Tumor Hybrid Cells: An Immunotherapeutic Agent [1,2]

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SUMMARY—Hybrid tumor cells were formed by the spontaneous and virus-induced fusion of EL-4 lymphoma cells with C3H fibroblasts. Some resultant hybrid cell lines specifically immunized C57BL/6 and (C57BL/6 × C3H)F_1 mice against subsequent lethal EL-4 challenge. The immune response to the hybrid tumor cells was superior to the response to irradiated EL-4 cells. The possibility of hybrid tumor cells as a specific immunotherapeutic agent was discussed.—J Natl Cancer Inst 52: 1541–1545, 1974.

IT IS RECOGNIZED that the tumor-associated transplantation antigens (TATA) of many animal and human neoplasms may evoke specific host immune responses (*1, 2*). The response may be cellular [immune lymphocytes (*3, 4*) or activated macrophages (*5*)] or humoral [specific antibody (*6–8*)]. If the host mounts an "effective" immune response, the tumor regresses; however, if the tumor progressively grows, the immune response is "ineffective"; i.e., it has not led to tumor rejection.

The proposed function of immunotherapy in neoplastic disease is to stimulate the host's immune system and to destroy the residual tumor cells after the tumor cell number has been reduced by surgery, irradiation, or chemotherapy. Immunotherapy may consist of either specific and/or nonspecific stimulation of the immune system. The use of nonspecific stimulants such as the attenuated tubercule bacillus, BCG, or *Corynebacterium parvum* has shown promise in animal and some human neoplastic diseases (*9–11*). Specific stimulation of the immune system has been attempted with killed tumor cells (*6, 9, 12, 13*), partially purified TATA (*14*), enzyme-treated cells (*15*), and chemically modified tumor cells (*16*). The use of viable cells, even after pretreatment, is limited, since too large an inoculum may give rise to local tumors. The number of killed cells that can be used is not limited; however, the response to killed cells or purified TATA is less than to viable cells (*6*).

A model immunotherapeutic agent would be a viable cell that expresses the TATA, enhances the immunogenicity of the TATA, and is nonmalignant. Cell fusion techniques permit the introduction of foreign transplantation antigens into tumor cells and the alteration of the tumor's growth characteristics. As shown by Britten, using Moloney virus-induced lymphomas (*17*), and Chen and Watkins with Ehrlich ascites tumor-fibroblast hybrid cells (*18*), foreign transplantation antigens may have a cellular carrier effect that increases the host's response to the TATA. Malignant cells fused with nonmalignant cells do not give rise to tumors in adult animals, though they may produce tumors in sublethally irradiated neonatal animals (*19, 20*). This paper reports the effectiveness of hybrid tumor cells as specific immunotherapeutic agents.

MATERIALS AND METHODS

Animals and tumors.—C57BL/6 (*H-2*^{b}), C3H/HeJ (*H-2*^{k}), and A/J (*H-2*^{a}) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. The F_1 offspring were bred in our animal quarters. Mice were immunized at the age of 10 weeks. The chemically induced lymphoma EL-4, carried in C57BL/6 female mice, and fibrosarcoma-1, FS-1, carried in A/J male mice, were obtained in their ascites form from Dr. G. Berke, Children's Hospital Medical Center, Boston, Massachusetts, and transferred weekly.

Cell fusion.—The EL-4 tumor was harvested from the peritoneal cavity with Hanks' balanced salt solution (HBSS), washed, then resuspended at a concentration of 5×10^6 cells/ml in HBSS. Clone 1-D fibroblasts (C3H origin), thymidine kinase-deficient, were continuously grown in Dulbecco's medium with 10% fetal calf serum, glutamine, and antibiotics (penicillin, streptomycin, Mycostatin, and kanamycin) (complete medium). After trypsinization, the fibroblasts were resuspended at a concentration of 1×10^6 cells/ml in HBSS. One ml of each cell suspension was mixed in a 13×75-mm test tube with 0.2 ml β-propiolactone-inactivated Sendai virus (Microbiological Associates, Bethesda, Md.), and the mixture was chilled at 4° C for 45 minutes (*21*). One ml complete medium was added, and the mixture was gently agitated at 37° C for 30 minutes. In each 65-mm tissue culture dish (Falcon Plastics, Los Angeles, Calif.), 0.5×10^6 fibroblasts were plated in complete medium with hypoxanthine (10^{-4}M), aminopterin (4×10^{-7}M), and thymidine (4×10^{-5}M) added (HAT medium) (*22*).

For spontaneous fusion experiments, 1×10^6 fibroblasts were plated in a 65-mm dish in complete medium; after 6 hours, the medium was removed and 5×10^6 EL-4 cells in 1 ml complete medium were added and incubated overnight (*23*). The next day the medium was changed to HAT medium. In all experiments the HAT medium was changed twice weekly. After 10 days, isolated colonies of cells were observed.

Cytotoxicity testing.—Fibroblast cytotoxicity was tested on confluent hybrid monolayers grown in Microtest plates (Falcon #3034, Falcon Plastics) (*24*).

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The medium was removed, 1 μl serum was added per well, and the plates were incubated at room temperature for 1 hour. Then 2 μl fresh rabbit serum diluted 4:1 with mouse serum was added to each well and the plates were incubated at 37° C for 1 hour. The plates were washed with HBSS and stained with 0.1% trypan blue, and the degree of cytotoxicity was determined. Mouse monospecific H-2 antisera were obtained from the Transplantation Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

Assay for malignancy.—The ability of the hybrid cells to produce tumors was assayed in (C57BL/6 × C3H)F_1 mice (*25*). Neonatal mice received 400 R total-body irradiation and then were given subcutaneous (sc) injections of 1×10^6 hybrid tumor cells in the right flank. Animals were observed for 6 months for the emergence of tumors. Some tumors were transplanted to adult F_1 mice.

Tumor challenge.—Animals were routinely challenged with 1×10^4 EL-4 tumor cells sc in the left groin region and observed weekly for tumor growth and death.

RESULTS

Hybrid Cell Formation

EL-4 cells were fused both spontaneously and with inactivated Sendai virus with clone 1-D fibroblasts. Colonies of cells were isolated in selective HAT medium. The EL-4 cells did not adhere to the tissue culture dishes, and the thymidine kinase-deficient clone 1-D cells were killed in the selective medium. The isolated cell lines were proved to be hybrid cells by fibroblast cytotoxicity testing with the use of monospecific mouse anti-H-2 antisera: antispecificity 33 (C57BL/6, EL-4) and anti-23 (C3H, clone 1-D). All the isolated cell lines exhibited complete cytotoxicity with antisera against both parental histocompatibility specificities.

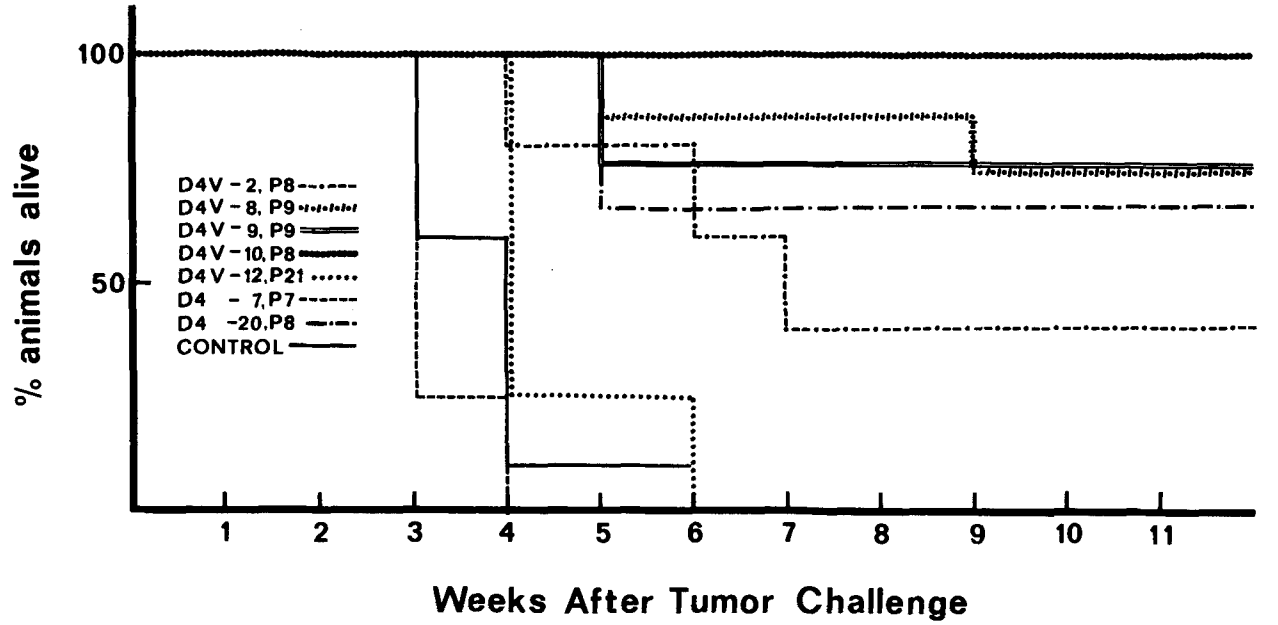
Expression of EL-4 TATA by Hybrid Tumor Cells

To determine if the hybrid cells expressed the EL-4 TATA and could immunize animals against EL-4, C57BL/6 mice were given intraperitoneal (ip) injections of 1×10^6 hybrid cells. Text-figure 1 shows the mortality curves of mice inoculated with both virally and spontaneously induced hybrid cell lines. Three virus-induced cell lines (D4V-8, -9, and -10) and 1 spontaneously induced line (D4-20) conveyed significant protection against subsequent lethal challenge with 1×10^4 EL-4 cells. If 1×10^2 EL-4 cells were injected sc, 100% death resulted in 4–6 weeks. One virus-induced line (D4V-2) gave intermediate protection, but 1 viral (D4V-12) and 1 spontaneous line (D4-7) gave no immunity. Surviving animals were resistant to repeat challenge with 1×10^5 EL-4 cells and, in some cases, 1×10^6 cells.

The virally and spontaneously induced hybrid cell lines were no different in their capacity to immunize C57BL/6 mice or in the frequency of protective hybrid cell lines.

The nonprotective hybrid cell lines possibly were derived from nontumor C57BL/6 cells. Cell line D4V-12, which was nonprotective, had been protective when first assayed. During subsequent continuous tissue culture passage, the immunizing capacity of the line was lost.

The specificity of the tumor protection afforded by the hybrid tumor cells was assessed in C57BL/6 × A/J (B6A) mice immunized with D4V-8 cells. The B6A mice were then challenged with EL-4 and FS-1, an



TEXT-FIGURE 1.—Mortality curves of C57BL/6 female mice given ip injections of 1×10^6 hybrid tumor cells and challenged 1 month later with 1×10^4 EL-4 cells sc. Test groups had 5 mice/group; controls (given saline), 10 mice. All animals died of tumors. P = tissue-culture passage number of cell line.

A/J strain-specific tumor. There was complete immunization against EL-4, but none against FS-1 (table 1).

Malignancy

The malignancy of the hybrid cells, i.e., the capacity to produce tumors in immunologically unresponsive hosts, was determined in sublethally irradiated neonatal F_1 mice. Approximately 20% of all animals developed tumors, regardless of the protective capacity of the hybrid cell lines (table 2). The resultant neoplasms were transplantable to adult F_1 but not C57BL/6 mice and microscopically were similar to the original EL-4 tumor, except that the cytoplasmic-to-nuclear ratio was increased. In 15 months, no adult mice produced tumors when inoculated with tissue culture hybrid cells.

The neonatal F_1 mice not developing lesions were tested for tumor immunity. Approximately half the animals were resistant to EL-4 tumor challenge.

Function of Foreign Histocompatibility Antigens

To negate the possible influence of the C3H histocompatibility antigens on host response to the hybrid tumor cells, the cell lines were assayed for their immunizing capacity in synergistic (C57BL/6 × C3H)-F_1 mice (table 3). There was no difference between the protection afforded in parental C57BL/6 and F_1 mice. The capacity of the hybrid cells to immunize against subsequent lethal tumor challenge was not totally dependent on histoincompatibility between the hybrid cells and the host.

TABLE 1.—*Specificity of immunity produced by EL-4/1-D hybrid cells*

Challenge tumor	Number of mice alive 60 days after tumor challenge/No. challenged*	
	Immunized	Nonimmunized
EL-4 (1×10^4 cells, sc)	4/4	0/4
FS-1 (1×10^6 cells, sc)	0/4	0/4

*(C57BL/6 × A/J)F_1 mice were immunized 1 month before tumor challenge with 1×10^6 D4V-8 hybrid cells (25th passage) ip.

TABLE 2.—*Ability of hybrid tumor cells to produce tumors*

Hybrid line (passage)	Number of animals developing tumors within 6 months/total No.*
D4V-2 (P10)	1/5
D4V-8 (P10)	1/5
D4V-9 (P8)	1/5
D4V-10 (P7)	2/9
D4V-12 (P20)	0/4
D4-20 (P6)	1/2

*(C57BL/6 × C3H)F_1 neonatal mice were irradiated with 400 R and given injections of 1×10^6 EL-4/1-D hybrid cells sc.

TABLE 3.—*Immunizing capability of EL-4/1-D hybrid cell line*

Method of fusion (passage)	Strains inoculated *	
	C57BL/6†	(C57BL/6× C3H)F_1†
Sendai virus:		
Lines D4V-1 (P6)	−	NT
-2 (P7)	+	+
-3 (P6)	−	−
-4 (P6)	−	−
-5 (P6)	NT	+
-6 (P7)	NT	+
-8 (P8)	+	+
-9 (P6)	+	+
-10 (P7)	+	+
-11 (P6)	NT	−
-12 (P6)	+	+
-13 (P7)	−	−
-14 (P6)	NT	−
-15 (P6)	−	NT
-20 (P6)	+	NT
Spontaneous:		
Lines D4-7 (P8)	+	−
-20 (P6)	+	−

*Animals were immunized by 1×10^6 hybrid cells sc and challenged with 10^4 EL-4 tumor cells sc 1 month later.

†−: Animals died with tumors; hybrid cells did not immunize. +: No animals died with tumors; hybrid cells did immunize. NT: Not tested.

Comparison of Hybrid Tumor Cells With Irradiated Tumor Cells

Since the hybrid tumor cells were equally protective in syngeneic and semiallogeneic animals, their protective ability might be due to the loss of malignancy achieved by the cellular fusion, which permitted immunization not usually possible with numbers of viable cells. The immunity might be equal only to that achieved with irradiated tumor cells with the same TATA.

Viable hybrid D4V-8 cells evoked a more efficient immune response than an equal number of EL-4 tumor cells given 5000 R (table 4); comparison with an equal number of viable tumor cells was not possible.

TABLE 4.—*Comparison of immunizing capacity of irradiated EL-4 cells and EL-4/1-D hybrid cells*

Immunizing cell	Number of animals alive 2 months after tumor challenge/No. challenged*
D4V-8 (P73)	19/21
EL-4 (5000 R)	2/11

*C57BL/6 mice were challenged with 1×10^4 EL-4 cells sc 1 month after the ip injection of 1×10^6 immunizing cells.

DISCUSSION

Specific immunotherapy has shown promise with some animal models; it has had limited use in human neoplastic disease. The use of viable tumor cells as the specific immunizing agent has several limitations. In syngeneic systems, the dose of cells is limited to minimize the possibility of tumor growth at the site of injection. In some animal models, viable cells

can be used where the specific tumor antigen is expressed on a background of allogeneic histocompatibility antigens (*19*). The histoincompatibility of the tumor cells permits their rejection, with an increased reactivity to the TATA. However, knowledge concerning the antigenic grouping of human tumor antigens is limited. Therefore, human cells cannot be used with allogeneic histocompatibility antigens with the certainty that they express the appropriate TATA. Killed tumor cells may be used; however, animal experiments have demonstrated that viable cells are superior to killed cells as immunotherapeutic agents (*6*).

Theoretically, a specific immunotherapeutic agent is a viable cell that expresses the TATA but is not malignant in the host to be immunized. An increase in the immunogenicity of the TATA is also desirable. Hybrid tumor cells fulfill many characteristics of this theoretical agent.

Hybrid cell lines were formed by the spontaneous and virus-induced fusion of EL-4 tumor cells and nonmalignant fibroblasts. Some hybrid lines could immunize C57BL/6 and (C57BL/6 × C3H)F_1 mice against subsequent lethal challenge. The ip and sc routes of immunization were equally effective. The use of inactivated Sendai virus raised questions concerning the effects of viral RNA introduction into the hybrid cells. No difference was evident between the spontaneously and virus-induced hybrid cells in their ability to immunize against EL-4.

The immunity achieved after hybrid cell immunization was specific for the parental tumor line and not a nonspecific stimulation of the host's immune system, since the immunized animals were not protected against an unrelated tumor, fibrosarcoma-1. The possibility that the immunity was due to a minor histocompatibility antigen difference cannot be completely excluded.

Chromosome loss continually occurs during long-term tissue culture of the hybrid cells. If the chromosome that controls TATA expression is missing, the hybrid tumor line will be unable to immunize (as in the D4V-12 line); the hybrid cell line could immunize in its sixth passage (table 3) but not in its 21st passage (text-fig. 1). For hybrid cells to be used as immunotherapeutic agents, it will be necessary to reclone periodically the cell lines and determine the continued presence of TATA on the hybrid cells.

Hybrid tumor cells formed by the fusion of malignant cells with nonmalignant fibroblasts are usually nonmalignant. After moderate chromosome loss in continuous tissue culture or by in vivo selection in immunologically unresponsive hosts, some hybrid cells may give rise to tumors. It is speculated that the loss of normal chromosomes of the nonmalignant parental cell permits the re-expression of the malignancy of the parental tumor line (*19*).

The EL-4 hybrid tumor cells could produce tumors in ≈20% of sublethally irradiated neonatal F_1 mice. The immunologically unresponsive F_1 mice permitted the growth of cells that had the appropriate chromosome loss. Recently, hybrid tumor cells that have lost the histocompatibility antigens of the nonmalignant parental line have been selected. These cells can produce tumors in sublethally irradiated mice of the parental tumor strain (*26*). If the hybrid tumor cells gave rise to tumors in normal adult animals, the usefulness of the hybrid cells as immunotherapeutic agents would be limited. No adult F_1 or C57BL/6 mice developed tumors after injection with tissue-culture hybrid tumor cells in 15 months of experiments. The hybrid cells did not produce tumors even after long-term culture, since the defense mechanisms of the adult animals rejected hybrid cells with malignant potential.

Tumors arising in the neonatal mice were transplantable into adult F_1 mice but not into C57BL/6 mice. If hybrid tumor cells were to be used in human disease, the rejection of the foreign histocompatibility antigens of the hybrid cells would be further security against local tumor growth.

From the observations of Chen and Watkins (*18*), the original hypothesis for the effectiveness of the hybrid tumor cells was that the foreign histocompatibility antigens introduced into the tumor by cell fusion operate as a cellular "carrier" effect. If histoincompatibility was necessary for the hybrids to be effective immunizing agents, then it would be anticipated that they would be effective in semiallogeneic parental tumor hosts (C57BL/6) but ineffective in syngeneic F_1 hosts. The F_1 hosts were equally protected as the parental tumor strain (table 3). The capability of the hybrid cells to immunize effectively against subsequent lethal tumor challenge did not totally depend on the histoincompatibility between the hybrid and the host.

In adult mice, the mmunizing capability of the hybrid tumor cells was presumably due to the change in the malignancy of the tumor produced by cell fusion. The hybrid tumor cells would allow the immunization of the host with the EL-4 TATA on a viable nonmalignant cell. The hybrid cells gave almost complete protection against the standard tumor challenge and were superior to irradiated tumor cells that gave almost no protection. The viable cells may be capable of longer survival and propagation in the inoculated animal and thus produce a more vigorous response.

Some hybrid cell lines formed by the fusion of EL-4 tumor cells with nonmalignant fibroblasts specifically immunize against subsequent lethal tumor challenge. The hybrid cells are nonmalignant, and the protection afforded is superior to that evoked by irradiated tumor cells. Hybrid tumor cells seem to hold promise in human disease. The patient's own tumor could be made nonmalignant by cell fusion with normal fibroblasts. The use of the patient's tumor will assure that the appropriate TATA is used for immunization.

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