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)

Malignant melanoma is the most commonly occurring oral tumor in dogs. 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 chemotherapy and radiotherapy appears to have limited value in local management of the disease. 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).

Immunotherapy targeting the melanoma differentiation antigen tyrosinase (essential to melanin synthe-
has been explored as a strategy for systemic treatment of melanoma. 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. In 1 study, 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, 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. 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.

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 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. 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.

**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
were conducted by use of statistical software. C Values of differences in survival time and in estimates of MST between vaccinates 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 control groups were determined by use of χ² tests and Wilcoxon rank-sum tests, respectively. All statistical analyses were conducted by use of statistical software. 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 \( (P = 0.23) \) 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 huTy 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 been administered. The remaining dogs were followed until dogs died or were lost to follow-up.

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.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vaccinates</th>
<th>Historical controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of dogs</td>
<td>58</td>
<td>53</td>
</tr>
<tr>
<td>No. of males</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>No. of females</td>
<td>28</td>
<td>19</td>
</tr>
<tr>
<td>Age (y)</td>
<td>Median (mean ± SD) 10.5 (10 ± 2.5)</td>
<td>11 (10.8 ± 3.4)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>5–16</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>Median (mean ± SD) 23.9 (23.6 ± 12.7)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>1.4–47</td>
</tr>
<tr>
<td>Stage II disease (No. of dogs)</td>
<td>44</td>
<td>34</td>
</tr>
<tr>
<td>Stage III disease (No. of dogs)</td>
<td>14</td>
<td>19</td>
</tr>
</tbody>
</table>

Vaccinates received 4 transdermal injections (given at 14-day intervals) of a vaccine containing plasmid DNA with a CDNA insert encoding human tyrosinase \(^{10,11} \) (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 dogs \(^{6} \) 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 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).

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.

<table>
<thead>
<tr>
<th>Disease stage</th>
<th>Dog No.</th>
<th>Surgical margins</th>
<th>Disease progression</th>
<th>Disease-free interval (d)</th>
<th>Survival time (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>1</td>
<td>C</td>
<td>Metastasis to contralateral mandible, progression to lungs</td>
<td>240</td>
<td>475</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>C</td>
<td>Metastasis to lungs</td>
<td>96*</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>I</td>
<td>Recurrence, with metastasis to regional LN, then lungs</td>
<td>102</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>I</td>
<td>Recurrence</td>
<td>243</td>
<td>530</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>I</td>
<td>Recurrence, with metastasis to lungs</td>
<td>157*</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>E</td>
<td>Metastasis to regional LN</td>
<td>207</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>NR</td>
<td>Recurrence, with metastasis to lungs</td>
<td>368</td>
<td>464</td>
</tr>
<tr>
<td>III</td>
<td>9</td>
<td>C</td>
<td>Recurrence, with metastasis to lungs</td>
<td>233</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>C</td>
<td>Metastasis to regional LN, then lungs</td>
<td>512</td>
<td>583</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>C</td>
<td>Multifocal metastasis</td>
<td>253</td>
<td>293</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>I</td>
<td>Recurrence</td>
<td>47*</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>I</td>
<td>Metastasis to contralateral mandible</td>
<td>161*</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>I</td>
<td>Recurrence</td>
<td>75*</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>NR</td>
<td>Recurrence, with metastasis to lungs</td>
<td>100*</td>
<td>100</td>
</tr>
</tbody>
</table>

*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.

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

<table>
<thead>
<tr>
<th>Outcome</th>
<th>No. (%) of dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Death attributable to MM</td>
<td>Vaccines</td>
</tr>
<tr>
<td>Death attributable to causes other than MM*</td>
<td>15 (26)</td>
</tr>
<tr>
<td>Lost to follow-up*</td>
<td>16 (28)</td>
</tr>
<tr>
<td>Removed from study*</td>
<td>10 (17)</td>
</tr>
<tr>
<td>Alive at the end of the study*</td>
<td>9 (16)</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
</tr>
</tbody>
</table>

*Dogs with this outcome were censored for analysis.
— = Not applicable.
Figure 1—Kaplan-Meier survival curves comparing survival time of dogs with spontaneously occurring oral MM in vaccinate (n = 58; dashed line) and historical control (63; solid line) groups over time. Dogs in both groups had stage II or III oral MM, for which locoregional disease control was achieved by means of surgery with or without fractionated radiation treatment. Vaccinates 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 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. Median survival time for historical controls was 324 days; MST for vaccinates 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 = Vaccinates. + = 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 vaccinate group were classified as having stage II and 14 as having stage III oral MM prior to study enrollment. Vaccinates 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 vaccinate 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 vaccinates with and without evidence of complete tumor excision (473 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 a firm subcutaneous swelling approximately 4 × 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
The concept that xenogeneic DNA vaccination could induce autoantibodies and autoreactivity unrelated to protein 2, induced autoantibodies and autoreactivity. In preliminary investigations of the same huTyr plasmid construct used in the present study, Bergman et al. 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 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.

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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 vaccines and historical control dogs. However, even though fewer vaccines (n = 8) than historical controls (11) were alive at the conclusion of the study, an MST had not been reached in the vaccine 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 vaccine 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 MM11 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.22,23

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