Increased susceptibility to metastasis during pro-oestrus/oestrus in rats: possible role of oestradiol and natural killer cells

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Summary It has been suggested that tumour development and immunocompetence are affected by the menstrual cycle and that sex hormones have been shown to modulate lymphokine production, neuroendocrine activity and immunity. In this study, we assessed natural killer cell activity and host susceptibility to metastasis during the oestrous cycle in the Fischer 344 inbred rat strain. Females were inoculated intravenously with MADB106 tumour cells, a syngeneic mammary adenocarcinoma cell line that metastasises only to the lungs. The susceptibility to metastatic development of this tumour was found to be significantly higher during pro-oestrus and oestrus than during metoestrus and dioestrus. Two days of exposure to oestradiol benzoate caused similar effects in ovariectomised females, and a single administration of progesterone reduced this effect of oestradiol to a statistically non-significant level. The tumour was found to be negative for oestrogen receptors, and its in vitro proliferation rate was not affected by oestradiol or progesterone. This suggests that the effects of sex hormones on the metastatic process are not attributable to a direct effect on tumour cells. Because the metastatic process of MADB106 tumour cells is known, and confirmed here, to be highly controlled by large granular lymphocyte/natural killer (LGL/NK) cell activity, we assessed their role in mediating the effects of the oestrous cycle. The number and activity levels of circulating blood LGL/NK cells (NK-R-P1 bright) were studied. Findings indicated oestrus-dependent alterations in the number of LGL/NK cells and suggested a diminished NK activity per LGL/NK cell during pro-oestrus/oestrus, the same phases that were characterised by higher susceptibility to metastatic development. These findings provide the first empirical evidence for a causal relationship between a short-term exposure to elevated oestradiol/low progesterone levels and decreased resistance to tumour metastasis, and it is hypothesised that an alteration in LGL/NK cell activity underlies these effects. Homologies and relevance to clinical phenomena are discussed.

Keywords: oestrous cycle; menstrual cycle; immunity; oestradiol; MADB106

The rate of tumour development has been associated with phases of the oestrous/menstrual cycle and with levels of sex hormones. In the mouse, spontaneous metastasis of an oestrogen-responsive tumour was reduced around ovulation (Ratajczak et al., 1988), and prolonged exposure to β-oestradiol (more than 8 weeks) increased experimental and spontaneous tumour metastasis (Hanna and Schneider, 1982). In humans, some studies, but not others, have associated certain periods of the menstrual cycle with a better long-term survival rate following a breast cancer resection (Badwe et al., 1991; Hrushesky, 1989; Saad et al., 1994; Senie et al., 1991; Spratt et al., 1993; Veronesi et al., 1994; for review see Davidson and Abeloff, 1993). For example, studies by Badwe et al. (1991) and Senie et al. (1991) reported a 3-fold increase in cancer recurrence and mortality in patients undergoing surgery during the unopposed oestrogen synthesis phase (elevated oestradiol/low progesterone levels, days 3–12 after the last menstrual period), relative to patients undergoing surgery during the rest of the menstrual cycle (approximately 45% vs 15% mortality rate respectively).

The effects of sex hormones on the development of malignancies can result from direct interaction with the neoplastic tissue (commonly via receptor systems for sex hormones) or can be mediated indirectly via various physiological mechanisms that affect tumour development (e.g. levels and affinity of intercellular adhesion molecules, vascular permeability and immune functions). The above-mentioned clinical phenomenon is reported to occur whether or not the excised breast tumours possess receptors for sex hormones (Badwe et al., 1991; Saad et al., 1994; Senie et al., 1991). Thus, it is likely that this phenomenon reflects an indirect effect of sex hormones on tumour development. Immune functions that control neoplasia, such as natural killer cell, macrophage and cytotoxic T cell activities, are potential mechanisms, and human and animal studies demonstrated oestrous/menstrual cycle-related alterations in cellular and humoral immunity (Sulke et al., 1985; White et al., 1982; Gruber et al., 1988; Rager et al., 1994). Sex hormones may directly impact anti-tumour activity of immune effector cells or may alter levels of immune modulators (interleukins, interferons and various hormones). Indeed, alterations in monocyte and macrophage release of interleukin (IL)-1, IL-2 and prostaglandins were reported during the oestrous/menstrual cycle and in response to in vivo and in vitro application of sex hormones (Lynch et al., 1994; Polan et al., 1988, 1989, 1990, 1994; Simon et al., 1993).

Natural killer (NK) cells are known to recognise and kill virally infected cells and tumour cells spontaneously (Herberman and Ortolando, 1981; Oldham, 1990) and have been shown to control metastatic growth of several types of tumour (Barlozzari et al., 1983; Gorelik et al., 1982; Hanna et al., 1985; Wiltrout et al., 1985; Schantz et al., 1987; Levy et al., 1987; Barlozzari et al., 1985; Ben-Eliyahu and Page, 1992). Few studies have reported fluctuation in NK-cell activity during the oestrous/menstrual cycle. In female mice, splenic NK activity was elevated around ovulation (Gruber et al., 1988; Hrushesky et al., 1988). In women, two studies have reported alterations in blood NK cytotoxicity during the menstrual cycle, although findings from these studies were not consistent (Sulke et al., 1985; White et al., 1982). None of the above-mentioned studies assessed the number of large granular lymphocyte (LGL)/NK cells within the population of splenocytes/monocytes tested for NK activity. Therefore, it is not clear whether changes in the number of LGL/NK cells

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or in the activity per cell account for the reported differences during the oestrous/menstrual cycle. To our knowledge, no consistent short-term in vivo or in vitro effects of oestradiol, progesterone or testosterone on NK activity have been reported. One study reported a lack of in vitro effects of these hormones on a cloned human NK cell line (Callewaert et al., 1991), and another study, using oestradiol and tamoxifen in mice, indicated dose-dependent alterations in NK cytotoxicity (Baral et al., 1991).

In the current study, we assessed the effects of the oestrous cycle and sex hormones on the number and activity levels of blood NK cells measured in vivo, and on the in vitro resistance to metastatic development of a mammary adenocarcinoma cell line, MADB106. This tumour line, syngeneic to the inbred F344 rats used in this study, metastasises readily in the lungs following intravenous inoculation, and thus constitutes a convenient model of breast cancer metastasis. The use of this tumour simulates a partial but well-defined subprocess of the overall metastatic process (Barlozzari et al., 1985; Ben-Eliyahu et al., 1991; Ben-Eliyahu and Page, 1992). There are three major advantages in employing the MADB106 tumour model in the current study: (1) The time interval that is most critical for the establishment of metastases is shorter than 24 h (Barlozzari et al., 1985); therefore, the efficiency of the host anti-metastatic activity can be correlated with the similarly short oestrous cycle phases in the rat. (2) Host control of the metastatic process (number of metastases established rather than size) is markedly dependent on the activity levels of LGL/NK cells during the first 24 h after tumour injection (Barlozzari et al., 1985; Ben-Eliyahu et al., 1991; Ben-Eliyahu and Page, 1992). Therefore, the resistance to metastatic development also reflects in vivo levels of LGL/NK cell activity and suggests the biological significance of alterations in NK activity that we directly measure in vitro. (3) As will be reported here, the MADB106 tumour does not express oestrogen receptors and its in vitro proliferation rate appears not to be affected by oestradiol or progesterone. Therefore, the use of this tumour advances the study of the indirect effects of sex hormones on tumour development (mediated via alteration in immunity or other physiological mechanisms).

In order to maximise the overlap between the time of the highest levels of sex hormones and the NK-sensitive period of the MADB106 tumour, we chose to inoculate the rats with tumour cells at the beginning of the light phase, when there is a sharp rise in the levels of most sex hormones on pro-oestrous day 1, and the cumulative metastatic activity of the host during the first 9 h after tumour inoculation. This assessment was conducted by measuring the percentage of tumour cells retained in the lungs 9 h after inoculation. We and others have reported high correlation between lung tumour cell retention within hours after tumour cell inoculation and the consequent number of lung metastases found 3 weeks later (Barlozzari et al., 1985; Ben-Eliyahu and Page, 1992). Also, in various studies using this tumour model and assessing the effects of surgery, stress, morphine or ethanol on lung tumour cell retention and on the number of lung metastases detected 3 weeks later, very similar effects in these two indices were observed, and changes in NK activity paralleled changes in susceptibility to the MADB106 metastasis (Ben-Eliyahu et al., 1991, 1993, 1996; Page et al., 1993, 1994; Yirmiya et al., 1991, 1992).

Materials and methods

Animals, oestrous cyclicity and hormonal pattern

Fischer 344 (F344) rats were purchased from Harlan Sprague Dawley (HSD), Indianapolis, USA, housed five per cage with free access to rats, water and kept under a 12:12 h light–dark cycle. Before all experiments, rats were acclimatised to the vivarium for a minimum of 4 weeks. The female rat has a 4–5 day oestrous cycle consisting of the following phases/days: pro-oestrous (pe), oestrus (e), meta-oestrous (me), dioestrous day 1 (de1), and a variably occurring dioestrous day 2 (de2). Oestradiol and prolactin levels, unlike those of progesterone and other sex hormones, begin to increase gradually during dioestrous (Lapolt et al., 1986; Nequin et al., 1979). Levels of most sex hormones, including oestradiol, progesterone, prolactin, LH and follicle-stimulating hormone (FSH), rise sharply and peak during the light phase of pro-oestrous and return to baseline levels before the oestrous day (Lapolt et al., 1986; Nequin et al., 1979).

Ovariectomy

Females were ovariectomised at 12 weeks of age under halothane anaesthesia through a midline abdominal incision (for procedure see D’Amour and Blood, 1954). Sutures were removed 10 days after surgery. Rats were allowed a minimum of 3 weeks for recovery.

MADB106 tumour cells

MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma (MADB100) chemically induced in the inbred F344 rat (Barlozzari et al., 1985). MADB106 cells were maintained in 5% carbon dioxide at 37°C in monolayer cultures in complete medium [RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 45 U penicillin G ml⁻¹, 0.045 mg streptomycin ml⁻¹, 2 mM L-glutamine, 0.1 mM non-essential amino acid and 1 mM sodium pyruvate] and separated from the flask (Falcon 3023) using 0.25% trypsin.

Radioiodination of tumour cells and lung clearance assessment

For lung clearance assessment, DNA radioiodination of tumour cells was accomplished by adding 0.4 μCi of [¹²⁵I]iododeoxyuridine ([¹²⁵I]IDUR) (ICN Radiomedicals, Irvine, CA, USA) per ml of complete media to the growing cell culture 1 day before harvesting the cells for injection. For tumour cell injection, rats were lightly anaesthetised with halothane and 4 × 10⁴ kg⁻¹ [¹²⁵I]IDUR-labelled MADB106 tumour cells were injected into the tail vein in approximately 0.5 ml phosphate-buffered saline (PBS). This procedure requires 1–2 min, after which rats quickly awake and behave normally. Nine hours later, rats were euthanised with halothane and their lungs removed to measure radioactive content in a gamma counter. Percentage radioactivity retained in the lungs is the ratio between radioactivity measured in the lungs and total radioactivity in the injected tumour cell suspension. Our previous studies with radioiodi labelled MADB106 tumour cells indicated that the level of radioactivity released from intact cells is negligible (Ben-Eliyahu and Page, 1992).

Selective in vivo depletion of LGL/NK cells

Two days before tumour inoculation or blood sampling, 1.5 mg kg⁻¹ Mab 3.2.3 (Pittsburgh Cancer Institute) was injected intravenously (i.v.) under light halothane anaesthesia. In vivo treatment of rats with Mab 3.2.3 selectively depletes LGL/NK cells and eliminates NK- and antibody-dependent non-MHC-restricted cell cytotoxicity without affecting other immune functions; T-cell function and percentage of T cells, monocytes and polymorphonuclear (PMN) cells are unaffected (Chambers et al., 1989, 1992; van den Brink et al., 1990). In a previous study, using this dose of the Mab 3.2.3, we showed a complete abolition of blood and splenic LGL/ NK cytotoxicity and a 100-fold increase in the lung retention and metastatic colonisation of MADB106 tumour cells (Ben-Eliyahu and Page, 1992). We have also used other monoclonal antibodies (R73, W3/25 and ED2), mouse serum and saline as controls for the administration of the depleting agent and found no differences between the effects of these controls and no injection (Ben-Eliyahu and Page, 1992).
Whole blood NK cytotoxicity assay

Rats were lightly anaesthetised with halothane, and blood was drawn into a heparinised syringe (20 units ml\(^{-1}\) blood, preservative free) by cardiac puncture. Exactly 1 ml of blood was washed once with PBS (4 x 10^6 blood volume) and twice with complete media (3 x blood volume, 15% FBS). For each wash, the mixture was centrifuged at 400 g for 10 min and the supernatant removed down to the original blood volume. For each of the six effector:target (E:T) ratios used, 100 μl of washed blood was placed into a well of a microtitre plate and 150 μl of \(^{51}\)Cr-labelled YAC-1 tumour cells in complete medium was then added on top of the blood. A concentration of 8 x 10^6 YAC-1 ml\(^{-1}\) was used for the lowest E:T ratio (approximately 8:1, leucocytes:YAC-1, depending on leucocyte concentration per ml of blood) and sequentially divided by two for higher E:T ratios (approximately 256:1 at the highest). Plates were centrifuged at 500 g for 10 min to create a buffy coat layer of leucocytes and target cells above the red blood cells before a 4 h incubation period. Following incubation, plates were centrifuged again and aliquots of 100 μl of the supernatant were recovered from each well for assessment of radioactivity in a gamma counter. The spontaneous and maximum release of radioactivity from tumour cells were measured separately for each of the six tumour concentrations, and percentage specific lysis was calculated for each E:T ratio using the standard formula: \([\text{experimental release - spontaneous release}]/\text{maximum release - spontaneous release}] \times 100\%

Flow cytometry

An aliquot of 100 μl of blood was combined with 50 μl of PBS (supplemented with 2% FBS and 0.1% sodium nitrite and 2 μg of the MAb 3.2.3 conjugated with fluorescein isothiocyanate (Pittsburgh Cancer Institute). Samples were kept in the dark at room temperature and incubated for 15 min before 2 ml of FAC's lysis solution (Becton Dickinson) was added. Ten minutes later, samples were centrifuged for 5 min at 500 g and the lysis solution aspirated. Cells were washed again with 2 ml of PBS (5 min centrifugation, 500 g) and resuspended in 300 μl of PBS for flow cytometry analysis using a FACScan (Becton Dickinson). The MAb 3.2.3 has been shown in the rat to recognise a surface antigen, NKR-P1, which is expressed exclusively on fresh and IL-2-activated LGL/NK cells (MAb 3.2.3 bright) and, to a much smaller degree, on polymorphonuclear (PMN) cells (MAb 3.2.3 dim) (Chambers et al., 1992). Our experience with this procedure has indicated that there is no overlap between the bright and the dim cells (Ben-Eliyahu et al., 1993). The number of white blood cells per ml of blood was assessed in each blood sample using a Coulter counter.

Counterbalancing, timing of procedures and statistics

The time and order of blood draw, drug administration and tumour injection were counterbalanced across groups and oestrous phases in all experiments. For each experiment, the procedure of either tumour inoculation or blood draw was completed within less than 2 h in all animals and was conducted during the first half of the light phase (otherwise noted). One factor ANOVA was conducted (repeated measures for NK cytotoxicity in experiment 4). Bonferroni post hoc tests or planned contrasts were used to identify specific differences. An alpha of 0.05 was set for all experiments.

Experiment 1: The effects of the oestrous cycle on host resistance to metastasis and LGL/NK sensitivity of the metastatic process

Oestrous cyclicity and phase were determined according to the method described in Everett (1989) based upon vaginal cellularity. Daily vaginal smears were performed in 6-month-old females (200 g, n = 34 cycling females) for 9 consecutive days during the first half of the light phase. On the tenth day, at the same time of vaginal cellularity sampling, each rat was inoculated with MADBI06 tumour cells. To compare males with females, and to establish a relationship between the dose of tumour injected and the percentage of tumour cell retention, similarly handled age-matched males (350 g) were injected with either the same dose, one-half or twice the dose of tumour cells per body weight (n = 6, 5 and 5 respectively). Tumour cell retention was assessed in all rats at 9 h after tumour injection. In a replicate study, 18 cycling females were used as described above. Six were selectively depleted of LGL/NK cells before tumour injection to verify the dependence of lung clearance on LGL/NK cells in this paradigm.

Experiment 2: The effects of oestriol and progesterone on host resistance to metastasis in ovariectomised females

Ovariectomised (OVX) females were injected subcutaneously (s.c.) with oestriol benzoate (EB) or progesterone (P) at different time intervals before tumour inoculation, and lung tumour retention was measured. Ovariectomised females (210 g, 15 weeks old, n = 57) were randomly assigned to the following groups and were given five successive daily injections of drugs/vehicle (Figure 2). One group received vehicle injections throughout the 5 day period (control); two groups received drugs only on the fifth day, one group receiving P (6 mg kg\(^{-1}\) in 0.25 ml of peanut oil), and the second group 16 μg kg\(^{-1}\) EB (in 0.25 ml of corn oil); three groups were also injected with an overall dose of 16 μg kg\(^{-1}\) EB that was given in two successive injections (8 μg kg\(^{-1}\) each), one group on days 1 and 2, the second group on days 4 and 5, and the third group also on days 4 and 5, but concomitantly with P (6 mg kg\(^{-1}\) on day 5. On the sixth day, all rats were inoculated with radiolabelled MADBI06 cells and euthanised with halothane 9 h later to assess lung tumour retention.

The doses of EB were chosen to induce high physiological levels of oestradiol for approximately 24-48 h after administration. In the intact female rat, average serum oestradiol levels increase from 15 pg ml\(^{-1}\) on oestrus/ metaoestrous days to 55 pg ml\(^{-1}\) on pro-oestrous day (Lapolt et al., 1986). The 15 pg ml\(^{-1}\) level found on oestroestrous day was elevated to 25 pg ml\(^{-1}\) within 12 h after the administration of 4 μg kg\(^{-1}\) EB, or elevated to 160 pg ml\(^{-1}\) after the administration of 80 pg ml\(^{-1}\) EB. These elevated levels decreased and approached baseline levels within 24 and 48 h respectively in OVX rats (Matt et al., 1986). Based on these reports and approximated linear relationships between levels of EB injected and induced blood levels of oestradiol, we injected a total of 16 pg kg\(^{-1}\) EB to OVX females, estimating the induced oestradiol levels to be approximately 40 pg ml\(^{-1}\). The dose of progesterone used is the standard for the induction of proceptivity in OVX females (following sensitisation with oestradiol). EB and progesterone were dissolved in the specific oil vehicle indicated in the literature reporting their serum levels.

Experiment 3: In vitro effects of oestriol and progesterone on MADBI06 tumour proliferation, and the levels of oestradiol and progesterone receptors on MADBI06

Oestradiol or progesterone at concentration of 10^{-8}, 10^{-6}, and 10^{-4} M or vehicle were added to the growing culture of MADBI06 cells, and cells were harvested and counted at 24 or 48 h after addition of these hormones. In a separate experiment, MADBI06 tumour cells were tested for oestradiol and progesterone receptors using the standard clinical procedure described in Geier et al. (1982), and the MCF-7 tumour line was tested for progesterone receptors as a positive control.
Experiment 4: Oestrous cycle and the number and activity level of circulating LGL/NK cells

Flow cytometry was used to assess the number of blood LGL/NK cells (MAb 3.2.3 ‘bright’) per ml of blood, and whole blood NK cytotoxicity assay was used specifically to assess LGL/NK cytotoxicity per ml of blood. Three different assessments of whole blood NK cytotoxicity were conducted (n = 70, 65 and 34; age = 12, 16 and 20 weeks; bw = 200–220 g). Blood was taken during the first half of either the dark phase (first study) or the light phase (second and third studies). To verify that the whole blood cytotoxicity assay specifically assesses LGL/NK cell-dependent cytotoxicity, rats selectively depleted of LGL/NK cells were also used in these experiments. Flow cytometry was conducted twice during the light phase, once using 20-week-old females (n = 34, bw = 220 g) and once using 10-week-old females (n = 9, bw = 210 g). Each assessment was conducted using cycling females that underwent daily vaginal cellularity sampling for the 9 days before blood withdrawal for either NK activity or flow cytometry.

Results

Experiment 1

The oestrous cycle and its effects on host resistance to metastasis Significant differences in tumour cell retention across the 5 oestrous days were evident (F(4,20) = 4.76, P < 0.01) (Figure 1). Specifically, rats in pro-oestrus and oestrus showed a higher percentage of tumour cell retention than rats in metoestrus and dioestrus (P < 0.05, Bonferroni post hoc test). The three groups of males injected with an increasing tumour load showed an increasing percentage of tumour cell retention (F(2,13) = 4.3, P < 0.05). Compared with the females, males injected with an equal number of tumour cells per body weight retained a lower percentage of tumour cells than females in pro-oestrus/oestrus [i.e. demonstrated a better lung clearance efficiency, (F(1,18) = 5.7, P < 0.05, planned contrast)], but similar levels of retention compared with females in metoestrus/dioestrus. The males injected with twice the dose of tumour cells per kg relative to females exhibited tumour cell retention midway between the low and high levels observed in females during metoestrus/dioestrus and pro-oestrus/oestrus respectively.

In the replicate study, similar oestrous effects were evident in normal cycling females (pro-oestrus/oestrus = 0.56% radioactivity retained, s.e.m. = 0.061; metoestrus/dioestrus = 0.40%, s.e.m. = 0.025; t0 = 5.5, P < 0.05). Oestrous phases were collapsed to pro-oestrus/oestrus vs metoestrus/dioestrus based on the findings from the first study and because of the small number of animals.

Oestrous cycles did not synchronise within or between cages in any of the studies conducted. More than 90% of females had regular 4–5 day cycles, the great majority of which had a 5 day oestrous cycle.

The effects of selective depletion of LGL/NK cells on lung tumour retention Selective depletion of LGL/NK cells resulted in approximately a 100-fold increase in tumour cell retention from 0.48% (s.e.m. = 0.045) in normal rats to 38% in the six depleted animals (ranging from 24% to 54%). The relationship between oestrous phase and tumour retention in this small group of LGL/NK-depleted animals was not tested for methodological reasons. The purpose of the depletion was merely to verify the LGL/NK sensitivity of the tumour.

Experiment 2

The effects of oestradiol and progesterone on host resistance to metastasis in OVX females The results showed that, compared with vehicle-injected rats, OVX rats receiving 16 µg kg⁻¹ EB given in two equal injections on the last 2 days, but not in one injection on the last day before tumour injection, exhibited a significant increase in tumour cell retention (F(1,18) = 2.6, P < 0.05, Bonferroni post hoc test) (Figure 2). No such increase was evident in the groups that

Figure 1 The percentage of tumour cell retention in males following injections of three different tumour loads, and in females in prooestrus (pe), oestrus (e), metoestrus (me), dioestrus 1 (de1) and dioestrus 2 (de2). Compared with females (○), males were injected with either an equal number of tumour cells per kg (---), half as many (-----), or twice as many (~-~) doubling tumour load). Females in pro-oestrus/oestrus showed a significantly higher percentage of cell retention than females in metoestrus/dioestrus. In males, the increasing tumour load resulted in a significant increase in tumour retention. Males injected with females’ load of tumour cells per bw (males) retained a significantly lower percentage of tumour cells than females in prooestrus/oestrus, but did not differ from females in metoestrus/dioestrus. In females, vertical bars represent s.e.m.; pe day is shown twice. Variance in the three male groups was similar to that observed in the five female groups (std = approximately 0.07%).
received the same 2 day treatment with EB either 4 days before tumour injection or 2 days before tumour injection together with progesterone on day 5.

Experiment 3
In vitro effects of oestradiol and progesterone on MADB106 tumour proliferation. Enumerating MADB106 tumour cells following 1 or 2 days of incubation indicated that the cells divided approximately every 18 h, and this rate was not affected by oestradiol or progesterone in the concentrations used.

Oestradiol and progesterone receptors on MADB106. The MADB106 tumour was found to be negative for oestradiol receptors (undetectable levels) and expressed low levels of progesterone receptors (19.4 ± 2.1 fmol mg⁻¹), which were significantly lower than in the MCF-7 tumour line (51.6 ± 4.6 fmol mg⁻¹ (tₙ = 6.3, P < 0.05).

Experiment 4
Oestrous cycle and LGL/NK cell number and activity levels. Females in pro-oestrus and oestrus exhibited a significantly greater number of LGL/NK cells (MAB 3.2.3³ bright) per ml of blood compared with animals in metoestrus/dioestrus, in each of the two replicate assessments (Table I includes averages and statistics). Despite this difference in LGL/NK number per ml of blood, there were no significant oestrous phase-related differences in NK activity per ml of blood in any of the three replications conducted. In fact, very similar levels were observed in each replication (Figure 3 illustrates one replication), suggesting a lower NK activity per LGL/NK cell during pro-oestrus and oestrus.

The blood from animals that were selectively depleted of LGL/NK cells showed no MAB 3.2.3³ bright cells and no cytotoxic activity. The number of LGL/NK cells per ml of blood across the two oestrous phases was higher in 10-week-old females than in 20-week-old females (Table I) (F(1,39) = 5.4, P < 0.05).

Discussion
The statistically significant relationship between the oestrous cycle and lung clearance observed in this study indicates a decrease in host resistance to metastasis during pro-oestrus and oestrus. To provide a measure of the biological significance of the differences in lung tumour retention among oestrous phases, it is noteworthy that a difference of similar magnitude was observed between the two groups of males injected with the lowest and the highest tumour load (i.e. a 4-fold increase in tumour load), (see Figure 1). Thus, it might be suggested that during metoestrus/dioestrus the host can resist four times as many tumour cells with the same rate of success as during pro-oestrus/oestrus. Furthermore, although the absolute differences in tumour cell retention between the oestrous phases are not large, the relatively low number of animals (n = 4–6 per group) needed to demonstrate significant differences (in both replications) testifies to the robust nature of the phenomenon.

The effects of oestradiol benzoate (EB) and progesterone on lung tumour retention in the OVX females and the time course of these effects suggest that: (1) oestradiol administration can simulate the increase in tumour cell retention during pro-oestrus/oestrus observed in Experiment 1; (2) more than 24 h of exposure to oestradiol is needed before its full tumour-enhancing effect is evident, and this effect dissipates within less than 3 days following discontinuation of oestriadiol treatment; and (3) progesterone, at the dose and timing administered, appears to attenuate the effects of oestradiol on tumour cell retention. This last suggestion is based upon the finding that progesterone administration to the EB-treated rats decreased the tumour-enhancing effects of EB by approximately 50% from a significant to a non-significant level.

In normal cycling rats, oestrous levels begin to increase during dioestrus, 24–48 h before pro-oestrus (Lapolt et al., 1986; Nequin et al., 1979). Taken together, the findings in OVX and in normally cycling females, suggest that the gradual increase in oestrous levels during the 2 dioestrus days, coupled with the surge of oestradiol during pro-oestrus, underlie the effects of the oestrous cycle on the resistance to tumour metastasis observed in Experiment 1. The termination of this effect may be initiated by the sharp increase in progesterone levels on pro-oestrus or may occur spontaneously within a day after the drop in oestradiol secretion. Nevertheless, the administration of EB or progesterone to OVX females is an imperfect simulation of the hormonal milieu of the normal cycling rat. Further studies using selective oestadiol antagonists in normal cycling rats are needed to provide additional support for this hypothesis.

Figure 3 Mean percentage specific killing on the different days of the oestrous cycle (± s.e.m.). Cytotoxicity against different numbers of YAC-1 target cells was assessed per 0.1ml of blood. No significant differences in cytotoxicity among oestrous days were found in this replicate or in the other two replications conducted. Taken together with the significant alteration in the number of LGL/NK cells per ml of blood during the oestrous cycle (Table I), these findings indicate a suppressed LGL/NK cell activity per LGL/NK cell during the pro-oestrus/oestrus phase.

Table 1 Means (± s.e.m) of LGL/NK cells per ml of blood (× 10⁶) on different days of the oestrous cycle in two replicate studies

| YAC-1 target cells × 100 | Specific killing (%) |
|-------------------------|----------------------|
| 1200                    | 50                   |
| 1300                    | 50                   |
| 1400                    | 50                   |
| 1500                    | 50                   |
| 1600                    | 50                   |
| 1700                    | 50                   |
| 1800                    | 50                   |
| 1900                    | 50                   |
| 2000                    | 50                   |
| 2100                    | 50                   |
| 2200                    | 50                   |
| 2300                    | 50                   |
| 2400                    | 50                   |
| 2500                    | 50                   |
| 2600                    | 50                   |
| 2700                    | 50                   |
| 2800                    | 50                   |
| 2900                    | 50                   |
| 3000                    | 50                   |
| 3100                    | 50                   |
| 3200                    | 50                   |
| 3300                    | 50                   |
| 3400                    | 50                   |
| 3500                    | 50                   |
| 3600                    | 50                   |
| 3700                    | 50                   |

Table 1 Means (± s.e.m) of LGL/NK cells per ml of blood (× 10⁶) on different days of the oestrous cycle in two replicate studies

| Replicate 1 | n = 34 | pe | 27.7 | 1.02 | 16.8 | 1.71 | 17.1 | 1.69 | 20.0 | F(1,39) = 2.81 |
|-------------|--------|----|------|------|------|------|------|------|------|----------------|
| (20-week-old rats) | | | | | | | | | | P < 0.05 |

| Replicate 2 | n = 9 | pe | 42.1 | 3.68 | 28.3 | 2.55 | F(1,39) = 10.2 |
|-------------|-------|----|------|------|------|------|----------------|
| (10-week-old rats) | | | | | | | P < 0.05 |
Several findings suggest that the in vivo effects of the oestrous cycle and its simulation by oestriadiol administration in OVX females are not mediated via a direct effect of sex hormones on MADB106 tumour cells. Firstly, MADB106 cells were found to be negative for oestriadiol receptors, and their proliferation rate in vitro was not affected by oestriadiol or progesterone. Secondly, whereas 2 days, but not 1 day, of oestriadiol treatment in OVX females were needed to induce its effects on MADB106 tumour retention, tumour cells were exposed to oestriadiol for an equal duration in both cases (i.e. for 9 h beginning from tumour inoculation to sacrificing the animal). Thus, the impact of oestriadiol on the animal rather than on the MADB106 tumour cells appears to determine host susceptibility to metastasis of this tumour. Therefore, it seems more likely that the effects of oestriadiol or the oestrous cycle are mediated via some alteration in the physiological milieu that affects metastatic development.

To support the suggestion that LGL/NK cells mediate the effects of the oestrous cycle on MADB106 tumour metastasis observed in this study, it is important to establish the role of LGL/NK cells in controlling this metastatic process, and the ability of the lung clearance assay (measured by lung tumour retention) to reflect the effects of the oestrous cycle. In this study, the number of decrease in lung metastases in rats selectively depleted of LGL/NK cells verifies the high sensitivity of the lung clearance assay for LGL/NK cell activity. We and others have also used other approaches to implicate LGL/NK cells in controlling lung tumour retention and the consequent metastatic colonisation of the MADB106 tumour (e.g. adoptive transfer of purified LGL cells and stimulation of LGL/NK cells), and have determined that the NK-sensitive period of this process is shorter than 1 day (Barlozzari et al., 1985; Ben-Eliyahu and Page, 1992). As reviewed in the Introduction, the short-term assessment of lung clearance is highly predictive of the actual number of lung metastases that will grow weeks later and correlates well with levels of NK cell activity in experimental conditions (Barlozzari et al., 1985; Ben-Eliyahu and Page, 1992; Ben-Eliyahu et al., 1991, 1993, 1996; Page et al., 1993, 1994, 1995; Yirmiya et al., 1991, 1992). Thus, the lung clearance assay constitutes a short-term index of the metastatic process and maximises the overlap between the LGL/NK-sensitive period of the MADB106 metastatic process and the similarly short oestrous phases in the rat. Therefore, it could be suggested that the decrease in lung clearance observed during pro-oestrus/oestrus reflects a decrease in LGL/NK cell activity during this period of the oestrous cycle. Nevertheless, because of the phylogenetic mechanisms that are involved, relatively, mediate the effects of the oestrous cycle or oestriadiol administration on tumour retention, more support and more direct evidence for the involvement of LGL/NK cells is needed.

To this end, we have directly assessed blood NK cell number and activity in vitro. Our findings indicated a significantly greater number of LGL/NK cells per ml of blood during pro-oestrus and oestrus in both replications, but no increase in LGL/NK cytotoxicity per ml of blood during these days in any of the three replications conducted. Taken together, these findings suggest that there is a decrease in blood NK activity per LGL/NK cell during pro-oestrus and oestrus. Although the blood seems to be an important immune compartment with respect to host anti-tumour NK activity in this tumour model (Ben-Eliyahu and Page, 1992), it is clear that other immune compartments (lung capillary beds, interstitial tissue and alveoli) could also play an important role. Interestingly, the same relationship between oestrous phases and LGL/NK cell number and activity was found in the spleen by Rager et al. (1994) using the standard splenic NK cytotoxicity assay and a different marker (5C6) for identifying LGL/NK cells. Thus, the findings of the present study in blood and those of Rager et al. (1994) in splenocytes suggest that sex hormones or other oestrous cycle-dependent hormonal factors induce a suppression of cytotoxicity per LGL/NK cell during pro-oestrus/oestrus, regardless of the specific location of these cells within the body. Therefore, and because these phases of the oestrous cycle were also characterised in experiment 1 by a higher susceptibility to the LGL/NK-sensitive MADB106 tumour, we hypothesise that decreased LGL/NK cell activity contributes to the observed oestrous effects on tumour metastasis.

The results of Experiment 3 also indicate that the number of LGL/NK cells per ml of blood is lower in older females (20 weeks old) than in younger females (10 weeks old) across oestrous phases (Table I). We previously conducted a longitudinal study and reported a similar decrease in females, but not in males. In the male F344 rat, LGL/NK number and activity continued to increase from 10 to 20 weeks of age (Page et al., 1995). This difference between the sexes may be attributed, among other things, to differences in chronic exposure to sex hormones and consequent differences in levels of various interleukins. Indeed, chronic exposure to oestriadiol or progesterone under natural conditions (e.g. pregnancy) (Gabrilovac et al., 1988) or artificial conditions (e.g. some oral contraceptives, implants of oestriadiol in ovariec-tomised rodents) (Baker et al., 1989; Hanna and Schneider, 1982; Seaman and Gindhart, 1979) was associated with NK cell suppression, as cell retention was lower in older females than in younger ones. At levels of prolactin (Hou and Zheng, 1988; Vidaller et al., 1992). Other studies have suggested a biphasic effect of oestrogens, with an early phase of increased NK activity and a long-term/higher dose reduction in NK activity (Screpanti et al., 1987). Thus, sex hormones may have both short-term (e.g. oestrous/menstrual) and long-term effects on LGL/NK number and activity.

Various mechanisms may mediate the effects of sex hormones on LGL/NK cell activity. Although NK cells do not appear to respond directly to sex hormones in vitro (Callewaert et al., 1991), changes in levels of interleukins, which are known to regulate LGL/NK cell activity, were reported to occur during the oestrous cycle and in response to sex hormones (Polan et al., 1994). The effects of sex hormones can also be mediated via interaction with the stress response. In mice, acute stress increased pituitary IL-1x content only during pro-oestrus (Nappi et al., 1994). Sex hormones and the oestrous cycle have been reported to affect baseline and stress levels of several stress hormones, including glucocorticoids (CORT), and such stress hormones are known to affect several immune functions, including NK activity (Ader et al., 1991). Specifically, during pro-oestrus, normal cycling rats maintained higher basal levels of CORT (Ragunath et al., 1971) and a high CORT response to stress (Viau and Meaney, 1991). Administration of oestradiol was shown to elevate basal and stress-induced levels of CORT in ovariec-tomised rats (Viau and Meaney, 1991).

The findings of the current study may have implications for the surgical management of breast cancer. Although a topic of intense debate in the clinical literature, several studies have indicated differential rates of cancer recurrence (and different rates of long-term mortality) as a function of the menstrual phase during which mastectomy is performed (Badwe et al., 1991; Hrushe sky et al., 1989; Saad et al., 1994; Senie et al., 1991; Spratt et al., 1993). Our study in rats and this clinical phenomenon have many characteristics in common. Specifically, in our study cancer development was found to be related to: (1) oestrous cycle; (2) the metastatic process; (3) high and positive progesterone levels; but (4) not related to a direct effect of sex hormones on the tumour. Similarly, the description of the clinical phenomenon provided by Badwe et al. (1991), Senie et al. (1991) and Saad et al. (1994) indicated: (1) an association between menstrual phase and the development of malignancies; (2) the involvement of the metastatic process, which is implicated by the reports that the clinical phenomenon occurred only in woman bearing tumours with evident metastatic potential (positive lymph nodes), and that mortality was associated with cancer recurrence; (3) an association of the high-risk period with increasing oestriadiol
levels and low progesterone levels; and (4) that the relationship between menstrual phase during which surgery is performed and subsequent metastasis occurred regardless of whether or not the excised tumour expressed receptors for sex hormones. These homologies between our findings and the suggested phenomenon in women undergoing surgery support the likelihood that this clinical phenomenon may indeed occur under certain conditions. Further, our findings suggest testable hypotheses as to the nature of the hormonal and immunological mechanisms underlying the clinical phenomenon and present an animal model that may be used to expand our understanding of this phenomenon.

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