DNA REPLICATION PATTERNS OF CANINE CHROMOSOMES IN VIVO AND IN VITRO

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I. INTRODUCTION

THE X chromosomes of man (GILBERT *et al.*, 1962) are, in the female, asynchronous in their DNA replication patterns. The same applies to other mammals such as the mouse (GALTON and HOLT, 1964 b), marsupial (WALEN, 1963) Chinese and Syrian hamsters (TAYLOR, 1962; GALTON and HOLT, 1964 a) domestic cow (MUKHERJEE and SINHA, 1963; GARTLER and BURT, 1964) donkey, horse and their hybrid, the mule (MUKHERJEE and SINHA, 1964) and a donkey-zebra hybrid (BENIRSCHKE *et al.*, 1964). This peculiar behaviour of the female sex chromosomes was discovered by means of autoradiographic studies of dispersed cells cultured in vitro. In this system, if the cells are in contact with a labelled DNA-precursor, tritiated thymidine, during the end of their synthetic period, at the subsequent metaphase it is found that one of the two X-chromosomes is synthesizing DNA while the other X and the autosomes have completed or are near to completing synthesis.

On the basis of genetical evidence in the mouse, LYON (1961, 1962) postulated that one of the two X-chromosomes of the normal mammalian female becomes genetically inactivated at an early stage of embryonic development and that this is either the paternal or maternal X in different cells of the same individual. The hypotesis was inspired by the cytological observation (OHNO and MAKINO, 1961) that one of the X-chromosomes of the female cell is in a condensed state (heteropycnotic) at prophase and forms the sex chromatin or Barr body of the

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female inter-phase nucleus. It is now evident that it is the late-replicating X-chromosome which forms the Barr body. The Lyon's hypothesis has been tested both cytologically and genetically in man (review in HARRIS *et al.*, 1963) and the mouse (OHNO and CATTANACH, 1962; RUSSEL, 1963) with results which are still partially controversial.

The study of the DNA duplication patterns of the X-chromosomes in normal individuals has so far been performed using *in vitro* systems. In this paper we report on such a study made on a female dog *in vivo* parallel with similar investigations *in vitro*.

Materials and methods

One female and one male Dalmatian dogs and a female of mixed origin were used. Sex chromatin was investigated in buccal mucosa smears by the method of KLINGER and LUDWIG (1957) and in cells cultured in vitro by the method of FRACCARO and LINDSTEN (1959). Drumsticks were studied in blood smears stained with May-Grünwald-Giemsa. Peripheral blood cultures were prepared and processed according to a modification of the method of MOORHEAD *et al.* (1960). Cultures of cells from solid tissues were made by a modification of the method of HSU and KELLOGG (1960).

Labelling of cells *in vivo* was performed in a female dog of 30 days of age, weighing 1460 g. Three millicuries of tritiated thymidine (Amersham, specific activity 14.8 c/mM) suspended in 6 ml of physiological saline were injected intravenously. After one hour Colcemide was injected at a dose of 0.1 mg/Kg of body weight. The first bone-marrow specimen was obtained by tibial puncture after three hours. The animal was killed after 4 hours and bone-marrow was obtained by flushing the diaphyses of the removed femurs with 95 % tri-sodium citrate. The cells were then incubated in the hypotonic solution for 20 min. at 37° C, centrifuged and fixed in a 3:1 mixture of absolute ethyl alcohol and glacial acetic acid. Cytological preparations were then prepared by the usual air-drying method. The slides were coated with AR 10 autoradiographic stripping film and exposed for 20 days and subsequently stained through the film with buffered Giemsa. For identification of individual chromosomes the grains were removed and bleached by a modification of the method of A. ROSS (Human Chromosome Newletter, n. 10, 1963).

Labelling of peripheral blood cells *in vitro* was performed by adding 0.28 μc of tritiated thymidine per ml of culture medium at 68 hours of culture. The cells were harvested at 71 and 72 hours and preparations

made and processed as described above. Exposure time was in this case 7 days.

Labelling of inter-phase nuclei was performed by adding 2 μc/ml of tritiated thymidine (specific activity 4.2 c/mM) to cultures of kidney and ovary cells growing on coverslips. After 30 min. the coverslips were removed from the culture vessels, fixed and stained with Feulgen, mounted with the cell layer upward on to microscope slides by mean of Euparal and exposed for 7 days after application of the stripping films.

II. RESULTS

1. Sex dimorphism in inter-phase nuclei

Nuclei of female oral mucosa were sex chromatin positive (Fig. 1 b) in a proportion which does not differ from the standard findings in the equivalent human material. Since the proportion of sex chromatin positive cells of oral mucosa are known to vary considerably according to the quality of smears and other technical factors we did not attempt to quantitize our findings. Female cells cultured in vitro from ovary and kidney explants were also found to be sex chromatin positive (Fig. 1 d).

Drumsticks were found in the neutrophil polymorphs of the female (Fig. 1 a). A count gave a finding of 13 drumsticks in the first 300 cells examined. No Barr bodies or drumsticks were observed in the male cells.

2. Somatic chromosomes

Consistent counts of 78 chromosomes were obtained in the tissues of both sexes. The karyotype of the dog has recently been studied in detail by one of us (GUSTAVSSON, 1964) and the present findings are in full agreement with the previous description. The female karyotype is characterized by the two X-chromosomes which are the only sub-metacentric in a complement of acrocentric chromosomes.

In most metaphase plates the two X-chromosomes differ in size, this difference often being immediately obvious as in Fig. 2. Chromosome measurements are in progress in order to detect eventual differences in the centromeric indexes of the two X-chromosomes. The Y chromosome of the male is the smallest of the complement and also sub-metacentric. Among the autosomes, one pair is conspicuous because of its length and easily recognizable (Fig. 2 a—d).

3. **Autoradiographic studies**

A. *Patterns of chromosome replication*

In a proportion of the bone-marrow cells which have been in contact with the tritiated thymidine in the living animal one of the two X-chromosomes was found to be heavily labelled in comparison with the rest of the complement (Fig. 3).

The same late-duplicating X has been clearly observed in a proportion of the blood cells labelled in vitro (Fig. 4). No late-duplicating X has been observed in male blood cells. As is also found in other types of material, the amount of grains on the late-duplicating "hot" X relative to the amount on the other chromosomes, varies considerably as a consequence of the differential contact with the labelled precursors of cells in different phases of their synthetic period.

No immediately obvious difference was found in the patterns of chromosome replication between the cells labelled in vivo and those labelled in vitro. We have the impression that in the bone-marrow labelled in vivo there are less cells with a late replicating X than in the blood in vitro. This impression should however be supported by a quantitative evaluation of the findings which is in progress. Quantitative analysis is also needed to confirm the impression that the majority of the "hot" X's in both types of cells are located at the periphery of the metaphase plate.

Analysis of the replication patterns of the autosomes is necessarily imprecise because it is impossible to individualize the homologues. We have noticed however, that among the shortest pairs there are two chromosomes which complete synthesis early while two other chromosomes in the same length range are late replicating. This indicates that patterns of synthesis are independent from chromosome length.

B. *Inter-phase nuclei*

As expected, labelling of inter-phase female nuclei gave a heterogenous picture. In all the preparations there were all gradients of labelling patterns, ranging from un-labelled nuclei to nuclei covered with grains. A large proportion of labelled nuclei showed a concentration of grain in correspondence to the chromatin massess of all dimensions. In several nuclei there was a particularly "hot" zone, in most instances located at the periphery of the nucleus. These were interpreted as labelled Barr bodies (Fig. 1 c).

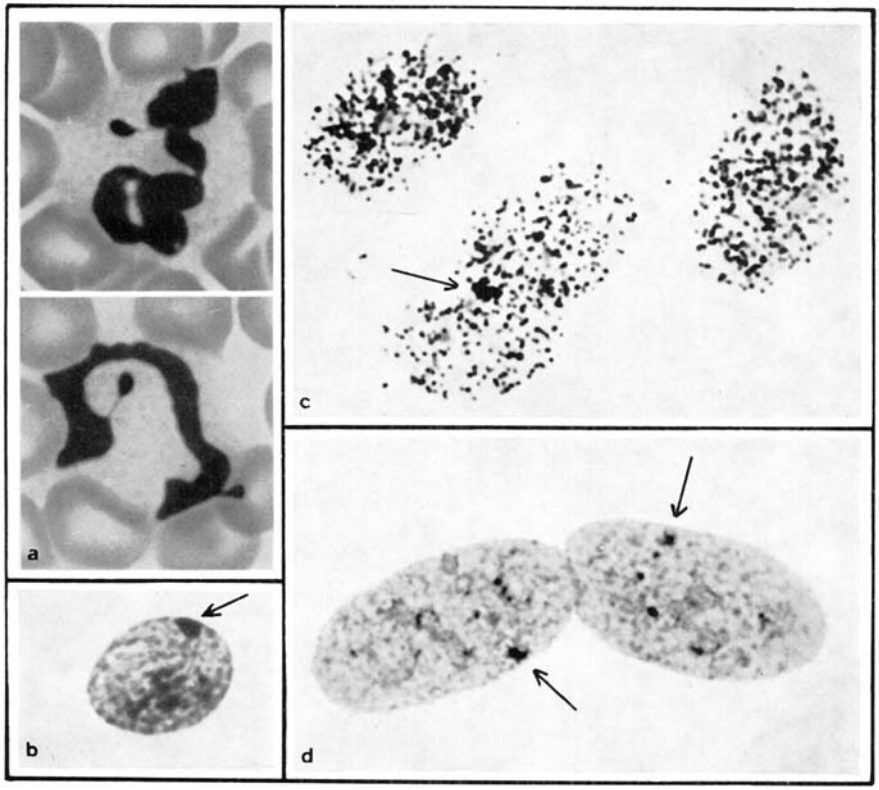

Fig. 1. Female dog. a) drumsticks (Reproduced at 2400×); b) Barr body (arrow) in an oral mucosa nucleus; c) tritiated thymidine labelled nuclei of kidney cells cultured in vitro. Arrow indicates heavily labelled Barr body. Reproduced at 1200×; d) sex chromatin (arrows) in nuclei from cultures of ovaries. Feulgen. Reproduced at 1200×.

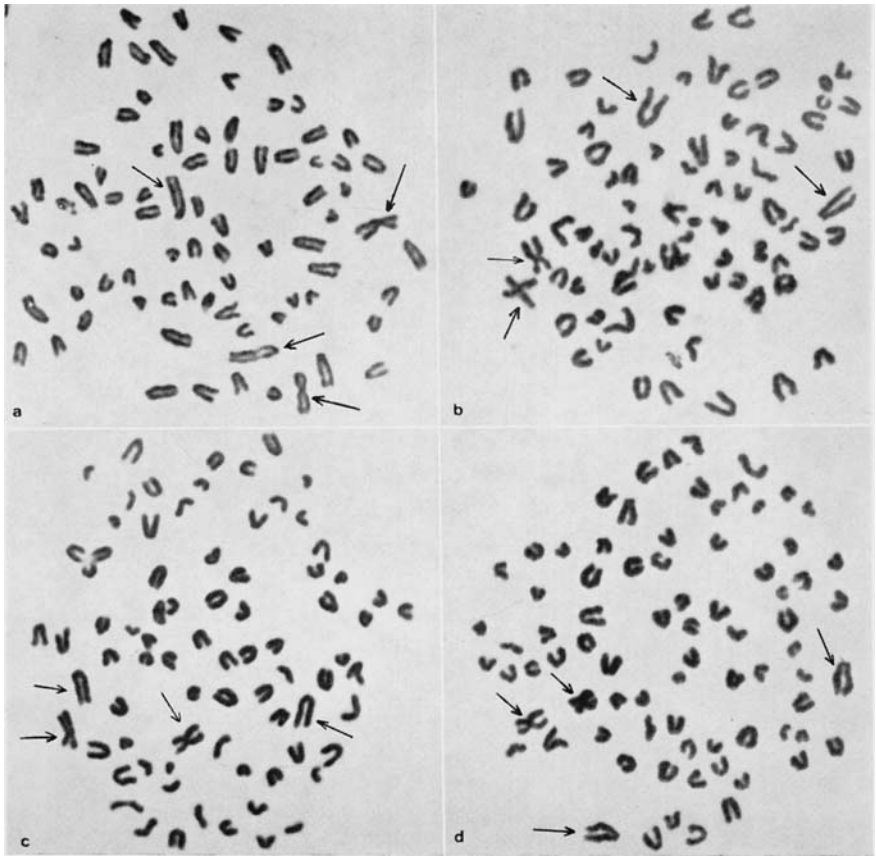

Fig. 2. Four metaphase plates of female dog lymphocytes cultured *in vitro*. Arrows indicate the two sub-metacentric X-chromosomes and the two characteristic autosomes of the longest pair. Acetic-orcein. Phase contrast. Reproduced at 1200×.

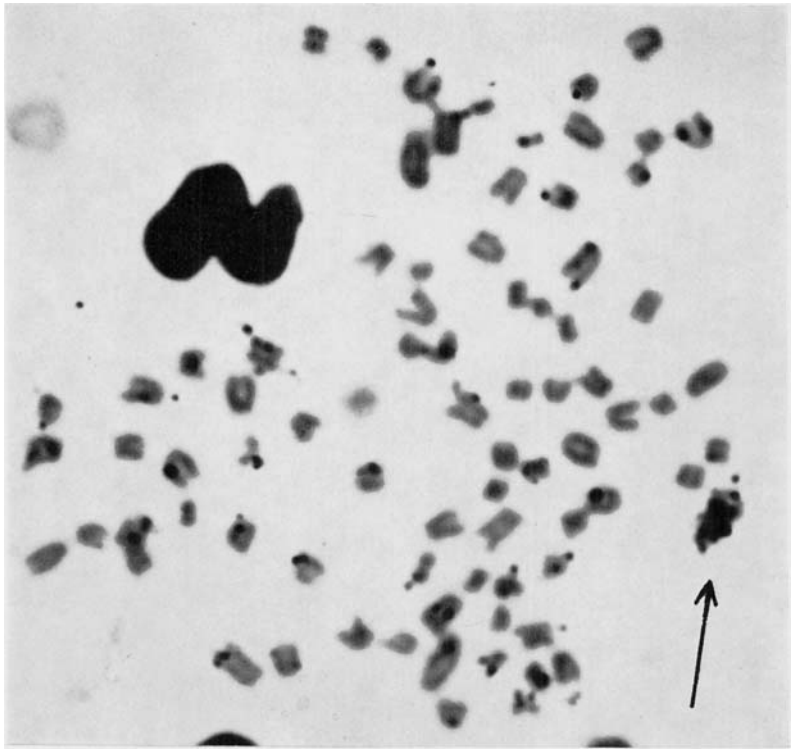

Fig. 3. Autoradiography of a bone-marrow cell exposed to tritiated thymidine *in vivo*. Arrow indicates a heavily labelled X-chromosome. Giemsa stain. Reproduced at 2000×.

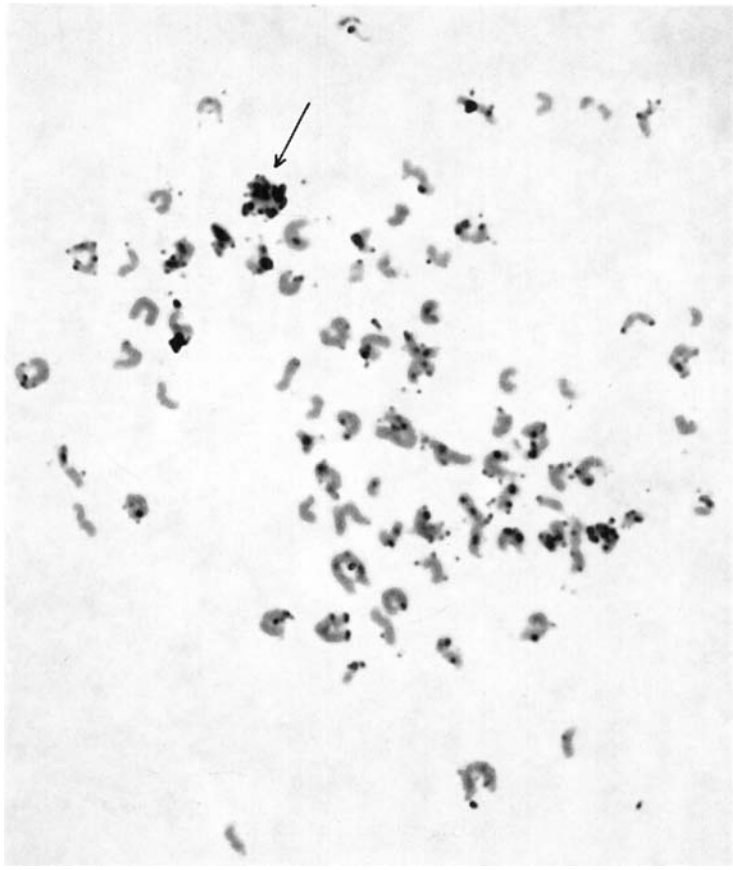

Fig. 4. Autoradiography of a blood cell exposed to tritiated thymidine *in vitro*. Arrow indicates a heavily labelled X-chromosome. Faint Giemsa stain. Reproduced at 1900×.

III. CONCLUSIONS

With this investigation we provide evidence that the asynchronous replication pattern of one of the two X-chromosomes so far observed *in vitro* in several mammalian species is also present *in vivo*. At the same time we add the dog to the list of animals in which DNA replication patterns have been investigated at the chromosomal level. We did not find any obvious difference between the replication patterns *in vivo* and *in vitro*, with the possible exception of a smaller proportion of cells with a late replicating X in the bone-marrow. This is probably due to a difference in the time for which the cells *in vivo* are in contact with the labelled precursor. This variable is obviously more difficult to control in the *in vivo* experiments.

The overall picture of the behaviour of the X-chromosome in the dog is not different from the one observed in man: the female dog displays clearly visible sex chromatin bodies, drumsticks and late replicating single-X-chromosome. The great advantage is, however, that the X-chromosomes are easily identifiable on simple morphological criteria. It is, therefore, possible to observe any difference in size between the two X-chromosomes in metaphase plates. For this reasons the dog cell should prove to be a useful research tool for biological studies of the mammalian X-chromosome.

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