

Safety and efficacy of a xenogeneic DNA vaccine encoding for human tyrosinase as adjunctive treatment for oral malignant melanoma in dogs following surgical excision of the primary tumor

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Objective—To evaluate the safety and efficacy of a vaccine containing plasmid DNA with an insert encoding human tyrosinase (ie, huTyr vaccine) as adjunctive treatment for oral malignant melanoma (MM) in dogs.

Animals—111 dogs (58 prospectively enrolled in a multicenter clinical trial and 53 historical controls) with stage II or III oral MM (modified World Health Organization staging scale, I to IV) in which locoregional disease control was achieved.

Procedures—58 dogs received an initial series of 4 injections of huTyr vaccine (102 µg of DNA/injection) administered transdermally by use of a needle-free IM vaccination device. Dogs were monitored for adverse reactions. Surviving dogs received booster injections at 6-month intervals thereafter. Survival time for vaccinates was compared with that of historical control dogs via Kaplan-Meier survival analysis for the outcome of death.

Results—Kaplan-Meier analysis of survival time until death attributable to MM was determined to be significantly improved for dogs that received the huTyr vaccine, compared with that of historical controls. However, median survival time could not be determined for vaccinates because < 50% died of MM before the end of the observation period. No systemic reactions requiring veterinary intervention were associated with vaccination. Local reactions were primarily limited to acute wheal or hematoma formation, mild signs of pain at the injection site, and postvaccination bruising.

Conclusions and Clinical Relevance—Results support the safety and efficacy of the huTyr DNA vaccine in dogs as adjunctive treatment for oral MM.

Impact for Human Medicine—Response to DNA vaccination in dogs with oral MM may be useful in development of plasmid DNA vaccination protocols for human patients with similar disease. (*Am J Vet Res* 2011;72:1631–1638)

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ABBREVIATIONS

MHC	Major histocompatibility complex
MM	Malignant melanoma
MST	Median survival time

Malignant melanoma is the most commonly occurring oral tumor in dogs.¹ Progression of the disease, like that of its counterpart in humans, is aggressive, and metastasis is frequently detected. Radical surgical excision has been the treatment of choice because oral MM has been reported to be poorly responsive to chemotherapy²⁻⁴ and radiotherapy appears to have limited value in local management of the disease.⁵ Dogs with stage II and III oral MM have been reported to have MSTs ranging from < 5 months to almost 1 year following aggressive local excision of the primary tumor (in stage II disease) and regional lymph nodes (stage III).^{1,6-8}

Immunotherapy targeting the melanoma differentiation antigen tyrosinase (essential to melanin synthe-

sis) has been explored as a strategy for systemic treatment of melanoma.⁹ Intramuscular administration of a vaccine containing xenogeneic plasmid DNA with a cDNA insert encoding human tyrosinase (ie, huTyr vaccine) in dogs results in the production of a tyrosinase protein that is 85% homologous to canine tyrosinase but varies enough from the canine protein to elicit an immune response.¹⁰ In 1 study,¹¹ 9 dogs with MM that were administered huTyr vaccine had substantially longer survival times than those typically described for dogs in similar stages of the disease after surgical removal of the tumor (and in some dogs, prior radiation treatment). Production of tyrosinase-specific antibodies that cross-reacted with the canine tyrosinase ortholog was reported to coincide with clinical response in 3 of these 9 dogs,¹⁰ suggesting that huTyr vaccine may elicit an immune response capable of overcoming host tolerance, and this response may be clinically effective against MM.

Because the tyrosinase antigen is transcribed and translated in the canine host, it is recognized and processed in the context of its relevant MHC and associated costimulatory molecules. The expression of class II MHC molecules in melanoma cells is upregulated during malignant transformation, whereas normal cutaneous melanocytes do not express class II MHC.¹² This greatly increases the density of class II MHC molecules presenting antigen on the melanoma cell surface, allowing the immune response to be preferentially directed toward the tumor cells. It has also been shown that antigen-specific interferon- γ T-cell responses in dogs are potentiated by delivery of huTyr vaccine through a needle-free transdermal delivery device.¹³

The huTyr vaccine used in the study reported here was conditionally licensed in 2007 by the USDA Center for Veterinary Biologics on the basis of demonstration of a reasonable expectation of efficacy,¹¹ and the purpose of the study reported here was to evaluate the safety and efficacy of this vaccine as adjunctive treatment for oral MM in dogs after locoregional disease control was achieved. We tested the hypothesis that adjunctive treatment with huTyr vaccine after surgical excision of primary tumors would result in increased survival time in dogs with stage II or III oral MM, compared with that in historical control dogs.

Materials and Methods

Animals—Between April 13, 2006, and October 11, 2007, dogs with histologically confirmed oral MM were prospectively enrolled in the study vaccine group at 5 specialty oncology practices in various geographic locations in the United States. These included Animal Medical Center, New York; North Carolina State University College of Veterinary Medicine, Raleigh, NC; Southwest Veterinary Oncology, Tucson and Gilbert, Ariz; Greater Houston Veterinary Specialists, Houston; and Animal Cancer Specialists, Seattle.

Dogs were considered eligible for inclusion in the study if they had stage II or III oral MM for which locoregional control had been achieved (ie, dogs had no gross evidence of MM at the excision site) and date of surgery for excision of the primary tumor was confirmed. Clinical disease staging (scale, I to IV) was

performed for all dogs according to a modification of World Health Organization guidelines whereby the criteria for size of the primary tumor were adjusted to match those used for the historical control dogs.⁶ Stage II oral MM was defined as a primary tumor with a diameter of 2 to 5 cm, without evidence of metastasis to lymph nodes or distant metastatic disease; stage III was defined as a primary tumor with a diameter > 5 cm or any size tumor with lymph node metastasis, without evidence of distant metastatic disease.

Pretrial evaluation included a complete physical examination, CBC, and serum biochemical analysis. The greatest length, width, and depth of tumors were measured when possible or measurements were estimated from medical records. Measurement methods were at the discretion of individual investigators. The extent of surgical margins was derived from available histopathology reports after samples were submitted to a veterinary diagnostic laboratory by the referring surgical institution and read by board-certified pathologists, and margins were assessed as complete (> 2 mm wide), incomplete (< 1 mm wide), or equivocal (1 to 2 mm wide). Dogs were evaluated for metastatic disease via 3-view thoracic radiography and cytologic examination of fine-needle aspirate samples or histologic evaluation of regional lymph node biopsy samples. Dogs that had undergone fractionated radiation treatment of the surgical site, regional lymph nodes, or both because of histopathologic evidence of incomplete surgical margins, metastasis to local lymph nodes, or both were not excluded from the study. Enrollment criteria did not permit systemic administration of long-acting steroids. Dogs receiving short-term topical steroid treatments during the postvaccination observation period were not excluded.

Written informed consent was obtained from owners prior to enrollment of their dogs in the study. This study was performed with approval from Merial's Institutional Animal Care and Use Committee and in accordance with applicable local animal use regulations. Permission to perform the multisite clinical trial was obtained from the USDA Center for Veterinary Biologics.

Historical control population—An external historical control group was chosen from the records of 2 previously reported clinical trials.⁶ Historical control dog data were collected at the University of Wisconsin School of Veterinary Medicine, and the raw data were provided by one of the authors (IDK). To minimize bias to the extent possible, data for these dogs were selected for inclusion in the control group on the basis of their having met the same criteria, prospectively, determined for enrollment of dogs in the vaccine group. The historical controls had received either a placebo or a post-surgical treatment that had no significant antitumor activity, compared with the placebo treatment.

Treatment protocol—Vaccinates received an initial series of 4 injections of the huTyr vaccine following surgical removal of the primary tumor (all dogs) and radiation treatment (in dogs that had histologic findings suggestive of incomplete surgical excision or metastasis to regional lymph nodes). One injection was administered approximately every 14 days, with minor

variations from the 14-day schedule caused by client scheduling needs. Surviving dogs were administered booster injections at 6-month intervals thereafter.

Following the initial vaccination series, dogs were evaluated approximately every 30 days for disease reoccurrence by means of physical examination, evaluation of regional lymph node aspirates, and 3-view thoracic radiography according to the standard protocol of each participating oncology practice. Dogs that survived > 1 year after surgery were then evaluated via the same methods on a quarterly basis. Follow-up was performed until dogs died or were lost to follow-up.

Vaccination—The huTyr vaccine included a plasmid vector with a supercoiled cDNA insert encoding human tyrosinase as described previously.^{11,a} All vaccinates received a dose of 102 µg of DNA in a 0.4-mL volume of injection. Vaccines were administered transdermally by use of a needle-free IM vaccination device^b according to the manufacturer's instructions. The injection site was located in the proximal half of the medial aspect of the thigh, caudal to the femur. Skin at the injection site was shaved and swabbed with alcohol prior to vaccine administration. Sequential vaccinations were administered to alternating hind limbs in the same manner.

Safety evaluation—All dogs were monitored for acute postvaccination reactions (eg, anaphylaxis, signs of pain, or wheal formation) or leakage from the injection site for ≥ 30 minutes after huTyr vaccine administration. Approximately 14 days after vaccine administration, injection sites were reexamined by the same investigator who administered the vaccine for evidence of residual injection-site reactions. In the interim, owners were asked to maintain a daily log of any signs of adverse systemic or local reactions. Owners were instructed to bring their dogs to the investigator for evaluation or to notify the investigator if adverse reactions were detected.

Statistical analysis—To allow comparisons between the vaccinate and historical control groups, the primary outcome measure, survival time, was defined as the time from date of surgery until date of death or last follow-up visit (for dogs lost to follow-up). Survival times were evaluated via Kaplan-Meier product-limit survival analyses. The log-rank test was used to test for differences in survival time and in estimates of MST between vaccinate and historical control groups. Within-group Kaplan-Meier survival analyses were used to investigate the effect of disease stage or extent of surgical margins on survival time. Dogs were classified as censored if they were removed from the study, lost to follow-up, died of causes other than MM, or were alive at the end of the study. An event was defined as euthanasia or death attributed to MM. Disease stage and follow-up time comparisons between vaccinates and historical controls were determined by use of χ^2 tests and Wilcoxon rank-sum tests, respectively. All statistical analyses were conducted by use of statistical software.^c Values of $P < 0.05$ were considered significant.

Results

Fifty-eight dogs met the criteria for prospective enrollment in the study, and 53 dogs met the criteria for

inclusion in the study as historical controls. No dogs were excluded on the basis of systemic steroid administration because none underwent this treatment.

Signalment—Age, weight, sex, and disease stage for vaccinates and historical control dogs were compared (Table 1). Age, weight, and sex were comparable between the 2 groups. Breed distribution was also similar between the 2 groups; vaccinates included 26 breed types (which included purebreds and mixed breeds) and historical controls comprised 23 breed types. The distribution of breeds among American Kennel Club group designations was similar between groups, with the sporting group most highly represented. There was no significant ($P = 0.25$) difference in disease stage between groups. Forty-four of 58 (76%) vaccinates had stage II oral MM, compared with 34 of 53 (64%) historical controls.

Surgery-to-vaccination interval—The mean interval between surgery and administration of the first dose of huTyr vaccine in treated dogs was 43 days (median, 35 days). Because most dogs were referred to oncology practices for surgery, a consistent time interval could not be established by study investigators. Additionally, the interval between surgery and first vaccine treatment in some dogs was extended to allow for removal of metastatic lymph nodes or for radiation treatment.

Outcomes and clinical response—Of 58 dogs initially enrolled in the vaccinate group, 15 died or were humanely euthanized because of MM and 16 died of other causes. Four dogs did not complete the initial series of 4 injections. Three of these 4 dogs died of unrelated causes or were removed from the study for treatment of other primary tumors and were censored for statistical analysis. One dog was euthanized because of metastatic MM after the first 2 doses of the vaccine had

Table 1—Sex*, age, weight, and disease stage⁶ of 111 dogs with spontaneously occurring oral MM for which locoregional disease control was achieved by means of surgery with or without fractionated radiation treatment.

Variable	Vaccinates	Historical controls
No. of dogs	58	53
No. of males	30	32
No. of females	28	19
Age (y)		
Median (mean ± SD)	10.5 (10 ± 2.5)	11 (10.8 ± 3.4)
Range	5–16	1–20
Weight (kg)		
Median (mean ± SD)	23.9 (23.6 ± 12.7)	NA
Range	1.4–47	NA
Stage II disease (No. of dogs)	44	34
Stage III disease (No. of dogs)	14	19

Vaccinates received 4 transdermal injections (given at 14-day intervals) of a vaccine containing plasmid DNA with a cDNA insert encoding human tyrosinase^{11,a} (102 µg of DNA/injection) administered transdermally by use of a needle-free IM vaccination device. Surviving dogs received booster injections at 6-month intervals thereafter. Historical control dogs⁶ were equivalent to vaccinates with respect to enrollment criteria and had participated in previous clinical trials in which they received either a placebo or a postsurgical treatment that had no significant antitumor activity, compared with the placebo treatment.

*Sex information for 2 historical control dogs was not available. NA = Not available.

been administered; this was included in the statistical analysis as an event.

Of 16 vaccinates that died of causes other than MM, only 3 had evidence of MM recurrence or metastasis at the time of death. Two dogs had pulmonary metastases diagnosed at necropsy, following accidental drowning of one and acute gastric dilatation-volvulus in the other. A third dog, euthanized for hind limb ataxia caused by multifocal intervertebral disk disease, had evidence of local recurrence without detectable metastases at the time of death. Three other dogs died of complications from other malignancies, including a cardiac tumor that was consistent with neuroendocrine neoplasia (assessed via immunohistochemistry), anaplastic sarcoma, and hemangiosarcoma, and 10 dogs died of age- or accident-related complications.

Ten vaccinates were lost to follow-up after 62 to 679 days of the study: 7 dogs were not returned for scheduled appointments, and the owners could not be contacted; 2 dogs were placed in this category because of the owners' lack of adherence to the follow-up examination schedule; and monitoring of 1 dog was taken over by the owner and primary care veterinarian. Nine dogs were removed from the study (7 to pursue alternate treatments for oral MM and 2 for treatment of

primary tumors unrelated to this disease). Eight dogs that received huTyr vaccine treatment were still alive at the time of the latest evaluation. Median follow-up time for censored vaccinates (437 days) was not significantly ($P = 0.92$) different from that of censored historical control dogs (321 days). Outcomes for vaccinates were compared with those of the historical control group (Table 2).

The primary outcome measure was survival time. None of 8 surviving vaccinates at the end of the study had any signs of MM. With few exceptions, dogs in either group that died of MM went from an apparently disease-free state to rapid progression between visits; because the endpoint for survival time (death) could be more reliably determined than onset of disease progression, Kaplan-Meier analysis was performed for survival time only. The disease-free interval and survival times, along with stage, surgical margins, and disease progression, were summarized for dogs that died of MM (Table 3).

Kaplan-Meier survival curves for vaccinates and historical control dogs were compared (Figure 1). The MST until death attributable to MM for historical controls was 324 days, whereas that for dogs that received the huTyr vaccine had not been reached at the time of the last data analysis. Survival time until death attributable to MM was determined to be significantly ($P < 0.001$) improved for dogs that received the huTyr vaccine, compared with that of historical controls. Because a direct comparison of MST (center of the distribution of survival times) between vaccinates and historical controls could not be made, it is useful to compare the lower (25th) percentiles of survival time (time beyond which 75% of the population could be expected to survive). The 25th percentile for vaccinates was 464 days (95% confidence interval could not be calculated for this value) and that of historical controls was 156 days (95% confidence interval, 94 to 228 days).

Thirty-four dogs in the historical control group had stage II and 19 had stage III oral MM according to the

Table 2—Outcomes for the same 111 dogs in Table 1.

Outcome	No. (%) of dogs	
	Vaccinates	Historical controls
Death attributable to MM	15 (26)	34 (64)
Death attributable to causes other than MM*	16 (28)	7 (13)
Lost to follow-up*	10 (17)	1 (2)
Removed from study*	9 (16)	—
Alive at the end of the study*	8 (14)	11 (21)
Total	58	53

*Dogs with this outcome were censored for analysis.
— = Not applicable.

Table 3—Characteristics and disease progression of oral MM in 15 vaccinates that died or were euthanized because of the disease during the study period.

Disease stage	Dog No.	Surgical margins	Disease progression	Disease-free interval (d)	Survival time (d)
II	1	C	Metastasis to contralateral mandible, progression to lungs	240	475
	2	C	Metastasis to lungs	96*	199
	3	I	Recurrence, with metastasis to regional LN, then lungs	102	310
	4	I	Recurrence	243	530
	5	I	Recurrence, with metastasis to lungs	157*	181
	6	I	Metastasis to lungs	207	240
	7	E	Metastasis to regional LN	368	464
	8	NR	Recurrence, with metastasis to lungs	223	290
III	9	C	Recurrence, with metastasis to lungs	233	235
	10	C	Metastasis to regional LN, then lungs	512	583
	11	C	Multifocal metastasis	293	293
	12	I	Recurrence	47*	52
	13	I	Metastasis to contralateral mandible	161*	177
	14	I	Recurrence	75*	165
	15	NR	Recurrence, with metastasis to lungs	100*	100

*Tumor recurrence or metastasis was detected in these dogs during the initial 4-injection treatment period.
C = Complete excision (margins > 2 mm wide). E = Equivocal (margins 1 to 2 mm wide). I = Incomplete excision (margins < 1 mm wide). NR = Not reported.

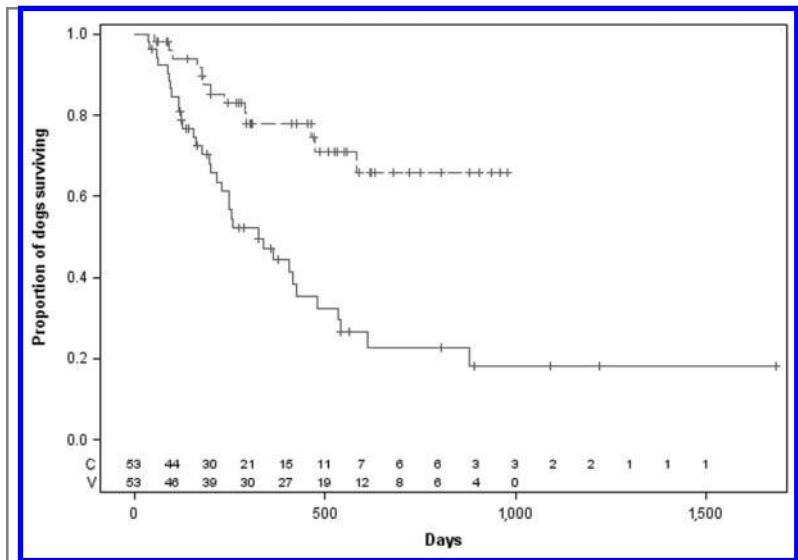


Figure 1—Kaplan-Meier survival curves comparing survival time of dogs with spontaneously occurring oral MM in vaccinee ($n = 58$; dashed line) and historical control (53; solid line) groups over time. Dogs in both groups had stage II or III oral MM,⁶ for which locoregional disease control was achieved by means of surgery with or without fractionated radiation treatment. Vaccinees received an initial series of 4 injections (given at approx 14-day intervals) of a vaccine containing plasmid DNA with a cDNA insert encoding human tyrosinase^{11,a} (102 μg of DNA/injection) administered transdermally by use of a needle-free IM vaccination device. Surviving dogs received booster injections at 6-month intervals thereafter. Historical control dogs⁶ were equivalent to vaccinees with respect to enrollment criteria and had participated in previous clinical trials in which they received either a placebo or a postsurgical treatment that had no significant antitumor activity, compared with the placebo treatment. Median survival time for historical controls was 324 days; MST for vaccinees had not been reached by the end of the study. Numbers along the x-axis indicate number of dogs at risk in each group at various time points; day 0 was the day the primary tumor was excised. C = Controls. V = Vaccinees. + = Censored dog.

described criteria. Disease stage was not significantly ($P = 0.58$) associated with survival time (MST, 324 and 338 days for dogs with stage II and stage III disease, respectively) for this group. Forty-four dogs enrolled in the vaccinee group were classified as having stage II and 14 as having stage III oral MM prior to study enrollment. Vaccinees that had stage II oral MM had a significantly ($P < 0.001$) greater survival time (MST not reached), compared with those that had stage III oral MM (235 days).

Evaluation of 51 available reports of postsurgical histologic examination of primary tumors from the vaccinee group revealed evidence of complete excision in 23 cases, whereas in 28 reports, excision was determined to be incomplete or equivocal. Kaplan-Meier survival probabilities for death attributable to MM were not significantly ($P = 0.64$) different between vaccinees with and without evidence of complete tumor excision (475 vs 464 days, respectively).

Safety evaluation—No dogs developed systemic adverse reactions that required veterinary intervention as a result of administration of the initial huTyr vaccine series. Reports of other adverse reactions were summarized (some dogs had > 1 type of reaction detected). Of 232 injections administered during the primary vaccination protocol, there were 84 (36.2%) incidents of signs of pain during injection; 72 (31.0%) of these were subjectively considered mild, and 12 (5.2%) were considered moderate. There were 2 (0.9%) incidents

of acute wheal formation and 5 (2.2%) incidents of wheals detected 30 minutes after vaccination. Formation of a droplet of what appeared to be serum at the injection site, forming a small (< 1 mm) encrustation, was not uncommon (115 [49.6%] observations) with leakage of fluid from the injection site in 11 (4.7%) cases. Additionally, there were 2 (0.9%) reports of acute hematoma formation in toy-breed dogs.

Few postvaccination reactions remained at follow-up examinations 2 weeks following injection. These included 1 (0.4%) unresolved bruise in a 6-kg Miniature Poodle, 2 (0.9%) incidents of a small encrustation at the injection site, and 3 (1.3%) occurrences of subjectively mild signs of pain on palpation of the injection site.

Twenty-seven owners reported that their dogs had ≥ 1 adverse reaction during ≥ 1 of the 14-day periods after vaccination. The number of owners reporting postvaccination reactions declined steadily from 16 after the first vaccination to 8 after completion of the fourth vaccination observation period. Reactions specific to the injection site included signs of pain on palpation (14 incidents), heat or swelling (14), lameness or stiffness of the injected limb (14), and bruising or erythema at the injection site (13). Nonspecific reactions that occurred within 48 hours after vaccination included lethargy or signs of depression (14 incidents), decreased appetite (4), polydipsia (5), vomiting (3), diarrhea (2), and anxiety, increased appetite, incontinence, and altered mentation (1 each). One dog had 2 incidents of sneezing and pruritis. Onset of 48 additional incidents of nonspecific reactions was reported during the observation period (3 to 14 days after vaccination). Some of these appeared to be preexisting conditions, and none were substantiated as being vaccine related by the attending clinical investigator.

Booster administration resulted in 2 reports of adverse reactions that developed within 2 days after vaccination. One dog developed bruising and edema of the medial aspect of the thigh between 12 and 24 hours following administration of the third booster vaccination. The dog was evaluated at an emergency clinic and was treated with analgesics and by increasing the dog's maintenance dose of diphenhydramine (which was being administered as treatment for atopy). Eighteen days following the vaccination, the reaction had resolved to form a firm subcutaneous swelling approximately 4 \times 2.5 cm at the injection-site area. Cytologic examination of an aspirate collected at the site was consistent with inflammation. The swelling completely resolved over the course of several weeks.

Another dog received a second booster vaccination 1 day before undergoing surgery for excision of a thoracic mast cell tumor ipsilateral to the vaccination

site. The day after surgery, mild lameness of the limb in which the vaccine was administered was detected, and this progressed to weakness and signs of pain in the same limb over the course of several days. On examination, the dog was weak and unable to stand. Vesicle formation and peeling of the skin was evident in the inguinal region, and edema and bruising extended ventrally below the hock and cranially along the ventral abdomen, with skin necrosis and sloughing of the caudal 3 mammae. The dog was humanely euthanized, and microbial culture of samples of the skin and liver yielded *Pseudomonas* spp.

Discussion

The study reported here was a result of collaboration between the Animal Medical Center and Memorial Sloan-Kettering Cancer Center and was part of an effort to develop human cancer treatments that circumvent the problem of immune tolerance to differentiation antigens expressed by cancer cells. In 1998, it was reported¹⁴ that immune tolerance to tyrosinase-related protein-1 (formerly called gp75) in mice could be overcome by means of vaccination with DNA encoding human tyrosinase-related protein-1, which resulted in immunity against the tumor mediated by autoantibodies. Results of another study¹⁵ revealed that vaccination against a xenogenic form of another tyrosinase-related protein, dopachrome tautomerase (formerly tyrosinase-related protein 2), induced autoantibodies and autoreactive cytotoxic T cells in mice and resulted in protection from syngeneic tumor challenge. On the basis of the concept that xenogenic DNA vaccination could induce antitumor responses, a phase I clinical trial¹¹ was performed to evaluate the use of huTyr vaccine in a small group of dogs with spontaneously occurring MM; results of that study led to conditional licensing of the vaccine. The purpose of the present study was to evaluate the safety and efficacy of the huTyr vaccine as adjunctive treatment for oral MM in dogs after locoregional disease control had been achieved.

Survival time for vaccinates were significantly ($P < 0.001$) improved, compared with those of historical controls. The MST of historical control dogs in the present study was 324 days. The MST of vaccinates had not been reached by the end of the study evaluation period; however, 25th percentiles of survival times for vaccinates and historical controls were 464 vs 156 days, respectively.

Eleven of 15 vaccinates that died of MM had survival times shorter than the MST of historical control dogs. Of these 11 dogs, 6 died or had evidence of recurrence or metastasis prior to completion of the initial 4-dose vaccination protocol. Seven of the 15 vaccinates that died of MM had stage III oral MM at vaccination, and 6 of these had a survival time < 324 days. Liao et al¹⁰ showed that administration of huTyr vaccine in dogs produced a detectable humoral response 3 to 9 months after completion of a 4-dose, biweekly protocol. It is possible that an immune response was induced but was not sufficient to overcome the disease progression among vaccinates that died of MM or that some dogs had immunologic deficiencies that predisposed them to a rapid spread of cancer and contributed to a

poor response to vaccination. Vaccination at an earlier stage would be more likely to allow for the development of an immune response capable of controlling disease progression, but the immunologic status of each dog may play an important role in vaccine response and disease progression.

Our intention was to minimize the time between surgery and initiation of the vaccination protocol, but because most vaccinates were referred to oncology practices for surgical treatment, this time interval was often not under control of the investigators. Additionally, the interval between surgery and first vaccination in some animals was extended to allow for removal of lymph nodes with metastatic disease or for radiation treatment prior to the initiation of the vaccination protocol; thus, the time between surgery and the first vaccination could not be standardized.

Disease stage classified according to World Health Organization criteria has been cited as a prognostic indicator for survival time in dogs with oral MM.^{1,7,8} Although there was no significant ($P = 0.58$) association between disease stage and survival time for the historical control group, vaccinates with stage II oral MM had significantly ($P < 0.001$) longer survival times than did vaccinates that had stage III oral MM. These results are based on small sample sizes (44 and 14 dogs, respectively), but they possibly support the notion that vaccine efficiency is enhanced by local disease control and the opportunity for development of an effective immune response that longer survival time in earlier stages affords.

In preliminary investigations of the same huTyr plasmid construct used in the present study, Bergman et al¹¹ emphasized the importance of minimizing residual disease and achieving locoregional disease control to maximize chances of long-term survival. However, in the study reported here, there was no significant difference in survival time between vaccinates that had histologic reports of complete versus incomplete or equivocal margins surrounding the excised tumor. Thus, it appears that, in the absence of disseminated microscopic disease, achievement of gross local disease control may be sufficient to allow an effective activation of the host immune response. These results should be interpreted with caution because creation of subgroups within the vaccinate group caused the Kaplan-Meier analysis to lose statistical power. Additionally, because histologic characterization of the tumor was not considered as a prospective variable, there was no requirement for assessment by a single pathologist, which may have introduced additional variability.

Adverse reactions following administration of huTyr vaccine to the diverse population of dogs in the present study were considered minimal; the most notable of these were bruising and hematoma formation in small- and toy-breed dogs early in the study. These were most likely attributable to investigators' lack of familiarity with the needle-free delivery device, rather than the vaccine itself. The previously reported overall safety of the vaccine and of the previously established delivery method^{11,16} was supported by results the present study.

Except for postvaccination reactions assessed by attending clinical investigators, evaluation of huTyr

vaccine safety and adverse reactions was made on the basis of owner-generated reports, which affected the consistency of these observations. It is noteworthy that the number of owner-reported adverse reactions declined throughout the vaccination series. This may have been a reflection of increased owner confidence in the vaccination procedure or of increased familiarity of the investigators with use of the needle-free device. It would be expected that reports of adverse reactions would increase with each subsequent treatment if the vaccine itself was the cause of the reactions. Additionally, it may have been difficult to distinguish between reactions associated with the vaccine and clinical signs indicative of other concurrent age- and disease-related abnormalities.

A limitation of the present study and others like it is the lack of a randomized study design. Recently, there has been a closer examination of the value of randomization against a standard-treatment control group in clinical trials rather than historical controls. The results of studies that use historical controls must be interpreted carefully because the likelihood of underestimating false-positive error rates (type I error) is increased.¹⁷ The historical control population cannot be totally equivalent to the study population by virtue of variability in response rates, changes in a population over time, and shifts in outcome as a result of the evolution of standard-of-care treatment over time.

In the study reported here, the historical control group was selected to minimize the creation of bias. Stage of disease, median follow-up time, breed type, sex, and age distribution were comparable between vaccinates and historical control dogs. However, even though fewer vaccinates (n = 8) than historical controls (11) were alive at the conclusion of the study, an MST had not been reached in the vaccinate group because censored dogs (those that died of other causes [n = 16], were lost to follow-up [10], or were removed from the study [9]) were more numerous than those that died of MM. Thus, the difference in outcome profiles of the 2 groups may also have contributed some statistical bias.

Although there are limitations inherent in the use of external controls, FDA guidelines for clinical trials indicate situations in which they are useful and valid, such as when a drug is intended to treat a serious illness for which there is no satisfactory treatment and a reasonable expectation for efficacy on the basis of theoretical considerations and early data has been shown for the new drug.¹⁸ Results of trials that use historical controls are persuasive when the study endpoint is definitive, the outcome of the treatment group is markedly different from that of the historical control group, a high level of significance for the treatment-control comparison is attained, and the control closely resembles the study group in all known relevant baseline, treatment (other than study drug), and observational variables. Other circumstances under which the so-called 1-arm study design may be preferred include when the response rate of the standard treatment is low, the test article has substantial activity as a single agent, and the sample size is small.¹⁹

We chose to limit Kaplan-Meier analysis in the present study to death attributable to MM rather than

disease-free interval because the former is more precisely quantifiable than the latter. Even when the described limitations concerning the study design are considered, the substantial difference with respect to survival times for death attributable to MM between the vaccinate and historical control groups likely outweighs bias that may have been introduced by the use of historical controls. Results of this study support previous research findings regarding safety and efficacy of the huTyr vaccine in dogs as adjunctive treatment for oral MM^{6,11} and extend the scale of those early studies to include multiple clinical settings. Successful use of the huTyr vaccine in dogs has led to its use in human trials,^{20,21} which supports a growing body of evidence that suggests dogs with spontaneously occurring cancer may be useful for determining the safety and efficacy of potential human treatments. Additionally, such studies make possible targeted immunotherapy of other diseases in veterinary medicine.^{9,22,23}

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- a. ONCEPT, Merial, Duluth, Ga.
 - b. Vitajet3, Bioject Medical Technologies Inc, Bedminster, NJ.
 - c. SAS, version 9.2, SAS Institute Inc, Cary, NC.
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