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EVALUATING METHODS TO ASSESS HUMORAL AND CELL MEDIATED IMMUNE RESPONSE IN CAPTIVE GREEN TURTLES (*CHELONIA MYDAS*)

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INTRODUCTION

Green turtles (*Chelonia mydas*) are endangered herbivorous marine reptiles that live in tropical and subtropical foraging pastures of algae and seagrasses throughout the world. A distinct population exists in Hawaii (Bowen et al., 1992) with the major nesting site being French Frigate Shoals in the Northwestern Hawaiian Islands (Balazs, 1980).

Green turtles are adversely impacted by man-made factors (Balazs and Pooley, 1994) and natural diseases like fibropapillomatosis. Fibropapillomatosis (FP) causes debilitating skin and internal tumors in green turtles worldwide (Herbst, 1994). In Hawaii, prevalence of FP is increasing or stable (Balazs, 1991). Fibropapillomatosis is probably caused by a virus (Casey et al., 1997; Quackenbush et al., 1998) although the role of toxicants has also been explored (Aguirre et al., 1994).

While green turtles with FP in Hawaii are thought to be immunosuppressed (Aguirre et al., 1995), relatively little information exists on immunology of free-ranging green turtles or the tools to evaluate their immune response. Benedict and Pollard (1972) determined that green turtles had three types of immunoglobulins. McKinney and Bentley (1985) found that green turtle lymphocytes could be isolated and stimulated in vitro. Herbst and Klein (1995) developed monoclonal antibodies against the different classes of green turtle immunoglobulins. Both Benedict and Pollard (1972) using radioelectrocomplexing and Herbst and Klein (1995) using enzyme-linked immunosorbent assay (ELISA) showed that green turtles will produce a detectable humoral immune response when exposed to a foreign antigen.

Ideally, evaluating immune response of free-ranging green turtles would measure cell mediated and humoral immune response to a known dose of antigen. This would require a minimum of one capture to immunize the animal, and a recapture of the same individual to measure immunologic response. Minimizing the number of times an animal needs to be exposed to the antigen to generate a detectable response would be of interest since chances of recapture decrease with each attempt. Since an adjuvant would probably be needed, evaluating its safety and efficacy in green turtles would be of interest.

Our objectives were to:

(1) Assess whether a single exposure to an antigen would produce a detectable immune response in captive healthy

green turtles using ELISA and lymphocyte stimulation test.

(2) Determine what adjuvant provided an optimal humoral and cell mediated immune response.

(3) Evaluate the clinical response of green turtles to different adjuvants.

(4) Compare the lymphocyte stimulation test using whole blood or isolated cells in two different culture medias.

METHODS

Study Animals

We captured eight turtles from Kaneohe Bay, Hawaii in July, 1997. Prior to placing in captivity, turtles were carefully examined for absence of fibropapillomas on the skin, eyes, or glottis because Kaneohe Bay is an area endemic for fibropapillomatosis (Balazs, 1991). Turtles were allowed to adapt to captivity for at least 1 month in 9 m^3 tanks (2-3 turtles/tank) with circulating seawater and were fed a daily diet of squid and mackerel with vitamin and mineral supplements. Tank temperatures (C) were recorded daily.

We chose chicken egg white lysozyme (EWL) (Sigma, St. Louis, Missouri) as an antigen because it is a complex protein to which the turtles were not likely to have been exposed. For adjuvants, we chose Freund's (DIFCO), Montanide ISA-70 (Seppic, Inc., Fairfield, New Jersey), and GERBU (CC Biotech, Poway, California). Freund's adjuvant was used to provide a direct comparison with past studies (Benedict and Pollard, 1972) involving experimental immunization of green turtles. ISA-70 (Yamanaka et al., 1992) and Gerbu (Grubhofer, 1995) were chosen for their propensity to elicit a less severe local inflammatory reaction than FCA. Egg white lysozyme dissolved in phosphate buffered saline (PBS) was mixed with adjuvants according to manufacturer instructions. On September 15, 1997, we injected six turtles (two for each adjuvant) with 1 mg/kg of EWL; a seventh turtle was injected with PBS and served as a control. Egg white lysozyme was delivered intramuscularly in the ventral rear leg muscle mass in the following volumes of adjuvants (½ in each leg): FCA-750 μl; ISA-70 3 cc and GERBU 5 cc. The dose of EWL was comparable to that used to elicit detectable immune response in green turtles after a single injection (Benedict and Pollard, 1972). On February 10, 1998, turtles received another dose of EWL (1 mg/kg) in appropriate adjuvant except that Freund's incomplete adjuvant was used in turtles that initially received FCA. The study ran until March 8, 1998.

Turtles were monitored daily for food consumption. Every 1-2 weeks, turtles were weighed (kg) with a spring scale and heparinized blood (18 USP heparin/ml) taken from the cervical sinus with a sterile syringe and 0.9 mm 2.57 cm needle (Owens and Ruiz, 1980). The injection site was visually examined and palpated for swelling or formation of granulomas. For each blood sample, we did hematocrit, estimated total solids, and did a complete blood count (Work et al., 1998).

Lymphocyte Stimulation Test

Cell mediated immune response was evaluated using methods adapted from McKinney and Bentley (1985). Cells were plated (100 µl/well) in 96-well microtiter plates (Costar, Cambridge, Massachusetts) in two types of media: Roswell Park Memorial Institute (RPMI) media 1640 (Gibco, Grand Island, New York) and RPMI-1640 supplemented with 5 mercaptoethanol (RPMI-ME). The latter was used because Farag and El Ridi (1986) reported enhanced proliferation of snake splenocytes in vitro in the presence of mercaptoethanol. Cells were supplemented with 100 µl of 0.25% Albumax I (Gibco) in RPMI along with the following mitogens/antigens plated in triplicate: Concanavalin A (ConA) at 0.1, 1 and 10 µg/well; phytohemaglutinin (PHA) at 0.01, 0.1, 1 and 10 µg/well; EWL at 0.001, 0.01. 0.1 and 1 µg/well. We chose PHA and ConA (Sigma) because they specifically stimulate T-cells in mammals (Benjamini and Leskowitz, 1994). PHA is also thought to stimulate T-like cells in alligators (*Alligator mississipensis*) (Cuchens and Clems, 1979c). Three control wells were supplemented with Albumax and RPMI or RPMI-ME only.

Plates were incubated for 90 hours in a humidified atmosphere at 32°C and 8% CO2. Cells were pulsed with 1 µCi of tritiated thymidine (Amersham Life Sciences Inc., Chicago, Illinois) in 50 µl RPMI/albumax, harvested 18 hours later, and radioactive decay (degradations/minute [DPM]) quantified on a liquid scintillation counter (Packard Instruments, Downers Grove, Illinois). Mean DPM of triplicate wells for each mitogen or antigen concentration was divided by mean DPM of triplicate control wells to calculate stimulation index.

Lymphocyte stimulation tests on whole blood were done using methods adapted from (Redig et al., 1984). Briefly, whole blood was diluted 1:20 in RPMI or RPMI-ME, plated, incubated, and harvested as above. Concentrations were 1, 5, 10 and 20 µg/well for ConA and 0.1, 1 and 10 µg/well for PHA.

Enzyme Linked Immunosorbent Assay (ELISA)

The globulin fraction from 12 ml of plasma from immature green turtles sampled from a fibropapilloma-free area was precipitated in half-saturated (50%) ammonium sulfate (Page and

Thorpe, 1996). The precipitated protein fraction was pelleted by centrifugation at 1500 x g for 10 minutes. The resulting fraction was resuspended in 50% saturated ammonium sulfate and repelleted three additional times before being redissolved in PBS. The dissolved globulin fraction was then desalted on a Sephadex G-25 column and concentrated, using an Amicon Microcon 100 protein concentration unit following manufacturer's directions. The total protein in the resulting sample was quantified using a Pierce BCA assay kit following manufacturer's instructions.

A sample containing 250 ug of protein was diluted 1:2 in electrophoresis sample buffer (Bio-Rad, Hercules, California) without mercaptoethanol and resolved on a 20 cm 5% acrylamide separatory gel under nonreducing conditions using a discontinuous system. Resolved protein bands were visualized by negative staining with copper (Bio-Rad). Two bands approximating 180 kDa, the size of full length green sea turtle IgY, were carefully excised, destained with Tris-glycine destain solution, minced, and electroeluted using the electroelution (Bio-Rad). The eluted protein specimens were collected, dialyzed against PBS, concentrated, diluted in reducing electrophoresis buffer, run on a 10% acrylamide minigel under reducing conditions, and stained with Coomassie brilliant blue 250R. The band which broke down into the appropriately-sized bands representing the heavy and light chains was identified as turtle IgY. A batch of purified heavy chain was prepared from the isolated intact IgY by electroelution from a 10% gel run under reducing conditions. Several batches of the IgY heavy chain were purified by nonreducing electrophoresis, excision, electroelution and dialysis using the isolated heavy chain as a molecular weight marker. Electrophoretic analysis of the pooled batches concentrated on a 10% acrylamide gel stained with Coomassie Brilliant Blue 250R demonstrated a single band at approximately 68 kDa.

Polyclonal rabbit anti-turtle IgY antisera was prepared at U.C. Davis utilizing the antibody production service offered through Animal Resources. Briefly, a New Zealand white rabbit was immunized 3 times with 150 ug of the IgY heavy chain preparation at 2-week intervals. Each immunization was given as multiple (4-5) subcutaneous injections. The initial dose was emulsified in an equivalent volume of Freund's complete adjuvant. Freund's incomplete adjuvant was employed in all subsequent immunizations. The rabbit was anesthetized with a ketamine xylazine cocktail 2 weeks after the third immunization and exsanguinated by cardiocentesis. The anti-IgY heavy chain titer was determined by solid phase ELISA using the Chequer-board assay technique (Campbell et al., 1984).

Humoral immune response in green turtles was evaluated using indirect ELISA (Saunders, 1979). Briefly, ELISA plates (Falcon, Becton Dickinson, Oxnard, California) were coated with 50 μl EWL

(5 μg/well) and air dried overnight at 27°C. Wells were blocked with 2% nonfat milk in 0.5M PBS, overlaid with 50 μl turtle plasma diluted 1:25 in 2% milk in duplicate wells, overlaid with rabbit anti-green turtle antibodies, which were detected with goat anti-rabbit antibodies conjugated to horseradish perodixase (Alpha Diagnostics, San Antonio, Texas). Color development was with 5 aminosalycilic acid, and optical density was measured at 450 nm wavelength with an ELISA plate reader. Titers for representative samples were assayed using serial twofold dilutions of plasma.

Data Analysis

Means of stimulation indices for all turtles for each mitogen concentration and media (MRPMI or MRPMI-ME) for cells or whole blood were used to determine the mitogen or antigen concentration where peak stimulation occurred. Because peak stimulation for each mitogen did not consistently occur at a set concentration, we calculated area under the curve for each mitogen for cell or whole blood. For each mitogen, mean area under the curve was compared between media or within media and between whole blood or cells using T-test. Coefficient of variation was used to assess variability of each type of media for each mitogen for whole blood or cells. Alpha for all comparisons was 0.05.

RESULTS

Animals

We observed no local inflammatory reaction after the first injection in any animal. After the booster, we palpated some mild edema at the injection site which slowly regressed in the ensuing 2-3 weeks. One animal subsequently developed a 4 mm fibropapilloma in the medial canthus of the right eye. This animal received EWL in FCA. Of eight turtles, three were removed from the study and released after supportive care. One refused to eat and was released 2 weeks after capture and prior to the start of the study. The control animal was released 2 months after injection after exhibiting progressive weakness of one front and one rear limb, loss of interest in food, anemia, heterophilia, and monocytosis. A third animal exhibited similar symptoms involving one front limb after receiving a booster of ISA-70 (Fig. 1). Remaining animals were unremarkable. All animals except the control animal gained an average of 5.8 kg throughout the study period. Tank temperatures ranged from 23-28°C (24.7±0.7).

Lymphocyte Stimulation Test

Mean viability of isolated cells in RPMI (95±3%) was significantly ($P < 0.001$) greater than in RPMI-ME (91±5%). Purity of isolated cells averaged 96±0.03% (range: 82-100%). Optimal mitogen concentrations for lymphocyte stimulation tests were similar for PHA but not ConA. Mean stimulation indices for whole blood were generally 5 to 10 times lower than those for isolated cells using comparable media (Fig. 2). Optimal concentration for egg white lysozyme was 1 μg/well for all except cells in MRPMI (data not shown). When compared to whole blood for a given media/mitogen, area under the curve was significantly ($P < 0.001$) greater for isolated cells; when comparing different media for a given mitogen, whole blood or cells in RPMI-ME gave significantly higher ($P < 0.001$) AUC than in RPMI. Variability was lower when isolated cells and RPMI-ME were used (Table 1). Antigen specific cell mediated response was seen only with turtles injected with FCA and did not correspond to antibody response (Fig. 3).

After primary immunization, antibody levels were similar for FCA or ISA-70, however, boosting with FIA gave higher titers. Only one animal had detectable antibodies after primary immunization with Gerbu, however, both responded after the booster (Fig. 3). Antibody titers were seldom higher than 1:25 after either primary immunization or after boosting (Table 2).

DISCUSSION

Both the control animal and one injected with EWL and ISA-70 developed similar clinical signs suggesting that factors other than the adjuvant or antigens were responsible for their debilitation. We suspect that repeated blood sampling may have contributed, however, other animals that underwent similar sampling regimes were unremarkable. The control animal was recaptured 5 months after release in Kaneohe Bay, had gained 4 kg, and appeared healthy with fully functional limbs. The lack of significant adjuvant associated reaction at the injection site along with weight gain of all but the control animal suggests that any of the three adjuvants could be used safely in green turtles.

Purity of isolated cells in our study was within the range found by McKinney and Bentley (1985). Viability of isolated turtle cells using our methods was comparable to that seen by others (Timms, 1979; Cuchens and Clems, 1979a; Confer, 1980). Like snakes (Farag and El Ridi, 1986) in vitro proliferation of green turtle cells in media supplemented with ME was greater than that of unsupplemented media. Stimulation indices for isolated turtle cells at peak mitogen concentration, particularly for RPMI-ME, were often 3-20 fold higher than those observed for peripheral blood lymphocytes from Florida green turtles (McKinney

and Bentley, 1985) or alligator (Cuchens and Clems, 1979b); thymocytes from the lizard *Chalcides ocellatus* (El Deeb and Saad, 1987); or splenocytes from the snake, *Psammophis sibilans* (Farag and El Ridi, 1986). We make such comparisons cautiously, however, particularly in the case of the snake and lizard since cells originating from spleens or thymus may differ from peripheral mononuclears in their in vitro response. Our optimal concentrations of conA and PHA for stimulation of green turtle lymphocytes agreed with those of McKinney and Bentley (1985); like them, we also saw reduced proliferation with conA compared to PHA.

For whole blood in RPMI, stimulation indices were similar to those observed for raptors (Redig et al., 1984) or turkeys (Nagaraja et al., 1980) but were about twice as high for whole blood in RPMI-ME. We suspect that a lower plated concentration of mononuclears was responsible for the lower stimulation indices seen in whole blood. Mean concentration of lymphocytes and monocytes in whole blood for all turtles was 15 X 10^6/ml which when diluted 1:20 and plated gave concentration of 3.5 X 10^4/ml, roughly 1/10 of what was plated for isolated cells.

Like Benedict and Pollard (1972), we showed that green turtles can mount an immune response after a single injection of antigen. Time to first detectable antibody response in our study (~6 weeks) was longer than that seen by Benedict and Pollard (1972) (4 weeks) or Herbst and Klein (1995) in Florida green turtles (3-5 weeks). Benedict and Pollard (1972) were able to detect antibodies through 74 weeks after a single inoculation of antigen. Herbst and Klein detected antibodies through 41 weeks after inoculation, however, their turtles were immunized multiple times. Temporal response of antibody of turtles in our study was the same regardless of adjuvant.

Titers in this study were low. Herbst and Klein (1995) using ELISA, seldom detected titers higher than 1:200 in turtles repeatedly immunized with DNP-BSA. In contrast, Benedict and Pollard (1972) using radioelectrocomplexing detected titers greater than 1:500 in some turtles immunized with DNP-BSA, suggesting that ELISA may be less sensitive than REC for detecting turtle antibodies. Like Benedict and Pollard (1972), we demonstrated that green turtles can mount an anamnestic response. The transitory drop in antibody levels after boosting was not encountered by Benedict and Pollard (1972) and may have been caused by massive removal of antigen by circulating antibodies.

If a primary immunization of green turtles is to be done, FCA or ISA-70 appear to produce similar antibody titers with no negative side effects. If the goal is to maximize antibody titers after a booster, FCA would be the adjuvant of choice. For a free-ranging study, we recommend either ISA-70 or FCA as either adjuvant can produce a detectable antibody response that will

last at least 4 months. We recommend doing in vitro cell assays using isolated lymphocytes in RPMI-ME. This will give the highest stimulation indices which would facilitate contrasts between groups of animals in the wild, given the inherent variability of the lymphocyte stimulation test. An added benefit of the LST using cells in RPMI-ME is that variability is lowest. While the ELISA may not be as sensitive as REC, antibody response can be detected and exposed versus nonexposed animals can be clearly differentiated even without a plate reader. It appears that an SI of >5 is suggestive of an antigen specific response in green turtles immunized with FCA.

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Table 1.--Area under the curve for MRPMI and MRPMI-ME, cells or whole blood and ConA or PHA for 7 turtles.

	n	Mean	SE	Min	Max	CV
ConA						
RPMI Cells	90	144	26	2	1155	168
RPMI Blood	84	28	3	3	200	110
RPMI-ME Cell	81	419	54	3	2860	116
RPMI-ME Blood	84	92	5	15	239	53
PHA						
RPMI cells	90	249	37	1	1991	142
RPMI blood	94	31	3	2	201	101
RPMI-ME cell	81	445	46	4	1827	92
RPMI-ME blood	87	88	5	10	237	52

Table 2.--Antibody response to EWL in serially diluted plasma ranging from 1:25-1:800. Optical density (450 nm) readings are means for two animals with increasing density indicating increasing antibody.

	DATE					
	9/15	10/28	12/12	01/30	02/19	03/09
Freunds						
1:25	0.02	0.09	0.15	0.18	0.16	0.32
1:50	0.01	0.06	0.12	0.15	0.11	0.29
1:100	0.01	0.03	0.09	0.12	0.06	0.25
1:200	0.00	0.00	0.05	0.08	0.04	0.22
1:400	0.00	0.01	0.02	0.05	0.00	0.19
1:800	0.01	0.01	0.02	0.05	0.01	0.18
ISA-70						
1:25	0.05	0.00	0.17	0.21	0.23	0.25
1:50	0.02	0.00	0.11	0.16	0.17	0.22
1:100	0.00	0.00	0.07	0.12	0.13	0.16
1:200	0.00	0.00	0.03	0.10	0.08	0.15
1:400	0.00	0.00	0.00	0.05	0.05	0.13
1:800	0.01	0.00	0.00	0.04	0.03	0.11
Gerbu						
1:25	0.03	0.03	0.10	0.09	0.04	0.22
1:50	0.01	0.01	0.06	0.06	0.01	0.18
1:100	0.01	0.01	0.05	0.05	0.01	0.16
1:200	0.01	0.00	0.04	0.03	0.00	0.13
1:400	0.01	0.00	0.02	0.01	0.00	0.10
1:800	0.01	0.00	0.01	0.00	0.02	0.10

*Note, for 3/9, n=1 for ISA-70

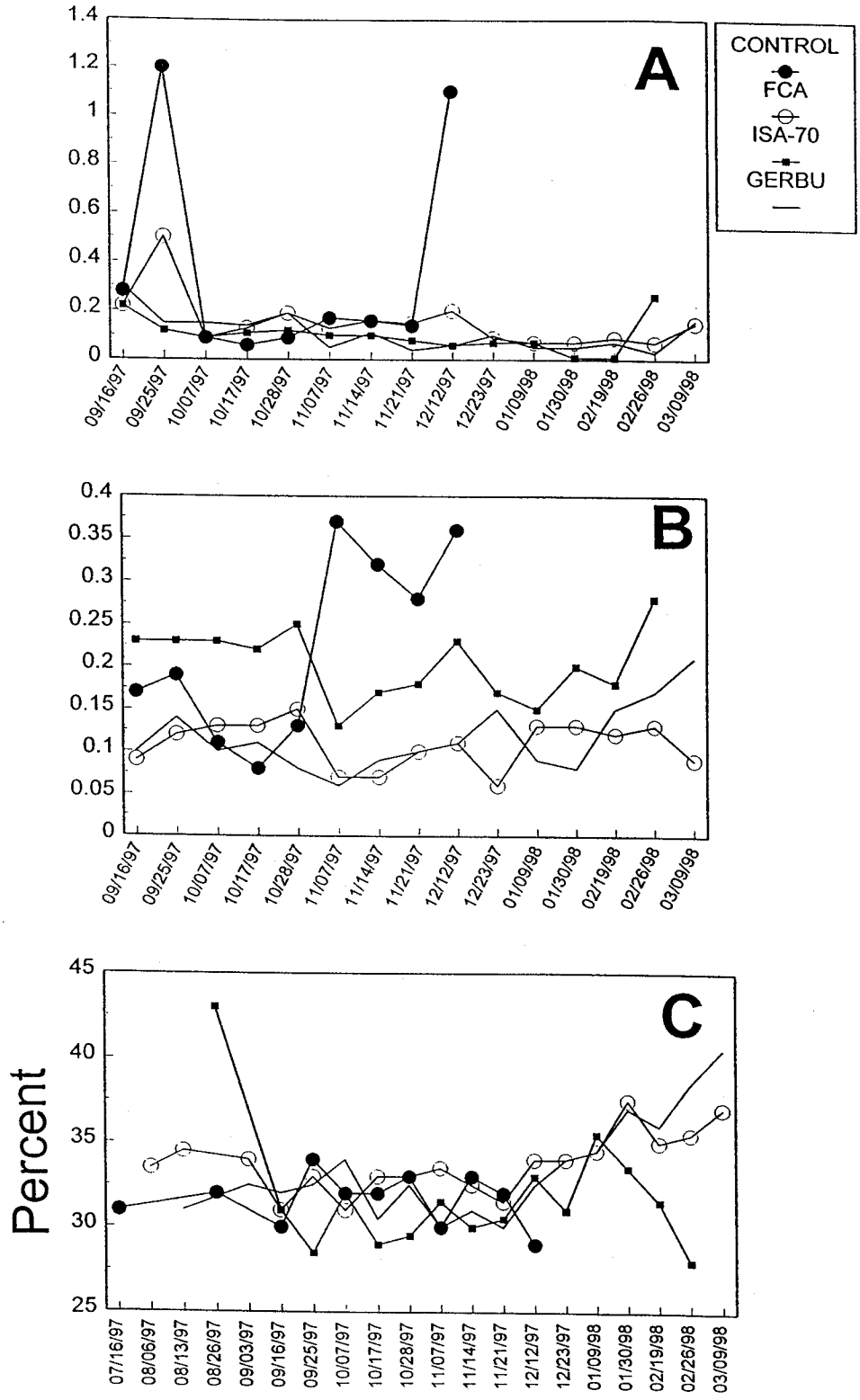

Figure 1.--Heterophil/lymphocyte ratio (A); monocyte/eosinophil ration (B) and hematocrit (C) of six turtles exposed to EWL and one control. N=7 for each point unless otherwise stated.

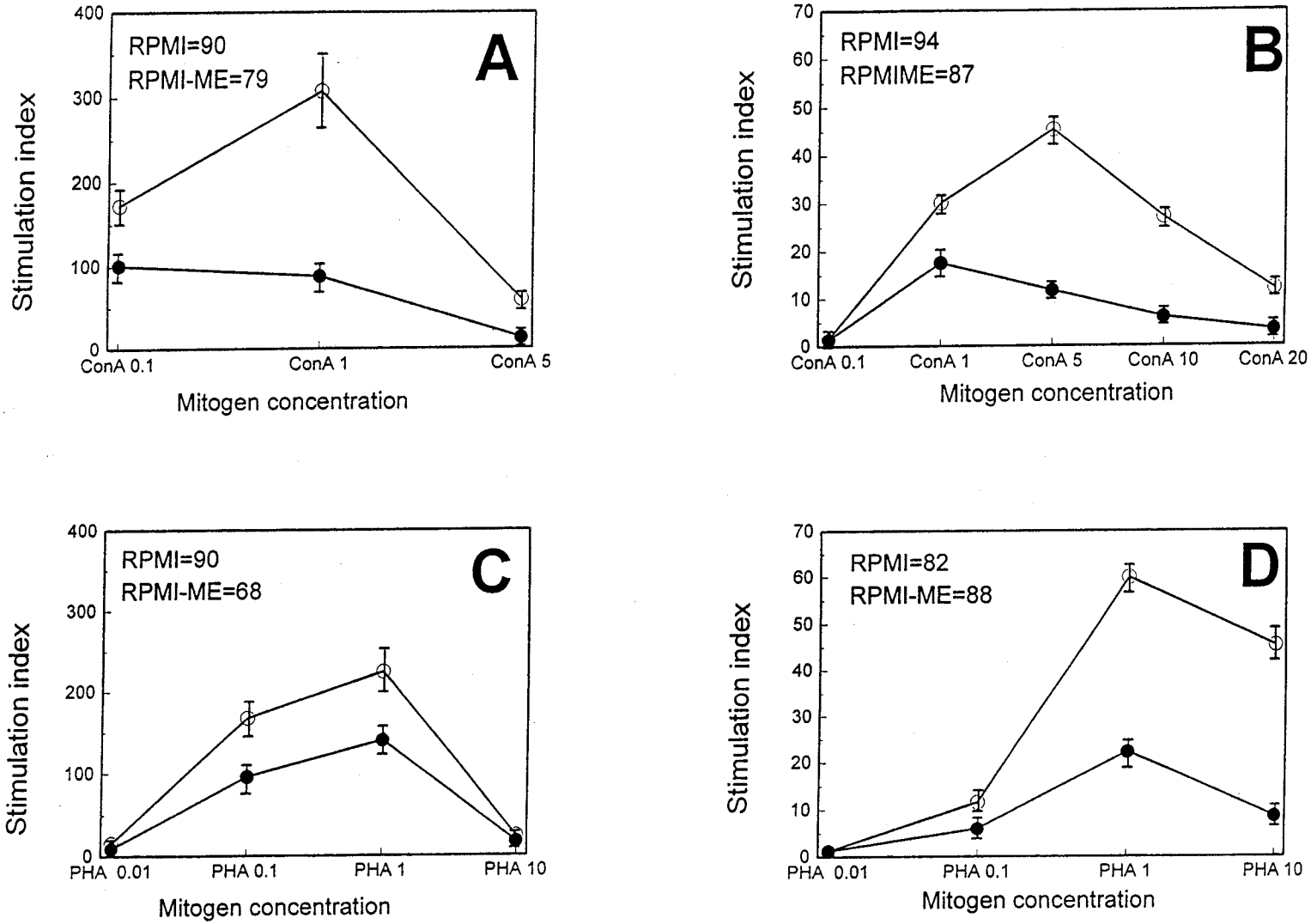

Figure 2.--Mitogenic response or isolated lymphocytes (A and C) of whole blood (B and D) conA or PHA in RPMI (dark circles) or RPMI-ME (open circles). Numbers after RPMI/RPMI-ME in each graph indicate a sample size for each point. Numbers for whole blood and PHA0.01 or ConA0.1 are 3 and 10 for RPMI-ME and RPMI respectively.

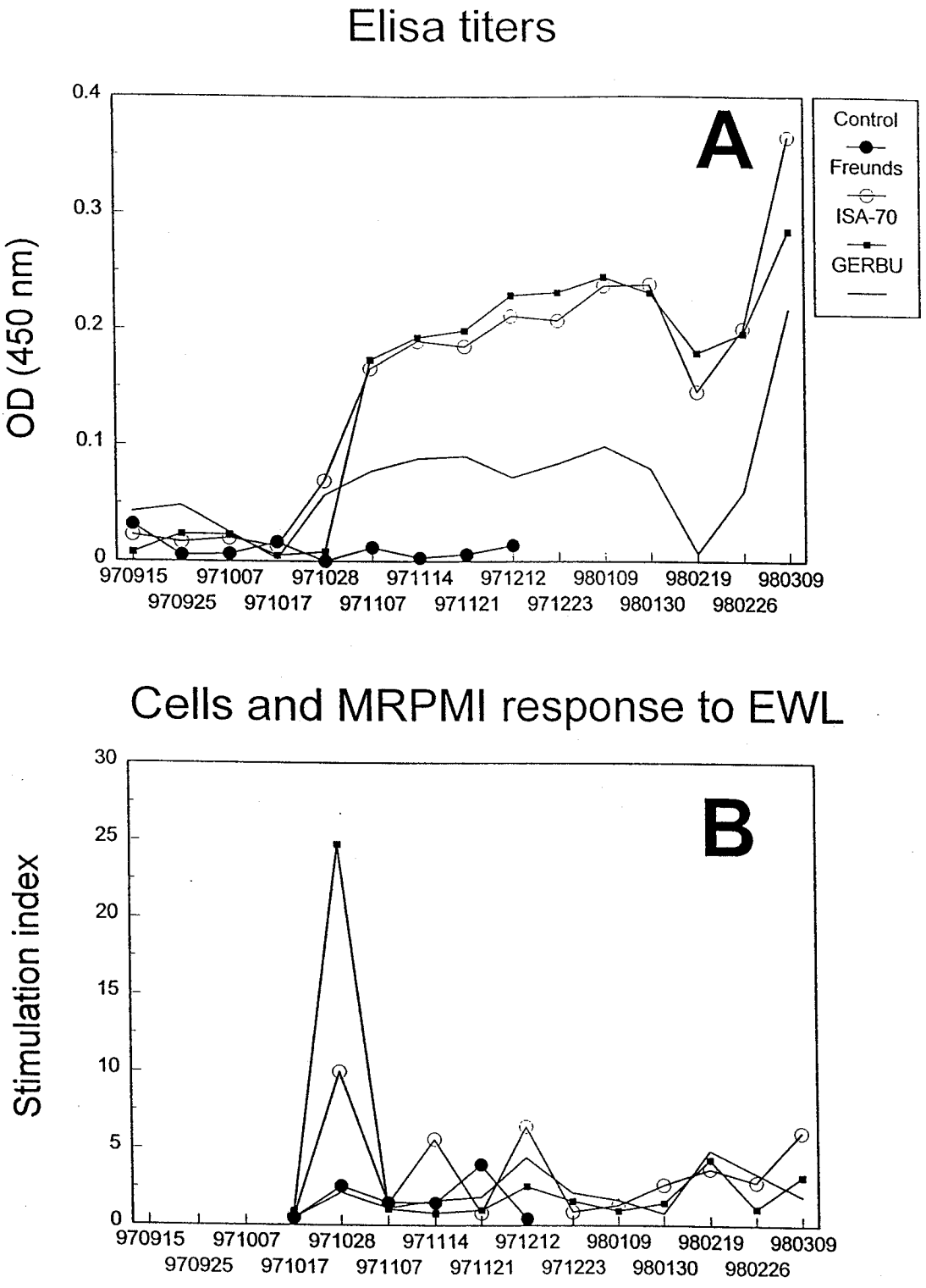

Figure 3.--Optical density (450 nm) of plasma of two pooled turtles/adjuvant diluted 1:25 for ISA-70, FCA or Gerbu (A); Stimulation indices for isolated lymphocytes in RPMI-ME in presence of EWL at 1 ug/well (B).