This is to certify that the

thesis entitled

The Squirrel Monkey (Saimiri sciureus) As A Model For
In Vivo Immunocontraceptive Testing

presented by

Donna Lynn Pierce

has been accepted towards fulfillment
of the requirements for

M.S. degree in Animal Science

W. Richard Dukelow

Major professor

Date June 5, 1986

THE SQUIRREL MONKEY (*SAIMIRI SCIUREUS*) AS A MODEL FOR IN VIVO IMMUNOCONTRACEPTIVE TESTING

By

Donna Lynn Pierce

A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Animal Science

1986

ABSTRACT

THE SQUIRREL MONKEY (SAIMIRI SCIUREUS) AS A MODEL FOR IN VIVO IMMUNOCONTRACEPTIVE TESTING

by

Donna Lynn Pierce

Fifty female squirrel monkeys (Saimiri sciureus) were each immunized with 200 ug of the 55 K macromolecule (ZP-3) from porcine zona pellucida. The effect of the ZP-3 antibodies on ovarian function and fertility of the immunized monkeys was monitored over a 19 month period. High antibody titers were found (75% binding levels as determined by radioimmunoassay) at approximately four months post-immunization and remained high (68% binding level) for the duration of the study. Initial disturbances in normal ovarian steroid secretion and function were found through hormone analyses and laparoscopic observations of ovaries. An interference in follicular growth was found through histological studies at 6-7 months post-immunization. No pregnancies occurred in the immunized monkeys during the first breeding season. Hormonal and laparoscopic data indicated a recovery in ovarian function at 10-15 months after the initial injection despite the presence of high titer levels to ZP-3. These findings demonstrate that purified porcine zona macromolecules have potential as an immunocontraceptive vaccine and should continue to be investigated.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following individuals for their personal and professional support over the last several years. My advisor, Dr. W.R. Dukelow, who made this all possible by providing me with a great opportunity and giving guidance and support when it was needed the most. I would like to thank Mrs. Lavonda Cleeves for her friendship (and the wild parties at her cottage!) since she has made my stay at the Endocrine Research Center even more enjoyable. My graduate committee, Dr. A.G. Sacco, Dr. D.E. Ullrey and Dr. J.A. King who provided their interest, help and encouragement which enabled this task to be completed. I would also like to thank all of my friends who helped me along the way especially Lori McCallum who is one of the nicest friends a person could ever have. I am eternally grateful to my entire family for their support, encouragement and LOVE in helping me fulfill a dream. Without them, this would not have been possible.

If we want to become all that is in us to become, we have to use everything we've got-our feelings, our intuition, our intelligence, and our will power-our whole self. If we do, the payoff is enormous.

*

We can all help ourselves to change, to grow, to become the person it is in us to be. We can learn to be our own best friend. If we do, we have a friend for life. We can buoy ourselves up, give ourselves comfort and sustenance the times when there is no one else. We are our best source of encouragement and good advice.

Mildred Newman and Bernard Berkowitz

*

The two greatest stimulants in the world are youth and debt.

Disraeli

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Introduction

The zona pellucida has been investigated for many years by a number of laboratories as a possible target antigen for immunocontraceptive purposes. The earliest experiments involved passive immunization of hamsters (Ownby and Shivers, 1972), mice (Jilek and Pavlok, 1975) and rats (Tsunoda and Chang, 1976) using antisera developed against ovarian antigens. Such antisera contained antibodies directed against the zona and were demonstrated to be effective in preventing fertilization.

Experiments describing the immunological similarity of antigens in porcine and human zonae (Sacco, 1977) indicated the potential of the porcine zona pellucida as a possible target antigen in the development of a contraceptive vaccine with human application. The cross-reactivity of antigens in the porcine zona pellucida with that of many other species, coupled with the availability of large numbers of porcine ovaries from slaughterhouses , has made this species extremely useful for immuncontraceptive studies.

Recently, studies utilizing porcine zona antigens in the active immunization of female rabbits (Wood et al., 1981; Dietl et al., 1982; Skinner et al., 1985) and dogs (Shivers et al., 1981; Mahi-Brown et al., 1982, 1985) have

been performed.

Preliminary work has been done in nonhuman primates to assess the anti-fertility effects of active immunization against zonae extracts (Shivers et al., 1978; Gwatkin et al.,1977).

The squirrel monkey (Saimiri sciureus) is widely used in research today due to its small size, ease of handling and simple housing requirements. In addition, a strong background of basic reproductive procedures exists for this species. Zonae from rodent species cross-react poorly with antisera produced against porcine zonae pellucidae (Sacco et al., 1981a,b). Rabbits would appear to be the next best alternative due to the cross-reactivity of rabbit and porcine zonae but antibodies against pig zonae do not inhibit homologous sperm-zona interaction in the rabbit system (Sacco et al., 1981a). Preliminary data from the squirrel monkey demonstrated adequate species cross-reactivity as well as in vitro contraceptive potential of antibodies to a purified porcine zona antigen (PPZA) (Sacco et al., 1983).

The data from these studies has emphasized the contraceptive effectiveness of antibodies formed against the zona pellucida. However, some of these investigations have noted disruptions in the menstrual cycle and reproductive hormonal profiles. Histological examination of ovaries from actively immunized rabbits with porcine zona

have indicated that the presence of zona antibodies were interfering with normal ovarian folliculogenesis (Skinner et al., 1985). Therefore, these recent studies have suggested that infertility observed following active immunization of females with zona antigens may be a consequence of zona antibodies inhibiting normal ovarian function as well as by preventing sperm-egg interaction. It must be noted that the studies described above were of short term duration and utilized crude or total zona prepartions as immunogens.

This investigation is unique in that is the first on-going, long term primate study using a large number of immunized monkeys (50) that were administered a highly purified 55K zona pellucida macromolecule as an immunogen.

The objectives of the present immunocontraceptive studies were:

1) To determine the longevity of the immune response.
2) To examine the anti-fertility effects of active heteroimmunization with ZP-3 in squirrel monkeys.
3) To assess the potential detrimental effects of such immunization on normal reproductive characteristics.

LITERATURE REVIEW

It is known that antiserum raised against zonae pellucidae from one species will cross react differently with zona of other species. This suggests that there are common antigens shared by the zonae of various animals and for immunocontraceptive purposes involving active immunization, the degree of cross reactivity and similarity of zona antigens is important. Antisera produced against porcine zonae antigens react poorly with rodent zonae (Sacco et al., 1981) so mice, hamsters and rats cannot be used to evaluate active immunization using porcine zona antigens (Sacco et al., 1981a,b). The level of cross reactivity of antibodies to rabbit zonae and pig zonae seems sufficient (Sacco et al., 1981b) but antibodies to porcine zonae do not inhibit homologous sperm-zona interaction. Results from these types of data suggest that a nonhuman primate might be the next likely test system to evaluate the contraceptive efficacy of active immunization with porcine zona antigens. The squirrel monkey was the species chosen based on preliminary results obtained by Sacco et al. (1983).

Studies have shown the porcine zona pellucida is biochemically and antigenically complex. When resolved by 2-dimensional polyacrylamide gel electrophoresis (2-D

PAGE), it is comprised of four major families of charge heterogeneous glycoproteins with presumptive molecular weights of 82K(ZP1), 61K(ZP2), 55K(ZP3) and 21K(ZP4) (Subramanian et al., 1981; Dunbar et al., 1981). In consideration of a contraceptive vaccine development using the porcine zona, the approach was to employ the smallest component of the porcine zona which was capable of producing antibodies exhibiting contraceptive properties. The 55K(ZP3) glycoprotein was selected for the following reasons: a) preliminary work expressed it was the best candidate for successful purification using chromatographic procedures, b) it is the most predominant glycoprotein accounting for 60% of the porcine zona, c) it appears to be the most immunogenic glycoprotein (Sacco et al., 1983) and, d) it probably is biologically active and exhibits sperm receptor activity (Sacco et al., 1984).

The first attempts to purify the 55K(ZP3) constituent resulted in a preparation that was free of the the other major zona glycoproteins but was not totally homogeneous (Yurewicz et al., 1983). This preparation called purified porcine zona antigen (PPZA) containing primarily the 55K component was further purified chromatographically to electrophoretic homogeneity and was referred to as ZP-3 (Yurewicz et al., 1984).

BASIC REPRODUCTIVE CHARACTERISTICS OF THE SQUIRREL MONKEY

Cycle Length

The cycle length of the squirrel monkey varies from 7-12 days with an average of 9 days. Earlier studies (Denniston, 1964; Srivastava et al., 1970; Richter, 1976) described cycle lengths as high as 25 days using cytological techniques to examine the vaginal cell cornification cycle. Hutchinson (1978) reported that cycle lengths might vary depending on the type of housing and whether the monkeys were housed individually or in groups. Rosenblum et al. (1967) cited a cycle length of 7-8 days using vaginal cell cornification cycles and presence of sperm in vaginal smears of females housed with males. This study was confirmed by Gould et al. (1973) and Travis and Holmes (1974) who reported a peak in the karypyknotic index at intervals of 10.9 days.

There have been many studies utilizing activity cycles and behavioral characteristics to confirm the 8-10 day period of the cycle (Richter, 1976; Latta et al., 1967; Jarosz et al., 1977; Wilson, 1977).

Early work examined urinary steroids as a measure of cycle length. Using animals showing a 10.9 day cycle length, Travis and Holmes (1974) divided the cycle into follicular and luteal phases based on a karyopyknotic index that peaked on day 5. They found

that pregnanediol excretion increased sharply on the sixth day of the cycle and the total amount excreted throughout the luteal phase was greater than that found for the follicular phase. With the development of radioimmunoasssay (RIA) techniques, small blood samples could be examined for circulatory steroid levels in the squirrel monkey throughout the cycle. It was discovered that these levels are strikingly higher than are found in old world monkeys and humans. Furthermore, this characteristic has been observed in other new world species. Estradiol levels peak at 503$\pm$57.5, and 81$\pm$8.2 pg/ml was found to be the minimum value. Serum progestins reached maximum concentrations of 399 $\pm$27.7 ng/ml, 3-4 days following the estradiol peak (Wolf et al., 1977; Wilson, 1977). In confirmation, Ghosh et al. (1982) determined the cycle length to be 8-9 days using hormonal measurements of serum estradiol 17-B, progesterone and luteinizing hormone(LH). Estradiol and LH increased markedly on day 4 of the cycle while progesterone peaked on day 6 indicating ovulation on day 5.

Recently, work was carried out to detect any seasonal changes in estradiol and progesterone in the Bolivian squirrel monkey (Diamond et al., 1984). The results indicated a 6-12 day cycle based on the time interval between consecutive estradiol peaks. It was noted that serum estradiol and progesterone concentrations were lower

during the nonbreeding season (April to November) with an absence of a preovulatory surge of estradiol.

Captive Breeding and Pregnancy Diagnosis

When squirrel monkeys have become acccustomed to their captive habitat, a conception rate of 50-60% can be expected (Kaplan, 1977; Dukelow, 1982). However, a high rate of stillbirths or abortions (16.6%) and neonatal death (34.3%) is observed which can possibly be attributed to a high maternal body weight to infant birth weight ratio (7:1). Many breeding systems house males and females in groups with a maximum female to male ratio of 10:1. When pregnancy occurs, the gestation period lasts from 135 to 175 days, with a mean length of 150 days (Jarosz et al., 1977; Kerber et al., 1977).

Various techniques have been used to detect pregnancy in the squirrel monkey (Table 1). Increased uterine size and chorionic gonadotropin occur in the first trimester and would be useful in detecting pregnancy as soon as possible. A pregnancy test kit developed by the National Institutes of Health can detect pregnancy in squirrel monkeys between 40 and 60 days of pregnancy with a 10% inherent risk of false negatives (Hodgen et al., 1978).

TABLE 1

Pregnancy detection in the squirrel monkey

Characteristic	Days After Conception	Reference
1. Uterine size increase(laparotomy)	20-25	Goss et al., 1968
2. Chorionic gonadotropin		
a. plasma	20-105	Nathan et al., 1966
b. urine	40-60	Hodgen et al., 1978
3. Uterine size increase(palpation)	42-56	Rosenblum, 1968
4. Diabetes insipidus	60	Clewe, 1969
	60-135	Travis and Holmes, 1974
5. Maternal weight gain	60-95	Hopf, 1967
	67-147	Travis and Holmes, 1974
6. Visual abdominal enlargement	74-102	Hopf, 1967
7. Fetal skeleton by x-ray	81	Nathan et al., 1966

Seasonality

Reproductive seasonality has been well documented in the squirrel monkey and is expressed by behavioral changes, dimorphic appearance of the adults ("fatted male syndrome") and hormonal variations. In their natural environment, squirrel monkeys mate from July to September with births occurring from January to March. The mating season shifts from January to March when squirrel monkeys are moved to the northern hemisphere with births occurring from June to September (Dumond, 1968). This marked change in the mating and birth seasons was correlated to the amount of rainfall, with the dry season triggering the onset of mating season. The humidity effect was corroborated by Baldwin and Baldwin (1971) in field studies.

In the laboratory, the seasonal effect of lowered ovulation is apparent in the female response to ovulation induction regimens (Harrison and Dukelow, 1973). Ovulation can be induced in the anovulatory months by increasing the dose of follicle stimulating hormone (FSH) before human chorionic gonadotropin (HCG) administration (Kuehl and Dukelow, 1975).

Seasonality has also been observed in the male and is termed the "fatted male syndrome" (Dumond and Hutchinson, 1967). It is associated with an increase in body size, fluffiness of pelage, maximum spermatogenesis and increased mating activity.

In establishing a breeding colony, adaptation to captivity must be taken into consideration. Some animals will adapt after nine months of captivity (Harrison and Dukelow, 1973) while others require as long as three breeding seasons to adjust (Lorenz et al., 1973).

Ovulation Induction

A wide variety of ovulation induction protocols have been effective in the squirrel monkey. Bennett (1967) used a regimen of five days pregnant mares serum gonadotropin (PMSG) followed by four days of a combination of PMSG and human chorionic gonadotropin (HCG) to mimic the luteal phase of the cycle. An average of 5.3 ovulations per ovary was observed with this regimen. Later, studies were performed to induce single or double ovulations in the squirrel monkey in an attempt to accurately time ovulation (Dukelow, 1970). The optimal treatment consisted of four days of 1 mg FSH followed by a single injection of 250-500 IU HCG on the fourth day.

Seasonality is observed in ovulation induction regimens as well. A seasonal responsiveness was reported with the FSH-HCG regimen (Harrison and Dukelow, 1973) but could be overcome by either increasing the dose of FSH or by extending the time of FSH administration during the anovulatory months of July, August and September (Kuehl and Dukelow, 1975a). The minimum effective dose of HCG was found to be between 100-250 IU (Dukelow, 1979), and increasing the dose had no effect during the anovulatory months (Kuehl and Dukelow, 1975a).

The question of whether oocytes produced from ovulation induction regimens are capable of fertilization

was also examined. It was shown that these oocytes were able to be fertilized both *in vivo* (Jarosz et al., 1977) and *in vitro* (Kuehl and Dukelow, 1975b; 1979; Chan et al., 1982).

Immunological Aspects of the Zona Pellucida- Consideration as a Target Antigen

The zona pellucida is a non-cellular, gelatinous layer surrounding all mammalian oocytes and preimplantation embryos. During oogenesis and unilaminar follicular growth , the zona pellucida is formed in the area between the developing oocyte and the surrounding granulosa cells. A controversy remains over the precise site of origin of zona pellucida material due to the biochemical complexity of the layer. Some researchers consider the granulosa cells surrounding the oocyte to be the sole site of zona synthesis (Oakberg and Tyrell, 1975; Haddad and Nagai, 1977) while others ascribe its origin to the oocyte (Chiquione, 1960; Flechon et al., 1984). A third viewpoint states that the granulosa cells may contribute protein and polysaccharide components while the oocyte contributes only polysaccharides (Hadek, 1965).

The zona pellucida has several roles in fertilization and early embryonic development. Its functions in fertilization include sperm recognition of the oocyte (Yanagimachi, 1972; Hanada and Chang, 1972) and prevention of polyspermy (Braden et al., 1954; Barros and Yanagimachi, 1972). The zona pellucida provides mechanical protection of the embryo during its journey through the oviduct (Gwatkin, 1963), osmotically regulates the environment (Piko, 1969) and maintains normal cleavage patterns (Mintz, 1962).

The structure of the zona pellucida has been examined by light microscopy and transmission and scanning electron microscopy. The zona appears as a translucent layer with a granular structure when viewed by light microscopy with thickness varying from 3 to 22 microns (Piko, 1969) and a diameter of approximately 60 to 170 microns (Wright et al., 1977). When viewed by transmission electron microscopy, a substructure of filaments permeated by numerous granulosa cell processes and egg microvilli are visible (Piko,1969). Scanning electron microscopy of zonae indicates an extensive fibrous network interspersed with numerous pores of various sizes with the largest pores at the outer surface (Dudkiewicz and Williams, 1977).

Biochemical studies on the zona pellucida have demonstrated neutral or weakly acidic glycoproteins to be the major constituent with amounts of sialic aid residues (Soupart and Noyes, 1964) and phosphate and sulfate esters (Dunbar et al., 1980). The structural integrity of the zona pellucida is maintained by both covalent and noncovalent bonds (Inoue and Wolf, 1974; Dunbar et al., 1980) and disruption of these bonds by heat or altering pH or ionic strength will result in a solubilized zona preparation. This zona preparation is maintained by covalent bonds which can be disrupted by reducing agents to prepare solubilized preparations of individual zona macromolecules.

The zona pellucida was considered as a target antigen

for use as a contraceptive based on the above information. It is also contains antigens specific to reproductive tissue and has an optimum location within the reproductive system to interrupt fertiliity since it represents a structure through which sperm must pass in order to reach and fertilize the egg. The zona pellucida is complex structurally and biochemically and is both antigenic and highly immunogenic. These characteristics would most likely lead to prevention of fertilization rather than affecting a developmental stage after fertilization. Finally, large quantities of isolated zonae material can be collected using new screening procedures (Dunbar et al., 1980; Oikawa, 1978).

Detection of Antibodies to Zona Pellucida

There are various methods used to measure and monitor antibody activity against ZP antigens. These methods include:

1) Zona precipitation reaction (ZPR)
2) Indirect immunofluorescence (II)
3) Prevention of zona digestion by proteolytic enzymes
4) Passive hemagglutination assay
5) Immunodiffusion and Immunoelectrophoresis
6) Radioimmunoassay (RIA)
7) Prevention of sperm attachment and penetration of the zona pellucida

Zonae pellucidae treated with antibodies to zonae pellucidae possess a precipitation layer on the outer surface of the zona which alters its light-scattering properties. When viewed through a bright-field microscope, a dark layer is observed on the surface of the zona. Antibody-treated zonae appear to be brighter than controls when viewed using dark-field microscopy (Sacco, 1981). It is believed that the binding of antibodies to the outer portion of the zona account for this change in light-scattering properties (Ownby and Shivers, 1972; Garvagno et al., 1974). Using light microscopy, Garavagno (1974) demonstrated that the precipitate was located only on the outside of the hamster zona. Flechon and Gwatkin (1980) used transmission electron microscopy to demonstrate that antibodies to bovine zonae pellucidae are present on both the internal and external surfaces of the zona pellucida. When the precipitation layer of antibody-treated zonae was

observed with an electron microscope, aggregations of fine to medium-coarse granules were seen to adhere to the fibrous zona network everywhere but around the pores (Dudkiewicz et al., 1976). The titer of the zona antisera is measured by the formation of the precipitation layer and is expressed as the reciprocal of the highest dilution of antiserum which produces the precipitation layer as compared to preimmune serum treated control zona (Sacco and Shivers, 1978).

The indirect immunofluorescence technique is also used to detect zona antibodies at the surface of the zona pellucida. Antibody-treated zonae fluoresce after treatment with a second fluorescein-labelled antibody that is directed against the zona antibody. The reciprocal of the highest antiserum dilution which causes a fluorescence on the zona as compared to those treated with a preimmune serum is termed the immunofluorescent titer (Sacco et al., 1983). This procedure is more sensitive than the ZPR and is able to detect antibodies to zona at titers insufficient to produce a precipitate.

There are reports in the literature (Ownby and Shivers, 1972; Sacco and Shivers, 1973) that the presence of zona antibody on the zona surface makes the zona resistant to digestion by proteases. Zona-bound antibodies can be detected by exposing antibody and control-treated zona-coated oocytes to proteolytic solutions and comparing

zona lysis times. Zona dissolution times are longer for antibody-treated zona as compared to untreated or control-treated zona (Sacco, 1981).

A passive hemagglutination assay has been used to detect ovarian antigens (Tsunoda and Chang, 1976) utilizing the method described by Herbert (1977). They reported antibodies to ovary homogenate were detectable in serum dilutions up to 2^{12}.

Early studies on immunoprecipitation (Ownby and Shivers, 1972; Sacco and Shivers, 1973) utilized antibodies to whole ovarian tissue and due to the heterogenity of the ovary, the exact ovarian antigens that were studied is difficult to determine. Recently, more precise methods have been developed that use antisera containing antibodies from isolated porcine and rabbit zonae (Dunbar et al., 1980; Dunbar and Raynor, 1980) and bovine zonae (Gwatkin et al., 1980). Immunoelectrophoresis techniques have been used to show a specific antigen in ovarian homogenates recognized by goat antiserum to isolated bovine zonae (Tsunoda et al., 1980). Using one-dimensional (Dunbar and Raynor, 1980) and two-dimensional (Woodard and Dunbar, 1981) gel electrophoresis, antibodies have been isolated against purified zona proteins. Immunoelectrophoretic techniques have demonstrated that there are multiple zona-specific antigens associated with porcine and rabbit zonae pellucidae and have allowed

characterization of these specific proteins.

The most sensitive technique for detecting antibodies to zona antigens is radioimmunoassay (RIA) (Palm et al., 1979; Gerrity et al., 1981; Subramanian et al., 1981). The presence of zona antigen can be examined using RIA either as a competitive inhibition assay or titration of zona antiserum of iodinated (^{125}I) solubilized zonae.

Sperm can be inhibited from attaching to the zona pellucida by exposing zona-coated eggs *in vitro* to anti-zona serum (Shivers et al., 1972). The degree of inhibition is directly related to the concentration of antibody added.

PRIMATE STUDIES ON IMMUNOCONTRACEPTION RELATIVE TO THE ZONA PELLUCIDA

Human Zona Pellucida Studies

Studies have been done to examine the antigenicity of the human zona pellucida for its potential use in regulating fertility (Sacco, 1977a). He reported that the human ovary contains at least one antigen not found in 21 other human tissues and fluids. Antiserum reacting with this ovarian antigen possessed antibody activity against the human zona pellucida as demonstrated by the ZPR. Cross-reactivity of human and porcine zonae pellucidae has been demonstrated using agar gel diffusion and immunofluorescent staining (Sacco, 1977,b; Shivers and Dunbar, 1977). Sacco et al. (1981) found antiserum to a purified pig zona antigen (PPZA) formed a precipitation layer on the surface of porcine and human oocytes and inhibited human sperm adherence *in vitro*. These results indicate that the porcine zona pellucida is a prime candidate as a target antigen for the development of a human contraceptive vaccine.

Investigations on the role of autoantibodies to zona pellucida was examined as a possible cause of idiopathic infertility. Using immunofluorescence techniques, anti-zona pellucida antibodies are found in the sera of infertile women (Shivers and Dunbar, 1977; Mori et al., 1978). Shivers and Dunbar (1977) speculated that zonae are continually exposed to autoantibodies through egg atresia

in the ovary and absorption of ovulated oocytes in the reproductive tract. Several investigators (Tsunoda and Chang, 1979; Nishimoto et al., 1980) have found autoantibodies to zona antigens to be present in the sera of aging women and animals. Nishimoto (1980) observed decreased zona binding activity in the sera of aging women after pre-absorption with red blood cells. However, when immunoflourescence techniques are used, zona binding activity has been observed in the sera of infertile women (Shivers and Dunbar, 1977) as well as in the sera of fertile males and females (Sacco and Moghissi, 1979; Dakhno et al., 1980). Sacco and Moghissi (1979) reported that several of the infertile females studied, whose serum possessed anti-zona activity, became pregnant. These successful pregnancies could be due to an insufficient titer level in the women to prevent fertilization. This was found to be the case in animal studies (Tsunoda et al., 1979; Sacco, 1979) where oocytes with bound zona antibodies were fertilized, indicating a minimal amount of zona antibody needs to be associated with the zona before fertilization is inhibited.

Nonhuman Primate Studies

Recently, work has been done with marmosets (Shivers et al., 1978), squirrel monkeys (Sacco et al., 1983) and cynomologus monkeys (Gulyas et al., 1983a,b) involving immunization with zona antigens. Initial studies (Shivers et al., 1978) demonstrated the cross-reactivity between human, chimpanzee, marmoset and porcine zona antigens as determined by the zona precipitation reaction and immunofluoresence methods. Following passive immunization in marmosets, sperm attachment to eggs was prevented *in vitro* and antibodies were located on the zonae of ovarian oocytes.

Fox et al. (1981) examined the antibody response using an enzyme-linked immunosorbent assay (ELISA) in marmosets innoculated with porcine zonae. Over a nine-week immunization period, the antibody response profile obtained by ELISA was similar to that found with indirect immunoflouresence on intact zona. The ELISA method was found to be useful when large numbers of samples were screened and was advantageous over previously used methods.

Cross-reactivity of human and squirrel monkey oocytes to a purified porcine antigen (PPZA) was shown by Sacco et al. (1983). Immunization of squirrel monkeys with PPZA resulted in production and maintenance of high antibody titers for at least one year. Antibody binding to monkey zonae was detected by the presence of a precipitation layer

on the surface of the zona. Pretreatment of human and squirrel monkey oocytes with anti-PPZA sera resulted in total inhibition of homologous sperm attachment *in vitro*. The *in vivo* effects of PPZA antibodies observed on squirrel monkey zonae were *in situ* binding of antibodies on the zonae forming a precipitate, and significantly fewer oocytes were collected via laparoscopy from immunized monkeys as compared to the controls.

Gulyas et al. (1983a) immunized cynomologus monkeys (*Macaca fascicularis*) with heat-solubilized pig zonae and examined the anti-fertility effects of the zona antibodies. A rapid solid-phase radioimmunoassay (Gulyas et al., 1983b) was used to monitor serum antibody titers which reached maximum levels 6 to 10 weeks after the initial immunization. Six of the twelve monkeys became pregnant at the time of maximum antiserum titers, although the remaining six that did not conceive had lower antibody titers. The hormonal status was altered in five of the six monkeys with the menses interrupted periodically and midcycle estradiol peak was absent for several cycles. The menses and midcycle estradiol peak returned to normal in five of the monkeys 3 to 5 months after the last booster injection. Histological studies showed atresia of small follicles and accumulation of luteal tissue at the end of the 18 month study.

MATERIALS AND METHODS

Animals

Adult squirrel monkeys (*Saimiri sciureus*) of Bolivian and Guyanese origin (South American Primates, Miami, Florida) were housed indoors on a 12:12 hour light:dark cycle. The temperature was maintained at 21 ± 3 °C and relative humidity was not controlled. During the summer months (June to October), the animals were maintained in large colony cages outdoors (Jarosz and Dukelow, 1976). The animals were fed a commercial, high protein monkey feed # 5047 (Ralston-Purina Co., St. Louis, Missouri) supplemented with apple slices and fresh water *ad libitum*.

A total of 100 sexually mature female squirrel monkeys were randomly divided into three treatment groups. Fifty monkeys received the ZP-3 antigen plus Freund's adjuvant, 25 females received the adjuvant only, and the remaining 25 were used as untreated controls.

Ovulation Induction Regimen

Mature female squirrel monkeys received an ovulation induction regimen consisting of four daily i.m. injections of follicle stimulating hormone (1 mg, F.S.H.-P, Burns-Biotec Laboratories Inc., Omaha, Nebraska) and a single i.m. injection of HCG (250 IU, A.P.L.® Ayerst Laboratories, Inc. New York, New York) on the fourth day (Dukelow, 1970; 1979). During the anovulatory months (July to September), five daily FSH injections, rather than four, were given

(Kuehl and Dukelow, 1975) followed by HCG.

Laparoscopic Recovery of Oocytes

The use of laparoscopy in reproductive studies has been extensively reviewed by Harrison and Wildt (1980). The laparoscopic technique for oocyte recovery in squirrel monkeys has been described (Dukelow et al., 1971; Dukelow and Ariga, 1976). The squirrel monkey was anesthetized with sodium pentobarbital (27 mg/kg body weight per adult female, i.m.) 15 to 16 hours after the HCG injection. A small midline incision was made with a scalpel and the trocar-cannula inserted. The trocar was then removed and the laparoscope (4 mm diameter, Karl Storz Co., Tuttlingen, West Germany) inserted. To improve viewing, the abdominal cavity was insufflated with carbon dioxide passed through the cannula. A 25 gauge needle and 1 ml tuberculin syringe were used to move the fimbria aside to expose the ovaries. The ovarian follicles were counted according to size (large >3 mm; medium 3mm to 1 mm; small <1 mm) and aspirations made using 25 gauge 5/8 inch needle. The oocytes were aspirated into 0.05 ml of TC-199 culture medium (with 25 mM Hepes buffer, Earle's salts and L-glutamine, Gibco Laboratories, Grand Island, New York) supplemented with 20% heat inactivated GG-free bovine serum albumin (Gibco Laboratories), 1 mM pyruvate (Sigma Chemical Co., St. Louis, Missouri), 100 mg Gentamicin sulfate (M.A. Bioproducts, Walkersville,Maryland) per ml and 1 unit

heparin per ml. The oocytes were placed into sterile 8-chambered tissue culture slides (Lab-Tek Products, Napierville, Illinois) and incubated at 37 C in a moist atmosphere of 5% CO_2 in air. The cultures were observed through an inverted microscope and the numbers of oocytes as well as the stage of maturation recorded for each monkey. The laparoscopy procedure was performed on all female monkeys before and after receiving either adjuvant or ZP-3 treatment.

Preparation and Administration of Vaccine

The first purified porcine zona antigen macromolecule (PPZA) consisting primarily of the 55 K component used to immunize squirrel monkeys (Sacco et al., 1983) was further purified chromatographically (Yurewicz et al., 1984) and referred to as ZP-3. Test tubes were prepared containing 350 ug of ZP-3 antigen in 5 ml of 0.1 M phosphate-buffered saline (PBS) and were frozen until needed. Each test tube contained enough antigen to inoculate 10 animals. The antigen was prepared for injection by thawing the test tube contents, pouring into a 20 ml Pyrex glass beaker and removing any remaining antigen from the test tube with a glass pipet. An equal volume (5 ml) of Freund's adjuvant (Sigma Chemical Co., St. Louis, Missouri) was added to the antigen in the beaker and emulsified using a 10 ml syringe and 18 gauge needle with a small piece of tygon tubing attached to the tip. The female squirrel

monkey was prepared for innoculation with a sedating dose of Vetalar® (10 mg/600 gm monkey, Parke-Davis, Morris Plains, New Jersey) and the hair on their backs was clipped from the shoulders to the mid-thoracic region. Each animal was injected intradermally with 1 ml of emulsion containing 35 ug ZP-3 antigen in multiple injection sites on the back (20-25 injection sites per one ml of emulsion). The injection regimen followed was three inoculations one week apart followed by a booster injection approximately 100 days later which contained 100 ug of ZP-3 antigen with Freund's adjuvant. The initial inoculations contained the antigen emulsified with Freund's complete adjuvant while the remaining inoculations were emulsified with Freund's incomplete adjuvant. The total amount of antigen injected per animal was 200 ug.

Blood Sampling Procedure

Blood samples were taken periodically to monitor antibody titer levels and hormonal status. The monkeys were sedated with Vetalar® (10 mg/600 gm monkey) and the femoral area cleaned with an alcohol swab. A 3 ml syringe with a 25 gauge needle was inserted into the femoral vein and 2-3 ml of blood withdrawn. The blood was stored overnight at 4°C and allowed to clot. The clot was centrifuged for 30 minutes at 4°C, the serum drawn off and stored at -20°C in 12x35 mm, 1/2 dram screw cap vials (Kimble, Toledo,Ohio).

Antiserum Titration

A control blood sample was obtained from each animal prior to inoculation. Beginning one week after the third inoculation, blood was collected on a weekly basis for 14 weeks and processed as described previously. Subsequent bleedings were taken at 1 month intervals to continue monitoring antibody response. The serum samples were sent to Wayne State University for analysis of the antibody response by radioimmunoassay (RIA) titration methods (Sacco et al., 1983).

Hormone Analyses

Daily blood samples were collected from monkeys in the three treatment groups during the months of May, August and November. Samples were taken at 9 AM each day for a period of 8-12 consecutive days to monitor hormonal status. The blood samples were processed as previously described and were later shipped to Wayne State University for analysis of serum estradiol and progesterone levels.

Statistical Analysis of Data

Analysis of variance (ANOVA) was used to evaluate data and the student-Newman-Keuls procedure was used to determine differences between groups. There was great variability in P values so log transformation of the data was performed before ANOVA. A chi-square test was used to examine differences between groups in the fertility study.

RESULTS

Laparoscopic Observations and Oocyte Yields

Hormonally-primed squirrel monkeys were subjected to laparoscopy in order to evaluate the possible adverse effects of immunization. At laparoscopy, the ovaries were observed, the number and size of the follicles counted and aspirated for oocyte recovery. Laparoscopic examinations were performed approximately four months after initial immunization (Experiment 1) and 15 months (Experiment 2). The results of laparoscopic observation and oocyte retrieval at the two time intervals are shown in Table 2. The number of follicles present and oocytes collected in the injected group were significantly less than in the control groups at four months post-immunization ($p<0.01$). At 15 months following initial immunization (Experiment 2), similar observations were noted in number of eggs retrieved and follicles present with the exception of large follicles where no significant difference was found ($p>0.05$) between ZP-3-injected and control groups. Visual inspection of the ovaries in the ZP-3-injected group at four months revealed the ovaries were small, whitish in color and non-responsive in appearance with respect to the ovulation induction regimen. During the time of experiment

TABLE 2

Laparoscopic observation of follicles and oocytes retrieved from ZP-3-injected, adjuvant-injected and untreated control monkeys at 4 and 15 months following initial immunization

Exp No.	Treatment Group	n	Mean Wgt. gms	Mean Number of Follicles[a] L	M	S	Eggs/ Monkey
1	Control	8	636	1.1	6.3	7.5	2.1
	Adjuvant	7	621	2.8	6.0	12.3	1.7
	ZP-3	20	591	0.3*	0.6*	3.5*	0.4*
2	Control	14	644	0.9	2.0	6.4	1.1
	Adjuvant	8	611	0.8	4.8	9.4	2.3
	ZP-3	34	642	0.2#	1.0*	3.8*	0.3*

[a]
L= > 2mm
M= 1-2mm
S= < 1mm
* sig. different from control ($p<0.01$)
# not sig. different from control ($p>0.05$)

2, most ovaries in the ZP-3-injected monkeys appeared morphologically similar to the animals in the control group.

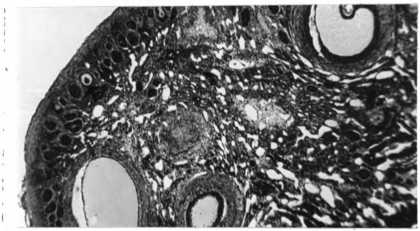
Ovarian Histology

Ovaries have been obtained from each of two monkeys in the ZP-3-injected and adjuvant-injected groups (total of 4 monkeys) and prepared for histological examination. Death occurred approximately 180-210 days following immunization with either ZP-3 or adjuvant alone. The ovaries were removed immediately after death, fixed in Bouin's solution and sections stained with hemotoxylin and eosin. Tissue sections from the adjuvant-injected animals exhibited normal ovarian histology (Figure 1). Numerous oocyte-containing primordial follicles of various size were observed in sections from one of the ZP-3-injected monkeys (Figure 1). The second ZP-3-injected animal had sections with oocyte-containing primordial follicles with a few primary follicles and corpora lutea.

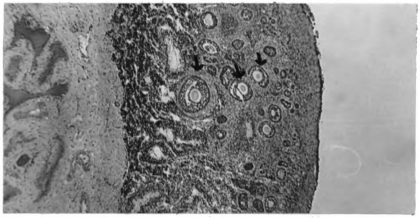
Antibody Titer Levels

A cumulative titration profile for all immunized monkeys is shown in Figure 2. Antibody titer levels increased significantly to approximately the 54% binding level during the first 56 days after initial immunization. During the next two months, titer levels continued to increase to the 65% binding level at which time a booster

A.



B.



C.

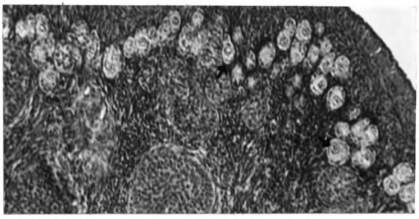


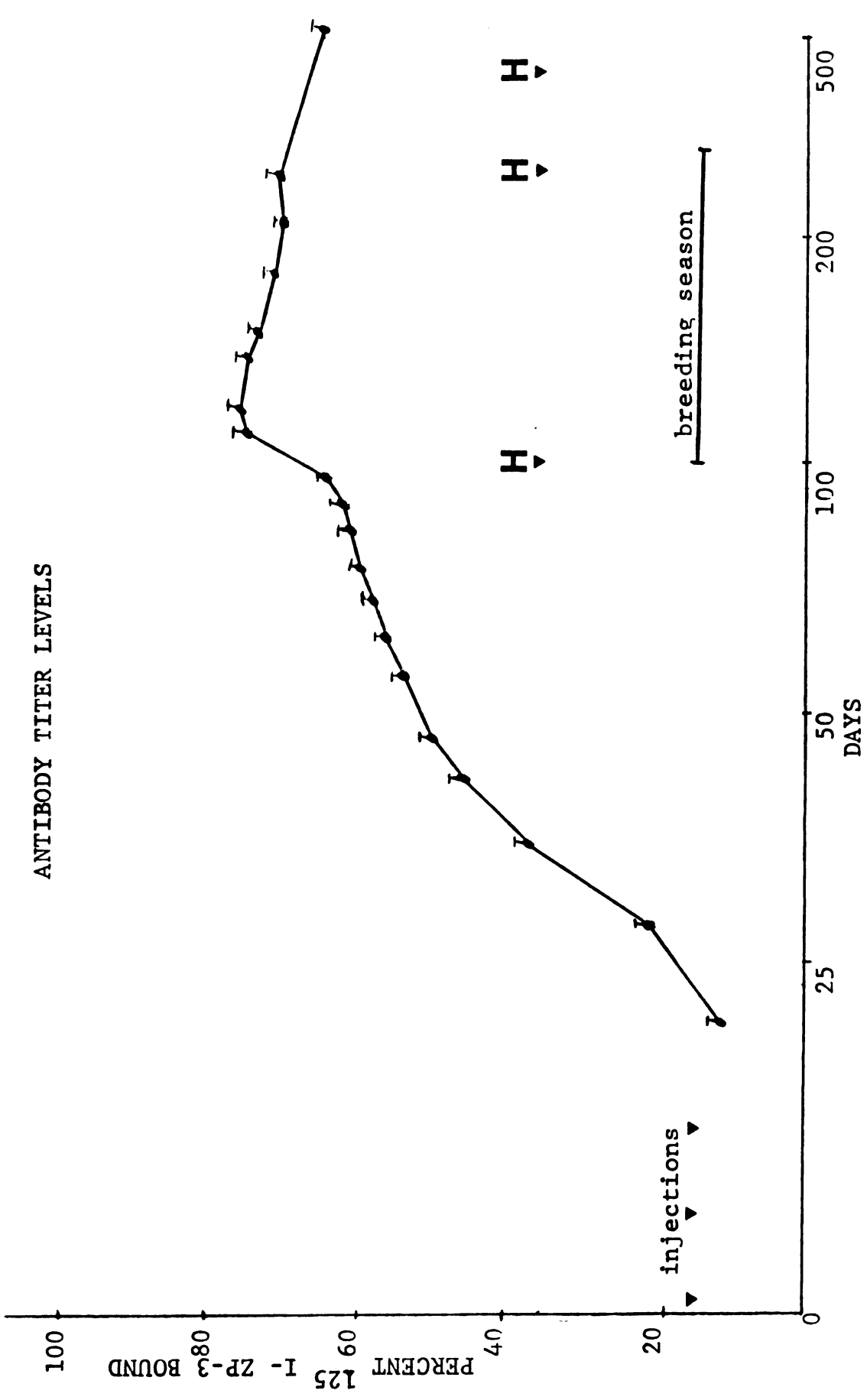
FIGURE 1

Tissue sections from adjuvant-injected and ZP-3 injected monkeys showing ovarian histology

A. Section from Adjuvant-injected monkey

B,C. Section from ZP-3 immunized monkeys

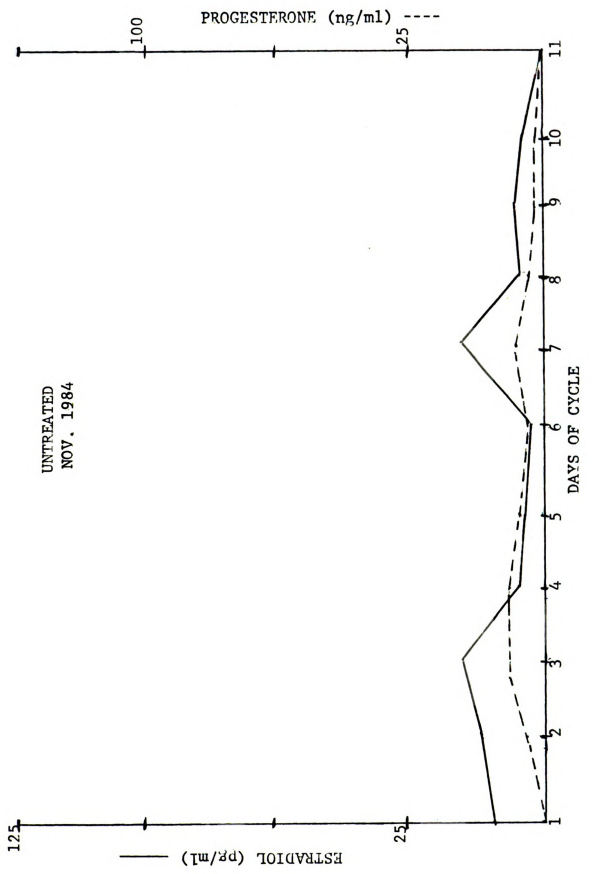
Arrows indicate oocytes within follicles

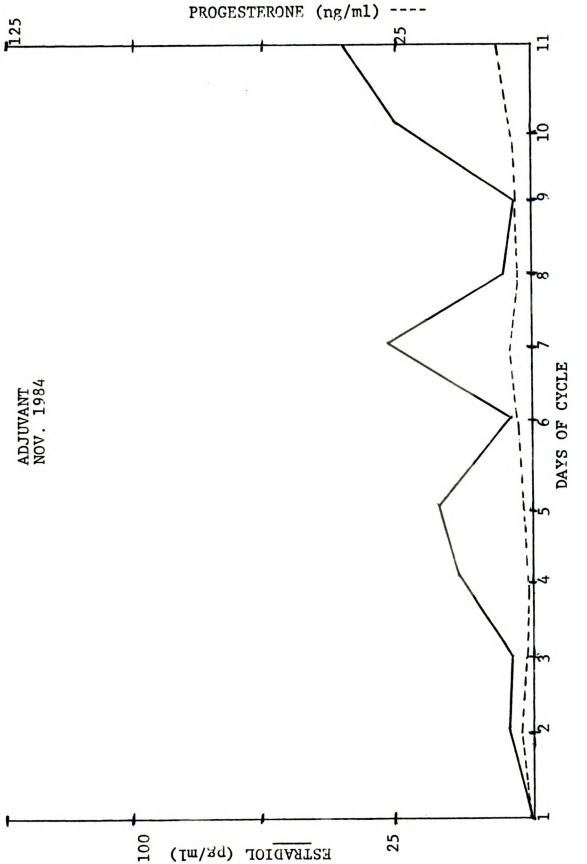

ANTIBODY TITER LEVELS
PERCENT 125 I- ZP-3 BOUND
100
80
60
40
20
0
25
50
100
200
500
DAYS
injections
breeding season

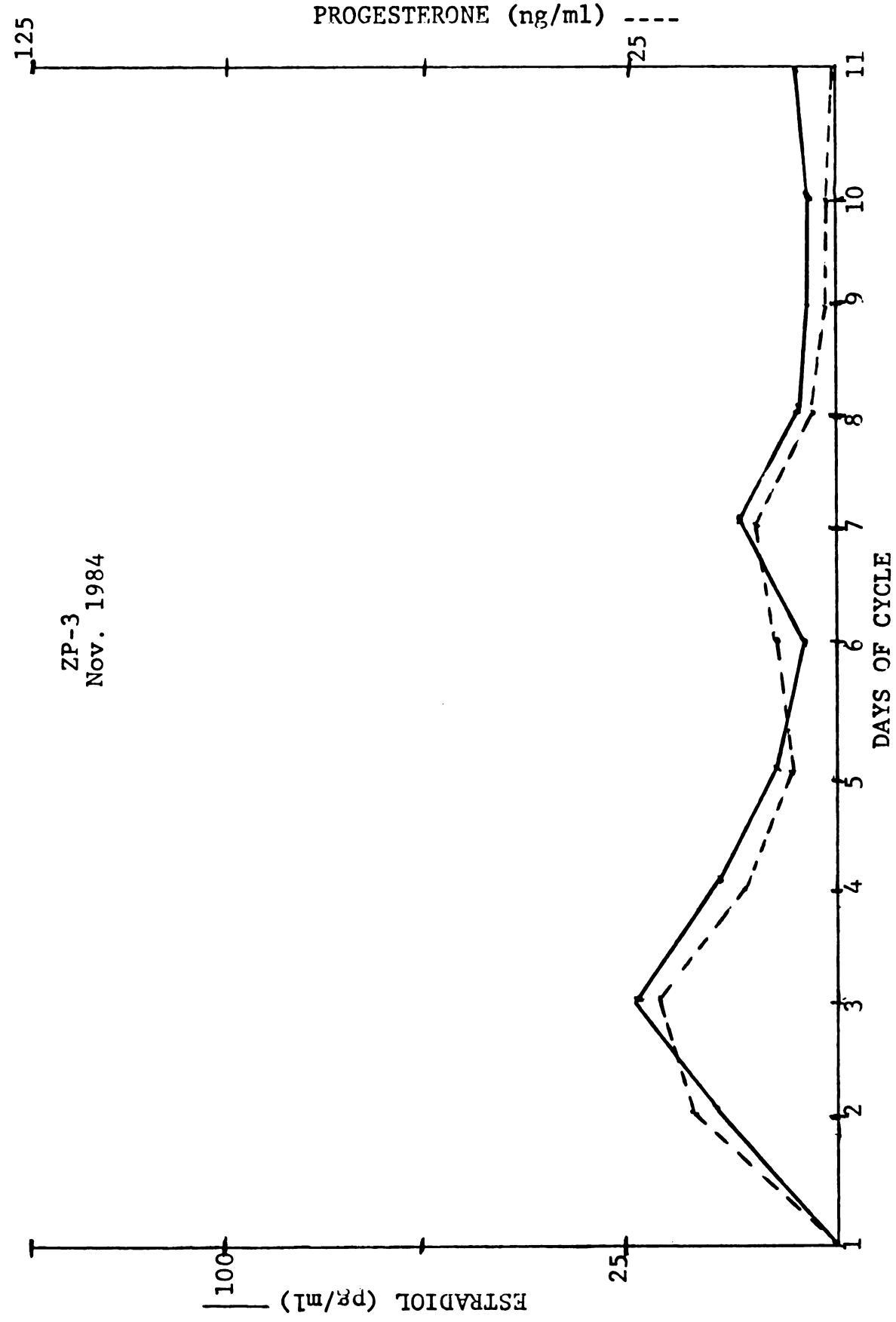
injection was administered. This resulted in further rapid increase in titers which peaked about 3 weeks following the booster at approximately the 77% binding level. In the absence of additional boosters, titer levels decreased slowly but remained at high levels (68% binding level) 493 days following the first immunization. The immune response was consistant among the 50 monkeys as evidenced by the small standard error (Figure 2). There was no anti-ZP-3 activity detected in sera from monkeys in the untreated control and Freund's adjuvant injected control groups.

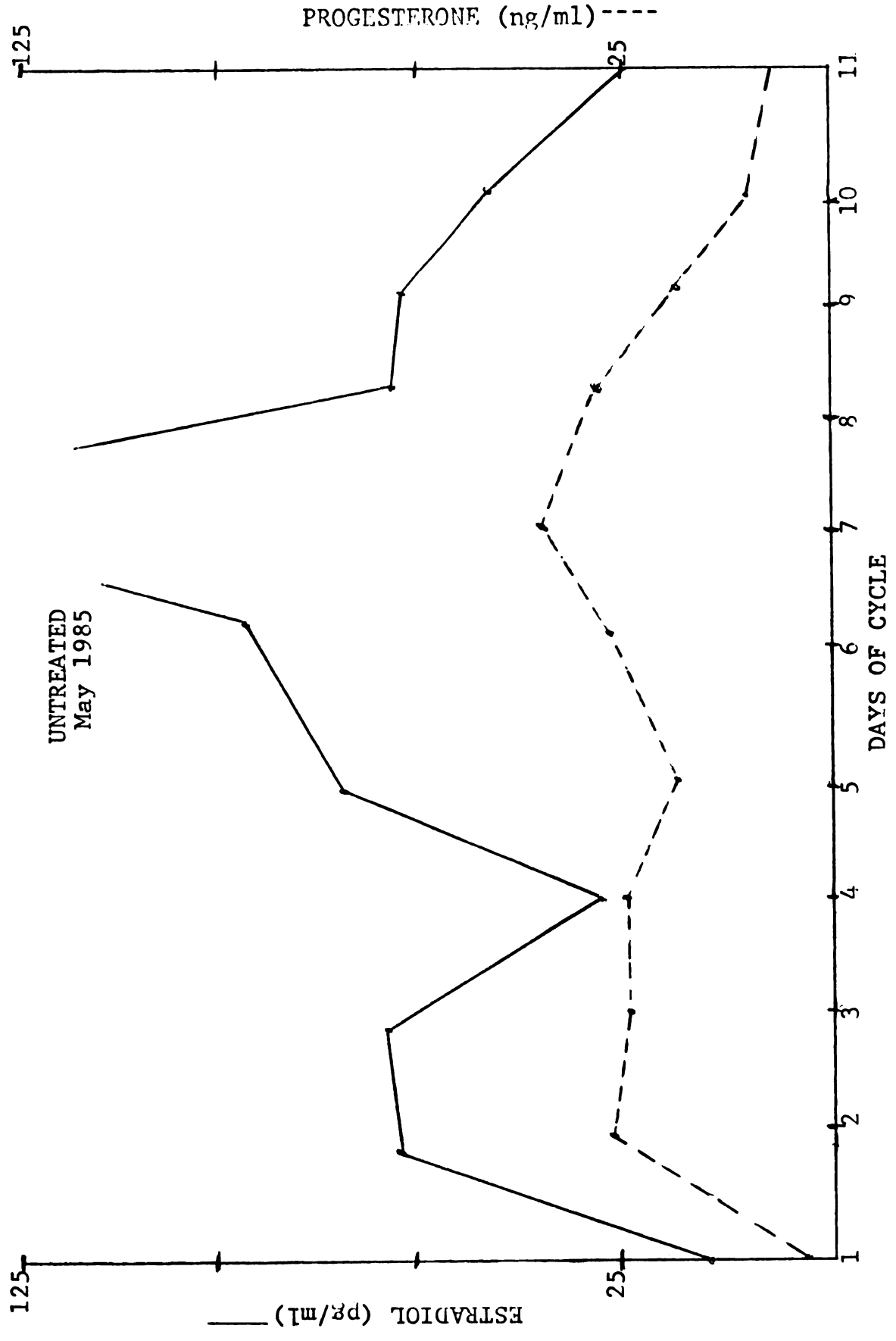
Hormonal Profiles

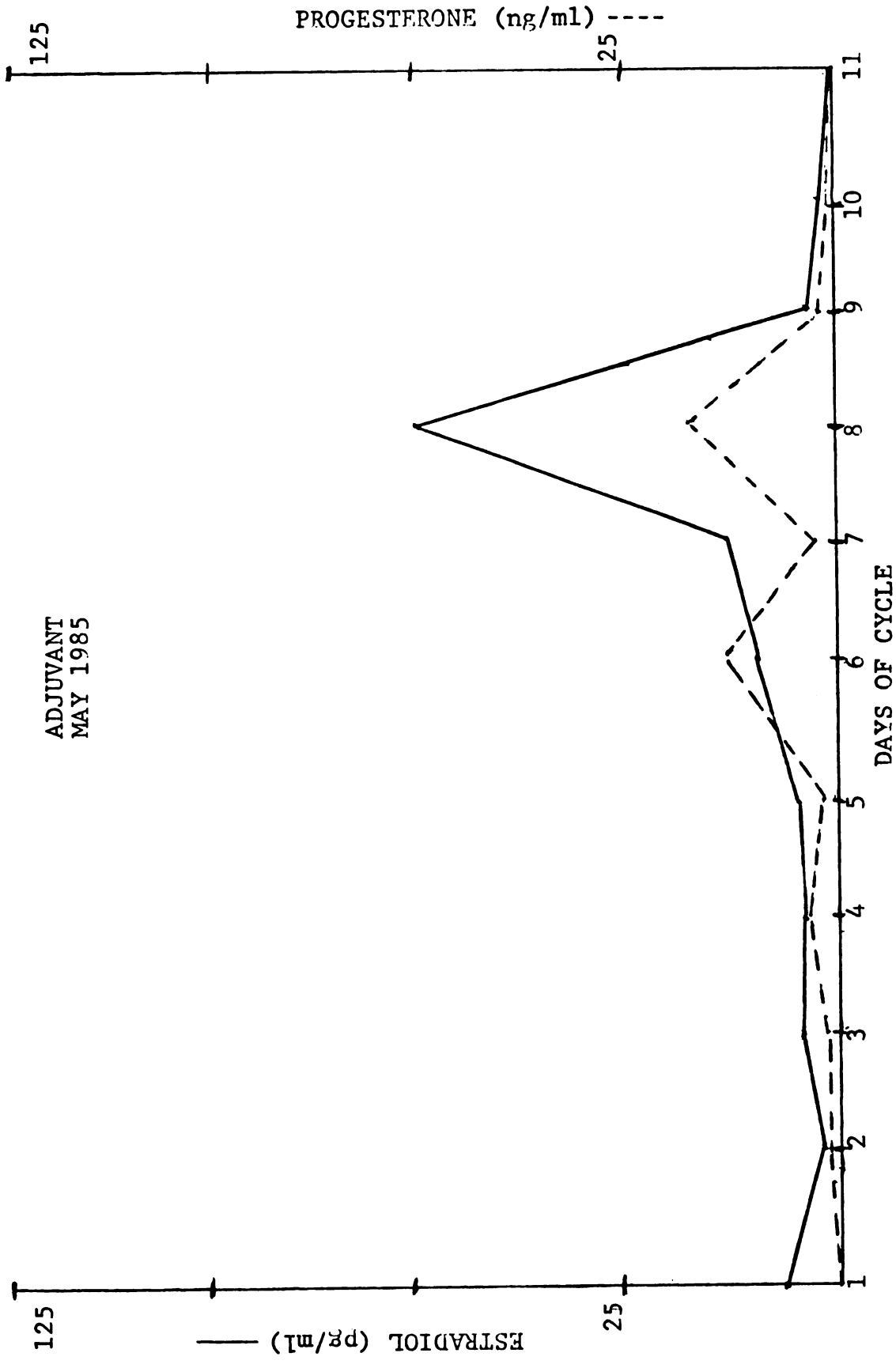
Series of daily blood samples were obtained from randomly selected monkeys in the three treatment groups, and estradiol (E_2) and progesterone (P) levels were measured by RIA. Figures 3 through 11 show the hormone profiles from untreated control, adjuvant-injected and ZP-3 injected monkeys during November, August and May. These samples represent hormonal status at approximately 114, 297 and 392 days following initial immunization, respectively. Cyclical trends were detected in estradiol and progesterone levels, although none of the monkeys had ovulatory cycles as evidenced by the peak E_2 and P levels (Table 3) obtained (Diamond et al., 1984). The maximum levels of E_2 were significantly greater in the control group ($p<0.05$) compared to adjuvant and ZP-3 injected groups during the bleeding period prior to the onset of breeding season.

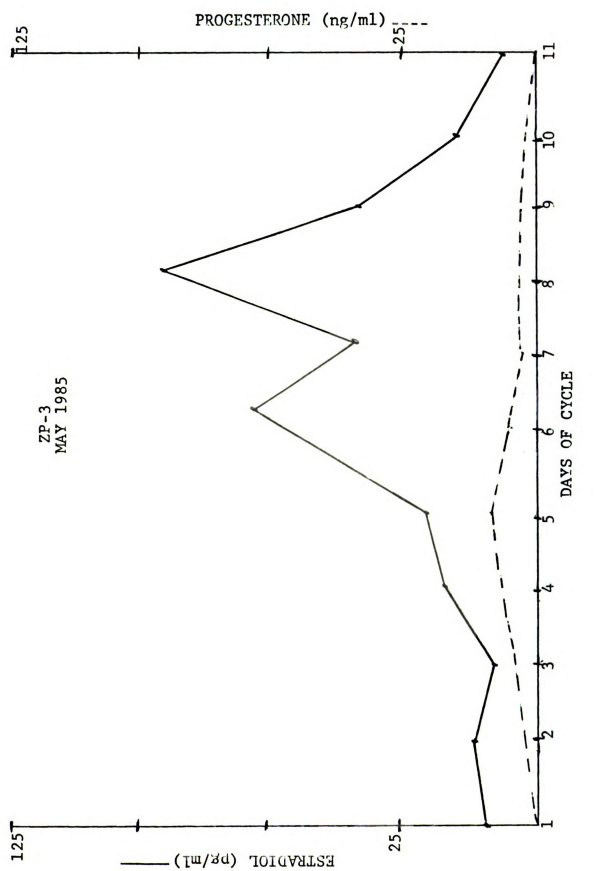

PROGESTERONE (ng/ml) ----
100
25
125
25
ESTRADIOL (pg/ml) ——
1
2
3
4
5
6
7
8
9
10
11
DAYS OF CYCLE
UNTREATED
NOV. 1984

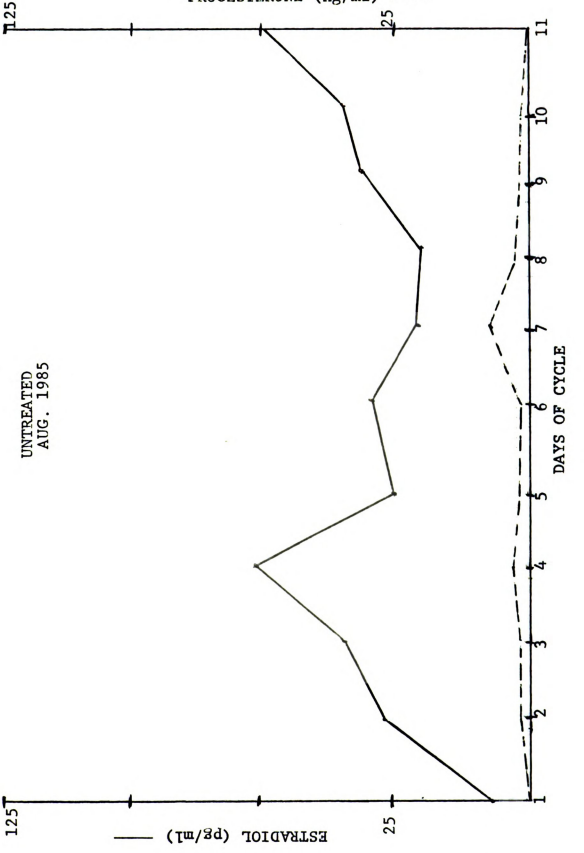

PROGESTERONE (ng/ml) ----
125
25
ESTRADIOL (pg/ml)
100
25
1
2
3
4
5
6
7
8
9
10
11
DAYS OF CYCLE
ADJUVANT
NOV. 1984

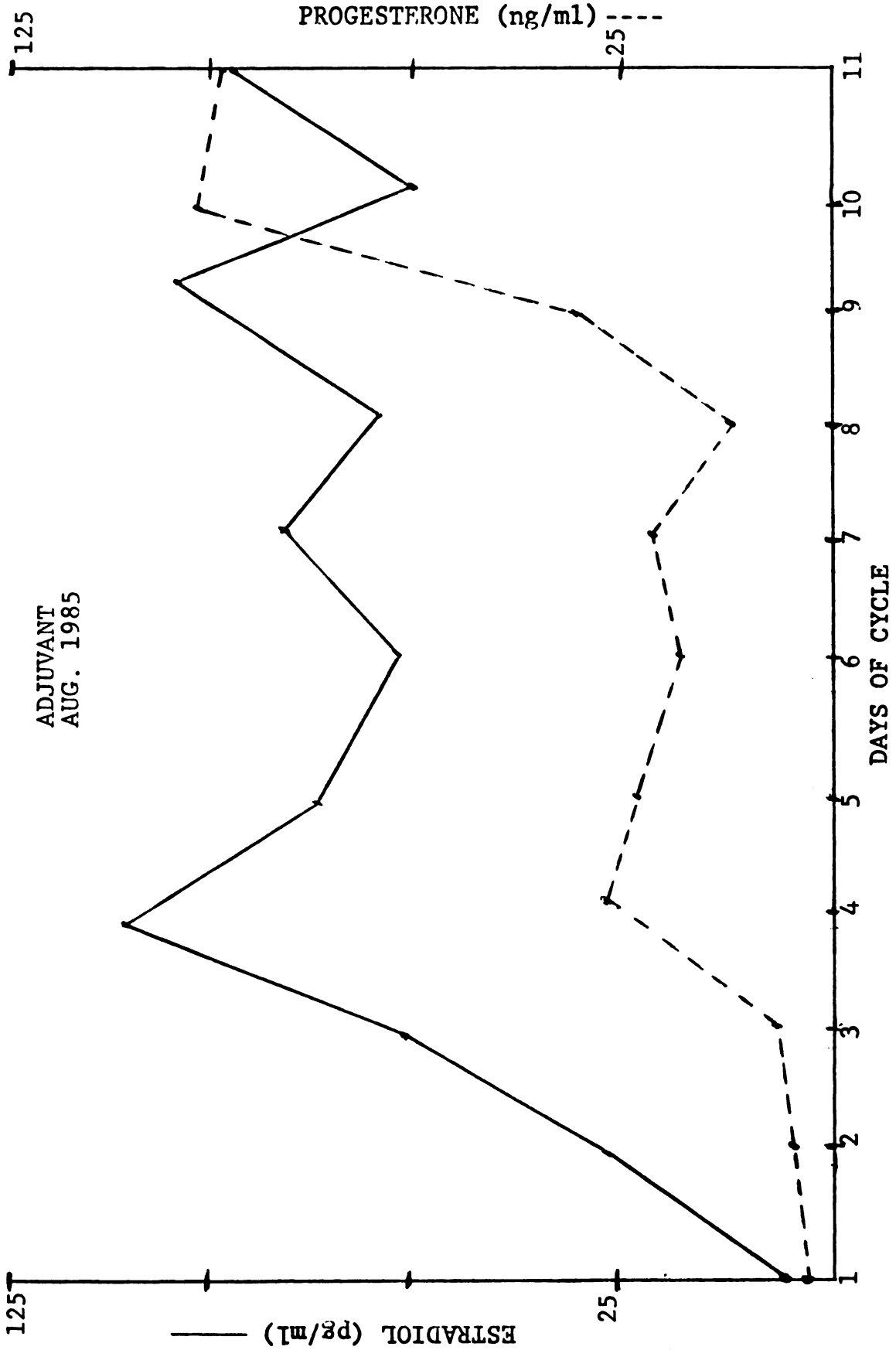

PROGESTERONE (ng/ml) ----
125
25
11
10
9
8
7
6
5
4
3
2
1
DAYS OF CYCLE
ZP-3
Nov. 1984
100
25
ESTRADIOL (pg/ml) ——


PROGESTERONE (ng/ml) ----
125
25
ESTRADIOL (pg/ml) ____
125
25
UNTREATED
May 1985
1
2
3
4
5
6
7
8
9
10
11
DAYS OF CYCLE


PROGESTERONE (ng/ml) ----
125
25
11
10
9
8
7
6
5
4
3
2
1
DAYS OF CYCLE
ADJUVANT
MAY 1985
125
25
ESTRADIOL (pg/ml) ——


PROGESTERONE (ng/ml)
125
25
ZP-3
MAY 1985
DAYS OF CYCLE
1
2
3
4
5
6
7
8
9
10
11
ESTRADIOL (pg/mL)
125
25


PROGESTERONE (ng/ml) ----
125
25
ESTRADIOL (pg/mL) ——
125
25
UNTREATED
AUG. 1985
1
2
3
4
5
6
7
8
9
10
11
DAYS OF CYCLE


PROGESTERONE (ng/ml) ----
125
25
11
10
9
8
7
6
5
4
3
2
1
DAYS OF CYCLE
ADJUVANT
AUG. 1985
125
25
ESTRADIOL (pg/ml) ——

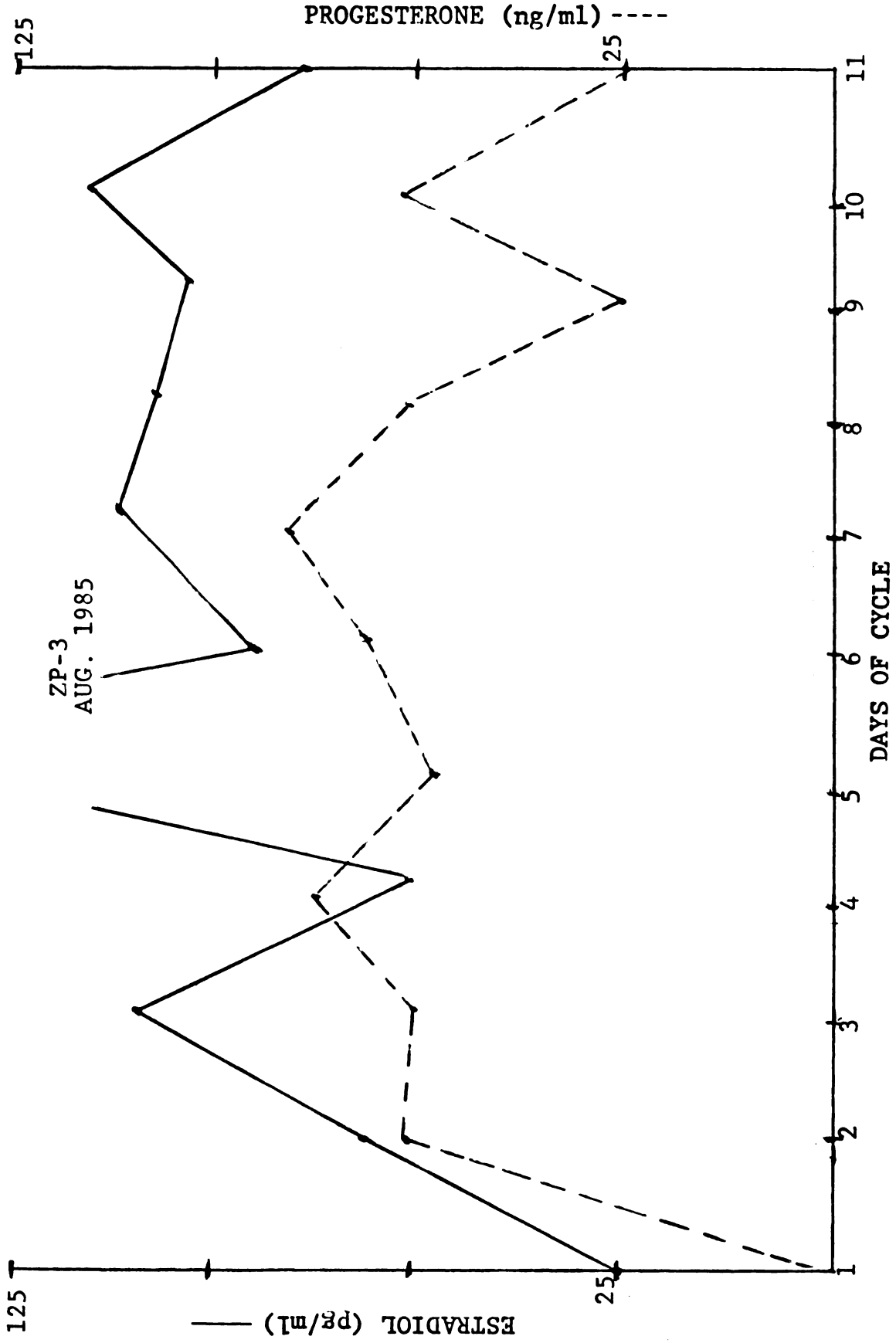

PROGESTERONE (ng/ml) ----
125
25
11
10
9
8
7
6
5
4
3
2
1
DAYS OF CYCLE
ZP-3
AUG. 1985
125
25
ESTRADIOL (pg/ml) ——

TABLE 3

Maximum estradiol and progesterone levels in blood samples from ZP-3, adjuvant-injected and untreated controls at 114, 297 and 392 days after initial injection

Treatment Group	(# Days after 1st injection)	Month of Bleeding NOV 114	MAY 297	AUG 392
Untreated	E2 (pg/ml)	77±13*(5)	57±0.5(6)	209±112(10)
	P (ng/ml)	2.8±1.0 (5)	5.7±0.5(6)	45.9±20(10)
Adjuvant	E2 (pg/ml)	35±5.5(5)	71±19.2(5)	256±81(10)
	P (ng/ml)	2.5±0.7(5)	22.4±7.6(5)	92.7±34(10)
ZP-3	E2 (pg/ml)	20±10(7)	95±23.2(7)	281±132(10)
	P (ng/ml)	20±54#(7)	16±3.9(7)	102.3±54 (10)

( )= number animals

* = signif. greater than adjuvant and ZP-3 injected groups ($p<0.05$)

= signif. greater than untreated and adjuvant injected ($p<0.05$)

Animals in the ZP-3 injected group exhibited significantly higher P levels than the untreated and adjuvant-injected animals ($p<0.05$).

Fertility Study

Matings began in December 1984 by placing a male of proven fertility with 10 females in a breeding cage. Male monkeys were rotated to different cages every two weeks throughout the breeding season. Through May 1985, 7 pregnancies were detected in the control group (Table 4) by palpation and confirmed by elevated progesterone levels. None of the pregnancies went to term due to either stillbirths or abortions. No pregnancies occurred in either the adjuvant-injected or the ZP-3-injected groups.

TABLE 4

The effect of ZP-3 immunization on fertility during the first breeding season

Treatment Group	n	No. Pregnant	% Pregnant
Control	24	7	29
Adjuvant	16	0*	0
ZP-3	34	0*	0

* ($p < 0.05$)

DISCUSSION

In this study, active immunization with porcine zona antigen demonstrated an inhibitory effect upon the fertility of the female squirrel monkey. Similar findings were reported in female dogs (Mahi-Brown et al., 1982, 1985) and cynomolgus monkeys (Gulyas et al., 1983b) after active immunization with porcine zona material. The squirrel monkey responded immunologically to the ZP-3 immunization regimen and produced antibodies to the porcine antigen possessing high antigen binding activity as determined by RIA. This study has shown that the circulating antibodies affected normal ovarian function by reducing the number of developing follicles and oocytes produced. In addition, reduced levels of estradiol and the variable patterns of progesterone secretion indicate disturbances in ovarian function. Previous work has shown that squirrel monkey antiserum to ZP-3 to inhibit sperm-zona interaction *in vitro* (Sacco et al., 1983a, 1984). However, these *in vivo* studies suggest that antibodies to the zona may be exerting their main effect at the ovarian level as revealed by cycle disturbances rather than preventing sperm attachment to the zona. Immunized monkeys recover from the ovarian disruptions by 10 months after the

initial injection as demonstrated by the elevated estradiol and progesterone levels between control groups and ZP-3 immunized group. Normal oocyte production in response to the ovulation induction regimen had not recovered in the ZP-3 immunized group at 15 months after initial injection since significantly fewer oocytes were retrieved despite the E2 and P recovery. The number of large follicles was not significantly different between the ZP-3 injected and control monkeys indicating that folliculogenesis may be slowly recovering in the immunized monkeys. Histological studies of ovaries from two of the ZP-3 injected monkeys showed only minor alterations on ovarian histology at 6 months after immunization. The data presented in this investigation indicate that the effects on ovarian function in the squirrel monkey in response to anti-zona antibodies are milder and probably reversible as compared to the rabbit (Skinner et al., 1984).

The differences in observations between the rabbit test system and the squirrel monkey could be due to species or the use of a purified zona macromolecule (ZP3) as immunogen. In rabbits, complete inhibition of follicular development resulted when heat-solubilized, total porcine zona proteins were used as the immunogen (Skinner et al., 1984). A follow-up study reported no effect on normal ovarian follicular development with the use of a purified zona protein (Skinner et al., 1984). In contrast to the

rabbit study, Gulyas et al. (1983) described a reversible infertility with intermittant distubances in menstrual cycles in response to immunization of cynomolgus monkeys with heat-solubilized total zona protein. The milder effect observed in the primate study following the use of total porcine zona as immunogen suggests a species difference in response to immunization.

Among individual monkeys,there was great variability in steroid levels, cyclicity and, follicle and oocyte production throughout the post-immunization period. It was apparent that the ZP-3 immunization did not result in complete inhibition of ovarian function since immunized monkeys did exhibit follicle and oocyte development despite the high antibody titer level.

Many of the reports previously discussed involving active immunization of monkeys (Gulyas et al.,1983), rabbits (Skinner et al., 1984) and dogs (Mahi-Brown et al., 1985) with porcine zona have cited disturbances in estrous cycles as well as disruptions in normal ovarian function and steroid secretion. The tissue specificity of zona antigens has been clearly demonstrated (Palm et al., 1979; Gerrity et al., 1981) so the effects caused by circulating antibodies are observed at the level of the ovary on zona components. Due to the controversy over the site of zona synthesis (Bleil and Wasserman, 1980; Wolgemuth et al., 1984), the exact cellular targets of these antibodies and

their mechanism of action on steroid secretion and normal ovarian function can only be hypothesized. Antibodies to the zona might interfere with the communication of the oocyte with its surrounding corona radiata cells. Another possibility is that the antibodies react with the zona or zona precursor material at the site of zona synthesis and ultimately destroy the oocyte. This would account for the lower number of follicles collected via laparoscopy and the alteration in steroid secretion.

The maximum steroid levels (E2 and P) for all three groups of monkeys was considerably lower than levels reported in the literature (Ghosh et al., 1983; Diamond et al., 1984; Aksel et al., 1985). The radioimmunoassay procedures used in all of these studies was identical. The short time of acclimatization to the laboratory, young age of the colony and stress of handling might account for the observed differences. An increase in the E2 and P levels was noted with the most recent bleedings indicating the monkeys in the colony are beginning to stabilize. Although the steroid levels reported here are lower than those previously cited in the literature, they are well within the range described. Also, none of the serial bleedings were performed during the breeding season when levels would normally be elevated in order to allow matings to occur undisturbed.

Early immunocontraceptive studies as well as this

investigation have demonstrated the contraceptive effectiveness of antibodies directed against zona pellucida macromolecules. Further work could examine the other zona glycoproteins for antigenic activity and modified forms of ZP-3 glycoporotein (deglycosylated, peptide fragments and enzyme digests). The use of other adjuvants might also be tested since Freund's adjuvant may have independently affected normal ovarian function in this study. Nevertheless, the findings of this study encourage further investigation of purified zona macromolecules for contraceptive vaccine development.

SUMMARY AND CONCLUSIONS

The squirrel monkey provides a model for the examination of the purified 55K porcine macromolecule (ZP-3) as a contraceptive vaccine. The following conclusions are indicated:

1) Immunization of female squirrel monkeys with the purified porcine zona macromolecule (ZP-3) results in the disruption of ovarian function. This disturbance is reversible as shown by the recovery in follicular development and hormone levels at 10-15 months post-immunization.
2) Histological data indicate an interference in folliculogenesis in the ZP-3 injected animals.
3) Production of high antibody titers (75% binding level) can be achieved in immunized monkeys at 120 days following initial injection. Anti-ZP-3 titers remained high (68% binding level) throughout the study.
4) Administration of the 55K macromolecule produced an anti-fertility effect on the immunized monkeys since no pregnancies occurred in this group. A 29% pregnancy rate was observed in the untreated control group.

Literature Cited

Ahuja, K.K. and S.J. Tzartes, 1981. Investigation of sperm receptors in the hamster zona pellucida by using univalent (Fab) antibodies to hamster ovary. J. Reprod. Fertil. 61, 257-264.

Aksel, S., E.J. Diamond, J. Hazelton, R.H. Weibe and C.R. Abee. 1985. Progesterone as a predictor of cyclicity in Bolivian squirrel monkeys during the breeding season. Lab Anim. Sci. 35, 54-57.

Baldwin, J.D. and J.I. Baldwin. 1971. Squirrel monkeys (Saimiri) in natural habitats in Panama, Columbia, Brazil and Peru. Primates 12, 45-61.

Barros, C. and R. Yanagimachi. Polyspermy-preventing mechanisms in the golden hamster eggs. J. Exp. Zool, 180, 251-266.

Bennett, J.P. 1967. The induction of ovulation in the squirrel monkey (Saimiri sciureus) with pregnant mares serum (PMS) and human chorionic gonadotropin (HCG). J. Reprod. Fertil. 13, 357-459.

Bleil, J.D. and P.M. Wassarman. 1980. Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. Cell 20, 873-882.

Braden A.W.H., C.R. Austin and H.A. David. 1954. The reaction of the zona pellucida to sperm penetration. Aust. J. Biol. Sci. 7, 391-409.

Butler, H. 1974. Evolutionary trends in primate sex cycles. In: Contributions to Primatology. Volume 3 (H. Kuhn, ed.) S. Karger, Basel. pp. 2-35.

Castellanos, H. and H.L. McCombs. 1963. The reproductive cycle of the new world monkey. Fertil. Steril. 19, 213-227.

Chan, P.J., R.J. Hutz and W.R. Dukelow. 1982. Nonhuman primate in vitro fertilization: Seasonality, cumulus cells, cyclic nucleotides, ribonucleic acids and viability assays. Fertil. Steril. 38, 609-615.

Chiquoine, A.D. 1960. Electron microscopic observations on the vitally stained ovary of the mouse. Anat. Rec. 136, 176.

Clewe, T.H. 1969. Observations on reproduction of squirrel monkeys in captivity. J. Reprod. Fertil.,Suppl. 6,151-156.

Dakhno, F.V., T. Hjort and V.I. Grischenko. 1980. Evaluation of immunofluorescence on pig zona pellucida for detection of anti-zona antibodies in human sera. J. Reprod. Immunol. 2, 281-291.

Denniston, R. 1964. Notes on the vaginal cornification cycle of captive squirrel monkeys. J. Mammol. 45, 471.

Diamond, E.J., S. Aksel, J.M. Hazelton, R.A. Jennings and C.R. Abee. 1984. Seasonal changes of serum concentrations of estradiol and progesterone in Bolivian squirrel monkeys (Saimiri sciureus). Amer. J. Primatol. 6, 103-113.

Dietl, J., J. Freye and L. Metler. 1982. Fertility inhibition using low-dose immunization with porcine zona pellucida. A.J.R.I. 2, 153-156.

Dudkiewicz, A.B., C.A. Shivers and W.L. Williams. 1976. Ultrastructure of hamster zona pellucida treated with zona-precipitating antibody. Biol. Reprod. 14, 175-185.

Dudkiewicz, A.B. and W.L. Williams. 1977. Fine structural observations of the mammalian zona pellucida by scanning electron microscopy. Scan. Elect. Micro. Vol 2, Proceedings Of the Workshop on Biomedical Applications. 317.

Dukelow, W.R. 1970. Induction and timing of single and multiple ovulations in the squirrel monkey (Saimiri sciureus) J. Reprod. Fertil. 22, 303-309.

Dukelow, W.R. 1978a. Reproduction in the squirrel monkey (Saimiri sciureus). Rec. Adv. Primatol. 2, 195-200.

Dukelow, W.R. 1978b.Laparoscopic research techniques in mammalian embryology. In, Methods of Mammalian Reproduction (J.C. Daniel, ed) Academic Press, New York pp. 438-460.

Dukelow, W.R. 1979. Human chorionic gonadotropin: induction of ovulation in the squirrel monkey. Science 206, 234-235.

Dukelow, W.R. 1983. Ovum recovery and embryo transfer in primates. In: Mammalian Egg Transfer (C.E. Adams, ed.) CRC Press, Boca Raton, Florida. pp. 155-174.

Dukelow, W.R.. 1986. The Squirrel Monkey. In: Universities Federation for Animal Welfare Handbook. (P. Troughton,ed.)MTP Press, London. (in press).

Dukelow, W.R. and S. Ariga. 1976. Laparoscopic techniques for biomedical research. J. Med. Primatol. 5, 82-99.

Dumond, F.V. and T.C. Hutchinson. 1967. Squirrel monkey reproduction: The "fatted" male phenomenon and seasonal spermatogenesis. Science 158, 1467-1470.

Dumond, F.V. 1968. The squirrel monkey in a semi-natural environment. In: The Squirrel Monkey, (L.A. Rosenblum and R.W. Cooper, eds) Academic Press, New York. pp. 87-145.

Dunbar, B.S. and B.D. Raynor. 1980. Characterization of porcine zona pellucida antigens. Biol. Reprod. 22, 941-954.

Dunbar, B.S. and C.A. Shivers. 1976. Immunological aspects of sperm receptors on the zona pellucida of mammalian eggs. In: Immunology of receptors (B. Cinader, ed.) New York. Marcel Dekker. pp. 509-519.

Dunbar, B.S., N.J. Wardrip and J.L. Hedrick. 1980. Isolation, physiochemical properties and the macromolecular composition of the zona pellucida from porcine oocytes. Biochem. 19, 356-365.

Dunbar, B.S., C. Lui and D.W. Sammons. 1981. Identification of three major proteins of porcine and rabbit zonae pellucidae by high resolution two-dimensional gel electrophoresis: comparison with serum, follicular fluid, and ovarian cell proteins. Biol. Reprod. 24: 1111-1124.

Flechon, J.E. and R.B.L. Gwatkin. 1980. Immunocytochemical studies on the zona pellucida of cow blastocysts. Gamete Res. 3, 141-148.

Fox, L.L., N. Gengozian, C.A. Shivers and P. Steg. 1981. Marmoset response to immunization with porcine zonae pellucidae. J. Med. Primatol. 10, 205-211.

Garavagno, A., J. Posado, C. Barros,and C.A. Shivers. 1974. Some characteristics of the zona pellucida antigen in the hamster. J. Exp. Zool. 189, 37-50.

Gerrity, M., E. Niu, and B.S. Dunbar. 1981. A specific radioimmunoassay for evaluation of serum antibodies to zona pellucida antigens. J. Reprod. Immunol. 3, 59-70.

Ghosh, M., R.J. Hutz and W.R. Dukelow. 1982. Serum estradiol 17-B, progesterone and relative luteinizing hormone levels in Saimiri sciureus: cyclic variation and the effect of laparoscopy and follicular aspiration. J. Med. Primatol. 11, 312-318.

Goss, C.M., L.T. Popejoy, J.L. Fusiler and T.M. Smith. 1968. Observations on the relationship between embryological development, time of conception and gestation. In: The Squirrel Monkey,(L.A. Rosenblum and R.W. Cooper,eds) Academic Press, New York. pp. 171-191.

Gould, K.G., E.M. Cline and W.L. Williams. 1973. Observations on the induction of ovulation and fertilization in vitro in the squirrel monkey (Saimiri sciureus). Fertil. Steril. 24, 260-268.

Gulyas, B.J., R.B.L. Gwatkin and L.c. Yuan. 1983a. Active immunization of cynomologus monkeys (Macaca fascicularis) with porcine zonae pellucidae. Gamete Res. 4, 299-307.

Gulyas, B.J., L.C. Yuan, R.B.L. Gwatkin and E.D. Schmell. 1983b. Response of monkeys to porcine zona pellucida as detected by a solid-phase radioimmunoassay. J. Med. Primatol. 12, 331-342.

Gwatkin, R.B.L. 1963. Studies on the zona pellucida of the mouse egg. J. Reprod. Fertil. 6, 325.

Gwatkin, R.B.L., D.T. Williams and D.J. Carlo. 1977. Immunization of mice with heat-solubilized hamster zonae: production of anti-zona antibody and inhibition of fertility. Fertil. Steril. 28, 871-877.

Haddad, A. and M.E.T. Nagai. 1977. Radioautographic study of glycoprotein biosynthesis and renewal in the ovarian follicles of mice and the origin of the zona pellucida. Cell Tiss. Res. 177, 347-369.

Hadek, R. 1965. The structure of the mammalian egg. In:Int. Rev. Cytol. (Bourne and Danielli, eds) Academic Press, New York. 19, 29-71.

Hanada, A. and M.C. Chang. 1972. Penetration of zona-free eggs by spermatozoa of different species. Biol. Reprod. 6, 300-309.

Harrison, R.M. and W.R. Dukelow. 1973. Seasonal adaptation of laboratory maintained squirrel monkeys (Saimiri sciureus). J. Med. Primatol. 2, 277-283.

Harrison, R.M. and D.E. Wildt. 1980. Animal Laparoscopy. Williams and Wilkins, Baltimore, Maryland.

Herbert, W.J. 1977. Passive hemagglutination. In: Handbook of Experimental Immunology. (D.M. Weir ed.) Oxford and Edinburgh. pp. 720-747.

Hodgen, G.D., S.J. Stolzenberg, D.C.L. Jones, D.F. Hildebrand and C.K. Turner. 1978. Pregnancy diagnosis in squirrel monkeys: hemagglutination test, radioimmunoassay and bioassay of chorionic gonadotropin. J. Med. Primatol. 7, 59-64.

Hopf, S. 1967. Notes on pregnancy, delivery and infant survival in captive squirrel monkeys. Primates. 8, 323-332.

Hutchinson, T.C. 1970. Vaginal cytology and reproduction in the squirrel monkey (Saimiri sciureus) Folia Primat. 12, 212-223.

Inoue, M. and D.P. Wolf. 1974. Comparative solubility properties of the zonae pellucidae of unfertilized and fertilized mouse ova. Biol. Reprod. 11, 558-565.

Jarosz, S.J., T.J. Kuehl and W.R. Dukelow. 1977. Vaginal cytology, induced ovulation and gestation in the squirrel monkey (Saimiri sciureus). Biol. Reprod. 16, 97-103.

Jilek, F. and A. Pavlok. 1975. Antibodies against mouse ovaries and their effect on fertilization in vitro and in vivo in the mouse. J. Reprod. Fertil. 42, 377-380.

Kaplan, J.N. 1977. Breeding and rearing squirrel monkeys (Saimiri sciureus) in captivity. Lab Anim. Sci. 27, 557-567.

Kerber, W.T., C.H. Conaway and D. Moore Smith. 1977. The duration of gestation in the squirrel monkey (Saimiri sciureus). Lab Anim. Sci. 27, 700-702.

Kuehl, T.J. and W.R. Dukelow. 1975. Ovulation induction during the anovulatory season in Saimiri sciureus. J. Med. Primatol. 4, 23-31.

Kuehl, T.J. and W.R. Dukelow. 1975. Fertilization in vitro of Saimiri sciureus follicular oocytes. J. Med. Primatol. 4, 209-216.

Kuehl, T.J. and W.R. Dukelow. 1979. Maturation and in vitro fertilization of follicular oocytes of the squirrel monkey (Saimiri sciureus). Biol. Reprod. 21, 545-556.

Lang, C.M. 1967. The estrous cycle of the squirrel monkey (Saimiri sciureus). Lab. Anim. Care. 17, 442-451.

Latta, J., S. Hopf and D. Ploog. 1967. Observations on mating behavior and sexual play in the squirrel monkey (Saimiri sciureus) Primates 8, 229-246.

Lorenz, R., C.O. Anderson and W.A. Mason. 1973. Notes on reproduction in captive squirrel monkeys (Saimiri sciureus). Folia Primatol. 19, 286-292.

Mahi-Brown, C.A., T.T.F. Huang and R.Yanagimachi. 1982. Infertility in bitches induced by active immunization with porcine zonae pellucidae. J. Exp. Zool. 222, 89-95.

Mahi-Brown, C.A., M.L. Nelson and R. Yanagimachi. 1984. Aberrant ovarian histology of bitches immunized against porcine zona pellucida. Biol. Reprod. 30 (Supplement 1), 105.

Mahi-Brown, C.A. R. Yanagimachi, J.C. Hoffman and T.T.F. Huang. 1985. Fertility control in the bitch by active immunization with porcine zonae pellucida: use of different adjuvants and patterns of estradiol and progesterone levels in estrous cycles. Biol. Reprod. 32, 761-772.

Mintz, B. 1962. Experimental study of the developing mammalian egg. Removal of the zona pellucida. Science 138, 594-596.

Mori, T., T. Nishimoto, H. Kohda, I. Takai, T. Nishimura and T. Oikawa. 1979. A method for specific detection of autoantibodies to the zona pellucida in infertile women. Fertil. Steril. 32, 67-72.

Nathan, T.S., L.A. Rosenblum, G. Limson and J.H. Nelson. 1966. Diagnosis of pregnancy in the squirrel monkey. Anat. Rec. 155, 531-536.

Nishimoto, T., T. Mori, I. Yamada and T. Nishimura. 1980. Autoantibodies to zona pellucida in infertile and aged women. Fertil. Steril. 34, 552-556.

Oakberg, E.F. and P.D. Tyrell. 1975. Labelling the zona pellucida of the mouse oocyte. Biol. Reprod. 12, 477-482.

Oikawa, T. 1978. A simple method for the isolation of a large number of ova from pig ovaries. Gamete Res. 1, 265-267.

Ownby, C.L. and C.A. Shivers. 1972. Antigens of the hamster ovary and effects of anti-ovary serum on eggs. Biol. Reprod. 6, 310-318.

Palm, V.S., A.G. Sacco, F.N. Syner, and M.G. Subramanian. 1979. Tissue specificity of porcine zona pellucida antigen(s) as tested by radioimmunoassay. Biol. Reprod. 21, 709-713.

Piko, L. 1969. Gamete structure and sperm entry in mammals. In: Fertilization, vol 2 New York, (Metz and Monroy eds.) Academic Press, New York, pp. 325-403.

Richter, C.P. 1976. Artifactual seven-day cycles in spontaneous activity in wild rodents and squirrel monkeys. J. Comp. Physiol. Psychol. 90, 572-582.

Rosenblum, L.A., T. Nathan, J. Nelson and I.CH. Kaufman. 1967. Vaginal cornification cycles in the squirrel monkey (Saimiri sciurea) Folia Primatol. 6, 83-91.

Rosenblum, L.A. 1968. Some aspects of female reproductive physiology in the squirrel monkey. In: The Squirrel Monkey, (L.A. Rosenblum and R.W. Cooper, eds.) Academic Press, New York. pp. 147-169.

Sacco, A.G. 1977. Antigenic cross-reactivity between human and pig zona pellucida. Biol. Reprod. 16, 164-173.

Sacco, A.G. 1979. Inhibition of fertility in mice by passive immunization with antibodies to isolated zonae pellucidae. J. Reprod. Fertil. 56, 533-537.

Sacco, A.G. 1981. Immunocontraception: Consideration of the zona pellucida as a target antigen. In: Obstetrics and Gynecology Annual(R.M. Wynn, ed.), Appelton-Century-Crofts, New York. pp. 1-26.

Sacco, A.G. and K.S. Moghissi. 1979. Anti-zona pellucida activity in human sera. Fertil. Steril. 31, 503-506.

Sacco, A.G. and C.A. Shivers. 1973. Effects of reproductive tissue-specific antisera on rabbit eggs. Biol. Reprod. 8, 481-490.

Sacco, A.G. and C.A. Shivers. 1978. Immunologic inhibition of development. In: Methods of Mammalian Reproduction (J.C.Daniel, ed) Academic Press, New York. pp. 203-228.

Sacco, A.G., M.G. Subramanian and E.C. Yurewicz. 1981a. Active immunization of mice with porcine zonae pellucidae: immune response and effect on fertility. J. Exp. Zool. 218, 405-418.

Sacco, A.G., E.C. Yurewicz, M.G. Subramanian and F.J. DeMayo. 1981b. Zona pellucida composition : species crossreactivity and contraceptive potential of antiserum to a purified pig zona antigen (PPZA). Biol. Reprod. 25, 997-1008.

Sacco, A.G., M.G. Subramanian, E.C. Yurewicz, F.J. DeMayo and W.R. Dukelow. 1983a. Heteroimmunization of squirrel monkeys (Saimiri sciureus) with a purified porcine zona antigen (PPZA): immune response and biologic activity of antiserum. Fertil. Steril. 39, 350-358.

Sacco, A.G., E.C. Yurewicz and S. Zhang. 1983b. Immunoelectrophoretic analysis of the porcine zona pellucida. J. Reprod. Fertil. 68: 21-31.

Sacco, A.G., M.G. Subramanian and E.C. Yurewicz. 1984. Association of sperm receptor activity with a purified pig zona antigen (PPZA). J. Reprod. Immunol. 6: 89-103.

Shivers, C.A., A.B. Dudkiewicz, L.E. Franklin and E.N. Fussell. 1972. Inhibition of sperm-egg interaction by specific antibody. Science 178, 1211-1213.

Shivers, C.A. and B.S. Dunbar. 1977. Autoantibodies to zona pellucida: a posible cause for infertility in women. Science, 197, 1082-1084.

Shivers, C.A., N. Gengozian, S. Franklin and C.L. McLaughlin. 1978. Antigenic cross-reactivity between human and marmoset zonae pellucidae, a potential target for immunocontraception. J. Med. Primatol. 7, 242-248.

Skinner, S.M. and B.S. Dunbar. 1984. Comparison of immunization using a purified zona pellucida (ZP) protein or total protein on ovarian follicular development. Biol. Reprod. 30 (Supplement 1) 73.

Skinner, S.M., T. Mills, H.J. Kirchick and B.S. Dunbar. 1984. Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gondaotropin-induced steoid secretion. Endocrinology 115, 2418-2432.

Srivastava, P.K., F. Cavazos and F.V. Lucas. 1970. Biology of reproduction in the squirrel monkey (Saimiri sciureus) I. The estrou cycle. Primates 11, 125-134.

Subramanian, M.G., E.C. Yurewicz and A.G. Sacco. 1981. A specific radioimmunoassay for the detection of a purified porcine zona pellucida antigen (PPZA). Biol. Reprod. 24, 933-943.

Takai, I., T. Mori, Y. Noda and T. Nishimura. 1981. Heteroimmunization with isolated human ova. J. Reprod. Fertil. 61, 19-24.

Travis, J.C. and W.N. Holmes. 1974. Some physiological and behavioural changes associated with oestrous and pregnancy in the squirrel monkey (Saimiri sciureus). J. Zool. Lond. 174, 41-66.

Tsunoda Y. and M.C. Chang. 1976a. In vivo and in vitro fertilization of hamster, rat, and mouse eggs after treatment with anti-hamster ovary antiserum. J. Exp. Zool. 195, 409-416.

Tsunoda, Y. and M.C. Chang. 1976b. The effect of passive immunization with hetero-and isoimmune anti-ovary antiserum on the fertilization of mouse, rat, and hamster eggs. Biol. Reprod. 15, 361-365.

Tsunoda, Y. and M.C. Chang. 1978. Effect of antisera against eggs and zonae pellucidae on fertilization and development of mouse eggs in vivo and in culture. J. Reprod. Fertil. 54, 233-237.

Tsunoda Y. and M.C. Vhang. 1979. the suppressive effect of sera from old female mice on in vitro fertilization and blastocyst developmewnt. Biol. Reprod. 20, 355-361.

Tsunoda, T., T. Sugie and J. Mori. 1979. Quantitative determination of titers of anti-zona serum. J. Exp. Zool. 207, 315-319.

Wilson, M.I. 1977. Characterization of the oestrous cycle and mating season of squirrel monkeys from copulatory behavior. J. Reprod. Fertil. 51, 57-63.

Wolf, R.H., R.M. Harrison and T.W. Martin. 1975. A review of reproductive patterns on new world monkeys. Lab Anim. Sci. 25, 814-821.

Wolf, R.C., R.F. O'Connor and J.D. Robinson. 1977. Cyclic changes in plasma progestins and estrogens in squirrel monkeys. Biol. Reprod. 17, 228-231.

Wolgemuth, D.J., J. Calenza, D.S. Bundman and B.S. Dunbar. 1984. Formation of the rabbit zona pellucida and its relationship to ovarian follicular development. Devel. Biol. 106, 1-8.

Wood, D.M. and Dunbar, B.S. 1981. Direct detection of two cross reactive antigens between porcine and rabbit zonae pellucidae by radioimmunoassay and immunoelectrophoresis. J. Exp. Zool. 217, 423-433.

Wright, R.W., P.T. Cupps, C.T. Gaskins and J.K. Hillers. 1977. Comparative solubility properties of the zona pellucida of unfertilized murine, ovine and bovine ova. J. Anim. Sci. 44, 850-853.

Yanagimachi, R. 1972. Penetration of guinea-pig spermatozoa into hamster eggs in vitro. J. Repro. Fertil. 28, 477-480.

Yurewicz, E.C., A.G. Sacco and M.G. Subramanian. 1983. Isolation and preliminary characterization of a purified pig zona antigen (PPZA) from porcine oocytes. Biol. Reprod. 29, 511-523.

Yurewicz, E.C., A.G. Sacco and M.G. Subramanian. 1984. Purification and characterization of glycoprotein antigens of the porcine zona pellucida. Fed. Proc. 43, 2039.

APPENDIX

Publications by the Author

Full Papers

1) Antigenic profile and impact of immunization with zona pellucida antigens in primates. A.G. Sacco, M.G. Subramanian, E.C. Yurewicz, D.L. Pierce and W.R. Dukelow. In: Immunological Approaches to Contraception and Promotion of Fertility. (G.P. Talwar, ed.) Plenum Press. New York. In press, 1986.

Abstracts

1) Ovulation control, sperm capacitation and fertilization. W.R. Dukelow, J.A. Kontio, R.D. Bates and D.L. Pierce. Proc. American Society of Primatologists. 1984.

2) Contraceptive potential of a ZP-3 antigen vaccine as tested in squirrel monkeys. D.L. Pierce, A.G. Sacco and W.R. Dukelow. Proc. Michigan Academy of Science. 1985.

3) Laparoscopic observation of the mouse uterus. W.R. Dukelow, D.L. Pierce, J.A. Kontio and L. Martin. Proc. Michigan Academy of Science.1985.

4) The effect of a ZP-3 antigen vaccine on the fertility of squirrel monkeys (Saimiri sciureus). D.L. Pierce, A.G Sacco, M.G. Subramanian and W.R. Dukelow. Proc. American Society of Primatology. 1985.

5) In vitro fertilization normality of squirrel monkey (Saimiri sciureus) embryos. W.R. Dukelow, D.L. Pierce, W.E. Roudebush, J.K. Graham and T. Asakawa. Proc. Fourth World Congress on In Vitro Fertilization. 1985.

6) Ovaries remain functional in squirrel monkeys immunized with porcine zona pellucida (ZP). A.G. Sacco, M.G. Subramanian, E.C. Yurewicz, D.L. Pierce and W.R. Dukelow. Society for Gynecological Investigation. 1986.

7) In vitro fertilization in nonhuman primates. W. R. Dukelow, W.E. Roudebush, D.L. Pierce, J.K. Graham and K. Sengoku. Proc. International Primatological Society. 1986.

8) Immunization of squirrel monkeys with porcine zona pellucida (ZP); effect on ovarian function. A.G. Sacco. M.G. Subramanian, E.C.Yurewicz, D.L. Pierce and W.R. Dukelow. Proc. 3rd International Congress of Reproductive Immunology. 1986.

VITA

Name:	Donna Lynn Pierce
Born:	August 9, 1961
Birthplace:	Washington, D.C.
Education:	Dulaney Senior High School Timonium, Maryland
	Michigan State University East Lansing, Michigan
Degrees Received:	Bachelor of Science (1984) Michigan State University