* KOPIE *

INGEKOMEN 0 8 FEB. 2010

Universiteit Utrecht

Heidelberglaan 8, Utrecht

VenC, Postbus 80125, 3508 TC Utrecht

Bestuursdienst

Vastgoed en Campusontwikkeling

Aan de Staatssecretaris van VROM
p/a RIVM/SEC/Bureau GGO
Postbus 1
3720 BA Bilthoven

Ons kenmerk
VenC/V&M 10.007
Faxnummer
[redacted]
Telefoon
[redacted]
E-mail
[redacted]
Blad
Blad 1 van 1

Datum
4 februari 2010
Onderwerp
Kennisgeving Introductie in het Milieu
faculteit Diergeneeskunde

Hierbij stuur dr. P.T. Odinot u namens dhr. dr.ir. J.A. Mol van de faculteit Diergeneeskunde van de Universiteit Utrecht de aanvraag van voorgenomen Introductie in het milieu – veterinaire toepassing als bedoeld in het Besluit genetisch gemodificeerde organismen Wet milieugevaarlijke stoffen. Deze aanvraag is getiteld: Vaccinatie met naakt nucleïnezuur coderend voor xenogenetisch humaan tyrosinase voor de inductie van specifieke afweer tegen melanoma cellen bij de hond.

Met vriendelijke groet,

Namens het College van Bestuur
Dr. P.T. Odinot
Universitaire bestuursdienst Vastgoed en Campus
Taakgroep Veiligheid en Milieu
Universiteit Utrecht

AANVRAAGFORMULIER INTRODUCTIE IN HET MILIEU:

VETERINAIRE TOEPASSINGEN VAN GENETISCH GEMODIFICEERDE ORGANISMEN

Indien u vragen heeft kunt u contact opnemen met Bureau GGO (email: bggo@rivm.nl, telefoon: 030-2742793).

INHOUDSOPGAVE

INTERNET www.bioveiligheid.nl

AFKORTINGEN

Regeling	Regeling genetisch gemodificeerde organismen
ggo	Genetisch Gemodificeerd Organisme

A. ALGEMENE GEGEVENS

A.1. Titel van de aanvraag:
Vaccinatie met naakt nucleïnezuur coderend voor xenogenetisch humaan tyrosinase voor de inductie van specifieke afweer tegen melanoma cellen bij de hond.

A.2. Geef een korte inhoudelijke beschrijving van de aanvraag.
Maligne melanoom is een redelijk frequent voorkomende spontane en agressieve tumor bij de hond. Ondanks agressieve lokale behandeling (chirurgie en radiotherapie) worden vaak metastasen op afstand gevonden. De tumorcellen zijn veelal ongevoelig voor chemotherapie. In de afgelopen jaren is vooruitgang geboekt voor wat betreft overlevingsduur bij honden met gemetastaseerd melanoom in de VS door deze te vaccineren met een DNA vaccin dat humaan tyrosinase, een melanoom –specifiek enzym, tot expressie brengt. Het gebruikte vaccin is inmiddels goedgekeurd door de FDA en beschikbaar op de Amerikaanse markt.
Het academisch dierenziekenhuis van het departement Geneeskunde van Gezelschapsdieren van de faculteit Diergeneeskunde in Utrecht wil in een pilot behandelingsstudie met door eigenaren gehouden honden (patiënten) ervaring opdoen met procedures en randvoorwaarden voor het ontwikkelen en toepassen van DNA vaccins. Nevendoel is dat in de nabije toekomst onderzoek kan worden geïnitieerd naar gentherapie van andere chemoresistente tumoren bij gezelschapsdieren.

A.3. Geef een korte beschrijving van de voorgenomen werkzaamheden.
Vergunning wordt aangevraagd voor opslag en toedienen van een DNA vaccin dat commercieel beschikbaar wordt gesteld. Patiënten zullen tijdens het toedienen verblijven in een ingeperkte ruimte die voldoet aan de DM-II inrichtingseisen. Vervolgens zal bij een goede conditie het dier worden ontslagen en medisch worden gevolgd. Afval zal volgens de standaard protocollen voor het werken met GGO's worden afgevoerd.

A.4. Beoogde einddatum:
In de proefperiode zal ervaring worden opgedaan met 10 honden. Per jaar worden enkele patiënten behandeld waardoor het behandel en ontwikkeltraject voor DNA vaccins een periode van ongeveer 5 jaar zal beslaan. Bij behandeling van de beoogde 10 honden binnen deze periode zal de evaluatie van het gehele project eerder plaatsvinden en worden afgerond.

DOEL VAN DE INTRODUCTIE IN HET MILIEU

A.5. Algemeen doel van de werkzaamheden die worden aangevraagd:
Behandelen van patiënten met en het ontwikkelen van DNA vaccins tegen chemoresistente tumoren bij de hond.

A.6. Specifiek doel van de werkzaamheden die worden aangevraagd:
Evaluatie van de klinische effectiviteit van DNA vaccinatie bij honden met gemetastaseerd melanoom.

VERGUNNINGAANVRAGER

A.8. **Rechtspersoon:**
Universiteit Utrecht

A.9. **Afdeling/vakgroep:**
Faculteit Diergeneeskunde
Departement Geneeskunde van Gezelschapsdieren

A.10. **Correspondentieadres:**
Yalelaan 104

A.11. **Postcode en plaatsnaam:**
3584 CM Utrecht

A.12. **Telefoonnummer:**
030-2531709

VERANTWOORDELIJK MEDEWERKERS (VM)

VERANTWOORDELIJK MEDEWERKER VOOR WERKZAAMHEDEN ANDERS DAN DE VETERINAIRE TOEPASSING VAN HET GGO (VM-I)

A.13. **Titel, voorletter, voorvoegsel, achternaam:**
Dr. Ir. J.A. Mol

A.14. **Instelling/bedrijf:**
Faculteit Diergeneeskunde, Universiteit Utrecht

A.15. **Afdeling/vakgroep:**
Departement Geneeskunde van Gezelschapsdieren

A.16. **Correspondentieadres:**
[redacted]

A.17. **Postcode en plaatsnaam:**
[redacted]

A.18. **Telefoon- en faxnummer:**
[redacted]

A.19. **E-mail adres:**
[redacted]

VERANTWOORDELIJK MEDEWERKER VOOR DE VETERINAIRE TOEPASSING VAN HET GGO (VM-II)

A.20. **Titel, voorletter, voorvoegsel, achternaam:**
Prof. Dr. J. Kirpensteijn

A.21. **Instelling/bedrijf:**
Faculteit Diergeneeskunde, Universiteit Utrecht

A.22. **Afdeling/vakgroep:**
Departement Geneeskunde van Gezelschapsdieren

A.23. **Correspondentieadres:**
[redacted]

A.24. **Postcode en plaatsnaam:**
[redacted]

A.25. **Telefoon- en faxnummer:**
[redacted]

A.26. **E-mail adres:**
[redacted]

MILIEUVEILIGHEIDSFUNCTIONARIS (MVF)

A.27. **Titel, voorletter, voorvoegsel, achternaam:**
Mw. Ing. M.G.J. Schmitz

Dr. P.T. Odinot

A.28. **Instelling/bedrijf:**
Faculteit Diergeneeskunde, Universiteit Utrecht

Bestuursdienst UU

A.29. **Afdeling/vakgroep:**
Arbo, Milieu en Huisvestingbeheer

Taakgroep Veiligheid en Milieu

A.30. **Correspondentieadres:**
[redacted]

A.31. **Postcode en plaatsnaam:**
[redacted]

A.32. **Telefoonnummer:**
[redacted]

A.33. **E-mail adres:**
[redacted]

PLAATS VAN UITVOERING

A.27. Op welke locaties wordt de voorgenomen toepassing uitgevoerd:

Toediening van het DNA vaccin zal plaats vinden in het dierenverblijf (voldoet aan de DM-II inrichtingseisen) van de kliniek voor Gezelschapsdieren, Yalelaan 108.

Detectie van plasmide DNA zal worden verricht op het onderzoekslaboratorium (ML-I) van het Departement Geneeskunde van Gezelschapsdieren, Yalelaan 104.

Het vaccin wordt geïmporteerd onder de verantwoordelijkheid van de apotheek van de faculteit Diergeneeskunde; Mevr. I.M. van Geijlswijk, Hoofd apotheek, Yalelaan 106, 3584 CM Utrecht.

ONDERTEKENING

Namens de Rechtspersoon
Naam:
Dr. P.T. Odinot **datum** 4-2-2010

MVF
Naam:
Mw. Ing. M.G.J. Schmitz **datum** 1-2-2010

VM I (niet veterinaire toepassing)
Naam:
Dr. Ir. J.A. Mol **datum** 28-1-2010

VM II (veterinaire toepassing)
Naam:
Prof. Dr. J. Kirpensteijn **datum** 28/01/2010

B. Beschrijving van het genetisch gemodificeerde organisme

B.1. Geef aan waaruit het genetisch gemodificeerde organisme dat aan de proefdier wordt toegediend bestaat.

O Naakt nucleïnezuur (onderdeel B.4.)

B.4. NAAKT NUCLEÏNEZUUR

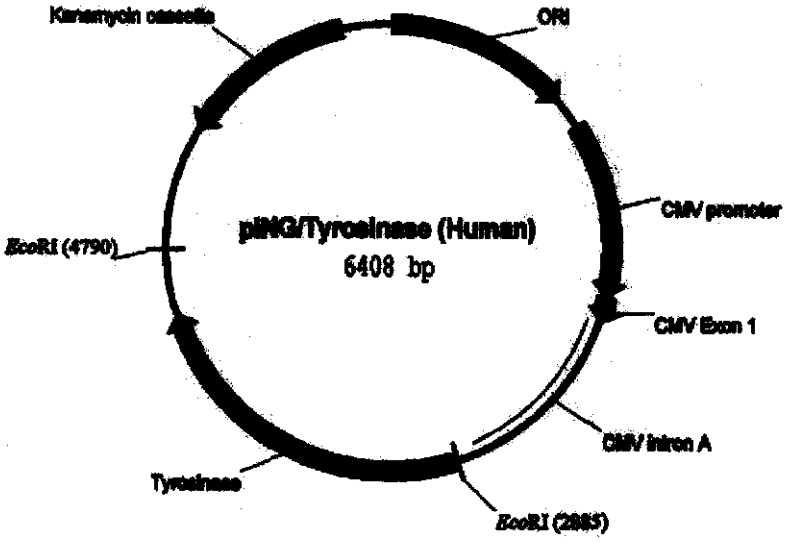
B.4.1. Bevat het nucleïnezuur sequenties van virale oorsprong die een interactie kunnen aangaan met genomen van autonoom replicerende virussen of andere micro-organismen.

Het plasmide bevat een CMV promoter afkomstig van het cytomegalie virus. CMV is een herpesvirus dat voornamelijk via de orale en seksuele route wordt overgedragen. De aanwezigheid van dit virus bij honden is uiterst onwaarschijnlijk. Gezien de wijze van toediening is het pING plasmide alleen transient aanwezig in spiercellen en mogelijk enige huidcellen op de plaats van injectie.
De kans dat het plasmide in aanraking komt darmbacteriën is eveneens zeer laag. Indien dit al gebeurd heeft opname van het plasmide, in de afwezigheid van kanamycine, geen groeivoordeel voor de darmbacterie en zal dus niet gehandhaafd worden en zal dit tot verlies van het plasmide leiden.

B.4.2. Geef een beschrijving van het genetisch gemodificeerde nucleïnezuur dat wordt toegepast.

Het plasmide (zie B.4.3.) bevat een ColE1 ori (origin of replication) die er voor zorgt dat het plasmide alleen in bacteriën van de familie *Enterobacteriaceae* kan repliceren en gehandhaafd blijft.
Daarnaast is een virale CMV promoter aanwezig.
Het plasmide brengt een humaan tyrosinase eiwit tot expressie. Tyrosinase katalyseert de omzetting van tyrosine naar het huidpigment melanine in huidcellen. In maligne melanoom komt het tyrosinase eveneens tot expressie. Door immunisatie met xenogenetisch tyrosinase, in dit geval afkomstig van de mens, wordt een immuunreactie opgewekt tegen endogeen honden tyrosinase waardoor het immuunsysteem maligne melanoom cellen gaat herkennen en zal verwijderen.

B.4.3. Geef een schematische weergave ('kaart') en een beschrijving van de opbouw van het genetisch gemodificeerde nucleïnezuur.



C. MILIEUGERELATEERDE GEGEVENS AFKOMSTIG UIT EERDERE EXPERIMENTEN

C.1. Geef een beschrijving van de resultaten welke afkomstig zijn uit eerdere studies met het GGO, en die van belang zijn voor de milieurisicobeoordeling.

Sinds 2000 tot heden is het genoemde vaccin al bij meer dan 1500 honden toegepast in de Verenigde Staten. Op grond van deze studies werd geconcludeerd dat DNA vaccinatie met het humaan tyrosinase construct:

1. Veilig is
2. Specifieke anti-tyrosinase immuunreacties oproept
3. Potentieel therapeutisch is met opvallend positieve resultaten in honden met stage II/III lokaal-regionaal gecontroleerde ziekte
4. Een aantrekkelijke kandidaat is voor verdere evaluatie in een adjuvante setting als Phase II studie

Artikelen van relevante literatuur worden gegeven in de bijlage.

Tevens is bijgesloten de risicoanalyse zoals die gemaakt is voor registratie in de VS voor zover het geen vertrouwelijke informatie bevat van de producent Merial Inc.

Inmiddels is na de conditionele licentie in 2007 op grond van voornoemde studies een volledige licentie afgegeven in 2009 door de USDA (United States Department of Agriculture).

http://www.fiercevaccines.com/story/vical-heralds-regulatory-approval-melanoma-vax-dogs/2010-01-12

D. PRODUCTIE VAN HET GGO OF NUCLEINEZUUR PREPARAAT

D.1. Geef aan onder welke verantwoordelijkheid productie van het GGO of nucleïnezuur preparaat wordt uitgevoerd.

De productie vindt plaats door Merial Inc. In de Verenigde Staten.
Zie bijgesloten document over de risicoanalyse.

D.2. In welke stappen van de productie vindt kwaliteitscontrole plaats, welke testmethoden worden gebruikt en hoe worden de tests uitgevoerd.

Zie bijgesloten document over de risicoanalyse.

D.3. Welke criteria worden aan een batch van het GGO gesteld voordat deze wordt *vrijgegeven voor de onderhavige toepassing.*

Zie bijgesloten document over de risicoanalyse.
Verdere kwaliteitsbewaking vindt plaats door de apotheek van de faculteit Diergeneeskunde, in overleg met het CBG (college ter beoordeling van geneesmiddelen) te Wageningen (www.cbg-med.nl). .

E. ASPECTEN BEHOREND BIJ DE VETERINAIRE TRIAL

E.1. Hoeveel proefdieren zullen opgenomen worden in het onderzoek.
Het betreft een pilot onderzoek waarbij het middel alleen wordt toegediend bij dieren die uitbehandeld zijn met de tot nu toe ter beschikking staande middelen, en serieus lijden wordt voorkomen dit ter beoordeling van de veterinaire specialist. In de komende periode worden maximaal 10 dieren voorzien waarna de behandeling zal worden geëvalueerd.

E.2. Welke doses worden toegediend en op welke tijdstippen gedurende de studie vindt toediening plaats.
Honden ontvangen een transdermale/intramusculaire vaccinatie met 500 µg plasmide DNA. Dieren worden 2x week gevaccineerd tot een maximum van 4 injecties.

E.3. Op welke wijze wordt het GGO preparaat aan het proefdier toegediend.
Plasmide DNA wordt transdermaal/intramusculair toegediend via een naaldvrij Biojector2000 systeem. De Bioinjector brengt de vloeistof onder CO2 druk in, is goedgekeurd door de FDA voor het toedienen van intramusculaire injecties en gebruikt voor DNA vaccinaties (zie referenties).

Aguiar, J. C., R. C. Hedstrom, et al. (2001). "Enhancement of the immune response in rabbits to a malaria DNA vaccine by immunization with a needle-free jet device." Vaccine 20(1-2): 275-80.
Williams, J., L. Fox-Leyva, et al. (2000). "Hepatitis A vaccine administration: comparison between jet-injector and needle injection." Vaccine 18(18): 1939-43.

Toediening vindt plaats in een dierenstal die voldoet aan de DM-II inrichtingseisen van het departement Geneeskunde van Gezelschapsdieren door daartoe bevoegd personeel. Eventueel vrij gekomen plasmide in de omgeving, inclusief mogelijke vloeistof op de huid van de hond zal worden afgenomen, gedecontamineerd en als biologisch afval worden afgevoerd.

E.4. Worden er monsters van het proefdier genomen die GGO's (kunnen) bevatten, en welke tests worden hiermee uitgevoerd.
Er worden geen spierbiopten of andere monsters genomen. Indien om welke reden ook bloedmonsters van het dier nodig zijn binnen 24 uur, dan zullen die op aanwezigheid van plasmide DNA worden gecontroleerd. De kans dat het plasmide DNA, anders dan in vrije vorm, in circulerende cellen zal zitten wordt nihil geacht.

E.5. Worden de proefdieren in isolatie gehouden of komen de proefdieren na behandeling met het GGO in contact met dieren die geen deel uitmaken van de onderhavige studie. Welke criteria worden gehanteerd voor het al dan niet implementeren van isolatie maatregelen. .

Toediening vindt plaats in een geïsoleerde ruimte waarbij geen contact met andere dieren kan plaatsvinden. Het risico dat het plasmide intact wordt uitgescheiden is zeer onwaarschijnlijk, temeer daar het plasmide zichzelf niet kan repliceren in het doeldier. Er zijn geen verdere isolatiemaatregelen noodzakelijk.

F. RISICO-ANALYSE

F.1. Geef aan volgens welk scenario het genetisch gemodificeerde organisme en/of een afgeleide van het nucleïnezuur preparaat zich vanuit het proefdier kan verspreiden in het milieu.

Na toediening van het plasmide wordt het deels opgenomen door spiercellen waar het zal leiden tot productie van humaan Tyrosinase. Het plasmide kan zichzelf echter niet repliceren.
Het plasmide dat aanwezig is in de bloedbaan zal daaruit verwijderd worden naar urine en feces. In het bloed en de darm wordt het vrije plasmide DNA door endonucleases afgebroken. Het risico van opname door darmbacteriën is uiterst gering en leidt zeker niet tot een groeivoordeel. Reden waarom eventueel opgenomen plasmide snel weer verwijderd zal worden uit de darmflora. Zelfs als het plasmide intact zou worden uitgescheiden is de opname door bodem bacteriën zeer onwaarschijnlijk.
De kleine patiënten populatie draagt bij aan het lage risico op verspreiding. Aan de hand van het pilot onderzoek wordt een schatting gemaakt van de patiënten aantallen op jaarbasis. Hierna wordt de aanvraag opnieuw ingediend.

F.2. Geef aan welke mogelijke nadelige effecten gepaard kunnen gaan met blootstelling van mens of milieu aan het GGO.

Omdat het plasmide zichzelf niet kan repliceren zal afgifte in het milieu uiterst gering zijn. Mocht het plasmide in het milieu terecht komen dan zijn de kansen dat het wordt opgenomen door bodem bacteriën laag.
Opname van het plasmide door betrokken medewerkers is zeer onwaarschijnlijk. In de ruimte wordt gepaste kleding gedragen en morsen van injectie vloeistof zal direct worden gedecontamineerd. Overigens zal productie van humaan tyrosinase bij de mens, mede gezien de ervaring van het toedienen van homoloog tyrosinase aan de muis, niet leiden tot een immuunreactie door de homologe aard van het tyrosinase.

F.3. Geef een inschatting van de kans dat de in F.2. beschreven nadelige effecten ook daadwerkelijk kunnen optreden.

Inmiddels is bij 1500 dieren over een periode van 9 jaar in de Verenigde Staten dit vaccin toegepast en is er geen melding gemaakt van enig nadelig effect. Op grond hiervan en van bovenstaande argumenten is de kans dat nadelige effecten optreden daarom uiterst onwaarschijnlijk te noemen.
Daarbij gaat het hier in Nederland om een pilot studie waarbij maximaal 10 dieren behandeld zullen worden. Als dieren uit de trial worden teruggetrokken zal dat geen additionele risico's geven.

F.4. Beschrijf de risico's die op kunnen treden ten gevolge van de toepassing van het GGO.

In principe ontstaan alleen getransfecteerde spiercellen in de patiënt. Om alle risico's van initiële verspreiding van het plasmide terug te dringen zal de vaccinatie daartoe plaats vinden in een dierenstal die voldoet aan de inrichtinseisen van een DM-II ruimte. Niet opgenomen op de huid aanwezig plasmide DNA zal worden verwijderd, gedecontamineerd en als biologisch afval worden behandeld.

G. RISICO MANAGEMENT

G.1. Welke criteria worden gehanteerd bij de selectie van proefdieren. En wat is het effect van deze criteria op de milieuveiligheid.
Het vaccin wordt alleen toegediend aan patiënten waarvoor chirurgie of bestraling geen optie is en waarbij serieus lijden, dit ter beoordeling van de veterinaire specialist, aanwezig is. De aantallen dieren die hierdoor in aanmerking komen voor behandeling zullen derhalve gering zijn en slechts enkele op jaarbasis bedragen. Hiermee worden mens en milieu dus ook minimaal belast.

G.2. Welke beperking van de omvang van de studie, in relatie tot het aantal proefdieren en de toe te passen dosering wordt toegepast in het kader van risico management maatregelen.
Zie G.1

G.3. Beschrijf welke maatregelen voorzien zijn ten aanzien van isolatie van het proefdier.
De patiënt wordt in een aparte ruimte, geïsoleerd behandeld.

G.4. Beschrijf welke maatregelen worden getroffen om verspreiding van het GGO naar derden (waaronder bij de studie en de proefdieren betrokken personeel) te voorkomen.
Betrokken personeel ontvangt allereerst instructie alvorens toegelaten te worden tot de DM-II faciliteit.
Ter plekke wordt beschermende kleding gedragen en handschoenen.
Na vaccinatie wordt gecontroleerd op de aanwezigheid van plasmide oplossing op of rond het dier.

G.5. Beschrijf welke maatregelen worden getroffen om verspreiding van het GGO naar derden (waaronder bij de studie en de proefdieren betrokken personeel) te voorkomen indien er sprake is van onverwachte gebeurtenissen zoals bijvoorbeeld ook de dood van een proefdier.

Het zonder naald toedienen van het vaccin verminderd het risico op prik accidenten. Door beschermende kleding en handschoenen wordt blootstelling van medewerkers voorkomen. Dieren worden behandeld door daartoe opgeleide dierverzorgers getraind in het voorkomen van bijt en krabincidenten (Zie ook F2).
Bij patiënten die na behandeling overlijden zal het ingebrachte plasmide net als het diereigen DNA door endogene nucleasen worden afgebroken.
Diereigenaren zullen tijdens de behandeling niet aanwezig zijn zodat die geen risico lopen.

G.6. Beschrijf van welke monsters verwacht kan worden dat zij GGO's kunnen bevatten, en geef voor die monsters aan hoe bemonstering plaatsvindt, en hoe de monsters verder worden verwerkt.
Van de dieren wordt een bloedmonster genomen. In principe komt hierin alleen vrij plasmide voor. Uit het monster zal DNA worden geïsoleerd voor de bepaling van de aanwezigheid van plasmide.

H. MONITORING EN AFVALVERWERKING

H.1. Op welke wijze wordt het GGO preparaat gedetecteerd na de toediening.
Standaard PCR techniek op humaan tyrosinase

H.2. Beschrijf hoe de monitoring wordt opgezet om eventuele verspreiding van het GGO waar te kunnen nemen.
Het DNA plasmide is niet infectieus en kan zichzelf ook niet repliceren in het doeldier. De kans dat er GGO's worden uitgescheiden is uiterst onwaarschijnlijk.

H.3. Geef een overzicht van de aard en hoeveelheid van het geproduceerde afval en beschrijf hoe het afval wordt afgevoerd.
Alle bij de injectie betrokken middelen zullen als biologisch afval worden afgevoerd of worden geautoclaveerd alvorens opnieuw te worden gebruikt. De afvalstroom bevat voornamelijk vrij plasmide DNA. Eventuele tijdens het verblijf in de DM-II ruimte geproduceerde excreta worden eveneens als biologisch afval aangemerkt en als zodanig worden afgevoerd.

H.4. Beschrijf welke maatregelen gehanteerd worden om verspreiding van het genetisch gemodificeerd organisme te voorkomen.
Behandeling van patiënten vindt plaats in een specifieke ruimte.

Bijlage: Literatuur

1. Bergman, P. J. (2007). "Canine oral melanoma." Clin Tech Small Anim Pract 22(2): 55-60.
2. Bergman, P. J. (2007). "Anticancer vaccines." Vet Clin North Am Small Anim Pract 37(6): 1111-9; vi-ii.
3. Liao, J. C., P. Gregor, et al. (2006). "Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma." Cancer Immun 6: 8.
4. Bergman, P. J., M. A. Camps-Palau, et al. (2006). "Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center." Vaccine 24(21): 4582-5.
5. Bergman, P. J., J. McKnight, et al. (2003). "Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial." Clin Cancer Res 9(4): 1284-90.

Advances in Brief

Long-Term Survival of Dogs with Advanced Malignant Melanoma after DNA Vaccination with Xenogeneic Human Tyrosinase: A Phase I Trial[1]

Philip J. Bergman,[2] Joanne McKnight, Andrew Novosad,[3] Sarah Charney,[4] John Farrelly,[5] Diane Craft, Michelle Wulderk, Yusuf Jeffers, Michel Sadelain, Ann E. Hohenhaus, Neil Segal, Polly Gregor, Manuel Engelhorn, Isabelle Riviere, Alan N. Houghton, and Jedd D. Wolchok

Donaldson-Atwood Cancer Clinic and Flaherty Comparative Oncology Laboratory, The E&M Bobst Hospital of the Animal Medical Center, New York, New York 10021 [P. J. B., J. M., A. N., S. C., J. F., D. C., A. E. H.], and Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [M. W., Y. J., M. S., N. S., P. G., M. E., I. R., A. N. H., J. D. W.]

Abstract

Purpose: **Canine malignant melanoma (CMM) is a spontaneous, aggressive, and metastatic neoplasm. Preclinical mouse studies have shown that xenogeneic DNA vaccination with genes encoding tyrosinase family members can induce antibody and cytotoxic T-cell responses, resulting in tumor rejection. These studies provided the rationale for a trial of xenogeneic DNA vaccination in CMM using the human tyrosinase gene.**

Experimental Design: **Three cohorts of three dogs each with advanced (WHO stage II, III, or IV) CMM received four biweekly i.m. injections (dose levels 100, 500, or 1500 μg, respectively/vaccination) of human tyrosinase plasmid DNA i.m. via the Biojector2000 delivery device.**

Results: **Mild local reactions at injection sites were the only toxicities observed, with no signs of autoimmunity. One dog with stage IV disease had a complete clinical response in multiple lung metastases for 329 days. Two dogs with stage IV disease had long-term survivals (421 and 588+ days) in the face of significant bulky metastatic disease, and two other dogs with locally controlled stage II/III disease had long-term survivals (501 and 496 days) with no evidence of melanoma on necropsy. Four other dogs were euthanized because of progression of the primary tumor. The Kaplan-Meier median survival time for all nine dogs was 389 days.**

Conclusions: **The results of this trial demonstrate that xenogeneic DNA vaccination of dogs with advanced malignant melanoma is a safe and potentially therapeutic modality. On the basis of these results, additional evaluation of this novel therapeutic is warranted in locally controlled CMM and advanced human melanoma.**

Introduction

CMM[6] of the oral cavity, nail bed, foot pad, and mucocutaneous junction is a spontaneously occurring, highly aggressive, and frequently metastatic neoplasm (1–4). CMM is a relatively common diagnosis representing ~4% of all canine tumors, and it is the most common oral tumor in the dog (3, 5, 6). CMM and advanced HM are diseases that are initially treated with aggressive local therapies, including surgery and/or fractionated radiation therapy; however, systemic metastatic disease is a common sequela (1, 3, 7). Also, CMM and HM are chemoresistant neoplasms (8–10). On the basis of these similarities, CMM is a good clinical model for evaluating new treatments for advanced HM (11).

Canine patients with advanced disease (WHO stage II, III, or IV) have a reported median ST of <5 months with aggressive local excision (1–3). Human patients with deep American Joint Committee on Cancer stage II or stage III disease (locally advanced or regional lymph node involvement) have at least a 50% chance of recurrence after surgical resection; patients with stage IV melanoma (distant metastases) have a median survival of <10 months, and most of these patients eventually die of melanoma (9, 12). Standard systemic therapy is dacarbazine chemotherapy in HM and carboplatin chemotherapy in CMM (8, 10). Unfortunately, response rates to chemotherapy in humans or dogs with advanced melanoma range from 8 to 28% with little evidence that treatment improves survival (8–10). It is evident that new approaches to this disease are desperately needed.

Active immunotherapy in the form of vaccines represents one potential therapeutic strategy for melanoma. The advent of DNA vaccination circumvents some of the previously encoun-

Received 10/11/02; accepted 12/6/02.
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[1] Supported, in part, by NIH Grants PO1 CA33049, P01 CA59350, and R01 CA56821 and by Swim across America, Mr. and Mrs. Quentin J. Kennedy Fund, Bioject, Inc., and Merial, Ltd.
[2] To whom requests for reprints should be addressed, at The E&M Bobst Hospital of the Animal Medical Center, 510 East 62nd Street, New York, NY, 10021. Phone: (212) 329-8674; Fax: (212) 702-0971; E-mail: Philip.bergman@amcny.org.
[3] Present address: Animal Diagnostic & Referral Clinic, 4444 Trinity Mills Road, Dallas, TX 75287.
[4] Present address: University of Illinois, Veterinary Clinical Medicine, 1008 West Hazelwood Drive, Urbana, IL 61802.
[5] Present address: Cornell University, Clinical Sciences NYS-CVM, Box 31, T3-008A VRT, Ithaca, NY 14853.

[6] The abbreviations used are: CMM, canine malignant melanoma; ST, survival time; HM, human melanoma; CR, complete response; PR, partial response; PD, progressive disease; ANA, antinuclear antibody; LDH, lactate dehydrogenase.

tered hurdles in vaccine development (13, 14). DNA is relatively inexpensive and simple to purify in large quantity. The antigen of interest is cloned into a bacterial expression plasmid with a constitutively active promoter. The plasmid is introduced into the skin or muscle with an intradermal or i.m. injection. Once in the skin or muscle, professional antigen-presenting cells, particularly dendritic cells, are able to present the transcribed and translated antigen in the proper context of MHC and costimulatory molecules. The bacterial and plasmid DNA itself contains immunostimulatory sequences that may act as a potent immunological adjuvant in the immune response (15, 16). In clinical trials for infectious disease, DNA immunization has been shown to be safe and effective in inducing immune responses to malaria and HIV (17, 18). Although DNA vaccines have induced immune responses to viral proteins, vaccinating against tissue-specific self-proteins on cancer cells is clearly a more difficult problem. One way to induce immunity against a tissue-specific differentiation antigen on cancer cells is to vaccinate with xenogeneic antigen or DNA that is homologous to the cancer antigen (19). It has been shown that vaccination of mice with DNA encoding cancer differentiation antigens is ineffective when self-DNA is used, but effective tumor immunity can be induced by orthologous DNA from another species (20).

We have chosen to target defined melanoma differentiation antigens of the tyrosinase family. Tyrosinase is a melanosomal glycoprotein, essential in melanin synthesis. The full-length human tyrosinase gene was shown to consist of five exons and was localized to chromosome 11q14-q21 (21). Immunization with xenogeneic human DNA encoding tyrosinase family proteins induced antibodies and cytotoxic T cells against syngeneic B16 melanoma cells in C57BL/6 mice, but immunization with mouse tyrosinase-related DNA did not induce detectable immunity (22). In particular, xenogeneic DNA vaccination induced tumor protection from syngeneic melanoma challenge and autoimmune hypopigmentation (23, 24). Thus, xenogeneic DNA vaccination could break tolerance against a self-tumor differentiation antigen, inducing antibody, T-cell, and antitumor responses. Sequence identity between partially sequenced canine tyrosinase (284 bp) and human tyrosinase (1888 bp) is 91% (GI 6513592 and GI 37508, respectively).

In this study, we demonstrate that human tyrosinase DNA vaccination of dogs with advanced malignant melanoma is safe and potentially active, warranting additional xenogeneic DNA vaccine investigations as Phase II studies in CMM and Phase I studies for HM.

Materials and Methods

Patient Population. From April 2000 to December 2000, nine dogs with previously histologically confirmed spontaneous malignant melanoma were treated with xenogeneic human tyrosinase DNA vaccination. Pretrial evaluation included complete physical examination, a complete blood count and platelet count, serum chemistry profile, urinalysis, LDH, antinuclear antibody, and three-dimensional measurements of the primary tumor if present (or maximal tumor size from medical records if patient was treated before pretrial considerations). For evaluation of metastatic disease, 3-view radiographs of the thorax were obtained, and regional lymph nodes were evaluated with fine needle aspiration/cytology and/or biopsy/histopathology. All dogs were clinically staged according to the WHO staging system of stage II (tumors 2–4 cm diameter, negative nodes), stage III (tumor > 4 cm and/or positive nodes) or stage IV (distant metastatic disease). The numbers of previous treatments with surgery, radiation, and/or chemotherapy were recorded. Dogs with WHO stage II (high grade), III, or IV histologically confirmed malignant melanoma were allowed entrance onto the study because of the lack of effective available systemic treatments. Additional entry criteria included an estimated life expectancy of ≥6 weeks, free of clinically detectable brain metastases, no previous therapy (surgery, radiation and/or chemotherapy) for at least 3 weeks, and no serious intercurrent medical illnesses. Written consent for entry onto this trial was obtained from each dog's owner before entry onto the study; this consent included request for necropsy upon death because of any reason. This study was performed under Animal Medical Center Institutional Review Board approval.

Table 1 Vaccine treatment and evaluation schedule

		Weeks				
	Pretreatment	0	2	4	6	8
Vaccine[a]		X	X	X	X	
Serology/PBMC's	X			X	X	X
Chest X-rays	X			X		X
Physical exam	X	X	X	X	X	X
CBC, Biochem Prof, LDH, anti-DNA antibody (ANA)	X			X		X

[a] 100, 500, and 1500 μg DNA dose levels.

Trial Design. Cohorts of three dogs each received increasing doses of xenogeneic plasmid DNA encoding human tyrosinase i.m. (100, 500, and 1500 μg/dose for each dose level) biweekly for a total of four vaccinations in the left semimembranosus/semitendinosus muscle (caudal thigh) region with the Biojector 2000 jet delivery device with no. 3 (i.m.) Bioject syringes. The Biojector2000 is a carbon dioxide-powered jet delivery device, which is Food and Drug Administration approved for administration of i.m. injections and has been used in DNA vaccine clinical investigations (25, 26). Subjective pain level responses and postvaccinal presence of a wheal or other reaction were assessed and recorded by the veterinarian administering the DNA vaccination. The dogs did not receive any concomitant treatments during the course of vaccination. Clinical safety patient rules were written such that the first dog in a new dose escalation group could not be entered until 2 weeks after the third dog from the previous dose group level received vaccination no. 1 without any subsequent evidence of serious toxicity. If any grade III or IV toxicities were noted, the size of the cohort of those dogs would be expanded for better delineation of the maximally tolerated dose. A schematic of the protocol is presented in Table 1.

Vaccine Information. Human tyrosinase cDNA was previously cloned and sequenced at Memorial Sloan-Kettering Cancer Center (21). The DNA has been inserted in the pING plasmid vector, which contains a cytomegalovirus promoter and kanamycin resistance selection marker. The vaccine was pro-

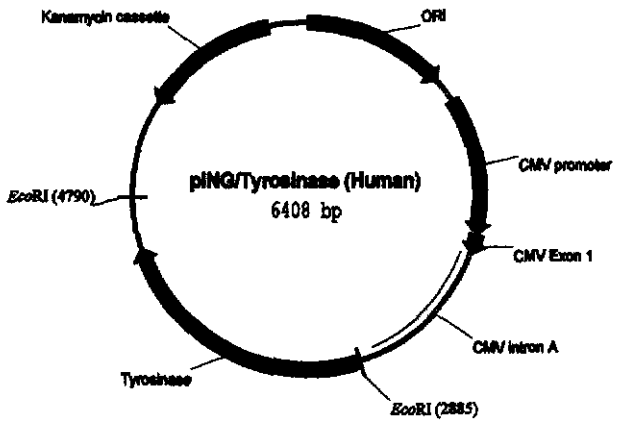

Fig. 1 Plasmid map of pING plasmid used for generation of human tyrosinase DNA vaccine given to nine dogs with advanced malignant melanoma.

duced and released from the Gene Transfer and Somatic Cell Engineering Facility at the Memorial Sloan-Kettering Cancer Center with permission from the United States Department of Agriculture. A schematic of the pING plasmid vector is presented in Fig. 1.

Clinical Response and Follow-Up Evaluations. The following criteria for antitumor effect were used: CR = disappearance of all clinical evidence of tumor on physical examination, radiographic examination, and biochemical evaluation for at least 1 month; PR = ≥50% decrease on physical and radiographic examination of the summed products of the perpendicular diameters of all measured lesions; stable disease = <50% decrease or <25% increase on physical and radiographic exams of the summed products of the perpendicular diameters of all measured lesions; or PD = ≥25% increase on physical and radiographic examination of the summed products of the perpendicular diameters of all measured lesions. After the fifth visit, 2 weeks after the fourth vaccination, serial rechecks with antitumor response evaluations continued to be performed as clinically indicated on an every 30–60-day interval. Historical abnormalities and problems found on physical examination were investigated as clinically indicated at each recheck visit. In addition, recheck evaluations continued as long as clinically necessary to determine ST for each dog. When requested by the dog's owner because of degradations in quality of life from advanced disease, euthanasia was performed with subsequent necropsy examination. A full necropsy was performed on all dogs that died or were euthanized with particular examination (gross and histopathological) of the primary tumor site, metastatic sites, left semimembranosus/semitendinosus vaccination site, and eyes.

Statistical Analysis. ST was defined as the time from receiving first xenogeneic human tyrosinase DNA vaccination until death. Median survival was calculated using the Kaplan-Meier product limit method using Statview statistical analysis software (27). Because of the limited number of cases evaluated in this Phase I clinical trial, appropriate nonparametric statistical testing (*e.g.,* Mann-Whitney *U* test, Spearman rank correlation, and so on) was used. All recorded variables (age, gender, neuter status, breed, weight, concurrent disease status, concurrent disease type, previous treatment, previous treatment type, number of previous treatments, and pain score) after each vaccination (none, mild, moderate, and severe), and toxicity from vaccination, ANA titer, change in ANA titer postvaccination, LDH level prevaccination, LDH level postvaccination, ST, survival censor, follow-up time, primary tumor location, WHO stage at diagnosis, WHO stage prevaccination, response at 2 week postvaccination, response at 6 months postvaccination, melanotic *versus* amelanotic malignant melanoma primary tumor, and DNA dose were evaluated when available for their effect on ST with Kaplan-Meier life table analysis and Cox proportional hazards analysis when appropriate. Dogs were censored if they were lost to follow-up or died because of disease other than malignant melanoma. In addition, all recorded variables were evaluated statistically for potential correlations and associations. To be evaluated for survival statistic purposes, dogs must have received at least three vaccinations; however, toxicity would be evaluated from dogs after entrance onto the trial independent of the number of vaccines received. A two-tailed P of <0.05 was considered statistically significant.

Results

Patient Demographics. The median age of the nine dogs receiving human tyrosinase DNA vaccination was 13 years (mean, 12 years; range, 9–14 years). The median weight was 13 kg (mean, 22 kg; range, 3.6–61 kg.). There were three spayed females, five castrated males, and one intact male dog. There were three mixed breed dogs, two Cocker Spaniels, and one each Siberian husky, Lhasa apso, Bichon frise, and German shepherd dog. All nine dogs received prior therapy before entrance onto the trial; seven dogs had a single previous surgery, two dogs had multiple previous surgeries, and three dogs received prior radiation therapy. Five dogs had primary malignant melanoma in the oral cavity, whereas three dogs had nail bed or footpad malignant melanoma, and one dog had an intraocular high-grade malignant melanoma treated with exenteration. Using a modified WHO staging scheme (3), four dogs had stage IV disease, two dogs had stage III disease, and three dogs had high-grade stage II disease. All nine dogs had a histological diagnosis of melanotic malignant melanoma verified by a veterinary pathologist. All nine dogs received all four biweekly vaccinations. Three dogs had concurrent nonmelanoma diseases but were still eligible for clinical trial; the concurrent diseases included histopathologically confirmed liver dysplasia, dermatopathy, and glaucoma. Table 2 presents an overview of the patient characteristics.

Toxicity. A total of 36 vaccinations were given to the nine dogs on this trial. Three, 26, and 7 vaccinations were subjectively assessed to have moderate, mild, and zero pain responses, respectively (Table 3). Immediately after vaccination, the injection site had at most a minimal to mild wheal with no other local toxicity subsequently noted. No systemic toxicity as assessed by physical examination, hematopathology, or serum chemistry was noted throughout the trial and on subsequent serial examinations after vaccinations were completed. One patient had an abnormally elevated LDH before vaccination that returned to normal after completion of four vaccinations. One

Table 2 Patient characteristics

Dog no.	DNA dose (μg)	Breed	Weight (lbs.)	Gender	Age (yr)	WHO stage	Local tumor control before vaccine	Response at 2 week recheck	Response at last recheck or death	Survival time (days)	Cause of death
1	100	Mixed	8	Female neutered	14	II	No	PD	PD	57	1[a]
2	100	Siberian husky	67	Male neutered	12	IV	No	CR	PD	389	1b
3	100	Bichon frise	18	Male neutered	13	II	Yes	NED[b]	NED	496	2
4	500	Lhasa apso	21	Male neutered	13	II	No	PD	PD	96	1
5	500	German shepherd	130	Male neutered	9	IV	No	PD	PD	421	3
6	500	Cocker spaniel	27	Female neutered	13	III	No	PD	PD	54	1
7	1500	Mixed	59	Female neutered	13	III	Yes	NED	NED	501	4
8	1500	Cocker	29	Male neutered	12	IV	No	PD	PD	126	1
9	1500	Mixed	80	Male intact	9	IV	No	SD	PD	588+	Alive

[a] Cause of death: 1, progressive local disease; b, concomitant sepsis; 2, hyperadrenocorticism complications; 3, progressive metastatic disease; 4, hepatocellular carcinoma.
[b] Response: NED, No evidence of disease; SD, stable disease.

Table 3 Toxicities

DNA dose/vaccination	Pain score = 0[a]	Pain score = 1	Pain score = 2	Local toxicity	Systemic toxicity
100 μg	0	11	1	None	None
500 μg	3	7	2	None	None
1500 μg	0	8	4	None	None

[a] Pain score 0 = none; pain score 1 = mild; pain score 2 = moderate.

patient had an abnormally elevated LDH after completion of the four vaccinations; this patient was diagnosed with liver dysplasia before entrance into the study and died with a ST of 501 days of hepatocellular carcinoma. For patients entering the trial with normal ANA titers ($n = 5$), there were no subsequent abnormal results. For those patients entering the trial with baseline abnormally elevated ANA titers ($n = 4$), there was no subsequent increase in ANA titers during or after the vaccination protocol. All dogs that died or were euthanatized had complete necropsies performed with particular attention and examination (gross and histopathological) of the primary tumor site, metastatic sites, previously vaccinated region, and the eyes. No toxicity was noted, except for two dogs having a mild pleocellular inflammatory infiltrate in the s.c. and i.m. regions of the vaccination site.

Response. Response data are shown in Table 2. At the latest evaluation, there was one stage IV dog alive (588+ days) with stable disease on this trial. This dog has an unchanged, solitary 4-cm pulmonary lesion that was confirmed to be melanoma via ultrasound-guided fine needle aspirate and cytological examination before entrance on the trial. One stage IV dog had a long-term stabilization of disease for 196 days with significant bulky pulmonary metastases. After experiencing PD, this dog went on to receive additional xenogeneic DNA vaccines (mouse tyrosinase-related protein 1/gp75 and then later murine tyrosinase) in subsequent clinical trials and was euthanatized at 421 days (28).

One stage III dog was alive for 496 days and was euthanatized because of complications from pituitary-dependent hyperadrenocorticism and was found to be free of any gross and histopathological evidence of melanoma on necropsy. An additional stage III dog was alive for 501 days and was euthanized because of complications from a subsequent hepatocellular adenocarcinoma (dog with histopathologically confirmed liver dysplasia before entrance onto study) and was found to be free of any gross or histopathological evidence of melanoma on necropsy. Both of these dogs were censored on Kaplan-Meier survival statistics at 496 and 501 days, respectively.

One stage IV dog had a CR 2 weeks after the fourth DNA vaccination that was durable for 329 days. This dog continued to have PD (increasing size of pulmonary metastases on thoracic radiographs) while being vaccinated; however, thoracic radiographs at the time of the fourth vaccination showed mild-moderate scalloping of the pulmonary metastases. Two weeks after the fourth vaccination, the pulmonary metastases disappeared with a subsequent long-term CR (Fig. 2). In clinical trials, cancer vaccines can take ~4–8 weeks or more to induce cellular and humoral immune responses and months for clinical responses (29, 30). The one dog experiencing a long-term CR fits into this temporal model of response. This dog eventually was euthanized because of complications from acute sepsis and was found to have a recurrent 2-cm malignant melanoma in the caudal pharynx ~3-cm caudal to the site of the original oral primary malignant melanoma. No evidence of the previously radiographically documented pulmonary metastases was found on gross and histopathological necropsy examination. Because of the presence of recurrent oral malignant melanoma, this dog was not censored and considered to be dead possibly because of melanoma, although there was no clinical evidence linking systemic sepsis to the local melanoma recurrence.

The Kaplan-Meier median ST for all nine dogs is 389 days (Fig. 3). No dogs were lost to follow-up, and the median follow-up time is 389 days, suggesting adequate data maturation. Two dogs were euthanatized at the 2-week recheck after the fourth vaccination because of progressive local disease. All four dogs with STs < 130 days were euthanized because of

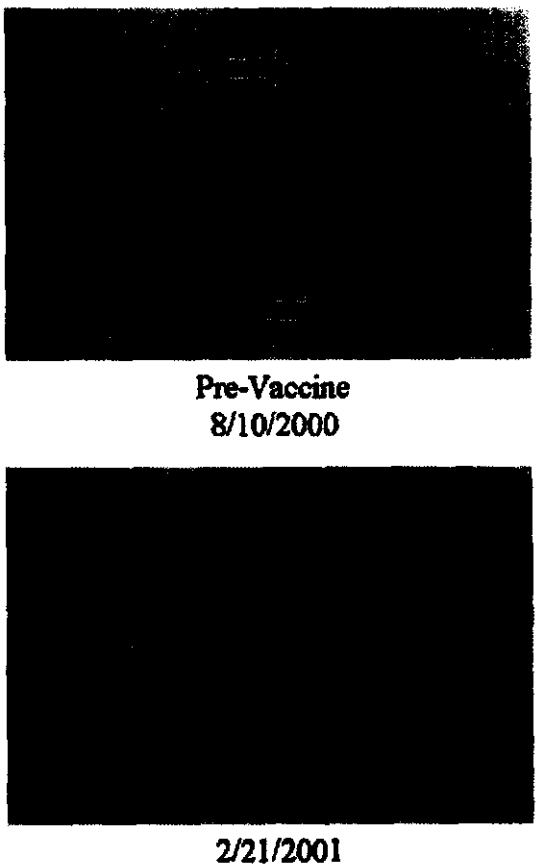

Fig. 2 Detail photographs of lateral chest radiographs from dog no. 2 (12-year-old neutered male Siberian husky; stage IV disease) placed on trial. *Top panel:* lateral chest radiograph before DNA vaccination documenting evidence of pulmonary metastases (note *arrows*). *Bottom panel:* lateral chest radiograph from same dog in *top panel* showing complete resolution of pulmonary metastases after four 100-μg human tyrosinase DNA vaccinations. The dog's complete response lasted for 329 days.

local tumor progression and not because of quality of life implications from metastatic disease. All four of these dogs did not have primary tumor control before entrance onto the study.

None of the recorded variables had a statistically significant association or effect on ST, likely because of the small number of patients. For association evaluation of continuous variables, a positive correlation was found for age and LDH level postvaccination (Kendall rank correlation; $P = 0.0333$), and a negative correlation was found for age and weight (Spearman rank correlation; $P = 0.0113$). For association evaluation of continuous and nominal variables, a positive association was found between increased LDH postvaccination and female gender (Mann-Whitney U test; $P = 0.0201$) as well as increased LDH prevaccination and no previous radiation therapy (Mann-Whitney U test; $P = 0.0389$). Statistically significant positive associations were found for the presence of a perivaccinal pain response between vaccination nos. 2 and 3, vaccination nos. 2 and 4, and vaccinations nos. 3 and 4 (χ^2; $P = 0.0111$, $P = 0.0111$, $P = 0.0027$, respectively).

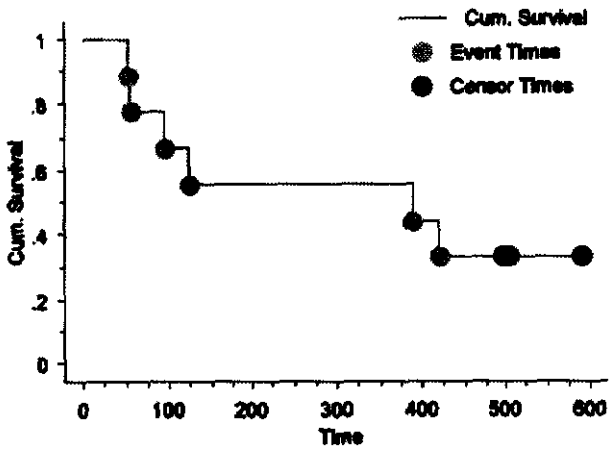

Fig. 3 Kaplan-Meier survival curve for nine dogs with advanced malignant melanoma treated with four biweekly xenogeneic human tyrosinase vaccinations. Kaplan-Meier median survival time is 389 days. Time as shown on X-axis is in days, and three dogs are censored at 496, 501, and 588 days.

Discussion

This clinical trial was designed to determine the safety and efficacy of xenogeneic DNA vaccination in advanced CMM. From the results of this study, we can conclude that xenogeneic human tyrosinase DNA vaccination of dogs is: (*a*) safe, based on minimal local toxicity and a lack of systemic toxicity; and (*b*) potentially efficacious, based on clinical antitumor responses and remarkably prolonged median STs. Historical Kaplan-Meier median STs for dogs with WHO stage II malignant melanoma treated with surgery is ≤5 months, and dogs with WHO stage III or IV have median STs of ≤2–3 months (2, 3, 31). The median ST of dogs treated with xenogeneic human tyrosinase DNA vaccination found in this study is 389 days, supporting clinical efficacy. These data warrant additional clinical evaluations in both CMM and HM.

Vaccine strategies to date in CMM have either used autologous tumor cell vaccines (with or without transfection with immunostimulatory cytokines), allogeneic tumor cell vaccines transfected with interleukin 2, or a bacterial super-antigen approach with granulocyte macrophage colony-stimulating factor or interleukin 2 as immune adjuvants (31–35). Although these approaches have produced clinical antitumor responses, the methodology for the generation of these vaccines is time consuming, sometimes dependent on patient tumor samples being established into cell lines, and fraught with the difficulties of consistency, reproducibility, and other quality control issues. Xenogeneic DNA vaccines are relatively easy to produce, inexpensive, break tolerance, and induce antitumor responses in mouse systems. In addition, DNA vaccines can induce both cellular and humoral immunity, and this combined immunity may be more effective than either arm alone. Importantly, the results of this study show that the xenogeneic model can be extrapolated to CMM as we believe this is the first report of xenogeneic DNA vaccination in spontaneous cancer. This study emphasizes how spontaneous canine cancers serve as an important bridge between preclinical studies in mouse model systems

and clinical trials in humans with cancer and additionally supports the synergy of collaborations between veterinary and human cancer centers.

It is believed that immunotherapy holds the most promise for cancer patients with minimal residual disease (24, 29, 36). The results of this trial support this long-held premise as cases of CMM without local tumor control before vaccination had STs ranging from 54 to 126 days ($n = 4$), whereas those dogs with good local tumor control via radical surgery and/or coarse fractionation radiation therapy (7) and no evidence of metastasis at the start of vaccination ($n = 2$) had STs of 496 and 501 days with both cases having no gross or histopathological evidence of melanoma at death. These data in addition to the aforementioned prolongation of median ST when compared with historical controls strongly argue for Phase II xenogeneic DNA vaccine investigations in locally controlled but not grossly metastatic CMM and HM settings.

Carboplatin is presently the therapy of choice for late-stage CMM; however, response rates are poor and predominately consist of short-term PRs with a median PR time of only 156 days (10). The results of this trial using xenogeneic human tyrosinase DNA vaccination in CMM appear to be substantially superior to carboplatin based on the lack of toxicity and increased median STs noted in this trial. In summary, we propose that xenogeneic tyrosinase DNA vaccination may be an effective treatment for stage II–IV CMM after locoregional control of primary tumor growth, presumably through inhibiting progression of metastases (and perhaps through therapeutic effects on established metastases in occasional patients).

CMM of the oral cavity, digit/footpad, and mucocutaneous junction appears clinically similar to aggressive HM because both diseases are chemoresistant, radioresistant, and share similar metastatic phenotypes and site selectivity. Importantly, CMM is a spontaneous syngeneic cancer occurring in outbred, immune-competent large mammals that live in the same environment that humans do. For these reasons, we believe CMM is a more clinically faithful therapeutic model for HM when compared with the more traditional mouse systems, and further use of CMM as a therapeutic model for HM is strongly encouraged.

Acknowledgments

We thank Shawn Takada, LVT, Maura Egan, LVT, and Claudia Wiley for technical support, as well as the technical assistance of Ping Song with construction of the pING vector.

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Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center

P.J. Bergman[a,b,*], M.A. Camps-Palau[a], J.A. McKnight[a], N.F. Leibman[a], D.M. Craft[a], C. Leung[a], J. Liao[a], I. Riviere[b], M. Sadelain[b], A.E. Hohenhaus[a], P. Gregor[b], A.N. Houghton[b], M.A. Perales[b], J.D. Wolchok[b]

[a] *Donaldson-Atwood Cancer Clinic & Flaherty Comparative Oncology Laboratory, The Animal Medical Center, 510 East 62nd Street, New York, NY 10021, USA*
[b] *Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA*

Available online 24 August 2005

Abstract

Introduction: Canine malignant melanoma (CMM) is an aggressive neoplasm treated with surgery and/or fractionated RT; however, metastatic disease is common and chemoresistant. Preclinical and clinical studies by our laboratory and others have shown that xenogeneic DNA vaccination with tyrosinase family members can produce immune responses resulting in tumor rejection or protection and prolongation of survival. These studies provided the impetus for development of a xenogeneic DNA vaccine program in CMM.
Materials and Methods: Cohorts of three dogs each received increasing doses of xenogeneic plasmid DNA encoding either human tyrosinase (huTyr; 100/500/1500 mcg), murine GP75 (muGP75; 100/500/1500 mcg), murine tyrosinase (muTyr; 5 dogs each at 100/500 mcg), muTyr ± HuGM-CSF (9 dogs at 50 mcg muTyr, 3 dogs each at 100/400/800 mcg HuGM-CSF, or 3 dogs each at 50 mcg muTyr with 100/400/800 mcg HuGM-CSF), or 50 mcg MuTyr intramuscularly biweekly for a total of four vaccinations.
Results: The Kaplan–Meier median survival time (KM MST) for all stage II–IV dogs treated with huTyr, muGP75 and muTyr are 389, 153 and 224 days, respectively. Preliminarily, the KM MST for stage II–IV dogs treated with 50 mcg MuTyr, 100/400/800 mcg HuGM-CSF or combination MuTyr/HuGM-CSF are 242, 148 and >402 (median not reached) days, respectively. Thirty-three stage II–III dogs with locoregionally controlled CMM across the xenogeneic vaccine studies have a KM MST of 569 days. Minimal to mild pain was noted on vaccination and one dog experienced vitiligo. We have recently investigated antibody responses in dogs vaccinated with HuTyr and found 2- to 5-fold increases in circulating antibodies to human tyrosinase.
Conclusions: The results of these trials demonstrate that xenogeneic DNA vaccination in CMM: (1) is safe, (2) leads to the development of anti-tyrosinase antibodies, (3) is potentially therapeutic, and (4) is an attractive candidate for further evaluation in an adjuvant, minimal residual disease Phase II setting for CMM.

Keywords: Melanoma; DNA vaccine; Xenogeneic; Canine; Comparative oncology

1. Introduction

Canine malignant melanoma (CMM) of the oral cavity, nail bed, foot pad and mucocutaneous junction is a spontaneously occurring, highly aggressive and frequently metastatic neoplasm [1]. CMM is a relatively common diagnosis representing ~4% of all canine tumors and it is the most common oral tumor in the dog [1,2]. CMM and advanced human melanoma (HM) are diseases that are initially treated with aggressive local therapies including surgery and/or fractionated radiation therapy; however, systemic metastatic disease is a common sequela [1,3–7]. Based on these similarities, CMM appears to be a good clinical model for evaluating new treatments for advanced HM.

Canine patients with advanced disease (WHO stage II, III or IV) have a reported median survival time of 1–5 months with standardized therapies [1,2,4]. A combination of

* Corresponding author. Tel.: +1 212 329 8674; fax: +1 212 702 0971.
E-mail address: philip.bergman@amcny.org (P.J. Bergman).

0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.08.027

hypofractionated radiation therapy and chemotherapy have a reported median survival time of 1 year in stage I oral CMM [8]. Human patients with deep AJCC stage II or stage III disease (locally advanced or regional lymph node involvement) have at least a 50% chance of recurrence after surgical resection; patients with stage IV melanoma (distant metastases) have a median survival of less than 10 months and most of these patients eventually die of melanoma [7,9,10]. Standard systemic therapy is dacarbazine chemotherapy in HM, and carboplatin chemotherapy in CMM. Unfortunately, response rates to chemotherapy in humans or dogs with advanced melanoma range from 8 to 28% with little evidence that treatment improves survival [11–13]. It is easily evident that new approaches to this disease are desperately needed and multiple methodologies have been reported to date [14–21].

Active immunotherapy in the form of vaccines represents one potential therapeutic strategy for melanoma. The advent of DNA vaccination circumvents some of the previously encountered hurdles in vaccine development. DNA is relatively inexpensive and simple to purify in large quantity. The antigen of interest is cloned into a bacterial expression plasmid with a constitutively active promoter. The plasmid is introduced into the skin or muscle with an intradermal or intramuscular injection. Once in the skin or muscle, professional antigen presenting cells, particularly dendritic cells, are able to present the transcribed and translated antigen in the proper context of major histocompatibility complex and costimulatory molecules. The bacterial and plasmid DNA itself contains immunostimulatory sequences that may act as a potent immunological adjuvant in the immune response. In clinical trials for infectious disease, DNA immunization has been shown to be safe and effective in inducing immune responses to malaria and human immunodeficiency virus [22,23]. Although DNA vaccines have induced immune responses to viral proteins, vaccinating against tissue specific self-proteins on cancer cells is clearly a more difficult problem. One way to induce immunity against a tissue specific differentiation antigen on cancer cells is to vaccinate with xenogeneic antigen or DNA that is homologous to the cancer antigen. It has been shown that vaccination of mice with DNA encoding cancer differentiation antigens is ineffective when self-DNA is used, but tumor immunity can be induced by orthologous DNA from another species [24,25].

We have chosen to target defined melanoma differentiation antigens of the tyrosinase family. Tyrosinase is a melanosomal glycoprotein, essential in melanin synthesis. The full length human tyrosinase gene was shown to consist of five exons and was localized to chromosome 11q14–q21 [26]. Immunization with xenogeneic human DNA encoding tyrosinase family proteins induced antibodies and cytotoxic T-cells against syngeneic B16 melanoma cells in C57BL/6 mice, but immunization with mouse tyrosinase-related DNA did not induce detectable immunity [24,27]. In particular, xenogeneic DNA vaccination induced tumor protection from syngeneic melanoma challenge and autoimmune hypopigmentation [28]. Thus, xenogeneic DNA vaccination could break tolerance against a self tumor differentiation antigen, inducing antibody, T-cell and anti-tumor responses.

2. Experimental design

From April 2000 to present, approximately 170 dogs with previously histologically confirmed spontaneous malignant melanoma were treated with xenogeneic DNA vaccinations. Pre-trial evaluation included complete physical examination, a complete blood count and platelet count, serum chemistry profile, urinalysis, lactate dehydrogenase, anti-nuclear antibody, and three-dimensional measurements of the primary tumor if present (or maximal tumor size from medical records *if patient* was treated prior to pre-trial considerations). For evaluation of metastatic disease, 3-view radiographs of the thorax were obtained and regional lymph nodes were evaluated with fine needle aspiration/cytology and/or biopsy/histopathology. All dogs were clinically staged according to the WHO staging system of stage II (tumors 2–4 cm diameter, negative nodes), stage III (tumor > 4 cm and/or positive nodes) or stage IV (distant metastatic disease). The numbers of previous treatments with surgery, radiation and/or chemotherapy were recorded. Dogs with WHO stage II, III or IV histologically confirmed malignant melanoma were allowed entrance onto the study due to the lack of effective available systemic treatments. Additional entry criteria included: an estimated life expectancy of 6 weeks or more, free of clinically detectable brain metastases, no previous therapy (surgery, radiation and/or chemotherapy) for at least 3 weeks and no serious intercurrent medical illnesses. Written consent for entry onto this trial was obtained from each dog's owner prior to entry onto the study; this consent included request for necropsy upon death due to any reason. These studies were performed under Animal Medical Center IRB approval.

Cohorts of three dogs each received increasing doses of xenogeneic plasmid DNA encoding either human tyrosinase (huTyr; 100, 500 or 1500 mcg), murine GP75 (muGP75; 100, 500 or 1500 mcg), murine tyrosinase (muTyr; 5 dogs at 100 or 500 mcg each), or muTyr ± HuGM-CSF (nine dogs at 50 mcg muTyr, three dogs each at 100, 400 or 800 mcg HuGM-CSF, or three dogs each at 50 mcg muTyr with 100, 400 or 800 mcg HuGM-CSF) intramuscularly biweekly for a total of four vaccinations in the left caudal thigh with the Biojector 2000 jet delivery device with #3 (intramuscular) Bioject syringes. The Biojector2000 is a carbon dioxide powered jet delivery device which is FDA approved for administration of intramuscular injections and has been used in DNA vaccine clinical investigations. At present, dogs are receiving 50 mcg muTyr as outlined above except with the Vitajet spring-loaded needle-free injection device. Subjective pain level responses and post-vaccinal presence of a wheal or other reaction were assessed and recorded by the veterinarian administering the DNA vaccination. The dogs did not receive any concomitant treatments during the course of vaccination.

3. Results

The signalments of dogs on this study have been similar to those in previously reported CMM studies. No toxicity was seen in any dogs receiving the aforementioned vaccines with the exception of minimal to mild pain responses at vaccination, one muGP75 dog experienced mild aural depigmentation, and one muTyr dog has experienced moderate foot pad vitiligo. Thirty-three dogs fit the sub-category of stages II–III loco-regionally controlled CMM across the xenogeneic vaccine studies. The Kaplan–Meier (KM) median survival time (MST) for this sub-population is 569 days with 25/33 dogs still alive. The KM MST for all stages II–IV dogs treated with huTyr, muGP75 and muTyr are 389, 153 and 224 days, respectively. The KM MST for stage II–IV dogs treated with 50 mcg muTyr, 100/400/800 mcg HuGM-CSF or combination muTyr/HuGM-CSF are 242, 148 and >402 days, respectively (median not reached, 7/9 dogs still alive). The results from dogs vaccinated with huTyr were published in 2003 [29].

4. Development of specific anti-tyrosinase humoral immune responses

We have recently begun investigating the humoral responses of dogs receiving HuTyr as a potential explanation for the long-term survivals seen in some of the dogs on this study. Utilizing standard ELISA with mammalian expressed purified human tyrosinase protein as the target of interest (kind gift of C. Andreoni & J.C. Audonnet, Merial, Inc.), we have preliminarily found 3/9 dogs with 2- to 5-fold post-vaccinal humoral responses compared to pre-immune sera. We are confirming these findings utilizing a flow-cytometric-based assay of pre- and post-vaccinal sera in permeabilized human SK-MEL melanoma cells expressing endogenous human tyrosinase and a canine melanoma cell line expressing endogenous canine tyrosinase (kind gift of Dr. L. Wolfe, Auburn University). Interestingly, the three dogs with post-vaccinal anti-HuTyr humoral responses are dogs with unexpected long-term tumor control. Additional studies are ongoing investigating the humoral responses from dogs on the other xenogeneic DNA vaccines as well as T-cell based assays utilizing ELISPOT and intracellular cytokine staining assays.

5. Conclusions

The results of these trials demonstrate that xenogeneic DNA vaccination in CMM is: (1) safe, (2) develops specific anti-tyrosinase humoral immune responses, (3) potentially therapeutic with particularly exciting results in stage II/III local-regional controlled disease and dogs receiving muTyr/HuGM-CSF combination, and (4) an attractive candidate for further evaluation in an adjuvant, minimal residual disease Phase II setting for CMM. In addition, CMM is a more clinically faithful therapeutic model for HM when compared to more traditional mouse systems as both human and canine diseases are chemoresistant, radioresistant, share similar metastatic phenotypes/site selectivity, and occur spontaneously in an outbred, immuno-competent scenario. Similarly, this work shows that veterinary cancer centers and human cancer centers can work productively together to benefit veterinary and human patients afflicted with cancer.

Acknowledgements

AMS House Officer & Dept. of Pathology staff, Merial, Inc. (Drs. T. Leard, B. Nordgren, J.C. Audonnet and C. Andreoni), Imclone Inc. (Dr. D. Hicklin), and Bioject Inc. (Dr. R. Stout).

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Clinical Techniques in
Small Animal Practice

Canine Oral Melanoma

Philip J. Bergman, DVM, MS, PhD

Melanoma is the most common oral malignancy in the dog. Oral and/or mucosal melanoma has been routinely considered an extremely malignant tumor with a high degree of local invasiveness and high metastatic propensity. Primary tumor size has been found to be extremely prognostic. The World Health Organization staging scheme for dogs with oral melanoma is based on size, with stage I = <2-cm-diameter tumor, stage II = 2- to <4-cm-diameter tumor, stage III = ≥4-cm tumor and/or lymph node metastasis, and stage IV = distant metastasis. Median survival times for dogs with oral melanoma treated with surgery are approximately 17 to 18, 5 to 6, and 3 months with stage I, II, and III disease, respectively. Significant negative prognostic factors include stage, size, evidence of metastasis, and a variety of histologic criteria. Standardized treatments such as surgery, coarse-fractionation radiation therapy, and chemotherapy have afforded minimal to modest stage-dependent clinical benefits and death is usually due to systemic metastasis. Numerous immunotherapeutic strategies have been employed to date with limited clinical efficacy; however, the use of xenogeneic DNA vaccines may represent a leap forward in clinical efficacy. Oral melanoma is a spontaneous syngeneic cancer occurring in outbred, immunocompetent dogs and appears to be a more clinically faithful therapeutic model for human melanoma; further use of canine melanoma as a therapeutic model for human melanoma is strongly encouraged. In addition, the development of an expanded but clinically relevant staging system incorporating the aforementioned prognostic factors is also strongly encouraged.
Clin Tech Small Anim Pract 22:55-60

KEYWORDS melanoma, oral melanoma, malignant melanoma, surgery, radiation therapy, chemotherapy, immunotherapy, xenogeneic DNA vaccination

The most common oral malignancy in the dog is melanoma.[1-4] Oral melanoma is most commonly seen in Scottish terriers, golden retrievers, poodles, and dachshunds.[2,5] Oral melanoma is primarily a disease of older dogs without gender predilection but may be seen in younger dogs.[5-7] Melanomas in dogs have extremely diverse biologic behaviors depending on a variety of factors. A greater understanding of these factors significantly helps the clinician to delineate in advance the appropriate staging, prognosis, and treatments. The primary factors that determine the biologic behavior of an oral melanoma in a dog are site, size, stage, and histologic parameters.[5-9] Unfortunately, even with an understanding of all of these factors, there are melanomas that have an unreliable biologic behavior, hence, the desperate need for additional research into this relatively common, heterogeneous, but frequently extremely malignant, tumor. Molecular biological aspects of melanoma have been previously reviewed.[10,11] This article will assume the diagnosis of melanoma has already been made, which in of itself can be fraught with difficulty, and will focus on the aforementioned biologic behavior parameters, the staging, and the treatment of canine oral melanoma.

Biologic Behavior

The biologic behavior of canine oral melanoma is extremely variable and best characterized on the basis of anatomic site, size, stage, and histologic parameters. On divergent ends of the spectrum would be a 0.5-cm haired-skin melanoma with an extremely low grade and likely to be cured with simple surgical extirpation, in comparison to a 5.0-cm high-grade malignant oral melanoma with a poor to grave prognosis. Similar to the development of a rational staging, prognostic, and therapeutic plan for any tumor, two primary questions must be answered: what is the local invasiveness of the tumor and what is the metastatic propensity? The answers to these questions will determine the prognosis, and to be discussed later, the treatment.

Site

The anatomic site of melanoma is highly, though not completely, predictive of local invasiveness and metastatic propen-

Donaldson-Atwood Cancer Clinic and Flaherty Comparative Oncology Laboratory, The Animal Medical Center, New York, New York.
Address reprint requests to Philip J. Bergman, DVM, MS, PhD, Diplomate ACVIM-Oncology, Head, Donaldson-Atwood Cancer Clinic and Flaherty Comparative Oncology Laboratory, The Animal Medical Center, 510 East 62nd Street, New York, NY 10021. E-mail: Philip.Bergman@amcny.org

1096-2867/07/$-see front matter
doi:10.1053/j.ctsap.2007.03.004

sity. Melanomas involving the haired-skin, which are not in proximity to mucosal margins, often behave in a benign manner.[1,12] Surgical extirpation through a lumpectomy is often curative, but histopathological examination is imperative for delineation of margins as well as a description of cytologic features. In haired-skin melanomas exhibiting histopathologic criteria of malignancy, the reader is referred to the grade discussion below. Oral and/or mucosal melanoma have been routinely considered an extremely malignant tumor with a high degree of local invasiveness and high metastatic propensity.[2,5-8] This biologic behavior is extremely similar to human oral and/or mucosal melanoma.[1,13] Melanoma is the most common oral tumor in the dog; additional neoplastic differentials include squamous cell carcinoma, fibrosarcoma, epulides/odontogenic tumors, and others.[1,2,4,14-16] Melanomas in the oral cavities of dogs are found in the following locations by order of decreasing frequency: gingiva, lips, tongue, and hard palate. While most melanomas are pigmented, amelanotic oral melanomas are noted clinically and have been previously reported.[17] In canine oral/mucosal melanomas with histological reporting suggestive of a benign lesion, the reader is referred to the grade discussion below.

The anatomic sites that split the opposite ends of the prognostic spectrum are of generally benign-acting haired-skin versus typically malignant and metastatic oral/mucosal melanomas include melanomas of the digit and foot pad. While these anatomic sites are not the primary focus of this discussion, dogs with melanoma of the digits without lymph node or further metastasis treated with digit amputation are reported to have median survival times of ~12 months, with 42 to 57% alive at 1 year and 11 to 13% alive at 2 years.[18,19] Unfortunately, metastasis from digit melanoma at presentation is reported to be ~30 to 40%, and the aforementioned outcomes with surgery suggest that subsequent distant metastasis is common even when no metastasis is found at presentation/digit amputation. The prognosis for dogs with melanoma of the foot pad has not been previously significantly reported; this author has found this anatomic site to be similar in metastatic propensity and prognosis to digit melanoma. Interestingly, human acral lentiginous melanoma (plantar surface of the foot, palms of the hand, and digit) has an increased propensity for metastasis.[20]

Size and Stage

For dogs with oral melanoma, primary tumor size has been found to be extremely prognostic. The World Health Organization staging scheme for dogs with oral melanoma is based on size, with stage I = <2-cm-diameter tumor, stage II = 2-cm- to <4-cm-diameter tumor, stage III = 4 cm or greater tumor and/or lymph node metastasis, and stage IV = distant metastasis (Table 1). Macewen and colleagues reported median survival times for dogs with oral melanoma treated with surgery to be approximately 17 to 18, 5 to 6, and 3 months with stage I, II, and III disease, respectively.[6] More recent reports suggest stage I oral melanoma treated with standardized therapies including surgery, radiation, and/or chemotherapy has a median survival time of approximately 12 to 14 months, with most dogs dying of distant metastatic disease, not local recurrence.[21,22] Other investigators have found dogs with stage I oral melanoma to have median progression-free survival times of 19 months.[23]

Table 1 Traditional World Health Organization TNM-based Staging Scheme for Dogs with Oral Melanoma

T: Primary tumor	
T1	**Tumor ≤2 cm in diameter**
T2	**Tumor 2-4 cm in diameter**
T3	**Tumor >4 cm in diameter**
N: Regional lymph nodes	
N0	**No evidence of regional node involvement**
N1	**Histologic/cytologic evidence of regional node involvement**
N2	**Fixed nodes**
M: Distant metastasis	
M0	**No evidence of distant metastasis**
M1	**Evidence of distant metastasis**
Stage I = T1 N0 M0	
Stage II = T2 N0 M0	
Stage III = T2 N1 M0 or T3 N0 M0	
Stage IV = Any T, any N and M1	

A variety of limitations exist with the present WHO staging scheme for canine oral melanoma. First, the size of the tumor is not standardized to the size of the patient. Therefore, a 1.8-cm oral melanoma without lymph node metastasis is a stage I melanoma in a Rottweiler as well as a Chihuahua. Further investigations with standardization to patient size are hereby encouraged. In addition, the histologic appearance and other histologically based indices of melanomas are not accounted for in the present WHO staging scheme, and proposed alternate schemes incorporating histologic criteria have unfortunately not gained widespread use for canine melanoma. For these reasons and others, various investigators have pursued other prognostic factors in canine oral melanoma to possibly develop alternative staging systems. These investigations have continued to find size to be extremely prognostic but have also found the following negative prognostic factors: lesser degree of extirpation and incomplete surgical margins; location (caudal mandibular and rostral maxillary do more poorly); tumor mitotic index >3; and bone invasion/lysis.[5,22] Prospective investigations including these variables into an expanded WHO staging system are hereby encouraged and ongoing at this author's institution.

The staging system for canine nonoral melanoma is less well defined to date. Henry and colleagues utilized the WHO TNM system for canine digital tumors, which defines T1 = tumor <2 cm and superficial; T2 = tumor 2 to 5 cm and minimum invasion; T3 = tumor >5 cm or invading subcutis; and T4 = tumor invading fascia or bone.[18] They reported that metastasis-free interval was significantly inversely associated with T-stage across all digit tumors. When specifically examining dogs with digit melanoma, there was one dog with T2, five dogs with T3, and four dogs with T4 tumors. Further studies defining staging schemes for canine nonoral melanoma with clinical variables and outcomes are also encouraged.

Grade and Histologic Parameters

Histopathologic grading of a tumor by the pathologist delineates degree of malignancy and grading systems vary across tumor types. The histological grade is commonly predictive of survival, metastatic rate, and other clinical variables in a

wide variety of tumors across species, including canine melanoma.[1,25,26] For example, in haired-skin melanomas exhibiting multiple histopathologic criteria of malignancy, such as increased mitotic rate, invasiveness, and/or poor differentiation, metastatic propensity is increased and the prognosis is reduced due to variability in outcomes postoperatively. Bostock reported that 45% of dogs with malignant skin melanomas died within 1 year, whereas 8% of dogs with "benign" skin melanomas died from their disease.[8] Furthermore, 10% of dogs with haired-skin melanoma with a mitotic index of 2 or less died from their tumor 2 years after surgery compared with >70% dogs dying from a tumor with a mitotic index of 3 or more. Dogs with haired-skin melanomas within 1 cm of mucosal margins have been minimally investigated to date; this author has had multiple patients with histologically benign, haired-skin melanoma within 1 cm of a mucosal margin develop subsequent distant metastatic disease. Additional investigation into patients with peri-mucosal melanoma is therefore encouraged.

The most exhaustive review of histologic findings in canine melanocytic neoplasms was recently published by Spangler and Kass.[9] In this article, 384 dogs with melanoma or melanocytoma (IDEXX submissions) had their tumors comprehensively histologically examined and statistically tested for association with malignant behavior (recurrence and/or metastasis) and median survival time via follow-up provided by the veterinarians submitting the samples. Significant negative prognostic factors included metastasis (ie, stage as discussed above), size/tumor volume, and a variety of histologic criteria such as mitotic index, nuclear atypia, tumor score, presence of deep inflammation, intralesional necrosis, and junctional activity. As expected, these investigators also found three primary anatomic-location mortality groups: (1) oral (19% of samples); (2) feet and mucosal surface of lips (19% of samples); and (3) cutaneous (59% of samples). Too few ocular melanomas were investigated to make recommendations. An unexpected finding from this investigation was the presence of 32% of dogs with oral melanoma without malignant behavior according to their criteria (no recurrence, no metastasis, and alive at the end of study or dead due to competing causes). This author sees no reason oral melanomas may not occasionally behave in a benign fashion; however, 32% is a significantly different frequency from all previous reports (which report a very small proportion to no benign oral melanomas), thereby warranting further study. Similarly, the number of benign-acting oral melanomas was relatively small (n = 22) and a variety of factors such as lack of necropsy, type of follow-up, lack of reporting of number of lost to follow-up cases, and last, the large number of cases disqualified for inclusion because of poor differentiation may have led to an increased frequency of benign-acting cases. Similarly, this author has seen in excess of 10 dogs in the last 4 years with a previous histopathologic diagnosis of benign oral melanoma present with florid distant metastasis. This is consistent with Bostock reporting three of seven dogs with "benign" oral melanoma going on to die of their disease.[8]

Spangler and Kass also reported that 38 and 12% of feet/mucosal surface of lips and cutaneous melanocytic tumors, respectively, behaved in a malignant fashion.[9] Four percent and 27% of those dogs that died of a foot/lip and cutaneous melanoma, respectively, had a tumor score that would have predicted benign behavior. On further review of those cases, there were no attributes found that would allow for prediction of malignant behavior. This suggests that additional testing is needed beyond routine light microscopy for delineation of malignant versus benign behavior for canine cutaneous melanoma. Laprie and coworkers reported the use of Ki-67 expression via immunohistochemistry in 68 canine cutaneous melanomas.[27] This group found that the predictive value of Ki-67 proliferative index (97%) was greater than the predictive value of classical histology (91%) for biologic behavior in canine cutaneous melanoma. This strongly suggests that the use of Ki-67 immunohistochemistry and possibly other proliferative markers (eg, AgNOR and others) in canine cutaneous melanoma should be commonly performed after the histopathologic diagnosis is made. The routine use of horseradish-peroxidase (HRP) -based immunohistochemistry (IHC) to characterize Ki-67 and other proliferative marker expressions is further complicated in melanoma due to strong baseline melanin pigmentation; investigations in this author's laboratory are ongoing to develop reliable methodologies through melanin bleaching and/or non-HRP-based IHC detection methodologies.

Staging

The staging of dogs with melanoma is relatively straightforward. A minimum database should include a thorough history and physical examination, complete blood count and platelet count, biochemical profile, urinalysis, three-view chest films, and local lymph node aspiration (ipsilateral and contralateral nodes for oral melanoma due to variability in draining patterns) with cytology whether lymphadenomegaly is present or not. Williams and Packer reported in dogs with oral melanoma that ~70% had metastasis when lymphadenomegaly was present, but more importantly ~40% had metastasis when no lymphadenomegaly was present.[28] Additional considerations should be made for abdominal compartment testing (eg, abdominal ultrasound) in all cases of canine melanoma, especially in cases with potentially moderately to highly metastatic anatomic sites such as the oral cavity, feet, or mucosal surface of the lips, as melanoma may metastasize to the abdominal lymph nodes, liver, adrenal glands, and other sites. The use of sentinel lymph node mapping and lymphadenectomy has been proven to be of diagnostic, prognostic, and clinical benefit in human melanoma.[29] Relatively few investigations have been reported to date for sentinel lymph node mapping and/or excision for dogs with malignancies[30-33] and this author strongly encourages additional investigation in this area and specifically with canine melanoma.

Treatment

The treatment for dogs with melanoma without distant metastatic disease on staging starts with local tumor control. This is generally best completed through surgical extirpation due to its speed, increased curative intent, and reduced cost compared with other modalities. The dose of surgery is generally based on the anatomic site of the melanoma, with cutaneous melanomas usually requiring lumpectomy and all other sites

requiring more aggressive and wide excision. While large resections such as partial mandibulectomy or maxillectomy carry an inherent level of morbidity, owner satisfaction rates are routinely considered high. It cannot be overstated the importance of complete staging when contemplating larger resections; the presence of distant metastatic disease would attenuate the use of more radical surgical procedures and convert the patient to medical and/or palliative care options.

Radiation therapy (RT) plays a role in the treatment of canine melanoma when the tumor is not surgically resectable, the tumor has been removed with incomplete margins, and/or the melanoma has metastasized to local lymph nodes without further distant metastasis. The use of smaller fractions of RT (eg, 3 to 4 Gy) given daily to every other day can allow for a greater total dose and fewer chronic RT reactions; however, melanoma appears comparatively resistant to these types of fractionation schemes.[24,34] Coarse fractionation schemes for canine melanoma utilizing 6 to 9 Gy weekly to every other week to a total dose of 24 to 36 Gy have been reported by a variety of investigators with complete remission rates of 53 to 69% and partial remission rates of 25 to 30%.[21-23,35,36] Unfortunately, recurrence and/or distant metastasis were common in all of these studies. Other modalities reported for local tumor control as case reports and/or case series have included intralesional cisplatin implants, intralesional bleomycin with electronic pulsing, and many others, but widespread use has not been reported to date.[37-39]

In dogs with melanoma in the aforementioned anatomic sites predicted to have a moderate to high metastatic propensity, or dogs with cutaneous melanoma with a high tumor score and/or increased proliferation index through increased Ki-67 expression, the use of systemic therapies is warranted. Rassnick and colleagues reported an overall response rate of 28% using carboplatin for dogs with malignant melanoma.[40] Unfortunately, only one dog had a minimally durable complete response, and the rest were nondurable partial responses. Similarly, Boria and coworkers reported an 18% response rate and median survival time of 119 days with cisplatin and piroxicam in canine oral melanoma.[41] Other reports using single-agent dacarbazine, melphalan, or doxorubicin suggest poor to dismal activity.[42-44] More recently and importantly, two studies suggest that chemotherapy plays an insignificant role in the adjuvant treatment of canine melanoma.[22,45] It can be argued that the studies performed to date to evaluate the activity of chemotherapy in an adjuvant setting for canine melanoma have been suboptimal due to a variety of reasons; however, the extensive human literature in this specific setting suggests melanoma is an extremely chemotherapy-resistant tumor.[46] It is clear that new approaches to the systemic treatment of this disease are desperately needed.

Immunotherapy represents one potential systemic therapeutic strategy for melanoma. A variety of immunotherapeutic strategies for the treatment of human melanoma have been reported previously, with typically poor outcomes due to a lack of breaking tolerance. Immunotherapy strategies to date in canine melanoma have used autologous tumor cell vaccines (with or without transfection with immunostimulatory cytokines and/or melanosomal differentiation antigens), allogeneic tumor cell vaccines transfected with interleukin-2 or GM-CSF, liposomal-encapsulated nonspecific immunostimulators (eg, L-MTP-PE), intralesional Fas ligand DNA, bacterial superantigen approaches with granulocyte macrophage colony-stimulating factor or interleukin 2 as immune adjuvants, and last, canine dendritic cell vaccines loaded with melanosomal differentiation antigens.[6,47-53] Although these approaches have produced some clinical antitumor responses, the methodologies for the generation of these products are expensive, time-consuming, sometimes dependent on patient tumor samples being established into cell lines, and fraught with the difficulties of consistency, reproducibility, and other quality-control issues.

The advent of DNA vaccination circumvents some of the previously encountered hurdles in vaccine development. DNA is relatively inexpensive and simple to purify in large quantities. The antigen of interest is cloned into a bacterial expression plasmid with a constitutively active promoter. The plasmid is introduced into the skin or muscle with an intradermal or intramuscular injection. Once in the skin or muscle, professional antigen-presenting cells, particularly dendritic cells, are able to present the transcribed and translated antigen in the proper context of major histocompatibility complex and costimulatory molecules. Although DNA vaccines have induced immune responses to viral proteins, vaccinating against tissue-specific self-proteins on cancer cells is clearly a more difficult problem. One way to induce immunity against a tissue-specific differentiation antigen on cancer cells is to vaccinate with xenogeneic (different species) antigen or DNA that is homologous to the cancer antigen. It has been shown that vaccination of mice with DNA encoding cancer differentiation antigens is ineffective when self-DNA is used, but tumor immunity can be induced by orthologous DNA from another species.[54]

At the Animal Medical Center in collaboration with human melanoma investigators at Memorial Sloan-Kettering Cancer Center (MSKCC), we have chosen to target defined melanoma differentiation antigens of the tyrosinase family. Tyrosinase is a melanosomal glycoprotein, essential in melanin synthesis. Immunization with xenogeneic human DNA encoding tyrosinase family proteins induced antibodies and cytotoxic T-cells against syngeneic B16 melanoma cells in C57BL/6 mice, but immunization with mouse tyrosinase-related DNA did not induce detectable immunity.[55] In particular, xenogeneic DNA vaccination induced tumor protection from syngeneic melanoma challenge and autoimmune hypopigmentation. Thus, xenogeneic DNA vaccination could break tolerance against a self-tumor differentiation antigen, inducing antibody, T-cell, and antitumor responses. We have investigated the use of human tyrosinase, murine gp75, murine tyrosinase, and murine tyrosinase ± human GM-CSF in dogs with advanced malignant melanoma.[56,57] The results of these trials demonstrate that xenogeneic DNA vaccination in canine malignant melanoma (1) is safe, (2) leads to the development of antityrosinase antibodies,[58] (3) is potentially therapeutic, and (4) is an attractive candidate for further evaluation in an adjuvant, minimal residual disease Phase II setting. To this end, we and our industrial sponsor, Merial Inc., are presently completing a multi-site USDA safety trial of human tyrosinase DNA vaccination in dogs with locoregionally controlled stage II/III malignant melanoma in the hopes of receiving a conditional license for widespread commercial use

with subsequent efficacy trials for full licensure. Human clinical trials at MSKCC utilizing murine and human tyrosinase DNA vaccination have recently initiated.

At the time this article was being formulated (October, 2006), there were three known sites with active immunotherapy trials for canine melanoma: The Animal Medical Center as discussed above, the University of Wisconsin–Madison (UW-Madison), and the University of Florida. While a number of immunotherapy approaches for melanoma and other tumors have been investigated and published by Macewen and colleagues, the present UW-Madison approach for canine melanoma immunotherapy is with a whole cell allogeneic tumor cell vaccine (ATCV). The canine melanoma cell line 17CM98 developed by Hogge and coworkers[48] is electroporated with human GM-CSF cDNA. Each ATCV aliquot is 2×10^7 transfected cells given ID once weekly ×4 and then every other week ×2 and monthly ×3. The primary contact at UW-Madison is Dr. Ilene Kurzman (kurzmani@svm.vetmed.wisc.edu). The present approach at the University of Florida is a GD3 ganglioside (tumor-associated carbohydrate antigen expressed on tumors of neuro-ectodermal origin such as melanoma and small-cell lung carcinoma) vaccine with MPL adjuvant and CpG motifs. There were no side effects with this vaccine in normal dogs, and no side effects to date in the 13 dogs accrued with resectable gross melanoma receiving three monthly vaccines. The primary contact at the University of Florida is Dr. Rowan Milner (milnerr@mail.vetmed.ufl.edu).

In summary, the future is looking brighter for canine melanoma on multiple fronts. We have a greater understanding of the prognostic aspects of this disease and it appears we are on the cusp of having a commercially available vaccine for treatment. It is hoped in the future that this same vaccine may also play a role in the treatment of melanoma in other species (eg, horses, cats, humans, etc.) due to its xenogeneic origins, and in melanoma prevention once the genetic determinants of melanoma risk in dogs are further defined. It is easy to see how the veterinary oncology profession is uniquely able to greatly contribute to advances for both canine as well as human melanoma, in addition to many other cancers with similar comparative aspects across species. This author and the fields of veterinary tumor immunotherapy and veterinary oncology are greatly indebted to the tireless work and seeds laid by the late Dr. Greg Macewen; he is greatly missed and this article is dedicated in his honor.

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Vet Clin Small Anim 37 (2007) 1111–1119

VETERINARY CLINICS

ELSEVIER SAUNDERS

Anticancer Vaccines

Philip J. Bergman, DVM, MS, PhD[a,b,*]

[a]Brightheart Veterinary Centers, 80 Business Park Drive, Suite 110, Armonk, NY 10504, USA
[b]Memorial Sloan-Kettering Cancer Center, New York, NY, USA

The term *immunity* is derived from the Latin word *immunitas*, which refers to the legal protection afforded to Roman senators holding office. Although the immune system is normally thought of as providing protection against infectious disease, the immune system's ability to recognize and eliminate cancer is the fundamental rationale for the immunotherapy of cancer. Multiple lines of evidence support a role for the immune system in managing cancer, including (1) spontaneous remissions in patients who have cancer and do not have treatment; (2) the presence of tumor-specific cytotoxic T cells within tumor or draining lymph nodes; (3) the presence of monocytic, lymphocytic, and plasmacytic cellular infiltrates in tumors; (4) the increased incidence of some types of cancer in immunosuppressed patients; and (5) documentation of cancer remissions with the use of immunomodulators [1]. With the tools of molecular biology and a greater understanding of mechanisms to harness the immune system, effective tumor immunotherapy is becoming a reality. This new class of therapeutics offers a more targeted, and therefore precise, approach to the treatment of cancer. The recent conditional licensure of a xenogeneic DNA vaccine for advanced canine malignant melanoma (CMM) strongly suggests that immunotherapy can play an extremely important role alongside the classic cancer treatment triad components of surgery, radiation therapy, and chemotherapy; we ardently look forward to immunotherapy playing a larger and larger role in the treatment of cancer in the future.

Any discussion about the potential usefulness of cancer immunotherapeutics predicates a more complete understanding of the principal players in the immune system, which is subsequently briefly reviewed here, before a further discussion on anticancer vaccines and other anticancer immunotherapy strategies.

TUMOR IMMUNOLOGY

The immune system is generally divided into two primary components: the innate immune response and the highly specific but more slowly developing adaptive or acquired immune response. Innate immunity is rapidly acting

0195-5616/07/$ – see front matter
doi:10.1016/j.cvsm.2007.06.005

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but typically not extremely specific and includes physicochemical barriers (eg, skin, mucosa); blood proteins, such as complement; phagocytic cells (macrophages, neutrophils, dendritic cells [DCs], and natural killer [NK] cells); and cytokines, which coordinate and regulate the cells involved in innate immunity. Adaptive immunity is thought of as the acquired arm of immunity that allows for exquisite specificity, an ability to remember the previous existence of the pathogen and to differentiate self from nonself, and, importantly, the ability to respond more vigorously on repeat exposure to the pathogen. Adaptive immunity consists of T and B lymphocytes. The T cells are further divided by *cluster of differentiation* (CD) and *major histocompatibility complex* (MHC) class into T helper cells (CD4 positive and MHC II), and T cytotoxic cells (CD8 positive and MHC I). B lymphocytes produce antibodies (humoral system) that may activate complement, enhance phagocytosis of opsonized target cells, and induce antibody-dependent cellular cytotoxicity (ADCC). B-cell responses to tumors are thought by many investigators to be less important than the development of T-cell–mediated immunity, but there is little evidence to support this notion fully [2]. The innate and adaptive arms of immunity are not mutually exclusive; they are linked by (1) the innate response's ability to stimulate and influence the nature of the adaptive response and (2) the sharing of effector mechanisms between innate and adaptive immune responses.

Immune responses can be further separated by whether they are induced by exposure to a foreign antigen (an "active" response) or if they are transferred through serum or lymphocytes from an immunized individual (a "passive" response). Although both approaches have the ability to be extremely specific for an antigen of interest, one important difference is the inability of passive approaches to confer memory. The principal components of the active/adaptive immune system are lymphocytes, antigen-presenting cells, and effector cells. Furthermore, responses can be subdivided by whether they are specific for a certain antigen or a nonspecific response whereby immunity is attempted to be conferred by upregulating the immune system without a specific target. These definitions are helpful because they allow methodologies to be more completely characterized, such as active specific and passive nonspecific, for example.

The idea that the immune system may actively prevent the development of neoplasia is termed *cancer immunosurveillance*. Sound scientific evidence supports some aspects of this hypothesis [3,4], including the following: (1) interferon (IFN)-γ protects mice against the growth of tumors; (2) mice lacking IFNγ receptor were more sensitive to chemically induced sarcomas than normal mice and were more likely to develop tumors spontaneously; (3) mice lacking major components of the adaptive immune response (T and B cells) have a high rate of spontaneous tumors; and (4) mice that lack IFNγ and B/T cells develop tumors, especially at a young age.

There are significant barriers to the generation of effective antitumor immunity by the host. Many tumors evade surveillance mechanisms and grow in immunocompetent hosts, as easily illustrated by the overwhelming

numbers of people and animals succumbing to cancer. There are multiple ways in which tumors evade the immune response, including the following: (1) immunosuppressive cytokine production (eg, transforming growth factor [TGF]-β, interleukin [IL]-10) [5,6]; (2) impaired DC function by means of inactivation ("anergy") or poor DC maturation through changes in IL-6/IL-10/vascular endothelial growth factor (VEGF)/granulocyte macrophage colony-stimulating factor (GM-CSF) [7]; (3) induction of cells called regulatory T cells (Treg), which were initially called suppressor T cells (CD4/CD25/CTLA-4/GITR/Foxp3-positive cells, which can suppress tumor-specific CD4/CD8+ T cells) [8,9]; (4) MHC I loss through structural defects, changes in B2-microglobulin synthesis, defects in transporter-associated antigen processing, or actual MHC I gene loss (ie, allelic, locus loss); and (5) MHC I antigen presentation loss through B7-1 attenuation (B7-1 is an important costimulatory molecule for CD28-mediated T-cell receptor and MHC engagement) when the MHC system in MHC I loss remains intact.

NONSPECIFIC TUMOR IMMUNOTHERAPY

Dr. William Coley, a New York surgeon in the early 1900s, noted that some patients who had cancer and developed incidental bacterial infections survived longer than those without infection [10]. Coley developed a bacterial "vaccine" (killed cultures of *Serratia marcescens* and *Streptococcus pyogenes* ["Coley's toxins"]) to treat people with sarcomas, which provided complete response rates of approximately 15%. Unfortunately, high failure rates and significant side effects led to discontinuation of this approach. His seminal work laid the foundation for nonspecific modulation of the immune response in the treatment of cancer. Nonspecific tumor immunotherapy approaches are numerous, and relevant examples are listed in Table 1 [11–34].

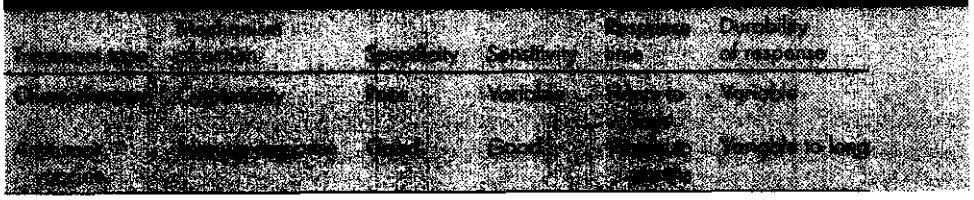
CANCER VACCINES

The ultimate goal for a cancer vaccine is elicitation of an antitumor immune response that results in clinical regression of a tumor or its metastases. Responses to cancer vaccines may take several months or more to appear because of the slower speed of induction of the adaptive arm of the immune system, as outlined in Table 2.

There are numerous types of tumor vaccines in phase I through III human trials across a wide range of tumor types. The immune system detects tumors through specific tumor-associated antigens (TAAs) that are recognized by cytotoxic T lymphocytes (CTLs) and antibodies. TAAs may be common to a particular tumor type; may be unique to an individual tumor; or may arise from mutated gene products, such as ras, p53, p21, or others. Although unique TAAs may be more immunogenic than the other aforementioned shared tumor antigens, they are not practical targets because of their narrow specificity. Most shared tumor antigens are normal cellular antigens that are overexpressed in tumors. The first group to be identified was termed *cancer testes antigens* because of expression in normal testes, but they are also found in

Table 1
Examples of neuropathic pain and [illegible] disorders

Table 2
Comparison of chemotherapy and antitumor vaccines



melanoma and various other solid tumors, such as the MAGE/BAGE gene family. This article highlights those tumor vaccine approaches that seem to hold particular promise in human clinical trials and some that have been tested to date in veterinary medicine.

A variety of approaches have been taken to focus the immune system on the aforementioned targets, including (1) whole cell or tumor cell lysate vaccines (autologous, or made from a patient's own tumor tissue; allogeneic, or made from individuals within a species bearing the same type of cancer; or whole cell vaccines from γ-irradiated tumor cell lines with or without immunostimulatory cytokines) [35–38]; (2) DNA vaccines that immunize with syngeneic or xenogeneic (different species than the recipient) plasmid DNA designed to elicit antigen-specific humoral and cellular immunity (to be discussed in more detail elsewhere in this article); (3) viral vector-based or other methodologies designed to deliver genes encoding TAAs or immunostimulatory cytokines [39,40]; (4) DC vaccines that are commonly loaded or transfected with TAAs, DNA or RNA from TAAs, or tumor lysates [41]; (5) adoptive cell transfer (the "transfer" of specific populations of immune effector cells to generate a more powerful and focused antitumor immune response); (6) cytokine approaches [42]; and (7) antibody approaches, such as monoclonal antibodies [43], anti-idiotype antibodies (an idiotype is an immunoglobulin sequence unique to each B lymphocyte; therefore, antibodies directed against these idiotypes are referred to as anti-idiotype), or conjugated antibodies. The ideal cancer immunotherapy agent would be able to discriminate between cancer and normal cells (ie, specificity), be potent enough to kill small or large numbers of tumor cells (ie, sensitivity) and, finally, be able to prevent recurrence of the tumor (ie, durability).

This author has developed a xenogeneic DNA vaccine program for melanoma at the Animal Medical Center in collaboration with human investigators from Memorial Sloan-Kettering Cancer Center and industrial partner Merial [44,45]. Preclinical and clinical studies by this author's laboratory and others have shown that xenogeneic DNA vaccination with tyrosinase family members (eg, tyrosinase, GP100, GP75, others) can produce immune responses resulting in tumor rejection or protection and prolongation of survival, whereas syngeneic vaccination with orthologous DNA does not induce immune responses [46]. These studies provided the impetus for development of a xenogeneic

DNA vaccine program in CMM. Cohorts of dogs received increasing doses of xenogeneic plasmid DNA encoding human tyrosinase (huTyr), murine GP75 (muGP75), murine tyrosinase (muTyr), muTyr with or without human GM-CSF (both administered as plasmid DNA), or muTyr "off study" administered intramuscularly biweekly for a total of four vaccinations. Minimal to mild pain was noted on vaccination, and one dog experienced vitiligo. The authors and his colleagues have recently investigated antibody responses in dogs vaccinated with HuTyr and found two- to fivefold increases in circulating antibodies to huTyr, which can cross react to canine tyrosinase, suggesting the breaking of tolerance [47]. The clinical results with prolongation in survival have been reported previously [44,45]. The results of these trials demonstrate that xenogeneic DNA vaccination in CMM (1) is safe, (2) leads to the development of antityrosinase antibodies, (3) is potentially therapeutic, and (4) is an attractive candidate for further evaluation in an adjuvant minimal residual disease phase II setting for CMM. A US Department of Agriculture (USDA) licensure study of huTyr in dogs with advanced malignant melanoma was initiated in April 2006, which led to USDA conditional licensure in March 2007. This is the first approved vaccine for the treatment of cancer across species in the United States. The xenogeneic DNA vaccine platform represents an interesting mechanism for exploration of immune responses and anticancer responses for other malignancies. To this end, the author and his colleagues have recently initiated a phase I trial of murine CD20 for dogs and cats with B-cell lymphoma [48].

Tumor immunology and immunotherapy is one of the most exciting and rapidly expanding fields at present. Significant resources are focused on mechanisms to stimulate an antitumor immune response maximally while minimizing the immunosuppressive aspects of the tumor microenvironment simultaneously. The recent elucidation and blockade of immunosuppressive cytokines (eg, TGFβ, IL-10, IL-13) or the negative costimulatory molecule CTLA-4 [49] may dramatically improve cell-mediated immunity to tumors. Immunotherapy is unlikely to become a sole modality in the treatment of cancer; the traditional modalities of surgery, radiation, and chemotherapy are extremely likely to be used in combination with immunotherapy in the future. Like any form of anticancer treatment, immunotherapy seems to work best in a minimal residual disease setting, suggesting that its most appropriate use is likely to be in an adjuvant setting with local tumor therapies, such as surgery or radiation. Similarly, the long held belief that chemotherapy attenuates immune responses from cancer vaccines is beginning to be disproved through investigations on a variety of levels [50,51]. In fact, mechanisms to induce cancer cell lysis through chemotherapy or other means after anticancer vaccination may induce increased cancer antigen presentation to an already primed immune system, thereby leading to a boosting of the immune response.

In summary, the future looks extremely bright for immunotherapy. Similarly, the veterinary oncology profession is uniquely able to contribute greatly to the many advances to come in this field. Unfortunately, what works

in a mouse often does not reflect the outcome in human patients who have cancer. Therefore, comparative immunotherapy studies using veterinary patients may be better able to "bridge" murine and human studies. To this end, a large number of cancers in dogs and cats seem to be remarkably stronger models for counterpart human tumors than presently available murine model systems. This is likely attributable to a variety of reasons, including but not limited to extreme similarities in the biology of the tumors (eg, chemoresistance, radioresistance, sharing metastatic phenotypes, site selectivity), spontaneous syngeneic cancer (typically versus an induced or xenogeneic cancer in murine models), and, finally, the fact that the dogs and cats spontaneously developing these tumors are outbred and immune competent and live in the same environment as human beings. The field of veterinary tumor immunotherapy is greatly indebted to the tireless work and seeds laid by MacEwen [52]. This author ardently looks forward to the time when immunotherapy plays a significant role in the treatment or prevention of cancer in human and veterinary patients.

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Cancer Immunity, Vol. 6, p. 8 (21 April 2006)
Submitted: 9 March 2006. Accepted: 9 March 2006.
Contributed by: AN Houghton

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Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma

Jack C. F. Liao[1], Polly Gregor[2,3], Jedd D. Wolchok[2], Francesca Orlandi[2], Diane Craft[1], Carrie Leung[1], Alan N. Houghton[2], and Philip J. Bergman[1]

[1]*Flaherty Comparative Oncology Laboratory, Donaldson-Atwood Cancer Clinic, The Animal Medical Center, 510 East 62nd Street, New York, NY 10021, USA*
[2]*Swim Across America Laboratory, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA*
[3]*Genitourinary Oncology Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA*

Keywords: dogs, melanoma, DNA vaccine, xenogeneic, humoral immunity

Antitumor immune responses can be elicited in preclinical mouse melanoma models using plasmid DNA vaccines encoding xenogeneic melanosomal differentiation antigens. We previously reported on a phase I clinical trial of human tyrosinase (huTyr) DNA vaccination of 9 dogs with advanced malignant melanoma (World Health Organization stages II-IV), in which we demonstrated the safety of the treatment and the prolongation of the expected survival time (ST) of subjects as compared to historical, stage-matched controls. As a secondary goal of the same study, we report here on the induction of tyrosinase-specific antibody responses in three of the nine dogs vaccinated with huTyr DNA. The antibodies in two of the three responders cross-react with syngeneic canine tyrosinase, demonstrating the ability of this vaccine to overcome host immune tolerance and/or ignorance to or of "self" antigens. Most interestingly, the onset of antibody induction in these three dogs coincides with observed clinical responses and may suggest a means to account for their long-term tumor control and survival.

Introduction

Canine malignant melanoma (CMM) of the oral cavity, nail bed, foot pad, and mucocutaneous junction is a spontaneous, highly aggressive neoplasm that can readily metastasize to the lymph nodes, liver, lung, and kidney (1, 2, 3, 4, 5, 6). Prognosis is often dependent on the clinical staging of the tumor; canine patients diagnosed with World Health Organization stage I oral CMM have a reported median ST of 1 yr with carboplatin chemotherapy and hypofractionated radiation (1, 7). Patients with advanced disease (World Health Organization stages II, III, or IV) have a reported median ST of less than 5 mo with surgical resection of the local tumor (2, 3, 4). Overall response rates of canine melanoma patients to chemotherapy are low, with little evidence that treatment improves ST (8). This is likely due to subsequent metastasis and resistance of residual tumor cells, despite aggressive therapies. Targeted treatment of microscopic disease should be considered a critical component in the overall management of CMM, in conjunction with current modalities for local/regional disease control.

Numerous immunologic strategies in preclinical models and clinical settings have been described as eliciting antitumor responses capable of tumor regression and rejection (9). Previous attempts in both human and veterinary oncology include the use of autologous tumor cells and/or cell extracts (with or without bacterial adjuvant) (10, 11, 12, 13), gene-modified tumor cells (14, 15, 16, 17, 18, 19, 20, 21), heat-shock proteins (22, 23), or tumor-specific peptides (24) as vaccines to activate humoral and/or T-cell immunity. Other approaches involve the induction of apoptosis by intratumoral Fas ligand (FasL) DNA injection (25) or vaccination with autologous DCs expressing xenogeneic tumor antigens in order to elicit antitumor responses (26). The utility of autologous tumor vaccines is often limited by the availability of tumor cell lines specifically established for each patient. Similarly, peptide-based vaccines can only be applied to discrete patient populations due to MHC haplotype restrictions. The main challenge in active immunization against malignant melanoma, nevertheless, is to selectively activate the immune repertoire that can recognize and target antigens on melanoma. The best-characterized antigens on melanoma include the melanosomal membrane proteins, which are also expressed on normal melanocytes.

Preclinical mouse models of melanoma demonstrate that mice vaccinated with xenogeneic (human) cDNAs encoding different melanosomal differentiation antigens (that is, TRP1/gp75, TRP2/DCT, pMel17/gp100, or tyrosinase) can generate specific antibody and T-cell responses against syngeneic tumor cells that protect against subsequent tumor challenge, whereas plasmid DNA encoding mouse antigens induces no tumor immunity (27, 28, 29, 30, 31). Hence, effective antitumor immunity can be induced *in vivo* when host immune tolerance/ignorance to/of the "self" differentiation antigens is overcome.

We have chosen to investigate xenogeneic DNA vaccines encoding tyrosinase as a means to induce immune responses in CMM patients. Tyrosinase is normally expressed in melanocytes to catalyze the rate-limiting step of melanin biosynthesis from tyrosine (32, 33). Tyrosinase, as well as other related glycoproteins, is a suitable target for CMM immunotherapy because of its restricted, tissue-specific expression. Full-length canine tyrosinase (NCBI protein database accession no. AAQ17535) also shares significant homology with that of human (NCBI accession no. AAA61242) and mouse (NCBI accession no. BAA00341) tyrosinase at 87.5% and 84.4% amino acid identity, respectively (Figure 1). Injections of xenogeneic tyrosinase DNA may therefore be a means to overcome canine

Figure 1

Tyrosinase is conserved from dog to mouse to man. Sequence comparison of tyrosinase from dog, mouse, and human shows a high degree of homology at the amino acid level. The calculated sequence identity by Vector NTI between canine tyrosinase and that of mouse and human is 84.4% and 87.5%, respectively, indicating evolutionary conservation of this protein. Dark gray highlights identical residues and light gray highlights conservative amino acid changes.

immune tolerance to the self-tyrosinase because of differences in the sequence that improve epitope recognition by MHC class I or the T-cell receptor. Sequence differences may also create class II-restricted helper epitopes and induce antityrosinase immune responses when expressed in vivo in dogs with CMM. We previously reported on the safety and prolonged survival of CMM patients immunized with xenogeneic huTyr DNA in a phase I, single-arm clinical trial (34).

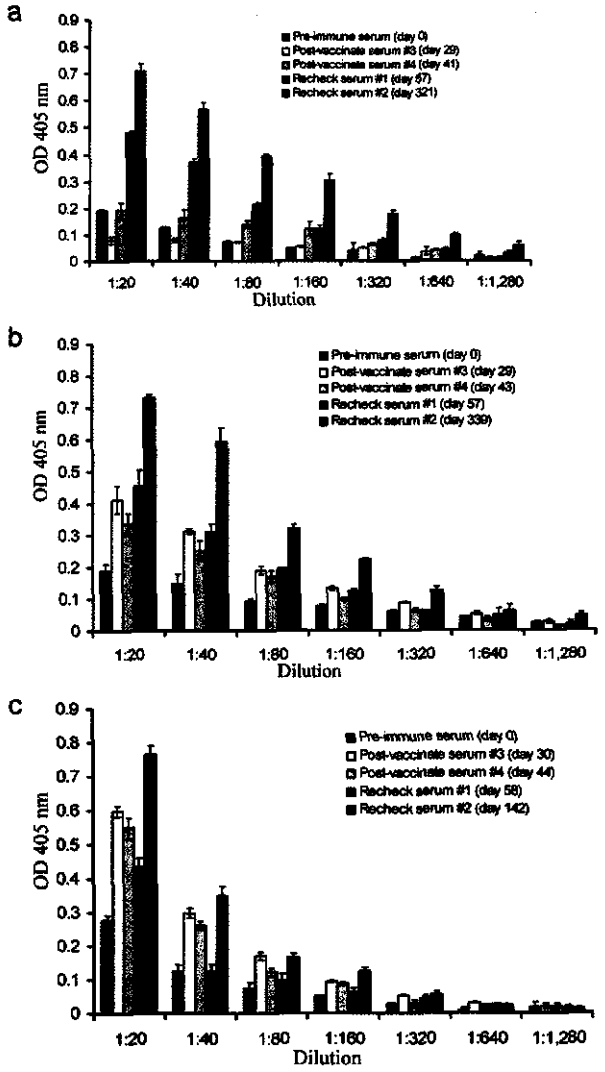
To validate the observed clinical efficacy of xenogeneic DNA vaccination as a therapeutic modality for CMM, the present study examines the humoral immune responses of the same three cohorts of dogs vaccinated with escalating doses (100 μg, 500 μg, and 1500 μg) of huTyr cDNA. Three of nine dogs have tyrosinase-specific antibodies induced after vaccination, with antibody titers as high as 1:1280 for one of the three dogs, compared to its preimmune serum and to the sera of normal, healthy dogs. The specificity of the antibodies generated is confirmed by the ability of the canine postimmune sera to detect both endogenous human and, more importantly, canine tyrosinase in cultured melanoma cell lines derived from both species. Temporal measures of the serum antibody level further indicate that the induced antibody response to the human antigen can be sustained for 3 to 9 months after the 4 biweekly immunizations. Most interestingly, these 3 dogs exhibited clinical responses with long-term tumor control; 1 of these dogs remains alive as of publication (for approximately 4 years) with an unchanged, cytologically-confirmed pulmonary metastasis. The induction of antibodies by the xenogeneic huTyr DNA vaccine, concurrent with observed antitumor responses in these CMM study subjects, supports the therapeutic feasibility of this treatment in preventing tumor dissemination, possibly through antibody-mediated immune responses. Nevertheless, further evaluation is warranted to fully elucidate its efficacy and the immunologic mechanisms of its action in an outbred, genetically heterogeneous population of large animals with spontaneous cancer. Other, related studies currently in progress are investigating the potential activation of T-cell responses by the huTyr vaccine, as well as other xenogeneic DNA vaccines that may contribute to the overall tumor regression/control of CMM.

Results

Measurement of the humoral response induced by vaccination

To measure the humoral immune response induced by the xenogeneic DNA vaccine, we analyzed canine sera for tyrosinase-specific antibodies by indirect ELISA. Of the 9 vaccinated dogs

Figure 2



Detection of tyrosinase-specific antibodies by ELISA. Representative results of three independent experiments for each patient--canine sera from patients A (panel a; 100 μg huTyr DNA dose), B (b; 100 μg huTyr DNA dose), and C (c; 1500 μg huTyr DNA dose)--were serially diluted at 1:20, 1:40, 1:80, 1:160, and 1:320, 1:640, and 1:1280. Each serum dilution was added in triplicate to individual wells (50 μl/well) containing recombinant huTyr (a-c) or myelin-basic protein, a nonspecific substrate (data not shown). The peak increase in tyrosinase-specific titer, compared to the preimmune serum, was detected for all patients at recheck 2: patient A at 1:1280, patient B at 1:640, and patient C at 1:320.

in the study, 3 had a measurable increase in postvaccine serum antibody binding to the mammalian-expressed, recombinant huTyr (Figure 2), but not to a nonspecific substrate, myelin-basic protein (data not shown). The level of antibody response, measured spectrophotometrically to reflect tyrosinase-specific antibody binding to the target substrate, ranged from two- to four-fold higher in the postvaccination sera than in the preimmune sera (Figure 2) or in the serum of a normal, healthy dog used as a control (data not shown). For dog A, initially diagnosed at stage IV with visible pulmonary metastases on thoracic radiographs, the onset of the antibody response was after the second vaccination (data not shown) with the 100-μg dose regimen. The induction of response, however, was not maintained by subsequent vaccinations, consistent with dog A's poor clinical prognosis and with the progression of pulmonary metastases. Interestingly, 2 weeks after the fourth vaccination, at a time when antibodies to tyrosinase were again detected in the sera (Figure 2a), this patient experienced a long-term remission with complete disappearance of the existing pulmonary metastases. As previously reported, dog A had a ST of 389 days and was euthanized due to complications from an acute septic episode. Subsequent gross and histopathological necropsy revealed a recurrent 2-cm malignant melanoma caudal to the site of the original oral primary malignant melanoma, but with no evidence of the previously documented pulmonary metastases (34). Evaluations of postvaccination sera taken at 2 weeks (recheck 1) and at 9 months (recheck 2) after the final vaccination indicated an antibody titer of 1:320 at recheck 1 and 1:1280 at recheck 2 (Figure 2a), which correlate with the positive clinical outcome after the completion of the vaccination series.

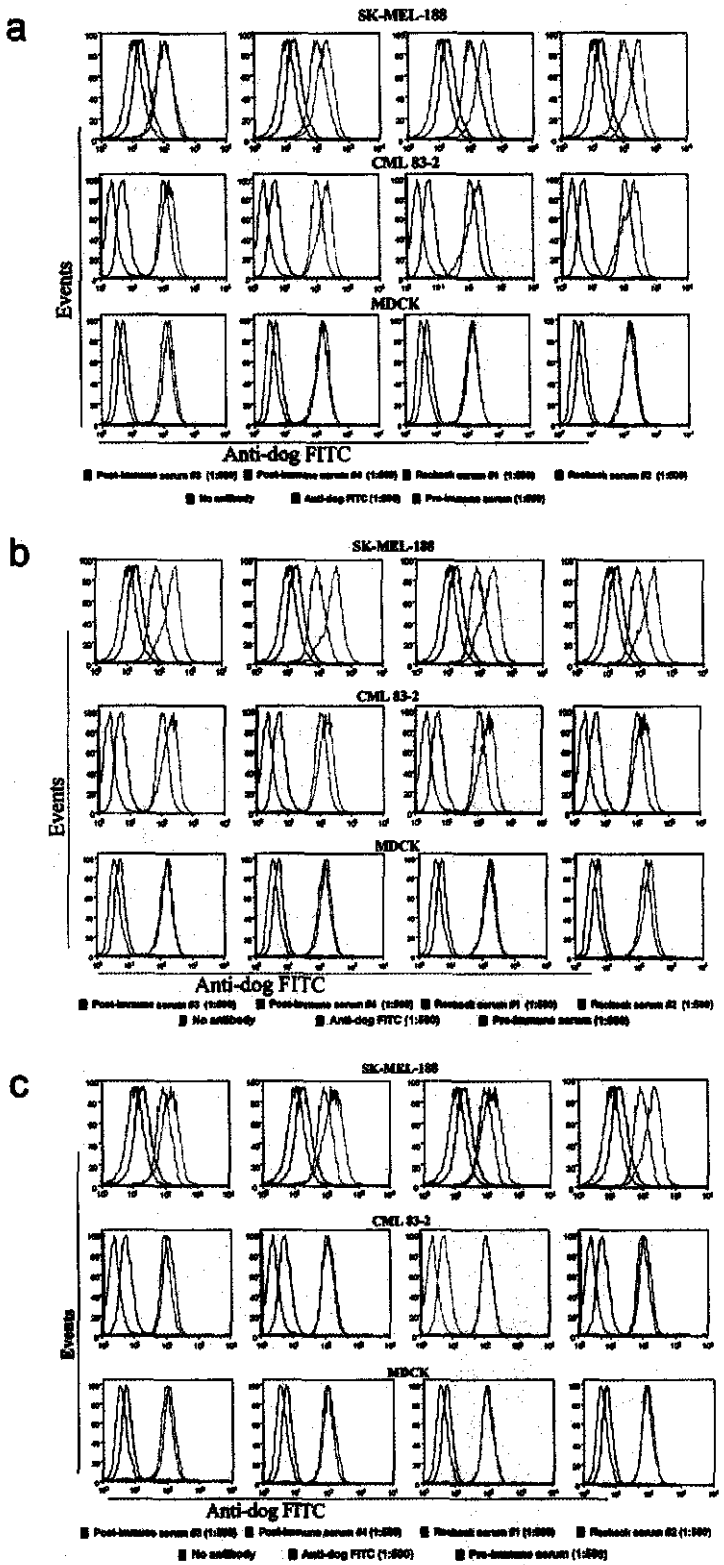
A similar induction of antibody response was detected for dog B, diagnosed with stage III melanoma, which had a reported ST of 496 d with no evidence of any gross or histopathological melanoma on necropsy (34). Consistent with the immune response in dog A, immunization of dog B at the same dose was sufficient to elicit tyrosinase-specific antibody production (Figure 2b), although the initial onset of the immune response occurred subsequent to the third vaccination. Despite this initial two-fold increase in antibody binding to the substrate, the highest level of response was not detected until 9 months after the final vaccination at recheck 2, with a titer of 1:640.

Although some of the dogs in the other cohorts (500 μg and 1500 μg doses) clearly experienced prolonged survival (34), we did not detect any measurable difference in tyrosinase-specific antibody titers between their pre- and postvaccination sera (data not shown). Of particular interest was dog C, initially diagnosed at stage IV with a solitary, cytologically confirmed 4 cm pulmonary metastasis (34). This dog developed an antibody response to the 1500 μg dose of DNA vaccine. Consistent with dogs A and B, the antityrosinase antibody response was detected for dog C after the third vaccination, and reached a maximum level 3 months after the final boost, at recheck 2 (Figure 2c). The measured antibody titer, however, was comparatively lower at 1:320, and gradually decreased to a level comparable to that of the preimmune and normal canine sera (Figure 2c and data not shown); this suggests that further vaccine boosts may be needed in order to maintain, if not increase, tyrosinase-specific antibodies. After the completion of the huTyr DNA vaccination program, dog C experienced minor progression of disease and received additional xenogeneic DNA vaccines (mouse tyrosinase-related protein 1/gp75 and, later, mouse tyrosinase). Studies to determine the immunologic response(s) to the multiple DNA vaccine regimens encoding different xenogeneic melanoma-associated antigens are ongoing, as dog C remains alive as of this publication, with a minimally changed pulmonary lesion since trial entry in 2000.

Confirmation of antityrosinase antibodies by flow cytometry

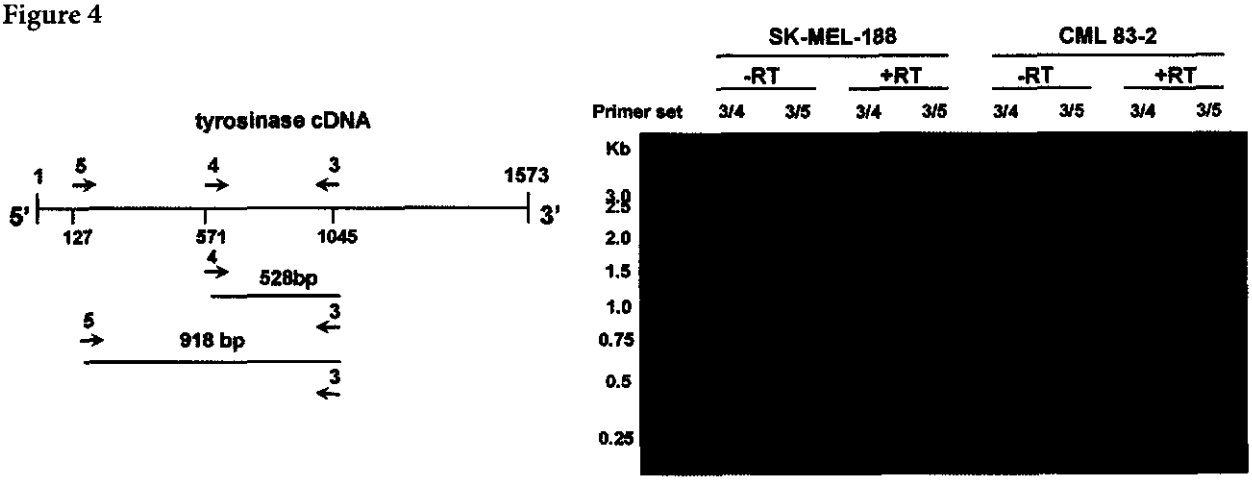
To confirm the induction of tyrosinase-specific antibodies capable of recognizing native tyrosinase in response to the DNA vaccination, a flow cytometry-based assay with cultured human SK-MEL-188 melanoma cells was used. Cells were incubated with diluted postvaccination or preimmune sera after cellular permeabilization and fixation and analyzed for recognition of endogenous intracellular tyrosinase by flow cytometry. Consistent with the detection of the tyrosinase substrate by ELISA, the postvaccination sera from dogs A, B, and C

Figure 3



Reactivity of canine postvaccinate sera to endogenous human and canine tyrosinase. Cultured human melanoma cells SK-MEL-188, canine melanoma cells CML 83-2, and canine MDCK cells were permeabilized and incubated with sera (diluted 1:500) from patients A (panel a; 100 μg huTyr DNA dose), B (b; 100 μg huTyr DNA dose), and C (c; 1500 μg huTyr DNA dose). Flow cytometry analysis showed an increase in mean fluorescence intensity for melanoma cells (but not canine MDCK cells) stained by the postvaccinate and recheck sera, and not by preimmune sera or secondary antibodies. This indicates specific recognition of cells expressing human or canine tyrosinase by the antibodies induced in the postvaccinate canine sera. Data shown are representative of two independent flow analyses for each patient.

Figure 4



Human and canine mRNA expression evaluated by RT-PCR. Expression of tyrosinase mRNA by human (SK-MEL-188) and canine (CML 83-2) melanoma cell lines was examined by RT-PCR. Conserved, tyrosinase-specific primer sets, 3/4 and 3/5 (schematic diagram), were used to amplify a 528 bp and an overlapping 918 bp cDNA fragment, respectively, indicating the expression of tyrosinase genes in both cell lines.

contained specific antibodies capable of binding endogenous huTyr, as shown by the increase in mean fluorescence intensity of the stained SK-MEL-188 cells (Figure 3 a-c, top panels) as compared to cells incubated with either preimmune sera or FITC-conjugated rabbit antidog secondary antibodies. Given the different experimental conditions and biological context of this methodology in detecting protein-protein interactions, serum samples from the other vaccinated patients were also analyzed for antityrosinase antibodies. There was no detectable difference in SK-MEL-188 binding between the pre- and postvaccination sera from dogs with no response by ELISA (data not shown), thereby demonstrating concordance between the ELISA and the flow cytometry assays.

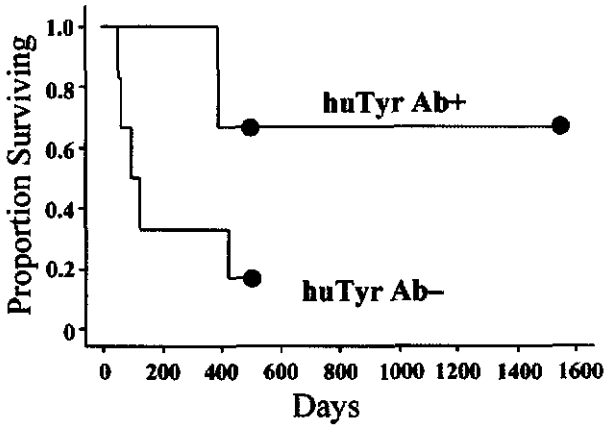
Detection of vaccination-induced autoantibodies to canine tyrosinase

Since the prior assays utilized xenogeneic (human) tyrosinase as a target, we were interested in determining whether the antibodies induced by DNA immunization could also recognize syngeneic canine tyrosinase. Canine melanoma cells CML 83-2, which express levels of tyrosinase mRNA comparable to those expressed by human SK-MEL-188 cells (Figure 4), were treated and analyzed by flow cytometry to assess the ability of vaccine-induced antibodies in dogs A, B, and C to bind endogenous canine tyrosinase. Consistent with the stained SK-MEL-188, we observed a modest increase in mean fluorescence intensity for CML 83-2 cells incubated with the same postvaccination sera from dogs A and B as compared to their preimmune sera (Figure 3 a and b, middle panels). In contrast, we did not detect any measurable difference in mean fluorescence intensity when CML 83-2 cells were incubated with either preimmune or postvaccination sera from dog C (Figure 3c, middle panel), despite the development and presence of antibodies that recognized the huTyr in this patient (Figure 3c, top panel). To further ensure that the antibody responses detected in these dogs are tyrosinase-specific, we included a canine MDCK kidney-derived epithelial cell line (a kind gift from Dr. A. Reilein) that does not express tyrosinase as a negative control for the flow-cytometric analyses. For dogs A and C (Figure 3 a and c, bottom panels), we did not observe any measurable change in fluorescence between MDCK cells stained with preimmune and postvaccinate serum samples. However, we detected a very slight increase in the mean fluorescence intensity for MDCK cells incubated with recheck serum 2 from dog B (Figure 3b, bottom panel). Taken together, these data demonstrate that xenogeneic huTyr DNA vaccination can induce antibodies capable of recognizing both xenogeneic and syngeneic tyrosinase.

Association of vaccine-induced humoral responses with patient ST

Kaplan-Meier survival analysis for patients A, B, and C ($n = 3$), when compared to the remaining population ($n = 6$) of humoral nonresponders (Figure 5), suggested an association between long-term survival (measured in days) and positive antibody responses. Given the small sample size, however, the association and/or effect of antibody production with overall survival did not reach statistical significance (log-rank $P = 0.148$ and Cox Proportional Hazards $P = 0.183$).

Figure 5



Kaplan-Meier survival curves for canine patients with ($n = 3$; huTyr Ab+) and without ($n = 6$; huTyr Ab-) positive humoral responses to the xenogeneic huTyr DNA vaccination. Dots denote patients that were censored from the analysis. The difference in ST between the two groups was not statistically significant ($P = 0.148$; log-rank analysis), possibly due to the small patient group size.

Discussion

In our previously published single-arm clinical trial, 9 dogs with advanced CMM (World Health Organization stages II-IV) were vaccinated with a xenogeneic DNA plasmid expressing huTyr, a melanosomal differentiation antigen. The trial demonstrated that the DNA vaccine is safe and efficacious for the vaccinated dogs, which had an increased median ST of 389 d (34), compared to historical controls of 1-5 months with conventional therapies (3, 4, 35). The results of the present study document an induction of antibody responses in 3/9 of the vaccinated CMM patients, a response rate comparable to other human DNA vaccine programs for malaria (36) and HIV (37). Consistent with preclinical mouse models, the induction of canine humoral responses by the orthologous human antigen to recognize self tyrosinase suggests the overcoming of canine immune ignorance or tolerance, as the tyrosinase-specific antibodies developed in response to the huTyr DNA vaccine are capable of cross-reacting with canine tyrosinase. Unlike some of the mouse melanoma models in which autoimmune coat hypopigmentation associated with xenogeneic DNA vaccines encoding various melanosomal differentiation antigens was seen (25, 26, 27, 28, 29), we did not observe any clinical signs of hypopigmentation in any of the dogs under current study. However, we have a single documented case of foot pad depigmentation in a dog vaccinated with DNA vaccine encoding murine tyrosinase (unpublished observation). The generation of canine tyrosinase, unavailable at present, would ultimately allow us to test the potential cross-reactivity of induced antibodies by the human antigen to recognize and target the specific self protein *in vitro*, as opposed to the xenogeneic antigen evaluated in the ELISA here.

With the initial priming followed by 3 biweekly boosts of DNA vaccine, the induced humoral responses were sustained for a relatively long period after the final vaccination. Positive responders had increased levels of antibody 3-9 months after the completion of the vaccination program at recheck 2 (Figure 2), which was associated with long-term tumor control. This study demonstrates the immunogenicity of huTyr DNA and suggests that the resulting humoral responses may persist long-term in some patients. Moreover, our findings are consistent with the temporal model of response for other cancer vaccines, which can take approximately 4 to 8 weeks, if not longer, to elicit specific humoral and/or cellular immune responses, and months for clinical antitumor responses (38, 39).

Current models suggest that xenogeneic DNA vaccines may exploit a preexisting repertoire of autoreactive B and T cells typically not responsive to tumor cells, which arise from self tissues and express self antigens. These autoreactive cells have the potential to target the same tumor cells when activated appropriately by orthologous antigens expressing subtle but distinct epitopes (40, 41, 42). The utility of this therapeutic modality in mounting antibody and/or T-cell-mediated immunity to tumor antigens has been clearly demonstrated in numerous preclinical mouse tumor models of the skin (25, 26, 27, 28, 29), lung (43), breast (43), and prostate (44, 45, 46, 47). Our findings are consistent with these models in that antitumor autoimmunity can be elicited in CMM patients in response to an orthologous antigen expressed *in vivo* from a plasmid DNA expression construct. We speculate that existing differences in protein sequences between human and canine tyrosinase may provide higher-affinity MHC class II-restricted peptides from the human protein to activate autoreactive CD4+ T helper cells with intermediate- or low-affinity receptors for this otherwise poorly immunogenic self antigen and may help generate antibodies against self tyrosinase. For our study, full-length cDNA encoding the huTyr was subcloned into a bacterial expression vector, pING, and constitutively expressed under the control of a CMV promoter (34). Insertion of the antigen encoding sequence in a bacterial expression vector provides the vaccine with an endogenous adjuvant in the form of unmethylated CpG nucleotide sequences, which activate APCs through toll-like receptor 9 (TLR9) (48, 49, 50, 51, 52, 53).

While the autoantibodies generated in dogs A-C are likely to have induced tumor regression and/or inhibited further metastases, the exact mechanism(s) of antibody-mediated tumor rejection in the canine system will require further elucidation. In particular, it remains unclear how tyrosinase, an intracellular melanosomal glycoprotein, can be recognized and targeted by antibodies in intact tumor cells. We speculate that a low-level, cell-surface expression of canine tyrosinase is the target for the autoantibodies, as detection and modulation of gp75/Trp1 (a tyrosinase-related melanosomal glycoprotein and a well-characterized melanoma antigen) expression in the presence of IFN-γ (54) has previously been described on the tumor cell surface of mouse melanoma models. The involvement of gp75-specific antibodies, transferred passively (54, 55, 56) or induced by active vaccination (27, 56), in mediating antitumor responses is directly linked to the host effectors (that is, NK1.1+ or macrophages) expressing activating Fc receptors (FcγR type I and III). Activated NK1.1+ cells may be directly involved in antibody-dependent cellular cytotoxicity or may modulate the activities of other effector-cell components (that is, macrophages or monocytes) for tumor cytotoxicity. Given the critical role for FcRs (and the inflammatory effectors) for effective antibody-dependent tumor cytotoxicity *in vivo*, a similar cascade of antibody-mediated FcγR engagement and activation of host effectors is likely conserved in the dog to mediate the observed clinical outcomes in response to the huTyr-induced autoantibodies.

Based on our studies, xenogeneic DNA vaccination should be considered as an adjunctive therapy for CMM patients with minimal residual disease and/or low tumor burden. Ultimately, long-term clinical efficacy with complete control, if not elimination, of microscopic tumor dissemination can only be achieved when the following have been optimized: (i) efficient uptake of the antigen, (ii) efficient antigen processing by APCs with migration to draining lymph nodes, (iii) precise antigen presentation by the APCs (that is, DCs), (iv) induction of appropriate helper and cytotoxic T cells systemically to target tumor cells, and (v) a persistent pool of memory helper and/or effector cells capable of challenging any subsequent tumor growth over time (40). We demonstrate here that xenogeneic DNA vaccine targeting tyrosinase can fulfill some of the above prerequisites to induce antigen-specific antibody responses in large, outbred animals with spontaneously occurring malignant melanoma.

The variability in the measured antibody responses among the vaccinated dogs likely reflects the need for further optimization of all the critical steps involved in vaccine-induced antitumor responses, as dogs A and B clearly developed cross-reactive autoantibodies to tyrosinase while the same humoral response to syngeneic tyrosinase was absent in dog C. It is equally plausible that the absence of detectable antityrosinase humoral responses in the other vaccinated patients with and/or without positive clinical outcomes may be attributable to the challenges we have discussed. It would be more informative if autologous melanoma tumor cells from each patient were readily available and accessible for us to determine the individual expression

level/profile of endogenous tyrosinase and to conduct similar comparative analyses, as discussed earlier. Likewise, functional measures of antibody-mediated cytotoxicity would better substantiate our observations and should be incorporated into future studies of DNA vaccine programs.

Equally important, although the present study demonstrates that induction of specific (auto)antibodies to an intracellular tumor antigen correlates with positive clinical outcome in some patients, we recognize that other components of the immune system, such as CD8+ CTLs, may become activated and contribute to the overall antitumor responses against CMM. To address this, we are currently engaged in the development of an assay to measure antigen-specific T-cell responses in these outbred dogs. Quantitative measures of potential cytotoxic T-cell responses should provide an additional, or alternative, mechanism to explain the positive antitumor effects in the CMM patients lacking detectable antibody response to the vaccine.

Abbreviations

CMM, canine malignant melanoma; huTyr, human tyrosinase; ST, survival time

Acknowledgements

We would like to thank Dr. Jean-Christophe Audonnet and Christine Andreoni (Merial, Inc., Lyon, France) for providing the huTyr, Dr. Robert P. Fisher (MSKCC, Memorial Sloan-Kettering Cancer Center, NY, USA) for providing the myelin-basic protein, Dr. Lauren G. Wolfe (Auburn University, Auburn, AL, USA) for providing the canine melanoma cell line, Dr. Amy Reilein (Weill Medical College, Cornell University, NY, USA) for providing the canine MDCK cell line, as well as Yongen Chang for her assistance in performing the tyrosinase cDNA amplification.

This work was supported in part by NCI grants CA59350, CA56821, and CA33049 to ANH; and by Swim Across America; the Mr. and Mrs. Quentin J. Kennedy Fund; the Lita Annenberg Hazen Award; the Louis and Anne Abrons Foundation; Mr. William H. Goodwin and Mrs. Alice Goodwin; the Commonwealth Foundation for Cancer Research; and The Experimental Therapeutics Center of Memorial Sloan-Kettering Cancer Center (MSKCC; to ANH and JDW).

Potential conflicts of interest: ANH, JDW, and PJB have submitted a patent application related to this work, and PJB has received speaker's fees from Merial, Inc.

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Materials and methods

Patient characteristics, vaccination protocols, vaccine development, and clinical outcomes of the canine patients in the xenogeneic huTyr DNA vaccine trial have been described in an earlier work (34).

Tissue culture

Human and canine melanoma cell lines SK-MEL-188 and CML 83-2 (kind gift of Dr. L.G. Wolfe, Auburn University, Auburn, AL, USA), respectively, as well as canine MDCK cell line (kind gift of Dr. A. Reilein, Weill Medical College, Cornell University, New York, NY, USA) were grown in RPMI 1640 (Mediatech, Inc., Herndon, VA, USA) supplemented with 7.5% heat-inactivated FCS, 1% nonessential amino acids, 1% L-glutamine, and 1% penicillin/streptomycin and maintained at 37°C with 5% CO2 until harvested.

RNA extraction and RT-PCR

Total RNA from SK-MEL-188 and CML 83-2 were isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA), following the manufacturer's instructions. Tyrosinase mRNA expression in each cell line was analyzed by RT-PCR. Briefly, tyrosinase mRNAs from each cell line were reverse transcribed from the isolated total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), followed by amplification of the tyrosinase cDNA from each first-strand cDNA synthesis reaction using conserved primer sets for both human and canine: Primer 3 (reverse): 5'-TGGCAGCTTTATCCATG GAA-3', Primer 4 (forward): 5'-AATGGATCAACACCCATGTT-3', and Primer 5 (forward): 5'-GGAGTCCCTGTGGCCAGCTT-3'. For cDNA amplification, the following thermal cycling conditions were used: denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and elongation at 72°C for 30 s, and a final extension at 72°C for 10 min. Amplified cDNA from each cell line was resolved and visualized on 1% agarose gel stained with ethidium bromide.

Antibody response

Canine sera were collected before the start of trial and after each vaccination. As previously described (34), approximately

2-4 ml of canine whole blood was collected in a VACUTAINER SST Gel & Clot Activator tube (Becton Dickinson, Franklin Lakes, NJ, USA) and spun for 15 min at 2000 rpm. Each serum was aliquoted and cryopreserved at -70°C until analysis.

Sera were analyzed by solid phase ELISA for reactivity against mammalian-expressed, recombinant huTyr protein as the specific target substrate. Briefly, high-affinity binding, 96-well plates (Sigma, St. Louis, MO, USA) were coated with recombinant huTyr expressed as secreted (and soluble) protein in CHO-easyC protein-free spinner culture (kind gift of C. Andreoni and Dr. J-C. Audonnet, Merial, Inc., Lyon, France), or purified myelin-basic protein (kind gift of Dr. R.P. Fisher, MSKCC) as negative control, overnight at 4°C. Coated plates were washed twice with 1x PBS (pH 7.4) and blocked with 3% BSA in 1x PBS (pH 7.4) for at least 1 h at room temperature. Plates were washed twice with 1x PBS after blocking and incubated with canine sera serially diluted in 1% BSA (in 1x PBS, pH 7.4) for 3 h at room temperature. To remove any nonspecific antibody or excess serum protein, plates were subsequently washed three times with 1x PBS containing 0.05% TWEEN-20 (PBST) and three times with 1x PBS. Alkaline phosphatase-conjugated antidog IgG antibody (Sigma) was diluted in 1% BSA (1:2500) and added to each well for 1 h incubation at room temperature. Plates were washed as described above to remove any unbound secondary antibody, followed by the addition/incubation of chromogenic substrate, *p-nitrophenyl phosphate* (Sigma). The colorimetric absorbance of each well was measured spectrophotometrically at 405 nm on the VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry

Human SK-MEL-188 and canine CML 83-2 and MDCK cells were trypsinized, counted, and resuspended in FACs* buffer (1x PBS supplemented with 1% heat-inactivated serum). For each staining and analysis, 1-3 x 105 cells were fixed with prechilled methanol for 30 min on ice, washed once with FACs* buffer, and then permeabilized with 0.1% triton X-100 (in 1x PBS) for 30 min at room temperature. Treated cells were washed twice with FACs* buffer and incubated with 50 µl diluted (1:500) canine sera for 1 h on ice. Stained cells were washed twice with FACs* buffer, followed by incubation with 50 µl diluted (1:500) FITC-conjugated rabbit antidog IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) on ice for 45 min. Stained cells were washed twice, resuspended in 120 µl FACs* buffer, and 10,000 events were counted using a FACscan flow cytometer (Becton Dickinson, San Jose, CA, USA). FloJo software (Tree Star Inc., Ashland, OR, USA) was used to analyze the changes in mean fluorescence intensity after vaccination. An increase (or positive shift) was interpreted as a measure of serum antibody-intracellular tyrosinase interaction.

Statistical analysis

ST, defined as the time from receiving the first xenogeneic huTyr DNA vaccination until death, was calculated using Kaplan-Meier life table analysis for patients with or without detectable humoral responses. For delineation of statistical significance between the two subgroups on survival, log-rank (Mantel-Cox) test was used; $P < 0.05$ was considered statistically significant. The Cox proportional hazards ratio was also calculated to evaluate whether the variable (antibody response) was associated with ST. Patients were censored upon death unrelated to melanoma or if still alive at the end of the study period, as previously reported (34).

Contact

Address correspondence to:

Philip J. Bergman
Tel.: + 1 212 329-8674
Fax: + 1 212 702-0971
E-mail: Philip.Bergman@amcny.org

Environmental Assessment
for Field Testing
Canine Melanoma Vaccine, DNA

I. Proposed Action

APHIS is considering granting authorization to ship an unlicensed Canine Melanoma Vaccine, DNA, for field testing. Merial, Inc., Athens, Georgia, has requested authorization to conduct clinical studies that will provide efficacy and safety data in dogs administered this vaccine. The efficacy trial will measure the sparing effect of the vaccine on dogs diagnosed with melanoma. The safety of the vaccine will be evaluated in animals participating in the studies.

Under the provisions of the Virus-Serum-Toxin Act of 1913, as amended in 1985, the USDA must ensure that veterinary biologics are pure, safe, potent, and efficacious and not worthless, contaminated, dangerous, or harmful. Accordingly, APHIS has conducted a risk analysis and has concluded that the safety risks to animals, public health, and the environment are low. A copy of the risk analysis with confidential business information removed is available upon request.

II. Background

Canine malignant melanoma (CMM) is a common and aggressive form of cancer in dogs, with usually a poor prognosis (1). Treatments for this condition have included surgery, chemotherapy, and radiation therapy (2). However, these therapeutic approaches have not been fully successful. Usually, CMM is detected at a late stage when excision is not likely to be complete and metastasis to regional lymph nodes has already occurred (1). The potential benefits of vaccination as a therapeutic option against CMM have been reported in the literature (3-6).

CMM is the most common malignant neoplasm of the oral cavity, and accounts for 7% of all malignant tumors in dogs. Other species affected by malignant melanoma include cats, horses, and humans (1). It is not as common in cats as in dogs, but the prognosis is equally grave. Gray horses commonly develop melanoma. Oral melanoma comprises only 1-2% of all human melanomas and is uncommon compared to the cutaneous form, which occurs with far greater frequency.

The experimental vaccine to be used in the proposed field tests is a replication-incompetent plasmid which has been genetically modified to contain a therapeutic gene. The phenotypic result of this genotypic modification is a requirement for growth factors not readily found in the external environment. Lacking these, its growth is attenuated, even with a suitable bacterial host.

The proposed field safety test will be conducted in at least three different geographical locations and the product will be used according to instructions on the product circular. The potential for escape and dispersal of the experimental vaccine from the proposed release sites is low. The personnel to conduct the study are experienced in canine health management.

III. Need for the Proposed Action

There are no CMM vaccines approved for use in dogs. Although CMM is a progressive fatal disease, there are reports in the literature suggesting that vaccination can potentially improve survival time following treatment of the primary tumor growth, presumably through inhibiting progression of metastases. A replication-incompetent DNA vaccine can potentially be both safe and efficacious, inducing both antibodies and cytotoxic T-cells in a broad immune response. A vaccine found to be successful against CMM may have wider application in treating melanoma in other animal species and in humans. It is expected that the data from these monitored field trials will confirm the safety of this melanoma DNA vaccine for use in dogs and within the environment of the United States.

IV. Areas of Concerns

The three areas of concern to APHIS are: 1) animal safety, 2) public health, and 3) environmental safety. APHIS has conducted a risk analysis to assess whether risks are associated with the proposal to field test this experimental vaccine. The safety characteristics of this vaccine have been thoroughly evaluated. The conclusions derived from the risk analysis for each of the areas of concern are summarized below.

A. Animal Safety

The risk to target animals is low. The experimental vaccine is highly purified plasmid DNA not capable of replication in mammalian cells. In a previous study, the vaccine was found to be safe when administered at the recommended dose and in excess of that dose, to dogs afflicted with melanoma. Plasmid DNA can persist at the site of injection but there have been no signs of pathology or toxicity associated with that persistence. The minimum age for dogs with melanoma, the target population, is 2 years old, so younger dogs will not be exposed. Regarding non-target species, because the vaccine is not infectious, cannot replicate in eukaryotic cells, and is not pathogenic, the risk to non-target species is considered to be low.

B. Public Health

The risk to public health is likewise low. In these proposed clinical studies, the possibility of human exposure is slight and limited to veterinary personnel. Should accidental self-injection occur, it is not expected to cause a public health concern. The plasmid vector has been used previously with other gene inserts to create vaccines against melanoma. These have been reported to be used in human subjects without adverse effect.

There is one veterinary DNA vaccine licensed for use in the United States. It is for use against West Nile Virus in horses and has been demonstrated to be safe and effective. There have been no reports of adverse effects or hazards to public health for this registered product.

C. Environmental Safety

The risks to the environment are low. The potential for escape and dispersal of this replication-incompetent DNA vaccine is restricted. The plasmid cannot replicate within the vaccinated animal. Thus, the shed/spread capabilities of the vaccine are very limited even under direct contact exposure. In the case of an accidental spill, the plasmid is unstable in the environment and rapidly degraded in the stomach of any animal that may ingest it. Should there be plasmid uptake by soil bacteria, the plasmid will not be maintained in the absence of its growth requirements. There are no expected adverse ecological effects associated with the proposal to conduct field testing with this experimental vaccine.

V. Alternatives

Two alternatives were considered. The only alternative considered, other than the preferred action alternative, is not to approve the proposed field tests, the "no action" alternative. We have considered the applicants' goals in light of the agency's public interest and responsibilities and any potential environmental impact. Based upon the results of our risk analysis and the potential applications for this vaccine in disease control, APHIS adopts the alternative that the proposed field tests be approved.

VI. Conclusion

Based upon the risk analysis documented in this EA, APHIS has determined that implementation of the proposal would not significantly affect the quality of the human environment and that the preparation of an Environmental Impact Statement is not required (Finding of No Significant Impact).

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Nucleic Acid-Mediated (Genetic) Vaccines

Risk Analysis for

Melanoma DNA Vaccine

(Product Code 9240.D0, Unlicensed)

[redacted]

[redacted]

Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for
Melanoma DNA Vaccine
CONFIDENTIAL

Table of Contents

Table of Contents (Cont.)

Executive Summary

Melanoma is a progressive fatal disease of dogs. Survival time is dependent upon many factors some of which are not well defined. Existing treatments include aggressive surgical intervention, chemotherapy and radiation; however, their effectiveness is questionable.

Merial has prepared this document to provide information on the Melanoma DNA Vaccine that is being developed for therapeutic immunization of dogs diagnosed with melanoma. The document provides a risk analysis consisting of the summary information format that describes the vaccine composition and use followed by a risk assessment of the safety of the product in animals, humans and the environment.

The experimental Melanoma DNA vaccine is highly purified plasmid DNA consisting of the [redacted] plasmid with the sequence of [redacted] plasmid was developed by [redacted] is not capable of replication in mammalian cells, because it's replication is restricted to E. coli [redacted]. Vaccines containing the [redacted] plasmid, and others [redacted] plasmid with similar gene inserts, have been tested in human subjects [redacted]. All of the studies to date, have indicated the vaccine is safe. No adverse effects on the test subjects or the environment have been observed.

Extensive testing of DNA vaccines over the last 10 years in animals and humans has demonstrated that the safety issues postulated during the early days of DNA vaccine technology (integration, autoimmunity, tolerance) have not materialized into real concerns. Biodistribution studies have demonstrated that after vaccination, although small amounts of plasmid are transiently and passively distributed throughout the body, accumulation in specific tissues does not occur. Plasmid levels in the tissues of vaccinated animals fall below the range detectable by PCR within weeks to months after vaccination. These facts in conjunction with the inability of the plasmid to replicate within the animal, indicate that the risk of plasmid shedding into the environment is exceedingly low.

In the event of an accidental spill into the environment, the risk to target and non-target animals is negligible as the plasmid is not infectious, it is not stable under environmental conditions and it would be unlikely to enter an animal via mucosal (oral) or dermal (skin) contact. The minute amount of plasmid, which might be ingested by an animal, would be degraded in the stomach and as such, be inconsequential. Similarly, the risk of uptake and maintenance by soil bacteria is inconsequential, as the plasmid would not be maintained in the absence of [redacted].

This product will be produced on [redacted] medium devoid of [redacted] and will be extensively purified and formulated without [redacted]. The risk of contamination with adventitious agents of significance to mammals will be virtually eliminated.

Merial proposes to conduct clinical studies that will provide efficacy and safety data in dogs administered this vaccine. The efficacy trial will evaluate the impact of the vaccine on [redacted] dogs diagnosed with melanoma. Safety will be assessed in the melanoma patients that are enrolled [redacted]. The Risk Analysis for the environmental release of Melanoma DNA vaccine associated with these clinical studies, results in a calculated risk rating of "Low" as regards risks to animal, human and environmental safety.

Collectively, the information presented in this document indicates that the Melanoma DNA vaccine will have a high safety profile in animals and pose an exceedingly low risk to the safety of humans, animals and the environment. Based on this Merial recommends that the CVB issue a "finding of no significant impact" for the Melanoma DNA vaccine and authorize initiation of the proposed clinical studies.

Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for Melanoma DNA Vaccine
CONFIDENTIAL

I. INTRODUCTION

A. Objective

Merial plans to develop and license a vaccine for melanoma that contains plasmid DNA encoding a gene [redacted]. The vaccine is intended for the therapeutic immunization of dogs diagnosed with melanoma. This Risk Analysis provides the technological information that is known and available to Merial at this time with respect to the proposed vaccine.

Merial submits this Risk Analysis in support of the request that APHIS grant a "Finding of No Significant Impact" (FONSI) for this DNA vaccine under the National Environmental Policy Act.

B. Proposal

[redacted]

Merial has prepared a master seed bacteria (MSB) containing [redacted] plasmid DNA. This MSB will be used to prepare purified plasmid DNA from which Merial intends to formulate vaccines for the purpose of conducting experimental trials to demonstrate safety and efficacy of the melanoma vaccine in support of USDA licensure.

II. CHARACTERIZATION OF THE BACTERIAL (REPLICATION) HOST CELL

The *E. coli* replication host cell is strain [redacted] obtained from [redacted]. Details of the host cell are provided throughout this document.

A. Identification and Source

[redacted]

[redacted]

B. Purity

B.1. 9 CFR 113.27 Purity Test Results

The purity of the host bacteria in the MSB was established according to 9CFR 113.27(d).

In addition, the genus and species identification (i.e. demonstrate that the MSB is an *E. coli*) was established according to 9CFR 113.100 using metabolic markers specific of *E. coli*. The Gram staining results obtained for purity testing also contributed to the identification of the MSB as *E. coli*.

The MSB was found to be pure when tested in accordance to the procedures described above. [redacted]

B.2. Lack of Endogenous Extraneous Plasmid(s) and Bacteriophage(s)

Most plasmids carry antibiotic resistance genes and are stable in the bacterial host cell in the presence of the appropriate antibiotic pressure. [redacted] plasmid containing the [redacted] gene is referred to in this document as [redacted]

The absence of contamination by an extraneous plasmid [redacted] also carrying the [redacted] resistance was demonstrated by a plasmid identity test. This test, as per 9CFR 113.100, was based on an enzymatic restriction map of purified plasmid. The restriction profile matched the theoretical profile established from the known [redacted] plasmid nucleotide sequence. The absence of extraneous DNA fragments after enzymatic digestion proves the absence of a contaminating plasmid. [redacted]

Specific receptors are required for bacteriophages to penetrate into bacteria. No receptor for bacteriophages has been described on the [redacted] strain. To the best of Merial's knowledge, there is no known bacteriophage susceptibility associated with the [redacted] strain.

C. Molecular Characterization

C.1. Conventional (non-engineered) Bacterial Replication Host

This section is not applicable to the [redacted] strain.

C.2. Genetically Engineered Bacterial Replication Host

C.2.a. Parental Strain

The cells were [redacted] lineage [redacted]

C.2.b. Summary of Construction Process

C.2.b.i. Cloning Strategies, Procedures, and Vectors

The [redacted] strain of *E. coli* was constructed from [redacted]

The [redacted] strain was constructed as follows: [redacted]

[redacted] was constructed by [redacted] introduction of [redacted]

[redacted]

C.2.b.ii. Deleted Genes

C.2.b.ii.1. Site and Function of Deleted Gene(s) (if known)

[redacted] genotype is [redacted]

The following genes were deleted:

[redacted]

Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for Melanoma DNA Vaccine
CONFIDENTIAL

C.2.b.ii.2. Consequences of Gene Deletion(s)

The consequences of the identified gene deletions are as follows:

- Deletion of [redacted] improves yield and quality of the plasmid preparations
- Deletion of [redacted] decreases homologous recombination to 0.1% of normal, thereby ensuring plasmid insert stability,
- Deletion of [redacted] mutation prevents cleavage by an endogenous endonuclease system
- Deletion of [redacted] renders the host strain more transformation efficient
- Consequences of [redacted] are less well understood.

C.2.b.ii.3. Inserted Gene(s)

a) [redacted]: allows the host to grow without supplemental [redacted].

D. Known Bacteriophage Susceptibility of Strain, (if applicable)

Specific receptors are required in order for bacteriophages to penetrate into bacteria. No receptor for bacteriophages has been described on the [redacted] strain. To the best of Merial's knowledge, there is no known bacteriophage susceptibility associated with the [redacted] strain.

E. Antibiogram: Endogenous Chromosomal Antibiotic Resistance Factors Allowed

No known endogenous chromosomal antibiotic resistance is associated to the strain.

F. Previous Use of Bacterial Replication Host

The [redacted] strain is a supercompetent bacteria commercialized for *in vitro* use in molecular biology laboratories.

III. CONSTRUCTION AND CHARACTERIZATION OF THE PLASMID CONSTRUCT

A. Parental Plasmid Source

A.1. Source(s) of Parental Sequences

The plasmid backbone [redacted] was constructed [redacted] using the following components: a eukaryotic promoter and enhancer [redacted], a [redacted] to facilitate cloning of a variety of DNA fragments, donor and acceptor splice sites and a [redacted] sequence [redacted], the [redacted] origin of replication and a gene conferring [redacted] resistance. With the exception of the [redacted] resistance gene, [redacted] all other gene segments were amplified by polymerase chain reaction (PCR), [redacted] and sourced from [redacted]

[redacted]

A.2. Phenotype Conferred on Bacterial Replication Host

The [redacted] plasmid confers [redacted] on the bacterial replication host.

A.3. Known Properties of Parental Plasmid

The [redacted] plasmid is a eukaryotic expression vector, which has been optimized for the expression of a transgene following direct injection into skeletal muscles.

In order to increase the transgene expression, optimal promoter-enhancer, intron and transcriptional termination signals have been engineered into this plasmid.

A.4. Known Hosts for Parental Plasmid

E. coli bacteria.

A.5. Replication and Expression Control Elements

A.5.a. Prokaryotic

A.5.a.i. Origin of Replication

The [redacted] plasmid uses the plasmid [redacted] origin of replication. This origin of replication is shared with numerous classical plasmids used in molecular biology laboratories [redacted].

A.5.a.ii. Bacterial Promoter(s), Enhancers, and Other Control Elements

The only prokaryotic transcription activity associated to [redacted] is related to the [redacted] gene. The expression of this gene is responsible for the [redacted] phenotype in *E. coli*. Since it is inactive in eukaryotic cells (i.e. in the cells of the vaccinated animal) there is no expression of the [redacted] gene in the vaccinated animal.

A.5.b. Eukaryotic (mammalian/avian/target host species) Control Elements

A.5.b.i. Promoters

The expression of the transgene in the vaccinated animal is driven by the strong [redacted] sequence [redacted]

In order to increase the transfer of nuclear transcripts into the cytosol, the [redacted] has been placed between the [redacted] sequences and the [redacted] region.

A.5.b.ii. Enhancers

As described above, the transgene expression is under the control of the enhancer/promoter sequences.

A.6. Identified Immunomodulatory Sequences and CpG Motifs (Immunostimulatory and Immunosuppressive)

Based on mouse data, specific CpG sequences within the plasmid could modulate the immune system and modify subsequent immune responses. Based on data obtained in other species, it is likely that the actual immunomodulatory sequences are species-specific (i.e. sequences identified in the mouse are not active in larger animals). Since sequences active *in vivo* in cats and dogs have not yet been identified, the effect of Immunomodulatory sequences in [redacted] that could have an impact in the target species of the contemplated DNA vaccine is not available.

A.7. Diagrammatic Representation of Parental Plasmid with Restriction Map

[redacted]

B. Inserted Sequences

B.1. Summary of Construction Process

B.1.a. Step-by-Step Construction Events

[redacted]

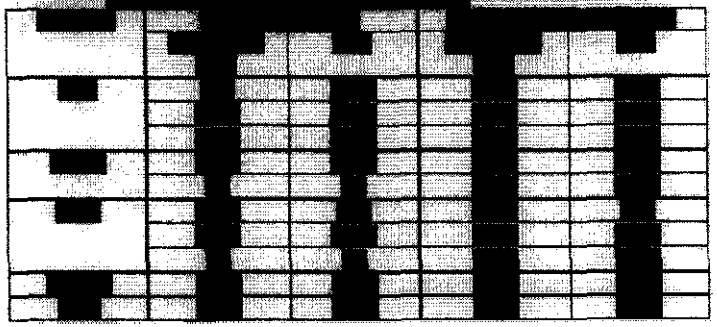
B.1.b. Parental and Intermediate (cloning) Vectors

The parental plasmid [redacted] as described in section III.A.1. The plasmid [redacted] was used as an intermediate vector to express the candidate cDNA [redacted] as described in Section III.B.1.a.. The [redacted] was digested by [redacted]

The plasmid [redacted] was the source of the [redacted] gene as described in Section III.A.1.

B.1.c. Loss or Gain of Restriction Sites

[redacted] restriction sites are gained in association with introduction of the [redacted] gene into the [redacted] plasmid. The new sites are [redacted]



B.2. Antigenic Gene(s)

B.2.a. Source and Sequence(s)

The [redacted] cDNA sequence was obtained from the [redacted] cDNA clone isolated from a [redacted] derived from the [redacted] melanoma cell line. [redacted]

B.2.b. Control Sequence(s) (promoter and/or enhancer)

There are no known promoters or enhancers in the antigenic gene sequence.

B.2.c. Known Biologic Properties in Parental Strain/Species

[redacted]

The [redacted] is contained in specialized organelles called melanosomes. The production of melanin is generally restricted to melanocytic cells containing melanosomes. These cells occur normally and are most commonly found in dermal and mucosal tissues. Melanosomes are abundant in typical melanoma cancer cells.

B.3. Immunomodulatory Gene(s) and Sequences:

Data is not available to confirm the presence of immunomodulatory sequences in the [redacted] gene.

Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for
Melanoma DNA Vaccine
CONFIDENTIAL

B.4. Selection Sequences (Kanamycin and Neomycin acceptable, other antibiotic resistance factors may be considered)

The [redacted] selection sequence is present.

B.5. Diagrammatic Representation of Target Protein

[redacted]

IV. PLASMID CONSTRUCT (Product)

A. Complete Characterization

A.1. Sequence

[redacted]

A.1.a. Antigenic Sequences

A.1.a.i. If Plasmid Does Not Encode Replicating Virus, Percentage Complete Viral Genome Encoded

The [redacted] plasmid does not encode a virus. It encodes the entire [redacted] protein as described in section III.B.5.

A.1.b. Ability of Plasmid to Recombine With Wild-Type Agent

The plasmid does not encode an infectious agent. The probability of a DNA molecule to integrate into the chromosome of muscle cells after intramuscular injection is at least 3000x lower than the natural mutation rate in an organism (Martin et al. Human gene Therapy, 10; 759-68, 1999; Ledwith et al., Intervirology, 43; 258-72, 2000).

A.1.b.i. Consequences of Recombinant Event

Consequences of a recombinant event involving the chromosomal DNA are subject to speculation. Problems associated with such events have not been reported, likely due to the extremely low probability of occurrence. Safety evaluations of injection sites have been performed. Plasmid DNA was found to persist in the muscle at the site of injection; however, no aberrant muscle pathology, muscle toxicity, or immune-mediated pathology was observed in the injected muscles (Parker et al., Human Gene Therapy, 10; 741-758, 1999; Manam et al, Intervirology, 43; 273-281, 2000).

A.1.c. Immunomodulator Sequences/Genes

Immunomodulator genes are not know to be present in [redacted]. Immunomodulator sequences (i.e., CpG) active *in vivo* in cats and dogs have not yet been identified. The effect of immunomodulatory sequences in [redacted] that could have an impact in the target species of the contemplated DNA vaccine is not known.

A.1.d. Other Immunologically Reactive Sequences

Other immunologically reactive sequences in [redacted] have not been recognized.

A.1.e. Transcription Binding and Termination Sequences

The [redacted] protein open reading frame is initiated by an [redacted] codon and terminated by the [redacted] codon. The [redacted] gene is under the control of an [redacted] and precedes a downstream region coding for a [redacted] termination sequence.

A.1.f. Polyadenylation Sites

The stabilization of the transgene mRNA is ensured by the presence of the bovine growth hormone termination and polyadenylation signals downstream of the transgene coding sequence.

A.1.g. Anchor or Secretory Sequences

[redacted] is protein bound in the melanosome membrane near [redacted] contained [redacted]

A.1.h. Sequences With Known Oncogenic Potential/Target Host Cell Genome Homology

The [redacted] does not contain known sequences of oncogenic potential and does not contain sequences of [redacted] origin.

A.2. Diagrammatic Representation of Plasmid DNA (Product size and restriction map)

[redacted]

A.3. Host Range for Replication

[redacted] can only replicate in *E. coli*. It cannot replicate within the vaccinated animal. Potential replication in other bacteria is unknown.

A.4. Host Range for Expression

Expression of the [redacted] can only occur in the bacteria host because it is under the control of a bacterial promoter.

Expression of the [redacted] protein cannot occur in the bacterial host (because it is under the control of an eukaryotic promoter). It will however, occur within the cells of the vaccinated animal into which the plasmid has been administered.

B. Purity of Plasmid DNA (Product)

B.1. 9 CFR 113.26

Purified plasmid will be tested for detection of viable bacteria and fungi in *accordance with 9 CFR 113.26.*

Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for
Melanoma DNA Vaccine
CONFIDENTIAL

B.2. Contaminants

B.2.a. Endotoxin Range

It is proposed that the amount of endotoxin present in the final product [redacted]

B.2.b. Cellular Components

B.2.b.i. Extraneous DNA

The presence of chromosomal DNA in the vaccine should not be a substantive issue because this vaccine is produced in an *E. coli* strain derived from the non-pathogenic [redacted] strain [redacted]. Furthermore, there is no data suggesting that residual chromosomal DNA is a safety issue in any of the existing, conventional USDA-licensed vaccines.

In order to support the consistency of its production process and demonstrate that the plasmid present in the melanoma DNA vaccine is well defined, Merial will demonstrate that only very small amounts of chromosomal DNA are present in the [redacted]

B.2.b.ii. RNA

Same comments as for *E.coli* chromosomal DNA impurities, bacterial RNA is not expected to impact the [redacted] DNA vaccine safety profile. In order to support the consistency of it's production process and demonstrate that the plasmid present in the melanoma DNA vaccine is well defined, Merial will demonstrate that only [redacted] amounts of bacterial RNA are present in the vaccine.

B.2.b.iii. Debris (organelles, proteins, lipids, etc.)

The presence of bacterial proteins in the vaccine is not considered to impact the safety of the [redacted] DNA vaccine for the following reasons:

- [redacted] vaccine will be produced on [redacted] medium [redacted]
- the only potential proteins in the vaccine will be derived from the *E. coli* host cell (i.e., derived from [redacted] bacteria), which does not contain known allergens
- the production process will not contain [redacted],
- no [redacted] will be used in the vaccine.

Based on the above, the likelihood of adverse reactions in the field should be minimal and protein contamination should have no impact on the *vaccine safety.*

In order to support the consistency of its production process and demonstrate that the plasmid present in the Melanoma DNA vaccine is well defined, Merial will demonstrate that [redacted]

B.3. Structural Integrity (circular plasmid)

As of today the importance of the ratio of supercoiled (sc) versus open circular (oc) plasmid isoforms on the efficacy of DNA vaccination is complex and only partially understood (Bergan et al. 2000; Middaugh et al., 1998; Evans et al.,2000).

In order to support the consistency of its production process and demonstrate that the plasmid present in the melanoma DNA vaccine is well defined, Merial will demonstrate that the plasmid ccc/oc in the final product meets or exceeds a minimal threshold of [redacted]

C. Stability

C.1. Genetic

The nucleotide sequence of the plasmid will be confirmed (on both strands) on the [redacted] and on [redacted] thereby demonstrating genetic stability.

[redacted]

C.2. Phenotypic

The phenotypic stability of the Melanoma DNA vaccine will be demonstrated via [redacted]. As the [redacted] plasmid is the active ingredient in the melanoma DNA vaccine, and as it will be a highly purified, well-defined plasmid, Merial proposes that the [redacted] DNA vaccine can be assessed based [redacted].

The intended parameters are:

[redacted]

D. Undesirable Immune Reaction(s) Based on Immune Reaction Targeted

D.1. TH1: Evidence of Autoimmunity Induction Potential

D.1.a. Autoimmunity in General

The development of melanoma occurs due to failure of the immune system to recognize proteins expressed on the malignant cells as foreign to the host animal. Induction of an immune response against one or more of these proteins is desirable in order to induce [redacted]. This may involve induction of an immune response against a protein otherwise considered to be "self" by the host animal (an auto-immune response). It this case, it is desirable to target a protein that does not result in a major adverse systemic or physiologic impact on the host.

[redacted] has approximately 85% homology with [redacted]. The difference between the two proteins is sufficient to induce an immune response in the dog against [redacted] protein, but similar enough that [redacted] on melanocytic cells will be targeted. In this respect, an auto-immune condition is induced to the benefit of the host. The side effect hypothesized would involve depigmentation of normal melanin laden tissues (refer to section III.B.2.c). [redacted] melanoma, this phenomena was not identified as an issue. [redacted] (personal communication, Apr 2004) has administered the [redacted] plasmid [redacted] to approximately 85 dogs. Two of these dogs exhibited mild depigmentation (ear and chin); otherwise, significant toxicities were not observed. If depigmentation does develop, the risk associated with this side effect is minimal in consideration of the short survival times and high mortality from melanoma.

D.1.b. Anti-DNA Antibodies

The possibility to induce anti-DNA antibodies through DNA vaccination has been presented as a theoretical critical issue in the early days of the technology development. Although no evidence of anti-DNA antibodies has been obtained in most laboratory animals, a single publication reports a slight increase of anti-DNA antibody titers when multiple injections of plasmid DNA were performed on lupus-prone mice (Gilkeson et al, J. Clin. Invest., 95; 1398-1402, 1995). However, these limited antibody titers had no impact on the onset and/or on the severity of the disease in this mouse model. We consider that this observation is anecdotal and likely to be specifically related to the mouse model used in this published study.

In human medicine, anti-DNA antibodies have been monitored over the course of numerous clinical trials but no clear seroconversion has been observed. [redacted]

As such, Merial (in agreement with the 2002 revised WHO Guidelines on *DNA vaccines, minutes of the 7th NAVSaC meeting held at NIBSC UK on* June 21, 2002) considers that the induction of anti-DNA antibodies is a theoretical issue, without practical implications for the development of a melanoma vaccine.

D.1.c. Tolerance

Another safety issue related to auto-immunity, which has been raised for DNA vaccines, is tolerance. It has been hypothetized that due to the low but long term expression of the transgene, DNA vaccination could induce *tolerance more easily than other vaccine technologies.*

Currently, there is a single publication reporting the induction of tolerance with a specific malaria antigen in newborn mice (Mor et al., J Clin. Invest; 98, 2700-5, 1997). Since this concern has not been confirmed in any other models, this appears to be a theoretical issue with no practical impact for the melanoma vaccine development. This position is in agreement with the 2002 revised WHO Guidelines on DNA vaccines, minutes of the 7th NAVSaC meeting held at NIBSC UK on June 21, 2002.

In the case of melanoma, we consider development of tolerance to not be an issue due to the preponderance of target antigen [redacted] in melanocytic cells associated with the tumor.

D.2. Th2: Allergic Reaction Induction Potential (activation of eosinophils and mast cells)

Although activation of eosinophils and mast cells following DNA vaccination has not been specifically assessed, there is no specific data to support the induction of allergic reactions following DNA vaccination in dogs.

Furthermore, any risk of protein related adverse effects (i.e. anaphylactic-like reactions) associated with the melanoma DNA vaccine is considered to be very low for the following reasons:

- the vaccine will be produced on [redacted] medium
- the only proteins in the vaccine will be derived from the *E. Coli* host cells that contain no known allergens
- the production process will be free of [redacted]
- the product will be a [redacted] vaccine.

V. ADMINISTRATION

A. Proposed Mode of Administration

A.1. Characterization of Live Bacterial Carrier (if applicable)

Not applicable.

A.2. Injection Routes of Administration

It is proposed to aseptically administer the melanoma DNA vaccine via the transdermal route using a needle free device.

B. Target Host Species

B.1. Minimum Age and Indications for Use

This vaccine is recommended for the vaccination of dogs diagnosed with melanoma. There is no minimum age targeted because these cancers tend to appear in mature animals and are not expected in neonatal or juvenile animals.

B.2. Target Site of Administration

The vaccine is to be injected aseptically into the proximal half of the medial thigh caudal to the femur. This site of administration will target primary deposition into intramuscular tissues.

B.3. Shed and Spread of Plasmid

B.3.a. In Target Host Species Tissues

Once administered (parenterally) into vaccinated animals, plasmids have been shown to be transiently distributed throughout the body (likely through blood transport and not through specific uptake and tissue retention). They are subsequently lost from most tissues within several days, probably by degradation (endonucleases). However, around the injection site (in the corresponding lymph node and in muscular tissues), plasmid DNA can generally still be amplified by PCR after several weeks (Parker et al., Human Gene Therapy, 10; 741-758, 1999; Manam et al, Intervirology, 43; 273-281, 2000).

Importantly, even though the plasmid DNA was found to persist in the muscle at the site of injection, no aberrant muscle pathology, muscle toxicity, or immune-mediated pathology indicative of an auto-immune disease was observed in the injected muscles. After a period of weeks to a few months, the plasmid will be cleared from the injection site area.

Distribution of plasmids to gonads has been inconsistent between studies. Although a transient migration into gonads cannot be ruled out, there is no substantive evidence to support any significant targeting and/or persistence in these organs.

B.3.b. In Environment

According to the literature, the risk of dissemination of plasmids in the environment from the vaccinated animal is exceedingly low because the plasmid cannot replicate within the vaccinated animal.

Furthermore, the accidental release of plasmids in nature should have no adverse effect because the risk that plasmids could be taken up by soil bacteria is very remote. Should this happen, the plasmid will not be maintained in the absence of [redacted]. As this [redacted] is not used in agricultural applications, the risk of having a plasmid disseminated by bacteria is exceedingly low. Minute amounts of plasmids, which could be accidentally ingested by animals will be degraded in their stomach and be of no consequence.

B.4. Duration of Detectable Expression

Long term expression (at least 1 month) of the transgene has been observed in the muscles of mice vaccinated intramuscularly with DNA vaccines (Hartikka et al, Human Gene Therapy, 7, 1205-17, 1996).

It is likely that the duration of detectable transgene expression is correlated to the persistence of plasmids in tissues. However, there are indications in the literature that the persistence/duration of expression in muscles is not directly related to the duration of immunity in DNA vaccinated animals (Hassett et al. J. Virol. 74: 2620-27, 2000).

Of note, plasmid DNA could be detected for more than 1 year in specific studies, as reported by Wolff et al. (Hum. Mol. Genet., 1; 363-369, 1992). The difference in the detectability of plasmid DNA across various studies using intramuscular injections may be due to the co-administration of chemicals that may alter plasmid biodistribution, persistence and expression.

B.5. Duration of Immunity

As discussed above, there is evidence that duration of immunity is not related to the persistence of plasmid in muscle. However, long lasting immunity (one year and more) has been described in different models of DNA vaccination.

Recently, the protection of monkeys 1 year after a single immunization with a rabies DNA vaccine has been reported, specifically demonstrating that DNA vaccine has the potential to trigger long-lasting immunity against rabies (Lodmell et al, Vaccine, 20; 838-44, 2002). Interestingly, since DNA vaccines trigger strong cell-mediated responses, the duration of immunity is frequently unrelated to the persistence of antibody titers.

B.6. Recommendations for Breeding Animals

B.6.a. Vertical Transfer to Germ Cells

Since, distribution to gonads of plasmids administered intramuscularly has been inconsistent between studies, the risk of plasmid reaching (and persisting in) gonads following intramuscular administration is very low. Accordingly, the risk of plasmid transfecting specific cells within the gonads is even lower and is likely to have no practical impact.

Canine melanoma patients usually represent the older segment of the pet population. These dogs are generally past prime breeding age. This further reduces any risk associated with exposure of germ cells to the plasmid DNA.

B.6.b. Vertical Transfer to Embryonic/Fetal Tissues or Reproductive Tissues

The risk of a plasmid becoming integrated into embryonic/fetal tissues or reproductive tissues has been theorized, but is believed to be without practical impact, as only a very small number of plasmid molecules enter into cells post injection.

Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for
Melanoma DNA Vaccine
CONFIDENTIAL

Furthermore, the probability of a DNA molecule to integrate into the chromosome of muscle cells after intramuscular injection is even lower [at least 3000x lower than the natural mutation rate in an organism [(Martin et al. Human gene Therapy, 10; 759-68, 1999; Ledwith et al., Intervirology, 43; 258-72, 2000)] The actual risk of integration into fetal tissues or reproductive tissues is believed to be exceedingly low.

Some experimental data indicates that, occasionally, when the plasmid was detected in gonads, it dissipated rapidly and was always extrachromosomal, confirming a low risk of germline transmission (Manam et al, Intervirology, 43; 273-281, 2000).

DNA vaccination *in utero* has been recently reported in the literature (Gerdts et al., J. Immunol., 168; 1877-85, 2002). However, since this experiment is based on the administration of the plasmid into the oral cavity of lamb fetus during surgery, it is unrelated to the transmission of plasmid from a pregnant animal to its offspring and is not relevant to the issue of plasmid vertical transfer.

VI. *IN VITRO* EXPRESSION IN CELL LINE (Potency Testing)

A. Characterization of Expression Cell Line

The qualitative expression assay for [redacted] will be performed by an [redacted] assay following transient transfection of [redacted] cells. Since the [redacted] cell line will only be used for QC tests [redacted] there are no specific requirements as per 9CFR [redacted]

B. Methods and Protocols for Expression Characterization

A functionality assay is required to demonstrate that the plasmid has the potential to express [redacted]. An IVGE assay will be established using an [redacted] ELISA following transient transfection of a [redacted] cell line. Transient transfection of [redacted] will be achieved using a [redacted] reagent to facilitate the uptake of the [redacted] DNA into tissue culture.

Following a [redacted] incubation period, the recombinant [redacted] will be labeled using a [redacted] and detected by [redacted]

C. Stability and Duration of Master Seed Expression

The stability of the MSB will be established by a sequencing of the [redacted] at both the [redacted] and [redacted] levels. The absence of mutations will demonstrate the stability of the seed lot system.

The MSB will be stored frozen in the presence of a cryo-protectant. Under these conditions, the MSB is expected to remain viable. It is proposed to test its viability by [redacted]

D. Target Copy Number of Plasmid

It is proposed to determine the number of plasmid molecules per bacteria of the MSB by quantification of the plasmid after extraction. The extraction will be performed [redacted] Quantification of plasmid [redacted] quantification of [redacted]

This quantification technique is plasmid-specific and will not be biased by the presence of bacterial chromosomal DNA. The plasmid copy number will be calculated based on the plasmid molecular weight and on bacteria numeration.

VII. *IN VIVO* EXPRESSION (target host species and laboratory animal model)

A. Characterization of Expression

A.1. Localization of Expression Protein (target host cell membrane, excreted, etc.)

The [redacted] protein expressed by [redacted] is a membrane-anchored antigen. It is expected to remain associated to the membrane of the cell in which *in vivo* expression occurs.

A.2. Methods and Protocols for Expression Characterization

The *in vivo* expression of the [redacted] protein will be characterized indirectly by its ability to induce [redacted]

[redacted]

A.3. Range of Time Plasmid Expressed and Consequences in Target Host Species

As previously discussed in Section V.B.4., the actual duration of plasmid expression *in vivo* is poorly understood and may vary greatly depending on the tissue that is analyzed. Furthermore, as also previously discussed, the duration of plasmid expression is likely to be only loosely related to the duration of immunity.

[redacted]

A.4. Shed and Spread of Expressed Protein

It is expected that the [redacted] protein will remain associated with the membrane of the cell in which expression occurs. No active shedding of the antigen is expected. Passive spreading related to the lysis of transfected cells cannot be ruled out, but this is unlikely, as transfected cells will probably evolve towards apoptosis rather than toward lysis. It is anticipated that most of the [redacted] protein will eventually be degraded within apoptotic bodies.

B. Characterization of Immunological Response

B.1. Targeted Response

B.1.a. Methods and Protocols for Assessment

DNA vaccination will trigger a broad immune response including both humoral (antibodies) and [redacted]

Specific techniques to assess [redacted] cell-mediated immunity in dogs include [redacted]

The therapeutic benefit provided by the vaccine will be confirmed in animal trials with [redacted]

B.1.b. Onset and Duration of Immunity

Onset of immunity induced by DNA vaccination is poorly understood. Although the induction of specific antibodies (by DNA vaccines in general) may be slower than with conventional inactivated-adjuvanted vaccine, there are indications, in the literature, that cell-based responses could be in place even in the absence of antibody responses (Siegrist et al. Eur. J. Immunol. 28: 4138-48, 1998).

B.2. Other Beneficial Immune Responses

None are anticipated.

B.3. Undesirable Immune Responses

No specific undesirable immune response is expected, as per Section IV.D.

VIII. RISK ASSESSMENT

Risk assessment may be defined as the process of identifying the likelihood of an adverse event occurring and the consequences if that adverse event occurs. Adverse events are defined as safety hazards to animals, public health, or the environment. A safety hazard is defined as a danger, risk, or peril; absence of predictability associated with an event; or an expected or unpredicted event.

A. Procedure

The risk assessment is conducted based on safety characteristics of the vaccine. The safety characteristics are based on empirical data and established scientific facts. The completion of a risk assessment requires that the vaccine microorganism is properly characterized. This information is provided in Section III of this document.

B. Hazard Identification

Hazard identification consists of identifying all possible adverse events related to animal safety, public health safety, and environmental safety relative to the recommended use of the DNA vaccine.

B.1. Animal Safety

B.1.a. Summary

The [redacted] DNA vaccine has been administered to [redacted] dogs [redacted] No adverse events were reported from dogs in this study at any dose level. [redacted] has administered the [redacted] plasmid [redacted] to approximately 85 dogs with melanoma. No significant toxicities were observed in these dogs; however, two dogs exhibited mild depigmentation (ear and chin).

It should be noted that the target population is adult dogs because cancer is not considered an issue in neonatal and juvenile animals. The comments in this section are made to address specific issues, which have been associated with DNA vaccination.

B.1.b. Target Animal Safety

B.1.b.1 Vaccination

B.1.b.1.a. Autoimmunity in General

The development of melanoma occurs due to failure of the immune system to recognize proteins expressed on the malignant cells as foreign to the host animal. Induction of an immune response against one or more of these proteins is desirable in order to induce stable disease or initiate tumor regression. This may involve induction of an immune response against a protein otherwise considered to be "self" by the host animal (an auto-immune response).

It this case, it is desirable to target a protein that does not result in a major adverse systemic or physiologic impact on the host.

[redacted] has approximately 85% homology with [redacted]. The difference between the two proteins is sufficient to induce an immune response in the dog against [redacted] protein, but similar enough that [redacted] on [redacted] cells will be targeted. In this respect, an auto-immune condition is induced to the benefit of the host. The side effect hypothesized would involve depigmentation of normal melanin laden tissues (refer to section III.B.2.c). In a [redacted] trial with the [redacted] vaccine in [redacted] dogs with melanoma, this phenomena was not identified as an issue. [redacted] has administered the [redacted] plasmid [redacted] to approximately 65 dogs with melanoma. No significant toxicities were observed in these dogs; however, two dogs exhibited mild depigmentation (ear and chin). If depigmentation does develop, the risk associated with this side effect is minimal in consideration of the short survival times and high mortality from melanoma.

B.1.b.1.b. Anti-DNA Antibodies

The possibility to induce anti-DNA antibodies through DNA vaccination was presented as a theoretical critical issue in the early days of the technology development. Although no evidence of anti-DNA antibodies has been obtained in most laboratory animals, a single publication reports a slight increase of anti-DNA antibody titers when multiple injections of plasmid DNA were performed on lupus-prone mice (Gilkeson et al, J. Clin. Invest., 95; 1398-1402, 1995). However, these limited antibody titers had no impact on the onset and/or on the severity of the disease in this mouse model. We consider that this observation is anecdotal and likely to be specifically related to the mouse model used in this published study.

In human medicine, anti-DNA antibodies have been monitored over the course of numerous clinical trials but no clear seroconversion has been observed. [redacted] As such, Merial (in agreement with the 2002 revised WHO Guidelines on DNA vaccines, minutes of the 7th NAVSaC meeting held at NIBSC UK on June 21, 2002) considers that the induction of anti-DNA antibodies is a theoretical issue, without practical implications for the development of a canine melanoma vaccine.

B.1.b.1.c. Tolerance

Another safety issue related to autoimmunity, which has been raised for DNA vaccines, is tolerance. It has been hypothetized that due to the low but long term expression of the transgene, DNA vaccination could induce tolerance more easily than other vaccine technologies.

Currently, there is a single publication reporting the induction of tolerance with a specific malaria antigen in newborn mice (Mor et al., J Clin. Invest; 98, 2700-5, 1997). Since this concern has not been confirmed in any other models, this appears to be a theoretical issue with no practical impact on the melanoma vaccine development. This position is in agreement with the 2002 revised WHO Guidelines on DNA vaccines, minutes of the 7th NAVSaC meeting held at NIBSC UK on June 21, 2002.

In the case of melanoma, we consider development of tolerance to not be an issue due to the preponderance of target antigen [redacted] in melanocytic cells associated with the tumor.

B.1.b.1.d. Th2: Allergic Reaction Induction Potential (activation of eosinophils and mast cells)

Although activation of eosinophils and mast cells following DNA vaccination has not been specifically assessed, there is no specific data to support the induction of allergic reactions following DNA vaccination in dogs.

Furthermore, any risk of protein related adverse effects (i.e. anaphylactic-like reactions) associated to the melanoma DNA vaccine is considered to be very low for the following reasons:

- the vaccine will be produced on [redacted] medium
- the only proteins in the vaccine will be derived from the *E. Coli* host cells that contain no known allergens
- the production process will be free of [redacted]
- the product will be a [redacted] vaccine.

B.1.b.1.e. Tumorigenesis by insertional mutagenesis

Consequences of a recombinant event involving the chromosomal DNA are subject to speculation. Problems associated with such events have not been reported, likely due to the extremely low probability of occurrence. Safety evaluations of injection sites have been performed. Plasmid DNA was found to persist in the muscle at the site of injection; however, no aberrant muscle pathology, muscle toxicity, or immune-mediated pathology was observed in the injected muscles (Parker et al., Human Gene Therapy, 10; 741-758, 1999; Manam et al, Intervirology, 43; 273-281, 2000). In addition, the [redacted] does not contain known sequences of oncogenic potential and does not contain sequences of [redacted] origin.

B.1.b.1.f. Risk for specific classes of target species

The melanoma DNA vaccine is unique compared to conventional vaccines in that the targeted class is dogs with melanoma. These animals generally represent older individuals in the canine population. Proulx, et.al., reported the median age for 140 dogs diagnosed with melanoma to be 11 years with a range of 2-19 years, (*Veterinary Radiology & Ultrasound*, 44(3):352-359, 2003). Therefore, neonatal and juvenile dogs are not the target class for this vaccine.

Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for
Melanoma DNA Vaccine
CONFIDENTIAL

With regard to vaccination of breeding animals, the risk of a plasmid becoming integrated into embryonic/fetal tissues or reproductive tissues has been theorized, but is believed to be without practical impact, as only a very small number of plasmid molecules enter into cells post injection. Furthermore, the probability of a DNA molecule to integrate into the chromosome of muscle cells after intramuscular injection is even lower [at least 3000x lower than the natural mutation rate in an organism [(Martin et al. Human gene Therapy, 10; 759-68, 1999; Ledwith et al., Intervirology, 43; 258-72, 2000)], and the actual risk of integration into fetal tissues or reproductive tissues is believed to be exceedingly low.

Some experimental data indicates that, occasionally, when the plasmid was detected in gonads, it dissipated rapidly and was always extrachromosomal, confirming a low risk of germline transmission (Manam et al, Intervirology, 43; 273-281, 2000).

DNA vaccination *in utero* has been recently reported in the literature (Gerdts et al., J. Immunol., 168; 1877-85, 2002). However, since this experiment is based on the administration of the plasmid into the oral cavity of lamb fetus during surgery, it is unrelated to the transmission of plasmid from a pregnant animal to its offspring and is not relevant to the issue of plasmid vertical transfer.

B.1.b.2. Vaccination/Challenge

This vaccine is utilized as a biotherapeutic in animals that already exhibit the target disease; there is no challenge. The therapeutic benefit provided by the vaccine will be confirmed in animal trials with dogs diagnosed with melanoma [redacted]

B.1.b.3. Reversion to Virulence

Since the melanoma DNA vaccine is not infectious and cannot replicate in eukaryotic cells, Merial is of the opinion that reversion to virulence is not a relevant issue for the melanoma DNA vaccine.

B.1.b.4. Purity Testing

B.1.b.4.a. MSB Purity Testing

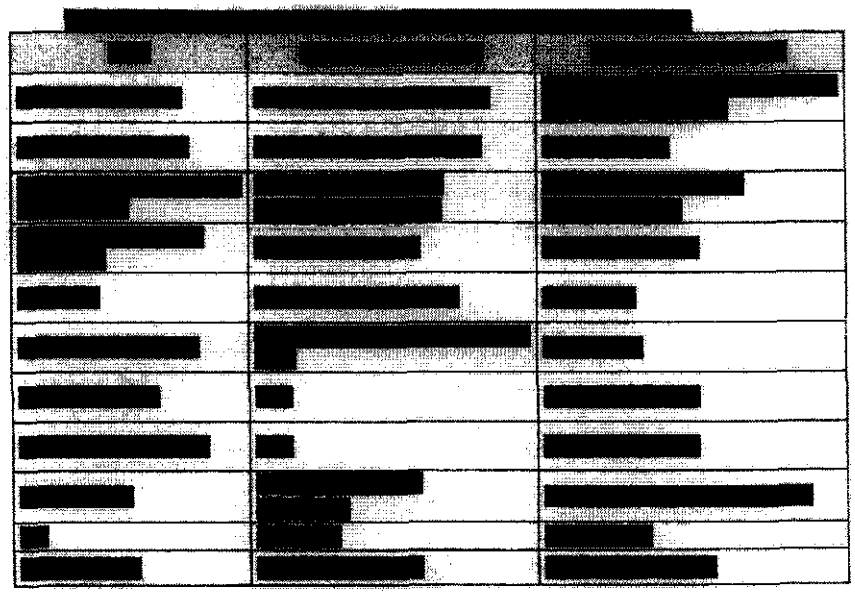
The purity of the host bacteria in the MSB has been established according to 9CFR 113.27(d).

The MSB was found to be pure when tested in accordance with the procedures described above. [redacted]

B.1.b.4.b. Vaccine Testing

The melanoma DNA vaccine will be released based on highly purified and well-characterized plasmid to ensure consistency of vaccine batch quality. [redacted]

[redacted]



B.1.b.5. Identity Testing

Identity of the plasmid in the DNA vaccine is based upon [redacted]

For the MSB host cell, the genus and species identification (i.e. demonstrate that the MSB is based on *E. coli*) has been established according to 9CFR 113.100 using metabolic markers specific of *E. coli*. The Gram staining results obtained for purity testing also contribute to the identification of the MSB as *E. coli*. [redacted]

B.1.b.6. Effect of Gene Manipulation on Pathogenicity

Since the melanoma DNA vaccine plasmid is not infectious and not pathogenic, Merial is of the opinion that the analysis of the effect of gene manipulation on pathogenicity is not a relevant issue for the melanoma DNA vaccine.

B.1.b.7. Genetic Stability

The nucleotide sequence of the plasmid will be confirmed (on both strands) on the [redacted] and on [redacted] thereby demonstrating genetic stability.

Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for
Melanoma DNA Vaccine
CONFIDENTIAL

[redacted]

B.1.b.8. Phenotypic Stability

The phenotypic stability of the [redacted] DNA vaccine will be demonstrated via [redacted]. As the [redacted] plasmid is the active ingredient in the melanoma DNA vaccine, and as it will be a highly purified, well-defined plasmid, Merial proposes that the [redacted] DNA vaccine can be assessed based on [redacted]. The intended parameters are: [redacted]

B.1.b.9. Alteration in Tissue Tropism

Since the melanoma DNA vaccine is not infectious and cannot replicate in eukaryotic cells, Merial is of the opinion that alteration of tissue tropism is not a relevant issue for a melanoma DNA vaccine.

B.1.b.10. Effect of Overdosing

[redacted] Merial will confirm safety in the target species as per 9 CFR 113.40 (2 dogs @10x dose).

B.1.c. Non-Target Animal Safety

B.1.c.1. Susceptible Non-Target Animals

Since the melanoma DNA vaccine is not infectious, Merial is of the opinion that susceptibility of non-target species is not a relevant issue for the melanoma DNA vaccine.

B.1.c.2. Virulence in Non-Target Animals

Since the melanoma DNA vaccine is not infectious, Merial is of the opinion that virulence in non-target species is not a relevant issue for the melanoma DNA vaccine.

B.1.c.3. Possible Outcome of Non-Target Animal Exposure

Since the melanoma DNA vaccine is not infectious, and does not replicate in the target species the probability of unexpected spread to non-target species is exceedingly low. Exposure of non-target species (including humans) is not an anticipated. However accidental exposure of non-target species may result in the development of an immune response to the [redacted] protein but no clinical disease or spread would be expected.

In addition, Merial (in agreement with the 2002 revised WHO Guidelines on DNA vaccines, minutes of the 7th NAVSaC meeting held at NIBSC UK on June 21, 2002) considers that the induction of anti-DNA antibodies is a theoretical issue, without practical implications for the development of the canine melanoma vaccine.

B.2. Public Health and Safety

B.2.a. Summary

Public health concerns associated with the use of the melanoma DNA vaccine are not expected.

B.2.b. Probability of Human Exposure

The probability of human exposure is low. The vaccine containing the plasmid [redacted] is for animal use only (under the prescription and supervision of qualified veterinary personnel). Administration of the injection by a qualified veterinarian further reduces the likelihood of accidental spread. The most likely exposure is through accidental self-injection and therefore limited to personnel administering the vaccine (veterinarian or veterinary technical personnel).

B.2.c. Pathogenicity of the Parent Microorganisms in Humans

The parental plasmid [redacted] is not pathogenic in humans because it is not infectious and it contains no sequence known to be involved in human pathology.

B.2.d. Virulence of the Vaccine Microorganism in Humans

Since the melanoma DNA vaccine is not infectious and it contains no sequence known to be involved in human pathology, Merial is of the opinion that the virulence of the vaccine microorganism in humans is not a relevant issue for this vaccine.

B.2.e. Possible Outcome of Human Exposure

The potential outcome of human exposure is the development of an immune response to the [redacted] protein which could theoretically lead to depigmentation of skin and hair. [redacted] has enrolled 18 human patients with melanoma in a [redacted] vaccine expressing [redacted]
No significant adverse events have been reported for these patients [redacted]

B.3. Environmental Safety

B.3.a Summary

According to the literature, the risk of dissemination of plasmids in the environment from the vaccinated animal is exceedingly low because the plasmid cannot replicate within the vaccinated animal. Furthermore, the accidental release of plasmids in nature should have no adverse effect because the risk that plasmids could be taken up by soil bacteria is very remote. Should this happen, the plasmid will not be maintained in the absence of [redacted]

As this [redacted] is not used in agricultural applications, the risk of having a plasmid disseminated by bacteria is exceedingly low. Minute amounts of plasmids, which could be accidentally ingested by animals will be degraded in their stomach and be of no consequence.

B.3.b. Environmental Distribution

The existence and extent of an environmental distribution of the melanoma DNA vaccine has not been established or reported.

B.3.c. Shed/Spread Capabilities

Merial is of the opinion that the melanoma DNA vaccine is associated with an exceedingly low risk of environmental release for the following reasons:

- The vaccine is to be administered [redacted] by a trained veterinarian. The small dose volume [redacted] minimize any risk of spill over during the injection. Visual observation of the procedure will guarantee that no vaccine is left on the skin of animal.
- The vaccine cannot be amplified in the vaccinated animal because it is based on a highly purified plasmid that has no eukaryotic origin of replication.
- Amount of plasmid per dose is very low [redacted].
- Once administered (parenterally) into vaccinated animals, plasmids have been shown to be transiently distributed throughout the body. They are subsequently lost from most tissues within several days, but may persist around the injection site (in the corresponding lymph node and in muscular tissues), for several weeks. Importantly, even though the plasmid DNA was found to persist in the muscle at the site of injection, no aberrant muscle pathology, muscle toxicity, or immune-mediated pathology indicative of an auto-immune disease was observed in the injected muscles. After a period of weeks to a few months, the plasmid will be cleared from the injection site area (Parker et al., Human Gene Therapy, 10; 741-758, 1999; Manam et al, Intervirology, 43; 273-281, 2000).

Based on the above Merial is of the opinion that the risk of plasmid shed/spread from a vaccinated animal into the environment is exceedingly small.

In the event of an accidental spill, there is no risk for target or non-target animals because the plasmid is (a) not infectious, (b) unstable in the environment and (c) extremely inefficient at penetrating into an organism after mucosal (e.g., oral) contact. Minute amounts that might be ingested by animals would be degraded in the stomach and be of no consequence.

B.3.d. Horizontal Gene Transmission / Recombination Potential

Horizontal gene transmission or recombination events would require that the plasmid survive in the environment. Merial is of the opinion that this risk is exceedingly low due to the remote likelihood that the plasmid will survive in the environment (Section VIII.B.3.g).

B.3.e. Potential for Transmission to Invertebrates

The potential for transmission to invertebrates is negligible. The plasmid [redacted] is not shed from vaccinated animals, therefore the only potential source of transmission to invertebrates would be an accidental spill. The ability of invertebrates to acquire the organism and to subsequently act as reservoirs or vectors is improbable given that the vaccine will be used in an animal hospital, the instability of plasmid in the environment, and it's lack of infectivity. Collectively, this should exclude the probability of transmission to invertebrates.

B.3.f. Host/Range Specificity

Since the melanoma DNA vaccine is not infectious, Merial is of the opinion that the host/range specificity is not a relevant issue for this vaccine.

B.3.g. Survivability of the Microorganism in the Environment

Possible establishment in the environment and specifically in soil bacteria surrounding the test site is highly unlikely because:

- Plasmid cannot replicate spontaneously in the environment,
- Bacteria don't efficiently take up plasmids without a prior *in vitro* chemical or physical treatment.

Furthermore, should such an event occur it would be without consequence because the plasmid will not be maintained in the absence of [redacted]. As this antibiotic is not used in agricultural applications, the risk of having a plasmid disseminated by environmental bacteria is exceedingly low.

B.3.h. Physical/Chemical Factors Affecting Environmental Dispersion

Only the simultaneous presence of a competent bacteria, [redacted] and the plasmid in one location could potentially enhance the dispersion of the melanoma DNA vaccine in the environment. However, this risk is considered to be exceedingly low since:

- [redacted] is not used in agricultural applications,
- the plasmid will be administered aseptically into the muscle of target animals by trained veterinarians.

B.3.i. Ecological Concerns

Due to the exceedingly low risk of survivability in the environment, Merial is of the opinion that the melanoma DNA vaccine poses no threat to any ecological system.

Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for
Melanoma DNA Vaccine
CONFIDENTIAL

C. Release Assessment

C.1. Contained release

Not applicable

C.2. Environmental release

C.2.a. Summary

The [redacted] has been subjected to environment release as a consequence of canine and human trials. [redacted] has administered the [redacted] plasmid [redacted] to approximately 85 dogs with melanoma. [redacted] has administered the [redacted] plasmid expressing [redacted] to 18 human melanoma patients in a FDA approved study.

Merial anticipates environmental release will occur in association with clinical trials. An efficacy trial will enroll client owned dogs diagnosed with melanoma.

C.2.b. Location of test site

For the efficacy study, veterinary clinics will be chosen based upon availability of canine melanoma cases and veterinary oncology specialists. Participating veterinary clinics will be identified in the 9 CFR 103.3 request to conduct the study and in the final study report.

C.2.c. Characteristics of Test Site

For the efficacy trial, veterinary clinics will serve as the focal point for each site and will be considered the "test sites" for this trial. Dogs diagnosed with melanoma enrolled in the studies are expected to come from typical households, located in the area of each participating test site. These animals will remain in the custody of their owners during the course of the study. Non-target species within the areas may include any other domestic animals located at each test animal's place of residence and the wildlife population endemic to each given locale.

C.2.d. Personnel

Personnel will consist of participating veterinarians, and participating Merial personnel. Only qualified personnel (based on their previous experience, training, and education) will handle the animals during vaccine administration. A list of participating veterinarians and Merial personnel and their qualifications will be provided to APHIS. Dogs enrolled in the efficacy study will remain in the custody of their owners.

C.2.e. Experimental Design

Efficacy: It is anticipated that dogs diagnosed with melanoma will be required for demonstration of efficacy. These dogs will receive doses of vaccine containing approximately of plasmid DNA at [redacted] Because the vaccine is a therapeutic for dogs previously diagnosed with melanoma, this trial will not include use of a challenge organism.

A detailed trial protocol will be submitted to CVB for approval prior to the conduct of the efficacy trial.

C.2.e.1. The Number of Animals:

Efficacy: It is anticipated that dogs diagnosed with melanoma are required to demonstrate efficacy of the vaccine.

C.2.e.2. Description of Animals:

Efficacy: client owned dogs diagnosed with melanoma.

C.2.e.3. Route of Administration:

The vaccine will be administered by the route in the medial thigh region using a needle free vaccination device.

C.2.e.4. The dose:

volume doses of vaccine, each containing approximately of plasmid DNA administered at intervals.

C.2.e.5. The total amount of test material:

Efficacy: With a maximum enrollment of dogs in the efficacy trial, doses of vaccine would be required.

C.2.e.6. Frequency and duration of exposure:

Each animal will received doses of vaccine administered at approximately [redacted]

C.2.e.7. Method of disposing waste:

Empty and used vaccine vials will be returned to Merial Ltd. Athens, GA for disposal by autoclaving. The needle free device disposables will be disposed of according to guidelines used in each veterinary clinic (i.e., autoclaving or incineration). All unused vials will be returned to Merial Ltd. Athens, GA., where they will be archived until licensure.

C.2.e.8. Decontamination of test site:

All materials used in the clinic that have had direct contact with vaccine fluid (needle free disposables, trays, etc.) should be discarded, autoclaved or disinfected with a disinfecting solution.

C.2.f. Potential Escape and Dispersal in the Environment

Merial is of the opinion that the melanoma DNA vaccine is associated with an exceedingly low risk of environmental release for various reasons:

The vaccine is to be administered by a trained veterinarian. The small dose volume will minimize any risk of spill over during the injection. Visual observation of the procedure will guarantee that no vaccine is left on the skin of animal.

- The vaccine cannot be amplified in the vaccinated animal because it is based on a highly purified plasmid that has no eukaryotic origin of replication.
- Amount of injected plasmid is very low .
- Low likelihood of Shed/spread of DNA from vaccinated animals

Based on the above Merial is of the opinion that the risk of plasmid escape and dispersal from a vaccinated animal into the environment is exceedingly small.

In the situation where plasmid accidentally spilled from the container, there is no risk for target or non-target animals because plasmid is (a) not infectious, (b) unstable in the environment and (c) extremely inefficient for penetration into an organism after mucosal (e.g., oral) contact. Minute amounts that could be ingested by animals will be degraded in the stomach and be of no consequence.

Based on the above Merial is of the opinion that the risk of plasmid escape and dispersal from an accidental spill into the environment is exceedingly small.

C.2.g. Potential for establishment in the environment:

Possible establishment in the environment and specifically in soil bacteria surrounding the test site is highly unlikely because:

- Plasmid cannot replicate spontaneously in the environment,
- Bacteria don't efficiently take up plasmids without a prior *in vitro* chemical or physical treatment.

Furthermore, should such an event occur it would be without consequence because the plasmid will not be maintained in the absence of . As this antibiotic is not used in agricultural applications, the risk of having a plasmid disseminated by environmental bacteria is exceedingly low.

C.2.g.1. The biological organisms found on the test site:

There will not be intentional introduction of known animal or human pathogens into the test sites. Non-pathogenic environmental organisms may be found and pathogenic organisms typical of veterinary clinics and places of residence of domestic animal species may be found.

C.2.g.2. Nutrient status:

There is no known nutrient(s) at the test site that will allow or promote the growth of plasmid DNA.

C.2.g.3. Physicochemical factors:
The plasmid is very sensitive to degradation and would rapidly be destroyed in the presence of nucleases, pH variations, and/or oxidation.

C.2.g.4. The presence of toxic chemicals and metabolites:
Toxic chemicals and metabolites should not be generated from the administration of the test article.

C.2.h. Monitoring
Adverse environmental events are not anticipated given the characteristics of the vaccine already described within this document. Based upon the low risk of shed and spread of the plasmid as described previously, Merial does not believe an environmental monitoring plan is necessary.

C.2.i. Contingency plans in case of adverse event
No adverse events are expected. Should some vaccine be accidentally spilled into the environment, Merial recommends exposing the plasmid to a mild acid solution (pH=6), which would almost immediately destroy the plasmid structure. There is a potential risk of accidental injection of the personnel administering the vaccine, or of skin contact with droplets of vaccine material leaking either from the injection site or the vial. In case of accidental human injection, medical professionals will be notified and an assessment requested.

In the unlikely circumstance that an adverse event is observed, the principle investigator will be notified immediately. Vaccination of the animals will be suspended and previous vaccinates will be examined carefully. Moreover, Merial will collect, analyze and evaluate the information and take appropriate actions to further mitigate the situation. If the decision is made that there is risk to personnel, animals, or the environment, APHIS will be notified with respect to planned actions to mitigate the potential impact of such an adverse event.

D. Risk Characterization

D.1. Likelihood ratings
Likelihood ratings are assigned for animal safety, public safety, and environmental safety based on the following criteria:

Low	=	An adverse event is unlikely to occur
Medium	=	An adverse event could possibly occur
High	=	An adverse event will most probably occur

Nucleic Acid-Mediated (Genetic) Vaccines Risk Analysis for
Melanoma DNA Vaccine
CONFIDENTIAL

D.2. Consequence ratings
Consequence rating are also assigned for animal safety, public health safety, and environmental safety based on following criteria:

Low	=	The consequences if the adverse event occurs will not be severe (the adverse event is self limiting and would have negligible impact).
Medium	=	The consequences if the adverse event occurs is moderately severe (the adverse event will have an impact, but it is not permanent, and can be treated).
High	=	The consequences if the adverse event occurs is severe (the adverse event will have an impact, is permanent, and can not be treated).

D.3. Degree of certainty ratings
Each likelihood and consequence rating is qualified by a degree of certainty rating that is based on following criteria

Certain	= The rating is supported by direct scientific evidence.
Moderately certain	= The rating is supported by indirect scientific evidence.
Uncertain	= The rating is not supported by scientific evidence.

D.4. Calculating the expected risk
Numerical values have been assigned to the likelihood, consequence, and degree of certainty ratings described above (Table 3). Each numerical value rating was derived from the importance placed on the rating of each category. The assigned numerical values are weighed to place emphasis on the severity of expected risk. These values reflect the professional judgment of the applicant (Merial Ltd.).

D.5. Risk Ratings
The risk ratings are based upon the likelihood, consequence, and degree of certainty ratings and expected risk for each category, as per guidance documentation provided by APHIS (Table 4) The total of 81 rating combinations are possible; e.g., Likelihood Low Moderately Certain, Consequence Low-Moderately Certain. Each combination has been assigned a risk rating of low, medium or high. The assigned ratings were weighed to place emphasis on the severity of the risk. Again, the severity of risk reflects the professional judgement of the applicant (Merial Ltd.). The low, medium, or high risk ratings are defined for the purpose of decision making as follows:

Low	=	Acceptable risk-very little concerns are associated with the proposal (does not justify denying the proposal)
Medium	=	Unacceptable risk-moderate concerns are associated with the proposal (either identify valid mitigative procedures or deny the proposal)
High	=	Unacceptable risk-major concerns are associated with the proposal (deny the proposal)

D.5.a. Risk to Animal safety
Likelihood Rating: Low (LL)
Degree of Certainty Rating: Certain (C)
Consequence Rating: Low (CL)
Degree of Certainty Rating: Certain (C)
Expected Risk Rating: 1.0000
Risk Characterization: LL.C.CL.C
Risk Rating: Low
Justification for rating: [redacted]

Conclusion and discussion of risk:
The risk to animal safety associated with the proposal to conduct efficacy and safety studies in dogs with the melanoma DNA vaccine, is low. The melanoma DNA vaccine cannot replicate in eukaryotic cells. It is also (a) not infectious, (b) highly purified and well characterized, and (c) extremely inefficient for penetration into an organism after mucosal (e.g., oral) contact. Minute amounts, which could be ingested by animals will be degraded in their stomach and be of no consequence.

D.5.b. Risk to Public Health and Safety
Likelihood Rating: Low (LL)
Degree of Certainty Rating: Certain (C)
Consequence Rating: Low (CL)
Degree of Certainty Rating: Certain (C)
Expected Risk Rating: 1.0000
Risk Characterization: LL.C.CL.C
Risk Rating: Low
Justification for rating: [redacted]

Conclusion and discussion of risk:
There are no public health concerns associated with the testing of the melanoma DNA vaccine. Human exposure will be limited to the qualified personnel administering the vaccine and the risk of exposure to these individuals is considered to be low.

D.5.c. Risk to Environmental safety:
Likelihood Rating: Low (L)
Degree of Certainty Rating: Certain (C)
Consequence Rating: Low (CL)
Degree of Certainty Rating: Moderately Certain (MC)
Expected Risk Rating: 0.7500
Risk Characterization: LL.C.CL.MC
Risk Rating: Low
Justification for rating: [redacted]

Conclusion and Discussion of risk:
The safety risks to environment are low. The vaccine will be kept in single dose sealed labeled glass vials in the participating clinic(s) and only veterinarian(s) and/or professional staff should handle it. In the case that vaccine is released into the environment, the inability of the vaccine to infect and replicate any eukaryotic species should eliminate any risk of persistence in the environment.

IX. Risk Management

A. Procedure
Risk management uses the information from the risk assessment, as well as, regulatory, social, and economic realities, to determine whether the proposed release should be approved. Risk management also includes the design and implementation of mitigative procedures to reduce or eliminate potential risks. If the risks to animals, public health, and the environment are low, the proposed study is approved. If the risk is high the request is denied. Requests to conduct studies that have been rated with medium risks are also denied, unless proper mitigation procedures are identified and implemented.

B. Recommendations
The safety risks to animals, public health, and the environment are low. A low risk rating is an acceptable risk, with very little concern associated with the proposal.

Table 3: Calculating the Expected Risk

VALUE RATINGS

	Likelihood (L)
Low (L)	LL = 1.00
Medium (M)	LM = 0.50
High (H)	LH = 0.10
	Consequence (C)
Low (L)	CL = 1.00
Medium (M)	CM = 0.10
High (H)	CH = 0.01

*If the Likelihood rating is Medium or High and the Consequence rating is also Medium or High use *Degree of Certainty Ratings I*; for all other combinations use *Degree of Certainty Ratings II*.

Degree of Certainty Ratings I

Certain (C)	C = 0.50
Moderately Certain (MC)	MC = 0.75
Uncertain (U)	U = 1.00

Degree of Certainty Ratings II

Certain (C)	C = 1.00
Moderately Certain (MC)	MC = 0.75
Uncertain (U)	U = 0.50

EXPECTED RISK

[(likelihood) x (degree of certainty)] x [(consequence) x (degree of certainty)] = Risk Rating

Table 4: Risk Ratings

Risk Characterization	Expected Rating	Risk Rating	Risk Characterization	Expected Rating	Risk Rating
LL.C.CL.C.	1.0000	L	LM.U.CM.C.	.0250	M
LL.C.CL.MC.	.7500	L	LM.C.CM.MC.	.0188	M
LL.MC.CL.C.	.7500	L	LM.MC.CM.C.	.0188	M
LL.MC.CL.MC.	.5625	L	LM.C.CM.C.	.0125	M
LL.C.CL.U.	.5000	L	LH.U.CM.U.	.0100	M
LL.U.CL.C.	.5000	L	LL.C.CH.C.	.0100	M
LM.C.CL.C.	.5000	L	LH.MC.CM.U.	.0075	M
LL.MC.CL.U.	.3750	L	LH.U.CM.MC.	.0075	M
LL.U.CL.MC.	.3750	L	LL.C.CH.MC.	.0075	M
LM.C.CL.MC.	.3750	L	LL.MC.CH.C.	.0075	M
LM.MC.CL.C.	.3750	L	LH.MC.CM.MC.	.0056	M
LM.MC.CL.MC.	.2813	L	LL.MC.CH.MC.	.0056	M
LL.U.CL.U.	.2500	L	LH.C.CM.U.	.0050	M
LM.C.CL.U.	.2500	L	LH.U.CM.C.	.0050	M
LM.U.CL.C.	.2500	L	LL.C.CH.U.	.0050	M
LM.MC.CL.U.	.1875	L	LL.U.CH.C.	.0050	M
LM.U.CL.MC.	.1875	L	LM.U.CH.U.	.0050	M
LM.U.CL.U.	.1250	L	LH.C.CM.MC.	.0038	M
LH.C.CL.C.	.1000	L	LH.MC.CM.C.	.0038	M
LL.C.CM.C.	.1000	L	LL.MC.CH.U.	.0038	M
LH.C.CL.MC.	.0750	L	LL.U.CH.MC.	.0038	M
LH.MC.CL.C.	.0750	L	LM.MC.CH.U.	.0038	M
LL.C.CM.MC.	.0750	L	LM.U.CH.MC.	.0038	M
LL.MC.CM.C.	.0750	L	LM.MC.CH.MC.	.0028	M
LH.MC.CL.MC.	.0563	M	LH.C.CM.C.	.0025	M
LL.MC.CM.MC.	.0563	M	LL.U.CH.U.	.0025	M
LH.C.CL.U.	.0500	M	LM.C.CH.U.	.0025	M
LH.U.CL.C.	.0500	M	LM.U.CH.C.	.0025	M
LL.C.CM.U.	.0500	M	LM.C.CH.MC.	.0019	H
LL.U.CM.C.	.0500	M	LM.MC.CH.C.	.0019	H
LM.U.CM.U.	.0500	M	LM.C.CH.C.	.0013	H
LH.MC.CL.U.	.0375	M	LH.U.CH.U.	.0010	H
LH.U.CL.MC.	.0375	M	LH.MC.CH.U.	.0008	H
LL.MC.CM.U.	.0375	M	LH.U.CH.MC.	.0008	H
LL.U.CM.MC.	.0375	M	LH.MC.CH.MC.	.0006	H
LM.MC.CM.U.	.0375	M	LH.C.CH.U.	.0005	H
LM.U.CM.MC.	.0375	M	LH.U.CH.C.	.0005	H
LM.MC.CM.MC.	.0281	M	LH.C.CH.MC.	.0004	H
LH.U.CL.U.	.0250	M	LH.MC.CH.C.	.0004	H
LL.U.CM.U.	.0250	M	LH.C.CH.C.	.0003	H
LM.C.CM.U.	.0250	M			