Brief Definitive Report

Tumor Cells Transfected with a Bacterial Heat-Shock Gene Lose Tumorigenicity and Induce Protection against Tumors

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Summary

The gene encoding a highly immunogenic mycobacterial heat-shock protein (hsp65) was transfected into the murine macrophage tumor cell line J774. The resulting hsp65-expressing cells (J774-hsp65) were no longer able to produce tumors in syngeneic mice. This loss of tumorigenicity was not mediated through T cells since the transfected cells did not produce tumors in athymic mice. If mice are first immunized with the J774-hsp65 cells and then challenged with the parent J774 cells, the mice do not develop tumors, indicating that the presence of the mycobacterial hsp65 protein greatly enhances immunological recognition of unique structures expressed by the parent tumor cells. This is further confirmed by the demonstration in vitro of T cells derived from J774-hsp65-immunized mice that are cytotoxic for the parent J774 cells. The results provide the basis for a novel strategy for enhancing the immunological recognition and decreasing the tumorigenicity of transformed cells.

Heat-shock proteins (hsp's) are molecular chaperones, mediating the assembly and folding of other proteins (1–5). They have been reported to be expressed at abnormal levels in tumor cells (6–9), and in a murine sarcoma model, the tumor-specific antigen has been shown to be a hsp (10). They are among the most immunogenic molecules known (11–14). The cause of this immunodominance is thought to reside in their universal distribution and high degree of sequence conservation, resulting in continuous priming of the immune system. Mycobacterial hsp's have been used as carrier molecules for circumventing the need for adjuvants to greatly enhance immune responses to unrelated antigens (15, 16).

Although spontaneously arising tumors are usually very weakly antigenic, their recognition by cytotoxic T cells and elimination in vivo can be greatly enhanced if they are engineered to express cytokine genes such as IL-2 (17) or IL-4 (18). Thus, such cells must express unique structures that can be targets for immunological recognition. In an alternative approach to enhance specific immunity to tumor cells, we have transfected a highly malignant macrophage tumor cell line with the mycobacterial 65-kD hsp-encoding gene (*hsp65*). We find that not only do hsp65-expressing tumor cells lose their tumorigenicity in mice, but immunization of mice with the transfected cells induces strong T cell–mediated cytotoxic responses against the parent untransfected cells, and such mice are protected against challenge with the parent cells. The transfer of cytokine genes for cancer therapy has been widely reported, and in some cases has reached the stage of clinical trials (19, 20); transfer of the mycobacterial 65-kD hsp gene to tumor cells represents a novel strategy for an immunotherapeutic or immunoprophylactic approach to cancer.

Materials and Methods

Cells and Cell Culture. Psi-CRE, J774G8 (J774; reference 21) cells and its transfected derivates (J774-vector and J774-hsp65) were maintained in DME with 10% newborn calf serum. Wehi 164 and Pu518 cells were maintained in RPMI 1640 with 12% FCS. All media contained 100 μg/ml streptomycin, 100 μg/ml penicillin, 1 mM L-glutamine, and 10 mM Hepes.

Transfection of J774 cells with the Mycobacterium leprae hsp65 Gene. The transfection was carried out as described previously (22, 23). Briefly, the *M. leprae* 65-kD hsp gene was cloned into the retroviral shuttle vector pZIPNeoSV(x). This was transfected by calcium phosphate precipitation into the virus-packaging cell line psi-CRE. The helper-free, replication-defective, ecotropic retroviruses obtained were used to infect J774 tumor cells. Infected J774-hsp65 cells were selected by culturing in 0.8 mg/ml neomycin (G418; Sigma Chemical Co., St. Louis, MO). Expression of the 65-kD hsp gene was assessed by Northern and Western blotting and by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) of cells after indirect labeling using mAb CIII8, which recognizes the *M. leprae* 65-kD protein, and FITC-labeled sheep anti-mouse $F(ab')_2$ antibody (Boehringer, Mannheim, Germany). J774 cells transfected with vector alone (J774-vector) were prepared in parallel.

mAbs and Immunofluorescence. mAbs against H-$2K^d$, Fcγ2 receptor, LFA-1, and Pgp-1 (CD44) were obtained from PharMingen

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/07/0343/06 $2.00
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(San Diego, CA), and anti-mouse CR3 was from Serotec (Oxford, UK). Anti-p53 mAbs 248 and 421 were provided by D. Lane (Dundee University, Dundee, UK) and anti-*M. leprae* hsp65 mAb IIIC8 was obtained from the World Health Organization Mycobacterial Monoclonal Antibody Bank (Dr. T. Shinnick, Atlanta, GA). Cells were labeled by standard procedures (22) for 30 min at 4°C and analyzed using a FACScan® microfluorimeter (Becton Dickinson & Co.).

Inoculation and Immunization of Mice. 6–12-wk-old female BALB/c, BALB/c athymic, or CBA mice were obtained from breeding colonies at the National Institute for Medical Research. For the production of tumors mice were inoculated intraperitoneally with the stated number of tumor cells taken from subconfluent cell cultures. Mice were immunized with J774 hsp65 cells by four intraperitoneal injections of 10^6 cells given at weekly intervals.

Assessment of Tumor Size. Since tumors grew in most abdominal organs, resulting in an unseparable aggregation of primary tumor and abdominal organs, tumor size was calculated by weighing the primary tumor and abdominal organs of the test mice, and subtracting the weight of abdominal organs for five age-matched normal controls.

Cytotoxicity Assays. Mice were immunized with four intraperitoneal injections of 10^6 cells at weekly intervals. On day 28, spleens were removed and single-cell suspensions prepared in RPMI 1640 plus 10% FCS. The cells were incubated for 6 d with J774-hsp65 cells γ irradiated by 30 Gy. B cells were removed by panning on rabbit anti-mouse IgG-coated plates. CD4 and CD8 T cell subsets were purified as described previously (24). Briefly, NK, CD4, and/or CD8 cells were removed by treatment of cells (10^7/ml) with 10 μg/ml PK136 (anti-NK1.1; PharMingen) and anti-L3T4 and/or anti-Lyt-2 (Becton Dickinson & Co.) mAbs at 4°C for 30 min, followed by incubation with a 1:10 dilution of rabbit complement at 37°C for 60 min, resulting in CD4 and CD8 populations that were >90% pure as judged by FACS® analysis. To measure cytotoxic activity, varying numbers of CD4 or CD8 effector cells were incubated with 2,000 target cells per well labeled with ^{51}Cr in triplicate wells for 6 h at 37°C in a V-bottomed 96-well microtiter plate. ^{51}Cr release was measured in wells containing effector T cells and target cells ($cpm_{experimental}$), wells containing target cells incubated in medium alone ($cpm_{spontaneous}$), and in wells containing target cells plus 0.1% Triton X-100 ($cpm_{maximal}$). Percent cytotoxicity was calculated using the formula: $100 \times [(cpm_{experimental} - cpm_{spontaneous}/(cpm_{maximal} - cpm_{spontaneous})]$. Target cells (J774-hsp65, J774, Pu518, or Wehi 164) were labeled with ^{51}Cr by incubating 10^6 cells with 3.7 MBq of ^{51}Cr in 0.5 ml RPMI containing 20% FCS for 2 h, then washing three times.

Results and Discussion

The murine macrophage tumor cell line J774 was transfected with the *M. leprae* hsp65-encoding gene as previously described (22, 23). Expression of the bacterial hsp was confirmed using mAbs directed to the bacterial hsp (not shown). J774 is a BALB/c-derived cell line; the tumorigenicity of the parent (J774) cell line and the hsp-transfected (J774-hsp65) cells was tested in euthymic and athymic BALB/c and in CBA mice. Euthymic BALB/c mice that received either the J774 cell line or the J774 cell line transfected with vector alone (J774-vector) developed tumors within 3 wk when given 10^6 cells (Table 1 and Fig. 1). These were large, highly malignant lymphoreticular neoplasms composed of histiocytic cells with many mitoses and cellular pleomorphisms with metastases in the spleen, liver, kidney, mesentery, and lung.

Table 1. *The Tumorigenicity of Transfected (J774-hsp65) and Parent (J774) Reticulum Sarcoma Cells in BALB/c, BALB/c Athymic, and CBA Mice*

Injected tumor cells	Dose	Tumor incidence		
		BALB/c	Athymic	CBA
J774	10^5	4/5	–	–
	10^6	9/10	5/5	0/10
	10^7	5/5	–	–
J774 vector	10^5	3/5	–	–
	10^6	14/15	5/5	–
J774-hsp65	10^6	0/10	0/10	0/10
	10^7	1/10	2/10	–
	5×10^7	0/5	–	–

Mice were injected intraperitoneally with the indicated number of tumor cells resuspended in 0.5 ml endotoxin-free PBS. Tumor cells were either the parent cell line (J774), the parent cell line transfected with the mycobacterial hsp65 gene (J774-hsp65), or the parent cell line transfected with the vector alone (J774-vector). Tumor incidence was determined by autopsy and histological examination 21 d after injection of cells.

BALB/c mice that received a similar or greater number of J774-hsp65 cells did not develop tumors. This loss of tumorigenicity of J774-hsp65 cells did not appear to be due to rejection by T cells, as a similar loss of tumorigenicity was seen in athymic mice, but could involve NK cell or antibody-mediated killing. It is unlikely to be due to a chance insertion of the foreign gene into a gene involved in tumorigenicity since we obtained exactly the same results with two independently derived hsp65-transfected J774 cell lines. Neither the parent cell line nor the transfected tumor cells produced tumors in CBA mice, confirming their inability to grow across an MHC barrier.

In an attempt to identify the underlying mechanism for their loss of tumorigenicity, J774-hsp65 cells were compared with the parent cell line for differences in in vitro growth rate, expression of MHC class I and II molecules, and a variety of cell markers (including Fc and C3b receptor, LFA-1, CD44, and p53) thought to play a role in cellular interactions, tumorigenicity, or metastatic potential, and for expression of cytokine genes. There was elevated intracellular expression of p53 in the hsp-transfected cells (data not shown). Lost tumorigenicity of the transfected cells could result from the interaction of the hsp with p53, the protein encoded by the p53 tumor suppressor gene. Mutations in the p53 gene resulting in dysfunctional p53 protein are the most frequently observed genetic lesions in tumors (25). P53 is a DNA-binding protein essential for normal cell cycle control, and hsp chaperones play a crucial role in its conformation and function (26, 27). Thus, the increased efficiency of chaperone activity in the hsp-65-transfected cells could result in the proper folding and conformation of ineffective p53 protein, thereby correcting its loss of tumor suppressor function. The association be-

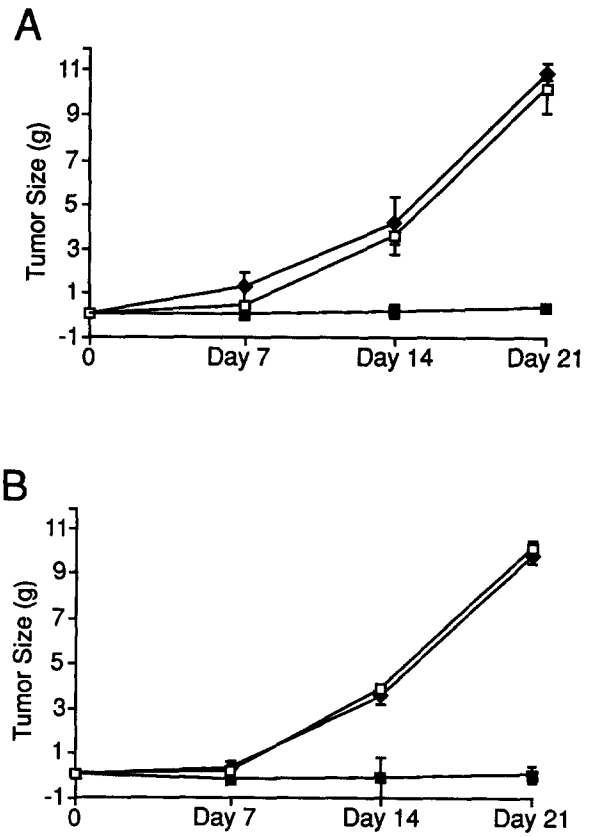

Figure 1. Growth of transfected tumor cells in normal (*A*) and athymic (*B*) BALB/c mice. Tumor cells were either the parent J774 cells (□), the parent cell line transfected with vector alone (J774-vector) (◆), or the parent cell line transfected with the mycobacterial hsp65 gene (J774-hsp65) (■). Mice were injected intraperitoneally with 10^6 cells, and tumor size was calculated by weighing the primary tumor and abdominal organs of the test mice and subtracting the weight of abdominal organs of five age-matched normal controls. For each cell type, 20 mice were inoculated. Five mice were killed on days 7 and 14, and 10 on day 21.

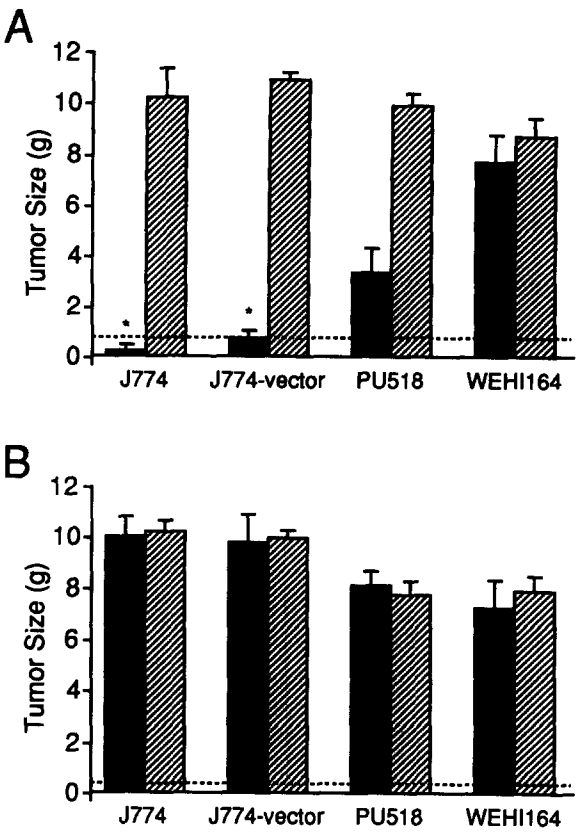

Figure 2. Growth of tumor cells in normal (*A*) and athymic (*B*) BALB/c mice that had been immunized with J774-hsp65 cells. Mice were immunized with four intraperitoneal injections of 10^6 J774-hsp65 cells at weekly intervals, and then challenged on day 28 with tumor cells of differing origin. The results show tumor size 21 d after challenge in immunized (■) and nonimmunized control (▨) mice. Tumor size was calculated by weighing the primary tumor and abdominal organs of the challenged mice, and subtracting the weight of abdominal organs of age-matched normal controls. The dotted line represents the mean + 2 SD for the weight of internal organs of the control mice. *A positive size is recorded, as the weight of internal organs of these mice exceeded those of control mice; however, no tumors could be detected in these mice, either macroscopically or histopathologically.

tween loss of tumorigenicity and elevated p53 expression is currently being investigated.

We next investigated whether mice that had received the transfected J774 cells showed any level of protection against developing tumors when challenged with the parent cell line. The results (Table 2 and Fig. 2) demonstrate a highly significant level of protection; J774 produced tumors in naive mice, but not in mice that had previously been immunized with J774-hsp65 cells. This was confirmed by autopsy and histological examination in which no evidence of tumor cells or autoimmune inflammation could be found. A second group of challenged mice were completely healthy 6 mo after challenge, and 80% have survived for >12 mo. The protection generated by J774-hsp65 cells appeared to be mediated by T cells, since immunized athymic mice all developed tumors after subsequent challenge.

To investigate the specificity of the protective effect, immunized mice were challenged with two other cell lines: Pu518, which is an unrelated, spontaneous reticulum sarcoma of BALB/c origin, and Wehi 164 cells, derived from a methyl cholanthrene–induced BALB/c sarcoma. The results (Table 2 and Fig. 2) show significant protection against challenge with Pu518, but not with Wehi 164 cells. When mice were immunized with irradiated J774 cells they were partially protected against subsequent challenge (Table 2). This indicates that J774 cells do have tumor-associated antigens that normally do not induce an effective immune response; the presence of the 65-kD hsp in transfected cells presumably enhances this response.

Since protection appeared to be T cell mediated, we investigated the relationship between protection in vivo and T cell–mediated cytotoxicity in vitro. Nonadherent spleen cells from mice immunized with J774-hsp65 cells were cultured in vitro with irradiated J774-hsp65 cells for 6 d. Purified populations of CD4 or CD8 T cells were tested for cytotoxicity using J774, J774-hsp65, Pu518, and Wehi 164 cells as targets (Figs. 3 and 4). Both CD4 and CD8 populations were highly cytotoxic for J774-hsp65 and for the parent J774 cells; deple-

Table 2. *The Effect of Immunization with Transfected Tumor Cells (J774-hsp65) on Subsequent Challenge with Reticulum Sarcoma Cells*

Immunization	Challenge	Tumor incidence	
		BALB/c normal	BALB/c athymic
J774-hsp65	J774	0/10	5/5
None	J774	9/10	5/5
J774-hsp65	J774-vector	0/15	5/5
None	J774-vector	10/10	5/5
J774-hsp65	Pu518	2/10	5/5
None	Pu518	5/5	4/4
J774-hsp65	Wehi 164	7/9	5/5
None	Wehi 164	5/5	4/4
Irradiated J774-vector	J774	4/9	5/5
None	J774	8/10	5/5

Mice, either normal or athymic BALB/c, were immunized with four intraperitoneal injections of 10^6 J774-hsp65 cells or γ irradiated (30 Gy) J774-vector cells, given at weekly intervals. On day 28 the mice were challenged intraperitoneally with 10^6 reticulum sarcoma cells of differing origins. Tumor incidence was determined by autopsy and histological examination 21 d after challenge.

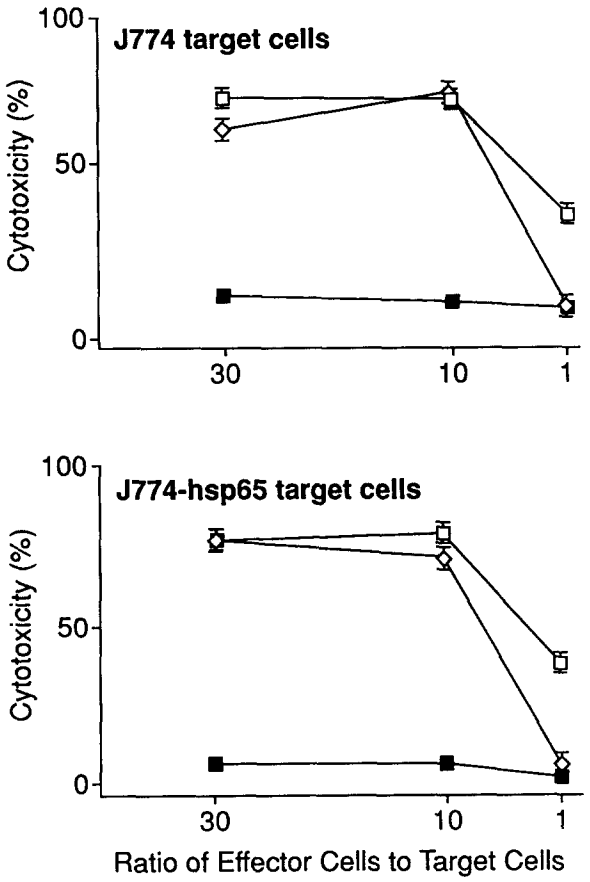

Figure 3. In vitro cytotoxic activity of splenocytes from mice immunized with J774-hsp65 cells. Mice were immunized with four intraperitoneal injections of 10^6 J774-hsp65 cells at weekly intervals. Spleen cells were cultured for 6 d with irradiated J774-hsp65 cells, and purified populations of CD4 (□) or CD8 (◇) cells were tested for cytotoxicity against J774-hsp65 and J774 cells. Cells depleted of both CD4 and CD8 cells were also included (■). The results are shown as the mean ± SD.

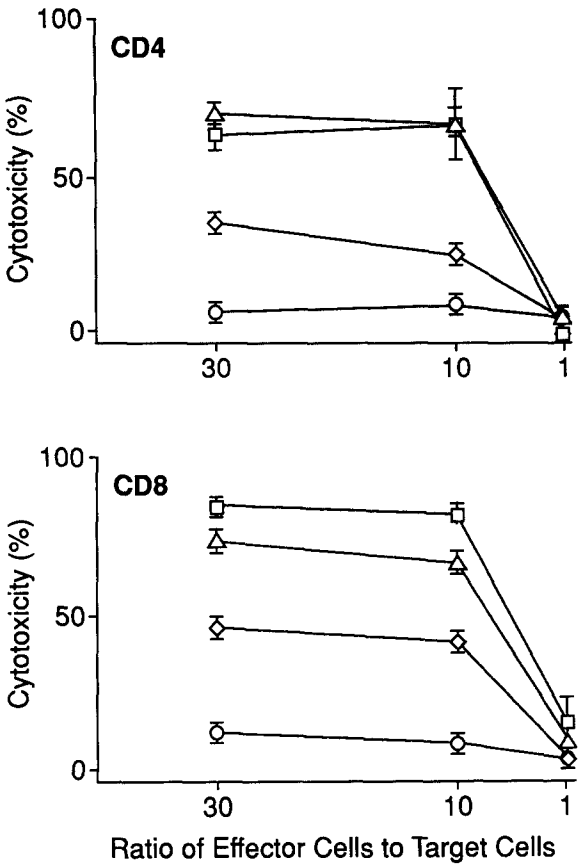

Figure 4. In vitro cytotoxic activity of splenocytes from mice immunized with J774-hsp65 cells. Mice were immunized with four intraperitoneal injections of 10^6 J774-hsp65 cells at weekly intervals. Spleen cells were cultured for 6 d with irradiated J774-hsp65 cells, and purified populations of CD4 or CD8 cells were tested for cytotoxicity against J774-hsp65 (□), J774 (△), Pu518 (◇), and Wehi 164 (○) cells. The results are shown as the mean ± SD.

tion of both CD4 and CD8 cells resulted in elimination of cytotoxic activity (Fig. 3), confirming that it was T cell mediated. CD4 and CD8 cells were also cytotoxic, although to a lesser degree, against Pu518, but not against Wehi 164 cells. Attempts to generate cytotoxic T cells from naive mice or from mice injected with J774 parent cells by in vitro stimulation with irradiated J774 cells failed, as insufficient T cells were obtained after the 6 d of in vitro stimulation. Thus, there appears to be a complete correlation between in vivo protection and the generation of cytolytic T cells capable of lysing these malignant reticulum sarcoma cells.

We assume that the highly immunogenic nature of the

65-kD molecule provides greatly enhanced, associated recognition of other, tumor-associated antigen(s). The use of a highly antigenic molecule such as purified protein derivative (PPD) (which contains the 65-kD hsp) to greatly enhance immune responses to associated antigens has been demonstrated previously (28, 29), and more recently the same effect has been achieved with recombinant mycobacterial hsp's (15, 16). As with superantigens, a genuine primary recognition of hsp65 has been reported. T cells from PPD-negative individuals (30), and from umbilical cord blood (31), are stimulated by the mycobacterial hsp65. In our experiments (data not shown), splenocytes from naive BALB/c mice do not proliferate in response to J774 cells, but do so strongly in response to J774-hsp65 cells, indicating a substantial change in the immunogenic properties of the cells after transfection with hsp65. The finding of crossprotection against an unrelated reticulum sarcoma, Pu518, suggests the presence of shared antigens on the two unrelated sarcomas. Shared tumor-associated antigens have been found in human melanomas (32) and murine sarcomas (33). Endogenous expression of the bacterial hsp65 appears to be essential for protection; BALB/c mice immunized with four weekly injections of 50 μg recombinant hsp65 (rhsp65) all developed large intra-abdominal tumors within 3 wk of challenge with J774 or J774-vector cells (data not shown). Similarly, preincubation of J774 cells with 50 μg/ml rhsp65 for 60 min, or injection of 50 μg of PPD as an adjuvant with J774 cells, failed to produce protection (data not shown).

Our results clearly show that transfection of the tumor cells with the bacterial hsp65 results in the induction of immunity against the parent tumor cell line; this immunity is reflected in the generation of cytotoxic T cells and protective immunity against the parent tumor. There is a great deal of current interest in the use of gene transfer, for example, of IL-2- or TNF-encoding genes, to increase the immunological recognition of cancer cells (19, 20). The rapid progress that is being made on in vivo transfection, and the targeting of transgene expression to tumor cells (34), could provide a role for the hsp65 gene in such an approach.

We thank Professor P. L. Lantos for carrying out the histological examination of the tumors and tissues.

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Received for publication 26 October 1992 and in revised form 10 March 1993.

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