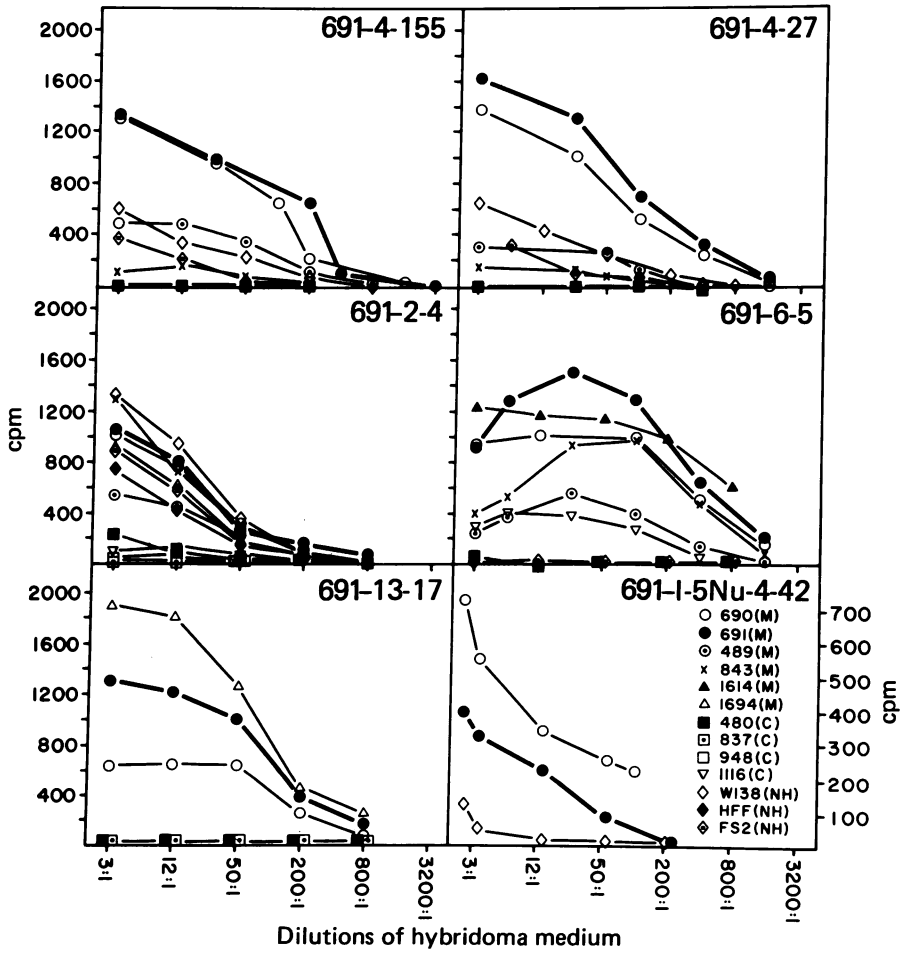

FIG. 1. Dilutions of hybridoma media were tested by radioimmunoassay for binding to cells of either human tumors or normal tissue. (M) = melanoma; (C) = colorectal carcinoma; (NH) = normal human cells.

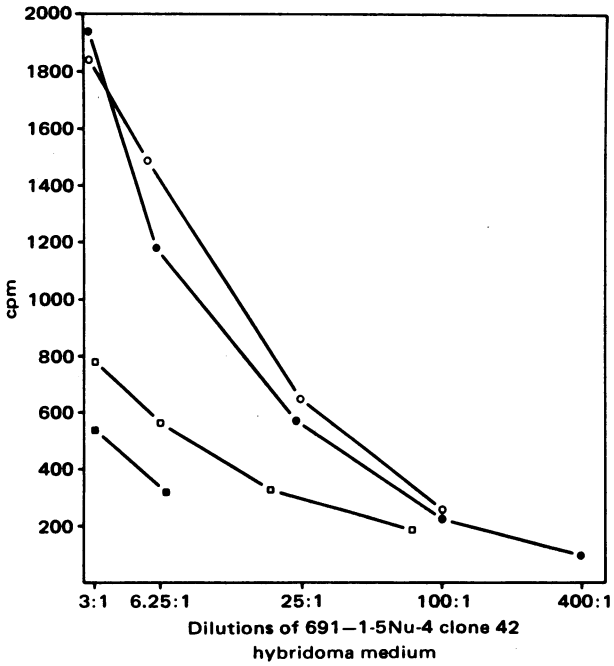

FIG. 2. Dilutions of medium of anti-691–I-5Nu-4 hybridoma clone 42 were tested by radioimmunoassay for binding to either *live* or *fixed* 691 and 691–I-5Nu cells, respectively. O, Live 691; ●, live 691–I-5Nu; □, fixed 691; ■, fixed 691–I-5Nu.

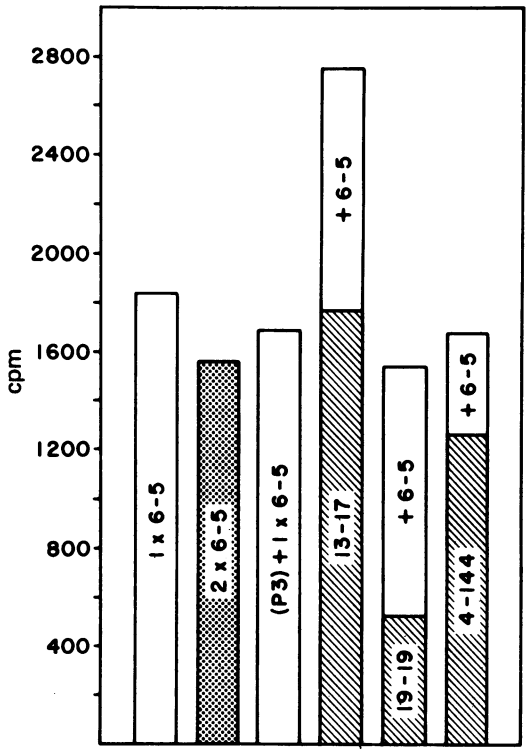

FIG. 3. Antibodies obtained from clones of four different hybridomas were tested by radioimmunoassay for binding to melanoma 691 in the following combinations: 691–6-5 antibody diluted with equal amounts of phosphate-buffered saline (1 × 6-5 white bar); 691–6-5 antibody at double concentration (2 × 6-5 dotted bar); 691–6-5 antibody plus equal amounts of P3 medium (P3 + 1 × 6-5 white bar); 691–13-17, 691–19-19, and 691–4-144 tested alone (slashed bar); 691–6-5 at single concentration of antibody mixed with 691–13-17, 691–19-19, and 691–4-144, respectively (part slashed, part white bar).

with regards to the maximum of cpm observed, there is clearly an additive effect of antibody binding in the above combinations. The results with mixtures of 691–4-144 and 691–6-5 antibodies are more difficult to interpret because the extent of additional binding with 691–6-5 is close to the limit of the experimental variability of the assay.

Suppression of Growth of Melanoma in *Nude* Mice. Hybridomas 691–4 and 691–6 and P3 tumors grew progressively in mice, ultimately killing the animals on or about the 36th day of the experiment. The 691–I-5Nu tumor grew progressively in mice receiving P3 cells, whereas growth of the tumor in hybridoma-bearing mice was inhibited (Fig. 4). Histological examination revealed only few inflammatory cells around the area of 691–I-5Nu necrotizing tumor in hybridoma-treated mice. Serum and ascitic fluid removed from mice injected with hybridoma cells showed a 500-fold increase in cpm in binding with 691 melanomas as compared to culture fluids of the same hybridomas.

DISCUSSION

In the present study, monoclonal antibodies were produced by hybrid cells obtained by fusion of P3 × 63 Ag8 myeloma cells with spleen cells from mice previously immunized with human melanoma cells. Antibodies secreted by individual hybrids were subsequently tested at various concentrations for their reaction with a panel of target cells in an indirect radioimmunoassay in which the amount of monoclonal antibody's binding to a given target cell was quantitated by means of a second, iodinated anti-mouse $F(ab')_2$ antibody (label). Obviously, the data from such analyses, when expressed in cpm (amount of label bound), depend not only on the amount of hybridoma antibody bound to target cell but also on the amount and specific radioactivity of the label used in the assay. However, because the same label was used in each assay, differences in cpm must reflect variations in the amount of a given hybridoma antibody bound to the various target cells. Differences in cpm might also reflect: (*i*) affinity of the given hybridoma antibody for the antigenic determinant expressed on the target cell (epitope); (*ii*) the distributions of determinants (monovalent versus multivalent antigen–antibody interaction); and (*iii*) by the total number of epitopes expressed by the target cells. The third variable is certainly of considerable importance in the present study, because the various target cells differed with regard to the amount of antibody that could be bound under saturating conditions (plateau values, Fig. 1).

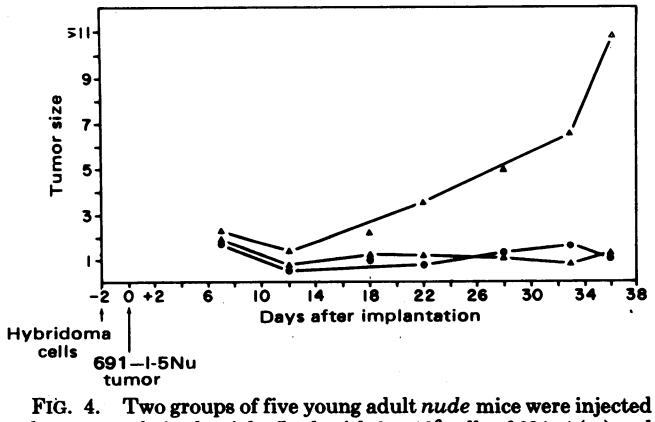

FIG. 4. Two groups of five young adult *nude* mice were injected subcutaneously in the right flank with 2×10^6 cells of 691–4 (●) and 2×10^6 cells of 691–6 (▲) hybridomas, respectively (see Table 1). Two days later, 1×10^7 cells of 691–I-5Nu tumor were implanted under the skin of the back of these mice. Three *nude* mice that received implants of 691–I-5Nu tumors 2 days after being injected with 1×10^7 P3 cells served as controls (△).

Despite the difficulty in interpreting accurately, at present, the various degrees of binding of monoclonal antibodies, detectable binding in a set of target cells can be taken as evidence for the presence of an identical or crossreactive antigenic determinant. Thus, the use of the various monoclonal antibodies enabled us to differentiate clearly between melanoma, colorectal carcinoma, and normal human cells, as well as between individual melanomas or colorectal carcinomas (unpublished data). For instance, 691–13 antibodies did not crossreact at all with heterologous tumors, whereas 691–2 antibodies crossreacted with normal human cells and possibly with some colorectal carcinoma cells. The present study also demonstrates that monoclonal antibodies can be used to delineate the topographical relationship between determinants by analysis of competitive or additive binding of monoclonal antibodies to a given target cell. It is interesting that the antibody combinations that delineate different epitopes on the melanomas (691–6 and 691–13 or 691–6 and 691–19) show additive binding. This indicates that the given epitopes are sufficiently separated to exclude steric inhibition. This does not indicate, however, whether the given epitopes are impressed on the same or on distinct cell-surface molecules. On the other hand, 691–6 and 691–4, the two antibodies that cannot be easily differentiated on the basis of their reaction with the panel of melanomas, seemed to bind competitively. The specificity of these two antobidies was confirmed by *in vivo* experiments in which the growth of melanoma hybrid tumor 691–I-5Nu was suppressed (Fig. 4).

The nature of the antigen(s) detected on the surface of the melanoma cells by their reactivity with some of the hybridoma antibodies is still unknown. Media of some of the hybridomas originating from lymphocytes of mice immunized against 691 melanoma crossreacted with 691–I-5Nu tumor and anti-691–I-5Nu-4 antibodies showed identical affinity for 691–1-5Nu and 691 tumor (Fig. 2). Because 691–I-5 tumor is a human–mouse hybrid containing only human chromosomes 14, 17, and 21, it does not express *HLA* haplotype and hence the hybridoma antibody in most cases is not directed against an HLA antigen. The fact that antibody secreted by either of the two hybridomas 691–6 or 691–4 suppressed growth of the hybrid (691–I-5Nu) tumor in mice may perhaps indicate that among various antigens coded by human chromosomes 14, 17, or 21 in the hybrid one or more may be involved in the phenotypic expression of malignancy and may ultimately be recognized by the monoclonal antimelanoma antibody.

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