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TECHNIQUES FOR EVALUATING HUMORAL AND CELL-MEDIATED IMMUNITY IN MULE DEER FAWNS *(Odocoileus hemionus)*[1]

BRUCE D. TRINDLE, LON D. LEWIS and LLOYD H. LAUERMAN

Abstract: Twenty mule deer fawns *(Odocoileus hemionus)* were removed from their dams 48 h after birth, and hand-reared. Methods for monitoring their immune capability are described. Passive humoral immunity was determined by serum protein electrophoresis. Active humoral immunity following Clostridium toxoid vaccination was determined by immunodiffusion. Cell-mediated immunity was assayed using contact sensitization to 1-nitro,2,4-dichlorobenzene (DNCB).

INTRODUCTION

Many studies, such as nutrition, determination of resistance to disease, etc., necessitate monitoring the animal's immune systems. To do this requires evaluation of both humoral and cell-mediated immunity; the two major components of the immune system. The techniques for doing this have been documented for man and many domestic ruminants,[1,2,5] but not for wild ruminants.

Humoral immunity is the presence of antibodies which may be passively or actively acquired. Most antibodies present at birth are passively acquired from the dam, either in-utero or absorbed from the colostrum after birth. In the ruminant, very few antibodies are obtained in-utero.[4] In addition, at birth they have little actively acquired humoral immunity from antibodies which they have produced themselves. The year prior to this study, jugular blood samples were taken from 26 mule deer fawns at birth. Immunoglobulin levels, as determined by serum protein electrophoresis, were an extremely low 0.4 ± 0.2 mg of gamma globulins/ml ($\bar{X} \pm$ SD). New born ruminants, therefore, are almost entirely dependent upon antibodies obtained from the colostrum for their humoral immunity.

In assessing humoral immunity in the neonate, it is necessary to: 1) measure the amount of its antibodies obtained from the dam, and 2) determine when it is first able to produce antibodies of its own to a specific antigenic challenge. Radial immunodiffusion may be used to quantitate immunoglobulin levels.[3] But, this requires specific antisera which are not available for mule deer. Total gamma globulin levels, most of which are immunoglobulins, may be determined, however, using serum protein electrophoresis. To determine the presence of active humoral immunity it is necessary to detect the presence of specific immunoglobulins produced in response to an antigenic challenge. This may be done by immunodiffusion reaction of serum with the specific antigen used for challenge.

Cell-mediated immunity in humans has been studied using contact sensitization to 1-nitro,2,4-dichlorobenzene (DNCB).[2] A relatively brief duration of binding to the skin is required for sensitization. Sensitization takes place in

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the regional lymph nodes and is elicited when sensitized lymphocytes come into contact with the DNCB bound to epidermal protein. This requires 7 to 21 days. When sensitization occurs there is a concentration dependent flare reaction at the site of DNCB binding. Some subjects require reapplication of a relatively small dose of DNCB for sensitization to occur. Approximately 90% of normal subjects can be sensitized to DNCB.[2]

DNCB also is a potent irritant which usually causes a marked erythema and edema within 12 h after applying a sensitizing dose. This effect subsides over 3 to 5 days and is followed by desquamation and pigmentation. Approximately 40% of the nonsensitized subjects will have a similar but milder reaction to the much smaller challenge dose applied 7-21 days later.[2] In such cases, histological sections of the site of DNCB application are needed to differentiate this irritative effect from true delayed hypersensitivity.

This paper describes the application of serum protein electrophoresis for measuring gamma globulin levels which should correlate with passively acquired humoral immunity, immunodiffusion for measuring immunoglobulin levels which assesses actively acquired humoral immunity, and DNCB skin sensitization for assessing cell-mediated immunity in mule deer fawns.

MATERIALS AND METHODS

Twenty fawns *(Odocoileus hemionus)* were removed from their dams 2 days after birth. The management of these does and fawns has been described.[6]

Humoral Immunity Assay

Jugular blood samples were taken at weekly intervals from 1 to 8 weeks of age. Serum protein electrophoresis [2] was conducted on the samples to determine the total amount of gamma globulins present. The amount of gamma globulins present in the first sample indicates the amount of humoral immunity which the fawn passively acquired from the doe's colostrum. To detect the presence of humoral immunity actively produced by the fawns themselves, they were vaccinated subcutaneously with 3 ml of Clostridium toxoid [3] at 4, 6, and 8 weeks of age, and their serum was analyzed by micro-immunodiffusion, as described by Crowle,[3] for the detection of precipitins to these toxoids. The weekly serum samples were assayed with toxins [4] corresponding to 5 of the 7 bacterial toxoids contained in the vaccine administered. The development of precipitation lines between the serum and toxin during the immunodiffusion technique is indicative of the presence in the serum of antitoxins produced by the fawns in response to the toxoids in the vaccine. The fawns were considered to be capable of active humoral immunity when serum antitoxins were first detected against two of the toxins.

Cell-Mediated Immunity Assay

Sensitizing doses of DNCB were applied at 7, and 23 days of age. Regardless of gross appearance of the skin from the sensitizing doses, a challenge dose was applied at 21 days of age. DNCB was applied to the right side of the neck at 7 and 21 days of age, and to the left side at 23 days of age. Two sensitizing doses of 2000 and 50 μg were used. All doses were dissolved in 0.1 ml of acetone. The time course for the procedures used in assessing cell-mediated immunity is given in Table 1.

Fawns were anesthetized[7] for each application of DNCB and each skin biop-

[2] As developed by Helena Laboratories, Beaumont, Texas.

[3] Jen-Sal Electroid 7, Jensen-Salsbery Lab., Kansas City, Mo. *(Clostridium chauvoei, septicum, novyi, sordellii,* and *perfringens* types C and D).

[4] Produced by Norden Laboratories, Lincoln, Nebraska.

TABLE 1. Procedures used for assessing cell-mediated immunity in mule deer fawns using 1-nitro,2,4-dichlorobenzene (DNCB) contact sensitization.

DNCB dose (μg)	Procedure conducted	Age of Fawns (days) 7	21	23	37
0	Skin biopsy taken	R*			
50	Skin color, condition, and thickness noted	R	R	R&L*	L
	DNCB applied topically	R		L	
2000	Skin color, condition, and thickness noted	R	R	R&L	L
	Skin biopsy taken			R	L
	DNCB applied topically	R		L	
200	Skin color, condition, and thickness noted			R	
	Skin biopsy taken			R	
	DNCB applied topically		R		

*R indicates the procedure was conducted on the right side of the neck, L that it was conducted on the left side of the neck, and the absence of either R or L that the procedure was not conducted at that time.

sy. A 12 cm square on the side of the neck being used was clipped, and thoroughly washed and disinfected. A skin biopsy was taken at 7 days of age as a control from one of the far corners of the clipped area. This and all subsequent biopsies were taken using an 8 mm diameter corneal punch and were preserved in buffered formalin. Biopsy sites were sutured and an antiseptic applied.

A clear plexiglass template with four holes (Fig. 1) was used to mark two areas for sensitization. The skin under the top two holes of the template was marked by pushing a toothpick previously dipped in india ink through holes in the skin made with a 20 gauge sterile hypodermic needle. The resulting tattoos allowed realignment of the template for later location of the skin under the bottom holes of the template where the sensitizing doses would be applied. The skin under the bottom holes was marked with a felt tip pen and the template removed. A double fold skin thickness was measured at each sensitizing site.

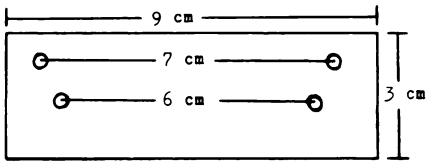

FIGURE 1: Plastic template for the application of sensitizing and challenge doses of DNCB.

A piece of rigid plastic tubing, 1 to 1.5 cm in length and 1.5 cm in diameter, was held firmly against the neck over the sensitizing mark. Vaseline may be used to improve the seal between the neck and the tubing to prevent leakage of the dose under the tubing. The two sensitizing doses were applied topically in the center

of the tubing using a 1 cc tuberculin syringe with a 20 gauge, 2.5 cm needle. The dose was allowed to evaporate before removing the tubing. The neck was then covered with a light cloth collar to screen out the sun and dirt.

The 200 μg challenge dose was applied in the same manner as the sensitizing doses with the site of application being no nearer than 5 cm from any previous application. Skin color and condition were noted and a double fold skin thickness was measured at each site prior to DNCB application and at the other times indicated in Table 1. Skin biopsies of the application sites were taken 14 or 16 days after applying the sensitizing doses, and 48 hours after applying the challenge dose (Table 1).

The degree of cell-mediated immunity was determined by histological examination of the skin biopsies. The intensity of reaction to DNCB was graded on a scale of 1 to 4, with 4 being the strongest reaction. Each biopsy was rated according to lymphocyte infiltration first and necrosis second (Table 2). Lymphocyte infiltration was calculated by finding the highest accumulations under 40× magnification and then in this area 3 to 4, 1 mm^2 fields were counted under 960× magnification and oil immersion to obtain an average lymphocyte count per field. Location of lymphocyte infiltration in relation to skin characteristics was recorded, e.g. vascular cuffing, folliculitis, and subepidermal or dermal accumulations. Necrosis was recorded as epidermal or dermal. The histologic grade of the three skin biopsies taken from each fawn were averaged to indicate that fawn's degree of cell-mediated immunity.

RESULTS

Two fawns died during the first week of life, and have been omitted from the results.

Humoral Immunity Assay

Total gamma globulin levels as determined by serum protein electrophoresis have been reported.[6] As an indication of active humoral immunity, the *Clostridium novyi* and *C. sordellii* toxins proved to be the most antigenic of the five *Clostridium* toxins used. They were able to stimulate the production of precipitins, which are considered to be antibodies, by the greatest number of fawns at the earliest age (Table 3). None of the fawns were able to produce precipitins against *perfringens* type D or *septicum* by 8 weeks of age (Table 3).

TABLE 2. Criteria used for ranking cell-mediated responses by histologic examination.

+4	Lymphocyte density of 20 or greater/square mm Vascular cuffing Folliculitis Subepidermal and dermal concentrations Epidermal and dermal necrosis
+3	Lymphocyte density of 13 - 19/square mm Folliculitis Subepidermal concentrations Epidermal and dermal necrosis
+2	Lymphocyte density of 4 - 12/square mm Epidermal necrosis
+1	Lymphocyte density of 3 or less/square mm Ordered appearance of skin features

TABLE 3. Age that mule deer fawns were first able to demonstrate precipitins against *Clostridium* toxoids.

Age in weeks	CLOSTRIDIUM TOXOID				
	Novyi	Sordellii	Perfringens type C	Perfringens type D	Septicum
5	0*	0	0	0	0
6	2	0	1	0	0
7	11	14	7	0	0
8	3	2	2	0	0

*Number of fawns from a total of 18, that produced precipitins at that age against that toxoid.

Cell-mediated Immunity Assay

Observation of the first applications of sensitizing doses at 23 days of age showed that all of the fawns had scab-like lesions, discoloration, and thickening of the skin where the 2000 μg dose was applied. At the site where the 50 μg dose was applied, six percent had scab-like lesions, 33% skin discoloration, and 6 percent skin thickening. The 200 μg challenge dose, 48 h after application, elicited the scab-like lesions in 17% of the fawns, skin discoloration in 100% and skin thickening in 67%. The second set of sensitizing DNCB doses applied at 23 days of age and examined 14 days later produced scab-like lesions, discoloration and thickening of the skin in all of the fawns where the 2000 μg dose was applied, while the 50 μg dose caused only skin discoloration and this in only 50% of the fawns.

The histological grading of the three skin biopsies taken at the site of application of the DNCB is given in Table 4.

DISCUSSION

Humoral Immunity Assay

The use of serum protein electrophoresis proved to be an effective tool for estimating serum antibody concentrations. In addition to the determination of total immunoglobulin levels, serum protein electrophoretic patterns allow monitoring of the animal's general health.

Assessing active humoral immunity in mule deer fawns requires the differentiation between existing antibodies and those actually produced by the animal's own immune system. The selection of the 7-way *Clostridium* toxoid vaccination used in challenging the fawn's immune system was chosen because of the wide variety of antigens it contains, and because two of those antigens coincided with the *C. perfringens* types C and D antitoxin vaccination given at birth. The 7-way *Clostridium* vaccination allows checking of the protection conferred by the antitoxin vaccination and still permits the detection of the fawn's active immune capability by stimulating it with 5 other bacterial toxoids. Also, the wide variety of bacterial toxoids present a stimulation to the fawn's immune system that would be less likely to be present in the colostral antibodies obtained from the doe. However, neither the *C. perfringens* types C or D antitoxin given, nor any possible antitoxins from the doe's colostrum against the 5 toxins the fawn's serum was tested for could be demonstrated by the immunodiffusion technique. The inability to detect these by immunodiffusion implies that this assay can only detect relatively high antibody titers. Titers of this magnitude

TABLE 4. Histologic grade of skin biopsies taken at the site of application of DNCB in mule deer fawns.

DNCB dose (μg)		2000	200	2000
Age at application (days)		7	21	23
Age at biopsy (days)		23	23	37
Histological grade	+4	2*	1	3
	+3	10	7	12
	+2	5	10	2
	+1	1	0	1

*Number of fawns from a total of 18 whose skin biopsy showed this histological grade, with +4 being the strongest reaction (see Table 2).

are more likely to be actively produced than passively obtained. Thus, this assay seems to be well adapted for detecting the presence of active humoral immunity.

Cell-mediated Immunity Assay

The technique of DNCB contact sensitization described by Catalona[2] utilized the visual appearance of skin at the sites of application to grade the cell-mediated response, along with histological confirmation. In this study the gross appearance of the skin at the site of application proved to be very similar between fawns, whereas histologic examination of the skin biopsies showed a substantial degree of variation in cellular reaction. For this reason the histological grading scheme was used, and is recommended, to determine the intensity of cellular reaction, and therefore, degree of cell-mediated immunity present. The observation of skin color, condition, and thickening was used to assure that sensitization to DNCB did occur. But, this alone, without histological confirmation, is not adequate to indicate the presence of cell-mediated immunity.

The 2000 μg DNCB dose was adequate for eliciting the delayed hypersensitivity response indicative of cell-mediated immunity. Because of the lack of consistent visual changes at the site of application of the 50 μg dose, biopsies were not taken at this site. Application of the challenge, regardless of the appearance of the sensitizing doses, was used to test the anamnestic response. An equal or more pronounced cellular reaction to the 10-fold smaller challenge dose than to the 2000 μg sensitizing dose indicates the presence of an anamnestic response.

The time course used for the application of DNCB and skin biopsies (Table 1) was selected to: 1) determine if cell-mediated immunity existed in the fawns at this early age, and 2) to determine if the cell-mediated reaction increased with age. Cell-mediated immunity was found to be present by 1 week of age. It did not appear to increase substantially with age as determined by reapplication of DNCB and histological examination of skin biopsies taken periodically throughout the first 8 weeks of life.

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