ARG4 translation start), and the probe is Nco I–Bgl II (−4.3 kb to −3.4 kb relative to *ARG4*). *THR4* centromere-proximal (Fig. 2B): Bgl II (nts 204996 to 215453), and the probe is Hind III–Hind III (nts 214047 to 214935). The band in lanes 1 and 17 through 23 of Fig. 2B apparently located just after the start of *YCR84w* is artifactual and is due to contamination of the probe with an adjacent fragment. *YCL14w-NFS1* (Fig. 2C): *Xho*I (nts 90286 to 99224), and the probe is Kpn I–Eco RI (nts 91185 to 91571). *YCL4w-YCL11c* (Fig. 2D): Xho I (nts 99224 to 118089), and the probe is Eco RI–Hind III (nts 100317 to 103054).

30. Diploid strains homozygous for *pho80ΔX::LEU2* or *pho4Δ25::LEU2* (L. W. Bergman, personal communication) were constructed by transforming *leu2* derivatives of the haploid parents of NKY1002 with the appropriate deletion or disruption. Yeast DNA was extracted at the indicated time after induction of meiosis, digested with Eco RI (Eco RI cuts 2.6 kb upstream and >10 kb downstream of the *PHO5* translation start site), displayed on a 1.2% agarose gel, transferred to membranes, and hybridized with an Eco RI–Cla I fragment (−2.6 kb to −2.2 kb). DSB positions were determined with external standards (Bst EII digests of λ DNA) and internal standards comprising Eco RI/Sal I, Eco RI/Bst EII, and Eco RI and Bam HI double digests of DNA from mitotic cells (Sal I, Bst EII, and Bam HI cut at nts +74, −185, and −542, respectively). DSB frequencies were determined as described above.

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Effective Tumor Vaccine Generated by Fusion of Hepatoma Cells with Activated B Cells

Yajun Guo, Mengchao Wu, Hen Chen, Xiaoning Wang, Guangluo Liu, Guanglo Li, Jing Ma, Man-Sun Sy

Fusion of BERH-2 rat hepatocellular carcinoma cells with activated B cells produced hybrid cells that lost their tumorigenicity and became immunogenic. Syngeneic rats injected with BERH-2–B hybrid cells became resistant to challenge with parental BERH-2 cells, and rats with established BERH-2 hepatomas were cured by subsequent injection of BERH-2–B cells. Both $CD4^+$ and $CD8^+$ cells were essential for the induction of protective immunity; however, only $CD8^+$ cells were required for the eradication of BERH-2 tumors. The generation of hybrid tumor cells that elicit antitumor immune responses may be a useful strategy for cancer immunotherapy.

Tumor cells may escape immune surveillance because they do not express signals that are essential for activation of the host immune system (*1*, *2*). At the molecular level, the defective signaling of tumor cells could be attributable to (i) down-regulation of major histocompatibility complex (MHC) molecules (*3*, *4*); (ii) alteration of antigen-processing pathways, resulting in an inability to present tumor-specific antigens to host T cells (*5*); (iii) absence of costimulatory or adhesion molecules that are essential for activation of the host immune system (6); or (iv) production of factors that modify host immune responses (*7*).

Activated B cells are the most effective antigen-presenting cells (*8*). We hypothesized that fusion of a tumor cell with an activated B cell would produce a hybridoma that both expressed tumor-specific antigens and had the machinery for antigen presentation and T cell activation.

BERH-2 is a chemical carcinogen–induced hepatocarcinoma from the Wistar rat (9). Cells derived from this tumor grow rapidly and form tumors in the liver of syngeneic animals. We obtained activated B cells from the spleens of rats injected 14 days earlier with bovine serum albumin in Freund's complete adjuvant (*10*). BERH-2 cells were fused with purified activated B cells by treatment with polyethylene glycol (PEG) (*11*). The fused cells were enriched by selection with a rabbit antiserum to BERH-2 cells and subsequent selection with a rabbit antiserum to rat B cells (*10*).

The parental BERH-2 cells expressed low amounts of MHC class I antigens and intracellular adhesion molecule–1 (ICAM-1) but were devoid of MHC class II antigens, leukocyte functional antigen–1 (LFA-1), and costimulatory molecule B7. In contrast, the BERH-2–B hybrid cell lines expressed MHC class II antigens, ICAM-1, LFA-1, and B7 (Fig. 1) and had enhanced expression of MHC class I antigens. These BERH-2–B cell lines have stably expressed both tumor and B cell antigens for more than 10 months.

All 10 rats injected intrahepatically with 2×10^6 parental BERH-2 cells developed liver tumors and died within 60 days (*12*). In contrast, 10 rats injected with the same number of BERH-2–B hybrid cells remained tumor-free for 180 days (*12*). All four hybrid

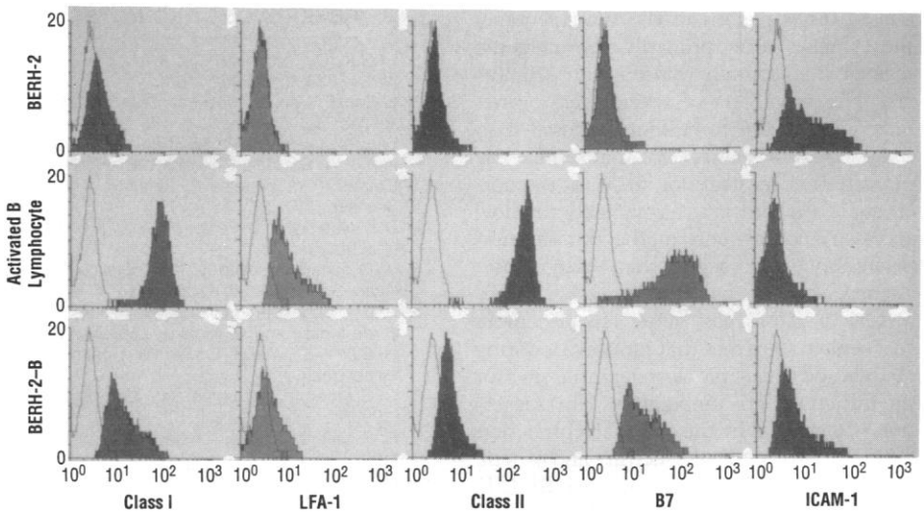

Fig. 1. Expression of MHC class I and class II antigens, B7, ICAM-1, and LFA-1 on BERH-2 cells, activated B cells, and BERH-2–B hybrid cells. Cells were washed with phosphate-buffered saline (PBS) and stained with monoclonal antibodies to rat MHC class I (OX-18), MHC class II (OX-6), ICAM-1 (IA 29), or LFA-1 (WT.1). To stain for rat B7, we used CTLA4-Ig, a soluble fusion protein containing the variable domain of the human CTLA-4 protein and the hinge, CH2, and CH3 domains of the human IgG1 constant region (*14*). Cells were incubated with the antibodies or chimeric protein for 30 min on ice. A mouse antibody to human CD3 (GH3, IgG2b) and a soluble human CD44-Ig chimeric protein were used as negative controls. Cells were washed three times. Fluorescein isothiocyanate (FITC)–conjugated goat antibody to mouse Ig or FITC-labeled rabbit antibody to human Ig was added for another 30 min on ice. Samples were then washed, fixed, and analyzed in a FACScan (Becton Dickinson, San Jose, California). Solid areas are cells stained with specific antibodies. Open areas are cells stained with control antibodies.

Y. Guo, M. Wu, H. Chen, X. Wang, G. Liu, G. Li, Tumor Immunology and Biotherapy Center, Eastern Institute of Hepatobiliary Surgery, Shanghai 200433, China.
Y. Guo, J. Ma, M.-S. Sy, Institute of Pathology, Case Western Reserve University, Cleveland, OH 44106.

cell lines we have examined lost their ability to form tumors in syngeneic rats. However, the hybrid tumor cells were able to grow and form tumors in nude mice (*12*).

Rats injected with hybrid BERH-2–B cells developed abundant lymphocytic infiltrates around the injected tumor cells (*12*). Most of the infiltrating cells were T lymphocytes. About 70% of these cells were $CD8^+$ and about 30% were $CD4^+$, as revealed by immunofluorescence staining of tissue sections with monoclonal antibodies specific for rat CD8 or CD4. There was no inflammatory response in animals injected with parental BERH-2 cells (*12*).

The rats that were immunized with BERH-2–B hybrid cells did not support growth of parental BERH-2 cells. All animals immunized with the BERH-2–B cells remained tumor-free for more than 150 days after challenge with BERH-2 cells. However, all rats immunized subcutaneously with BERH-2 cells or irradiated BERH-2 cells and then challenged intrahepatically with BERH-2 cells developed tumors and died within 60 days (Fig. 2A). Immunization with BERH-2–B cells could also eradicate established hepatomas. The rats that were injected with parental BERH-2 cells and then treated with BERH-2–B cells survived for more than 120 days. In contrast, rats injected and treated with the parental BERH-2 cells all died within 42 days (Fig. 2B). In other experiments, we surgically implanted small fragments of a BERH-2 hepatoma into the liver of rats (*9*). After 10 days, a fraction of the tumor-implanted animals were injected with BERH-2–B cells and the others were injected with parental BERH-2 cells. All rats injected with BERH-2 cells died within 50 days. Two of the eight rats injected with BERH-2–B hybrid cells developed tumors and died at 71 and 74 days after tumor implantation. The other six animals lived for more than 180 days after tumor implantation (Fig. 2C).

We next determined whether tumor rejection induced by BERH-2–B cells was mediated by $CD4^+$ or $CD8^+$ T cells. Rats were depleted of $CD4^+$ or $CD8^+$ cells by antibody treatment before injection of BERH-2–B cells. BERH-2–B cells were able to form tumors in CD4- and CD8-depleted rats (Table 1). In another experiment, rats were immunized with BERH-2–B cells, depleted of $CD4^+$ or $CD8^+$ cells 14 days later, and then challenged with BERH-2 cells. Tumors developed in rats depleted of $CD8^+$ cells but not in those depleted of $CD4^+$ cells (Table 1). Thus, both $CD4^+$ and $CD8^+$ cells appear to be necessary for the induction of immunity, but after the induction of the immune response, $CD8^+$ cells can mediate tumor cell destruction in the absence of $CD4^+$ cells. These results differ from those obtained with murine melanomas, where it has been shown that tumor cells transfected with the B7 gene can directly stimulate naive $CD8^+$ T cells without the participation of $CD4^+$ T cells (*6*).

To investigate whether the immunity induced by BERH-2–B cells was tumor-specific, we tested the effect of these cells on NBT-II,

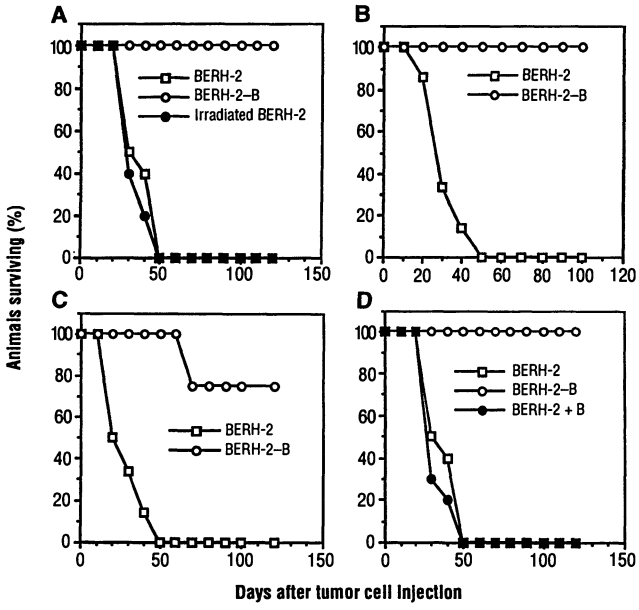

Fig. 2. Induction of protective immunity with BERH-2–B hybrid tumor cells or nonselected BERH-2–B hybrid tumor cells. (**A**) Three groups of female Wistar rats (eight per group) were injected subcutaneously with 2×10^6 BERH-2 cells, BERH-2–B cells, or irradiated (5000 roentgens) BERH-2 cells. After 2 weeks, all three groups were challenged with 5×10^6 BERH-2 cells injected intrahepatically. This experiment was repeated twice with identical results. (**B**) Fourteen rats were injected intrahepatically with 2×10^7 BERH-2 cells. Ten days later, eight of the injected rats were immunized with a subcutaneous injection of 5×10^6 BERH-2–B hybrid cells. The other six rats were injected subcutaneously with the same number of BERH-2 cells. This experiment was repeated twice with identical results. (**C**) Fourteen rats were intrahepatically implanted with a small fragment (0.3 mm by 0.5 mm) of BERH-2 tumor. After 10 days, eight of the animals were injected subcutaneously with 5×10^6 BERH-2–B hybrid cells. The other six rats were injected subcutaneously with the same number of BERH-2 cells. This experiment was repeated twice with identical results. (**D**) Three groups of rats (eight per group) were injected subcutaneously with 5×10^6 BERH-2 cells, 5×10^6 BERH-2 cells fused with 5×10^6 activated B cells, or 5×10^6 BERH-2 cells mixed with 5×10^6 activated B cells in the presence of PEG. Fused cells were washed three times with PBS, resuspended in PBS, and injected subcutaneously. Two weeks later, all rats were challenged with 5×10^6 BERH-2 tumor cells intrahepatically. This experiment was repeated three times with comparable results.

Table 1. Effects of depletion of $CD4^+$ or $CD8^+$ T cells on the growth of BERH-2–B and BERH-2 cells in vivo.

Antibody specificity	Treatment protocol		Number of animals with tumors
	Ab, then immunize*	Immunize, then Ab†	
None	–		0/6
CD4	+		4/6
CD8	+		5/6
Control Ab	+		0/6
CD4		+	0/5
CD8		+	5/5
Control Ab		+	0/5

*Female Wistar rats were treated with purified mouse monoclonal antibodies (Abs) to rat CD4 (OX-38) or CD8 (OX-8) or a control mouse monoclonal antibody to diethylthiamine pentaacetic acid. Each animal received 500 μg of the purified antibody intravenously twice per week for 3 weeks. Two days before injection of tumor cells, peripheral blood lymphocytes were obtained from individual, treated rats and stained with antibodies to CD4 or CD8 to verify the depletion of $CD4^+$ or $CD8^+$ T cells. Treatment with the antibody to CD4 depleted >95% of the $CD4^+$ cells, treatment with the antibody to CD8 depleted ~95% of the $CD8^+$ cells, and treatment with the control antibody did not alter the number of $CD4^+$ or $CD8^+$ cells. Three days after the last injection of antibodies, all rats were injected intrahepatically with 5×10^6 BERH-2–B tumor cells. †Female Wistar rats were immunized with 2×10^6 BERH-2–B cells injected subcutaneously. Two weeks after immunization, animals were treated with an antibody to CD4, an antibody to CD8, or a control antibody. The depletion of each cell type was verified by immunofluorescence staining. Three days after the last injection of the antibodies, all animals received 5×10^6 BERH-2 cells intrahepatically. These experiments were repeated twice with comparable results.

Table 2. Specificity of the immune response elicited by BERH-2–B hybrid tumor cells. Female Wistar rats were injected subcutaneously with 2×10^6 BERH-2–B cells. Two weeks after immunization, one group of rats was injected intrahepatically with 5×10^6 BERH-2 cells. Another group of rats was injected subcutaneously with 5×10^6 NBT-II rat bladder carcinoma cells (obtained from American Type Culture Collection). Tumors developed locally in the subcutaneously injected site in all rats immunized with BERH-2–B tumor cells and challenged with NBT-II tumor cells. All animals in this group died within 45 days after tumor cell challenge. This experiment was repeated twice with identical results.

Immunization cells	Challenge cells	Number of animals with tumors
BERH-2–B	BERH-2	0/8
BERH-2–B	NBT-II	8/8

a bladder carcinoma that grows rapidly in syngeneic Wistar rats. Immunization with BERH-2–B cells did not inhibit the growth of NBT-II cells in vivo (Table 2). In addition, $CD8^+$ T cells from rats immunized with BERH-2–B lysed BERH-2 cells but not NBT-II cells in vitro (*12*).

Finally, we determined whether in vitro selection of hybrid tumor cells was obligatory for the induction of tumor immunity. After fusing BERH-2 tumor cells with activated B cells, we washed the mixture of cells and injected them subcutaneously into syngeneic rats without prior in vitro selection. The efficiency of the fusion ranged from 30 to 50%. For controls, we injected BERH-2 tumor cells mixed with activated B cells in the absence of PEG. All animals were then injected with the parental BERH-2 cells intrahepatically. Only animals immunized with tumor cells fused with activated B cells were protected from tumor formation. Simply mixing tumor cells with activated B cells was not effective in inducing protective immunity (Fig. 2D), nor was treating BERH-2 tumor cells with PEG alone (*12*).

In summary, a BERH hepatocarcinoma–specific vaccine in rats can be made by fusing tumor cells with syngeneic, activated B cells. In addition to MHC class II and B7 antigens, BERH-2–B cells may express other cell surface molecules that are essential for the stimulation of host T cells. Production of B cell–specific cytokines by hybrid tumor cells may be important in the elicitation of host immune responses (*13*). BERH-2 cells fused with activated T cells were unable to stimulate BERH-2–specific immune responses (*12*). Preliminary experiments suggest that tumor cells fused with activated allogeneic B cells are also immunogenic and can induce protective immunity (*12*).

In order to induce protective immunity, the hybrid tumor cells must retain their capacity to express tumor-specific antigens. In addition, the hybrid tumor cells must be able to process and present tumor-specific antigens so as to activate host T cells. Whether this approach can be used in other tumor models remains to be determined. Our observation that protective immunity can be induced by tumor cells fused with activated B cells without in vitro selection may have broad clinical applications and may provide a useful strategy for cancer immunotherapy.

REFERENCES AND NOTES

1. M. E. Osband and S. Ross, *Immunol. Today* **11**, 193 (1990).
2. L. M. Muul, P. J. Spiess, E. P. Director, S. A. Rosenberg, *J. Immunol.* **138**, 989 (1987).
3. J. T. George *et al.*, *Cancer Res.* **53**, 2374 (1993).
4. B. E. Eliot, D. A. Carlow, A. Rodricks, A. Wade, *Adv. Cancer Res.* **53**, 181 (1989).
5. N. P. Restifo *et al.*, *J. Exp. Med.* **177**, 265 (1993).
6. L. Chen *et al.*, *Cell* **71**, 1093 (1992); S. E. Townsend and J. P. Allison, *Science* **259**, 368 (1993).
7. J. Trojan *et al.*, *Science* **259**, 94 (1993).
8. R. W. Chestnut and H. M. Grey, *J. Immunol.* **128**, 1075 (1981); A. Lanzavecchia, *Immunol. Rev.* **8**, 773 (1990).
9. Y. J. Guo *et al.*, *J. Mil. Med. Coll. People's Liberation Army* **3**, 272 (1988).
10. We prepared polyclonal antisera by injecting rabbits subcutaneously with BERH-2 hepatoma cells or purified activated B cells from Wistar rats, each mixed with Freund's complete adjuvant. Activated B cells were purified by panning with plates coated with purified goat antibody to rat immunoglobulin (IgG). After repeated boosting for 2 months, the antisera were collected and purified by passage over a protein G column. Finally, the antisera were repeatedly absorbed with either BERH-2 hepatoma cells or rat B cells.
11. J. Alan and T. Robin, *Immunochemistry in Practice* (Blackwell, New York, ed. 2, 1988), chap. 2. Tumor cells were fused with activated B cells at a ratio of 1 tumor cell to 10 activated B cells. Fusions were carried out with 50% PEG in Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} at pH 7.4.
12. Y. Guo *et al.*, unpublished results.
13. E. R. Fearon *et al.*, *Cell* **60**, 397 (1990); R. I. Tepper, P. K. Pattengale, P. Leder, *ibid.* **57**, 503 (1989); T. Blankenstein *et al.*, *J. Exp. Med.* **173**, 1047 (1991); Y. Watanabe *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9459 (1989).
14. P. L. Linsely *et al.*, *J. Exp. Med.* **174**, 561 (1991).

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Parallel Neuronal Mechanisms for Short-Term Memory

Earl K. Miller* and Robert Desimone

Although objects that have just been seen may persist in memory automatically for a time and interact passively with incoming stimulation, some tasks require that the memory be actively maintained and used. To test for the existence of separate automatic and volitional mechanisms of short-term memory, recordings were made from neurons in the inferior temporal cortex of monkeys while the monkeys held a sample picture "in mind" and signaled when it was repeated in a sequence of pictures, ignoring other stimulus repetitions. Some neurons were suppressed by any picture repetition, regardless of relevance, whereas others were enhanced, but only when a picture matched the sample. Short-term memory appears to reflect the parallel operation of these two mechanisms—one being automatic and the other active.

Combined evidence from psychology and neuroscience has cleaved long-term memory into two functionally independent systems: an explicit system for facts and events, and an implicit system for the learning of perceptual and motor skills and habits (*1*). Psychological studies suggest that there may be more than one neural system mediating short-term memory (STM) as well. Some theoretical accounts, for example, posit that incoming stimuli are automatically held in some type of short-term storage buffer but may, in addition, be voluntarily maintained by active rehearsal mechanisms (*2*). We sought neurophysiological evidence for multiple STM mechanisms in recordings from the anteroventral portion of the inferior temporal (IT) cortex, a region important for visual memory in primates, including humans (*3*).

Nearly all behavioral and physiological studies of memory in the IT cortex have used some variation of the delayed matching-to-sample (DMS) task, in which the subject indicates whether a test stimulus matches a previously shown sample stimulus. The memory of the sample has a lasting effect on many IT neurons, because their response to subsequent test items is suppressed according to how well they match the sample—a property we have termed "adaptive mnemonic filtering" (*4, 5*). Because the sample is behaviorally relevant in DMS tasks, it is commonly assumed that it is actively maintained in memory (that is, "working memory"), interacting with the neural processing of incoming test stimuli; however, it is also possible that all stimuli, relevant or not (including, but not limited to, the sample), automatically linger in memory for a time, interacting with incoming stimuli. For example, if one actively searches for a repetition of the sample number 3897 in the following series—1436 3482 3482 3897—one may automatically detect the repeated but irrelevant number 3482, in addition to detecting the specific repetition of the sample number. Thus, detection of stimulus repetition in DMS tasks might be mediated by either automatic or active mnemonic mechanisms, or both.

To distinguish among these possibilities, we tested two monkeys with two types of trials (Fig. 1). The first type, standard trials, were conventional DMS trials identical to those used in our previous studies of adaptive

Laboratory of Neuropsychology, Building 49, Room 1B80, National Institute of Mental Health, Bethesda, MD 20892.

*To whom correspondence should be addressed.