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UNIVERSITY OF SOUTHAMPTON

IMMUNOLOGICAL STUDIES OF RAT METRIAL GLAND CELLS

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by

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ABSTRACT

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IMMUNOLOGICAL STUDIES OF RAT METRIAL GLAND CELLS

by Robert Ian Craggs

Using a collagenase digestion technique, viable single cell suspensions have been obtained from metrial glands of the pregnant rat uterus and from metrial glands of the deciduomata of the pseudopregnant rat uterus. The cell suspensions have been examined for a series of immunological markers to help define a functional role for the metrial gland.

Immunoglobulin (Fcγ) receptors have been detected on the fibroblast-like stromal cells of the metrial gland using an EAγ rosetting technique in which SRBCs were sensitised with rabbit or rat anti-SRBC IgG. Considerably more cells formed EAγ rosettes with rabbit sensitised SRBCs than with rat sensitised SRBCs. Metrial gland cells from day 13 multiparous animals formed significantly more EAγ rosettes than cells from day 13 uniparous animals. Rosette formation with both sensitising antibodies was inhibited by low concentrations of monomeric or heat aggregated IgGs. Rabbit IgG inhibited rosette formation by rabbit sensitised SRBCs to a greater extent than rat IgG and rat IgG inhibited rosette formation by rat sensitised SRBCs to a greater degree than rabbit IgG. EAγ rosette formation by peritoneal macrophages was only inhibited with heat aggregated IgGs.

Surface IgG has been detected on a small, but significant, proportion of metrial gland cells using an Fabγ fraction of a rabbit anti-rat IgG antibody. This finding has been discussed in relation to the idea that granulated metrial gland cells are derived from lymphocyte-like precursors.

Cytoplasmic IgG has been located in cell smears from the metrial gland, using the indirect fluorescent antibody technique, and the number of positive cells has been quantified. This number showed a close correlation with the number of cells containing diastase-fast PAS positive granules in cell smears. Recent observations on tissue sections revealed that it is the granulated metrial gland cells which contain the IgG.

The Fcγ receptors, surface IgG and cytoplasmic IgG all appear to be on different cell types and there do not appear to be any differences between the metrial gland cells observed in pregnancy and those observed in the deciduomata of pseudopregnancy. Several possible functions are proposed for the metrial gland and are discussed in relation to the immunology of pregnancy.

Abbreviations

ADCC - antibody dependent cellular cytotoxicity
AFP - alphafoetoprotein
C' - complement
Con A - concanavalin A
CMI - cell mediated immunity
DAP - decidualisation associated protein
DNase - deoxyribonuclease
DNP - α-2,4-dinitrophenyl-polylysine
E - erythrocyte
EAγ - erythrocyte-antibody (IgG)
EAμ - erythrocyte-antibody (IgM)
EAC - erythrocyte-antibody-complement
EPF - early pregnancy factor
Fabγ and $F(ab')_2$ - antigen binding fractions of IgG
Fcε, Fcγ and Fcμ regions - C terminal constant regions of Ig heavy chains
FCS - foetal calf serum
FITC - fluorescein isothiocyanate
GAA/E - glacial acetic acid in ethanol
GVH - graft versus host
HBSS - Hank's balanced salt solution
hCG - human chorionic gonadotrophin
hPL - human placental lactogen
Ig - immunoglobulin
Classes IgE, IgG and IgM.
For structure see Winklehake (1978)
MES - 2-(N-morpholino)-ethane sulphonic acid
MHC - major histocompatibility complex
In man = HLA
In mice = H-2
In rats = Ag-B
MIF - migration inhibition factor
MLC - mixed lymphocyte culture
MP - multiparous
NBF - neutral buffered formalin

PAM - pregnancy associated α_2-macroglobulin
PAP - peroxidase anti-peroxidase
PAS - periodic acid Schiff
PBS - phosphate buffered saline
PFC - plaque forming cells
PG-E - prostaglandin-E
PHA - phytohaemagglutinin
PPD - purified protein derivative
PZP - pregnancy zone protein
$\hat{s}$ - sensitised
SAMC - saturated alcoholic mercuric chloride
SEM - standard error of the mean
SIg - surface immunoglobulin
SRBC - sheep red blood cell
TA - trophoblast antigen
TBM - trophoblast basement membrane
UV - ultraviolet.

Chapter 1.

Introduction

Chapter 1

Introduction

The immunological success of pregnancy relates to the ability of the foetus to establish contact with the mother without its antigenically foreign tissues inducing a tissue rejection reaction in the mother.

Recently the metrial gland of the pregnant rat uterus has been implicated as one of the features which might be involved in the immunological complexities of pregnancy. This idea is based on a number of observations. Firstly, it has been suggested that the granulated cells of the metrial gland may be derived from lymphocyte-like precursors. Secondly, immunoglobulin has been detected in the granulated metrial gland cells and thirdly, immunoglobulin (Fcγ) receptors have been found on fibroblast-like stromal cells isolated from the metrial gland. However, the metrial gland may not be solely related to the immunological success of pregnancy because a similar structure develops in the deciduomata of pseudopregnancy.

It is the intention of the work reported here to characterise cells isolated from the rat metrial gland in an attempt to understand its function. Initially a comparison of the number of granulated metrial gland cells in histological sections and in single cell suspensions was made to assess the validity of using single cell suspensions *in vitro* to make deductions about cells *in situ*. An examination was then made for a series of immunological markers in an attempt to identify any similarities between the cells of the metrial gland and other cell types, particularly those of the haemopoietic or lymphoid series. Observations on the presence of immunoglobulin (Fcγ) receptors have been extended to include work on metrial gland cells from multiparous animals and from the metrial gland of the deciduomata of pseudopregnancy. Further characterisation of the immunoglobulin (Fcγ) receptors has involved observations on the inhibitory effects of normal rat sera and native or heat aggregated immunoglobulins on the ability of the cells to form erythrocyte-antibody (EAγ) rosettes. Finally smears of cell suspensions have been examined for their ability to bind antibodies directed against immunoglobulin and other serum proteins.

Chapter 2

Literature survey

Chapter 2

Literature survey

The metrial gland of the rat and the mouse

The metrial gland of the pregnant rat uterus arises in the mesometrial triangle at about day 9 of pregnancy (Dickson and Bulmer, 1961). Differentiation proceeds and reaches a maximum in cell numbers by day 14 (Larkin and Schultz, 1968) after which time there is a regression towards parturition at approximately day 22 of pregnancy (Baker, 1948). Two types of cell can be characterised in the metrial gland. The most obvious of these is the granulated metrial gland cell, described in the rat by Weill (1919) and Gérard (1925). These cells measure up to 25μm in diameter (Peel and Bulmer, 1977) with one or two nuclei (Gérard, 1925), which are round or kidney shaped (Smith, 1966b). Their cytoplasm contains a large amount of glycogen (Gérard, 1925) and clusters of diastase-fast periodic Schiff (PAS) positive granules (Wislocki et al.,1957). These granules measure between 1 - 2μm in diameter (Peel and Bulmer, 1977). The other major cell type in the metrial gland is a fibroblast-like stromal cell. These two cell types do not account for all the cells present and the other cell types will be discussed later with reference to the possible origin and function of the metrial gland. The cellular and vascular components of the metrial gland are supported by a connective tissue matrix composed mainly of collagen fibres (Dixon and Bulmer, 1971; Larkin, 1972).

There are no reports of granulated metrial gland cells in the non-pregnant rat uterus. Granulated metrial gland cells are present in the mesometrial decidua (the decidua basalis) by day 7 of pregnancy and are first seen in the mesometrial triangle by day 8 (Baker, 1948). During all stages of its existence the rat metrial gland is a well vascularised structure and there are reports of granulated metrial gland cells being seen within the lumina of maternal blood vessels (Baker, 1948; Bridgman, 1948; Peel and Stewart, 1979), and from day 9 of pregnancy granulated metrial gland cells have been seen within the ectoplacental cone and in the cone-shaped area adjacent to the apex of the ectoplacental cone (Dickson and Bulmer, 1961).

In the mouse, cells similar to those described in the rat were first observed by Jenkinson (1902). The nuclear and cytoplasmic features of the granulated metrial gland cells in the mouse are essentially similar to those described for the rat, with the exception that the cells can measure up to 50μm in diameter and the granules can measure up to 5μm

in diameter (Stewart and Peel, 1977). In the mouse, granulated metrial gland cells are found in the pre-implantation uterus from day $2\frac{3}{4}$ of pregnancy, and following implantation on day 4 of pregnancy, they are observed in large numbers in the mesometrial decidua (Smith, 1966a; Stewart and Peel, 1978). As in the rat, granulated metrial gland cells are seen within the lumina of maternal blood vessels in the metrial gland and the decidua basalis. They are also present in the maternal blood spaces around the trophoblastic giant cells and in the labyrinthine placenta (Smith, 1966a; Stewart and Peel, 1978).

The main difference between the metrial gland of the mouse and the rat is that in the mouse the metrial gland forms only a small flattened region at the apex of the mesometrial triangle (Finn and Porter, 1975) whereas in the rat it is a discrete entity above the decidua basalis. In the mouse the metrial gland and the decidua basalis persist together until parturition, although they undergo extensive degeneration in the latter stages of pregnancy (Jenkinson, 1902). In the rat, however, the decidua basalis has degenerated by day 17 of pregnancy (Holmes and Davis, 1948; Dickson and Bulmer, 1960) leaving the metrial gland overlying the fibrinoid capsule and the giant cells of the junctional zone trophoblast (Bulmer and Dickson, 1961).

Granulated cells of other species

Cells similar to the granulated metrial gland cells of the pregnant rat uterus have been found in the pregnant hamster uterus (Orsini, 1954; Pijnenborg et al., 1974; Peel et al., 1979b) and the pregnant rabbit uterus (Ancel and Bouin, 1911; Mossman, 1926; Selye and McKeown, 1935). Granulated cells with a similar structure and distribution to those of the pregnant rodent uterus have also been found in some insectivors (van der Horst, 1950). However, in none of these species does there appear to be a discrete area of metrial gland tissue.

Whether or not the endometrial granulocytes described in the human (Asplund and Holmgren, 1947) and the monkey (Bartelmez et al., 1951) are similar to the granulated metrial gland cells in the rat remains to be established. However, there are two major differences. Firstly, in both the human and the monkey, endometrial granulocytes are found in the normal menstrual cycle as well as during pregnancy (Asplund and Holmgren, 1947; Bartelmez et al., 1951). Secondly, as described by Cardell et al. (1969) in the monkey, these cells are only about 10μm in diameter with cytoplasmic granules of around 0.5μm in diameter. The granules of the monkey granulocytes were found to be diastase-resistant PAS positive

(Dallenbach-Hellweg et al., 1966) and Cardell et al. (1969) suggested that these cells were equivalent to the granulated metrial gland cells of the rat. A similar assumption was made by Jaeger and Dallenbach-Hellweg (1969) about the endometrial granulocytes in the human, based on ultrastructural observations, despite the fact that human endometrial granulocytes were PAS negative (Dallenbach-Hellweg and Nette, 1964). Recently there has been a report that describes PAS positive granules in human endometrial granulocytes (Bulmer et al., 1980). There is, however, no discrete area of metrial gland tissue in either species.

The metrial gland of the deciduomata of pseudopregnancy

After induction of deciduomata in pseudopregnant rats, the differentiation that occurs at the mesometrial aspect of the uterus and within the mesometrial triangle parallels the formation of the decidua basalis and the metrial gland of normal pregnancy (Selye and McKeown, 1935; Wolfe and Wright, 1942; Velardo et al., 1953). The metrial gland of deciduomata reaches its largest size at day 14 of pseudopregnancy (Lobel et al., 1965), which is similar to that reported for the metrial gland of normal pregnancy (Larkin and Schultz, 1968). Peel et al. (1979a) have confirmed that the ultrastructure of the granulated cells of the deciduomata is also similar to that described in normal pregnancy.

The origin and function of the metrial gland cells

The origin of the granulated metrial gland cells is the subject of some controversy. It was proposed that some of the granulated cells may have been derived from a foetal origin (Dickson and Bulmer, 1961). However, their presence in the deciduomata of pseudopregnancy (Velardo et al., 1953) means that many must be of maternal origin. Some reports suggest that the granulated metrial gland cells probably migrate to the metrial gland from the decidua basalis, where they first appear in considerable numbers (Selye and McKeown, 1935; Dickson and Bulmer, 1961). However, it is now generally agreed that the granulated metrial gland cells arise in situ in the decidua basalis and in the metrial gland from precursor cells. It is the identity of these precursor cells about which there has been debate. Larkin and Schultz (1968), in support of early workers (Baker, 1948; Ellis, 1957), proposed that the precursor cells of the granulated cells arose from the fibroblast-like stromal cell

population. Larkin and Cardell (1971) supported this hypothesis and proposed that the precursor cell was small and round, with a large nucleus, a limited amount of cytoplasm containing many ribosomes and capable of glycogen synthesis. However, no evidence was presented which could link the precursor cells to the fibroblast-like stromal cells. Dixon and Bulmer (1971) proposed that the two main cell types of the metrial gland were both derived from a divergent line of undifferentiated connective tissue cells, but no evidence was presented to support this hypothesis.

An alternative hypothesis for the origin of the granulated metrial gland cells was proposed by Smith (1966b) who suggested that the precursor might be a cell of the lymphocytic series. This observation was illustrated by camera lucida drawings of the cell types present in the metrial gland. More recently the ultrastructure of a series of cells has been described which may represent stages in the differentiation of granulated metrial gland cells from lymphocyte-like precursors. These observations have been made on the uteri of pregnant rats (Peel and Bulmer, 1977), pseudopregnant rats (Peel et al., 1979a), pregnant mice (Stewart and Peel, 1977) and pregnant hamsters (Peel et al., 1979b).

The function of the granulated metrial gland cells, whether they are within the metrial gland itself, in the decidua basalis or the trophoblast still remains to be established. A number of authors have suggested that they might provide nutrition, in the form of glycogen, for the developing embryo (Jenkinson, 1902; Gérard, 1925; Selye and McKeown, 1935; Baker, 1948). Selye and McKeown (1935) provided supportive evidence by showing an accumulation of granulated metrial gland cells subsequent to the removal of the foetus from the implantation site. Ancel and Bouin (1911) were the first to propose an endocrine function for the granulated cells. Since then many other workers have proposed a variety of functions for possible holocrine secretions from granulated metrial gland cells. A role in the involution of the uterus related to the degenerative processes of late pregnancy and the immediate postpartum period has been proposed (Baker, 1948; Ellis, 1957; Larkin, 1972; Peel and Stewart, 1979). Numers (1953) suggested that granulocytes had a role in preventing the coagulation of menstrual blood in the human uterus. Ancel and Bouin (1911) and Dickson and Bulmer (1961) proposed a role for the metrial gland cells in preparing the mammary glands for lactation or possibly a role in maintaining the corpora lutea of pregnancy (Dickson and Bulmer, 1961;

Smith, 1966b). Bloch (1964) suggested that the holocrine secretion may disrupt the circular smooth muscle fibres at the base of the mesometrium to allow differentiation of the decidua basalis and the metrial gland.

The hypothesis that the metrial gland may produce relaxin was first proposed by Bridgman (1948) and has been supported by a number of authors (Velardo et al., 1953; Wislocki et al., 1957; Dallenbach and Dallenbach-Hellweg, 1964; Dallenbach-Hellweg et al., 1965). Dallenbach and Dallenbach-Hellweg (1964) and Dallenbach-Hellweg et al. (1965) proposed that the relaxin is involved with the dissolution of connective tissue fibres within the uterus during pregnancy as well as serving a similar function during menstruation in the human uterus. These workers used a fluorescent antibody technique to detect the relaxin in human endometrial granulocytes and rat granulated metrial gland cells. However, there are several observations which are not consistent with the production of relaxin by granulated metrial gland cells. Dallenbach-Hellweg et al. (1965) were unable to obtain positive fluorescent staining for relaxin on rat ovary using their antiserum, despite the fact that the ovary has been shown to be the richest source of relaxin (Steinetz et al., 1959). In addition extracts of metrial gland cells have been reported to lack relaxin activity (Bloom et al., 1958; Larkin, 1974). Therefore it would seem likely that if there is relaxin present in the granulated metrial gland cells, it is present in an inactive form (Larkin, 1974).

In an attempt to find a possible function for the granulated metrial gland cells they have been examined for certain enzymes. It has been shown that the granules are associated with various hydrolytic enzymes, including non-specific esterases (Bulmer, 1968; Stewart, 1980) and it is proposed that these might serve a lysosomal function (Peach and Bulmer, 1965). However, phagocytosis is not a predominant feature of the granulated metrial gland cells (Bridgman, 1948; Sharma and Peel, 1979) and it is difficult to visualise a purely intracellular activity for these enzymes (Bulmer, 1968).

The most recently held view about a function for the cells of the metrial gland is that they might play a role in the immunology of pregnancy. This idea was first proposed by Smith (1966b) after she had traced a possible origin for the granulated metrial gland cells from cells of the lymphocytic series and the morphological evidence of Peel and co-workers has supported these ideas. The metrial gland may be

further implicated in the immunology of pregnancy by the report of cytoplasmic immunoglobulin (Ig)G in some of the cells of the metrial gland of normal pregnancy (Bulmer and Peel, 1977a, b). These IgG positive cells were later identified as granulated metrial gland cells (Mitchell et al., 1980). Bernard et al. (1977) also reported the presence of cytoplasmic IgG in cells of the mouse decidua, but they did not identify the cells.

Further support for a possible immunological role for the metrial gland in pregnancy has come from the observation that some cells display surface receptors for the Fcγ region of IgG (Bray et al., 1978). These Fcγ receptors were detected on cells in suspension using an erythrocyte-antibody (EAγ) rosetting technique. Using the PAS reaction, after digestion of glycogen with diastase, on 1μm sections prepared from embedded suspensions of rosetting cells it was shown that the Fcγ receptors were on a proportion of the fibroblast-like stromal cells, and were not present on the granulated metrial gland cells (Bray et al., 1978). Further characterisation of the Fcγ receptor positive cells (Bray and Craggs, 1979) revealed that they resemble Fcγ receptors on certain mouse decidual cells (Bernard et al., 1978; Dillon, 1979), yolk sac endodermal cells in the rabbit (Wild and Dawson, 1977) and the mouse (Elson et al., 1975), and certain human foetal placental cells (Jenkinson et al., 1976; Johnson et al., 1976; Matre and Johnson, 1977).

Immunology and pregnancy

In view of the potential immunological role of the metrial gland in the maintenance of the foetal allograft during pregnancy it is necessary to summarise briefly some of the observations about the immunocompetence of the mother during pregnancy. The events which precede implantation and the associated immunological changes are reviewed by Beer and Billingham (1976) and Johnson (1976).

The implantation of the blastocyst into the hormonally prepared wall of the uterus is a naturally occurring 'tissue transplant' in which the embryo, with its range of paternally inherited histocompatibility antigens, should elicit an immunological rejection reaction. However, the embryo survives in the uterus for a period in excess of that normally required for a rejection reaction to occur. Therefore it would appear that some form of immunoregulatory mechanism suppresses the maternal rejection reaction against the histo-incompatible foetus (Medawar, 1953).

The criteria for a primary allograft rejection reaction have been summarised by Billington (1979). Alien histocompatibility antigens on the graft need to be presented in sufficient quantity and strength for recognition to occur via an established vascular pathway so as to produce an effective immune response by cells of the host. The resulting cellular response requires access to the antigenic sites on the graft. It can be inferred that if any part of this series of events is blocked or missing then the response is reduced or prevented.

Examination of mouse embryos from the 2 cell stage to the pre-implantation blastocyst have shown that H-2 and non-H-2 (major and minor) histocompatibility antigens are present before implantation (Palm et al., 1971; Muggleton-Harris and Johnson, 1976; Krco and Goldberg, 1977). Histocompatibility antigens are also present on the post-implantation embryo and placenta, but there seems to be some differential expression of these antigens on trophoblast cells according to the stage of pregnancy examined (Sellens, 1977; Sellens et al., 1978; Carter, 1978). Despite the presence of the histocompatibility antigens it seems that the trophoblast may be capable of protecting the embryo from being rejected. The classic experiments of Kirby (1960; 1962) have shown that allogeneic trophoblast cells and blastocysts transplanted to extra-uterine sites develop without rejection: even xenogeneic grafts survive without being rejected (Kirby, 1962). Whether it is because of the sensitisation of the mother, as a result of shedding of trophoblast cells into the maternal circulation (Beer and Billingham, 1971) which relates to the ability to prevent cellular recognition or whether the histocompatibility antigens are too weak or of too low a density remains to be established.

Maternal immune responses during pregnancy

That the mother responds to paternal antigens on foetal tissues is inferred from the fact that there is an increase in the weight of the para-aortic lymph nodes draining the rodent uterus during pregnancy (Beer and Billingham, 1971; Maroni and de Sousa, 1973; Forster et al., 1979; Chatterjee-Hasrouni et al., 1980). This increase is more marked in allogeneic pregnancies than in syngeneic pregnancies. There is also a gradual increase in the weight of the spleen in both syngeneic and allogeneic pregnancies (Maroni and de Sousa, 1973; Forster et al., 1979; Chatterjee-Hasrouni et al., 1980). Reports on the weight of the thymus show considerable variation, with Maroni and de Sousa (1973) and

Chatterjee-Hasrouni et al. (1980) observing a decrease in weight as pregnancy proceeds, whereas Forster et al.(1979) observe an increase in the weight of the thymus as pregnancy proceeds. Allogeneic lymph node cells and spleen cells from multiparous mice show an increased ability to mount a graft versus host (GVH) reaction when injected into paternal strain mice (Sorens, 1967) indicating that the response is cumulative. Sera from multiparous mice can sometimes enhance the growth of tumours from paternal strain mice (Kaliss and Dagg, 1964) and haemagglutination tests also show that pregnant mice have antibodies to paternal H-2 antigens (Herzenberg and Gonzales, 1962). Recently it has been shown that mice pregnant to allogeneic males do not only produce a rejection reaction but they also show an enhancing reaction to paternal antigens (Chaouat et al., 1979a). In fact the general rule appears to be that the pregnant female produces both immunoglobulins and lymphocytes as a response to paternal antigens.

The protective role of the immune response in pregnancy involves the production of enhancing antibodies (Chaouat et al., 1979b) and suppressor T cells (Chaouat and Voisin, 1980). Enhancing antibodies can be eluted from the murine placenta and have been shown to be of sub-class IgG_1. These antibodies have activity directed against paternal antigens in that they can rebind in vitro to placentae and thymocytes bearing paternal antigens (Chaouat et al., 1979a). In mice, it has been shown that the amount of IgG detected within the placenta increases with multiparity (Voisin and Chaouat, 1974). This placental bound IgG is capable of enhancing paternal strain tumours growing in the maternal strain (Chaouat et al., 1979a). A similar blocking IgG molecule has been shown to be present in the sera of pregnant women and is absent from the sera of women who chronically abort (Rocklin et al., 1976).

The production of a suppressor cell response has been supported by the observation that maternal spleens contain cells which when present in small numbers promote graft rejection but when present in large numbers enhance tumour survival (Chaouat et al., 1979a). These cells have been found to be a sub-set of T lymphocytes (Chaouat et al., 1979a; Chaouat and Voisin, 1980; Suzuki and Tomasi, 1979a) and a sub-set of non-T cells (Suzuki and Tomasi, 1979a). The T cells are capable of suppressing the late phase of mixed lymphocyte cultures (MLC)(Chaouat and Voisin, 1980) while both sub-sets can reduce the numbers of plaque

forming cells (PFC) producing IgM in a primary antibody response to sheep red blood cells (SRBC) both in vivo and in vitro (Suzuki and Tomasi, 1979a). Further evidence to support the role of T cells in the maternal response to pregnancy is shown by the effect of anti-thymocyte serum on pregnancy. Rats injected with anti-thymocyte serum one week before mating in general produce no young and show a complete lack of development of foetal, placental or decidual tissues, including the metrial gland (Gusdon, 1971). Smith and Powell (1977) have shown that female mice of the C57B strain can be made tolerant to skin grafts of male H-Y antigens by multiparity and this tolerance can be transferred to virgin females by thymocytes, but not by B lymphocytes or serum. However, in the athymic nude (nu/nu) mouse, pregnancy proceeds with both maternal decidua and metrial gland developing as normal (Stewart and Peel, 1980). Despite the lack of a thymus it is generally agreed that some T cells are present in the nude mouse (Raff, 1973; Loor et al., 1975; Scheid et al., 1975).

Of interest to cell suppression systems is the fact that when human maternal lymphocytes are allowed to react with lymphocytes of the foetus in MLC their reactivity is only half that seen between maternal and paternal lymphocytes (Granberg et al., 1979; Birkland and Kristoffersen, 1980a). Both sets of authors conclude that the reduced response of the mother to her child is based on the one haplotype difference between the mother and foetus rather than the occurrence of specific tolerance. There is further reduction in the MLC response of maternal lymphocytes to her child's lymphocytes when the MLC is carried out in autologous serum. These observations correspond to a decreasing response during pregnancy or an increasing tolerance towards the foetal allograft (Birkland and Kristoffersen, 1980a). Birkland and Kristofferson (1980b) also present evidence from in vitro studies that there is an increased stimulation of maternal peripheral blood cells by concanavalin A (Con A) during pregnancy. Con A has been accepted as a stimulator of T cells (Stout and Herzenberg, 1975). Conversely, responses to purified protein derivative (PPD) and other B cell stimulants were lower than seen in non-pregnant female blood (Birkland and Kristoffersen, 1980b). From these observations it is proposed that suppressor T cell function is increased and B lymphocyte function is decreased during pregnancy (Birkland and Kristoffersen, 1980b). Indirect evidence that there is an increased suppressor T cell function during pregnancy comes from Gall (1977) who reports that there is altered cell mediated immunity (CMI)

during pregnancy, such that pregnant women have increased susceptibility to certain diseases. However, Rocklin et al. (1979) report that the various components of the maternal immune system are essentially intact during pregnancy.

Beer et al. (1976) have proposed the existence of enhancing antibodies and suppressor T cells in multiparous mice to explain the occurrence of suppressed haemagglutinin formation and weakened cellular immunity to paternal strain allografts. However, this suppression is only induced by normal pregnancies in utero; allogeneic intrarenal pregnancies lead to normal haemagglutinin synthesis. Therefore it appears that both the site and route of antibody presentation are important to the host's response and as such the absolute degree of immunosuppression induced in the mother during pregnancy still remains to be established.

Beer and Billingham (1974) have reported that skin allografts transplanted to the pre-implantation uterus or the pseudopregnant uterus in both the rat and the mouse induce a decidual reaction, but are still rejected and in fact this rejection reaction is only delayed by a few days when compared to a normal orthotopic allograft. In contrast Dodd et al. (1980) report that skin allografts placed in a blastocyst free decidualised horn of a rabbit survive at least twice as long as orthotopically placed allografts, nor do they show signs of being rejected. Beer and Billingham (1974) and Dodd et al. (1980) both agree that decidual tissue provides no protection to intra-uterine grafts in a pre-sensitised host. It appears that one of the roles played by the decidua is to restrict the penetration of the invasive trophoblast, such that trophoblast transplanted to the spleen, kidney or testis grows as a highly invasive tissue within the host tissue for some days longer than it would in utero (Kirby and Cowell, 1968). McLean and Scothorne (1970) have shown that most of the uterine endometrium in the rabbit lacks a lymphocytic drainage. Therefore it may be that the decidua ensures that the antigens are presented via an intravenous route; a mechanism which may favour the production of enhancing antibodies (Dodd et al., 1980).

Antigens in relation to the morphology of the pregnant uterus

Amongst the mammals there is considerable variation between species in the precise arrangement of the foetus and its membranes (Steven and

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Morriss, 1975). Even within the same species there is a changing morphology so that as development progresses different membranes come into direct contact with the maternal circulation. Thus in considering the antigens which may have provoked the maternal immune response it is necessary to bear in mind their relationship to the maternal circulation (Figs. 1 and 2).

a) Histocompatibility antigens

In the murine placenta, paternally derived major histocompatibility (H-2) antigens have been shown to be accessible to the maternal circulation by the absorption of monoclonal anti-foetal H-2 antibody from the maternal circulation (Wegmann et al., 1979). This process was shown to be Fc independent and it is suggested that the antigens are on the materno-foetal interface. In vitro cell culture systems have shown that murine trophoblast cells possess H-2 antigens (Sellens, 1977; Sellens et al., 1978; Chatterjee-Hasrouni and Lala, 1979) and both maternal and paternal antigens (Carter, 1978) as detected by mixed haemadsorption. H-2 and non-H-2 histocompatibility antigens have been found on the mouse yolk sac endoderm (Jenkinson and Billington, 1974) at day 14 of pregnancy when the yolk sac is in direct contact with the maternal tissues of the uterus, but not in direct contact with the maternal circulation.

Ferguson and Palm (1977) were unable to find major histocompatibility antigens (Ag-B) on the trophoblastic giant cells or the cytotrophoblast cells of rat placentae, cells which are in direct contact with either the maternal circulation or maternal tissue. Minor histocompatibility antigens (non-Ag-B) were detectable on cytotrophoblast cells and Ag-B antigens were only detected on a population of fibroblasts (Ferguson and Palm, 1976; 1977).

Immunofluorescent studies on immature and mature human placentae using anti-HLA (Faulk et al., 1977) or anti-β-2-microglobulin (Johnson and Faulk, 1978; Faulk and Johnson, 1977) have failed to label trophoblast cells of human chorionic villi. (β-2-microglobulin is a protein associated with the HLA antigens; Jones et al., 1975.) Positive fluorescence can, however, be detected on stromal cells and the vascular endothelium of the foetal blood vessels. Ogbimi et al. (1979) have also reported a lack of β-2-microglobulin in soluble fractions of human syncytiotrophoblast microvillous plasma membranes.

The fact that major histocompatibility antigens cannot be detected on human trophoblast (Faulk et al., 1977; Faulk and Johnson, 1977; Johnson and Faulk, 1978) or on rat trophoblast (Ferguson and Palm, 1977) is not in agreement with the work on murine placentae (Wegmann et al., 1979). Whether this lack of detection relates to a mechanism for protecting the foetus, or whether the antigens are in some way masked (Kirby et al., 1964) or are only present at concentrations which cannot be detected by existing techniques remains to be established. The expression of histocompatibility antigens by trophoblast cells might in fact be beneficial to the survival of the foetus, in that they may stimulate the production of enhancing antibodies and/or suppressor T cells (Chaouat et al.,1979a). Another possibility is that histocompatibility antigens on the trophoblast may bind potentially harmful antibodies produced by the mother and prevent them from entering the foetal circulation (Voisin and Chaouat, 1974; Chaouat et al., 1979a).

b) Trophoblast specific antigens

Trophoblast specific antigens may be involved in eliciting an immune response for the protection and maintenance of the placenta. Trophoblast specific antigens have been detected on trophoblast, stromal and endothelial cells of human chorionic villi as well as on viable human lymphocytes and certain human cell lines (Faulk et al., 1978). By the use of absorption studies, Faulk et al. (1978) define two categories of trophoblast membrane antigens, trophoblast antigen one (TA_1) present only on trophoblast cells and certain human cell lines and TA_2 present on trophoblast, stromal and endothelial cells and lymphocytes. The hypothesis is proposed that in order to maintain normal pregnancy an antibody response to TA_2 is necessary and the resultant antibodies block the recognition and cytotoxic potential of the TA_1 antigens (Faulk et al., 1978). The presence of a blocking antibody needed to maintain human pregnancy is similar to that reported by Rocklin et al. (1976). TA_1 and TA_2 are not of the HLA system (Faulk and Johnson, 1977; Faulk et al., 1977). Instead it appears that the trophoblast antigens are species and organ specific (Faulk et al., 1979) but cross react with certain transformed cell lines and certain lymphocyte surface antigens (Faulk et al., 1979; McIntyre and Faulk, 1979). Recently it has been shown that some lymphoblastoid cells from human bone marrow, thymus and peripheral blood express on their surfaces certain human placental cell

Figures 1 and 2

Fig. 1: Diagrammatic representations of transverse sections through the implantation site from the pregnant rat uterus showing the relationship of the placenta and foetal membranes to the maternal tissues.

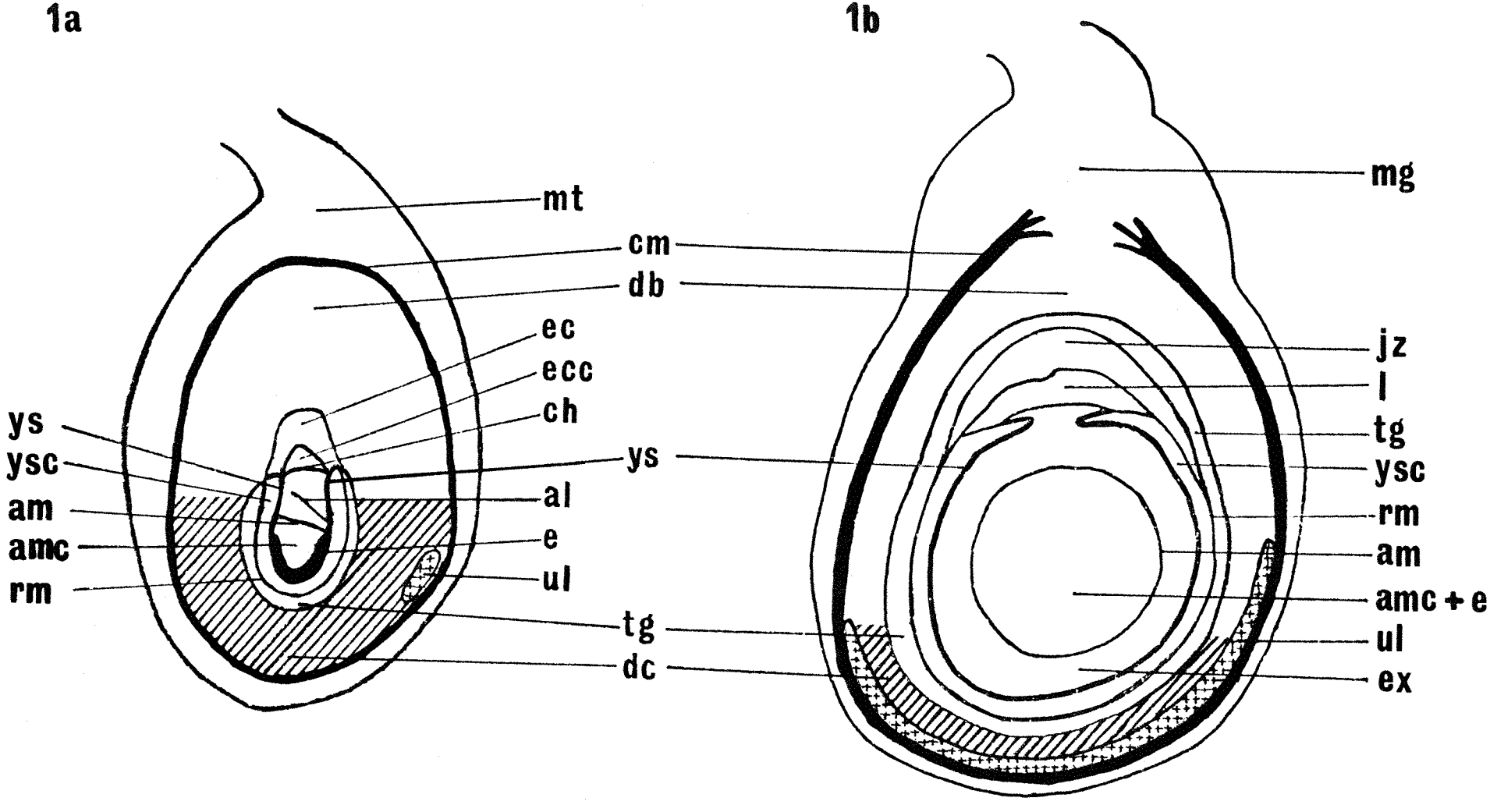
Fig. 1a : day 9 of pregnancy
Fig. 1b : day 12 of pregnancy
Fig. 1c : day 15 of pregnancy
Fig. 1d : day 19 of pregnancy.

Crosses = uterine lumen.

Cross-hatching = decidua capsularis.

al. allantois; alm. allantoic mesoderm; am. amnion; amc. amniotic cavity; ch. chorion; cm. circular muscle; db. decidua basalis; dc. decidua capsularis; e. embryo; ec. ectoplacental cone; ecc. ectoplacental cavity; ep. endovascular plasmodium; ex. exocoel; jz. junctional zone trophoblast; l. labyrinthine trophoblast; mg. metrial gland; mt. mesometrial triangle; rm. Reicherts membrane and parietal yolk sac; tg. trophoblastic giant cells; ul. uterine lumen; yz. visceral yolk sac; ysc. yolk sac cavity.

Adapted from Bulmer and Dixon (1961) and Sharma and Peel (1979).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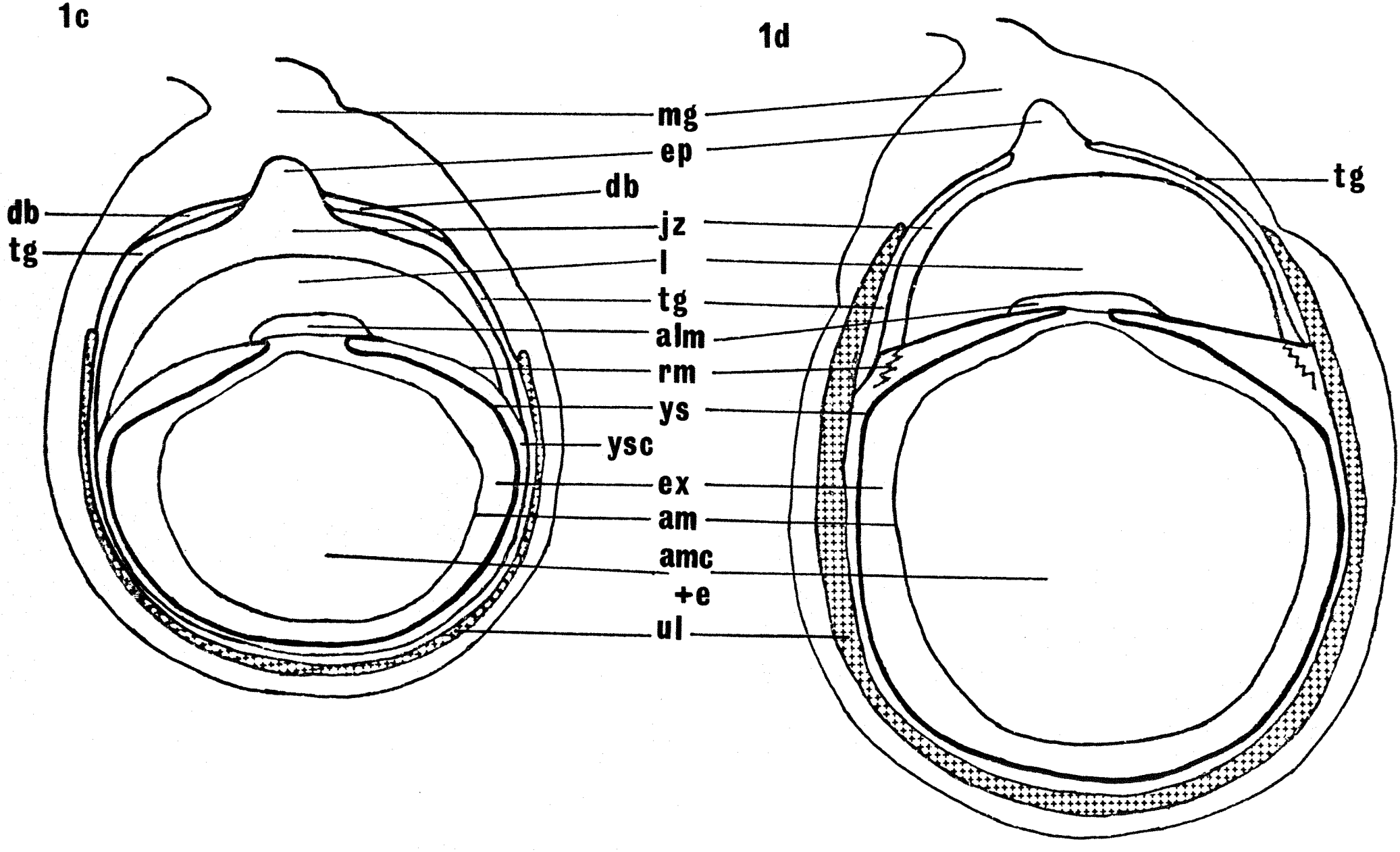
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Fig. 2: Diagrammatic representations of the implantation site in the pregnant human uterus showing the relationship of the placenta and foetal membranes to the maternal tissues and the changes seen in transverse sections of the chorionic villi of the placenta.

Fig. 2a : approx. week 5 of pregnancy

Fig. 2b : approx. week 15 of pregnancy.
The arrangement at term (week 40) is essentially the same as seen here.

Fig. 2c : T.S. chorionic villus week 5 of pregnancy

Fig. 2d : T.S. chorionic villus week 15 of pregnancy

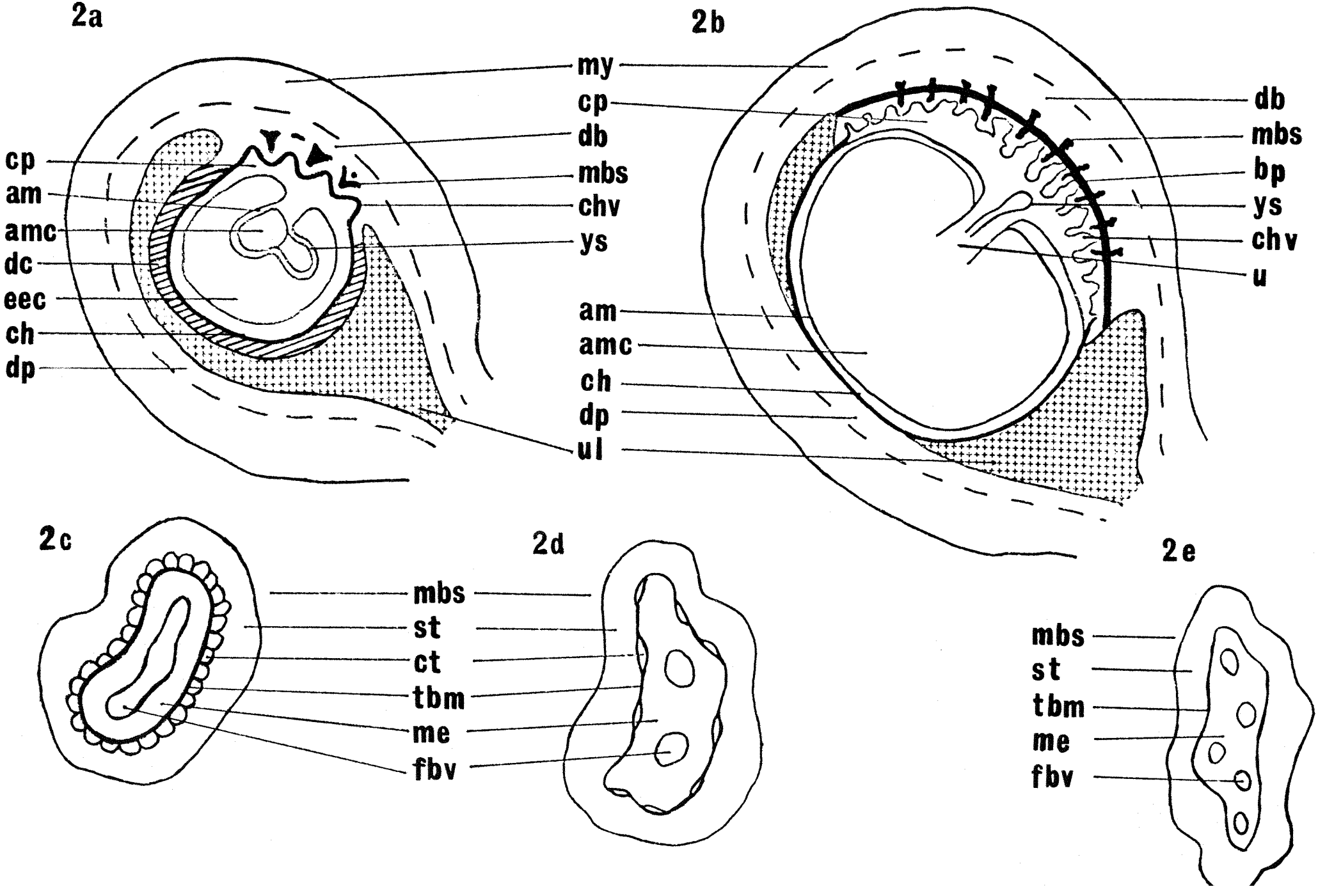
Fig. 2e : T.S. chorionic villus week 40 of pregnancy.

Crosses = uterine lumen

Cross-hatching = decidua capsularis.

am. amnion; amc. amniotic cavity; bp. basal plate; ch. chorion; cp. chorionic plate; chv. chorionic villi; ct. cytotrophoblast; db. decidua basalis; dc. decidua capsularis; dp decidua parietalis; eec. extraembryonic coelom; fbv. foetal blood vessel; mbs; maternal blood space; me. mesenchymal cells, Hofbauer cells and stroma; my. myometrium; st. syncytiotrophoblast; tbm. trophoblast basement membrane; u. umbilicus; ul. uterine lumen; ys. yolk sac.

Adapted from Hamilton et al. (1972) and Steven and Morris (1975).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surface antigens (Hamilton et al., 1980) and the proposition has been made that oncofoetal antigens are involved in the maintenance of the placenta and that they may also be used by neoplastic cells to avoid detection by the host's immune system.

Immunoglobulin, complement and complement receptors in pregnancy

If there are major histocompatibility antigens or trophoblast specific antigens on the placenta then it is reasonable to assume that the mother may respond to them at an immunological level. Therefore the placenta has been examined for components of immune reactions. In particular it has been examined for immunoglobulin, complement and complement receptors. However, the presence of IgG within the placenta or yolk sac must be interpreted with care. This is because many species transfer passive immunity from mother to young, across the placenta or yolk sac, via an Fcγ receptor mechanism (Billington and Wild, 1979).

IgG and certain components of the complement system have been detected on a variety of tissues during pregnancy. IgG and the third component of complement (C'_3) have been found on the apical aspect of all trophoblast cells in immature villi (Johnson and Faulk, 1978) but not on trophoblastic cells of mature villi (Faulk and Johnson, 1977). In the stroma of the villi in immature placentae there was no detection of IgG or C'_3, but they were found on the fibrinoid areas along with the fourth component of complement (C'_4) (Johnson and Faulk, 1978). By term, however, IgG was detected on the stroma of the villi, but there was no C'_3. IgG and C'_3 were absent from the fibrinoid areas leaving only C'_4 detectable (Faulk and Johnson, 1977). IgG was also found on the trophoblast basement membrane (TBM) of mature placentae (Faulk et al., 1974; Faulk and Johnson, 1977) but only a small amount was detectable on the TBM of immature placentae (Johnson and Faulk, 1978). C'_3 was found on the TBM of both mature (Faulk and Johnson, 1977) and immature (Johnson and Faulk, 1978) placentae. Wood et al. (1978b) localised IgG on the trophoblastic villi at all stages of pregnancy, but proposed that the positive area for IgG was the area of cells beneath the TBM rather than the TBM, which only stained positively for C'_3. Localisation of IgG within the stromal and fibrinoid areas reported by Wood et al. (1978b) was similar to that described by Faulk and Johnson (1977) and Johnson and Faulk (1978).

Elution of IgG from human placentae showed specific binding to TBM, but not to basement membrane of lung, thyroid or kidney (Faulk et al., 1974). This could relate to either the presence of placental specific antigens within the TBM or to the observation of Wood et al. (1978b) that the eluted IgG may be bound to cells beneath the TBM rather than to the TBM itself. IgG eluted from human placentae has been shown to be of maternal origin and to inhibit MLC: the blocking activity resided in the $F(ab')_2$ region of the antibody (Faulk et al., 1974). Gusdon (1971) has reported the existence of a pregnancy specific antibody to human placental lactogen which is a non-cytotoxic, non-complement fixing IgM. This is an unusual report considering that Johnson et al. (1976) were unable to show any IgM bound to human placentae, but one which may still have a consequence in the immunological enhancement of pregnancy (Gusdon, 1971).

In the mouse, IgG has been detected in the implantation site and shows variable distribution as development proceeds (Bernard et al., 1977). In particular the trophoblast at day 5 of pregnancy shows both surface and cytoplasmic IgG. On day 7 of pregnancy the embryo and ectoplacental cone are negative but the visceral endoderm, Reichert's membrane, the trophoblast giant cells and the outer part of the decidual capsule are all positive for IgG. At day 8 - 10 of pregnancy some decidual cells are also positive for IgG (Bernard et al., 1977). In the decidua and metrial gland of the rat it is the granulated metrial gland cells which have been shown to be positive for cytoplasmic IgG (Mitchell et al., 1980). Whether or not the immunoglobulin in the rodent uterus serves an immunosuppressive function remains to be established. Dillon (1979) has reported the appearance of complement receptors on murine decidual cells, after 24 hours in culture, from day 13 and day 14 of pregnancy. At earlier stages of pregnancy complement receptors are absent from murine decidual cells (Bernard et al., 1978; Dillon, 1979). There have been no reports of complement in the pregnant rodent uterus.

Fcγ receptors in pregnancy

There are two key roles proposed for the function of Fcγ receptors in pregnancy. They are thought to be involved in the selective transfer of immunity from the mother to her young (Brambell, 1970). The foetus receives protective antibodies from its mother so that it can combat infections it may encounter on leaving the uterus whilst it undergoes its

own immunological education (Billington and Wild, 1979). Fcγ receptors may also bind soluble antigen-antibody complexes in a way that could cause masking of antigens on the cell surface, thereby preventing access of lymphocytes in the cell mediated immune response (Sjogren et al., 1971; Kerbel and Davis, 1974; Elson et al., 1975). A similar masking mechanism has been proposed for the survival of certain tumours which promote the production of enhancing antibodies (Hellström and Hellström, 1974).

a) Yolk sac

The Fcγ receptors on yolk sac endodermal cells of murine (Elson et al., 1975) and rabbit (Wild and Dawson, 1977) tissue have been demonstrated using EAγ rosetting techniques on cells obtained by trypsin digestion. These receptors have been shown to be related to the transmission of homologous and heterologous species immunoglobulins across the rabbit yolk sac endoderm. Thus, although bovine IgG is non-selectively endocytosed, it does not bind to the Fcγ receptors and is not transported to the foetal circulation. Human IgG, however, appears to bind to the Fcγ receptors with high affinity and is transported to the foetal circulation (Wild and Dawson, 1977). A similar series of results was presented by Tsay et al. (1980) who used ^{125}I labelled IgG in their binding studies on formalin fixed discs of yolk sac membranes. In contrast to the rosette inhibition study of Wild and Dawson (1977), Tsay et al. (1980) show that the human IgG binds with a lower affinity. One conclusion which can be drawn from these observations is that the binding of different species IgGs to Fcγ receptors on rabbit yolk sac endoderm parallels the route of transmission of IgG from mother to young in that species. In general the IgGs from species which transmit IgG prenatally via the yolk sac or the chorioallantoic placenta (Billington and Wild, 1979) show a greater degree of binding to rabbit yolk sac endoderm than does IgG from species which transmit IgG on a non-selective basis across the post-natal gut (Brandon, 1976). It has also been proposed that the Fcγ receptors on murine yolk sac may bind IgG to prevent antibody or antigen recognition by maternal immune cells and thereby suppress the possibility of a CMI attack (Elson et al., 1975).

b) Placental tissues

Johnson et al. (1975), using fluorescein labelled, heat aggregated, human IgG or soluble immune complexes on cryostat sections of human foetal

placenta, showed binding on only the endothelial cells of the foetal stem vessels within the chorionic villi. There was no observable inhibition of this binding by monomeric IgG and the suggestion was made that the binding had a specificity for altered rather than native IgG. In a continuation study (Johnson et al., 1976) it was shown that the receptor present on the endothelial cells was specific for the heat aggregated Fcγ portion of all four sub-classes of human IgG. There was no binding of native IgG, Fabγ or $F(ab')_2$ or of heat aggregated albumin, IgM or IgA_2. Contrary to these reports Matre et al. (1975) showed that EA complexes bound to the syncytiotrophoblast by an Fcγ dependent mechanism. This EAγ binding was inhibited by both heat aggregated and monomeric IgGs. These features are similar to those described on mouse yolk sac and placenta (Elson et al., 1975). Using refined techniques Matre and Johnson (1977) described two populations of Fcγ receptors in the human placenta. Those on the endothelium of foetal stem vessels were demonstrable by fluorescein conjugated, heat aggregated, IgG and by haemadsorption of EAγ complexes, whereas those on the syncytiotrophoblast were demonstrable only by EAγ binding. The results were essentially the same on first trimester placentae as on full term placentae. The hypothesis was proposed that the Fcγ receptors present on trophoblast are not detectable by fluorescein aggregates due to being of low affinity and low density. However, it is these receptors that may be involved in the active transport of IgG from the mother to the foetus. The receptors on the endothelial stem cells may serve to bind complexes or aggregates formed in the placenta following the transfer of maternal IgG. The reasoning behind this suggestion is that all four sub-classes of human IgG are transported to the foetus (Wang et al., 1970), and may contain maternal alloantibody. It is during transport across the placenta that this antibody would first meet its antigen and form immune complexes (Johnson et al., 1976). Thus the Fcγ receptors on the foetal endothelial cells, with their high affinity for complexed human IgG but not for native IgG, are in an ideal situation to prevent immune complexes entering the foetal circulation.

Jenkinson et al. (1976) have shown the presence of Fcγ receptors on syncytiotrophoblast and chorionic membranes, using an EAγ rosetting method on single cell suspensions of human first trimester and term, placentae and chorionic membranes. As these receptors were detected on first trimester placentae and transmission of IgG across the human placenta does not begin until after the twenty-second week of gestation (Gitlin,

1974), it would seem likely that the Fcγ receptors may be involved in other functions. A similar function to that described for endothelial cells (Matre and Johnson, 1977) is proposed for the receptors on the syncytiotrophoblast (Jenkinson et al., 1976), with the added possibility that by binding complexes they may also interfere with lymphocyte recognition of surface determinants. The Fcγ receptors on the chorion may function in a similar manner to those described on the rodent and rabbit yolk sac and will probably take up IgG from the uterine fluid and pass it into the chorionic vessels and onto the foetus (Jenkinson et al., 1976). Confirmation of the work by Jenkinson et al. (1976) has come from observations made by Wood et al. (1978a), on both frozen sections and on single cell suspensions. Fcγ receptors were localised on the syncytiotrophoblast and they had as high an affinity for 'native' IgG as they had for immune complexes. A similar type of receptor was also found on the endothelial cells of the foetal vessels (Wood et al., 1978a).

Therefore Fcγ receptors for both complexed and native IgG have been identified on the interfaces between the maternal circulation and foetal tissue (trophoblast) and between foetal tissue (endothelial cells) and the foetal circulation. Consequently it is possible for an immunoregulatory system to operate at both interfaces.

The cells of the stromal region of the villi express Fcγ receptors which bind EAγ complexes and native IgG, but only bind native IgG at very high concentrations (Wood et al., 1978a). These cells appear to be macrophage-like based on their morphology, phagocytic activity and their non-specific esterase staining (Wood et al., 1978b). The proposed function of these macrophages within the placenta is that they are probably a second line of defence. They could remove anti-foetal immune complexes from the placenta and may also suppress the immune response to foetal transplantation antigens (Calderon and Unanue, 1974; Wolf et al., 1977). Their presence in the placenta may be due to the release of a chemotactic factor as a result of the activation of the complement (Wood et al., 1978b) bound to the TBM (Faulk and Johnson, 1977; Wood et al., 1978b).

Hofbauer cells found within the placenta are also defined as macrophages (van Furth et al., 1972) but they are much larger than the stromal macrophages described by Wood et al. (1978b). They are most obvious in the trophoblastic villi and basal plate of immature or oedematous placentae (Fox, 1967). Moskalewski et al. (1975) have shown

that Hofbauer cells form EAγ rosettes which can not only bind homologous antibody but also bind heterologous cytophilic antibodies. Matre and Johnson (1977) also detected EAγ binding to cells within the mesenchymal stroma of the chorionic villi and suggested that these were Hofbauer cells. The Hofbauer cells probably represent an early line of defence which exists before and during the period of foetal macrophage infiltration into the stroma of the villi.

Recently it has been shown that plasma membrane preparations of human placental syncytiotrophoblast bind EAγ complexes in an Fcγ dependent manner and that this binding can be inhibited by monomeric IgG (van der Meulen et al., 1980). The relative degree of inhibition by different species immunoglobulins shows a close parallel to the results obtained for the transmission of IgG across the rabbit yolk sac (Wild, 1979; Tsay et al., 1980) and is further circumstantial evidence that species variations in the adjunctive functions of the Fcγ portion of IgG may relate to its route of transmission from mother to young.

c) Maternal tissues

As mentioned earlier, Fcγ receptors have been found on murine decidual cells (Bernard et al., 1978; Dillon, 1979) and on cells from the rat metrial gland (Bray et al., 1978; Bray and Craggs, 1979). The function of these Fcγ positive cells is unknown. Because of their position *in situ*, it is unlikely that they relate to the transport of maternal IgG to the foetus. It is more likely that they are involved in the removal of immune complexes and cytotoxic antibodies from the maternal circulation.

Non-specific factors in the immunosuppression of pregnancy

There are a great many reports of factors in pregnancy which possess immunosuppressive properties. These include alphafoetoprotein, oestrogen and progesterone, the placental hormones, certain pregnancy specific proteins and some normal serum proteins.

a) Alphafoetoprotein (AFP)

The immunosuppressive properties of AFP are reputed to inhibit primary and secondary antibody synthesis *in vitro* (Murgita and Tomasi, 1975a) and cellular responses to mitogens or allogeneic lymphocytes in one-way MLC (Murgita and Tomasi, 1975b; Lester et al., 1978; Figueredo et al., 1979).

Not all reports, however, show that AFP is immunosuppressive in function: Sourbiran et al. (1979) report that AFP stimulates mitogenic and MLC reactions. Figueredo et al. (1979) relate the contrasting results to variations in techniques. Suzuki and Tomasi (1979b) have shown that whole mouse amniotic fluid exerts a greater degree of in vitro suppression on T cell dependent proliferation than purified AFP. The suggestion is made that there are at least two immunological inhibitors present in mouse amniotic fluid (Suzuki and Tomasi, 1979b). As AFP in mouse amniotic fluid has an oestrogen binding capacity (Mizejewski et al., 1979) it may be that both AFP and oestrogen play an interrelated role in the suppression of immune responses.

b) Oestrogen, progesterone and placental hormones

The immunsuppressive effects of oestrogen and progesterone are somewhat in doubt as it has been shown that each can have either a stimulatory or an inhibitory effect on the CMI system (Gusdon, 1976; Gall, 1977). Stimson and Hunter (1975) have suggested that the effect of oestrogen may be indirect, acting via a thymic factor which alters the immune response. They report a decreased number of T cells and an increased number of both B and null cells in the blood of rats receiving injections of oestrogen.

Human chorionic gonadotrophin (hCG) inhibits phytohaemagglutinin (PHA) induced lymphocyte transformation (Contractor and Davis, 1973; Adcock et al., 1973) and human placental lactogen (hPL) has been shown to be twice as immunosuppressive as hCG (Contractor and Davis, 1973). It has been reported, however, that the greater the degree of purity of hCG used in blastogenic tests, the lower its degree of immunosuppression (Caldwell et al., 1975; Kitzmiller and Rocklin, 1980). Carter (1978) has reported that hCG, oestrogen and progesterone all fail to inhibit haemadsorption techniques used for the detection of maternal and paternal antigens on in vitro cultures of mouse primary trophoblastic cells.

c) Pregnancy associated proteins

Various pregnancy associated proteins have been described in serum which are reputed to possess immunosuppressive activity. The existence of a pregnancy zone protein (PZP) has been described, which has a dose dependent immunosuppressive activity on lymphocyte transformation tests (Beckerman et al., 1971; von Shoultz, 1974; Gusdon, 1976). The

electrophoretic mobility and immunsuppressive properties of PZP appear to be the same as the pregnancy-associated α_2-macroglobulin (PAM) described by Stimson (1978). There is also a report of an early pregnancy factor (EPF), detected before the establishment of the placenta and before the appearance of hCG, PZP or PAM in the maternal serum (Morton et al., 1977), which inhibits EAγ rosette formation by normal human lymphocytes. Horne et al. (1976) have described a pregnancy specific β_1-glycoprotein, produced by syncytiotrophoblast, which is immunosuppressive to PHA stimulation of lymphocytes, but not to Con A stimulation.

d) Transferrin

Of interest in the field of immunosuppression during pregnancy is the proposed role of transferrin. In addition to its many other functions transferrin has been found bound to receptors on human trophoblast (Galbraith et al., 1978; Faulk and Galbraith, 1979a) and it is believed that it might hinder immunosurveillance by maternal lymphocytes (Faulk and Galbraith, 1979a, b). It is also proposed that some human lymphoblastoid cell lines and other transformed cells bind human transferrin (Galbraith et al., 1980). The blocking mechanism proposed for transferrin is similar to that proposed for blocking antibody to trophoblast antigens (Faulk et al., 1978).

e) Products of the maternal placenta

Extracts of rat decidual cells obtained from the deciduomata of pseudopregnancy (Globerson et al., 1976) have been shown to inhibit *in vitro* antibody responses to α-2,4-Dinitrophenyl-polylysine (DNP). This suppression is not species specific and it is thought to act via T cell suppression. Henney et al. (1972) showed that prostaglandin-E (PG-E) can inhibit lymphocyte mediated cytotoxicity and Globerson et al. (1976) proposed that PG-E was probably responsible for the *in vitro* antibody suppression seen in their system. Since then it has been confirmed that rat decidual cells in culture produce large amounts of PG-E (Vladimirsky et al., 1977).

Using a radio-actively labelled amino acid assay system, decidual cells from normal pregnancy and the deciduomata of pseudopregnancy in rats have been shown to produce a pregnancy specific 'decidualisation

associated protein'(DAP) (Bell, 1979a). In normal pregnancy this synthesis is localised in the endometrium and the myometrium, especially in the mesometrial triangle which contains the metrial gland. Synthesis in all areas of the implantation site falls dramatically from about day 14 of pregnancy onwards. Bell (1979a) speculates that DAP might be involved in the establishment of the placenta in the rat, but acknowledges that there must be a stimulus, other than implantation, for its production in the deciduomata of pseudopregnancy. In a further study Bell (1979b) has shown that DAP and α_2 acute phase macroglobulin are identical as assessed by immunochemical techniques. Therefore DAP may also play an immunosuppressive role in pregnancy.

As to how all these various non-specific factors and their immunosuppressive effects in vitro relate to the in vivo situation of pregnancy remains to be established.

Chapter 3.

Cell numbers and relative proportions of granulated metrial gland cells

Chapter 3

Cell numbers and relative proportions of granulated metrial gland cells

Introduction

The metrial gland of pregnancy and the deciduomata of pseudopregnancy are continuously changing structures (Bridgman, 1948; Velardo et al., 1953) and therefore a record of the total number of cells isolated from each animal was made and expressed as the number of cells per implantation site in pregnancy and the number of cells per uterine horn in pregnancy and pseudopregnancy. In pregnancy the number of intact foetuses was also recorded to check on the degree of foetal mortality as pregnancy proceeded.

In dealing with an isolated cell suspension from any tissue it is necessary to know how the cells *in vitro* reflect the cells *in situ*. Therefore a comparison has been made between the percentage of granulated metrial gland cells in smears of cell suspensions and the percentage of granulated metrial gland cells found in tissue sections. The granulated metrial gland cells were chosen for counting because they are the most easily distinguishable cell type in the metrial gland and also because they are the largest cells present and as such may be the most sensitive to the isolation procedures.

Materials and methods

Animals

Wistar rats from a randomly bred closed colony were used. Nulliparous or tetraparous rats were mated overnight and day 0 of pregnancy was taken as the day on which spermatozoa were detected in vaginal smears.

Pseudopregnancy was induced by cervical stimulation of nulliparous rats in oestrous (day 0 of pseudopregnancy). Vaginal smears were examined at day 4 to check that the animals were pseudopregnant and if so, deciduomata were induced by the insertion of a silk thread into each uterine horn.

Isolation of metrial gland cells

Cells from the metrial glands of primiparous, multiparous and pseudopregnant rats were prepared by the method of Bray et al. (1978).

The uterine horns were removed under aseptic conditions and the metrial glands dissected free of the surrounding tissues in Hank's balanced salts solution without magnesium and calcium (HBSS, Flow). The tissue from each animal was pooled and minced with scissors. The minced tissue was washed twice by decantation and then digested by constant stirring for 90 min. at 37^0C in $10cm^3$ of 0.25% collagenase (Type I, Sigma) containing 10% heat inactivated foetal calf serum (FCS, Flow). Cells were collected from the mixture by filtration through a 100 mesh steel gauze and the filtrate washed twice by centrifugation (150 x g for 10 min.). Resultant cell suspensions were assessed for viability by nigrosine exclusion and counted in a haemocytometer; only cell preparations showing >90% viability were used. Cells were resuspended in HBSS/FCS at 1 x $10^6/cm^3$ for all subsequent procedures.

Cell numbers were subjected to a one way analysis of variances or a Student's 't' test.

Histological examination of granulated metrial gland cells

Whole conceptuses from three day 12 and three day 15 uniparous pregnant rats were fixed in 10% neutral buffered formalin (NBF). Tissues were processed to wax and sectioned at 5μm. A series of twelve transverse sections was prepared from each conceptus, with a 200μm interval between each section. The first section was taken near the entry of the umbilical vessels to the placenta and further sections were taken towards the interconceptual region. All sections were reacted by the PAS technique, after diastase digestion, combined with a haematoxylin counterstain. Each section was then analysed by using a 25 spot random eye piece, at a magnification of 320, to calculate the percentage of granulated metrial gland cells as a proportion of the nucleated cells present per implantation site (Chalkley, 1943). Fields were counted in two horizontal and two vertical runs across each section (Fig. 6).

A Student's 't' test was used to compare the results.

Smears of cell suspensions

Cell smears of metrial gland cells were prepared from uniparous rats as described by Bray et al. (1978), fixed in 10% NBF, reacted by the PAS technique, after incubation in fresh spittle, followed by

a haematoxylin counterstain. The mean percentage of granulated metrial gland cells was established by examining 1000 nucleated cells on each of three smears from each animal.

The cell counts from the various days examined were subjected to a one way analysis of variance. A Student's 't' test for unmatched samples was used to compare the number of granulated metrial gland cells seen in histological sections and in smears of single cell suspensions.

Results

Fig. 3 shows the number of viable cells obtained from the metrial gland of normal pregnancy. There is a gradual decrease in the number of viable cells obtained from each implantation site as pregnancy proceeds. There is no decrease in the average number of foetuses per pregnancy as gestation proceeds. There is no significant difference between the results observed at day 13 of uniparous pregnancy and those observed at day 13 of multiparous pregnancy. Fig. 4 shows the number of viable cells obtained from the metrial gland tissue of the deciduomata of pseudopregnancy. The total number of cells obtained from the metrial gland tissue of each uterine horn of the deciduomata of pseudopregnancy is always considerably less than the number of cells obtained per uterine horn from the corresponding day of normal pregnancy. There does not appear to be much change in the number of cells obtained per uterine horn over the days of pseudopregnancy examined, except at day 11 of pseudopregnancy when considerably more cells were isolated.

The percentage of granulated metrial gland cells detected in cell smears remains fairly constant (Fig. 5) and there were no significant differences between any of the days of pregnancy examined. This is despite the fall in the total number of cells obtained as normal pregnancy proceeds (Fig. 3). However, the number of granulated metrial gland cells in tissue sections (Fig. 7) increases significantly between day 12 and day 15 of uniparous pregnancy ($P<0.01$). A comparison between the percentage of granulated metrial gland cells seen in cell smears (Fig. 5) and those seen in tissue sections (Fig. 7) reveals that there is no significant difference at day 12 and that the difference is only significant at the $P<0.05$ level at day 15 of pregnancy.

Figures 3 - 7

Fig. 3. Viable cell yields from metrial gland tissue of pregnancy following collagenase treatment

Day of pregnancy	Number of rats	Mean number of conceptuses (± SEM)	Mean number of cells/ metrial gland x10^6 (± SEM)	Mean number of cells/ uterine horn x10^6 (± SEM)
12	11	11.9 ± 0.6	2.57 ± 0.1	15.3 ± 1.2
13	28	10.9 ± 0.4	2.56 ± 0.1	14.4 ± 0.9
13 (MP)	3	10.3 ± 0.9	2.41 ± 0.7	12.4 ± 3.6
14	23	11.4 ± 0.5	2.23 ± 0.1	13.0 ± 0.9
15	24	11.6 ± 0.4	2.19 ± 0.1	12.8 ± 0.9
16	2	12.5 ± 1.5	1.78 ± 0.2	11.1 ± 0.2
17	3	11.7 ± 0.9	1.14 ± 0.7	6.7 ± 0.8
19	12	9.5 ± 0.8	0.7 ± 0.7	3.5 ± 0.5
20	1	9.0	0.4	1.8

SEM - Standard error of the mean

MP - Multiparous pregnancy

Fig. 4. Viable cell yields from metrial gland tissue of the deciduomata of pseudopregnancy following collagenase treatment

Day of pseudopregnancy	Number of rats	Mean number of cells/ uterine horn $x10^6$ (± SEM)
11	3	7.6 ± 1.1
12	8	4.4 ± 0.6
13	9	4.1 ± 0.7
14	3	4.9 ± 1.8

Fig. 5. Percentage of granulated metrial gland cells in cell smears

Day of pregnancy	Number of rats	Mean percentage of granulated cells (± SEM)
12	6	6.4 ± 0.8
13	7	6.2 ± 0.7
14	5	7.8 ± 0.7
15	6	8.7 ± 0.9
16	2	8.4 ± 0.1

Fig. 6: Diagrammatic transverse section through the metrial gland at day 12 of pregnancy showing the pattern of lines scanned in counting granulated metrial gland cells (cells containing diastase-fast PAS positive granules) as a proportion of the nucleated cells present. Cell counts were made using a random point eye piece (25 spot) in a method similar to that described by Chalkley (1943).

cm. circular muscle; db. decidua basalis; mg. metrial gland.

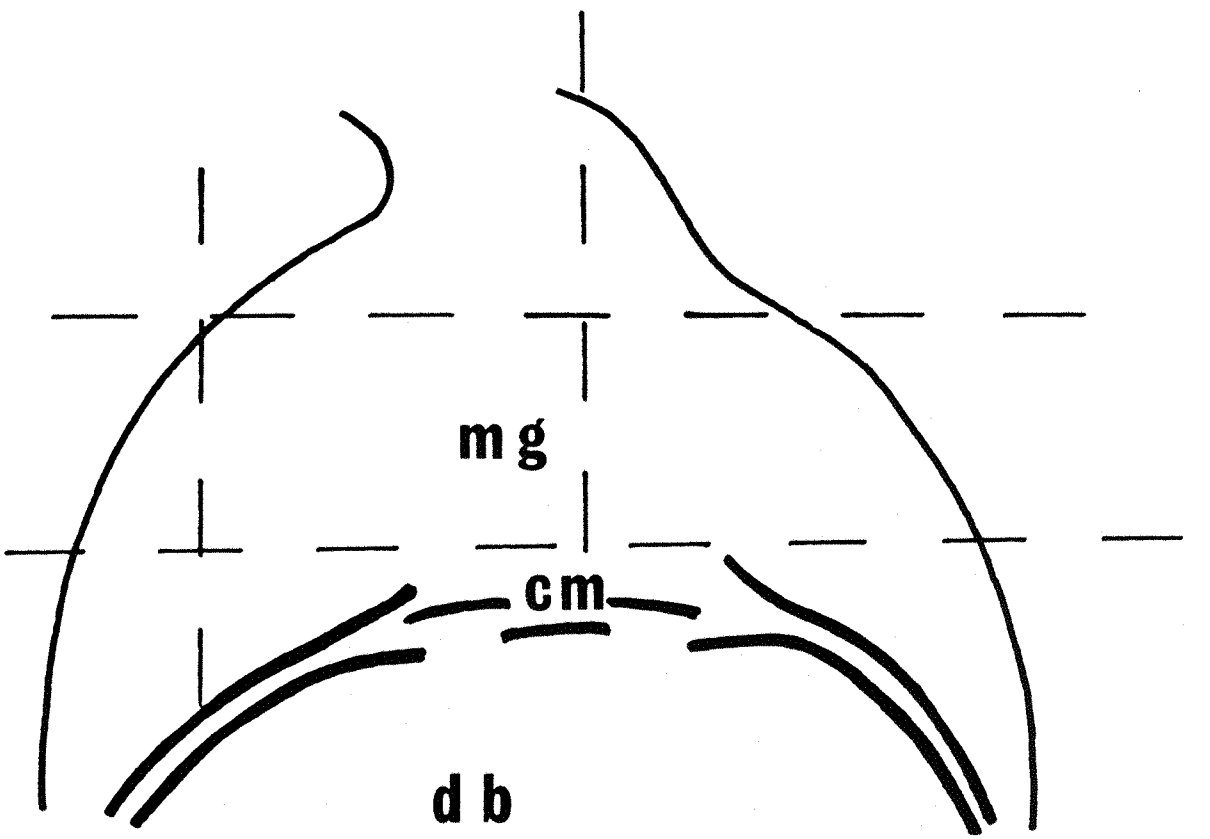

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Fig. 7. Percentage of granulated metrial gland cells as a proportion of the nucleated cells observed in tissue sections

Day of pregnancy	Number of rats	Mean percentage of granulated cells (± SEM)
12	3	9.0 ± 0.1
15	3	12.3 ± 0.7

Chapter 4

Examination for immunological markers on cells from the metrial gland

Chapter 4

Examination for immunological markers on cells from the metrial gland

Introduction

A possible immunological role for the metrial gland in pregnancy has been inferred from the morphological and cytological evidence reported earlier (Smith, 1966b; Peel and Bulmer, 1977; Bray et al., 1978; Mitchell et al., 1980). Therefore it was decided to examine cells from the metrial gland for a range of immunological markers in an attempt to relate the metrial gland cells to other cells whose immunological characteristics are already defined.

In a preliminary part of this study Fcγ receptors were found on cells from the rat metrial gland (Bray et al., 1978; Appendix III). However, Fcγ receptors are also found on a wide variety of other cell types. Their distribution on several tissues in pregnancy has been described earlier (p. 16-20). They are also reported on macrophages and monocytes (Huber and Fudenberg, 1968), B lymphocytes, T lymphocytes and a third population of lymphocytes (Shevach et al., 1973; Dickler, 1976), neutrophils (Scott, 1979; An, 1980), myelin sheaths, neurones and glia (Aarli et al., 1975), skeletal muscle (Aarli and Tønder, 1974), Kupffer cells (Huber et al., 1969), hepatocytes (Frommel and Rachman, 1979), fibroblasts (Papamichail et al., 1979) and on cells from the choroid plexus (Braathen et al., 1979). Many of these cell types have other surface markers that are well defined. Macrophages and monocytes, as well as being defined by their Fcγ receptors (Huber and Fudenberg, 1968; Arend and Mannik, 1972), also have a receptor for complement which can be detected by rosette formation using red blood cells sensitised by antibody and complement (EAC rosettes)(Huber et al., 1968). Although B lymphocytes possess Fcγ receptors (Dickler and Kunkel, 1972) they are not readily detectable by the use of EAγ rosetting techniques (Shevach et al., 1973). However, B lymphocytes can be characterised by their membrane incorporated surface immunoglobulin (SIg)(Raff, 1970) and by the fact that most B cells also form EAC rosettes (Lay and Nussenzweig, 1968; Bianco et al., 1970). T lymphocytes can be defined by their ability to form non-immune rosettes (E rosettes) with unsensitised red blood cells (Jondal et al., 1972; Steele, 1979) and

by their ability to form EA rosettes with red blood cells sensitised by either IgG or IgM (EAγ and EAμ rosettes respectively)(Moretta et al., 1975). Examination for Fcμ receptors (EAμ rosette formation) is best observed after overnight culture (Moretta et al., 1975).

The possibility that the cells of the metrial gland may be derived from or related to other cell types has been examined by looking for SIg and by examining their ability to form E, EAγ , EAμ and EAC rosettes. All rosette formation tests were also carried out after overnight culture of the cells.

The Thy-1 antigen used to identify T cells in the mouse (Raff, 1971) is not confined to T cells in the rat (Douglas, 1972; Acton et al., 1974; Williams, 1975; Hunt et al., 1977) and was therefore not looked for as a T cell marker in these studies.

Materials and methods

Metrial gland cells from animals at days 12, 13, 14 (x2), 16 and 19 (x2) of pregnancy were prepared as described in Chapter 3.

Preparation of rat peritoneal exudate, thymus and spleen cells and human peripheral blood lymphocytes

Peritoneal exudate cells were collected as described by Bray and Craggs (1979) from non-pregnant, pregnant and pseudopregnant animals. Cells were washed twice in HBSS/FCS at 500xg for 10 min., assessed for viability by nigrosine exclusion and resuspended at 2 x $10^6/cm^3$. Cells from the thymus and spleen were prepared from pregnant and pseudopregnant animals by removing the surrounding tissues and mincing the organ through 100 mesh steel gauze. The resultant cell suspensions were prepared as described for the peritoneal exudate, but were resuspended at 5 x $10^6/cm^3$. 18 hour cultures of †human peripheral blood lymphocytes were washed as described for the rat peritoneal exudate and resuspended at 2 x $10^6/cm^3$.

Surface markers used to characterise cell types

All observations were on cells from uniparous animals, except observations for surface IgG which were also carried out on cells from

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† Kindly supplied by Dr. D. Jones, Experimental Pathology, University of Southampton

pseudopregnant animals. Some cells were cultured for 18 hours, in RPMI 1640 (Flow) plus 10% FCS, 2% penicillin and streptomycin (5000 iμ/cm^3 and 5000 mcg/cm^3 respectively, Flow) and 1% L-glutamine (200 mM, Flow) at 37°C on a cell shaker. Subsequently they were washed in 5 cm^3 RPMI plus 0.1 cm^3 of 0.4% deoxyribonuclease-I (DNase-I, Sigma) to remove cell clumps caused by nuclear material, spun at 150xg for 5 min. and given two further washes in HBSS/FCS before being resuspended, tested for viability and used in the various tests.

a) E rosette formation

E rosette formation was carried out according to the method of Jondal et al. (1972). Cell preparations (0.1 cm^3) were incubated with 0.1 cm^3 of a 0.7% suspension of washed SRBCs (Gibco - Biocult) in 0.9% saline at 37°C for 5 min., spun at 150xg for 5 min. and incubated on ice for 2 hours. The supernatant was removed, the pellet gently resuspended and duplicate counts of 100 cells were made for each sample in a haemocytometer. Control rosette formation was performed at room temperature, spun at 150xg for 5 min. and cells counted immediately. In both cases cells binding 5 or more SRBCs were counted as positive.

b) EAγ rosette formation

Washed SRBCs (7.0% suspension) were sensitised with a subagglutinating dose (1:64) of rabbit or rat anti-SRBC IgG (see Appendix I for preparation procedures) and washed OxRBCs* (7.0% suspension) were sensitised with a subagglutinating dose (1:32) of rabbit anti-OxRBC IgG* by incubation at 37°C for 2 hours. Sensitisation was confirmed in a Coombs-type assay using goat anti-rabbit IgG (1:8) or rabbit anti-rat IgG (1:4). Control RBCs were unsensitised . Sensitised or control RBCs (0.1 cm^3 of a 0.7% suspension) were mixed with an equal volume of cell preparations at room temperature, contrifuged at 150xg for 5 min. and the pellet gently resuspended. Cell counts were made as described for E rosette formation.

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* Kindly supplied by Dr. J. Smith, Tenovus Research Laboratory, Southampton.

c) EAμ rosette formation

Washed OxRBCs* (7.0% suspension) were sensitised with an equal volume of rabbit anti-OxRBC IgM* (1:16) for 30 min. at 37^{0}C and resuspended as a 1.0% solution. Sensitised OxRBCs (0.1 cm^3) were mixed with 0.1 cm^3 of cell preparations, spun at 150xg for 5 min. and incubated on ice for 30 min. Cell counts were made as described for E rosette formation.

d) EAC rosette formation

Washed OxRBCs* (7.0% suspension) were sensitised with rabbit anti-OxRBC IgM* (as described above) and further incubated with an equal volume of AKR strain mouse serum † (1:4), as a non-lytic source of complement, for 30 min. at 37^{0}C (Lay and Nussenzweig, 1968). Sensitised OxRBCs (0.1 cm^3 of a 1.0% solution) were added to 0.1 cm^3 of cell preparations, incubated at room temperature for 5 min., spun at 150xg for 5 min and counted as described for E rosette formation.

e) Surface IgG (SIg) localisation

Cell suspensions were prepared from the metrial gland at days 12, 13, 14, 15, 17 and 19 of pregnancy and from the metrial gland of the deciduomata of pseudopregnancy at days 11, 12, 13 and 14 of pseudopregnancy. Cell suspensions were examined for SIg by the method described previously (Mitchell et al., 1980). Equal volumes of cells were reacted at 4^{0}C in HBSS/FCS plus 0.01% sodium azide (BDH) with either whole molecule rabbit anti-rat IgG (1:160 to 1:320, Wellcome) or with an Fabγ fraction of the same molecule (1:80 to 1:160) (see Appendix II for preparation procedure). The primary layer was followed by a fluorescein conjugated (FITC) sheep anti-rabbit IgG (1:20 to 1:40, Wellcome). Cells were washed three times (150xg for 5 min) in medium between each procedure, mounted in glycerol/PBS and examined under incident ultraviolet light. Cells showing surface fluorescence were counted in duplicate samples of 100 cells. Controls consisted of examining cells for autofluorescence or when reacted in the presence of FITC antibody alone.

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* Kindly supplied by Dr. J. Smith, Tenovus Research Laboratories, Southampton.

† Kindly supplied by Prof. G. Stevenson, Tenovus Research Laboratories, Southampton.

A paired 't' test was used to determine any significant difference between the number of Fabγ positive cells and the number of cells showing rings of fluorescence when reacted with the FITC antibody alone.

Results

Unsensitised SRBCs or OxRBCs were consistently negative for control rosette formation with all cell types tested (Fig. 8). All rat tissues incubated with SRBCs at 37^{0}C and then at 4^{0}C failed to form spontaneous (E) rosettes. A high proportion of the human peripheral blood cells formed E rosettes (Fig. 8).

EAγ rosettes were formed by a proportion of cells from all tissues, including a small percentage of thymocytes (Fig. 9). Using SRBCs or OxRBCs sensitised with rabbit anti-SRBC IgG or rabbit anti-OxRBC IgG respectively, approximately the same proportions of cells formed EAγ rosettes. However, when rat anti-SRBC IgG was used, considerably fewer cells from the rat tissues formed EAγ rosettes. This effect was not observed on the human peripheral blood cells. The effects of culturing the rat metrial gland cells and the thymocytes for 18 hours did not reduce the number of EAγ rosettes formed, whilst there was a reduction in the number of rosettes formed by peritoneal exudate and spleen cells (Fig. 9).

Examination of metrial gland cells for EAμ or EAC rosette formation was negative at time zero and after 18 hours in culture (Fig. 10). Positive results for EAμ rosette formation were observed on peritoneal exudate cells and a very small proportion of spleen cells (irrespective of time in culture) and on human peripheral blood cells (Fig. 10). EAC rosettes were formed by peritoneal exudate cells and spleen cells (irrespective of time in culture), and human peripheral blood cells (Fig. 10).

Using the whole molecule rabbit anti-rat IgG on viable single cell suspensions prepared from rat metrial glands between day 12 and day 19 of uniparous pregnancy, followed by FITC sheep anti-rabbit IgG, between 20.5 and 29.0 percent of cells (Fig. 11) showed rings of surface fluorescence (Fig. 12). There is a decrease between day 12 and day 14 of pregnancy after which time the percentage remains fairly constant (Fig. 11). When the Fabγ portion of rabbit anti-rat IgG was used in

the sandwich test, between 1.0 and 3.0 percent of cells showed surface fluorescence (Fig. 11). A small percentage of cells also showed rings of fluorescence after reaction with FITC sheep anti-rabbit IgG alone (Fig. 11). However, using a paired 't' test, the percentage of cells showing rings of fluorescence after the Fabγ antibody was significantly higher ($P<0.001$) than the number after the FITC antibody on its own. Spleen and thymus cells were used as controls (Fig. 11).

Observations on cells from the metrial gland of the deciduomata of pseudopregnancy (Fig. 13) also showed that the whole molecule rabbit anti-rat IgG bound to a proportion of cells between day 11 and day 14 of pseudopregnancy (18.0 - 29.0%). There is a drop from day 11 to day 12 followed by a rise during day 13 and day 14 of pseudopregnancy (Fig. 13). The binding of the Fabγ portion of the rabbit anti-rat IgG was at a similar level to that seen in normal pregnancy and it is at a significantly higher level ($P<0.01$) than the degree of binding produced by the FITC sheep anti-rabbit IgG alone (Fig. 13). Spleen and thymus cells were used as control tissues (Fig. 13).

Figures 8 - 13

Fig. 8. Mean percentage (± SEM) of cells forming control rosettes or E rosettes

Cells	Number of samples	Hours in culture	Mean % control rosettes		Mean % E rosettes
			SRBC	OxRBC	SRBC
Metrial	7	0	0.5 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
gland	4	18	0.5 ± 0.2	0.6 ± 0.1	0.5 ± 0.2
Peritoneal	4	0	0.4 ± 0.2	0.6 ± 0.1	0.5 ± 0.2
exudate	1	18	0.5	1.0	0.5
Spleen	5	0	0.7 ± 0.2	0.6 ± 0.2	1.0 ± 0.2
	2	18	0.7 ± 0.2	0.5 ± 0.0	0.7 ± 0.2
Thymus	5	0	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
	2	18	0.2 ± 0.2	0.2 ± 0.2	0.5 ± 0.0
Human peripheral blood	2	18	0.2 ± 0.2	0.2 ± 0.2	60.5 ± 1.0

Fig. 9. Mean percentage (± SEM) of cells forming EAγ rosettes

Cells	Number of samples	Hours in culture	Mean % EAγ rosette formation: rabbit ŝ SRBC	rat ŝ SRBC	rabbit ŝ OxRBC
Metrial gland	7	0	26.9 ± 1.6	15.4 ± 1.5	25.8 ± 1.4
	4	18	22.4 ± 3.3	10.4 ± 1.9	21.9 ± 2.3
Peritoneal exudate	4	0	59.3 ± 4.3	27.5 ± 2.5	58.0 ± 3.2
	1	18	25.0	18.0	25.0
Spleen	5	0	43.3 ± 2.0	28.2 ± 2.5	42.2 ± 2.6
	2	18	26.6 ± 1.6	10.4 ± 3.4	29.2 ± 1.7
Thymus	5	0	3.1 ± 0.5	2.2 ± 0.2	2.8 ± 0.2
	2	18	3.9 ± 0.4	0.7 ± 0.2	3.2 ± 0.2
Human peripheral blood	2	18	20.5 ± 2.5	20.2 ± 1.2	21.0 ± 1.5

Fig. 10. Mean percentage (± SEM) of cells forming EAμ rosettes and EAC rosettes

Cells	Number of samples	Hours in culture	Mean % EAμ rosette formation	Mean % EAC rosette formation
Metrial gland	7	0	0.8 ± 0.1	0.4 ± 0.1
	4	18	1.0 ± 0.4	0.1 ± 0.1
Peritoneal exudate	4	0	7.1 ± 5.6	32.1 ± 5.9
	1	18	5.7	31.5
Spleen	5	0	1.5 ± 0.7	8.7 ± 0.9
	2	18	1.7 ± 0.2	5.7 ± 1.2
Thymus	5	0	0.2 ± 0.1	0.2 ± 0.1
	2	18	0.2 ± 0.2	0.2 ± 0.2
Human peripheral blood	2	18	28.2 ± 1.2	21.5 ± 1.5

Fig. 11. Mean percentage (± SEM) of cells showing surface fluorescence from the metrial gland of uniparous pregnancy

Day of pregnancy	Number of rats	SAR(FITC) (cross-hatching)	Fabγ + SAR(FITC) (crosses)	RARa + SAR(FITC) (open bars)
12	2	1.0 ± 0.5	2.0 ± 1.0	29.0 ± 0.5
13	6	1.0 ± 0.2	2.2 ± 0.6	25.6 ± 0.9
14	2	1.0 ± 0.5	2.7 ± 0.2	20.5 ± 0.5
15	3	0.3 ± 0.2	0.9 ± 0.4	20.5 ± 0.8
17	3	0.5 ± 0.3	1.7 ± 0.3	21.3 ± 0.9
19	3	1.0 ± 0.3	3.1 ± 0.7	22.2 ± 1.3
Spleen	7	ND	38.8 ± 1.6	40.3 ± 1.2
Thymus	7	ND	1.0 ± 0.4	1.7 ± 0.4

RARa - Whole molecule rabbit anti-rat IgG

Fabγ - Fabγ portion of rabbit anti-rat IgG

SAR(FITC) - Fluorescein conjugated sheep anti-rabbit IgG

ND - Not done

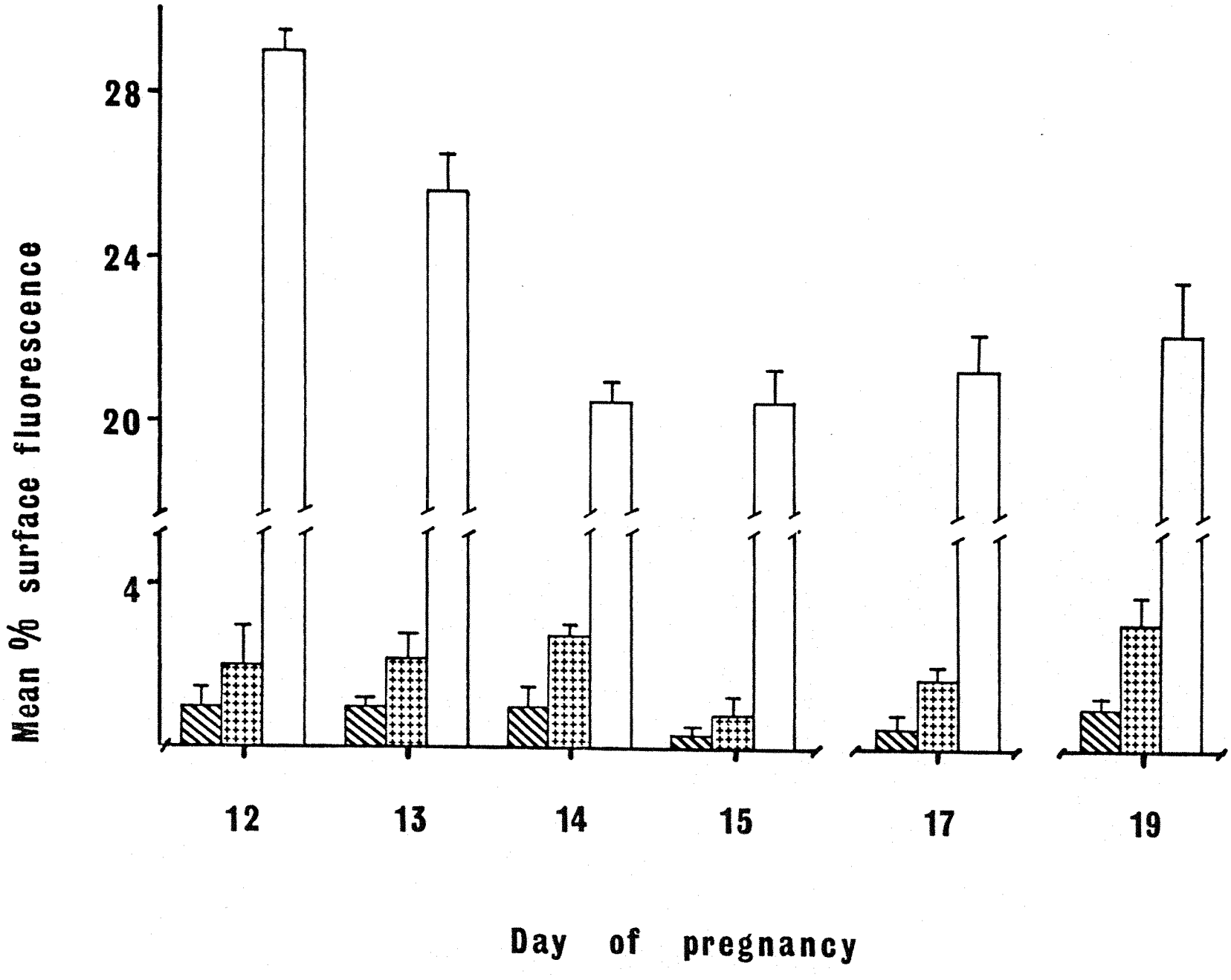

Mean % surface fluorescence
28
24
20
4
12
13
14
15
17
19
Day of pregnancy

Fig. 12: A preparation of metrial gland cells suspension (day 13 of pregnancy) showing a ring of fluorescence on a cell reacted with whole molecule rabbit anti-rat IgG (12a) and the corresponding phase-contrast appearance in which two cells are negative for fluorescence (12b). x950.

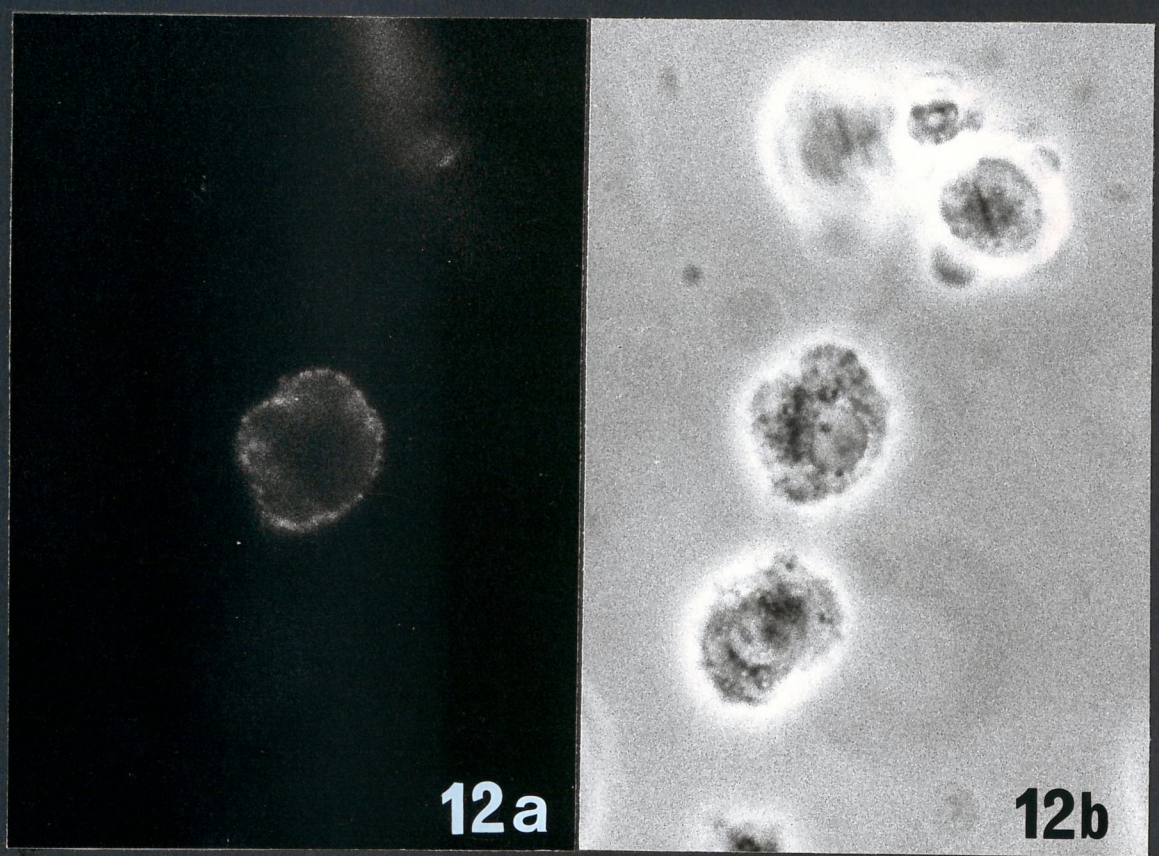

12a
12b

Fig. 13. Mean percentage (± SEM) of cells showing surface fluorescence from the metrial gland of the deciduomata of pseudopregnancy

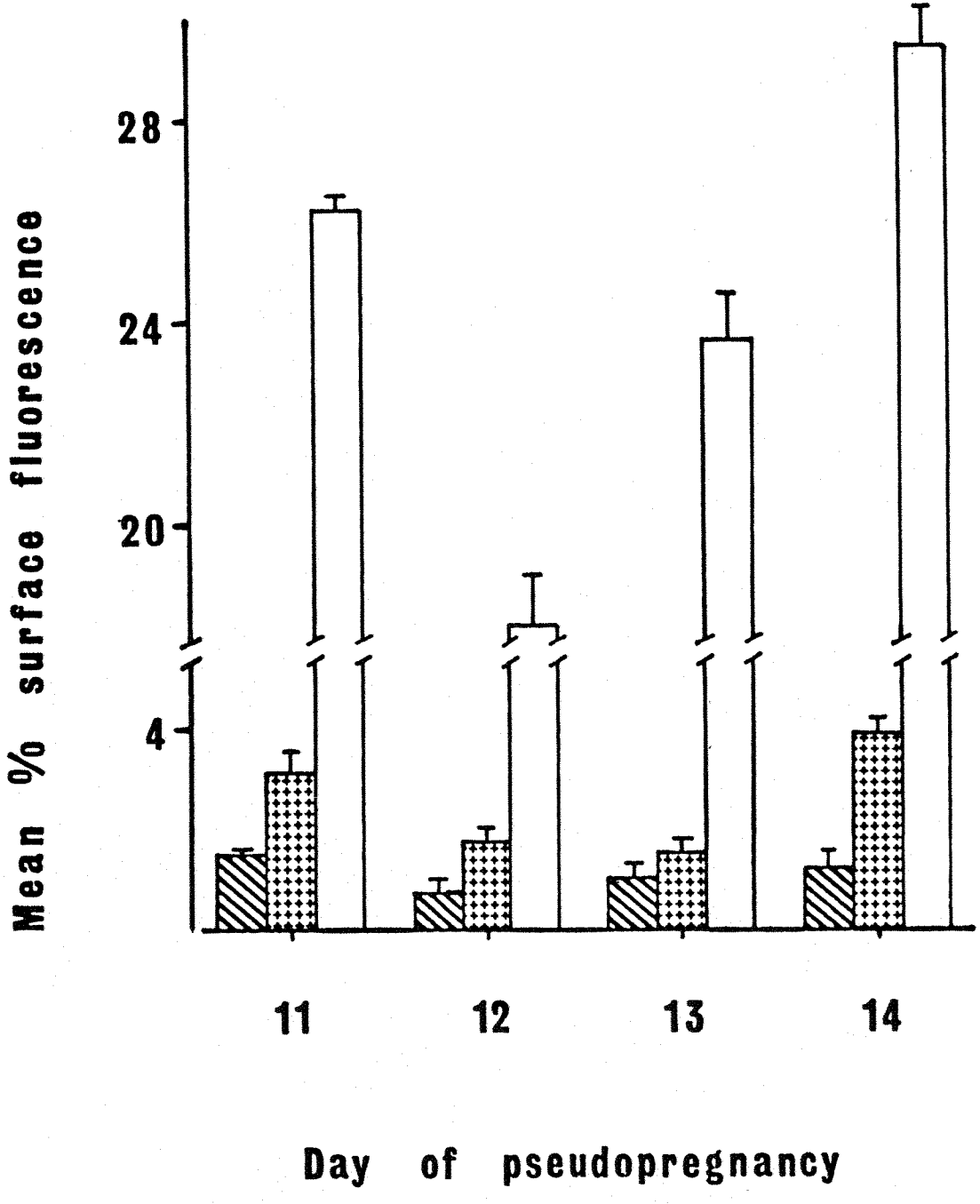
Day of pseudopregnancy	Number of rats	SAR(FITC) (cross-hatching)	Fabγ + SAR(FITC) (crosses)	RARa + SAR(FITC) (open bars)
11	2	1.5 ± 0.0	3.1 ± 0.4	26.5 ± 0.2
12	2	0.7 ± 0.2	1.7 ± 0.2	18.0 ± 1.0
13	3	1.0 ± 0.3	1.5 ± 0.2	23.7 ± 0.9
14	2	1.2 ± 0.2	3.9 ± 0.1	29.2 ± 0.7
Spleen	3	ND	41.0 ± 3.0	45.5 ± 3.5
Thymus	3	ND	0.5 ± 0.5	1.0 ± 1.0

RARa - Whole molecule rabbit anti-rat IgG

Fabγ - Fabγ portion of rabbit anti-rat IgG

SAR(FITC) - Fluorescein conjugated sheep anti-rabbit IgG

ND - Not done


Mean % surface fluorescence
28
24
20
4
11
12
13
14
Day of pseudopregnancy

Chapter 5

Quantification of the metrial gland Fcγ receptors and their inhibition characteristics

Chapter 5

Quantification of the metrial gland Fcγ receptors and their inhibition characteristics

Introduction

As an extension of the work by Bray and Craggs (1979; Appendix III) the Fcγ receptor-bearing cells of the metrial gland have been quantified between day 12 and day 20 of uniparous pregnancy, at day 13 of multiparous pregnancy and between day 11 and day 14 of the deciduomata of pseudopregnancy. This quantification has involved the use of both rabbit and rat sensitising antibodies to SRBCs to compare the number of cells forming EAγ rosettes with heterologous and homologous sensitising antibodies.

Bray and Craggs (1979; Appendix III) also compared the abilities of homologous (rat) and heterologous (rabbit, human and bovine) monomeric immunoglobulins to inhibit EAγ rosette formation by metrial gland cells and peritoneal exudate cells. Rosette formation by metrial gland cells was inhibited to a greater degree by low concentrations of rabbit, rat and human immunoglobulins than rosette formation by peritoneal exudate cells. In this study the work using monomeric immunoglobulins has been repeated and has been extended to include the inhibitory effects of various rat sera and heat aggregated immunoglobulins on the ability of cells to form EAγ rosettes with either rabbit or rat sensitised SRBCs. The work using heat aggregated immunoglobulins has been carried out on cells from uniparous, multiparous and pseudopregnant animals.

On the basis of an ultrastructural study Larkin (1972) suggested that there was an increase in the number of macrophages present in the metrial gland from day 15 of pregnancy and that they might be derived from stromal cells. Previously it has been shown that macrophage Fcγ receptors are not inhibited by monomeric IgGs (Bray and Craggs, 1979), but are inhibited by heat aggregated IgGs (Matre et al., 1975) when using the EAγ rosetting technique. Therefore attention has been paid to a comparison between the ability of monomeric and heat aggregated immunoglobulins to inhibit EAγ rosette formation by metrial gland cells at day 19 of uniparous pregnancy.

In another study the effects of incubating cells with inhibiting immunoglobulin at different temperatures and of washing the cells after incubation have been examined for their ability to alter the inhibition characteristics of the Fcγ receptors found on the metrial gland cells. This is because a population of lymphocytes defined by Froland and Natvig (1973) possess an Fcγ receptor which binds serum IgG at 4^0C and releases it again at 37^0C. They are therefore sometimes called 'L' cells because of the labile nature of the membrane IgG (Horwitz and Lobo, 1975).

Resistance to trypsin treatment, shown by Fcγ receptor-bearing cells from the metrial gland of normal pregnancy (Bray and Craggs, 1979) has been examined on cells from the metrial gland and peritoneal exudate of pregnancy and from the metrial gland of the deciduomata of pseudopregnancy. This is in a further attempt to define a possible macrophage or B cell relationship for the Fcγ positive cells of the metrial gland as the Fcγ receptors found on macrophages and B lymphocytes are resistant to trypsin (Cline et al., 1972; Lay and Nussenzweig, 1968).

Peritoneal exudate, spleen and thymus cells have been treated with collagenase to see how the enzyme digestion technique used for metrial gland cells affects subsequent EAγ rosette formation.

Materials and methods

Metrial gland cells were prepared as described in Chapter 3. Peritoneal exudate, thymus and spleen cells were prepared as described in Chapter 4. To act as extra controls some samples of peritoneal exudate, thymus and spleen cells were incubated in 0.25% collagenase as described for the preparation of the metrial gland cells.

EAγ rosette formation

EAγ rosette formation was performed as described in Chapter 4 using rabbit or rat sensitised SRBCs. Additional observations have been made to examine metrial gland cells from day 12 to day 20 of uniparous pregnancy, day 13 of multiparous pregnancy and day 11 to day 14 of pseudopregnancy.

Results were subjected to a one way analysis of variance and Duncan's (1955) new multiple range test using Kraemer's (1956) modification for unequal groups, or to a Student's 't' test for unmatched groups.

Inhibition experiments on EAγ rosette formation

Cells from the metrial gland or peritoneal exudate were incubated with equal volumes (0.1 cm^3) of various rat sera, monomeric IgGs or heat aggregated IgGs at room temperature for 30 min., before addition of 0.1 cm^3 of rabbit or rat sensitised SRBCs. Cells were counted as described for E rosette formation (Chapter 4). Percentage rosette inhibition was calculated as 100 - (percentage of rosettes formed in the presence of competing solution/percentage of rosettes formed in the absence of competing solution x100).

Rat sera were obtained by cardiac puncture from animals under ether anaesthesia and diluted to 1/5 and 1/10 with 0.9% saline. Monomeric IgGs were prepared in 0.9% saline from lyophilised samples and spun at 2000xg for 60 min. to remove aggregates. Heat aggregated IgG was prepared by incubating samples of IgG at 60^0C for 45 min. Protein concentrations were established by absorption at 280 nm. Rabbit, rat, human and bovine IgGs were used and were pure as defined by immunoelectrophoretic analysis (Miles).

Effects of temperature and washing on the inhibition of EAγ rosette formation

Cells from the metrial gland and peritoneal exudate of day 13 to day 15 uniparous animals were incubated at 4^0C and 37^0C in heat aggregated human IgG (7 mg/cm^3). After intervals of 30, 60 and 120 min., four samples were taken and two were washed three times in HBSS/FCS (150xg for 5 min.) to remove unbound or loosely bound IgG. All four samples were mixed with rabbit sensitised SRBCs and examined for rosette formation.

Effects of trypsin on EAγ rosette formation

Cells from the metrial gland and peritoneal exudate of uniparous rats and metrial gland cells from the deciduomata of pseudopregnant rats were incubated in a solution of 0.25% trypsin (Flow) at 37^0C for 30 min., washed twice to remove the trypsin and tested for rosette formation with rabbit or rat sensitised SRBC.

Results

EAγ rosettes were formed when cells from the metrial gland between day 12 and day 20 of uniparous pregnancy, day 13 of multiparous pregnancy and between day 11 and day 14 of pseudopregnancy were mixed with SRBCs

sensitised with the IgG fraction of a rabbit anti-SRBC antiserum or a rat anti-SRBC antiserum (Fig. 14 and Fig. 15). In normal pregnancy (Fig. 14) there were more rosettes formed by using the rabbit sensitising antibody than by using the rat sensitising antibody. However, both antibodies show a similar decrease in the percentage of cells forming rosettes between day 12 and day 15 of pregnancy ($P<0.01$). Using the rat sensitising antibody there are significant increases between day 15 and day 17 of pregnancy ($P<0.05$) and between day 17 and day 19 of pregnancy ($P<0.001$). However, using the rabbit sensitising antibody there is no significant increase until after day 17 of pregnancy, when the increase to day 19 is significant at the 1.0% level. Both sensitising antisera show a significantly higher ($P<0.001$) number of cells forming rosettes at day 13 of multiparous pregnancy than at day 13 of uniparous pregnancy.

The percentage of cells forming rosettes from the metrial gland of the deciduomata of pseudopregnancy (Fig. 15) is similar to that observed for normal pregnancy (Fig. 14). Rabbit sensitised SRBCs demonstrate more rosette forming cells than do rat sensitised SRBCs. Both sensitising antibodies show a decrease between day 11 and day 12 ($P<0.01$ and $P<0.05$ for rabbit and rat sensitised SRBCs respectively) and an increase between day 12 and day 14 ($P<0.01$ and $P<0.05$).

The percentage EAγ rosette formation produced by both rabbit and rat sensitising antibodies on cells from other rat tissues is shown in Fig. 16. Peritoneal exudate, spleen and thymus cells from uniparous and non-pregnant animals showed no significant difference in the number of cells forming EAγ rosettes and have therefore been grouped together as control cells. The only significant change observed by pretreating control cells with collagenase was an increase in the number of cells from the peritoneal exudate (Fig. 16) forming EAγ rosettes with rabbit sensitised SRBCs ($P<0.01$). There is no significant difference between the number of cells forming rosettes when cells are taken from control animals or from pseudopregnant animals (Fig. 16).

Incubation of cells from the metrial gland of uniparous pregnancy, from the metrial gland of the deciduomata of pseudopregnancy and from the peritoneal exudate of pregnancy with trypsin had no effect on subsequent EAγ rosette formation, irrespective of the antibody used to sensitise the SRBC.

In further controls there was no change in the number of rosettes formed when a mixture of metrial gland cells and sensitised SRBCs were

incubated for 60 minutes before counting nor if the metrial gland cells were prepared in collagenase and medium from which FCS had been omitted.

Fig. 17 shows the effects on EAγ rosette formation by preincubating cells from the metrial gland of day 14 uniparous pregnancy with normal male serum, non-pregnant female serum, uniparous female serum or multiparous female serum. The degree of inhibition was not markedly different for any of these sera and was approximately the same with both rabbit or rat sensitised SRBCs.

When rabbit, rat, human or bovine IgGs were used in inhibition assays on metrial gland cells and peritoneal exudate cells the following results were observed. Monomeric rabbit and human IgGs on day 14 uniparous metrial gland cells (Fig. 18) inhibited rosette formation by rabbit sensitised SRBCs to a high degree, even at 1.0 mg/cm^3. Rat IgG inhibited at a lower level and bovine IgG produced little or no inhibition. In contrast using rat sensitised SRBCs the rat and human IgG inhibited rosette formation to a higher degree, and considerably less inhibition was produced by rabbit IgG, even at 7.0 mg/cm^3. Bovine IgG produced no inhibition and even had an enhancing effect. Day 14 peritoneal exudate cells showed only a minimal degree of inhibition when incubated with the monomeric immunoglobulin preparations (Fig. 19).

Using heat aggregated IgGs, metrial gland cells from days 12, 13, 14, 15 and 19 of uniparous pregnancy (Figs. 20, 21, 22, 23 and 24), day 13 of multiparous pregnancy (Fig. 25) and day 12 and day 13 of the deciduomata of pseudopregnancy (Fig. 26 and 27) were all inhibited in a similar manner to that seen for monomeric IgGs on metrial gland cells from day 14 of uniparous pregnancy (Fig. 18). The only exception being that at days 13, 14 and 15 (Figs. 21, 22 and 23) of uniparous pregnancy there is less inhibition by heat aggregated rat IgG at 1.0 mg/cm^3 than by heat aggregated rabbit IgG (1.0 mg/cm^3) of rosettes formed by rat sensitised SRBCs. However, in general rabbit IgG inhibits rosette formation by rabbit sensitised SRBCs to a greater extent than rat IgG and rat IgG inhibits rosette formation by rat sensitised SRBCs to a greater extent than rabbit IgG. Human IgG inhibits rosette formation at a fairly high level irrespective of the sensitising antibody and bovine IgG produces a range of effects from limited inhibitions, through no inhibition to a slight enhancing effect.

The effect of heat aggregated IgGs on cells from the peritoneal exudate of uniparous animals (Fig. 28), similar cells treated with collagenase (Fig. 29) and peritoneal exudate cells from pseudopregnant

animals (Fig. 30) was to increase dramatically the amount of inhibition compared to that seen with monomeric IgGs (Fig. 19). The pattern of inhibitions observed corresponds to those seen for both monomeric and heat aggregated IgGs on cells from the metrial gland; rabbit IgG inhibiting rosette formation by rabbit sensitised SRBCs to a greater degree than rat IgG and rat IgG producing a greater inhibitory effect than rabbit IgG on rosette formation by rat sensitised SRBCs.

In an experiment to compare inhibiting effects of both monomeric and heat aggregated IgGs on cells from day 19 of uniparous pregnancy (Fig. 31) to their inhibiting effects on peritoneal exudate cells from the same animal (Fig. 32) it can be seen that there is very little variation from the results already reported. The metrial gland cells (Fig. 31) are inhibited by both monomeric and heat aggregated IgGs while the peritoneal exudate cells (Fig. 32) are only inhibited by the heat aggregated IgGs. However, the monomeric IgGs at 3.0 mg/cm^3 do not seem to inhibit metrial gland cell rosette formation with rabbit sensitised SRBCs to the same extent as do the heat aggregated IgGs (Fig. 31). Monomeric human IgG also produces less inhibition than heat aggregated human IgG at 3.0 mg/cm^3 with rat sensitised SRBCs (Fig. 31).

Figs. 33 and 34 show the inhibitory effect of heat aggregated human IgG at 7.0 mg/cm^3 on rosette formation by metrial gland and peritoneal exudate cells with rabbit sensitised SRBCs at 4^0C and 37^0C. The control value obtained for rosette inhibition at room temperature by the same immunoglobulin is shown by the asterisk (Figs. 33 and 34). Figs. 33 and 34 also show the percentage inhibitions obtained when cells were washed three times to remove free or loosely bound inhibiting IgG before rabbit sensitised SRBCs were added. At both 4^0C and 37^0C and with both metrial gland cells and peritoneal exudate cells the percentage of rosette inhibition tends to increase with time towards the value obtained when cells have not been prewashed before rosetting. Incubation in PBS produced only a limited amount of inhibition of rosette formation.

Figures 14 - 34

Fig. 14. Mean percentage (± SEM) of cells forming EAγ rosettes from the metrial gland of pregnancy

Day of pregnancy	Number of rats	Mean % EAγ rosette formation	
		Rabbit ŝ SRBC (●)	Rat ŝ SRBC (■)
12	4	29.7 ± 0.9	12.9 ± 0.5
13	11	26.9 ± 0.7	9.8 ± 0.7
13 (MP)	3	38.5 ± 1.1	19.1 ± 2.6
14	8	25.3 ± 1.3	9.5 ± 1.1
15	16	24.1 ± 0.8	9.0 ± 0.6
17	3	21.5 ± 1.9	12.6 ± 1.5
19	10	28.3 ± 1.1	20.2 ± 1.3
20	1	34.2	28.7

MP - Multiparous pregnancy

ŝ - Sensitised

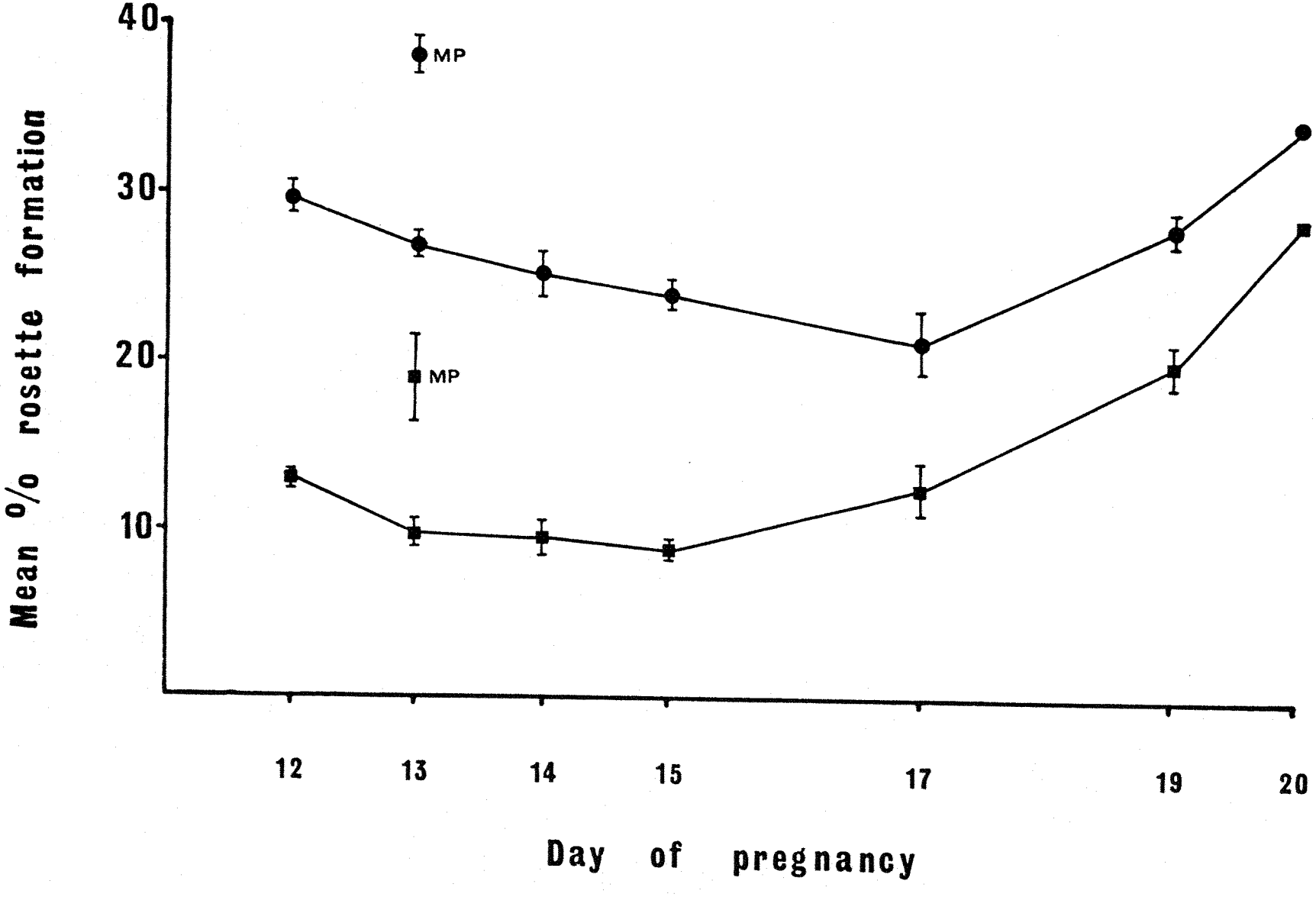

40
30
20
10
Mean % rosette formation
MP
MP
12
13
14
15
17
19
20
Day of pregnancy

Fig. 15. Mean percentage (± SEM) of cells forming EAγ rosettes from the metrial gland of the deciduomata of pseudopregnancy

Day of pseudopregnancy	Number of rats	Mean % EAγ rosette formation	
		Rabbit ŝ SRBC (●)	Rat ŝ SRBC (■)
11	3	27.8 ± 0.7	16.1 ± 0.3
12	8	21.2 ± 1.3	11.8 ± 0.9
13	9	25.3 ± 1.1	12.9 ± 1.1
14	3	31.2 ± 0.8	17.8 ± 0.4

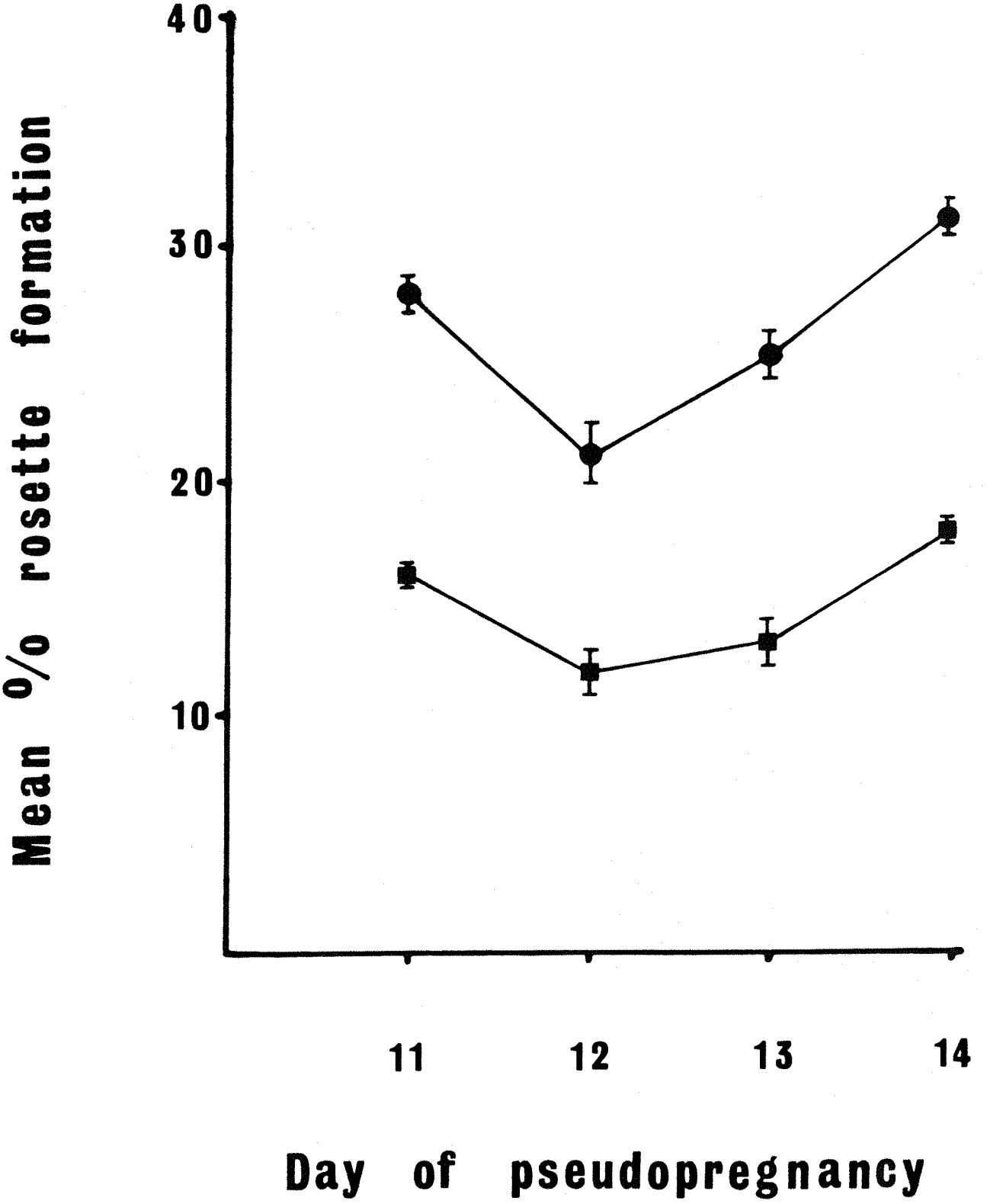

40
30
20
10
Mean % rosette formation
11
12
13
14
Day of pseudopregnancy

Fig. 16. Mean percentage (± SEM) of cells forming EAγ rosettes from control tissues

Tissue	Number of rats	Mean % EAγ rosette formation	
		Rabbit ŝ SRBC	Rat ŝ SRBC
PE*	18	52.9 ± 3.2	20.5 ± 3.4
PE*(C)	7	74.5 ± 2.4	22.5 ± 7.5
PE(PP)	9	63.6 ± 4.6	23.3 ± 4.7
Spleen*	11	40.1 ± 1.5	26.2 ± 1.6
Spleen*(C)	3	33.0 ± 1.1	20.5 ± 2.5
Spleen (PP)	2	45.2 ± 7.2	29.2 ± 3.2
Thymus*	11	3.2 ± 0.3	2.1 ± 0.3
Thymus *(C)	3	2.2 ± 0.4	1.2 ± 0.3
Thymus (PP)	2	2.2 ± 0.2	2.2 ± 0.2

PE - Cells of the peritoneal exudate

C - Cells treated with collagenase

PP - Cells from pseudopregnant animals

* - Cells from control rats; uniparous or non-pregnant.

Fig. 17. Day 14 uniparous metrial gland cells. Mean percentage (± SEM) rosette inhibition produced by various rat sera. Normal male (●), non-pregnant female (■), uniparous female (▼), multiparous female (♦).

Inhibiting serum	Dilution	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Normal male	1/10	2	6.1 ± 4.1	12.5 ± 6.0
	1/5	2	27.3 ± 1.2	28.9 ± 6.6
	Neat	2	67.1 ± 8.0	79.2 ± 1.5
Non-pregnant female	1/10	2	12.9 ± 0.6	31.2 ± 2.2
	1/5	2	29.7 ± 1.1	52.0 ± 3.6
	Neat	2	69.5 ± 8.0	73.9 ± 3.5
Uniparous female day 13	1/10	2	8.9 ± 1.3	11.3 ± 1.3
	1/5	2	36.5 ± 3.8	45.9 ± 8.9
	Neat	1	65.3	74.1
Multiparous female day 13	1/10	2	9.7 ± 5.6	18.9 ± 0.5
	1/5	2	30.5 ± 4.0	39.3 ± 6.0
	Neat	2	55.3 ± 4.3	65.4 ± 2.4

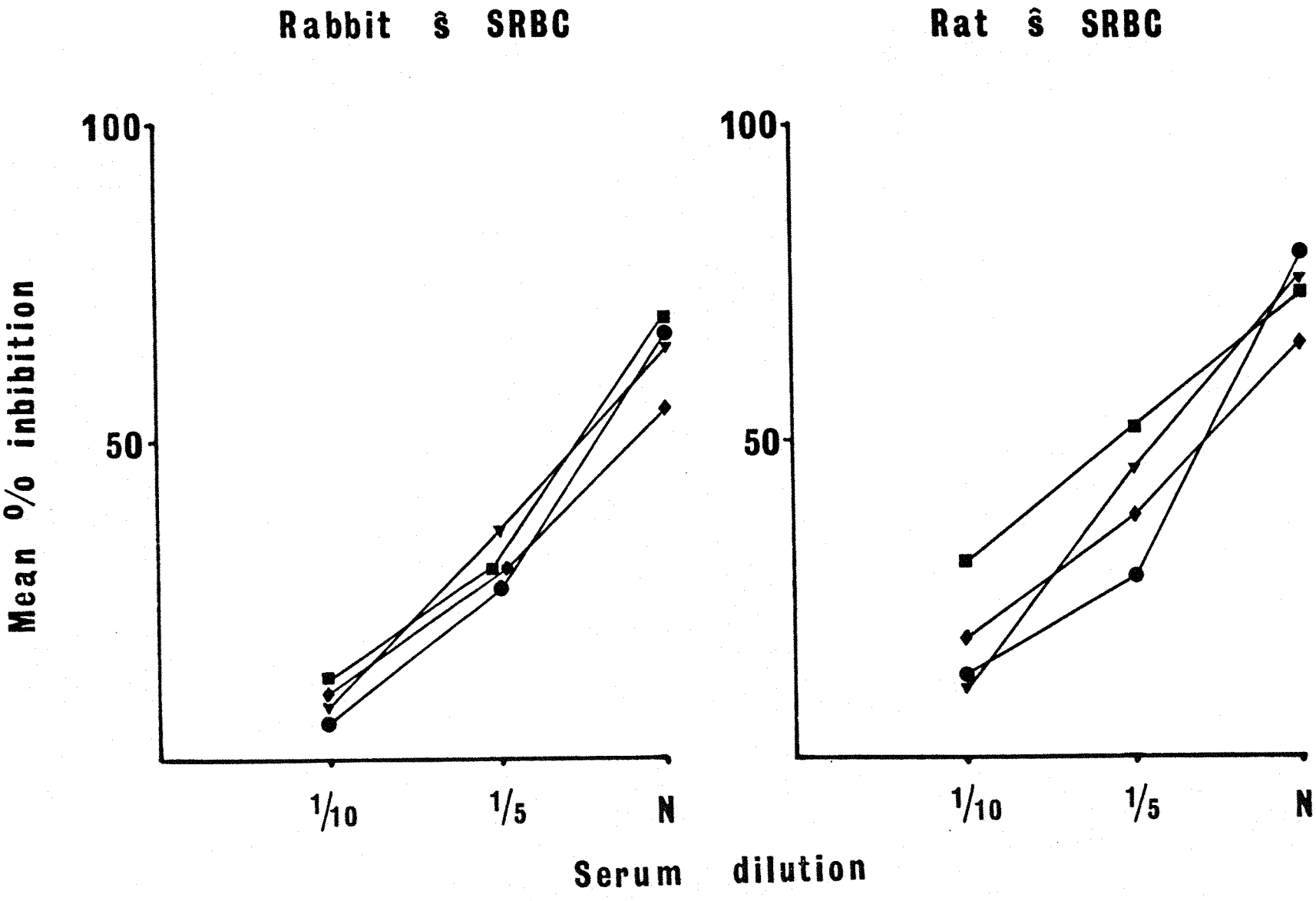

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
Mean % inhibition
1/10
1/5
N
Serum dilution

Fig. 18. Day 14 uniparous metrial gland cells. Mean percentage (± SEM) rosette inhibition produced by monomeric immunoglobulins.

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	3	90.5 ± 1.9	49.2 ± 1.1
(♦)	3	3	89.1 ± 1.0	40.2 ± 8.5
	1	3	89.0 ± 0.6	17.3 ± 3.3
Rat	7	3	73.3 ± 9.0	89.8 ± 2.9
(■)	3	3	51.7 ± 6.4	81.8 ± 3.7
	1	3	15.9 ± 2.6	74.6 ± 3.9
Human	7	3	93.7 ± 3.4	77.3 ± 9.4
(●)	3	3	87.2 ± 0.7	70.0 ± 5.7
	1	3	63.6 ± 3.7	52.3 ± 1.8
Bovine	7	3	14.9 ± 2.6	(-) 7.6 ± 8.8
(▼)	3	3	(-) 2.2 ± 9.3	(-) 0.3 ± 6.4
	1	ND	-	-

ND - Not done

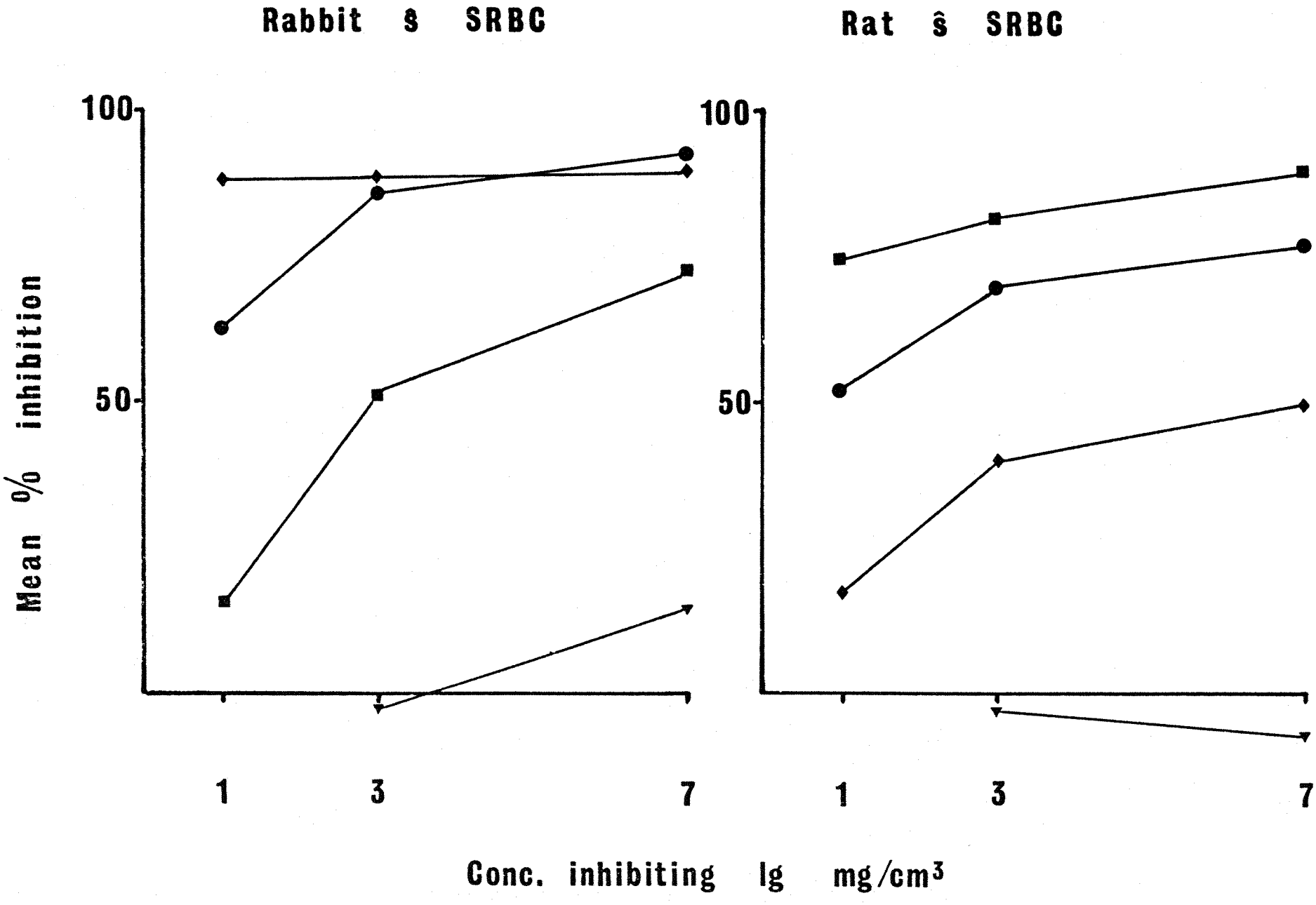

Rabbit ♂ SRBC
Rat ♀ SRBC
100
50
100
50
Mean % inhibition
1
3
7
1
3
7
Conc. inhibiting lg mg/cm³

Fig. 19. Day 14 uniparous peritoneal exudate cells. Mean percentage (± SEM) rosette inhibition produced by monomeric immunoglobulins.

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	ND	-	-
(♦)	3	2	2.9 ± 1.3	5.9 ± 2.3
	1	2	1.3 ± 0.6	3.7 ± 1.1
Rat	7	2	14.8 ± 1.8	16.4 ± 0.5
(■)	3	2	4.4 ± 2.8	6.7 ± 0.7
	1	2	2.3 ± 2.3	2.3 ± 1.3
Human	7	ND	-	-
(●)	3	2	19.7 ± 0.4	19.4 ± 0.7
	1	2	10.5 ± 1.7	8.6 ± 1.6
Bovine	7	ND	-	-
(▼)	3	2	(-) 9.4 ± 9.4	(-) 2.0 ± 5.0
	1	ND	-	-

ND - Not done

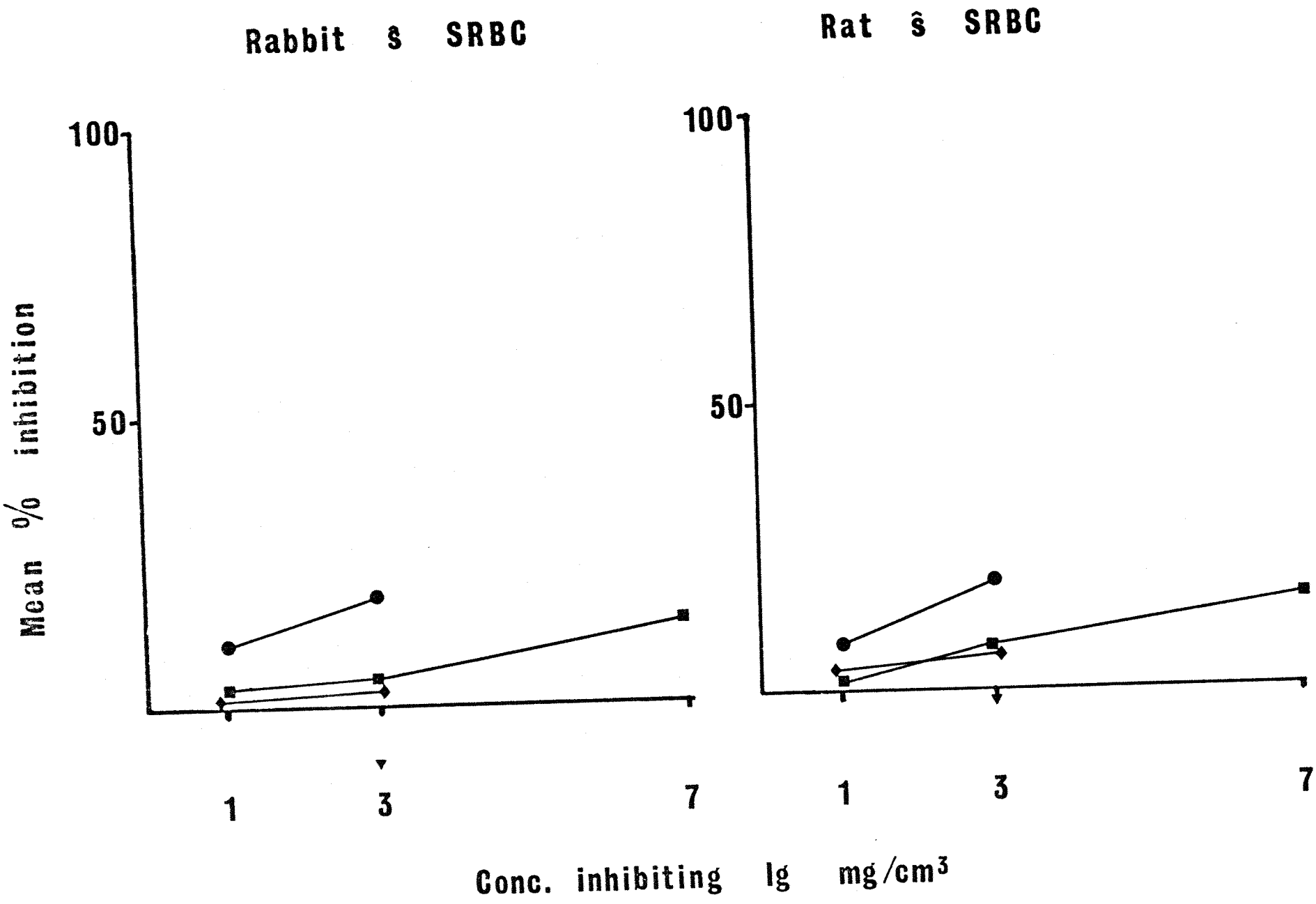

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
Mean % inhibition
1
3
7
Conc. inhibiting lg mg/cm3

Fig. 20. Day 12 uniparous metrial gland cells. Mean percentage (± SEM) rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	3	86.7 ± 2.7	67.9 ± 8.9
(♦)	3	3	79.9 ± 0.1	60.7 ± 6.8
	1	3	80.7 ± 3.7	56.5 ± 6.7
Rat	7	3	50.7 ± 3.2	81.3 ± 2.6
(■)	3	3	47.1 ± 4.5	68.2 ± 2.9
	1	3	38.8 ± 3.8	62.5 ± 8.4
Human	7	3	91.8 ± 2.3	69.2 ± 6.7
(●)	3	3	84.9 ± 3.3	67.7 ± 10.7
	1	3	81.8 ± 4.2	60.5 ± 5.9
Bovine	7	3	40.5 ± 4.5	32.8 ± 2.4
(▼)	3	3	33.7 ± 6.2	7.8 ± 2.9
	1	3	20.9 ± 11.4	5.1 ± 3.2

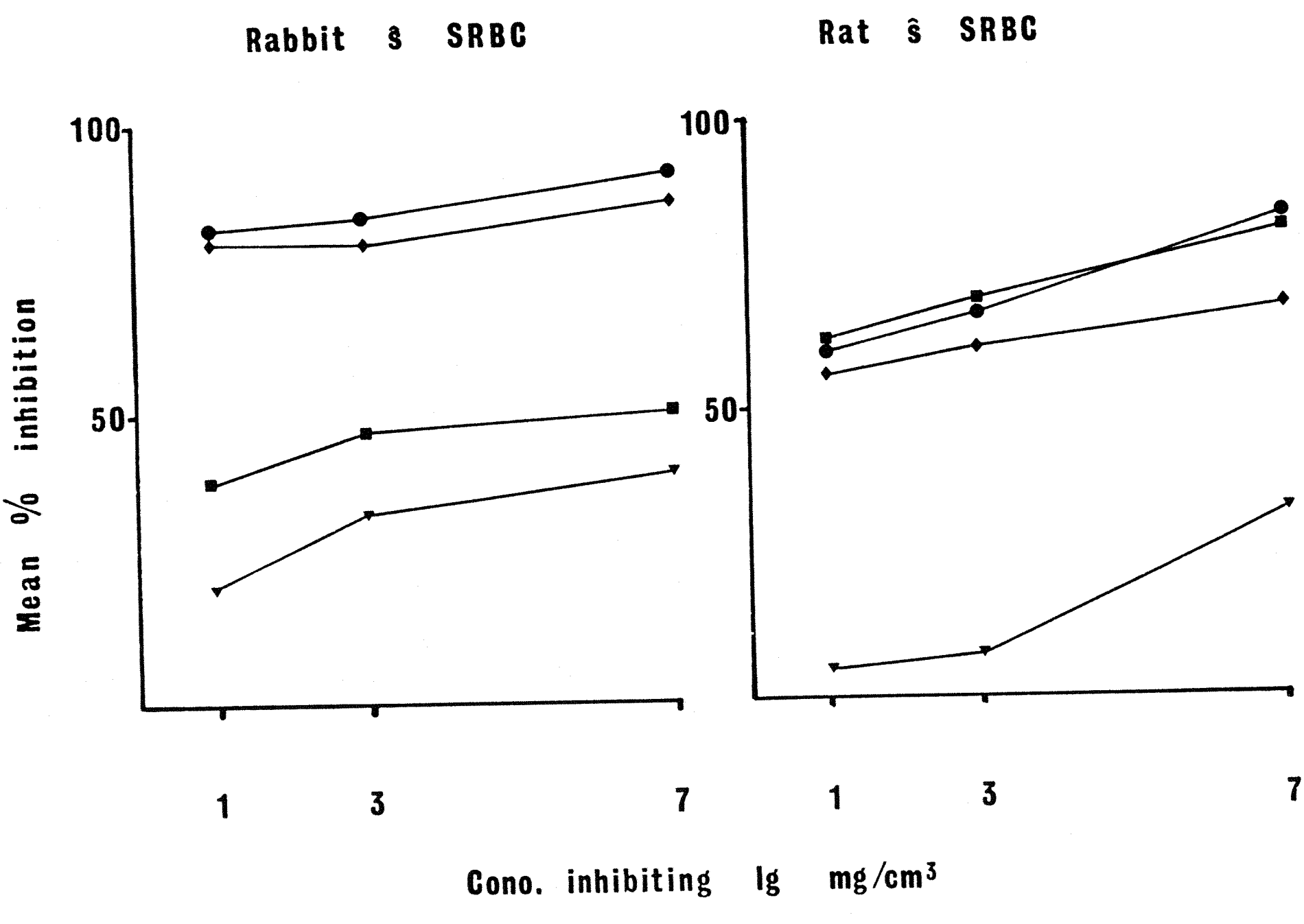

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
100
50
Mean % inhibition
1
3
7
1
3
7
Cono. inhibiting lg mg/cm3

Fig. 21. Day 13 uniparous metrial gland cells. Mean percentage (± SEM) rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	5	87.3 ± 1.7	59.0 ± 14.1
(♦)	3	5	82.3 ± 2.1	55.9 ± 16.0
	1	5	76.2 ± 3.8	54.6 ± 13.5
Rat	7	5	58.1 ± 6.0	71.1 ± 10.9
(■)	3	5	36.2 ± 6.8	58.0 ± 10.7
	1	5	18.7 ± 6.1	41.2 ± 8.2
Human	7	5	92.0 ± 1.3	80.2 ± 4.6
(●)	3	5	85.0 ± 1.9	78.9 ± 8.5
	1	5	70.3 ± 4.6	66.9 ± 8.6
Bovine	7	4	37.7 ± 3.9	29.4 ± 9.0
(▼)	3	4	26.9 ± 5.9	11.7 ± 10.2
	1	4	26.1 ± 4.9	0.9 ± 7.3

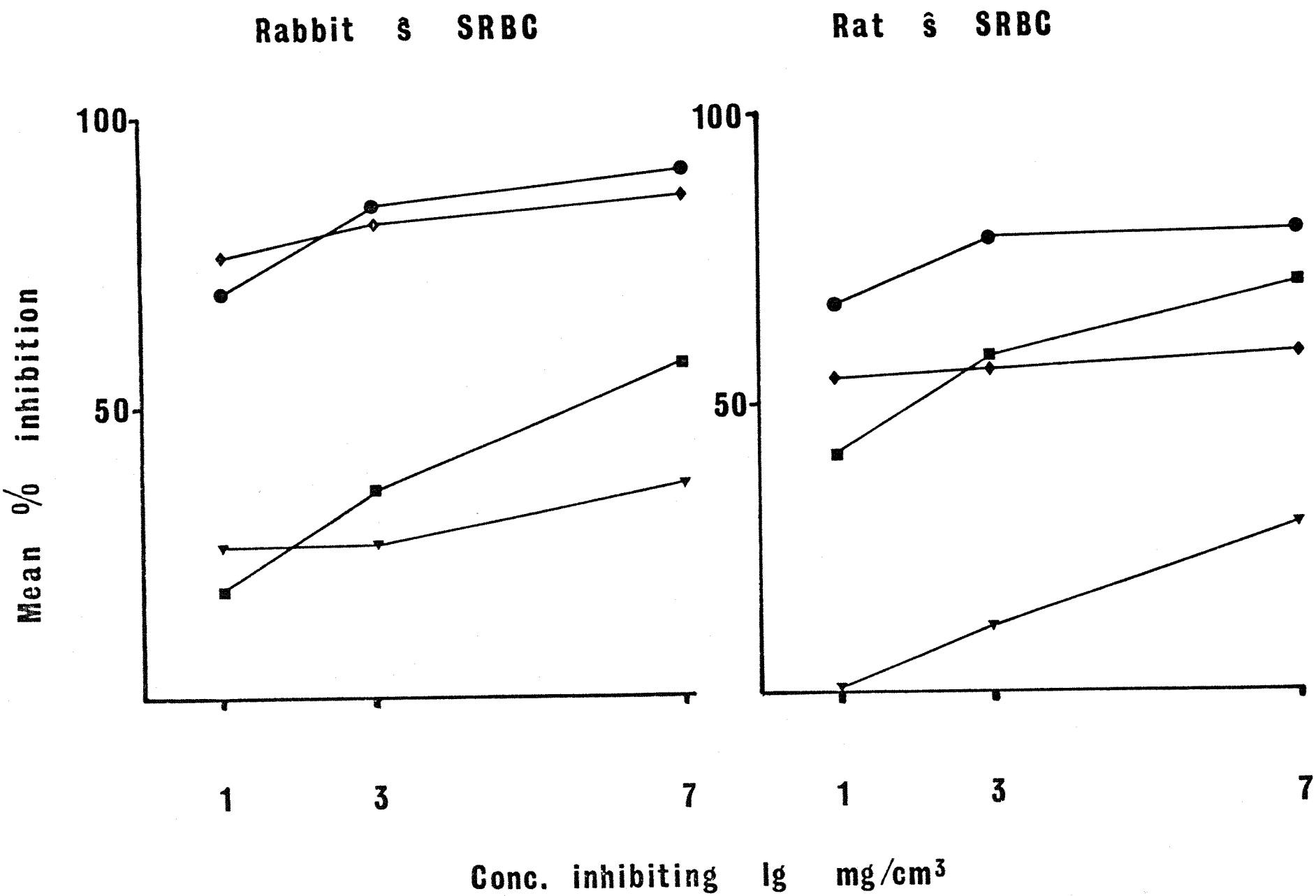

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
100
50
Mean % inhibition
1
3
7
1
3
7
Conc. inhibiting lg mg/cm3

Fig. 22. Day 14 uniparous metrial gland cells. Mean percentage (± SEM) rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/cm³	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	3	80.6 ± 4.2	66.1 ± 4.6
(♦)	3	3	79.3 ± 1.7	55.0 ± 1.2
	1	3	75.9 ± 5.6	54.5 ± 3.3
Rat	7	3	57.9 ± 10.7	78.1 ± 6.6
(■)	3	3	50.5 ± 13.5	55.1 ± 4.2
	1	3	37.7 ± 18.5	46.9 ± 7.6
Human	7	2	87.5 ± 2.3	76.8 ± 3.7
(●)	3	2	84.7 ± 5.1	80.1 ± 4.4
	1	2	74.1 ± 7.4	64.2 ± 6.5
Bovine	7	4	22.7 ± 7.4	21.0 ± 8.4
(▼)	3	4	4.3 ± 8.4	16.1 ± 13.5
	1	4	0.2 ± 12.4	(-) 3.4 ± 12.1

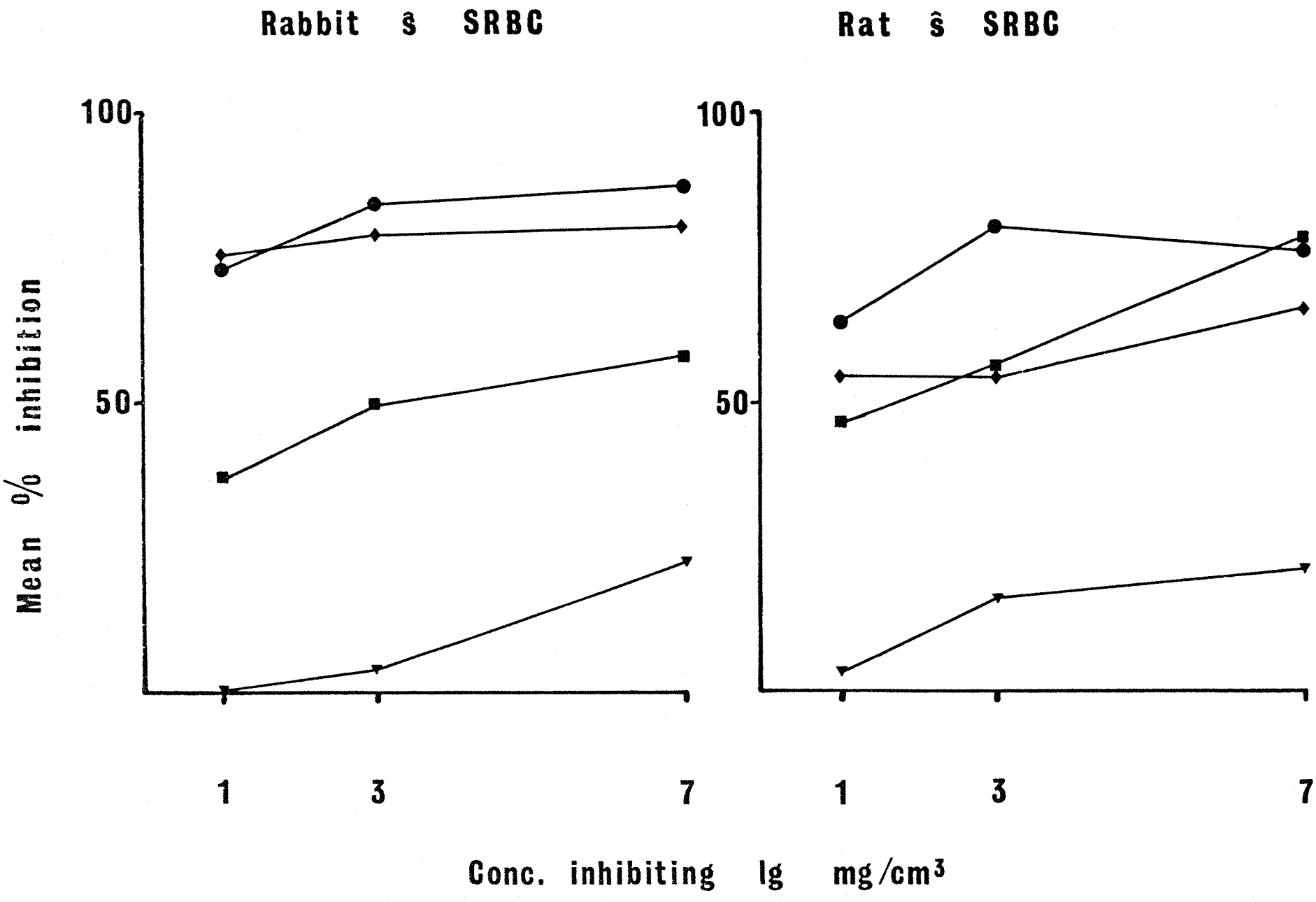

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
100
50
Mean % inhibition
1
3
7
1
3
7
Conc. inhibiting lg mg/cm3

Fig. 23. Day 15 uniparous metrial gland cells. Mean percentage (± SEM) rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	5	85.4 ± 1.7	36.5 ± 4.7
(♦)	3	5	79.5 ± 1.9	31.6 ± 5.8
	1	5	72.4 ± 4.4	33.6 ± 7.6
Rat	7	5	41.8 ± 8.4	65.8 ± 11.3
(■)	3	5	39.1 ± 6.9	50.1 ± 8.3
	1	5	14.4 ± 5.1	31.4 ± 6.5
Human	7	2	85.5 ± 6.9	72.3 ± 12.3
(●)	3	2	81.8 ± 5.6	78.4 ± 1.5
	1	2	66.0 ± 6.5	55.7 ± 5.7
Bovine	7	2	24.5 ± 5.5	1.5 ± 11.5
(▼)	3	2	3.0 ± 10.0	11.5 ± 9.5
	1	2	5.4 ± 9.6	(-) 1.1 ± 8.8

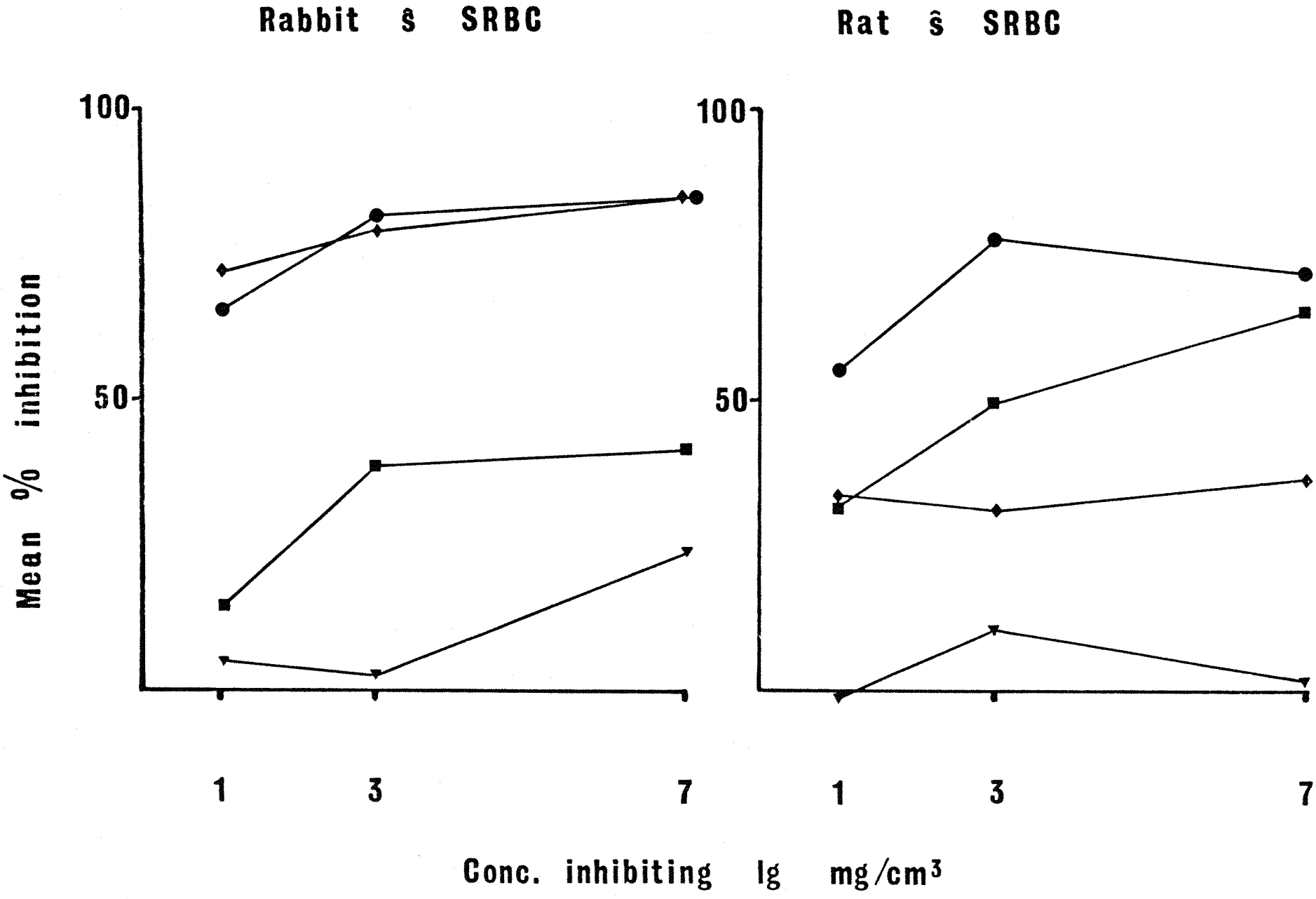

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
100
50
Mean % inhibition
1
3
7
1
3
7
Conc. inhibiting lg mg/cm³

Fig. 24. Day 19 uniparous metrial gland cells. Percentage rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC	Rat ŝ SRBC
Rabbit	7	1	76.4	50.6
(♦)	3	1	72.8	35.5
	1	1	76.4	50.6
Rat	7	1	51.0	97.9
(■)	3	1	18.2	93.6
	1	1	14.6	87.1
Human	7	1	89.1	85.0
(●)	3	1	56.4	93.6
	1	1	56.4	82.8
Bovine	7	1	(-) 3.9	(-) 5.7
(▼)	3	1	(-) 1.8	(-) 3.2
	1	1	(-) 2.8	1.1

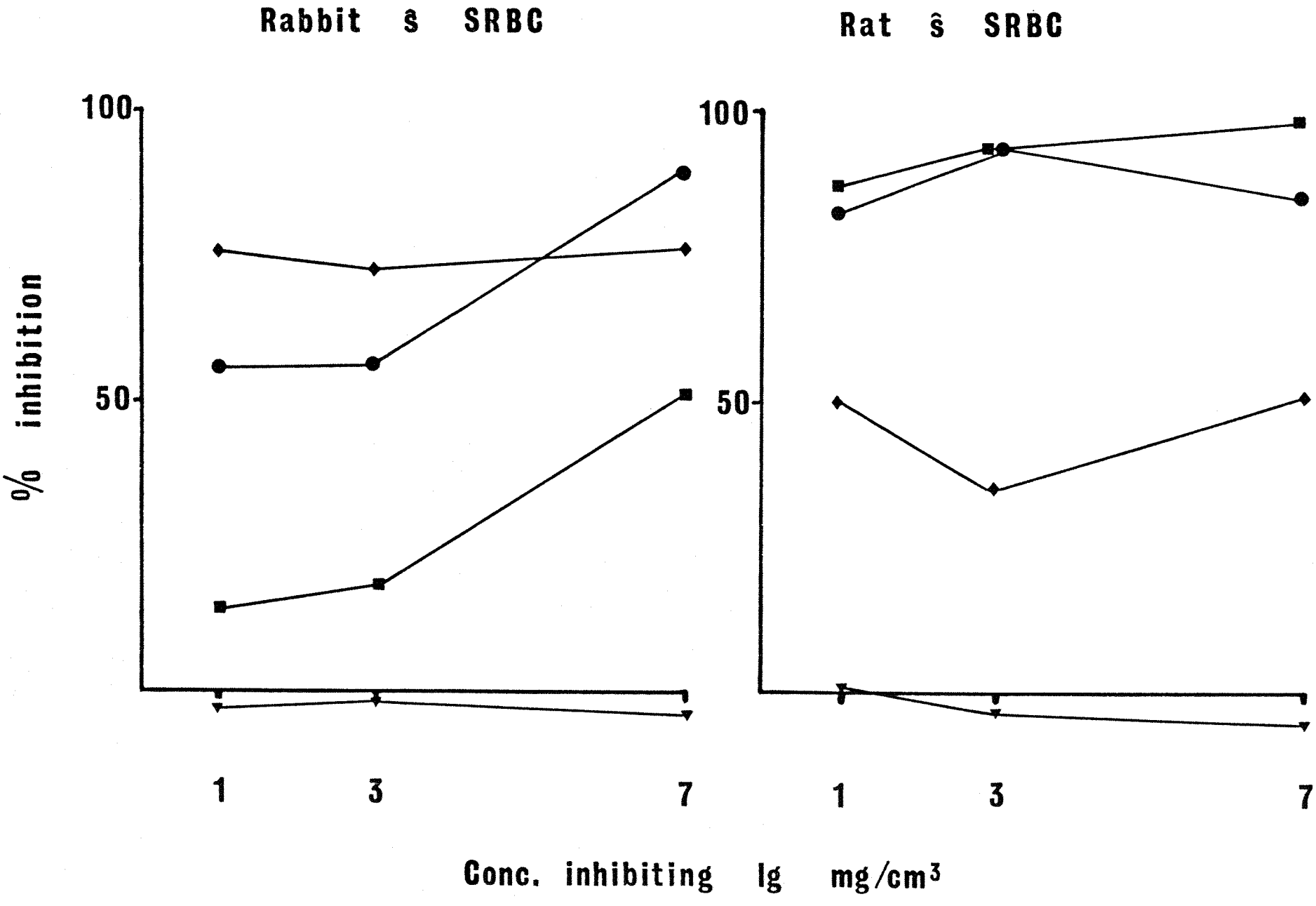

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
100
50
% inhibition
1
3
7
1
3
7
Conc. inhibiting lg mg/cm3

Fig. 25. Day 13 multiparous metrial gland cells. Percentage rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC	Rat ŝ SRBC
Rabbit	7	1	76.9	50.9
(♦)	3	1	74.2	33.4
	1	1	60.6	15.8
Rat	7	1	41.5	72.0
(■)	3	1	27.9	61.5
	1	1	21.1	43.9
Human	7	1	79.6	61.5
(●)	3	1	76.9	54.4
	1	1	63.3	29.9
Bovine	7	1	10.3	26.4
(▼)	3	1	(-) 2.0	(-) 1.2
	1	1	18.4	12.3

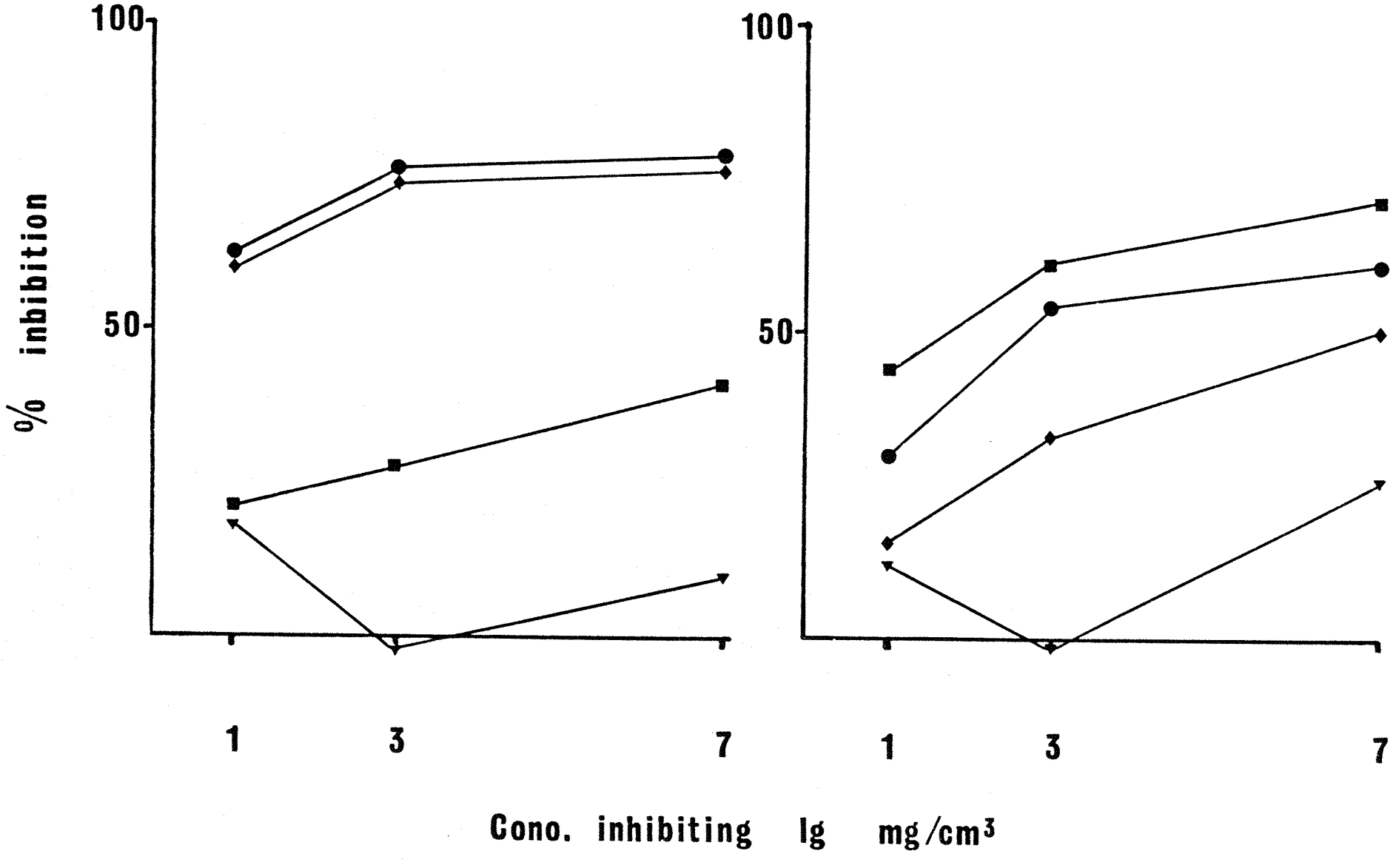

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
100
50
% inhibition
1
3
7
1
3
7
Cono. inhibiting lg mg/cm3

Fig. 26. Day 12 metrial gland cells from the deciduomata of pseudopregnancy. Mean percentage (± SEM) rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/om^3	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	2	72.0 ± 1.9	61.8 ± 4.4
(♦)	3	ND	-	-
	1	2	59.7 ± 8.2	30.1 ± 5.5
Rat	7	2	35.8 ± 13.8	74.9 ± 9.1
(■)	3	ND	-	-
	1	2	20.6 ± 4.6	48.3 ± 6.2
Human	7	2	78.7 ± 1.3	70.1 ± 5.8
(●)	3	ND	-	-
	1	2	57.2 ± 7.2	51.6 ± 6.1
Bovine	7	2	36.0 ± 10.0	16.4 ± 9.0
(▼)	3	ND	-	-
	1	2	17.6 ± 7.6	1.4 ± 8.6

ND - Not done

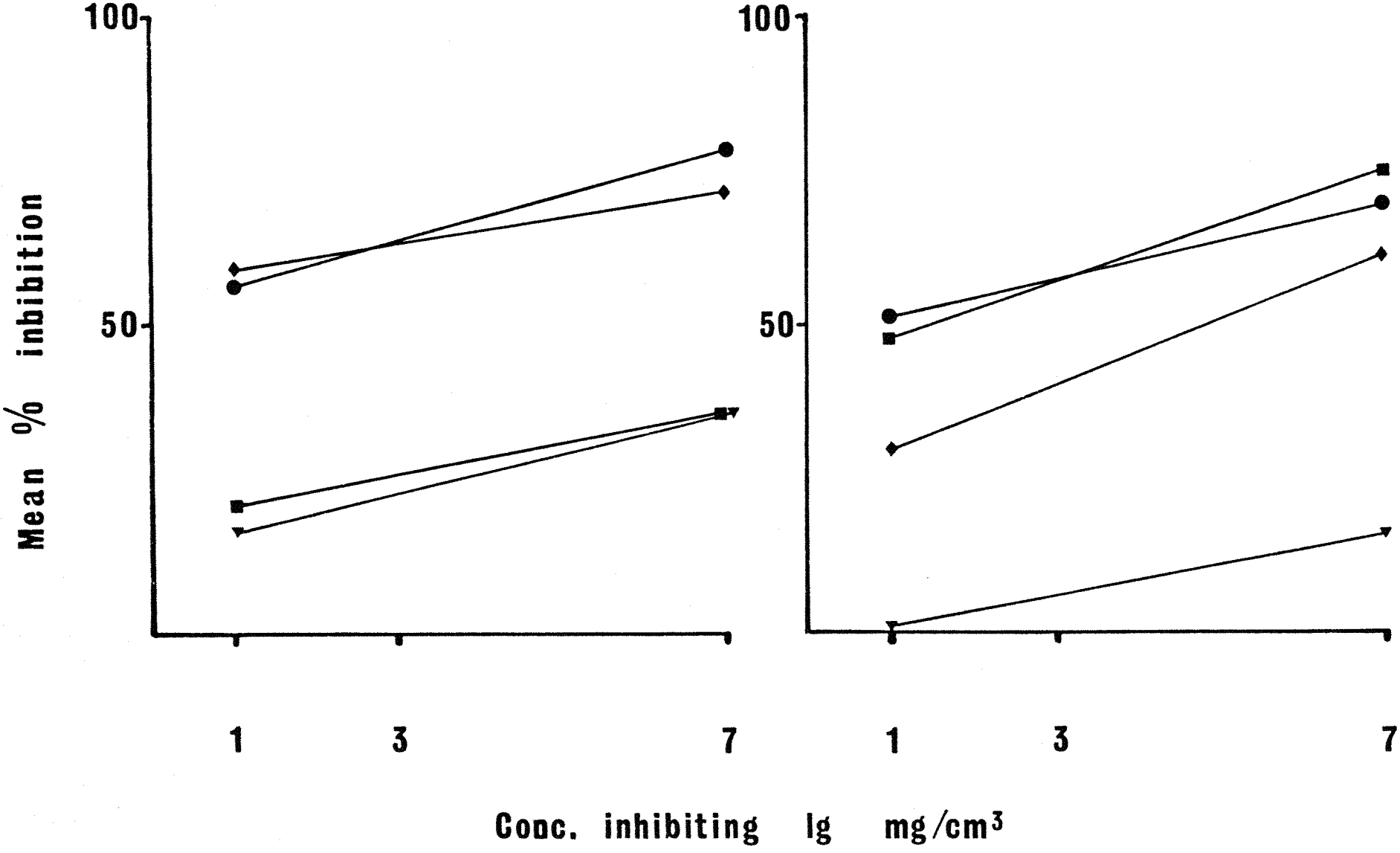

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
100
50
Mean % inhibition
1
3
7
1
3
7
Conc. inhibiting lg mg/cm3

Fig. 27. Day 13 metrial gland cells from the deciduomata of pseudopregnancy. Mean percentage (± SEM) rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	3	70.9 ± 6.8	55.4 ± 6.4
(♦)	3	2	66.7 ± 6.3	52.5 ± 2.5
	1	3	55.0 ± 5.7	35.4 ± 12.2
Rat	7	3	29.6 ± 9.3	68.4 ± 7.2
(■)	3	2	20.4 ± 5.0	54.2 ± 7.4
	1	3	15.7 ± 4.6	50.0 ± 9.1
Human	7	3	82.8 ± 5.3	78.4 ± 5.0
(●)	3	1	82.0	87.5
	1	3	61.6 ± 5.0	53.5 ± 10.0
Bovine	7	3	13.8 ± 7.2	(-) 0.4 ± 13.8
(▼)	3	1	1.0	0.0
	1	3	(-) 11.3 ± 5.4	(-) 5.1 ± 2.6

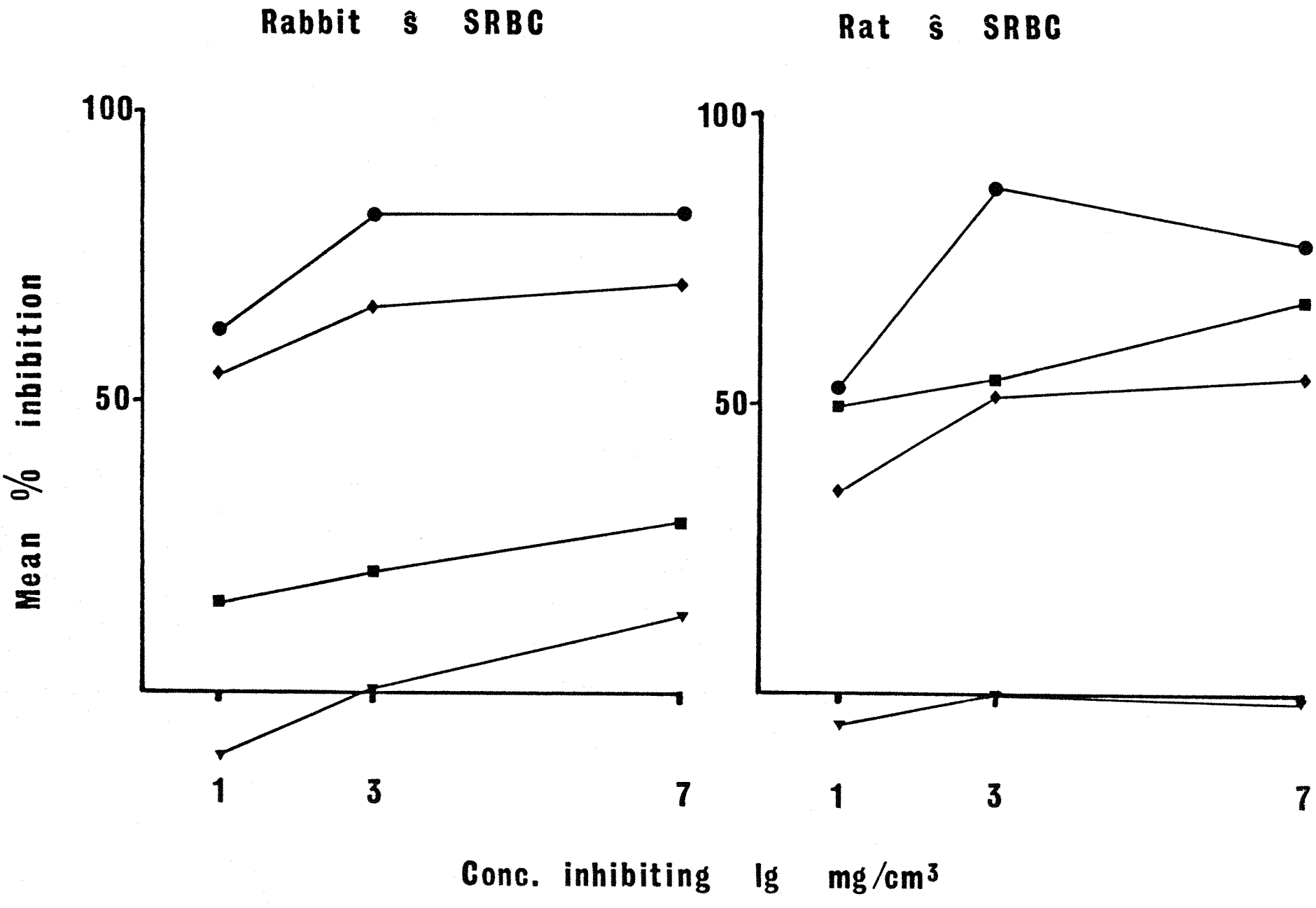

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
100
50
Mean % inhibition
1
3
7
1
3
7
Conc. inhibiting lg mg/cm3

Fig. 28. Uniparous peritoneal exudate cells. Mean percentage (± SEM) rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	3	67.7 ± 14.1	46.9 ± 2.1
(♦)	3	3	67.6 ± 13.8	37.8 ± 7.4
	1	3	66.4 ± 17.7	31.5 ± 10.3
Rat	7	3	42.0 ± 16.9	69.9 ± 10.5
(■)	3	3	35.3 ± 18.5	77.0 ± 12.5
	1	3	14.7 ± 8.2	50.0 ± 12.2
Human	7	2	95.4 ± 1.9	78.8 ± 3.8
(●)	3	2	94.0 ± 0.5	83.1 ± 8.5
	1	2	69.5 ± 17.9	53.8 ± 11.3
Bovine	7	3	44.9 ± 11.4	44.2 ± 16.8
(▼)	3	3	23.0 ± 17.9	27.2 ± 9.3
	1	3	(-) 3.5 ± 13.2	11.9 ± 11.7

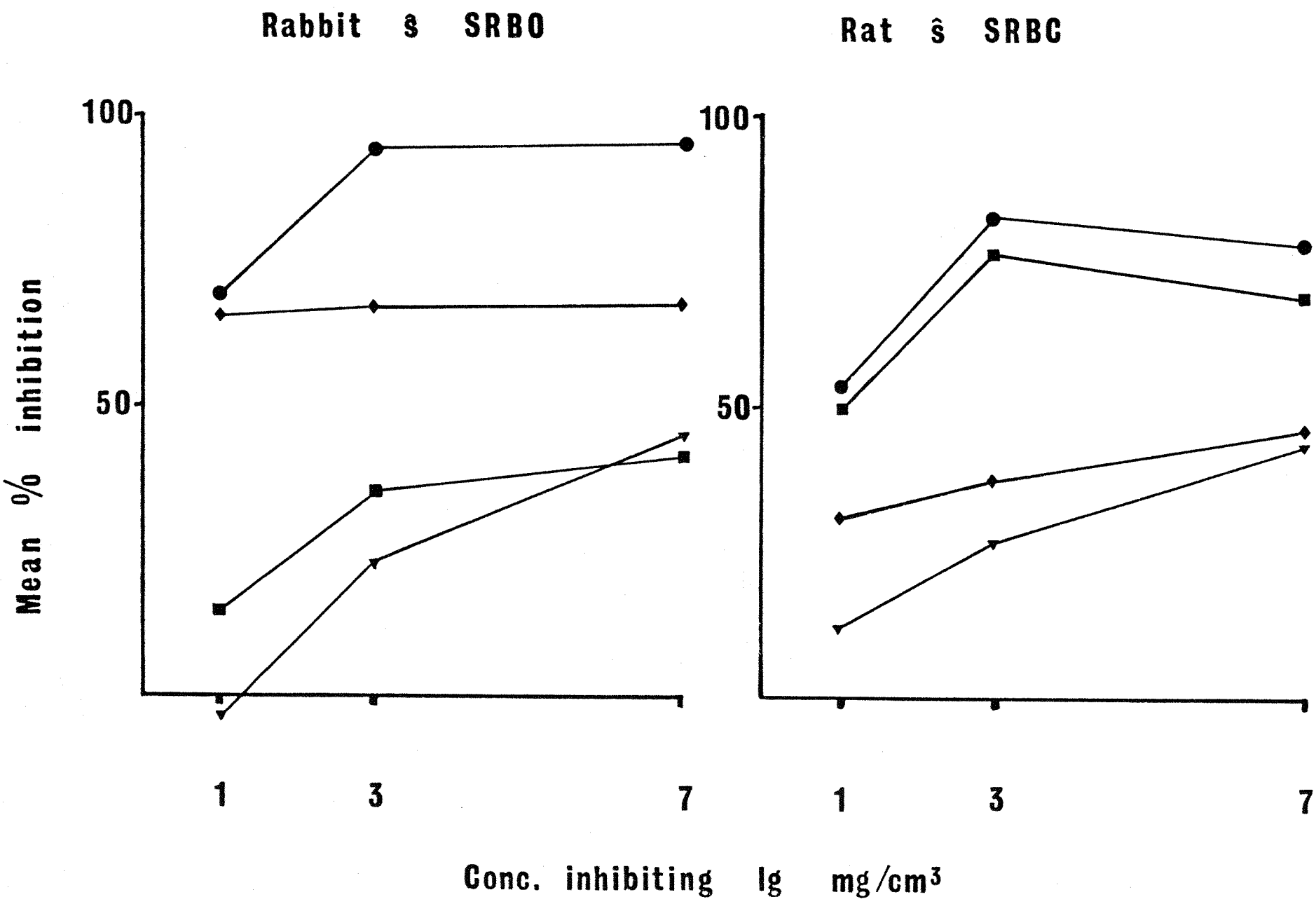

Rabbit ŝ SRBO
Rat ŝ SRBC
100
50
100
50
Mean % inhibition
1
3
7
1
3
7
Conc. inhibiting lg mg/cm3

Fig. 29. Uniparous peritoneal exudate cells treated with collagenase. Mean percentage (± SEM) rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/cm³	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	3	90.0 ± 7.6	38.3 ± 10.9
(♦)	3	3	79.7 ± 17.9	24.7 ± 10.5
	1	3	76.9 ± 18.3	37.7 ± 4.9
Rat	7	3	46.0 ± 1.6	98.0 ± 2.0
(■)	3	3	29.0 ± 6.8	78.9 ± 8.9
	1	3	2.0 ± 3.7	48.2 ± 14.6
Human	7	2	97.0 ± 1.5	89.0 ± 9.0
(●)	3	2	90.9 ± 5.2	79.1 ± 9.1
	1	2	79.5 ± 1.7	72.2 ± 12.2
Bovine	7	3	15.9 ± 0.8	62.9 ± 5.0
(▼)	3	3	3.6 ± 7.7	45.2 ± 1.9
	1	3	(-) 2.0 ± 5.9	20.8 ± 15.5

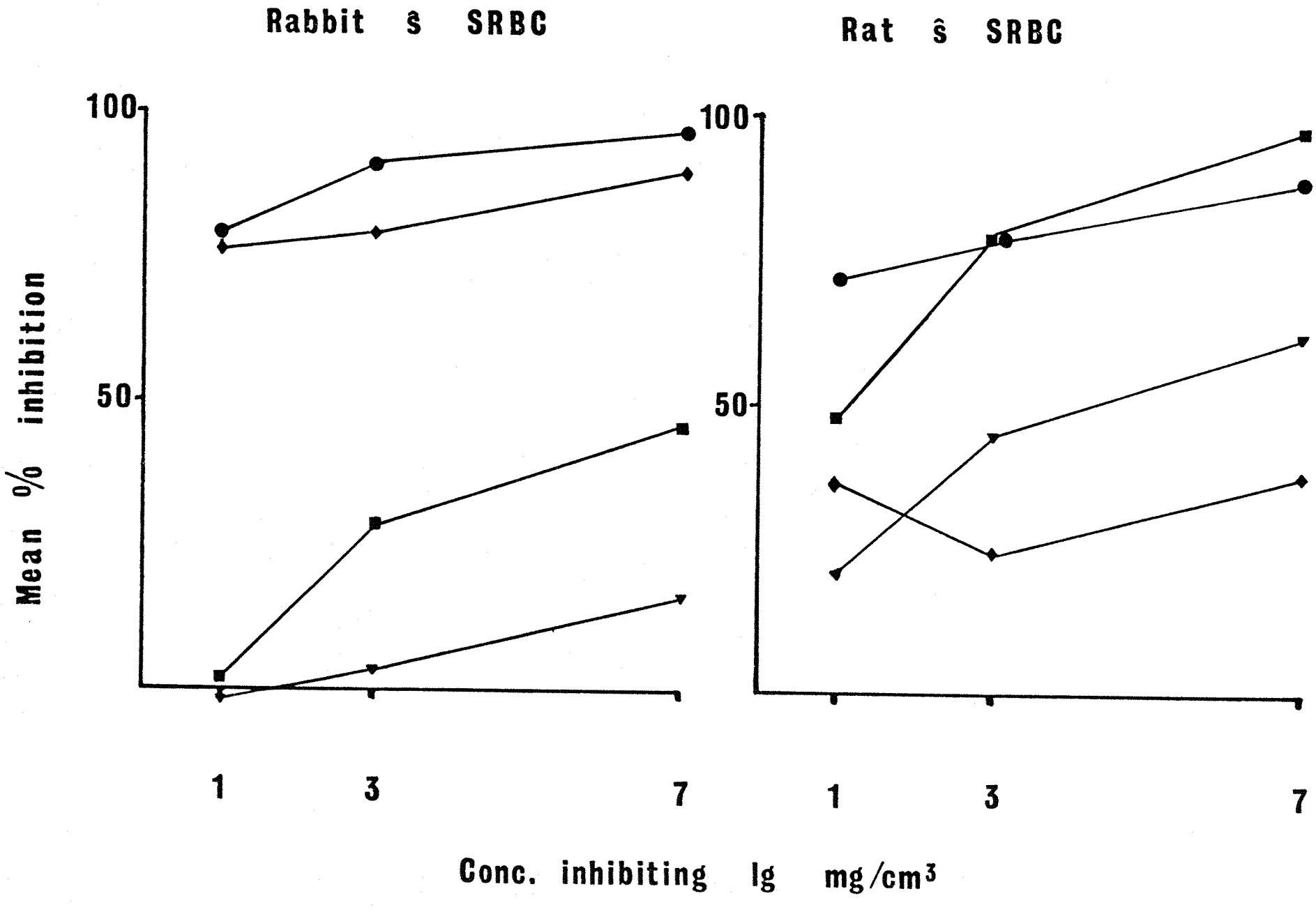

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
100
50
Mean % inhibition
1
3
7
1
3
7
Conc. inhibiting lg mg/cm3

Fig. 30. Peritoneal exudate cells from rats with deciduomata. Mean percentage (± SEM) rosette inhibition produced by heat aggregated immunoglobulins.

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC mean ± SEM	Rat ŝ SRBC mean ± SEM
Rabbit	7	3	64.2 ± 2.3	73.9 ± 5.5
(♦)	3	2	58.9 ± 3.9	66.6 ± 0.8
	1	3	41.6 ± 7.1	53.9 ± 9.7
Rat	7	3	29.6 ± 4.9	85.5 ± 5.8
(■)	3	2	22.3 ± 2.5	67.0 ± 4.5
	1	3	17.2 ± 6.5	64.2 ± 8.2
Human	7	3	86.9 ± 1.4	87.6 ± 1.7
(●)	3	1	84.1	91.9
	1	3	45.6 ± 4.0	75.1 ± 7.9
Bovine	7	3	21.2 ± 6.8	61.8 ± 3.9
(▼)	3	1	7.1	59.2
	1	3	1.9 ± 2.8	29.7 ± 9.2

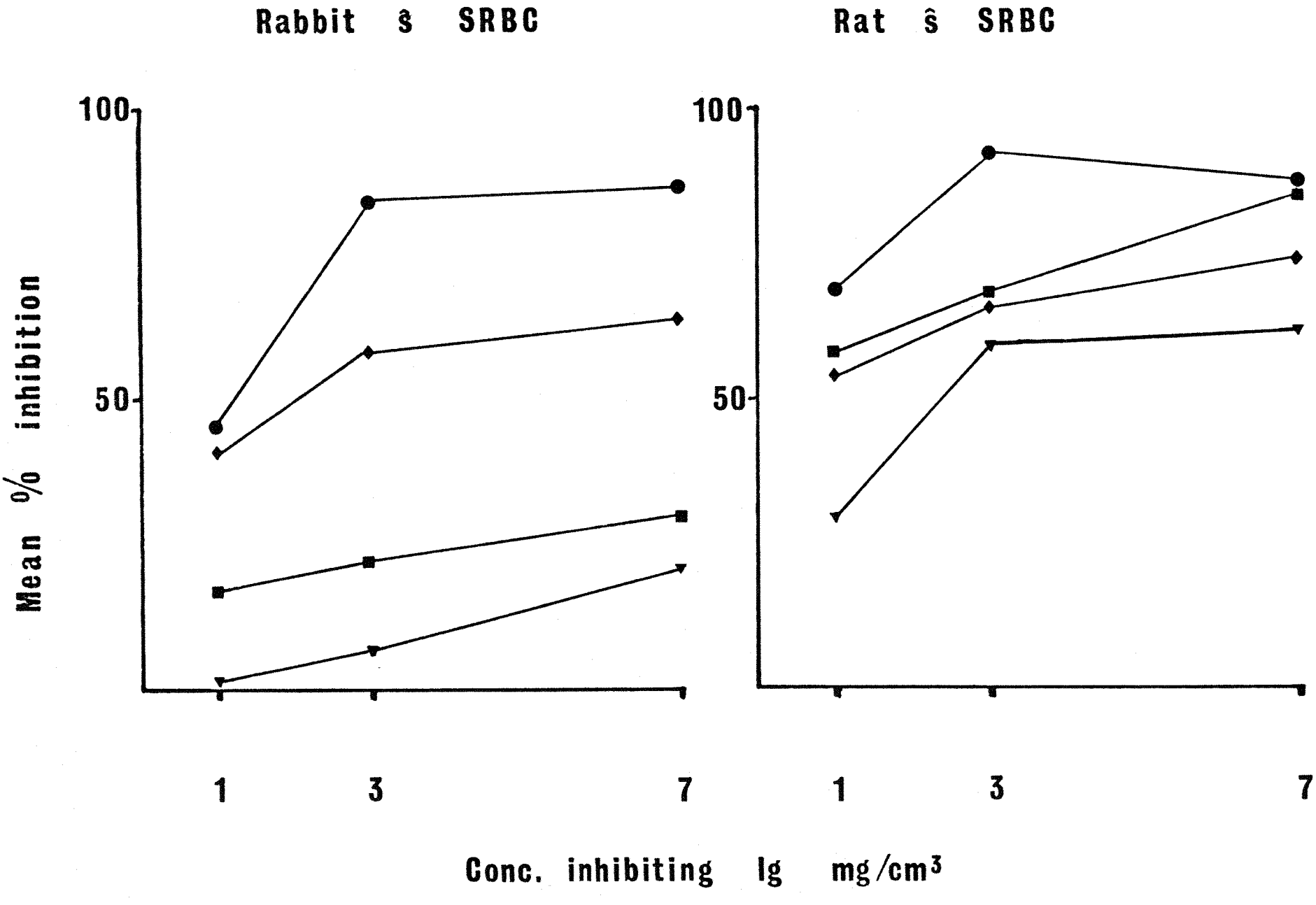

Rabbit ŝ SRBC
Rat ŝ SRBC
100
50
100
50
Mean % inhibition
1
3
7
1
3
7
Conc. inhibiting lg mg/cm3

Fig. 31. Day 19 uniparous metrial gland cells. Comparison of percentage rosette inhibition produced by monomeric or heat aggregated immunoglobulins

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC		Rat ŝ SRBC	
			Mono	HA	Mono	HA
Rabbit	3	1	63.6	77.3	47.7	43.2
(♦)	1	1	42.4	54.5	31.8	40.9
Rat	3	1	29.8	51.5	75.0	79.5
(■)	1	1	30.3	30.3	68.2	65.9
Human	3	1	56.1	77.5	63.6	84.1
(●)	1	1	39.4	39.4	54.5	75.0

Mono - monomeric IgG

HA - heat aggregated IgG

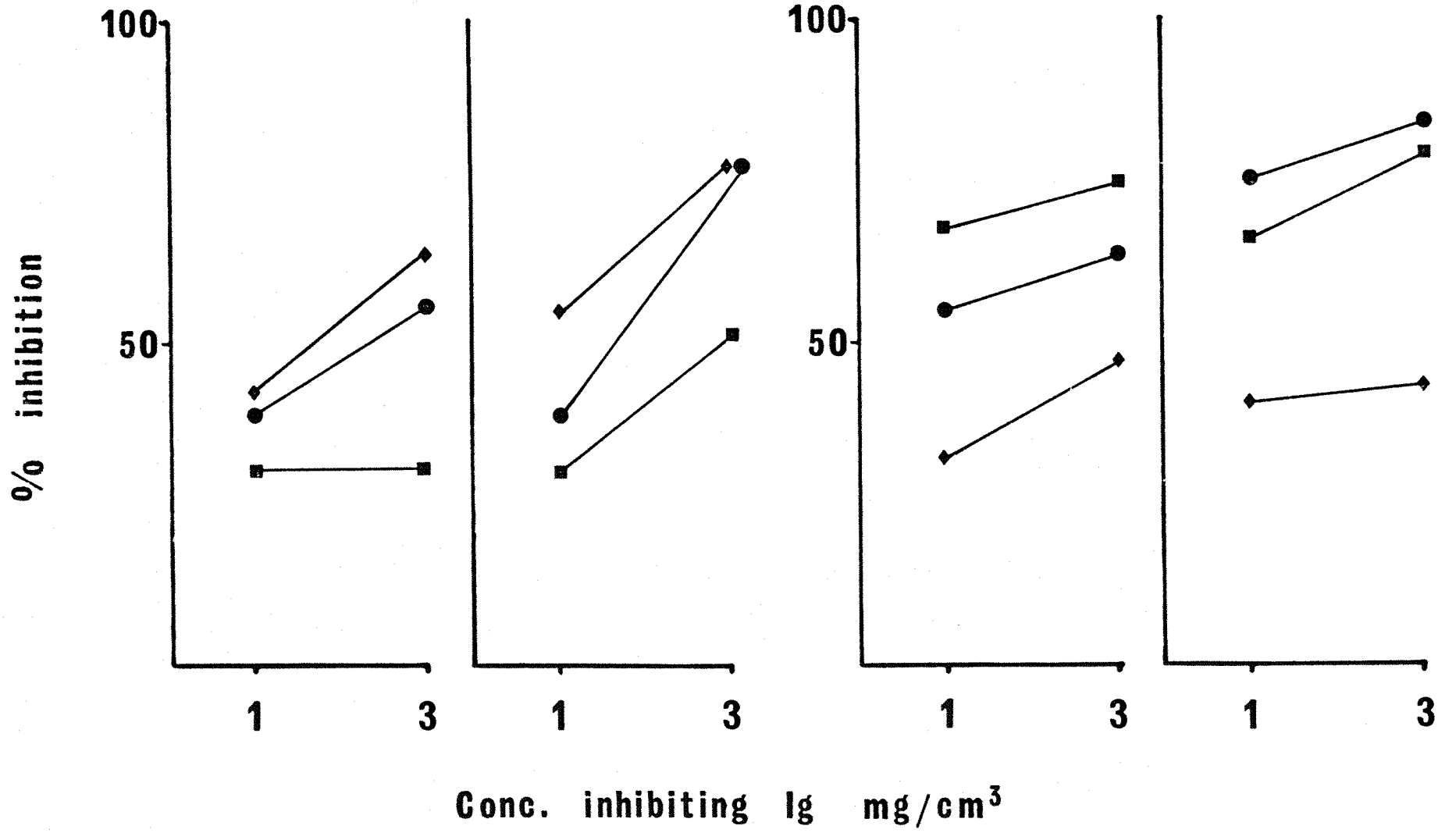

Rabbit ŝ SRBC
mono
HA
Rat ŝ SRBC
mono
HA
100
50
% inhibition
1
3
1
3
1
3
1
3
Conc. inhibiting Ig mg/cm3

Fig. 32. Day 19 uniparous peritoneal exudate cells. Comparison of percentage rosette inhibition produced by monomeric or heat aggregated immunoglobulins

Inhibiting IgG	mg/cm^3	Number of rats	Rabbit ŝ SRBC		Rat ŝ SRBC	
			Mono	HA	Mono	HA
Rabbit	3	1	21.9	70.0	27.0	74.0
(♦)	1	1	5.6	46.8	23.8	44.5
Rat	3	1	14.2	66.5	20.6	85.7
(■)	1	1	4.7	46.8	15.9	57.1
Human	3	1	9.9	70.0	27.0	65.1
(●)	1	1	9.9	41.6	17.5	61.9

Mono - monomeric IgG

HA - heat aggregated IgG

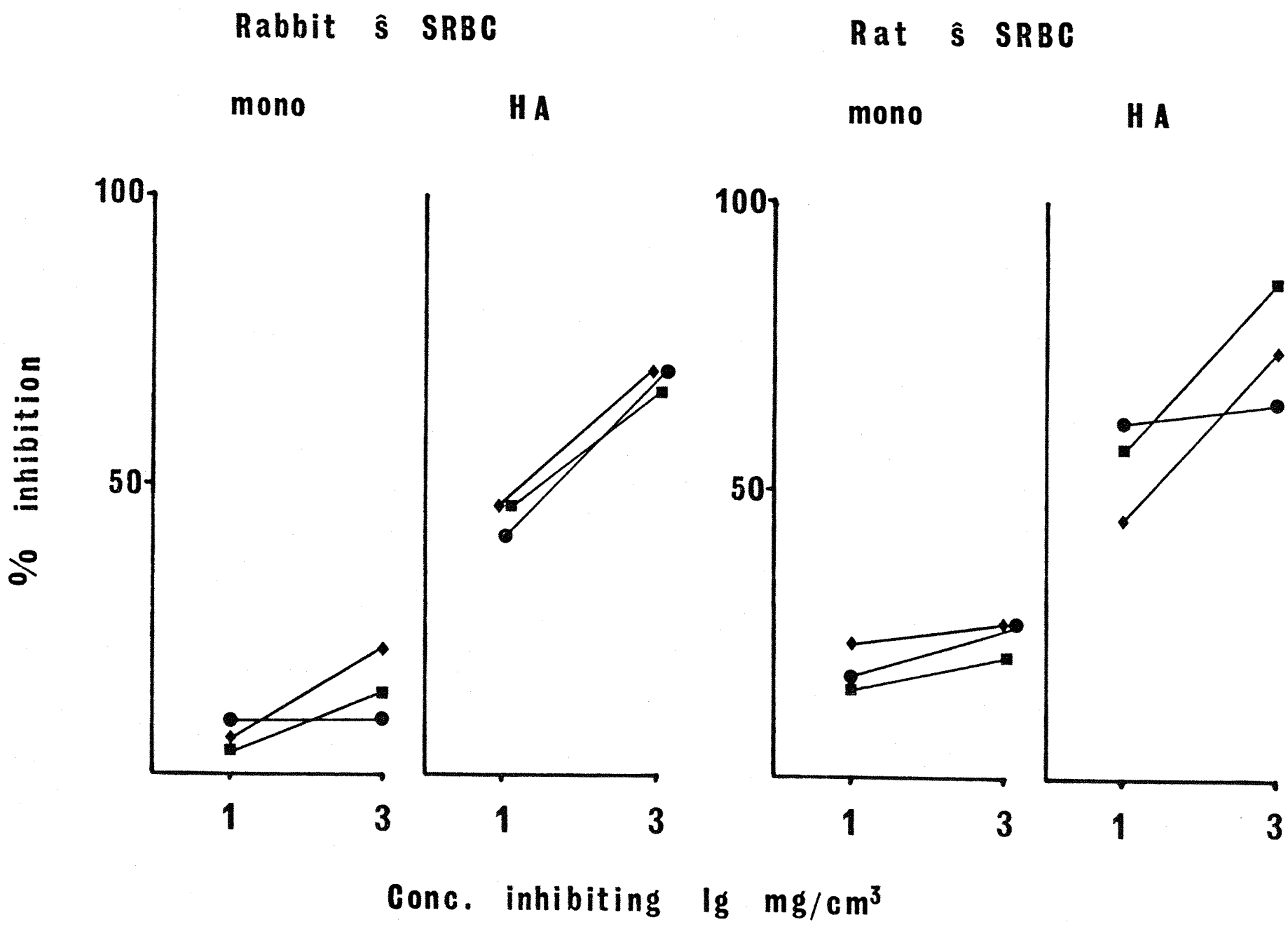

Rabbit ŝ SRBC
mono
HA
Rat ŝ SRBC
mono
HA
100
50
% inhibition
1
3
1
3
100
50
1
3
1
3
Conc. inhibiting lg mg/cm3

Fig. 33. Mean percentage (± SEM) rosette inhibition produced by heat aggregated human IgG (@ 7mg/cm^3) on metrial gland cells

Incubation time/min	4°C		37°C	
	Unwashed (♦)	Washed (●)	Unwashed (♦)	Washed (●)
Human IgG				
30	75.3 ± 4.1 (3)	45.3 ± 9.1 (3)	83.4 ± 4.9 (3)	40.9 ± 11.5 (4)
60	82.1 (1)	46.8 ± 4.3 (2)	80.8 (1)	63.8 ± 7.9 (4)
120	ND	61.7 ± 0.5 (2)	ND	79.1 ± 4.1 (4)
PBS		(▼)		(▼)
30	ND	(-) 6.7 (1)	ND	2.6 ± 0.3 (2)
60	ND	8.9 (1)	ND	0.7 ± 6.0 (3)
120	ND	8.9 (1)	ND	1.8 ± 4.3 (3)

Numbers in brackets = number of samples observed
ND - Not done
PBS - Phosphate buffered saline (pH7.2)
* - Mean % rosette inhibition at room temperature from Figs. 21-23.

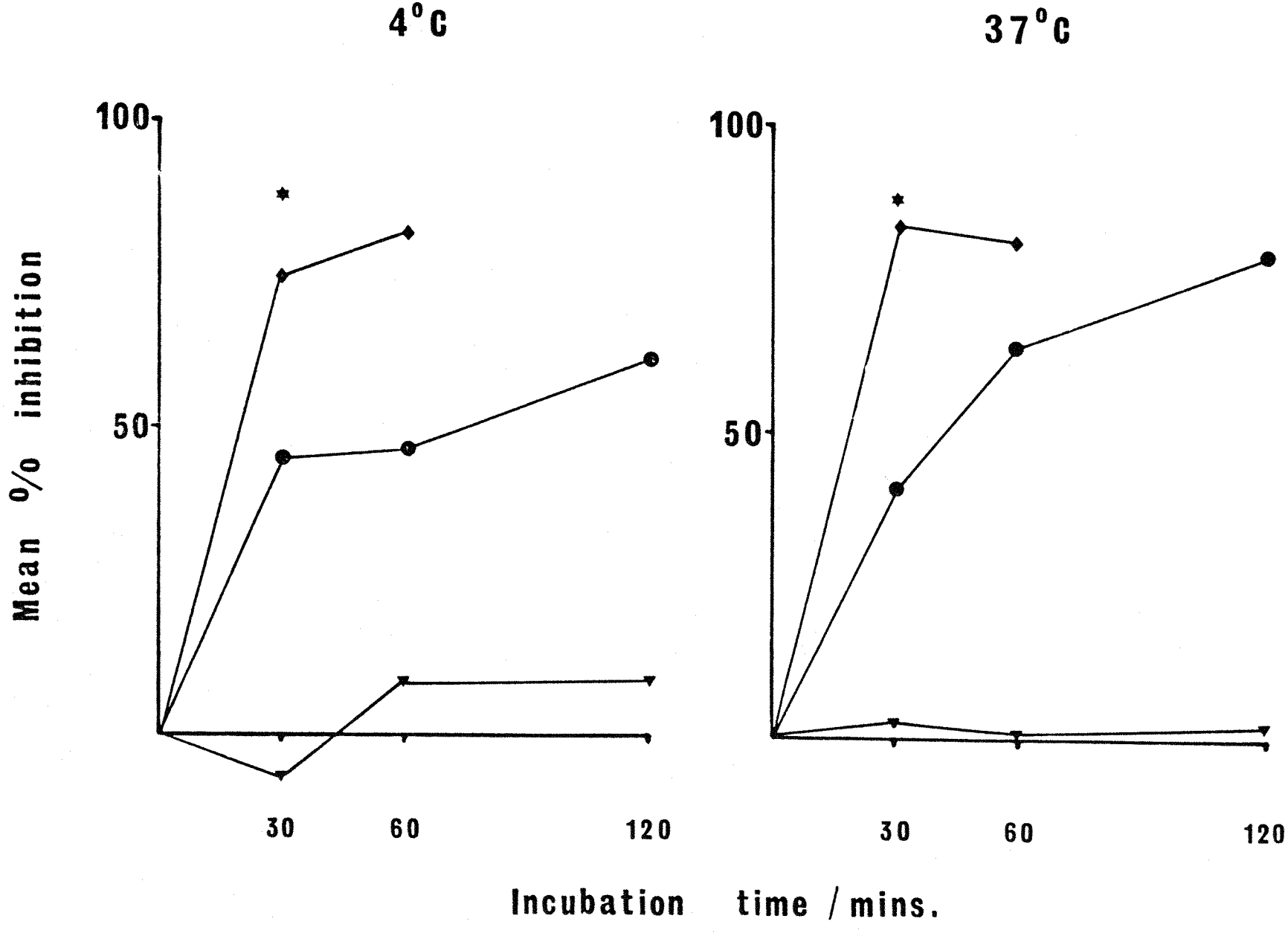
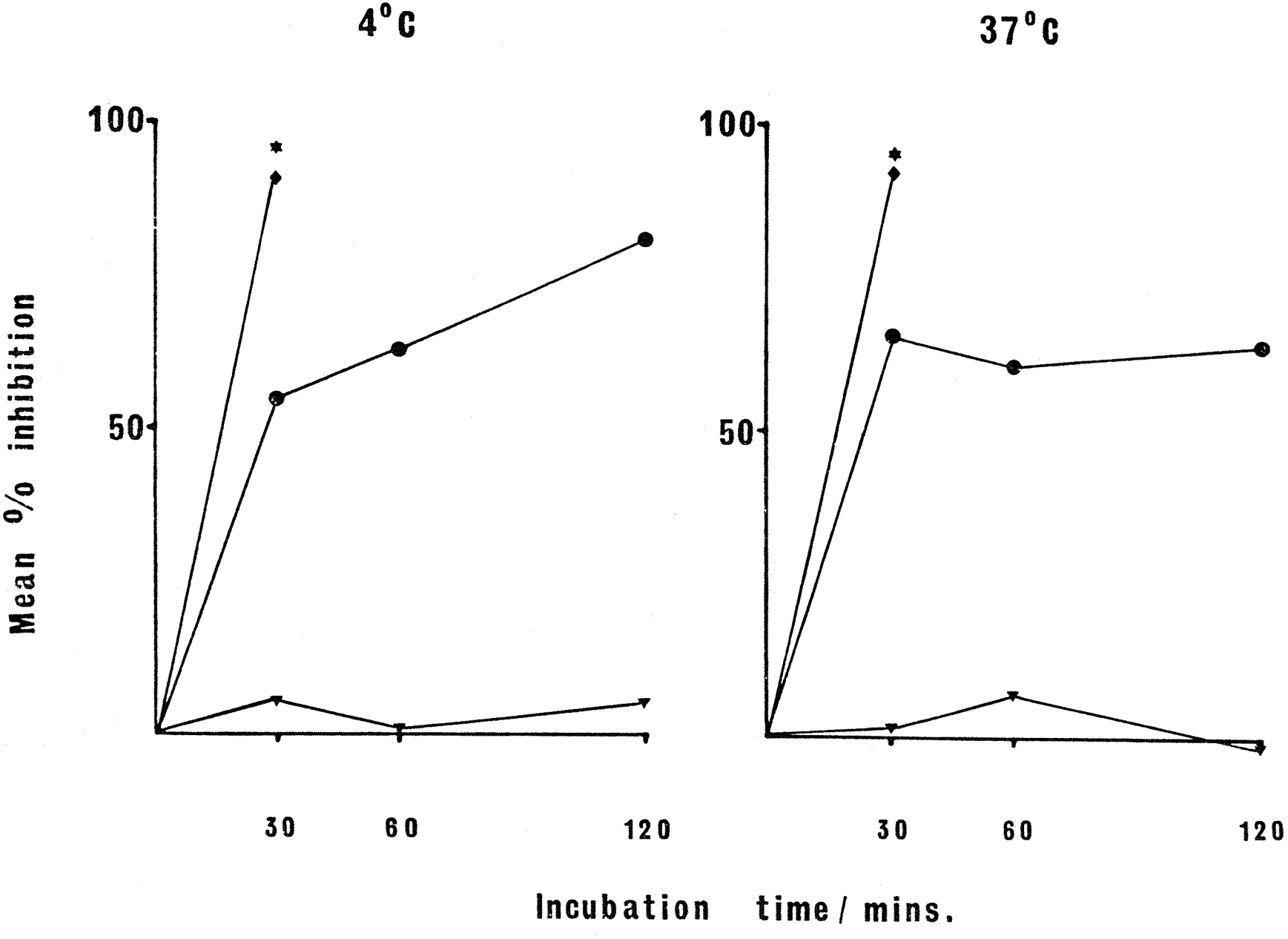

4°C
37°C
100
50
Mean % inhibition
30
60
120
Incubation time / mins.

Fig. 34. Mean percentage (± SEM) rosette inhibition produced by heat aggregated human IgG (@ 7mg/cm^3) on peritoneal exudate cells

Incubation time/min	4°C		37°C	
	Unwashed (♦)	Washed (●)	Unwashed (♦)	Washed (●)
Human IgG				
30	90.5 (1)	55.0 ± 2.8 (2)	92.1 (1)	66.1 ± 4.8 (3)
60	ND	63.1 ± 10.7 (2)	ND	61.1 ± 9.2 (3)
120	ND	80.7 ± 12.4 (2)	ND	63.7 ± 5.0 (3)
PBS		(▼)		(▼)
30	ND	5.6 (1)	ND	2.1 ± 2.6 (2)
60	ND	0.0 (1)	ND	7.4 ± 0.3 (2)
120	ND	5.6 (1)	ND	(-) 0.8 ± 2.3 (2)

Numbers in brackets = numbers of samples observed
ND - Not done
PBS - Phosphate buffered saline (pH7.2)
* - Mean % rosette inhibition at room temperature from Fig. 28.


4°C
37°C
100
50
Mean % inhibition
30
60
120
30
60
120
Incubation time / mins.

Chapter 6

Examination for IgG and other serum proteins in cell smears from the metrial gland

Chapter 6

Examination for IgG and other serum proteins in cell smears from the metrial gland

Introduction

Immunoglobulin G (IgG) was first observed in the rat metrial gland of normal pregnancy using fluorescein labelled or enzyme-linked antibody techniques on tissue sections (Bulmer and Peel, 1977 a, b). The suggestion was made, on the basis of a phase contrast study, that the IgG containing cells were the granulated cells of the metrial gland (Bulmer and Peel, 1977a). Recently this suggestion has been substantiated by the use of a technique which has shown that the IgG is located in the cells which contain diastase-fast PAS positive granules (Mitchell et al., 1980). As part of the work by Mitchell et al. (1980), smears of single cell suspensions were used to quantify the number of cells containing cytoplasmic IgG. In this study the quantification of cells containing cytoplasmic IgG has been made between day 12 and day 19 of uniparous pregnancy, at day 13 of multiparous pregnancy and between day 11 and day 14 of pseudopregnancy. The quantification of cytoplasmic IgG has involved the use of a number of different antisera directed against rat IgG because of the variable nature of reaction observed on sections of metrial gland tissue when using different antisera in the peroxidase anti-peroxidase (PAP) technique (Mitchell, personal communication).

Comparisons have been made between the number of cells containing diastase-fast PAS positive granules (Fig. 5) and the number of cells containing cytoplasmic IgG and between the number of rosette forming cells which contain IgG to try to define further the cell populations present in the metrial gland.

In an attempt to establish whether the IgG in the cells of the metrial gland is present due to non-specific endocytosis, cell smears have been examined using antisera directed against AFP, transferrin and albumin. Serum albumin might be present if non-specific uptake is occurring and the uptake of AFP and transferrin may be significant because they are thought to play a role in the immunosuppression of pregnancy. Tissue sections of implantation sites have been used for control purposes.

In an attempt to understand the function of the metrial gland cell Fcγ receptors, cells were washed after incubation with human IgG and smears prepared after rosette formation. The cells were then examined to see if endocytosis of human IgG had occurred and whether or not it was related to cells forming EAγ rosettes.

Materials and methods

Metrial gland cell suspensions were prepared as described in Chapter 3.

Detection of serum proteins in cell smears from the metrial gland

Smears of cell suspensions were prepared as described by Bray et al. (1978). Smears were then fixed in 5% glacial acetic acid in ethanol (GAA/E) at -20°C for 18 hours before being subjected to the indirect fluorescent antibody technique. Rabbit anti-rat IgG preparations were obtained from Wellcome, Miles (H and L chain specific) and Nordic (H and L chain specific). An Fabγ fragment of rabbit anti-rat IgG was prepared as described in Appendix II. All primary antisera were used at dilutions between 1:80 and 1:160 and the FITC labelled sheep anti-rabbit IgG (Wellcome) was used at a 1:20 dilution. Smears were washed three times in PBS (pH 7.2) between each application of antiserum, mounted in glycerol/PBS and the number of cells showing cytoplasmic fluorescence was counted in duplicate samples of 100 cells. Control observations included examination of smears for autofluorescence, use of the FITC conjugate only, substitution of the primary antiserum by non-immune rabbit serum and the preparation of cell suspensions and smears in the absence of FCS to eliminate any non-specific uptake of possible contaminating IgG by metrial gland cells from FCS.

The results were analysed as described for EAγ rosette formation (Chapter 4).

Smears from six uniparous rats (four day 14 and two day 15) were prepared after rosette formation with rabbit sensitised SRBCs. These smears were fixed in 95% ethanol at 4°C (to avoid lysis of the SRBC) and stained for IgG as described above. 1000 cells from each animal were scored as positive or negative for cytoplasmic IgG and for rosette formation.

Cell smears, fixed in 5% GAA/E, were also examined by the indirect fluorescent antibody technique for the presence of AFP, transferrin and albumin (sheep anti-rat AFP (1:40, Nordic) and FITC rabbit anti-sheep

IgG (1:20, Nordic), rabbit anti-rat transferrin (1:64, Cappel) and FITC sheep anti-rabbit IgG (1:20, Wellcome), rabbit anti-rat albumin (1:80, Nordic) and FITC sheep anti-rabbit IgG (1:20, Wellcome)]. Similar controls were preformed as described for the localisation of IgG. As further controls, tissue sections of implantation sites, fixed in 5% GAA/E, were examined for AFP, transferrin and albumin using the peroxidase anti-peroxidase (PAP) technique of Sternberger (1974). The primary and secondary antibodies were used, as described above, but at lower dilutions (1:500 and 1:80 respectively) with either rabbit or goat PAP (1:200, Nordic) as the final layer.

Localisation of human IgG

Metrial gland cells were incubated in heat aggregated human IgG (7.0 mg/cm^3) or PBS as described on Page 36 in Chapter 5. After washing the cells, EAγ rosettes were formed with rabbit sensitised SRBCs. Smears of rosettes were fixed in 95% alcohol at 4^{0}C and were stained for human IgG using the indirect fluorescent antibody technique [rabbit anti-human IgG (1:100, Behringwerke) and FITC sheep anti-rabbit IgG (1:20, Wellcome)].

Results

Smears prepared from single cell suspensions of metrial gland tissue from pregnancy contained cells with cytoplasmic IgG (Fig. 35) and there was no difference between the various primary antisera used. The number of positive cells detected using whole molecule rabbit anti-rat IgG (Wellcome, Nordic or Miles) and the Fabγ fraction of this antibody are shown in Fig. 36. There was no significant difference between the percentage of cells containing IgG at day 13 of multiparous pregnancy when compared to day 13 of uniparous pregnancy. Using whole molecule rabbit anti-rat IgG antisera there were more cells containing IgG at day 13 than at day 14 ($P<0.01$) and the percentages of cells containing IgG at each of days 13, 15 and 17 was higher than at day 19 ($P<0.01$). Using the Fabγ portion of a rabbit anti-rat IgG the percentages of cells containing IgG at both day 13 and day 17 were significantly higher than the percentages at day 14 and day 19 ($P<0.05$).

Smears of cells from the metrial gland of the deciduomata of pseudopregnancy also contained a proportion of cells with cytoplasmic

IgG (Fig. 37). Irrespective of the primary antiserum used there was no significant difference in the number of positive cells observed at any of the days of pseudopregnancy examined.

Control smears showed no autofluorescence and omission of primary antiserum or its substitution by non-immune rabbit serum did not produce any fluorescence. Cells prepared in the absence of FCS showed cytoplasmic IgG although their appearance under phase-contrast illumination showed signs of degeneration.

Comparison of the number of cells containing cytoplasmic IgG from uniparous pregnancy (Fig. 36) to the number of cells containing diastase-fast PAS positive granules (Fig. 5) showed no significant difference. Examination of rosetting populations for cytoplasmic IgG showed that the percentages of rosette forming cells and cells containing cytoplasmic IgG were as expected for the day of pregnancy examined, but less than one percent of cells were positive for both (Fig. 38).

The percentage of cells positive for cytoplasmic AFP, transferrin and albumin are shown in Figs. 39 and 40. Examination for AFP was negative in all metrial gland cell smears, however, the yolk sac in tissue sections acted as a positive control (Fig. 41). The proportion of cells showing positive fluorescence for transferrin and albumin (Figs 39 and 40) was always higher than the proportion of cells containing cytoplasmic IgG (Figs. 36 and 37). The yolk sac (Figs. 42 and 43), metrial gland and trophoblastic giant cells in sections were positive for transferrin and albumin. Control treatments of cell smears and tissue sections were all negative.

Smears of rosettes, formed after incubation of cells in human IgG, had only a limited number of cells and it was not possible to quantify the observations. However, human IgG appears within cells after incubation at 37^{0}C (Fig. 44) and some positive cells were still capable of forming EAγ rosettes (Fig. 45). Incubation at 4^{0}C produced fewer cells which had taken up the human IgG and instead there appeared to be cells where the human IgG had remained on the cell surface. Smears of cells incubated in PBS were negative when reacted for the presence of human IgG.

Figures 35 - 45

Fig. 35: A smear of a metrial gland cell preparation (day 13 of pregnancy) showing cytoplasmic IgG in a binucleate cell (35a) and the corresponding phase-contrast appearance in which smaller cells are negative (35b). x950.

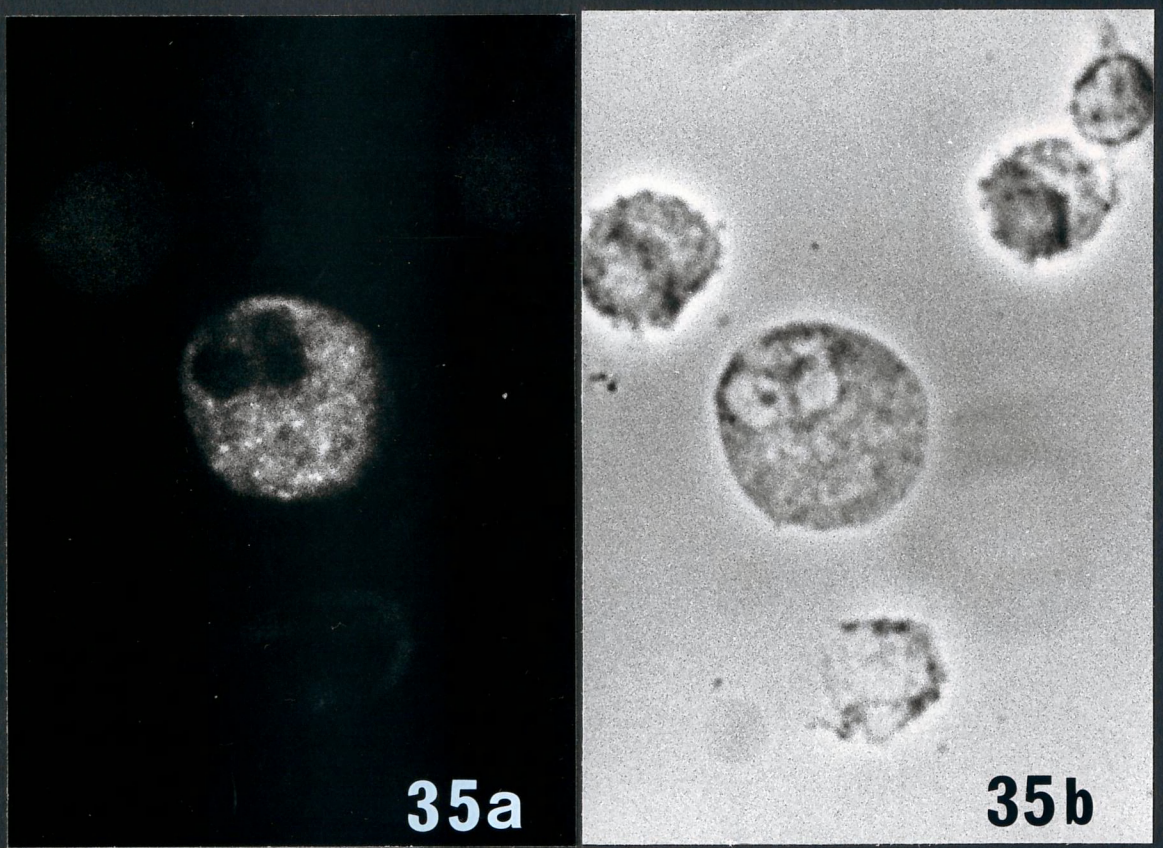

35a
35b

Fig. 36. Mean percentage (± SEM) of cells showing internal IgG from the metrial gland of pregnancy

Day of pregnancy	Number of rats	RARa + SAR(FITC)	Number of rats	Fabγ + SAR(FITC)
12	11	6.4 ± 0.4	4	7.7 ± 0.4
13	23	8.1 ± 0.4	12	8.2 ± 0.5
13 (MP)	3	9.2 ± 0.4	3	10.5 ± 2.5
14	12	6.4 ± 0.3	4	6.2 ± 0.6
15	21	7.2 ± 0.3	9	7.2 ± 0.5
17	3	8.3 ± 0.3	3	9.3 ± 0.6
19	7	5.4 ± 0.5	7	5.9 ± 0.4

RARa - Whole molecule rabbit anti-rat IgG
Fabγ - Fabγ portion of rabbit anti-rat IgG
SAR(FITC) - Fluorescein conjugated sheep anti-rabbit IgG
MP - Multiparous pregnancy.

Fig. 37. Mean percentage (± SEM) of cells showing internal IgG from the metrial gland of the deciduomata of pseudopregnancy

Day of pseudopregnancy	Number of rats	RARa + SAR(FITC)	Fabγ + SAR(FITC)
11	3	6.7 ± 0.4	8.8 ± 0.6
12	7	7.6 ± 0.8	9.7 ± 1.2
13	6	8.3 ± 0.6	8.8 ± 0.5
14	3	7.0 ± 0.1	7.9 ± 0.6

RARa - Whole molecule rabbit anti-rat IgG

Fabγ - Fabγ portion of rabbit anti-rat IgG

SAR(FITC) - Fluorescein conjugated sheep anti-rabbit IgG

Fig. 38: A smear of a metrial gland cell preparation (day 14 of pregnancy) after rosette formation with rabbit sensitised SRBCs. This is one of the very few cells which show both cytoplasmic IgG and EAγ rosette formation. x1350.

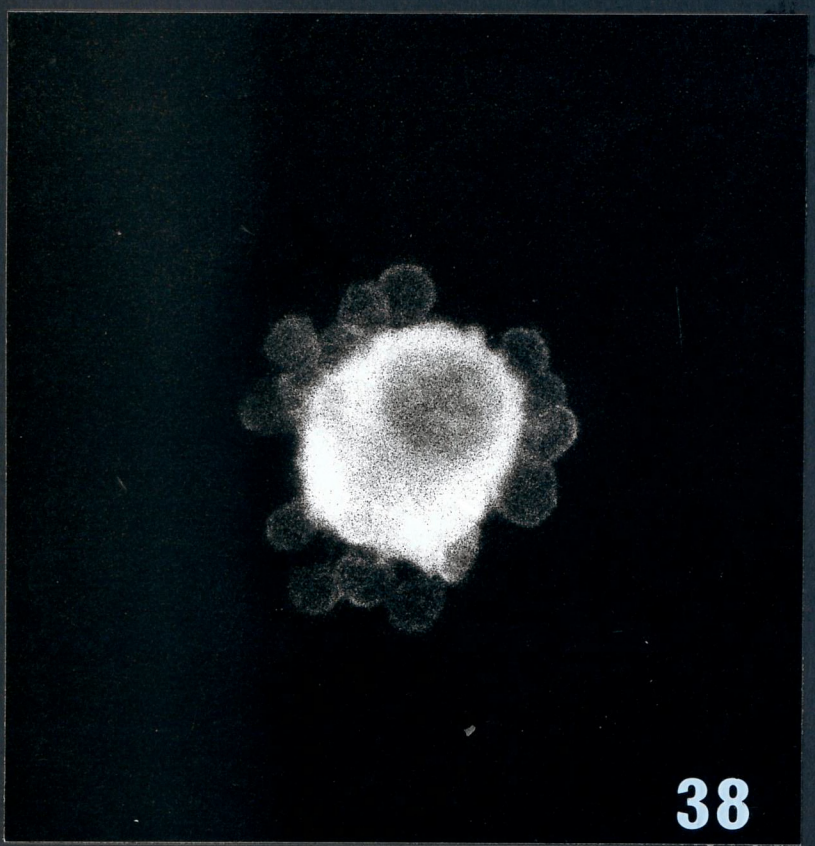

38

Fig. 39. Mean percentage (± SEM) of cells showing cytoplasmic fluorescence for serum proteins from the metrial gland of pregnancy

Day of pregnancy	Number of rats	SARa-AFP RAS(FITC)	RARa-Trans SAR(FITC)	RARa-Alb SAR(FITC)
12	1	0.0	ND	20.0
13	2	0.0	12.2 ± 0.2	13.7 ± 0.1
13 (MP)	1	0.0	12.5	15.5
14	1	0.0	ND	11.9
15	4	0.0	16.6 ± 2.6	10.6 ± 1.9
17	3	0.0	17.2 ± 1.4	12.5 ± 2.6
19	7	0.0	13.7 ± 2.5	16.6 ± 3.4

SARa-AFP - Sheep anti-rat alphafoetoprotein
RARa-Trans - Rabbit anti-rat transferrin
RARa-Alb - Rabbit anti-rat albumin
RAS(FITC) - Fluorescein conjugated rabbit anti-sheep IgG
SAR(FITC) - Fluorescein conjugated sheep anti-rabbit IgG
ND - Not done
MP - Multiparous pregnancy.

Fig. 40. Mean percentage (± SEM) of cells showing cytoplasmic fluorescence for serum proteins from the metrial gland of the deciduomata of pseudopregnancy

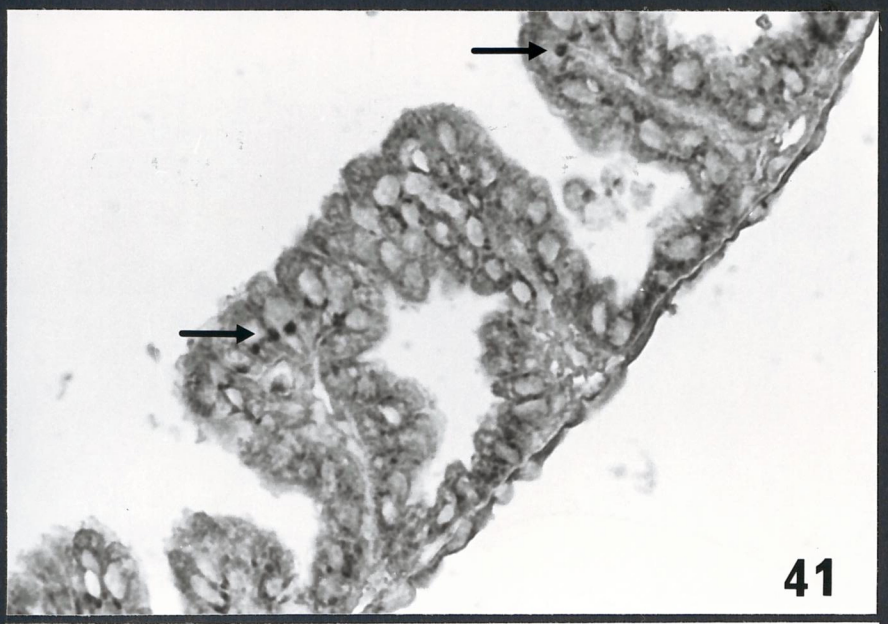
Day of pseudo-pregnancy	Number of rats	SARa-AFP RAS(FITC)	RARa-Trans SAR(FITC)	RARa-Alb SAR(FITC)
11	1	0.0	12.5	18.5
12	3	0.0	14.2 ± 2.7	15.0 ± 0.5
13	3	0.0	21.7 ± 1.8	ND

SARa-AFP - Sheep anti-rat alphafoetoprotein
RARa-Trans - Rabbit anti-rat transferrin
RARa-Alb - Rabbit anti-rat albumin
RAS(FITC) - Fluorescein conjugated rabbit anti-sheep IgG
SAR(FITC) - Fluorescein conjugated sheep anti-rabbit IgG
ND - Not done.

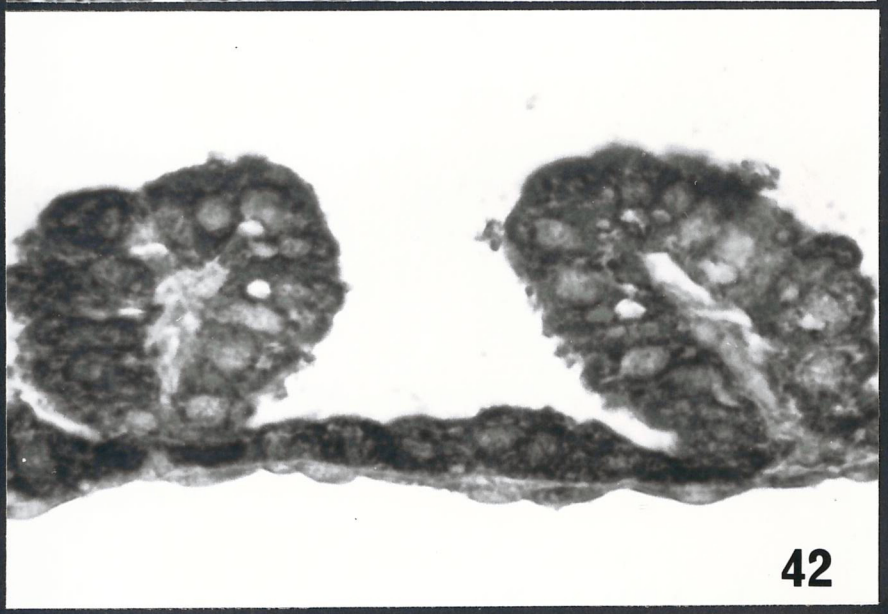
Fig. 41: Rat visceral yolk sac (day 13 of pregnancy) reacted for AFP by the PAP method. The reaction product is in the mesodermal cells and there is also some in vesicles in the endodermal cells (arrows). The basement membrane is negative. x600.

Fig. 42: Rat visceral yolk sac (day 13 of pregnancy) reacted for transferrin by the PAP method. Cytoplasmic reaction product is in both the mesodermal and endodermal cells. The basement membrane is negative. x650.

Fig. 43: Rat visceral yolk sac (day 13 of pregnancy) reacted for albumin by the PAP method. Cytoplasmic reaction product is seen in most of the endodermal cells. The mesodermal cells and basement membrane are negative. x650.



41



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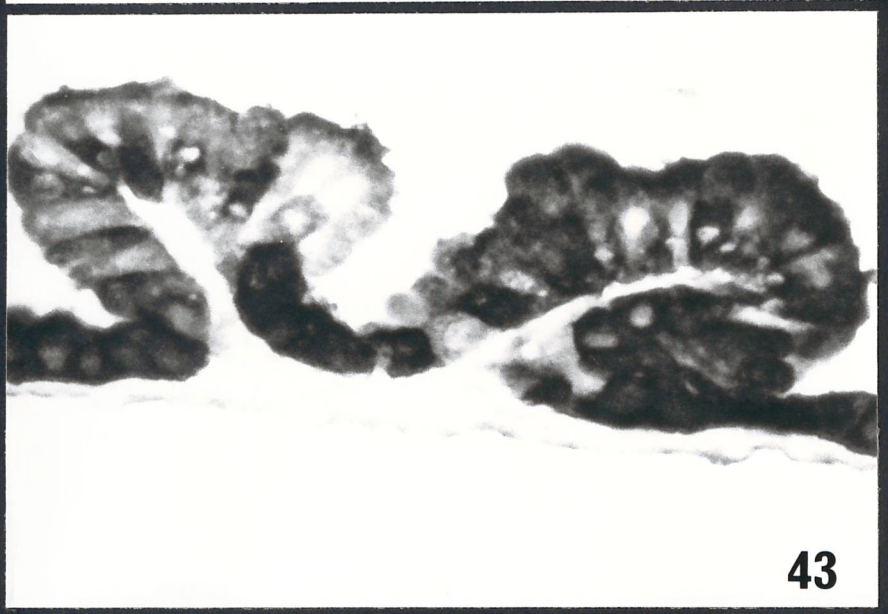
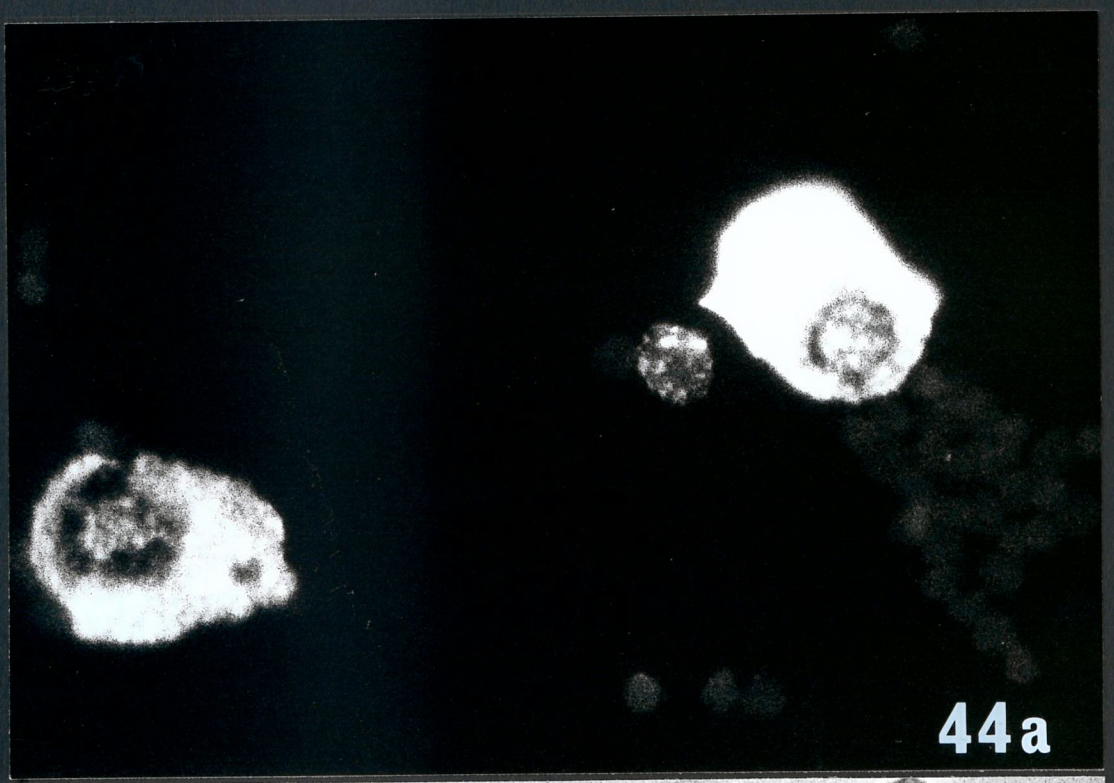
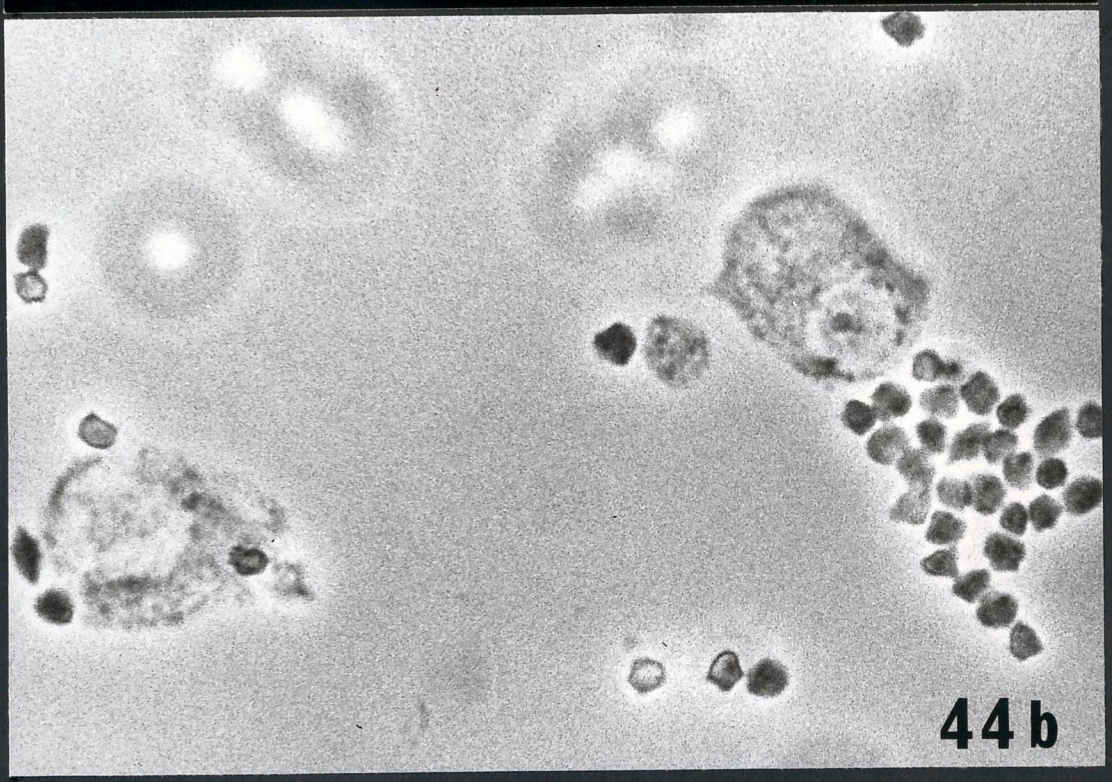


43

Fig. 44: A smear of metrial gland cells (day 15 of pregnancy) prepared after incubation in heat aggregated human IgG at 37^0C for 30 min. and after rosetting with rabbit sensitised SRBCs. The smear shows human IgG within cells (44a) and the corresponding phase-contrast appearance (44b). x1200.

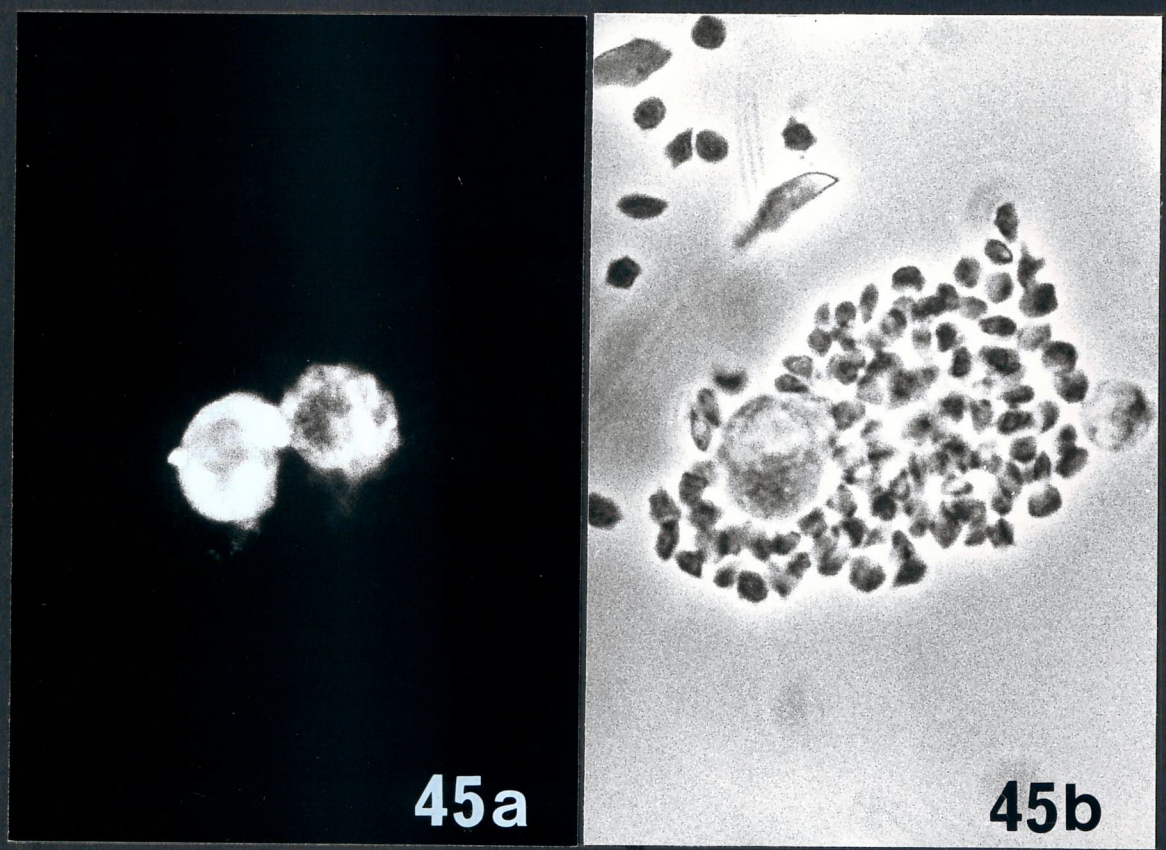


44a



44b

Fig. 45: A smear of metrial gland cells (day 15 of pregnancy) prepared after incubation in heat aggregated human IgG at 37^{0}C for 30 min. and after rosetting with rabbit sensitised SRBCs. The cells containing human IgG (45a) are also forming EAγ rosettes when examined under phase-contrast illumination (45b). x950.


45a
45b

Chapter 7.

Discussion

Chapter 7

Discussion

Cells from the metrial gland

Using a collagenase digestion technique (Bray et al., 1978) it has been possible to obtain viable single cell suspensions from rat metrial gland tissue and despite the small number of cells extracted from each animal, the technique has proved to have a high degree of reproducibility at each day of pregnancy examined (Fig. 3). The number of cells obtained per implantation site or per uterine horn decreases from day 12 to day 20 of pregnancy (Fig. 3). It is possible that the decreasing number of cells obtained as pregnancy proceeds may reflect a variation in cell susceptibility to lysis during collagenase digestion. However, it may be that there is a decrease in cell numbers as pregnancy proceeds. The number of cells obtained per uterine horn from the metrial gland tissue of the deciduomata of pseudopregnancy does not show a continuous decrease over the days of pseudopregnancy examined (Fig. 4). However, the number of cells obtained at day 11 of pseudopregnancy is nearly twice the number obtained at any of the other days of pseudopregnancy. The difference between the number of cells obtained from the metrial glands of pregnancy and from the metrial glands of the deciduomata of pseudopregnancy may be accounted for by the fact that in the deciduomata reaction the whole uterus does not respond to the induction stimulus at a uniform rate (personal observations) nor are there discrete sites of response along the length of the uterus. Thus although the general histological and ultrastructural features of the metrial gland of the deciduomata of pseudopregnancy and of the decidual reaction of pregnancy do not seem to differ (Velardo et al., 1953; Peel et al., 1979a), the gross morphological features and the number of cells extracted per horn at corresponding days are certainly not identical.

Smears of the cell suspensions obtained from the metrial gland of pregnancy have enabled the percentage of granulated metrial gland cells present between day 12 and day 16 of pregnancy to be recorded (Fig. 5). It appears that the percentage remains fairly constant despite the fall in the total number of cells obtained as pregnancy proceeds (Fig. 3). This, therefore, represents a fall in the absolute number of granulated metrial gland cells obtained from the days of pregnancy examined. Comparison of the number of granulated metrial gland cells

in smears of cell suspensions (Fig. 5) to the number in histological sections (Fig. 7) showed no significant difference at day 12 while at day 15 the number of granulated metrial gland cells observed was greater in tissue sections ($P<0.05$). The reason for this discrepancy is probably due to cell susceptibility to lysis during the extraction procedures. Of the non-granulated cells found in smears of metrial gland tissue some are polymorphonuclear leucocytes but most are small round basophilic cells. The majority of these cells must be the fibroblast-like stromal cells, although some will be endothelial cells and some may be the proposed precursor cells of the granulated metrial gland cells (Peel and Bulmer, 1977).

Cell surface markers

Cells from the metrial gland have been examined for surface markers which might imply that they have a relationship to phagocytes or cells of the lymphocytic series. A proportion of the cells from the metrial gland of pregnancy have been shown to possess a receptor for the Fcγ portion of IgG, which can be detected by EAγ rosette formation (Fig. 9) and a small, but significant, proportion possess SIg (Fig. 11). The IgG receptors on cells from the metrial gland have been shown to be specific for the Fcγ region of IgG by the fact that SRBCs sensitised with the $F(ab')_2$ fraction of a rabbit anti-SRBC IgG failed to form EAγ rosettes (Bray et al., 1978). Tests on the ability of cells from the metrial gland of pregnancy to form E, EAμ and EAC rosettes were all negative (Figs. 8 and 10), thus implying that certain surface markers or receptors are absent from these cells.

a) Fcγ receptors

Using SRBCs sensitised with rat antibody consistently fewer metrial gland cells formed EAγ rosettes than when SRBCs or OxRBCs sensitised with rabbit antibody were used (Figs. 9, 14 and 15). One explanation for these results is that different sub-classes of IgG might have been raised in the preparation of the sensitising antibodies. There are no reports of different sub-classes existing in the rabbit IgG fraction, whereas there are at least four sub-classes of rat IgG (Bazin et al., 1974). It is possible that the metrial gland cell Fcγ receptors have a preference for a sub-class of rat IgG which was poorly represented in the immune sera. It has been reported that the Fcγ receptors on murine macrophages preferentially bind sub-classes IgG_{2a} and IgG_{2b} but

have a low affinity for IgG_1 and human macrophages bind IgG_1 and IgG_3 to a greater extent than IgG_2 and IgG_4 (Dorrington, 1977). An alternative idea to explain the variation in rosette formation by the rat and rabbit sensitising antibodies is that there might be one type of Fcγ receptor which has a greater preference for heterologous than for homologous immunoglobulins. There may also be at least two types of Fcγ receptor, one for homologous and one for heterologous immunoglobulins. The Fcγ receptors on the peritoneal exudate, thymus and spleen cells (Figs. 9 and 16) are similar to those seen on the metrial gland cells (Figs. 9, 14 and 15) with more rosettes formed by rabbit sensitised SRBCs than by rat sensitised SRBCs. It may be significant that in the only tests where both the sensitising antibodies were heterologous there was no variation in the percentage of EAγ rosette formation (Fig. 9, human peripheral blood). It is also possible that in observing Fcγ receptors on cells from rat tissues it may be only the observations using rat sensitising antibody which bear any functional significance, while those observations involving the use of the rabbit sensitising antibody may be only incidental to the system under test.

Rosette formation using both sensitising antibodies produced a similar pattern of a decrease in the percentage of rosettes formed between day 12 and day 15 of pregnancy, followed by an increase again from day 15 or day 17 to day 19 of pregnancy (Fig. 14). This increase takes place at a time when the absolute number of metrial gland cells extracted is decreasing (Fig. 3). Both sensitising antibodies show a significantly higher number of cells forming rosettes at day 13 of multiparous pregnancy than at day 13 of uniparous pregnancy (Fig. 14). Although the function of the Fcγ receptors is not understood, this may reflect an increased response by the mother to foetal antigens as a result of previous pregnancies and may relate to the idea that the maternal immune response to foetal antigens in allogeneic matings is beneficial to the development of the foetus and the successful outcome of pregnancy (Hamilton et al., 1976; Chaouat et al., 1979a).

Inhibition studies using various rat sera showed no significant difference between the types of sera used (Fig. 17). This observation is in contrast to <u>in vitro</u> cytotoxicity tests, where embryonic or tumour target cells pretreated with multiparous rat serum can inhibit the cytotoxic activity of lymph node cells, whereas pretreatment of target

cells with virgin female serum, does not inhibit cytotoxicity (Baldwin et al., 1974; Baldwin and Embleton, 1974). This inhibition is believed to be mediated by Fcγ receptors on the target cells binding enhancing antibodies present in the multiparous serum.

Previous inhibition studies using monomeric immunoglobulins established that rosette formation by cells from the metrial gland, with rabbit sensitised SRBCs, was inhibited to a greater degree by rabbit and human IgGs than by rat IgG, while bovine IgG had a slight enhancing effect (Bray and Craggs, 1979; Appendix III). The work reported here has attempted to characterise further the Fcγ receptors on cells from the metrial gland by inhibition studies on rosette formation using SRBCs sensitised with either rabbit or rat antibodies. Using monomeric IgGs (Fig. 18) it has been possible to repeat the results obtained by Bray and Craggs (1979) and to show that when using an inhibiting IgG which is from the same species as the antibody used to sensitise the SRBCs, there is a greater degree of inhibition by that IgG than by heterologous IgGs. In summary, rabbit IgG inhibits rosette formation by rabbit sensitised SRBCs to a greater degree than rat IgG and rat IgG inhibits rosette formation by rat sensitised SRBCs to a greater degree than rabbit IgG (Fig. 18). Human IgG inhibits rosette formation with approximately equal effect in both systems and bovine IgG has little or no effect on rosette formation (Fig. 18). Using heat aggregated IgG in the inhibition assays, metrial gland cells from uniparous pregnancy (Figs. 20 - 24), multiparous pregnancy (Fig. 25) and from the deciduomata of pseudopregnancy (Figs. 26 and 27) respond in a similar manner to metrial gland cells from uniparous pregnancy incubated with monomeric IgGs (Fig. 18).

Cells from the metrial gland of the deciduomata of pseudopregnancy which possess Fcγ receptors (Fig. 15) occur at approximately the same proportions as seen in pregnancy (Fig. 14) and show similar inhibition characteristics with heat aggregated IgGs (Figs. 26 and 27). The pattern of EAγ rosette formation (Fig. 15) is also similar to that seen in pregnancy (Fig. 14) except that it occurs over a shorter time period. These observations present further evidence to support the fact that the metrial gland cells of the deciduomata resemble the metrial gland cells of the normal decidual reaction (Velardo et al., 1953; Peel et al., 1979a).

Control tissues (Fig. 16) show no corresponding variations in the percentages of rosette formation between the days of pregnancy or

pseudopregnancy examined. Rosette formation by peritoneal exudate cells shows very little inhibition after incubation with monomeric IgGs (Fig. 19) irrespective of the antibody used to sensitise the SRBCs whereas heat aggregated IgGs (Figs. 28 - 30) produce a pattern of inhibitions similar to that described for monomeric and heat aggregated IgGs on metrial gland cells (Figs. 18 and 20 - 27). These observations on peritoneal macrophages correspond to similar studies where it has been shown that EAγ rosette formation by macrophages is only inhibited by heat aggregated IgGs (Matre et al., 1975) and not by soluble (monomeric) IgGs (Matre et al., 1975; Wild, 1979). Macrophage Fcγ receptors will only bind radio-actively labelled monomeric IgGs when they are not in competition with aggregated IgGs or EAγ complexes (Unkless and Eisen, 1975).

Previously it has been reported that the Fcγ receptors on cells from the metrial gland of pregnancy were resistant to treatment with trypsin (Bray and Craggs, 1979). This observation has been confirmed and extended to peritoneal exudate cells and to metrial gland cells from the deciduomata of pseudopregnancy. These observations show that metrial gland cell Fcγ receptors are similar to those reported on macrophages (Lay and Nussenzweig, 1968; Wild, 1979) and lymphocytes (Cline et al., 1972). Recently it has been shown that rabbit yolk sac endodermal cells extracted using the MES buffer of Pearce (1975), followed by mechanical separation, show an increase in the percentage of cells forming EAγ rosettes over cells prepared by trypsin digestion (Wild, 1979). These newly exposed Fcγ receptors have been shown to be trypsin sensitive (Wild, 1979). Anderson and Grey (1978) reported that there are two types of Fcγ receptors on macrophages, one of which is resistant to trypsin and the other which is sensitive to trypsin. It is possible, therefore, that the percentage of EAγ rosette forming cells in the metrial gland might be increased if MES buffer and mechanical isolation were used to prepare single cell suspensions, instead of collagenase digestion, and that any newly exposed Fcγ receptors might then be sensitive to trypsin. Pretreatment of control cells with collagenase (Fig. 16) has shown that there is only an increase in the percentage of EAγ rosette formation when peritoneal exudate cells are subsequently combined with rabbit sensitised SRBCs. In no case does exposure to collagenase cause a decrease in the percentage of cells forming EAγ rosettes (Fig. 16).

Previously it has been shown that the granulated metrial gland cells, which make up approximately 10 percent of the isolated cell population (Fig. 5), do not possess Fcγ receptors as detected by EAγ rosette formation (Bray et al., 1978; Appendix III). Instead it appears to be a proportion of the fibroblast-like stromal cells which are the Fcγ receptor bearing cells. It is still possible, however, that there are other populations of cells bearing Fcγ receptors in the metrial gland, which may be similar to the Fcγ receptors on B lymphocytes. These receptors are not readily detected by EAγ rosette formation (Shevach et al., 1973) instead they require the use of fluorescein conjugated aggregates of IgG or radio-actively labelled complexes (Dickler, 1976) and these techniques have not been applied in the work reported here.

b) Surface immunoglobulin (SIg)

When the Fabγ fraction of a rabbit anti-rat IgG antibody was used in the indirect immunofluorescent technique, a small, but significant, number of cells from the metrial gland of both pregnancy (Fig. 11) and the deciduomata of pseudopregnancy (Fig. 13) showed rings of surface fluorescence. Therefore a small proportion of metrial gland cells appear to bear SIg. These cells may represent B lymphocytes (Raff, 1970) but their size, which is usually larger than small lymphocytes, suggests that they may be the proposed precursor cells of the granulated metrial gland cells (Mitchell et al., 1980; Appendix III). Using the whole molecule rabbit anti-rat IgG antibody in the indirect immunofluorescence test, approximately 24.0 percent of cells showed rings of surface fluorescence (Figs. 11 and 13). The majority of this binding can be accounted for by its attachment to the Fcγ receptors on a proportion of the fibroblast-like stromal cells and provides further evidence for the ability of the Fcγ receptors to bind monomeric immunoglobulins. Using whole molecule rabbit anti-rat IgG (Fig. 13) the percentage of positive cells (i.e. Fcγ receptor bearing) observed in the metrial gland from the deciduomata is similar to the percentage showing EAγ rosette formation with rabbit sensitised SRBCs (Fig. 15). Both sets of observations show a decrease from day 11 to day 12 followed by an increase from day 12 to day 14 of pseudopregnancy. However, during pregnancy there is no increase in the percentage of metrial gland cells showing binding of whole molecule rabbit anti-rat IgG from day 17 to day 19 of pregnancy (Fig. 11), unlike the

observations on EAγ rosette formation (Fig. 14). If the two methods are detecting the same population of Fcγ receptors then this observation may relate to a change in the Fcγ receptor positive cells. This change could be in functional specificity or in the cell types which make up the Fcγ receptor positive cells. No such variation was detected in metrial gland cells from the deciduomata of pseudopregnancy but the observations may not have been extended over a long enough period.

Despite the fact that SIg and Fcγ receptors on B cells have been shown to be independent (Abbas and Unanue, 1975; Birch et al., 1978), the possibility cannot be excluded that the rings of fluorescence produced by the Fabγ molecule may represent serum IgG bound to Fcγ receptors rather than IgG incorporated into the surface membrane. Examination of cells obtained from murine decidual tissues has failed to detect SIg using a peroxidase labelled Fabγ fraction of a rabbit anti-mouse IgG antibody (Bernard et al., 1978). The difference between the results reported here and those of Bernard et al. (1978) may relate to the presence of Fcγ receptor bound IgG or to a difference in sensitivity between the two test systems.

Cytoplasmic localisation of serum proteins in cells from the metrial gland

a) Immunoglobulin (Ig)G

Immunoglobulin (Ig)G has been localised in cells in smears from the metrial gland of pregnancy (Fig. 36). Bray et al. (1978) showed that the cells forming EAγ rosettes were not the granulated metrial gland cells and in this work it has been possible to show that the vast majority of the Fcγ receptor bearing cells do not contain IgG. During pregnancy there appears to be fluctuations in the numbers of cells containing IgG with a decrease at day 19 of pregnancy as parturition approaches (Fig. 36). The results obtained using different primary antisera in the indirect immunofluorescent antibody technique were essentially the same. Comparison of the percentage of cells containing cytoplasmic IgG in smears (Fig. 36) to the percentage of cells containing diastase-resistant PAS positive granules in smears (Fig. 5) showed no significant difference and subsequently it has been possible to detect both features within the same cell type in tissue sections (Mitchell et al., 1980; Appendix III). There is no significant difference between the number of cells containing IgG at day 13 of multiparous pregnancy and the number at day 13 of uniparous pregnancy (Fig. 36).

Cytoplasmic IgG was also detected in cell smears from the metrial gland of the deciduomata of pseudopregnancy (Fig. 37). This is not in agreement with an earlier report on tissue sections from the deciduomata of pseudopregnancy (Bulmer and Peel, 1977b). However, they pointed out that their failure to detect IgG may have been due to differences between the fixation characteristics of the IgG in the metrial gland of the deciduomata and that in the metrial gland of pregnancy. Using saturated alcoholic mercuric chloride (SAMC) as a fixative, Mitchell et al. (1980) have been able to overcome some of the problems of fixation and recently Mitchell (1980) has been able to demonstrate IgG in cells containing diastase-resistant PAS positive granules from the metrial gland of the deciduomata of pseudopregnancy. There are no observable fluctuations in the numbers of cells containing IgG in the metrial gland of the deciduomata (Fig. 37), a fact which may relate to the IgG having limited functional activity in the absence of the embryonic tissues and placenta. Whether or not the IgG found in the metrial gland of pregnancy and that found in the metrial gland of the deciduomata has the same functional specificity remains to be established. However, because IgG can be found in the granulated metrial gland cells of the deciduomata, the stimulus for its presence must be regarded as being derived from the induction of the decidual reaction in pregnancy and pseudopregnancy rather than as a response to stimulation by embryonic antigens in pregnancy.

b) AFP, transferrin and albumin

In examining cell smears from the metrial gland of pregnancy (Fig. 39) and of the deciduomata of pseudopregnancy (Fig. 40) it was found that transferrin and albumin were present in a proportion of the cells. In pregnancy AFP was seen in the yolk sac (Fig. 41) but was not seen in the metrial gland in tissue sections, nor in smears of metrial gland cells (Fig. 39). AFP can, however, be detected at low levels in maternal serum by immunoelectrophoresis (Lai et al., 1976). Staining for AFP in the deciduomata would not be expected because it is well established that in rodents AFP is synthesised by the foetal liver (Lai et al., 1976; Dziadek and Adamson, 1978) and by the visceral yolk sac endoderm (Dziadek and Adamson, 1978). The detection of transferrin in cell smears from metrial gland tissues

(Figs. 39 and 40) does not agree with the report by Faulk and Galbraith (1979b) in which they were unable to detect transferrin in implantation sites from mice. The detection of cytoplasmic albumin in cell smears from metrial gland tissues (Figs. 39 and 40) may relate to the fact that AFP and albumin have been shown to possess sequence homologies of amino acids (Ruoslahti and Terry, 1976) and that this might suggest a similar functional role for albumin in the maternal compartment as for AFP in the foetal compartment (Ruoslahti and Terry, 1976). The detection of albumin and transferrin in the metrial gland cells of the deciduomata (Fig. 40) is indicative that a stimulus, other than the presence of the foetus and placenta, causes their appearance in the rat metrial gland.

At present there is no evidence to show whether the albumin and transferrin occur in the same cell types or to suggest how these cell types relate to the granulated metrial gland cells which contain IgG. There are, however, more cells containing albumin and transferrin (Figs. 39 and 40) than there are cells containing IgG (Figs. 36 and 37). There is no evidence to show whether the IgG, albumin and transferrin are endocytosed or synthesised by the cells in which they are localised. However, in vivo injections of markers have revealed that it is the fibroblast-like stromal cells which are capable of endocytosis, rather than the granulated metrial gland cells (Bridgman, 1948; Sharma and Peel, 1979): an observation which is supported by the ultrastructural appearance of the fibroblast-like stromal cells (Peel and Bulmer, 1977).

Comparison of cells from the metrial gland to:-

a) Cell types in pregnancy

The metrial gland in the pregnant rat uterus is not directly in contact with any tissues derived from the implanting blastocyst until late in pregnancy. However, as the maternal blood vessels to and from the foetal placenta pass through the metrial gland it is in an ideal position to monitor the components of maternal blood and tissue fluid. The Fcγ receptors observed in pregnancy have been described earlier (P.16) and have been compared to the Fcγ receptors on the metrial gland as an earlier part of this study (Bray and Craggs, 1979; Appendix III). The main functions of the Fcγ receptors in pregnancy are associated with the transport of IgG from mother to young and in

the protection of the placenta and membranes. The Fcγ receptors on metrial gland cells are not in a position which would facilitate transport of IgG nor are they in direct contact with maternal blood where they could have a direct influence on the protection of the foetus. However, the aim of the rest of the discussion is to compare the relationships of the cells in the metrial gland to other cell types.

b) Macrophages and monocytes

The morphological heterogeneity of macrophages and monocytes is probably related to a number of different cell lines differentiating from a common stem cell (von Furth et al., 1972). However, they can be characterised as mononuclear phagocytes by their ability to exhibit immune and non-immune phagocytosis, by their high affinity, trypsin resistant Fcγ receptors, by their receptors for complement (Berken and Benacerraf, 1966; Huber et al., 1968; Lay and Nussenzweig, 1968) and by their content of non-specific esterase (Yam et al., 1971).

Some metrial gland cells are similar to macrophages and monocytes in that they possess Fcγ receptors (Bray et al., 1978) which are resistant to trypsin (Bray and Craggs, 1979). However, the inhibition characteristics of the Fcγ receptors on cells from the metrial gland (Figs. 18 and 20 - 27) differ from those on peritoneal macrophages (Figs. 19 and 28 - 30). Another observation which argues against the Fcγ receptor bearing cells from the metrial gland being closely related to mononuclear phagocytes is that no EAC rosettes were formed (Fig. 10) and therefore it appears that no complement receptors are present. It has also been shown that the localisation of non-specific esterase in the metrial gland is in the granulated metrial gland cells (Bulmer, 1968), which are not the cells which possess Fcγ receptors. However, some of the fibroblast-like stromal cells of the metrial gland are similar to macrophages in that they display endocytosis of injected markers (Bridgman, 1948; Sharma and Peel, 1979). This is probably the same cell type which is seen to endocytose the heat aggregated human IgG (Figs. 44 and 45). However, unlike macrophages metrial gland cells do not display non-immune phagocytosis in that they do not readily take-up carbonyl iron (unpublished observations). Therefore if the Fcγ receptor positive cells from the metrial gland are related to or derived from the stem cells of mononuclear phagocytes, they differ from any other previously defined macrophages and monocytes.

It has been suggested that there is an increase in the number of macrophages present in the implantation site as pregnancy proceeds (Larkin, 1972). Therefore it is possible that the function of Fcγ receptor bearing cells detected in the metrial gland may not only be related to the period of pregnancy or pseudopregnancy at which they are detected, but that they may also be related to the postpartum development of macrophages (Lobel and Deane, 1962; Padykula and Tansey, 1979). Evidence reported here suggests that towards parturition there is a change in the characteristics of the Fcγ receptors on cells from the metrial gland. Firstly there is an increase in the percentage of cells forming EAγ rosettes between day 15 and day 19 of pregnancy (Fig. 14) although there is a continuing decrease in the actual number of cells extracted over that period (Fig. 3). When the binding of whole molecule rabbit anti-rat IgG (Fig. 11) is compared to EAγ rosette formation (Fig. 14) there is no corresponding increase in the percentage of cells showing rings of fluorescence between day 15 and day 19 of pregnancy. This observation might reflect either a reduced avidity of certain Fcγ receptors for monomeric IgG or a change in the cell population bearing Fcγ receptors. The net effect of this change is that there appears to be an increase in the percentage of cells which only bind heat aggregated IgG. Further support for this change may be reflected in the results shown in Fig. 31 from which it is suggested that metrial gland cells at day 19 of pregnancy are inhibited to a lower degree by monomeric IgGs than by heat aggregated IgGs.

The observations between day 11 and day 14 of pseudopregnancy do not show any significant difference between the percentage of cells forming EAγ rosettes (Fig. 15) and the percentage of cells showing surface fluorescence with whole molecule rabbit anti-rat IgG (Fig. 13). Despite the fact that observations on metrial gland cells from the deciduomata of pseudopregnancy have not been extended to the equivalent of day 19 of pregnancy, there is still the possibility that the absence of the foetus and placenta in deciduomata fails to stimulate the differentiation of macrophages.

c) B lymphocytes

B lymphocytes can be distinguished by the presence of SIg (Raff, 1970), complement receptors (Bianco et al., 1970) and Fcγ receptors (Dickler, 1976). The Fcγ receptors on B cells are resistant to trypsin

and can be detected by EAγ rosette formation (Cline et al., 1972). However, the Fcγ receptors on B cells are not as readily detectable by EAγ rosette formation (Shevach et al., 1973) as they are by using fluorescein conjugated heat aggregated IgG (Dickler and Kunkel, 1972) or by radio-actively labelled soluble immune complexes (Basten et al., 1972a). The Fcγ receptors on B lymphocytes are not inhibited by monomeric IgGs, even at high concentrations (Basten et al., 1972b; Dickler and Kunkel, 1972).

The detection of a small proportion of cells from the metrial gland bearing SIg in both pregnancy (Fig. 11) and the deciduomata of pseudopregnancy (Fig. 13) may represent a B cell associated line in the metrial gland. It is also possible that the SIg is present on the proposed precursor cells for the granulated metrial gland cells (Peel and Bulmer, 1977) or it may be serum IgG bound to Fcγ receptors (Mitchell et al., 1980). To test the hypothesis that the SIg is membrane incorporated, rather than Fcγ receptor bound, it would be necessary to use a method used to define B cells in lymphoid populations (Birch et al., 1978). This would involve treating metrial gland cells with pronase to remove all cell surface associated IgG and then culturing the cells over 48 hours to see if immunoglobulin re-appeared in the cell membrane.

The absence of a receptor for complement on cells from the metrial gland (EAC rosette formation, Fig. 10) and the fact that the trypsin resistant Fcγ receptors on metrial gland cells bind monomeric IgG (Fig. 18) argue against the presence of B cell associated lines in the metrial gland.

d) T lymphocytes

T lymphocytes can be distinguished by their ability to form E rosettes (Jondal et al., 1972) and by the presence of a receptor for either IgM (Fcμ) or for IgG (Fcγ) (Moretta et al., 1975).

It has not been possible to demonstrate E rosette formation by cells of the metrial gland using SRBCs (Fig. 8). Recently, however, Steele (1979) has shown that murine thymocytes and splenic T cells do not form E rosettes with SRBCs but will form E rosettes with mouse red blood cells. E rosette formation has been shown to be enhanced by pretreating target cells with neuraminidase (Tønder et al., 1974). Therefore it may be of value to investigate E rosette formation using

rat red blood cells after metrial gland cells have been pretreated with neuraminidase. Examination of cells from the metrial gland for IgM receptors (EAμ, Fig. 10) has proved to be negative, even after overnight culture. Therefore the only T cell characteristic for which cells from the metrial gland have been examined is the presence of an Fcγ receptor detectable by EAγ rosette formation (Fig. 9).

The proportion of human T cells which bear Fcγ receptors are the ones which suppress B cell responses to pokeweed mitogen in vitro (Moretta et al., 1977). It is possible that the T cells with Fcγ receptors may be related to the suppressor T cells found during the maternal immune response to pregnancy (Chaouat et al., 1979a; Suzuki and Tomasi, 1979a; Chaouat and Voisin, 1980). It is possible then that the Fcγ receptor bearing cells in the metrial gland may be related to and/or perform a similar function to the suppressor T cells in pregnancy.

e) Third population lymphocytes

The third population of lymphocytes was first defined by Froland and Natvig (1973) and is known by a variety of names (Dickler, 1976). In this thesis they are referred to as 'L' cells (Horwitz and Lobo, 1975) and are defined as being negative for E rosette formation and lacking complement receptors. They are also non-adherent and negative for non-specific esterase but have an avid Fcγ receptor, which is detectable by EAγ rosette formation and is resistant to trypsin (Froland and Natvig, 1973; Horwitz and Lobo, 1975; Lobo and Horwitz, 1976; Horwitz and Garrett, 1977). The 'L' cell Fcγ receptors bind monomeric IgG from normal serum (Froland and Natvig, 1973; Winchester et al., 1975). This binding is optimal at 4^0C and the IgG is released again at 37^0C.

Comparison of the Fcγ receptor bearing cells from the metrial gland to 'L' cells shows that neither cell type is capable of forming E or EAC rosettes and that their Fcγ receptors are resistant to trypsin and capable of binding both monomeric and complexed IgGs. Inhibition assays carried out with heat aggregated human IgG were as efficient at inhibiting EAγ rosette formation by metrial gland cells at 37^0C as they were at 4^0C (Fig. 33). In fact at 37^0C it was shown that heat aggregated IgG was readily endocytosed by metrial gland cells (Fig. 44), some of

which were still capable of forming EAγ rosettes (Fig. 45). Therefore it appears that metrial gland cell Fcγ receptors seem to show a close similarity to 'L' cells.

f) Other cell types

Neutrophils possess an Fcγ receptor detectable by EAγ rosette formation (Scott, 1979). This Fcγ receptor is trypsin resistant (An, 1980) but is only capable of binding heat aggregated IgGs in inhibition studies (Scott, 1979; An, 1980). Therefore neutrophil Fcγ receptors differ from those on metrial gland cells. Neutrophils are phagocytic granulocytes and there are two types of characteristic granules in their cytoplasm (Murphy et al., 1977). This makes them distinct from both the granulated cells and the Fcγ receptor bearing cells in the metrial gland.

Mast cells possess independent receptors for IgG (Fcγ) and IgE (Fcε) (Daëron et al., 1980). The Fcγ receptors on mast cells only bind heat aggregated or complexed IgGs and not monomeric IgGs (Daëron et al., 1980). Although no attempt has been made to identify IgE receptors in this study, it is apparent that the Fcγ receptors on metrial gland cells differ from those on mast cells. Histochemical studies have suggested that there might be a similarity between the granules in the granulated metrial gland cells and those in mast cells, but subsequent examination revealed that there was no histamine in the granules of the granulated metrial gland cells (Bulmer, 1968). Therefore there is no evidence to suggest that either of the major cell types in the metrial gland is related to mast cells or function in a manner similar to mast cells.

The recent report of Fcγ receptors on cultured mouse fibroblasts (Papamichail et al., 1979) shows that they are capable of binding both monomeric and heat aggregated IgGs at concentrations which correspond to those used in the inhibition tests reported here. Therefore there is support for the idea that the Fcγ receptors on cells in the rat metrial gland (Bray et al., 1978) might be on a proportion of the fibroblast-like stromal cells. The Fcγ receptors detected on cells from murine decidual tissue (Bernard et al., 1978; Dillon, 1979) may also be on fibroblast-like cells.

Possible functions of the metrial gland

Examination of cells from the metrial gland for immunological markers has revealed the presence of Fcγ receptors, SIg and cytoplasmic IgG and these three features seem to occur on different cell types. The most likely relationship in the metrial gland is that the small population of cells which bear SIg (Figs. 11 and 13) might be the precursors of the IgG containing granulated metrial gland cells. This idea presupposes that the granulated metrial gland cells are similar to plasma cells, differentiating from precursor cells to synthesise IgG. If this is the case then it is possible to account for the lack of Fcγ receptors on granulated metrial gland cells by the observation that small lymphocytes differentiating into IgG secreting plasma cells lose their Fcγ receptors (McConnell and Hurd, 1976). If the IgG in the granulated metrial gland cells is endocytosed, it is either done so non-specifically or by Fcγ receptors which are not detectable by the tests used here. However, endocytosis is not a prominent feature of the granulated metrial gland cells, instead it appears to be the fibroblast-like stromal cells which take up injected markers (Sharma and Peel, 1979). It is also the fibroblast-like stromal cells which possess the Fcγ receptors (Bray et al., 1978). Despite the suggestion that granulated metrial gland cells differentiate from the fibroblast-like stromal cells (Larkin and Schultz, 1968), there is no evidence to support this hypothesis. In fact the only relationship between these two cell types appears to be a close morphological association observed in ultrastructural studies (Peel and Bulmer, 1977).

As a result of the work described in this thesis, a number of functions can be proposed for the metrial gland.

a) Phagocytosis

Phagocytic cells, especially macrophages, are capable of ingesting antibody coated particles or of binding soluble immune complexes by their Fcγ receptors (Dorrington, 1977). It may be the role of the Fcγ receptors on cells in the metrial gland to bind and remove immune complexes which have formed in maternal tissue fluid. The position of the Fcγ receptor bearing cells in the metrial gland is not one which allows them close association with maternal blood vessels passing to and from the placenta and therefore it is unlikely that they are involved with removing immune complexes from the maternal circulation. The increased percentage of cells with Fcγ receptors observed in multiparity (Fig. 14)

may be associated with sensitisation of the mother by her previous pregnancies and may allow a more efficient monitoring of pregnancy.

The fate of ingested proteins in the metrial gland remains unknown, but one possibility is that they are used as a source of energy. Larkin and Schultz (1968) have suggested that stromal cells may differentiate into granulated metrial gland cells, the granules of which have been proposed to be lysosomal in nature (Peach and Bulmer, 1965). If the uptake of immune complexes triggers differentiation of stromal cells to granulated metrial gland cells then it is necessary to establish the stimulus which induces the development of the deciduomata of pseudopregnancy.

b) Immunoregulation

The suppression of antibody synthesis is regulated by the Fcγ portion of IgG in a feedback mechanism (Kerbel and Davis, 1974). Both monomeric and heat aggregated immunoglobulins have been shown to inhibit plaque forming cells producing antibodies to T dependent and T independent antigens in vitro (La Via and La Via, 1978). This inhibitory effect on the humoral immune response has been attributed to Fcγ receptor bearing lymphocytes, detected by EAγ rosette formation (Ryan and Henkart, 1976 and 1977; Yodoi et al., 1978; Masuda et al., 1978; Mijama et al., 1979). The proposition has been made that it is the 'L' cells which mediate this suppression by binding immune complexes or antibody to their Fcγ receptors (Horwitz and Garrett, 1977). The similarity of the Fcγ receptors in the metrial gland to those on 'L' cells suggests that metrial gland cells might also be involved in immunosuppression in pregnancy. If the Fcγ receptors on metrial gland cells are involved in the control of the maternal immune response, then the increased number of Fcγ receptor bearing cells observed in multiparity (Fig. 14) may allow elevated levels of antibody before feedback suppression of antibody synthesis becomes operative. This idea may explain the increased amount of IgG observed in the placentae of multiparous mice (Voisin and Chaouat, 1974) although it does not account for the origin of the IgG. If granulated metrial gland cells are synthesising some of the IgG found in the placenta, there is no increase in the number of cells containing IgG with multiparity (Fig. 36), although there may be a higher rate of synthesis by each individual cell.

Suppressor T cells have been shown to possess Fcγ receptors (Moretta et al., 1977) and it is possible that the Fcγ receptor bearing cells in the metrial gland may function in a manner similar to and/or be a subpopulation of the suppressor T cells which have been shown to be present in pregnancy (Chaouat et al., 1979a; Suzuki and Tomasi, 1979a; Chaouat and Voisin, 1980).

c) Cytotoxicity

Several different cell types, all of which possess Fcγ receptors, are capable of displaying antibody dependent cellular cytotoxicity (ADCC). These include T cells (Moretta et al., 1978), K cells, phagocytes and the non-immune, lymphocyte-like null or 'L' cells (Kerbel and Davis, 1974; Dickler, 1976). It is possible that the Fcγ receptor bearing cells in the metrial gland may also carry out ADCC. The most likely targets for this action are the trophoblastic cells sloughed off into the maternal circulation (Beer and Billingham, 1971). As to how the Fcγ receptor bearing cells, which are essentially tissue cells, come into contact with the trophoblastic cells in the maternal circulation would need to be established.

An alternative hypothesis for a cytotoxic function of metrial gland cells is possible if the granulated metrial gland cells synthesise the IgG detected within them (Mitchell et al., 1980). If this IgG has specificity directed against foetal antigens, then the localisation of granulated metrial gland cells around maternal blood vessels and within maternal blood spaces (Peel and Stewart, 1979) is ideal for the IgG to bind to trophoblastic cells in the circulation and to effect cellular cytotoxocity by ADCC or complement activation. If this is the case then it is unlikely that the IgG detected within the metrial gland cells of the deciduomata of pseudopregnancy has the same specificity as that seen in pregnancy.

d) Enhancement of foreign tissues

It is possible that the granulated metrial gland cells may be involved in the production of enhancing antibody and this antibody may be presented to Fcγ receptors (Elson et al., 1975; Jenkinson et al., 1976; Wood et al., 1978a) or antigens (Faulk et al., 1978; Wegmann et al., 1979) on the surface of trophoblast tissues. The Fcγ receptors of tumour cells may protect the tumour by binding non-cytotoxic antibody and in so doing mask antigenic determinants and prevent

recognition of the tumour by circulating lymphocytes of the host (Kerbel and Davis, 1974). Support for the idea that enhancing antibodies are present during pregnancy comes from the work of Voisin and Chaouat (1974) who eluted sub-class IgG_1 from murine placentae: this is a non-cytotoxic, non-complement fixing antibody. There is also the report that a blocking antibody is needed to maintain human pregnancy (Rocklin et al., 1976). It is believed that the rate of sensitisation of the mother by trophoblast sloughing off into the maternal circulation is at an optimal rate to elicit immunological enhancement rather than a rejection reaction (Hellström and Hellström, 1974; Billington, 1979). Further support for the idea that blocking antibodies are produced during pregnancy comes from the observations made by Beer et al. (1976) that in rats allogeneic pregnancy does not lead to the production of haemagglutinins in maternal blood despite the ease with which they can be detected after orthotopic or intra-uterine allografts and during pregnancy in the mouse. They suggest that rats may make blocking antibodies more readily than mice during pregnancy and are therefore more capable of suppressing haemagglutinin formation. The site of enhancing antibody synthesis during pregnancy is assumed to be plasma cells in the spleen and lymph nodes, but it is also possible that enhancing antibody may be made by the granulated metrial gland cells. This enhancing antibody need not necessarily have specificity directed against foetal antigens. This might explain the appearance of IgG in the granulated metrial gland cells of the deciduomata of pseudopregnancy as being in response to the stimulus for the production of the deciduomata, rather than to the presence of embryonic antigens.

Conclusion

Using an enzyme digestion technique single cell suspensions from the metrial gland of pregnancy and from the metrial gland of the deciduomata of pseudopregnancy have been shown to be similar when examined for a number of immunological markers. Populations of cells have been observed which possess Fcγ receptors, SIg or cytoplasmic IgG. All three features appear to be on different populations of cells. The Fcγ receptor bearing cells which make up a large proportion of the fibroblast-like stromal cells, have been compared to other cell types which possess Fcγ receptors and they show similarities to suppressor T cells, 'L' cells and/or fibroblasts. The small

proportion of cells which bear SIg may represent the proposed lymphocyte-like precursors of the granulated metrial gland cells. The cells which contain the cytoplasmic IgG have been shown to be the granulated metrial gland cells.

From a functional point of view a number of ideas have been proposed to explain how the Fcγ receptors and cytoplasmic IgG may relate to the theories about the maternal immune response to pregnancy. The main problem is to account for the similarity of the observations made on cells from the metrial gland of pregnancy to those made on the metrial gland of the deciduomata of pseudopregnancy. Understanding of the problem will be furthered by finding answers to the following questions. Is the IgG in the granulated cells endocytosed or synthesised in situ? Does the IgG in the metrial gland of the deciduomata have the same specificity as that in the metrial gland of pregnancy? What are the functions of the Fcγ receptors and are they related to the IgG containing cells? Are the Fcγ receptors and cytoplasmic IgG related to the maintenance of the foetal allograft or do they have some other physiological function?

Appendices I to III

Appendix I

Preparation of rabbit and rat anti-SRBC immunoglobulins

Antibodies to SRBCs were raised in two New Zealand White rabbits and six Wistar rats. Intraperitoneal injections of a 50% suspension of washed SRBCs in 0.9% saline were given at weekly intervals for three weeks. Rabbits received 1.0 cm^3 per injection and rats received 0.2 cm^3 per injection. The titres of the antisera were checked by an agglutination test and all animals were exsanguinated after four weeks. Rabbit and rat IgG fractions were prepared from pooled rabbit and rat sera as described by Stevenson and Dorrington (1970) for human IgG. The antisera were precipitated twice by 1.6 M ammonium sulphate at pH 8.0, dissolved in and dialysed against 0.03 M sodium phosphate buffer, pH 7.3 and then passed through a column of DEAE cellulose (DE52, Whatman) equilibrated with the same buffer. The rabbit and the rat IgG fractions were dialysed against PBS, pH 7.3 and concentrated by Amicon filtration to 3.4 mg/cm^3 and 4.4 mg/cm^3 for the rabbit and the rat anti-SRBC respectively. Protein concentration was determined by UV absorption at 280nm. The sub-agglutinating dose was determined by using doubling dilutions (down to 1/256) of the antisera mixed with an equal volume of a 7.0% suspension of washed SRBCs in 0.9% saline. Sensitisation of the SRBCs at the sub-agglutinating dose (1:64 for both antisera) was confirmed in a Coombs-type assay using goat anti-rabbit IgG (1:8) or rabbit anti-rat IgG (1:4) with the appropriately sensitised SRBCs.

Appendix II

Preparation of the Fabγ fraction of rabbit anti-rat IgG

Rabbit anti-rat IgG antibodies were raised in three New Zealand White rabbits. A primary injection of 1.0 mg/cm^3 rat IgG,* emulsified in Freund's complete adjuvant (Difco) was distributed amongst three or four subcutaneous sites in the nape of the neck. A booster dose (1.0 mg/cm^3) was given five weeks later. The titre of the antisera was checked by a precipitation method. All animals responded identically and were exsanguinated after six weeks. The sera were pooled and rabbit IgG was prepared as described by Stevenson and Dorrington (1970) for human IgG (Appendix I). Fabγ was prepared by the modified method of Nisonoff et al. (1960) as described by Stevenson et al. (1975). Rabbit IgG at approximately 10.0 mg/cm^3 in 0.03 M phosphate buffer, pH 7.3 was dialysed against 0.07 M acetate buffer, pH 4.0 and then digested at 37^{0}C for 18 hours by pepsin (Sigma) at 0.1 mg/ml. The proteins were then passed through a column of Sephadex G150 (Pharmacia) equilibrated in 0.2 M tris-HCl pH 8.0. The F(ab')$_2$ and any undigested IgG was eluted before the pepsin and small proteolytic fragments. The F(ab')$_2$ was then reduced with 0.01 M dithiothreitol (BDH) for 30 minutes at room temperature and alkylated by the addition of 0.022 M iodoacetamide (BDH) for 10 minutes at room temperature. This mixture was passed through a Sephadex G150 column and the Fabγ separated from any undigested IgG. The Fabγ was then dialysed against PBS, pH 7.3 and concentrated by Amicon filtration to approximately 16.0 mg/cm^3 as determined by UV absorption at 280 nm.

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* Kindly supplied by Dr. R.A. Robbins, Cancer Research Campaign Laboratories, Nottingham.

Appendix III

Reprints

The following published papers were included in the bound thesis. These have not been digitised due to copyright restrictions, but the links are provided.

Bray, J., Stewart, I. & Craggs, R.(1978) **The demonstration of cells bearing Fc receptors in the metrial gland of the pregnant rat uterus** Cell Tissue Res: 192: 89. https://doi.org/10.1007/BF00231025

Bray, J. & Craggs, R.(1979) **The characterisation of Fc receptors on cells from the metrial gland of the pregnant rat uterus** Journal of Reproductive Immunology. 2: 109-115. https://doi.org/10.1016/0165-0378(79)90011-1.

Mitchell, B.S. & Craggs, R & Peel, S. (1980). **Localisation of immunoglobulin (IgG) within the rat metrial gland** Journal of reproductive immunology. 2: 235-244. https://doi.org/10.1016/0165-0378(80)90029-7.

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