in vivo *22*: 565-570 (2008)

Frequency of the Cancer-resistant Phenotype in SR/CR Mice and the Effect of Litter Seriation

JANNE KOCH[1], ANNA BOSCHIAN[1], JANN HAU[1] and KLAUS RIENECK[1,2]

[1]*Department of Experimental Medicine, University of Copenhagen and University Hospital, 2200 Copenhagen N;*
[2]*Department of Clinical Immunology, H:S Blood Bank, 2034, Copenhagen University Hospital, DK-2100 Ø Copenhagen, Denmark*

Abstract. *The SR/CR mouse model of cancer resistance was fortuitously discovered in 1999. It is resistant to a number of different cancer cell lines and the heritable phenotype was demonstrated on different genetic backgrounds. The cancer resistance is transferable to other strains of mice by adoptive transfer of innate immune cells. We independently, for the first time, confirm the findings of the SR/CR phenotype of cancer resistance to the S180 cell line in mice of two different genetic backgrounds: BALB/c and C57BL/6. The SR/CR mice were screened by intraperitoneal injection of S180 cells. The frequency of the SR/CR phenotype in the present study was 30% for the BALB/c strain and 22% for the C57BL/6 strain in the first litters, but the overall frequency was 8% for both strains. A frequency of about 30% was reported in the original US colony. A litter seriation effect on the frequency of the SR/CR phenotype was recorded. The phenotype frequency in the first-born litters was similar to that recorded in the founder colony in the US. There was no significant difference in the frequency of the SR/CR phenotype between the two genders, but the overall frequency of the SR/CR phenotype was significantly higher in litters from SR/CR mice on a BALB/c background compared to litters from SR/CR mice on a C57BL/6 background.*

Cancer is a frequent disease in developed countries and has a high mortality rate, especially when multiple metastases have been established. It is well recognised that the immune system plays a role in immunosurveillance of carcinogenic development as suggested by Burnet more than 50 years ago (3). In selected haematological patients, allogeneic bone marrow transplantation has had success as a curative treatment in humans (1). The current concept is that T-lymphocytes tag minor histocompatibility antigens, alternatively "tumour-specific" antigens for destruction of tumour cells (7).

In the SR/CR (spontaneous regression/complete resistance) mouse model, fortuitously discovered in 1999 by Cui and co-workers (4, 5), cells of the innate immune system have been shown to mediate cancer resistance. Originally, a single mouse unexpectedly survived administration of high numbers of carcinogenic S180 cells that are normally lethal to mice of all strains tested. This survival trait was successfully established on a BALB/c background as well as on a C57BL/6 background, and the trait named SR/CR. Significant proportions of mice with the SR/CR phenotype have been shown to be resistant to various tumour cell lines which cause cancer in other mouse strains (4).

Cells of the SR/CR mouse specifically target and kill injected cancer cells and thereby prevent cancer development without harming normal host cells. The lifespan of the SR/CR mouse is normal and there are no signs of autoimmunity.

The genetic basis of the SR/CR phenotype is unknown but the phenotype is heritable reminiscent of a mendelian trait.

Intriguingly, the response to injected cancer cells varies with age and older SR/CR mice, when injected with cancer cells for the first time, do develop cancer that regresses completely after two to three weeks. Very old mice completely lose resistance to cancer cells, unless they have been challenged at a younger age (4).

By several methods, including adoptive cell transfer to wild type mice, the tumour resistance of the SR/CR mouse has been shown to be mediated by three innate immune cell populations *i.e.* neutrophils, NK cells and macrophages. Established tumours in wild type-mice regress following transfer of these three cell types from SR/CR mice (8).

Correspondence to: Klaus Rieneck, Department of Clinical Immunology, H:S Blood Bank, 2034, Copenhagen University Hospital, Blegdamsvej 9, DK-2100 Ø Copenhagen, Denmark. e-mail: rieneck@dadlnet.dk

Key Words: Cancer-resistant phenotype, SR/CR, litter seriation.

Mice of the Prunt inbred strain subjected to chemically induced fibrosarcomas exhibited a marked difference in survival time correlated with litter seriation *i.e.* whether they originated from early or late litters, and litter seriation variation in response to induced cancer has been demonstrated for other cancer types as well (2, 11, 12, 13). Stevens demonstrated a two-fold higher frequency of testicular teratomas in mice in second and later litters compared to the frequency in the first litter (11).

The aims of the present study were: i) to analyse the frequency of the SR/CR phenotype in the offspring from translocated SR/CR mice in an independent laboratory, ii) to analyse the variation in the frequency of the SR/CR phenotype between offspring from SR/CR mice on a BALB/c and a C57BL/6 backgrounds, and iii) to examine whether the phenotype exhibited seriation variation, *i.e.* varied between successive litters.

Materials and Methods

Animals, housing and husbandry. The founder mice (four males on BALB/c background and four males on C57BL/6 background) were imported from Dr. Zheng Cui's laboratory (Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, NC, USA). At arrival they were 6 months old. Two weeks after the arrival of the SR/CR mice at the facility in Copenhagen, Denmark, the mice were injected with 5×10^6 S180 cells intraperitoneally (*i.p.*) and weighed two times a week the following months. None of the eight male SR/CR mice gained weight or showed signs of ascites development within this period.

Each of the SR/CR mice was housed in breeding trios with 7 to 8-weeks-old standard female BALB/c mice (Charles River, Massachusetts, USA) for the SR/CR mice on BALB/c background, and 7 to 8-weeks-old standard female C57BL/6 mice (Taconic, Ejby, Denmark) for the SR/CR mice on C57BL/6 background. The breeding system applied was continuous, which means that the same females were housed with the male mice continuously, and when litters were 3-4 weeks old they were weaned, removed from the breeding cages and housed in single sex groups (n=8).

All mice were housed in IVC racks (Techniplast, Varese, Italy) in ventilated polycarbonate type III cages (Techniplast). They were housed under standard conditions: 12 hour artificial light-dark cycles, temperature was maintained at 21±1˚C, and the relative humidity was 30-60%. The bedding material was Aspen chips (Tapvei, Oy, Kortteinen, Finland) and shredded cardboard and cardboard hides were used as environmental enrichment. Acidified tap water and standard rodent pellet diet (Brogaarden, Gentofte, Denmark) were supplied *ad libitum*.

Preparation of S180 cells. Maintenance of the S180 cells: The S180 cells were a generous gift from Dr. Cui's laboratory and upon arrival were submitted to a MAP 18 panel test to screen for potential murine pathogenic microorganisms (Taconic, USA). In a few early experiments, the S180 cells were maintained as *in vitro* cultures in DMEM (Invitrogen, Taastrup, Denmark) with glutamax I and Hepes. Gentamycin was added to a final concentration of 50 μg/ml and 10% FCS (Invitrogen). Medium was changed every second day and cells were seeded at densities of $4\text{-}5\times10^5$ viable cells/ml. When S180 cells reached a density of $1\text{-}2\times10^6$ viable cells/ml they were replated. Cells were frozen in 10% DMSO (Sigma-Aldrich, Brøndby, Denmark) and 20% FCS at densities of about 5×10^6 cells /ml and stored in liquid nitrogen. In later experiments, S180 cells were maintained in ascitic fluid in wild-type BALB/c mice (Charles River Laboratories, Boston, USA).

Preparation of S180 cells from frozen batches. S180 cells were thawed according to a minor modification of standard protocols (current protocols in Immunology, supplement 21 A.3G.2, 1996) and washed 3 times in 30 ml sterile PBS. The cells were counted in a haemocytometer (Reichert, NY, USA) using Trypan Blue (Sigma-Aldrich, Vallensbæk, Denmark), adjusted to 100,000 cells ml in sterile PBS, kept at room temperature and used for *i.p.* injection within 2 hours. The tube containing the cells was inverted several times during aliquoting to meticulously keep the cells in suspension.

Preparation of S180 cells from ascitic fluid: Mice with ascitic fluid were euthanized by cervical dislocation and the ascitic fluid was immediately removed by aspiration with a sterile 19-G needle and a 10 ml syringe. The S180 tumour cells were counted and prepared as described above without further manipulation.

Screening of the mice for the cancer-resistant phenotype. The offspring from the SR/CR mice were injected using a 25-G needle and a 2 ml syringe with 2×10^5 S180 cells *i.p.* at 6-9 weeks of age and weighed after the injection. After injection, the mice were observed daily and weighed twice a week.

When mice showed signs of accumulation of ascitic fluid and had gained more than 5 g in weight they were euthanized by cervical dislocation. The autopsy findings were haemorrhagic ascitic fluid and at microscopy S180 cells were observed in large numbers.

Three of the injection sessions with 2×10^5 S180 cells were performed with thawed *in vitro* cultured S180 cells and these injections are marked in Tables I and II. All the other injections of S180 cells were performed with S180 cells obtained from ascitic fluid.

Some of the injections were performed on anaesthetized mice and this is noted in the tables. The anaesthesia was a Hypnorm (10 mg/ml) (VetaPharma Ltd, Leeds, UK) and Midazolam (5 mg/ml) (Hameln pharmaceuticals, Hameln, Germany) mixture administered subcutaneously.

Statistics. The results of the screenings of the litters from the SR/CR mice for the SR/CR phenotype were analysed by the Chi-square test.

Ethics. The animal experiments and the laboratory animal facilities were licensed by the Danish authorities. The animals were inspected daily by educated laboratory animal technicians.

Results

Cells. The S180 cell line tested negative for all unwanted murine vira and Mycoplasma in the MAP 18 panel.

Frequency of the SR/CR phenotype. The pups from the matings of the SR/CR BALB/c male mice with standard BALB/c female mice were analysed for the SR/CR phenotype. The overall frequency of the SR/CR phenotype in the offspring was 10/73 (13.7%) (Table I).

Table I. *In the first pooled litter, litter 1, all the first litters produced by the matings of the imported BALB/c male SR/CR mice with standard BALB/c female mice were tested for the SR/CR phenotype by i.p. injections of S180 cells. Same procedure was repeated with the next pooled litters from the SR/CR mice and standard BALB/c female mice.*

Pooled litters screened for the SR/CR phenotype	Number of mice tested	Age in weeks at injection of 2×10^5 S180 cells	Number (percentage) of mice surviving 2×10^5 S180 cells
1	27*	8	8 (30%)
2	13*^	9	0 (0%)
3	7	6	1 (14%)
4	10	6	0 (0%)
5	16	6	1 (6%)

*These mice were injected with thawed S180 cells. ^These mice were anaesthetized prior to injection of S180 cells.

Table II. *In the first pooled litter, litter 1, all the first litters produced by the matings of the imported C57BL/6 male SR/CR mice with standard C57BL/6 female mice were tested for the SR/CR phenotype by i.p. injections of S180 cells. Same procedure was repeated with the next pooled litters from the SR/CR mice and standard C57BL/6 female mice.*

Pooled litters screened for the SR/CR phenotype	Number of mice tested	Age in weeks at injection of 2×10^5 S180 cells	Number (percentage) of mice surviving 2×10^5 S180 cells
1	18*^	6	4 (22%)
2	9^	6	0 (0%)
3	19	6	0 (0%)
4	29	7	1 (3%)
5	31	8	0 (0%)

*These mice were injected with thawed S180 cells. ^These mice were anaesthetized prior to injection of S180 cells.

The pups from the matings of the SR/CR C57BL/6 male mice with standard C57BL/6 female mice were analyzed for the SR/CR phenotype. The overall frequency of the SR/CR phenotype in the offspring was 5/106 (4.7%) (Table II).

In total, the number of SR/CR mice regardless of background strain surviving the injection of 2×10^5 S180 cells was 15/179 (8.4%).

The Chi-square test showed a significant difference ($p<0.001$) when comparing the present overall frequency of the SR/CR phenotype of 8.4% with the rate of 30% reported by Cui *et al*. (4).

Frequency of the SR/CR phenotype between BALB/c and C57BL/6 mice. When comparing the frequency of mice exhibiting the SR/CR phenotype in the litters from the SR/CR mice on BALB/c (13.7%) and C57BL/6 (4.7%) backgrounds, the Chi-square test showed a significant difference ($p<0.05$).

Frequency of the SR/CR phenotype among the different successive litters in each strain. When comparing the frequency of SR/CR phenotype mice in the first two screenings of the BALB/c litters, the Chi-square test showed a significant difference ($p<0.01$) between the frequencies of SR/CR phenotype in these first two successive litters.

Comparing the SR/CR phenotype frequency of 30% in the first litter of the BALB/c mice with the frequency of the phenotype in all the subsequent BALB/c litters which was 2/46 (4.3%), the Chi-square test also showed a significant difference ($p<0.01$) between these frequencies.

When the first two litters of the C57BL/6 SR/CR mice were compared in the same way, there was no significant difference between the numbers of SR/CR phenotype mice in these first two successive litters.

Comparing the frequency of the SR/CR phenotype of 22% in the first litter of the C57BL/6 mice with the frequency of the phenotype in all the subsequent C57BL/6 litters which was 1/88 (1.1%), the Chi-square test showed a significant difference ($p<0.001$) between these frequencies.

Gender frequency. There was no difference in the frequency of the phenotype between male and female pups of the two strains examined. In both tables, approximately the same number of male and female pups was screened for the SR/CR phenotype. For both BALB/c and C57BL/6, 40% of the mice with the SR/CR phenotype were females and 60% were males.

Discussion

This is the first report on the frequency of the SR/CR phenotype in a colony outside the U.S. The overall frequency of the SR/CR phenotype in the present study was 8.4%, which is significantly lower than that in the US colony (30%).

A significant seriation impact was observed on the frequency of the SR/CR phenotype in pups from the first litters compared to the later litters regardless of the genetic background. If testing for the phenotype was restricted to the offspring of the very first litters produced, the frequency of the SR/CR phenotype would be 20%-30%, which is in good agreement with the performance of the US colony.

A possible explanation for the litter seriation phenomenon is that there may be a negative correlation between the time span from the male's injection with the S180 cells and the frequency of the offspring exhibiting the phenotype. If this hypothesis is true, one explanation could be that the resistance is transferred by viral material in the SR/CR male mice. When the male mice have been inoculated with S180 cells, the viral material may be stimulated to replicate in the male resulting in its presence in the ejaculate. Entering the female, it may eventually have been transferred transplacentally to the foetuses/pups. The amount of viral material being transferred

from the male to the female through mating may decrease with time after inoculation of the cell preparation, and less and less of this material may be transferred to subsequent litters. The phenotype would thus be dependent on exposure to S180 cells in order to penetrate. This notion is easy to test experimentally.

The observed seriation effect in the SR/CR phenotype frequency is difficult to reconcile with a genomic mutation.

A change in the phenotype frequency following translocation to another facility is in accordance with the findings by many independent groups, who have demonstrated that a phenotype and its frequency often change when a model is translocated to a new facility (6).

It can be surmised that an epigenetic phenomenon could be responsible for the phenotype. Recently, a mode of epigenetic inheritance dependent on zygotic transfer of RNA molecules has been described (10) and regulatory RNAs may be involved in the SR/CR phenotype.

We have confirmed the SR/CR phenotype in offspring from male SR/CR mice but not from female SR/CR mice. However, the two genders among the offspring show the resistant phenotype at a similar frequency and there is *a priori* no reason to presume that one gender more efficiently transfers the phenotype.

In the present study, there was a significant difference in the frequency of the SR/CR phenotype between SR/CR mice on a BALB/c background and a C57BL/6 background, indicating that the frequency of the SR/CR phenotype was higher on the BALB/c background. We have at present no reason to believe that the higher frequency of the SR/CR phenotype in BALB/c mice should be due to a biological difference between BALB/c and C57BL/6 mice. With the rather small number of mice tested, it cannot be ruled out that there is no a true difference, however, testing this will require a larger number of mice.

There was no difference in the frequency of the SR/CR phenotype expressed after administration of either thawed *in vitro* cultured S180 cells or S180 cells obtained from ascitic fluid (See Tables I and II). It may be speculated that S180 cells maintained in ascitic fluid could better retain the malignant phenotype than thawed *in vitro* cultured cells, since they have been stimulated by *in vivo* factors just prior to injection. This could result in a lower frequency of the SR/CR phenotype when injecting with S180 cells from ascitic fluid. However, when comparing the frequencies of the SR/CR phenotype between the first two pooled litters in Table I, the Chi-square test shows a significant difference, though both groups were injected with thawed *in vitro* cultured S180 cells. This difference in the frequency of the SR/CR phenotype is more likely a result of the seriation effect between the litters.

It could be speculated that injection of S180 cells more reliably induces ascites when the mice are anaesthetized as the S180 cells in anaesthetized mice would be more evenly distributed in the abdominal cavity and leakage minimized. We had no problems detecting the mice without the SR/CR phenotype and did not observe leakage from the injection site in the abdominal musculature after *i.p.* injection of the S180 cells.

When looking at a possible connection between the age of the litters at the first injection with S180 cells and the frequency of the SR/CR phenotype, no obvious correlation was found, as both litters older and younger than the group with the highest frequency of the SR/CR phenotype showed lower frequency of the SR/CR phenotype.

This mouse model gives an insight into the interplay between cancer and the innate immune system. Through more detailed immunological and genetic examination of this mouse model, the molecular basis of the SR/CR phenotype may be unravelled.

In conclusion, we have confirmed the resistance of the SR/CR mouse to challenge with S180 cells in two genetic backgrounds and observed a seriation effect that warrants further studies.

Acknowledgements

The SR/CR mice as well as the S180 cell line were generously donated by Dr. Zheng Cui, Wake Forest University, NC, USA.

We thank Jonathan Adams at Wake Forest University for his kind help with the shipment of the SR/CR mice to our animal facility and the demonstration of the different laboratory techniques.

This work was kindly supported by Aase og Ejnar Danielsens Fond, the Beckett Fond and the Dagmar Marshall Fond.

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Received March 6, 2008
Revised May 14, 2008
Accepted May 21, 2008