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THE 14TH EUROPEAN COLLOQUIUM ON CYTOGENETICS OF DOMESTIC ANIMALS

The 14th European Colloquium on Cytogenetics of Domestic Animals took place at the University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic from 27th to 30th June 2000. The organizers were the Veterinary Research Institute, the Faculty of Veterinary Medicine of the University of Veterinary and Pharmaceutical Sciences, the Cytogenetic Section of the Biological Society of the Academy of Sciences of the Czech Republic, and the J. G. Mendel Museum.

The first European Colloquiums on Cytogenetics of Domestic Animals were organized by Professor G. W. Rieck in 1970 and 1975 in Giessen. Since 1980 the colloquiums have been organized biannualy in various countries. The subsequent meetings were held in Jouy-en-Josas, France, in 1977 (organizer: Dr. C. P. Popescu); in Uppsala, Sweden, in 1980 (organizer: Dr. I. Gustavsson); in Milano/Gargano, Italy, in 1982 (organizer: Dr. G. Succi); in Zürich, Switzerland, in 1984 (organizer: Dr. G. Stranzinger); in Warsaw, Poland, in 1986 (organizer: Dr. P. Sysa); in Bristol, the United Kingdom, in 1988 (organizer: Dr. S. E. Long); in Tolouse/Auzeville, France, in 1990 (organizer: Dr. G. Echard); in Utrecht, The Netherlands, in 1992 (organizer: Dr. A. A. Bosma); in Frederiksberg/Copenhagen, Denmark, in 1994 (organizer: Dr. K. Christensen); in Zaragoza, Spain, in 1996 (organizer: Dr. M. V. Arruga); and in Budapest, Hungary, in 1998 (organizer: Dr. A. Kovacs).

The final decision at the meeting was that the next colloquium would be organized by Dr. L. Iannnuzzi in Italy in 2002.

Although they are called European Colloquia, these meetings have traditionally welcomed cytogenetics from the whole world. This year's meeting was attended by participants from 20 European countries (Austria, Croatia, Czech Republic, Denmark, Finland, France, Germany, Hungary, Italy, Lithuania, Poland, Romania, Russia, Slovakia, Spain, Sweden, Switzerland, Ukraine, United Kingdom and Yugoslavia), as well as from Japan, Argentina, and Thailand.

The program of the 14th European Colloquium on Cytogenetics of Domestic Animals provides an evidence of the high scientific level of this branch. This issue contains mostly texts of oral presentations. Papers by authors who preferred publication in other journals have not been included.

The members of the organizing committee wish to thank Prof. Gerald Stranzinger of the ETH, Zurich, and the Faculty of Veterinary Medicine of the Zurich University for providing funds used for covering expenses associated with the stay of invited speakers, and conference fees of several participants who were unable to obtain a support from local sources.

The organizers appreciate the interest in this meeting and wish full success in further work to all participants.

Jiří R u b e š
Veterinary Research Institute, Brno, Czech Republic

PROGRAM OF 14TH EUROPEAN COLLOQUIUM ON CYTOGENETICS OF DOMESTIC ANIMALS

Brno, Czech Republic, June 27-30, 2000

Wednesday, 28 June

INTRODUCTION

Popescu P.: Domestic Animal Cytogenetics – Present status and future development.

Pliska V.: Links between hormones, cell signalling, and chromosomes: A non-cytogeneticist's view on cytogenetics.

MOLECULAR CYTOGENETICS I

Oral presentations

Breen M., Hitte C., Holmes N. G., Mellersh C. S, Suter N., Bristow A. E., Andre C., Boundy S., Gitsham P., Bridge W. L, Dickens H. F., Ryder E., Thomas R., Curson A., Benke K., Vignaux F., Jouquand S.,Sampson J., Galibert F., Ostrander E. A., Binns M. M.: Development of chromosome specific reagents for the dog (Canis familiaris) and their use to facilitate chromosome identification and to anchor integrated linkage and radiation hybrid maps of the dog genome.

Hayes H., Di Meo G. P., Gautier M., Laurent P., Eggen A., Iannuzzi L.: Localisation by FISH of the 31 Texas nomenclature type I markers to both Q- and R-banded bovine chromosomes.

Brunner R., M., Seyfert H. M., Goldammer T., Schwerin M.: Genomic organisation of the STAT5 locus as revealed by fiber-FISH.

Posters

Pinton A., Ducos A., Berland H., Séguéla A., Brun-Baronnat C., Darré A., Darré R., Pinton P., Yerle M.: Characterisation of chromosomal rearrangements in pigs using microdissection and FISH.

Kubíčková S., Černohorská H., Řezáčová O., Rubeš J.: Laser microdissection and laser pressure catapulting for the generation of bovine chromosome-specific paint probes.

Antonacci R., De Iaco R., Massari S., Ciccarese S.: FISH-mapping of TCRB locus in ruminants.

CLINICAL CYTOGENETICS

Oral presentations

Iannuzzi L., Di Meo G. P., Perucatti A., Zicarelli L.: Clinical cytogenetics in river buffalo (*Bubalus bubalis*): sex chromosome anomalies and reproductive disturbances.

Stranzinger G. F.: Some rare cytogenetic findings in our research and routine work.

Mäkinen A., Katila T., Gustavsson I., Hasegawa T.: Some practical approach to horse cytogenetic studies.

Posters

Suwattana D., Koykul W., Chovanaikul V., Joerg H., Stranzinger G.: The 2/7 Robertsonian translocation and telomere FISH in Fea's muntjac *(Muntiacus feae).*

Di Meo G. P., Molteni L., Perucatti A., De Giovanni A., Incarnato D., Succi G., Schibler L., Cribiu E. P., Iannuzzi L.: Chromosomal characterisation of three Robertsonian translocations in cattle by using G-, R- and C-banding and FISH-technique.

Ducos A., Berland A., Pinton A., Séguéla A., Brun-Baronnat C., Darré A., Darré R.: Chromosomal control of domestic animal populations in France.

Di Meo G. P., Perucatti A., Ferrara L., Iannuzzi L.: A pericentric inversion in cattle Y chromosome.

Hanada H., Awata H., Hodate K., Takeda K., Onishi A., Takahashi M.: Mosaic trisomy in a Holstein cow with dwarf phenotype.

Christensen K., Skúli P.: A case of trisomy 22 in a live Hereford heifer.

Kosarcic S., Kovacs A., Kosarcic D., Kovacevic M., Karakas P., Nemes Z., Marton F., Bodo I.: Cattle chromosome investigations in Yugoslavia.

Arruga M. V., Monteagudo L. V.: A case of XX/XY cell populations in sheep.

Pawlak M., Rogalska-Niznik N., Cholewinski G., Switonski M.: 64,XX/63,X0 mosaicism in four sterile mares.

Switonski M., Slota E., Pietrzak A., Klukowska J.: Chimerism 78,XX/77,XX,Rb in a bitch, revealed by chromosome and microsatellites studies.

Anistoroaei R.: XX sex reversal in a pig.

Tiranti I. N., Genghini R., Guarda I., Gonzáles Q. H., Wittouck P.: Chromosomal and morphological studies of pig intersexes.

MEIOTIC STUDIES – CYTOGENETICS OF GERM CELLS AND EMBRYOS

Oral presentations

Switonski M.: Synaptonemal complexes – its molecular nature and appearance in spermatocytes carrying chromosome abnormalities.

Rejduch B., Slota E., Sysa P., Kwaczyńska A.: Analysis of synaptonemal complexes in the bulls and rams, carriers of XY/XX chimerism.

Sosnowski J., Waroczyk M., Switonski M.: Aneuploidy in pig oocytes matured *in vitro*.

Rubeš J., Vozdová M., Machatková M., Kubíčková S., Zudová D., Jokešová E.: Aneuploidy in porcine oocytes matured *in vitro*: multi-color fluorescence *in situ* hybridization using probes for chromosomes 1 and 10.

Poster

Révay T., Tardy E. P., Tóth A., Kovács A., Salgó A.: Sexing bovine cells by FISH with a synthetic Y probe.

CHROMOSOME NOMENCLATURE

Iannuzzi L. (co-ordinator): "*International System for Chromosome Nomenclatures of Domestic Bovids (ISCN-DB), 2000*".

Thursday, 29 June

COMPARATIVE CYTOGENETICS

Oral presentations

Chowdhary B. P., Raudsepp T.: From chromosomes to Comparative Genomics: cytogenetics sets the pace.

Fillon V., Vignoles M., Crooijmans R. P. M. A., Seguela A., Gellin J., Vignal A.: Avian Comparative Cytogenetics by FISH.

Iannuzzi L., Di Meo G. P., Perucatti A., Schibler L., Incarnato D., Ferrara L., Cribiu E. P.: Seventy-seven type I loci were comparatively FISH-mapped in river buffalo and sheep R-banded chromosomes.

Yang F., Graphodatsky A. S., O'Brien P. C. M., Milne B. S., Squire M., Trifonov V., Solanky N., Perelman P., Colabella M., Serdukova N., Rens W., Kawada S. I., Sargan D. R., Ferguson-Smith M. A.: Comparative maps of the dog, red fox, Arctic fox, raccoon dog, American mink, cat, and human built by chromosome painting and their phylogenetic implications.

Rogalska-Niznik N., Schelling C., Dolf G., Schlapfer J., Pienkowska A., Switonski M.: Localisation of three canine microsatellites on the blue fox *(Alopex lagopus)* chromosomes.

Posters

Thomas R., Breen M., Holmes N. G., Binns M. M.: An integrated physical, radiation-hybrid and comparative map of chromosome five of the domestic dog *(Canis familiaris)*.

Pinton P., Schibler L., Y., Lahbib-Mansais Cribiu E., Gellin J., Yerle M.: FISH mapping of 113 new anchor loci in pigs

CANCER CYTOGENETICS

Oral presentations

Michalová K., Zemanová Z.: Chromosome abnormalities in human cancer.

Mayr, B.: Cytogenetic and tumor suppressor gene studies on feline soft tissue tumors.

Poster

Milne B., Thomas R., Hoather T., O'Brien, P., Stiduorthy M., Dunn K., Benke K., Desson J., Fergusson-Smith M., Breen M., Sargan D.: Comprehensive cytogenetic analysis of a canine chondrosarcoma by Fluorescence in-situ Hybridisation (FISH)

INVITATION TO PARIS 2001

Popescu P.: Invitation to 3rd European Cytogenetics Conference.

Friday, 30 June

MOLECULAR CYTOGENETICS II

Oral presentations

Haas O. A.: The expanding universe of FISH: technological aspects and practical applications.

Raudsepp T., Skow L., Womack J. E., Chowdhary B. P.: *Radiation hybrid analysis in the horse: current status.*

Christensen K.: Mink Radiation Hybrid cell panel, generation and results.

Suwatana D., Koykul W., Mahasawangkul S., Kanohanapa S., Joerg M., Stranzinger G.: The GTG -banded karyotype and telomere FISH in Asian elephants (*Elephas maximus*).

Lahbib-Mansais Y., Leroux S., Yerle M., Robic A., Gellin J.: New Anchorage Markers (TOASTs) on the Porcine Radiation Hybrid Map.

Posters

Hiendleder S., Kaupe B., Wassmuth R., Janke A.: Mitochondrial DNA phylogeny of wild sheep with different chromosome numbers.

Graphodarskaya D. A., Joerg H., Stranzinger G.: Characterisation of the MSHR gene of different cattle breeds.

Kovacs B. Zs., Stranzinger G.: X chromosom inactivation studies in the Swiss Eringer breed.

Zhdanova N., Ivanova F., Kuznetsov S., Svischeva G., Aksenovich T.: Radiation induced map of p arm of porcine chromosome 2.

Morisson M., Bocs S., Galan M., Plisson-Petit F., Pinton P., Yerle M., Vignal A.: Towards a whole genome radiation hybrid panel in Chicken.

MUTAGENESIS – CHROMOSOME INSTABILITY – ECOGENOTOXICOLOGY

Oral presentations

Slota E., Danielak-Czech B., Pietraszewska J., Kozubska-Sobocinska A.: Preliminary identification of the fragile X in two crossbreed cows.

Dianovský J., Šiviková K.: Sister-chromatid exchanges induced by metal containing emission in sheep peripheral lymphocytes after *in vivo* and *in vitro* exposure.

Kiselyova T. Yu., Lazarev N. M., Podoba E. B.: Studying chromosomal alterations in cattle after the Chernobyl accident.

Posters

Lioi M. B., Barbieri R., Santoro A., Ambrosio V., Borzacchiello G., Roperto F.: Chromosome instability in cattle grazing on bracken-infested lands in South of Italy.

Jaszczak K., Parada R., Dziedzic R.: Cytogenetic monitoring of several hare populations.

Miceikiene I., Kucinskiene J.: Influence of biological and ecological factors to the instability of cattle genome.

Genghini R., Tiranti I., Wittouck P., Carbó L.: Genotoxicity of vaccine against classic swine fever in pig farms of Argentina.

Lioi M. B., Santoro A., Barbieri R., Lioi M., Zeni O., Scarfì M. R.: Cytogenetic damage induced by ochratoxin A in bovine lymphocyte cultures *in vitro*.

Muramatsu M., Miyazaki H., Muramatsu S., Yoshizawa M., Fukui E., Utsumi S.: Mitomycin C-induced sister chromatid exchanges in equine lymphocytes.

Šiviková K., Dianovský J.: A decrease of sister chromatid exchanges induced by carbon tetrachloride in cultured sheep peripheral lymphocytes after vitamin E and selenium supplementation.

GENOMIC ORGANIZATION OF THE STAT5 LOCUS AS REVEALED BY FIBER-FISH

R. M. Brunner, H.-M. Seyfert, T. Goldammer, M. Schwerin

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ABSTRACT: The STAT-transcription factors form a family of signal transducers and activators of transcription mediating the action of a variety of peptide hormones and cytokines *via* the Janus-type kinases. At least seven distinct STAT-encoding genes have been identified, STAT1-6. STAT5 being encoded by two genes, known as STAT5A and STAT5B. STAT factors arose from a common ancesteral gene by repeated gene duplication events. We had detected many copies of pseudogenes (9 from among 14 isolates) during isolation of the bovine genes. Since our previous gene mappings had shown that both genes must be located in close vicinity on BTA19 we used fiber FISH to verify true overlap of new isolates with proven STAT5A or B gene isolates. However, extended areas (several kbp) of the isolates revealed identical restriction maps and sequences with previously identified authentic gene isolates, but grossly diverging restriction maps in other segements. Therefore, the results of restriction mapping were proven by an independent technique, the DNA-fiber-FISH hybridizations. Indeed, the STAT5B-encoding gene resides only about 40 kbp downstream of the STAT5A-encoding gene in correspondence to restriction mapping. While it is clear that the STAT5B gene is located 3′ from the STAT5A gene, the 5′–3′orientation of the STAT5B-encoding is unclear, since we did not determine the relative position of the 5′-region of the latter gene. Thus, while the entire locus comprises about 85 kbp as revealed by these hybridizations, it might be a little larger.

STAT-transcription factors; fiber FISH; cattle

INTRODUCTION

The STAT-transcription factors form a family of signal transducers and activators of transcription mediating the action of a variety of peptide hormones and cytokines *via* the Janus-type kinases (Ihle, 1996; Darnell, 1997). Properties of these factors are the activation of the latent cytoplasmic proteins through phosphorylation of serine (Zhang et al., 1995; Wen et al., 1995) and a distinct tyrosine residue (Shuai et al., 1992; Zhong et al., 1994) upon ligand binding of the cell. The factors dimerize and translocate into the nucleus where they bind to characteristic DNA-sequence motifs (Stahl et al., 1995; Darnell, 1997). At least seven distinct STAT-encoding genes have been identified (Copeland et al., 1995), STAT1-6, with STAT5 being encoded by two genes, known as STAT5A and STAT5B. STAT factors arose from a common ancestral gene by repeated gene duplication events as evidenced by sequence similarities of their protein encoding regions. At the N-terminus a string of some 130 amino acids forms a binding domain for several factor molecules (Vinkemeyer et al., 1996; Xu et al., 1996), followed by a bundle of four α-helices (Becker et al, 1998) and the DNA-binding domain (Horvath et al., 1995; Moriggl et al., 1996).

STAT5A and STAT5B, are involved in signal transduction not only of interleukins 2, 3, 5 and 12, but also of erythropoeitin, granulocyte-macrophage-colony-stimulating factor and the peptide hormones prolactin and growth hormone (Huo, 1995; Liu, 1995; Mui, 1995; Azam et al., 1995; Pallard, 1995; ; Mui, 1996; Ofir, 1997; Gollob, 1998). Originally, it has been identified as the signal transducer of the prolactin signalling pathway (Wakao et al., 1994). Therefore, it was named as 'mammary-gland-factor (MGF)'. Subsequent analyses showed its homology to other known STAT factors making the MGF factor to the STAT5 prototype. In model systems, STAT5 was shown to confer the stimulating effects of prolactin for gene expression to a variety of mammary gland expressed genes including the rat β–casein, mouse Whey Acidic Protein, rabbit αs1-casein and ovine β-lactoglobulin (Groner et al., 1994; Burdon, 1994; Li and Rosen, 1995; Jolivet, 1996). For gene activation, it homo- or heterodimerizes with the closely related STAT5B factor (Kirken et al., 1997), first known from mouse and human (Huo et al., 1995; Mui et al., 1995; Lin et al., 1996). The STAT5 factors interact synergistically with the glucocorticoid hormone (Stöcklin et al., 1996) receptor and the insulin receptor (Chen et al., 1997), focusing the signals of these hormones to promoters harboring STAT5-binding sites.

Our previous gene mappings had shown that both genes must be located in close vicinity on BTA19 (Goldammer, 1997). However, we had detected many copies of pseudogenes (9 from among 14 isolates) during isolation of the bovine genes. Since extended areas (several kbp) of such isolates eventually revealed identical restriction maps and sequences with previously identified authentic gene isolates, but grossly diverging restriction maps in other segements, it was necessary to verify true overlap of isolates.

We applied the technique of hybridization to mechanically stretched DNA fibers (fiber FISH) to analyze the structure of the bovine STAT5 locus as well as to prove for overlapping clones or homologous pseudogenes.

MATERIAL AND METHODS

Probes

Three λ clones served as hybridization probes (Tab. I). The clones were labeled either by biotin-16dUTP or digoxygenin-11dUTP (Boehringer Mannheim) using the Gibco-BRL nick translation kit. Various probe combinations were used for several FISH experiments [(1) mgf18 + mgf19; (2) mgf4 + mgf18; (3) mgf4 + mgf19; (biotin label underlined)].

I. Clones used for fiber FISH exeriments, exons comprised, lengths of the λ clones insert (entirely sequenced) in comparison to the length as measured by the FISH signal. Mean ($\bar{x}$) and standard deviation (s) of n analyzed fluorescence images are presented for each probe

Probes	Exons comprised	Insert size (kb)	Size calculated from fiber measurements in kb ($\bar{x}$) ± s (n)
STAT5A			
mgf18	1–4	7.0	8.7 ± 2.3 (80)
mgf19	11–19	13.0	17.7 ± 8.3 (77)
STAT5B			
mgf4	5–10	16.0	9.9 ± 3.7 (50)

Fiber preparation

Bovine peripheral lymphocytes were isolated by standard density gradient centrifugation procedure and embedded in 1% (w/v) NuSieve low melting point agarose in PBS at about 10^7 cells per ml. The agarose blocks were digested under permanent shaking in freshly made 2% (w/v) Proteinase K in 0.5 M EDTA, pH 8.0 containing 0.25% (w/v) Na-lauroyl-sarcosine at 50 °C for 3 days. After digestion the blocks were clear, not fragmented and sank to the bottom of the tube. Tubes were cooled on ice for 10 min to harden the blocks. To proof the digestion results, 1/3 of a block was placed on one end of a poly-L-lysin coated slide, covered with 20 µl H_2O and heated on a warming plate at 80 °C for 90 s. The melted drop was then spread slowly over the slide using a second slide as drawing spatula, air dried and mounted with DABCO-antifade containing 0.2 µg/ml DAPI. If nuclei were no longer detectable, the blocks were washed 4 times in TE-buffer, pH 8.0 and covered with 40 ml TE containing 40 µl fresh made phenylmethylsulfonylfluoride (PMSF – 40% w/v in ethanol). The blocks were incubated at 50 °C for 1 h under permanent shaking, washed 4 times in TE and stored at 4 °C in TE until use. Preparation of fiber slides for hybridization was exactly the same as described above for control of digestion. Only slides with long, straight and nearly parallel fibers were used for hybridization.

The coverslips were removed by washing in 2 x SSC, and the slides were dehydrated by passing through an ethanol-series.

In situ hybridization and detection

The *in situ* hybridization protocol followed Trask (1991). Biotin labeled DNA was detected with two layers Texas-Red conjugated avidin and anti-avidin (Vector). Digoxygenin labeled probes were detected by applying 1 layer each of monoclonal mouse anti-digoxin antibody (Dianova), sheep anti-mouse-DIG antibody (Boehringer Mannheim) and sheep anti-DIG-FITC antibody (Boehringer Mannheim), respectively.

The slides were covered with DABCO-antifade containing 0.2 µg/ml DAPI and examined under an NIKON FXA epifluorescence microscope equipped with an Texas-Red/FITC double band pass filter (Chroma Technologies).

Image analysis

Image analysis and measuring of signal distances was performed with the McProbe fluorescence image analysis system (PSI). We first calculated the relative length of signal and gaps as described by Sjöberg et al. (1997) to avoid differences from the pixel shape. Afterwards, we estimated the width of the gap between the red and green signal in kb by comparison of the measured TR or FITC signal width and the known length of the probe from restriction mapping as probe size standard (PSS). To prove the accuracy of our estimation we also used to calculate the size of the TR and FITC signal from the measured with of the opposite colored signal and it's known probe length from restriction mapping, respectively.

RESULTS AND CONCLUSIONS

To assess the organisation and extent of the bovine *STAT5* locus two-colour fiber FISH experiments applying various combinations of probes were performed. Probes labeled with digoxygenine and biotin, respectively were found to hybridize equally well. All hybridizations revealed clear, distinct signals and low background. Representative fiber-FISH are shown in Colour Plate, Fig.4. In Tab. I means of measured lengths of labeled DNA fibers after FISH are summerized. During measurements of sizes of labeled DNA a variation in the degree of DNA stretching became apparent. This might reflect differences in the visualization of the dotted fiber signal ends. However, according to Nishio et al. (1996) another reason could be inhomogeneous stretching of DNA fibers. Standard deviations of these measurements were slightly higher than those obtained in previous experiments (e.g. Brunner et al., 1998).

STAT 5B was found to be separated by about 38 kb of DNA from the STAT5A as represented by the gap between clone mgf4 and mgf18. While it is clear that the STAT5B gene is located 3′ from the STAT5A gene, the 5′–3′orientation of the STAT5B-encoding is unclear, since we did not determine the relative position of the 5′-region of the latter gene. Thus, while the entire locus comprises about 85 kbp as revealed by these hybridizations, it might be a little larger. Crosshybridization of probes was barely detectable. There was no detectable signal outside the shown locus giving hints to closely related pseudogenes. Our results did not resolve the orientation of the locus with regard to telomere and centromere of BTA19. Taken together, the fiber FISH and restriction data allowed to establish a detailled physical map of the entire STAT5 locus (see Colour Plate, Fig. 4).

Acknowledgement

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THE GTG-BANDED KARYOTYPE AND TELOMERE FISH IN ASIAN ELEPHANTS (*ELEPHAS MAXIMUS*)

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ABSTRACT: Lymphocyte cultures were performed for chromosome preparations from 70 Asian elephants (*Elephas maximus*) held captive in the province of Lampang, Thailand. The elephant karyotype was demonstrated using Giemsa staining and GTG banding techniques. The diploid number ($2n$) was 56 or 28 chromosome pairs, consisting of 6 pairs of bi-armed chromosomes and the rest of 21 chromosome pairs were telocentric. The chromosomes were classified and numbered according to size, centromere position and banding patterns. Chromosome one exhibited a secondary constriction on the proximal third of the p-arm whereas chromosomes 2 to 6 showed faint bands probably due to their heterochromatic constitution. The X chromosome was medium submetacentric with distinctive bands while the Y was a small telocentric chromosome with a dark band around the centromere. An idiogram of total 216 bands was established according to the GTG-bands which will be useful for physical gene mapping. Telomeres of Asian elephants' chromosomes were localized using a fluorescein-conjugated peptide nucleic acid (PNA) probe containing a telomeric sequence (TTAGGG). Fluorescence in situ hybridization (FISH) revealed the locations of four spots of telomeres on all including the sex chromosomes. Our observations demonstrated that telomeric DNA sequences have been well conserved in the Asian elephants similar to those shown in almost all vertebrates.

Asian elephant; karyotype; telomeres; FISH

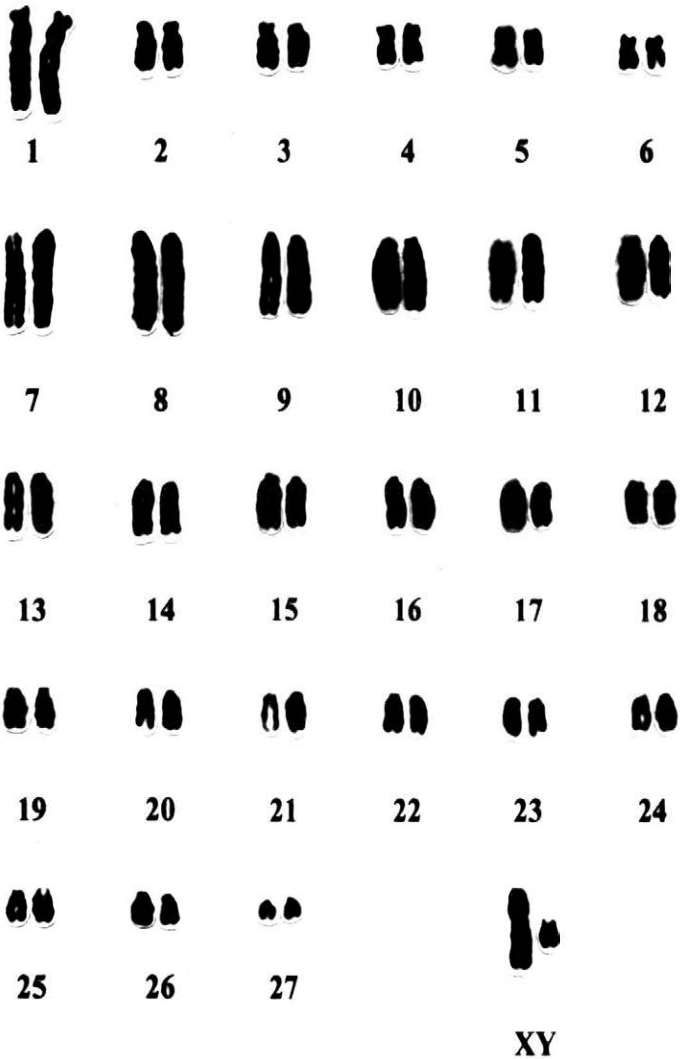
INTRODUCTION

The Asian elephant (*Elephas maximus*) once widespread in countries of Asia are morphologically and genetically different from their African counterparts (*Loxodonta africana*) in several aspects. Their superior intelligence and docility have been exploited as beasts of burden for centuries. Genetics of the Asian elephant has been described in terms of chromosome morphology and number, DNA fingerprints and electrophoretic variations (Hungerford, 1966; Hartl et al., 1995). This paper investigates the cytogenetics of the Asian elephant in conventional and GTG-banded preparations, as well as the localization of telomeres using fluorescence *in situ* hybridization (FISH).

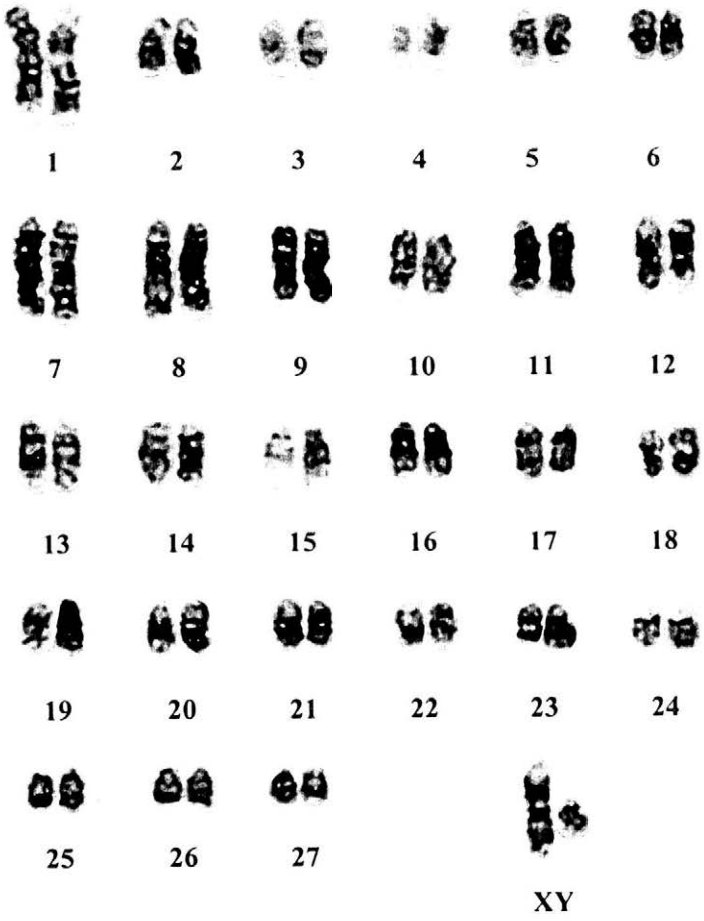
MATERIALS AND METHODS

Thirty-five heparinized blood samples were collected from male and female Asian elephants (*E. maximus*) held captive in the province of Lampang, Thailand. Lymphocyte cultures were performed using a mixture of RPMI 1640, 15% fetal bovine serum and pokeweed mitogen (40 µg/ml). After three days incubation at 37 °C CO_2, the cells were treated with colchicine and harvested. After hypotonic treatment (0.075 M KCl), cells were fixed and the metaphase spreads were stained with 2.5% Giemsa solution and banded with GTG-banding techniques (Seabright, 1971). Approximately 15–30 well-banded chromosome spreads of each sample were photographed and analyzed to establish the karyotype and idiogram.

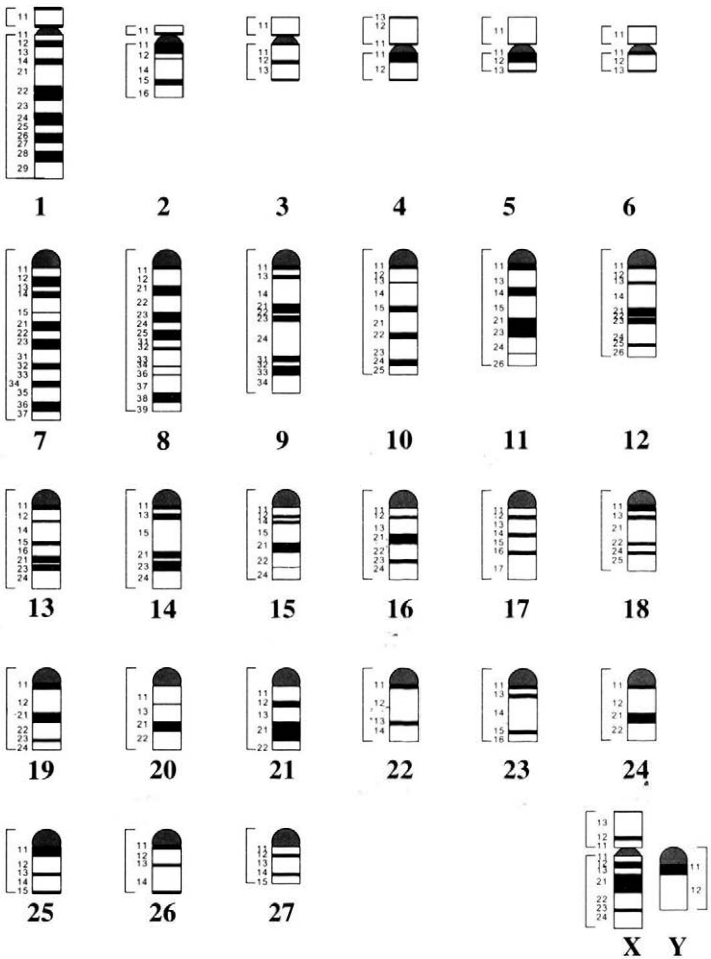
Metaphase cells were subjected to Fluorescence in situ hybridization (FISH) using Telomere PNA probe (DAKO A/S, Denmark). In brief, the metaphase spreads were fixed in 3.7% formaldehyde prior to washing in Tris-Buffered Saline (TBS) and treated with Pre-Treatment solution. The Telomere PNA Probe/FITC was introduced onto the slide and the denaturation of DNA was allowed to take place in the 80 °C incubator for 3 min, followed by the hybridization of PNA probe into the telomeric sequences (30–60 min, RT). After rinsing and washing, the chromosomes were viewed and photographed under a fluorescence microscope.



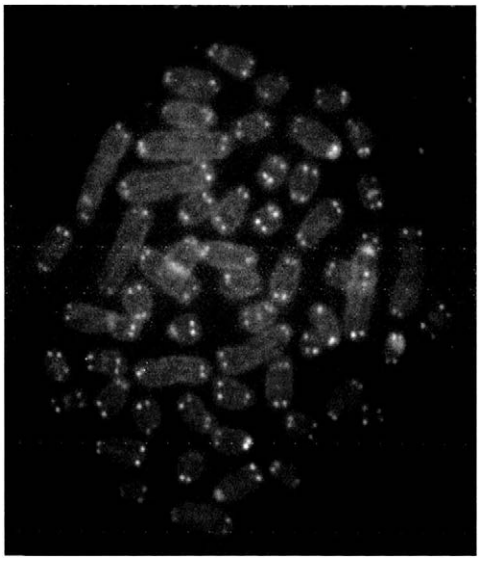
1. The karyotype of Asian elephant (*E. maximus*), $2n = 56$, XY) (Conventional Stain)



2. The GTG-banded karyotype of Asian elephant (*E. maximus*), $2n = 56$, XY



3. Idiogrammatic representation of GTG-banded karyotype of Asian elephant (*E. maximus*), $2n = 56$, XY



4. A metaphase spread of male Asian elephant (*E. maximus*), $2n = 56$, XY, demonstrated the fluorescent signals on the teminal arms of each chromosome

RESULTS AND DISCUSSION

The diploid chromosome number of the Asian elephant was 56 which is in accordance with the investigations previously reported (Hungerford et al., 1966; Norberg, 1969; Hartl et al., 1995). However, the standard karyotype for the Asian elephant has not been established. In the present study, the Asian elephant karyotype was, for the first time, proposed based on the basis of chromosome size and morphology (Fig. 1). The autosomal set was divided into 2 groups: one contained bi-armed chromosomes and the other carried telocentric chromosomes. The X chromosome was a medium-sized submetacentric while the Y was a small telocentric chromosome. A GTG-banded karyotype (Fig. 2) and its representative idiogram with the haploid 216-band resolution level were then constructed (Fig. 3). This idiogram will allow the correct identification of elephant chromosomes and will be useful for further gene mapping and molecular cytogenetic studies.

Fluorescence *in situ* hybridization of the telomeric sequence (TTAGGG) revealed spots of positive signals at the four ends of every metaphase chromosome (Fig. 4). Localization of telomeres in this study has proved that functional telomeres on each chromosome had been successfully conserved as previously demonstrated in all vertebrates tested (Meyne et al., 1989; Lejnine et al., 1995).

Acknowledgements

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POLYMORPHISM IN THE MSHR GENE OF DIFFERENT CATTLE BREEDS

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ABSTRACT: The melanocyte stimulating hormone receptor (MSHR) is involved in coat colour determination in mammals by controlling the relative amounts of eumelanin (black/brown) and phaeomelanin (red/yellow). MSHR is activated by alpha melanocyte stimulating hormone (α-MSH). However, alleles of the MSHR gene have been described, which encode for a constitutively active receptor resulting in the production of eumelanin and a non-functional receptor resulting in the production of phaeomelanin. In this study the complete coding sequences of the MSHR gene from Holstein, Red Holstein, Simmental and Brown Swiss cattle were amplified, cloned into a plasmid vector and sequenced. Four different variants are characterised: a deletion of one G at position 310 or 311, a T/C base substitution at position 296, a T/C base substitution at position 667 and a duplication of 12 nucleotides at position 650. Combinations of the polymorphic sites result in 5 different alleles with the deduced amino acid sequences. The allele containing a deletion and therefore a frameshift of the open reading frame was found in red animals. The allele containing a Proline at position 99, an Arginine at position 223 and missing a duplication was found in black animals. Three different alleles were found in Brown Swiss. All of them contained a Leucine at position 99 and no deletion, the other two sites were polymorphic. The obtained results support our hypothesis, that the allele with a Proline at position 99 encodes a constitutively active receptor, while the allele with a frameshift encodes an inactive receptor and the alleles found in Brown Swiss produce the receptors controlled by α-MSH stimulation. The stimulation of the cells expressing the different MSHR alleles with α-MSH will be done in view of finding functional differences between the alleles.

MSHR; polymorphism; cattle

INTRODUCTION

The coat colour in mammals is dependent on the presence of the pigment melanin in both skin and hair. The melanin granules are produced in melanocyte cells. Two different forms of melanin have been found to exist, eumelanin (black/brown) and phaeomelanin (red/yellow) (Searle, 1968). The relative amounts of eumelanin and phaeomelanin are primarily controlled by the two genetic loci, *Extension* and *Agouti*. In mammals dominant alleles of *Extension* are associated with black coat colour, while the recessive alleles of this locus lead to production of the red/yellow pigment (Searle, 1968; Jackson, 1997). Dominant alleles of *Agouti* cause a yellow coat colour in mice, and the recessive allele is associated with black coat colour (Jackson, 1997). Molecular genetic studies have revealed that the *Extension* locus encodes a melanocyte stimulating hormone receptor (Robbins et al., 1993), which is involved in the eumelanin and phaeomelanin production (Cone et al., 1996). The synthesis of eumelanin and phaeomelanin from tyrosine is controlled by several enzymes and the rate limiting enzyme in this process is tyrosinase, which is regulated both transcriptionally and posttranscriptionally by intracellular cyclic Adenosine Monophosphate (cAMP). Basal tyrosinase activity leads to the production of phaeomelanin, while eumelanin synthesis results from increased tyrosinase activity. The amount of intracellular cAMP in melanocytes is controlled by MSH, which binds to the melanocyte stimulating hormone receptor (MSHR), also known as the melanocortin-1 receptor (MC1R). Stimulated MSHR activates adenylate cyclase, thereby increasing the amount of cAMP in the cell, which activates the tyrosinase resulting in eumelanin synthesis. The *Agouti* signalling peptide acts as an antagonist of MSH by binding to the receptor and thereby preventing the MSHR-MSH interaction, resulting in phaeomelanin synthesis (Bultman et al., 1992).

Comparative genetic and population studies postulate the following alleles of the *Extension* locus (Searle, 1968):

$\mathbf{E^D}$– dominant extension of black

E – normal extension of black

I. MSHR specific primer sequences

Primer	Sequence 5´→3´	Ta (°C)
MC1-RF2 (forward)	ACGATGCCTGCACTTGGCTCCCAG	58 °C
MC1-RR1 (reverse)	CCTCACCAGGAGCACTGCAGCAC	58 °C
MSHR-P9 (forward)	GGAGGTGTCCATCTCTGACGG	56–60 °C
MSHR-P10 (reverse)	CCGGGCCAGCATGTGGACGTA	56 °C
MSHR-P12 (reverse)	CTGCTGACCACGGACGCCTG*	60 °C
MSHR-P13 (forward)	CCTGCCAGCATGCACGGGGC**	58 °C
MSHR-P14 (reverse)	GGCTGCGGAAGGCATAGATGAG	58 °C
MSHR-P15 (forward)	GCCAGCATCCTCACCAGCCTG	60 °C
MSHR-P16 (reverse)	Joe TGGGGCGCTGCCTCTTCTGG***	60 °C

Ta (°C) – annealing temperature
* G in the original MSHR sequence is replaced in the primer sequence by an A to erase the AciI digestion site in the PCR product
** C in the original MSHR sequence is replaced in the primer sequence by an A to erase the MspI digestion site in the PCR product
*** The primer MSHR-P16 was labelled with the fluorescent dye Joe (green)

e^{br} – black and yellow (brindled)
e – restriction of black (yellow and red)

Klungland et al. have characterised three alleles of the extension (**E**) locus, encoding for MSHR, in Norwegian and Icelandic cattle; a dominant acting $\mathbf{E}^{D}$ allele producing black coat colour, a recessive **e** allele giving red coat colour, and wild-type allele $\mathbf{E}^{+}$ producing a wide variety of different colours (Klungland et al., 1995, 2000). The homozygoty for single nucleotide deletion identical to the deletion reported in Norwegian cattle (Klungland et al., 1995) was shown to be associated with red coat colour in Holstein cattle (Joerg et al., 1996).

In this study we investigated the differences in MSHR alleles sequences in Swiss cattle breeds.

MATERIAL AND METHODS

Animals

Simmental, Red Holstein, Holstein, Brown Swiss (see Colour Plate, Figs. 1a, b, c, d) and Simmental x Holstein cattle were used in this study. Genomic DNA was isolated from blood and semen samples.

Cloning and Sequencing

The complete coding sequence of the MSHR gene with the size 954 bp was amplified with the primers MC1RF2 and MC1RR1 (Tab. I). The primers were designed based on the published sequences of the bovine MSHR (accession numbers Y13957 and S71017). The obtained PCR fragments were cloned into pGem®-T easy vector (Promega) and sequenced using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer) and the ABI Prism™ System 377 DNA Sequencer (Applied Biosystems, Perkin Elmer Corp., Foster City, Calif). The obtained sequences were analysed with the GCG package (Devereux et al., 1984).

PCR-RFLP assays

310/311 G deletion: PCR was carried out with the primers MSHRP9 and MSHRP10 (Tab. I). PCR products were digested with MspI. The digested DNA fragments were separated in a 1% agarose gel.

T296C base substitution: PCR was carried out with the primers MSHRP9 and MSHRP12 (Tab. I). PCR products were digested with AciI. The digested DNA fragments were separated in a 2% agarose gel.

T667C base substitution: PCR was carried out with the primers MSHRP13 and MSHRP14 (Tab. I). PCR products were digested with MspI and the digested DNA fragments were separated in a 2% agarose gel.

AFLP assay

PCR was carried out with the primers MSHRP15 and MSHRP16 (Tab. I), followed by fragment analysis performed with the ABI Prism™ System 377 DNA Sequencer (Perkin Elmer).

RESULTS AND DISCUSSION

The coding sequence of the MSHR gene was amplified from DNA obtained from a Simmental x Holstein cow, cloned into a plasmid vector and sequenced. Two types of sequences were obtained: the allele containing a deletion of a G at position 310/311 and the allele without the deletion and with a C at position 296 (Figs. 2 and 3).

```
E^D ATGCCTGCAC TTGGCTCCCA GAGGCGGCTG CTGGGTTCCC TTAACTGCAC  50
E1  ATGCCTGCAC TTGGCTCCCA GAGGCGGCTG CTGGGTTCCC TTAACTGCAC  50
e   ATGCCTGCAC TTGGCTCCCA GAGGCGGCTG CTGGGTTCCC TTAACTGCAC  50
E2  ATGCCTGCAC TTGGCTCCCA GAGGCGGCTG CTGGGTTCCC TTAACTGCAC  50

E^D GCCCCCAGCC ACCCTCCCCT TCACCCTGGC CCCCAACCGG ACGGGGCCCC 100
E1  GCCCCCAGCC ACCCTCCCCT TCACCCTGGC CCCCAACCGG ACGGGGCCCC 100
e   GCCCCCAGCC ACCCTCCCCT TCACCCTGGC CCCCAACCGG ACGGGGCCCC 100
E2  GCCCCCAGCC ACCCTCCCCT TCACCCTGGC CCCCAACCGG ACGGGGCCCC 100

E^D AGTGCCTGGA GGTGTCCATC CCTGACGGGC TCTTTCTCAG CCTGGGGCTG 150
E1  AGTGCCTGGA GGTGTCCATC CCTGACGGGC TCTTTCTCAG CCTGGGGCTG 150
e   AGTGCCTGGA GGTGTCCATC CCTGACGGGC TCTTTCTCAG CCTGGGGCTG 150
E2  AGTGCCTGGA GGTGTCCATC CCTGACGGGC TCTTTCTCAG CCTGGGGCTG 150

E^D GTGAGTCTCG TGGAGAACGT GCTGGTAGTG GCTGCCATTG CCAAGAACCG 200
E1  GTGAGTCTCG TGGAGAACGT GCTGGTAGTG GCTGCCATTG CCAAGAACCG 200
e   GTGAGTCTCG TGGAGAACGT GCTGGTAGTG GCTGCCATTG CCAAGAACCG 200
E2  GTGAGTCTCG TGGAGAACGT GCTGGTAGTG GCTGCCATTG CCAAGAACCG 200

E^D CAACCTGCAC TCCCCCATGT ACTACTTTAT CTGCTGCCTG GCTGTGTCTG 250
E1  CAACCTGCAC TCCCCCATGT ACTACTTTAT CTGCTGCCTG GCTGTGTCTG 250
e   CAACCTGCAC TCCCCCATGT ACTACTTTAT CTGCTGCCTG GCTGTGTCTG 250
E2  CAACCTGCAC TCCCCCATGT ACTACTTTAT CTGCTGCCTG GCTGTGTCTG 250

E^D ACTTGCTGGT GAGCGTCAGC AACGTGCTGG AGACGGCAGT CATGCCGCTG 300
E1  ACTTGCTGGT GAGCGTCAGC AACGTGCTGG AGACGGCAGT CATGCTGCTG 300
e   ACTTGCTGGT GAGCGTCAGC AACGTGCTGG AGACGGCAGT CATGCTGCTG 300
E2  ACTTGCTGGT GAGCGTCAGC AACGTGCTGG AGACGGCAGT CATGCTGCTG 300

E^D CTGGAGGCCG GTGTCCTGGC CACCCAGGCG GCCGTGGTGC AGCAGCTGGA 350
E1  CTGGAGGCCG GTGTCCTGGC CACCCAGGCG GCCGTGGTGC AGCAGCTGGA 350
e   CTGGAGGCC. GTGTCCTGGC CACCCAGGCG GCCGTGGTGC AGCAGCTGGA 349
E2  CTGGAGGCCG GTGTCCTGGC CACCCAGGCG GCCGTGGTGC AGCAGCTGGA 350

E^D CAATGTCATC GACGTGCTCA TCTGCGGATC CATGGTGTCC AGCCTCTGCT 400
E1  CAATGTCATC GACGTGCTCA TCTGCGGATC CATGGTGTCC AGCCTCTGCT 400
e   CAATGTCATC GACGTGCTCA TCTGCGGATC CATGGTGTCC AGCCTCTGCT 399
E2  CAATGTCATC GACGTGCTCA TCTGCGGATC CATGGTGTCC AGCCTCTGCT 399

E^D TCCTGGGTGC CATTGCTGTG GACCGCTACA TCTCCATCTT CTACGCCCTG 450
E1  TCCTGGGTGC CATTGCTGTG GACCGCTACA TCTCCATCTT CTACGCCCTG 450
e   TCCTGGGTGC CATTGCTGTG GACCGCTACA TCTCCATCTT CTACGCCCTG 449
E2  TCCTGGGTGC CATTGCTGTG GACCGCTACA TCTCCATCTT CTACGCCCTG 450

E^D CGGTACCACA GTGTTGTGAC ACTGCCCCGA GCGTGGAGGA TCATTGCGGC 500
E1  CGGTACCACA GTGTTGTGAC ACTGCCCCGA GCGTGGAGGA TCATTGCGGC 500
e   CGGTACCACA GTGTTGTGAC ACTGCCCCGA GCGTGGAGGA TCATTGCGGC 499
E2  CGGTACCACA GTGTTGTGAC ACTGCCCCGA GCGTGGAGGA TCATTGCGGC 500

E^D CATCTGGGTG GCCAGCATCC TCACCAGCCT GCTCTTCATC ACCTACTACA 550
E1  CATCTGGGTG GCCAGCATCC TCACCAGCCT GCTCTTCATC ACCTACTACA 550
e   CATCTGGGTG GCCAGCATCC TCACCAGCCT GCTCTTCATC ACCTACTACA 549
E2  CATCTGGGTG GCCAGCATCC TCACCAGCCT GCTCTTCATC ACCTACTACA 550

E^D ACCACAAGGT CATCCTGCTG TGCCTCGTTG GCCTCTTCAT AGCTATGCTG 600
E1  ACCACAAGGT CATCCTGCTG TGCCTCGTTG GCCTCTTCAT AGCTATGCTG 600
e   ACCACAAGGT CATCCTGCTG TGCCTCGTTG GCCTCTTCAT AGCTATGCTG 599
E2  ACCACAAGGT CATCCTGCTG TGCCTCGTTG GCCTCTTCAT AGCTATGCTG 600

E^D GCCCTGATGG CCGTCCTCTA CGTCCACATG CTGGCCCGGG CCTGCCAGCA 650
E1  GCCCTGATGG CCGTCCTCTA CGTCCACATG CTGGCCCGGG CCTGCCAGCA 650
e   GCCCTGATGG CCGTCCTCTA CGTCCACATG CTGGCCCGGG CCTGCCAGCA 649
E2  GCCCTGATGG CCGTCCTCTA CGTCCACATG CTGGCCCGGG CCTGCCAGCA 650
```

E^D	. . . . .	. . TGCCCGGG	GCATTGCCCG	GCTCCAGAAG	AGGCAGCGCC	688
E1	. . . . .	. . TGCCCGGG	GCATTGCC**T**G	GCTCCAGAAG	AGGCAGCGCC	688
e	. . . . .	. . TGCCCGGG	GCATTGCCCG	GCTCCAGAAG	AGGCAGCGCC	687
E2	TGCCCGGGGC	ATTGCCCGGG	GCATTGCCCG	GCTCCAGAAG	AGGCAGCGCC	700
E^D	CCATTCATCA	GGGCTTTGGC	CTCAAGGGCG	CTGCCACCCT	CACCATCCTG	738
E1	CCATTCATCA	GGGCTTTGGC	CTCAAGGGCG	CTGCCACCCT	CACCATCCTG	738
e	CCATTCATCA	GGGCTTTGGC	CTCAAGGGCG	CTGCCACCCT	CACCATCCTG	737
E2	CCATTCATCA	GGGCTTTGGC	CTCAAGGGCG	CTGCCACCCT	CACCATCCTG	750
E^D	CTGGGCGTCT	TCTTCCTCTG	CTGGGGCCCC	TTCTTCCTGC	ACCTCTCGCT	788
E1	CTGGGCGTCT	TCTTCCTCTG	CTGGGGCCCC	TTCTTCCTGC	ACCTCTCGCT	788
e	CTGGGCGTCT	**C**CTTCCTCTG	CTGGGGCCCC	TTCTTCCTGC	ACCTCTCGCT	787
E2	CTGGGCGTCT	TCTTCCTCTG	CTGGGGCCCC	TTCTTCCTGC	ACCTCTCGCT	800
E^D	CATCGTCCTC	TGCCCCCAGC	ACCCCACCTG	TGGCTGCATC	TTCAAGAACT	838
E1	CATCGTCCTC	TGCCCCCAGC	ACCCCACCTG	TGGCTGCATC	TTCAAGAACT	838
e	CATCGTCCTC	TGCCCCCAGC	ACCCCACCTG	TGGCTGCATC	TTCAAGAACT	837
E2	CATCGTCCTC	TGCCCCCAGC	ACCCCACCTG	TGGCTGCATC	TTCAAGAACT	850
E^D	TCAACCTCTT	CCTGGCCCTC	ATCATTTGCA	ACG**T**CATTGT	GGACCCCCTC	888
E1	TCAACCTCTT	CCTGGCCCTC	ATCATTTGCA	ACGCCATTGT	GGACCCCCTC	888
e	TCAACCTCTT	CCTGGCCCTC	ATCATTTGCA	A**T**GCCATTGT	GGACCCCCTC	887
E2	TCAACCTCTT	CCTGGCCCTC	ATCATTTGCA	ACGCCATTGT	GGACCCCCTC	900
E^D	ATCTATGCCT	TCCGCAGCCA	GGAGCTCCGG	AAGACGCTCC	AAGAGGTGCT	938
E1	ATCTATGCCT	TCCGCAGCCA	GGAGCTCCGG	AAGACGCTCC	AAGAGGTGCT	938
e	ATCTATGCCT	TCCGCAGCCA	GGAGCTCCGG	AAGACGCTCC	AAGAGGTGCT	937
E2	ATCTATGCCT	TCCGCAGCCA	GGAGCTCCGG	AAGACGCTCC	AAGAGGTGCT	950
E^D	GCAGTGCTCC	TGGTGA	954			
E1	GCAGTGCTCC	TGGTGA	954			
e	GCAGTGCTCC	TGGTGA	953			
E2	GCAGTGCTCC	TGGTGA	966			

2. Comparison of the coding sequences of the four bovine MSHR alleles. The non identical nucleotides are shown in grey. The first stop codon occuring in e allele after deletion is shown in italics

The deletion of 310/311G was identical to the deletion previously described in Red Holstein cattle (Joerg et al., 1996) and Red Norwegian cattle (Klungland et al., 1995). Therefore the allele, containing the 310/311G deletion was designated as **e**. Comparison of the sequenced **e** allele with the sequences of the other alleles also revealed two base substitutions at positions 748 and 869 (Fig. 2). The stop codon in the **e** allele is 486bp upstream from the stop codon in the other alleles (Fig. 2), and therefore these substitutions located after the stop codon have no effect on the protein structure.

The allele containing the nucleotide C at position 296 and subsequently a Proline at position 99 of the deduced amino acid sequence was designated as $\mathbf{E^D}$, because this base substitution is identical to the mutation reported in black Norwegian cattle (Klungland et al., 1995). Another base substitution found in the $\mathbf{E^D}$ allele at position 872, in which case the substituted T encodes for a Valine, while in other alleles the C encodes for an Alanine.

Cloning and sequencing of the MSHR gene in Brown Swiss cattle revealed two other alleles, designated as **E1** and **E2**. The allele **E1** contains a T at position 667 and therefore a Tryptophan at position 223 of the deduced amino acid sequence. The alleles $\mathbf{E^D}$, **e** and **E2** have a C and therefore an Arginine at this position (Figs. 2 and 3). The allele **E2** shows a duplication of twelve nucleotides at position 650 (Fig. 2) and subsequently a duplication of four amino acids at position 220 (Fig. 3).

The results of the sequencing were confirmed with the RFLP and AFLP assays for the four polymorphic sites: 310/311G deletion, T296C base substitution, C667T base substitution, 650 duplication.

310/311G deletion: The allele with the 310/311G deletion showed one band of 531 bp, and the allele without the 310/311G deletion showed two bands 201 bp and 331 bp. A heterozygote animal therefore reveal three bands of 531 bp, 331 bp and 201 bp (Joerg et al., 1996).

T296C base substitution: The allele with a C at position 296 after digestion with AciI should give 3 DNA fragments 50 bp, 90 bp and 97 bp. It was not possible to see the 7 bp difference in an agarose gel electropho-

E^D	MPALGSQRRL	LGSLNCTPPA	TLPFTLAPNR	TGPQCLEVSI	PDGLFLSLGL	50
E1	MPALGSQRRL	LGSLNCTPPA	TLPFTLAPNR	TGPQCLEVSI	PDGLFLSLGL	50
E2	MPALGSQRRL	LGSLNCTPPA	TLPFTLAPNR	TGPQCLEVSI	PDGLFLSLGL	50
e	MPALGSQRRL	LGSLNCTPPA	TLPFTLAPNR	TGPQCLEVSI	PDGLFLSLGL	50
E^D	VSLVENVLVV	AAIAKNRNLH	SPMYYFICCL	AVSDLLVSVS	NVLETAVMPL	100
E1	VSLVENVLVV	AAIAKNRNLH	SPMYYFICCL	AVSDLLVSVS	NVLETAVMLL	100
E2	VSLVENVLVV	AAIAKNRNLH	SPMYYFICCL	AVSDLLVSVS	NVLETAVMLL	100
e	VSLVENVLVV	AAIAKNRNLH	SPMYYFICCL	AVSDLLVSVS	NVLETAVMLL	100
E^D	LEAGVLATQA	AVVQQLDNVI	DVLICGS.MV	SSLCFLGAIA	VDRYISIFYA	150
E1	LEAGVLATQA	AVVQQLDNVI	DVLICGS.MV	SSLCFLGAIA	VDRYISIFYA	150
E2	LEAGVLATQA	AVVQQLDNVI	DVLICGS.MV	SSLCFLGAIA	VDRYISIFYA	150
e	LEAVSWPPRR	P.	. . WCSSWTM	SSTC . . . . SS	ADPWCPA . .	
E^D	LRYHSVVTLP	RAWRIIAAIW	VASILTSLLF	ITYYNHKVIL	LCLVGLFIAM	200
E1	LRYHSVVTLP	RAWRIIAAIW	VASILTSLLF	ITYYNHKVIL	LCLVGLFIAM	200
E2	LRYHSVVTLP	RAWRIIAAIW	VASILTSLLF	ITYYNHKVIL	LCLVGLFIAM	200
e	. . . . SASWVP	LLW . . . TATS	PSSTPCGTTV	L*HCPERGGS	LRPSGWPASS	
E^D	LALMAVLYVH	MLARACQH..	. . ARGIARLQ	KRQRPIHQGF	GLKGAATLTI	246
E1	LALMAVLYVH	MLARACQH..	. . ARGIAWLQ	KRQRPIHQGF	GLKGAATLTI	246
E2	LALMAVLYVH	MLARACQHAR	GIARGIARLQ	KRQRPIHQGF	GLKGAATLTI	250
e	PACSSSPTTT	TRSSCCASLA	S . . . . S*LC	WP*WPSSTST	CWPGPASMPG	
E^D	LLGVFFLCWG	PFFLHLSLIV	LCPQHPTCGC	IFKNFNLFLA	LIICNVIVDP	296
E1	LLGVFFLCWG	PFFLHLSLIV	LCPQHPTCGC	IFKNFNLFLA	LIICNAIVDP	296
E2	LLGVFFLCWG	PFFLHLSLIV	LCPQHPTCGC	IFKNFNLFLA	LIICNAIVDP	300
e	ALPGSRRGSA	PFIRALASRA	L .PPSPSCWA	SSSSAGAPSS	CTSRSSSSAP	
E^D	LIYAFRSQEL	RKTLQEVLQC	SW*~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	317
E1	LIYAFRSQEL	RKTLQEVLQC	SW*~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	317
E2	LIYAFRSQEL	RKTLQEVLQC	SW*~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	321
e	STPPVAASSR	TST. . . . . S	SWPSSFATSL	WTPSSMPSAA	RSSGRRSKRC	

3. Comparison of the deduced amino acid sequences of the four bovine MSHR alleles. Non identical aminoacids in ED, E1 and E2 are designated with grey. The stop codons in are shown with the stars

resis, however the fragments 90 bp and 97 bp were visible as a single band with double intensity. The allele with a T at position 296 gives two DNA fragments 90 bp and 147 bp. Heterozygote animals showed three bands: 50 bp, (90 + 97) bp and 147 bp.

C667T base substitution: After digestion with MspI the allele with a C at position 667 gave two DNA fragments of 241 bp and 25 bp. The band of 25 bp was not visible in agarose gel. The allele with a T at position 667 gave one DNA fragment of 266 bp. Heterozygote animals revealed two bands of 266 bp and 241 bp.

650 duplication: The allele containing a duplication of 12 nucleotides showed a band of 193bp and the allele without the duplication band of 181 bp.

II. Combinations of the four polymorphic sites and predicted genotype in Swiss cattle

Breed	Phenotype	Deletion	T296C	C667T	Duplication	Predicted genotype
Brown Swiss	brown	–/–	TT	TC	–/–	**E1/E3**
Brown Swiss	brown	–/–	TT	TT	–/–	**E1/E1**
Brown Swiss	brown	–/–	TT	TC	du/-	**E1/E2**
Brown Swiss	brown	–/–	TT	CC	du/du	**E2/E2**
Holstein	black	–/–	CC	CC	–/–	**E^D/E^D**
Holstein/Simmental	black	de/–	CT	CC	–/–	**E^D/e**
Simmental	red	de/de	xx	xx	xx	**e/e**
Holstein	red	de/de	xx	xx	xx	**e/e**
Simmental	red	de/–	TT	CC	–/–	**E3/e**

xx – means, that this polymorphic site was not tested
de – represents presence of the deletion
du – represents the presence of duplication
the dashes (–) show the absence of the deletion and duplication

The C872T base substitution has not yet been tested with an RFLP assay and for that reason it is not known, whether it is really polymorphic or because it results from a mistake from Taq polymerase during amplification. Valine and alanine belong to the same class of non polar amino acids, therefore this polymorphism is not expected to have an important influence on the protein structure and receptor function.

The four polymorphic sites (310/311G deletion, T296C base substitution, C667T base substitution and 650 duplication of twelve nucleotides) were tested with RFLP and AFLP assays in 12 Brown Swiss animals and in four black animals.

None of the investigated Brown Swiss animals contained a 310/311G deletion or a C at position 296. The C667T base substitution and the 650 duplication were polymorphic inside the breed. According to the performed assays one more allele of MSHR (**E3**) exists. A presumptive **E3** allele contains a C at position 667 and no duplication (Tab. II).

Three black Holstein animals were genotyped as $\mathbf{E^D/E^D}$, one crossbred Holstein x Simmental cow was $\mathbf{E^D/e}$. These data are consistent with the results reported by Klungland (Klungland et al., 1995) and the hypothesis that $\mathbf{E^D}$ encodes a constitutively active receptor.

RFLP assays for the 310/311G deletion are routinely performed in our group for genotyping cattle as it was shown to be associated with red coat colour (Joerg et al., 1996). A red bull was genotyped as **E3/e** incidentally. This phenomenon can be explained, either by the presence of another mutation in the **E3** allele of that bull, causing a non-functional receptor or by the influence of a dominant *Agouti* allele.

The obtained results are consistent with the common hypothesis that the $\mathbf{E^D}$ allele causing the black coat colour encodes a constitutively activated receptor, the **e** allele encodes a non functional receptor, and both of them are not dependent on MSH stimulation and are epistatic over *Agouti* (Klungland et al., 1995). The so called "wild type" or **E** allele is sensitive to both MSH and *Agouti*, therefore in **Ee** and **EE** animals a variety of colours from red to black are possible. In Brown Swiss cattle the **E** allele was shown to be present in at least three forms.

Stimulation of the cells expressing different alleles of MSHR with MSH and measuring the resultant cAMP production will be performed to show differences between alleles on the functional level.

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CHIMERISM 78,XX/77,XX,RB IN A BITCH, REVEALED BY CHROMOSOME AND MICROSATELLITE STUDIES

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ABSTRACT: A 5-years-old mix breed bitch was subjected to cytogenetic evaluation due to infertility. Two cell lines were identified: 78,XX (96% of cells) and 77,XX (4% of cells). In the second line apart from two bi-armed X chromosomes, an additional bi-armed chromosome was present, which was poor in centromeric constitutive heterochromatin (C-band). It was assumed that this chromosome represents Robertsonian translocation (Rb), in which two medium size acrocentrics were involved. To resolve whether the studied case represented mosaicism or chimerism, a microsatellite polymorphism study was applied. Altogether nine microsatellite loci were analysed. At three loci more than two alleles were found. This indicated that the studied bitch was chimeric and it is suggested that this status was established during foetal life due to vascular anastomoses.

chimerism; dog; microsatellite; Robertsonian translocation

INTRODUCTION

The appearance of more than one cell line in an individual brings a question about the origin of this abnormality. There are two possibilities: mosaicism or chimerism. These two phenomena are defined as follows (King and Stansfield, 1990): (a) a **mosaic** is an individual composed of two or more cell lines of different genetic or chromosomal constitution, both cell lines being derived from the same zygote while (b) a **chimera** is an individulal composed of a mixture of genetically different cells being derived from genetically different zygotes.

The composition of two or more cell lines can be revealed by the use of chromosome studies or/and genetic marker polymorphism study. Unfortunately, the identification of different karyotypes very often is not sufficient (with an exception of XX and XY cell lines) for the correct classification of the case.

In the intersex dog, karyotypes composed of two cell lines mainly XX/XY were described several times (Genero et al., 1998). This type of abormality, caused by the exchange of hemopoietic stem cells through placental anastomoses, is responsible for infertility in some species, including the dog. On the other hand, studies on canine neoplasm showed that mosaicism, with a cell line carrying an extra bi-armed chromosome, is quite a frequent phenomenon (Mellink et al., 1989; Reimann et al., 1994).

Cytogenetic and molecular genetic analyses of a bitch demonstrating two leukocyte cell lines are presented in this report.

MATERIAL AND METHODS

Case report

An infertile 5-years-old mix breed bitch, phenotypically normal, was subjected for cytogenetic analysis.

Cytogenetic analysis

Chromosome studies on in vitro cultured leukocytes were carried out. Air-dried chromosome spreads were Giemsa-stained, QFQ-banded and CBG-banded.

Microsatellite polymorphism studies

Polymorphism of nine microsatellite loci, namely: AHT136, CPH1, CPH3, CPH11, 2004, 2010, 2159, 2168 and 2319, was studied. DNA was isolated from blood cells. PCR amplified fragments were analysed with the use of the ALFexpress authomatic sequencer.

RESULTS

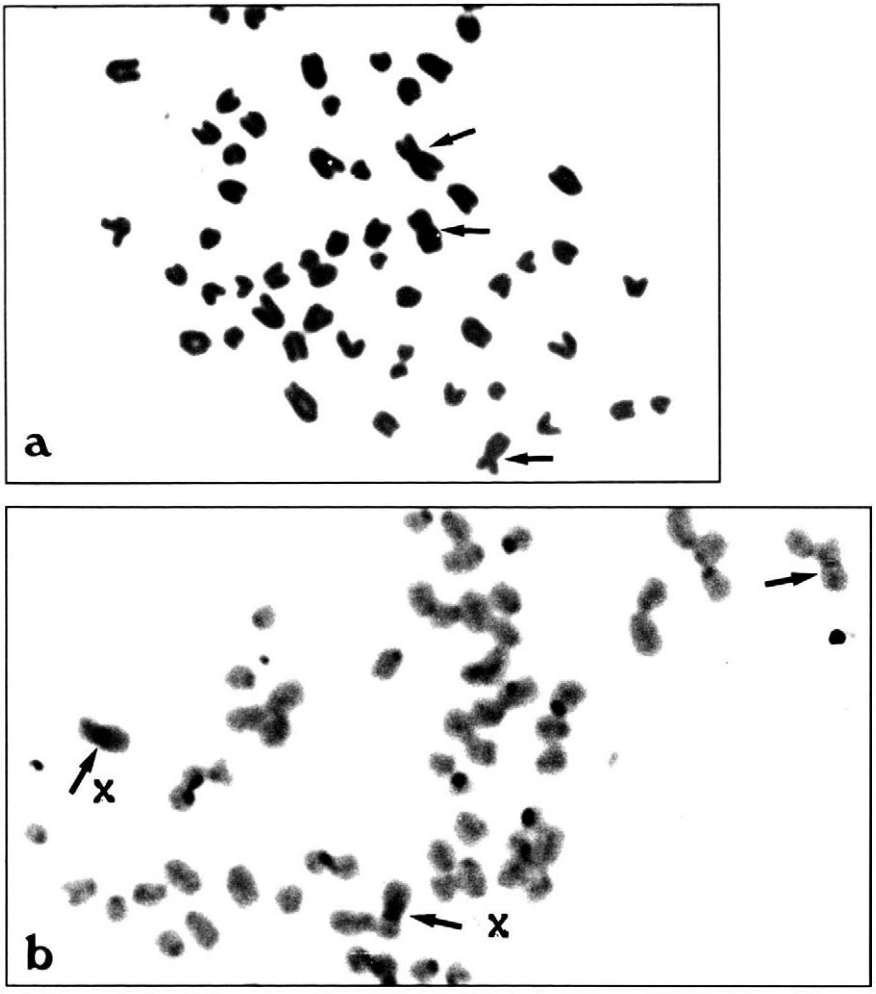
Among 200 Giemsa stained chromosome spreads, 96% demonstrated a normal female karyotype – 78,XX and in 4% of the cells an abnormal chromosome set was found – 77,XX (Fig. 1a). In the second cell line a marker chromosome was present. The marker chromosome was bi-armed and its size and morphology were similar to those of the X chromosome. The application of CBG banding technique revealed a lack of the centromere constitutive heterochromatin block on the

marker chromosome (Fig. 1b). Unfortunately, we did not obtain good quality QFQ banded spreads, so it can only be suggested that two medium sized autosomes were involved in this Robertsonian translocation (Rb). On the basis of cytogenetic analysis it was not possible to draw a conclusion whether the studied case represented mosaicism or chimerism.

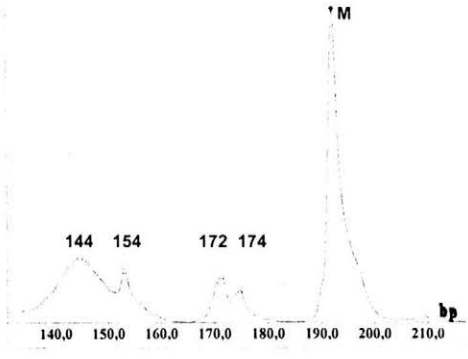
A detailed analysis of nine microsatellites revealed the presence of more than two alleles at three loci (Tab. I). At one locus (CPH11) four alleles were found (Fig. 2) and at two loci (2010 and 2168) three alleles were present. Unfortunately, it was not possible to study the parents of the bitch. From the above results we concluded that the bitch was chimeric.

DISCUSSION

Cytogenetic and molecular genetic studies, carried out on blood cells of the studied bitch, revealed the presence of two cell lines, among which one was carrying Robertsonian translocation. Unfortunately, the identification of chromosomes involved in the translocation was not possible. The application of C-banding technique facilitated the distinguishing between the X chromosomes and the additional bi-armed one, due to the presence of an extended C-band block on the long arm of the X chromosome (Pathak and Wurster-Hill, 1977; Switonski et al., 2000). Robertsonian translocations are the most common structural rearrangements found in



1. Metaphase spreads (2 *n* = 77,XX,Rb) of the chimeric bitch: (a) Giemsa staining, three bi-armed chromosomes, including two X chromosomes, are indicated by arrows; (b) CBG banding, the additional bi-armed chromosome, the effect of the Robertsonian translocation (Rb) is indicated by an arrow, two X chromosomes are also shown



2. Alleles found at locus CPH11 in the chimeric bitch; allele sizes (bp) and internal standard (M) are shown

1. Alleles identified at nine loci in the chimeric bitch

Locus	Alleles (size in bp)
AHT 136	146, 154
CPH1	123, 129
CPH3	170, 180
CPH11	144, 154, 172, 174
2004	233, 241
2010	220, 228, 236
2159	154, 178
2168	209, 225, 229
2319	307, 311

dogs, but their negative effect on fertility was not reported (Larsen et al., 1979; Mayr et al., 1986; Stone et al., 1991). This type of abnormality was also identified in cancer cells, for instance in mammary carcinoma (Mellink et al., 1989; Reimann et al., 1994). Microsatellite polymorphism analysis showed that this bitch was chimeric. Since the study was carried out on DNA isolated from blood cells, it was not possible to state whether this case represented a whole-body chimerism or only a blood cell chimerism.

Blood cell chimerism, a result of the exchange of hemopoietic stem cells between twin fetuses through placental anastomoses, is a very well known phenomenon in cattle. In case of heterosexual twins, the XX/XY chimerism is related to the infertility of the female cotwin, called freemartin in cattle (Popescu, 1990). Similar consequences are observed in XX/XY chimeric ewes (Long, 1990). On the other hand, chimeric mares are often fertile (Power, 1990; Bugno et al., 1999; Jaszczak et al., 1999). From the limited number of reports it can be tentatively concluded that XX/XY chimeric bitches are infertile (Genero et al., 1998). Since in the studied case two female cell lines were found it can not be considered as a cause of her infertility. At present it is difficult to estimate the incidence of placental anastomoses in dogs; however, an increasing interest in paternity control in this species, with the use of microsatellite markers, may bring in the near future more data concerning this issue.

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STUDY ON THE ORIGIN OF 64,XX/63,X KARYOTYPE IN FOUR STERILE MARES

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ABSTRACT: Twenty four mares, demonstrating fertility disturbances, were cytogenetically investigated. In four mares (17%) the presence of two cell lines – 64,XX and 63,X – was found. Paternity testing for two of the above mares, based on blood typing and protein polymorphism, confirmed the status of mosaicism. No relationship between the occurrence of the X monosomy mares and the age of their parents was found.

X monosomy; chimerism; horse

INTRODUCTION

The most frequent chromosomal abnormality in the horse is the X monosomy which may occur in the pure – 63,X or mosaic – 64,XX/63,X forms (Power, 1990).

Univocal distinguishing between chimerism and mosaicism, on the basis of leukocyte chromosome studies, can be difficult. In case of the presence of XX and XY cell lines, the chimeric classification is obvious. But in other cases – XX/X0, XY/X0 or XY/X0/XXY – the situation is not as clear. For instance, two cell lines XX and X0 can appear due to: (a) non-disjunction of chromatids of the X chromosome in the course of early embryonic development of XX or X0 zygotes, leading to mosaicism or (b) exchange through anastomoses of hemopoietic cells between homosexual twins XX and X0, leading to chimerism. The application of genetic markers polymorphism study is very helpful in the identification of blood cells chimerism, for instance in cattle (Glowatzki-Mullis et al., 1995; Rejduch et al., 1998).

From the horse breeding point of view, it is very important to reveal whether the age of parents may influence the incidence of aneuploidy, specially the most common X monosomy. There is very little data concerning this issue (Trommershausen-Smith et al., 1979).

In this study further data are provided on the origin of the X monosomy in mares.

MATERIAL AND METHODS

Altogether 24 mares were subjected to cytogenetic analysis due to various fertility problems: sterility, lack of oestrus, miscarriages, still birth and birth of malformed foals.

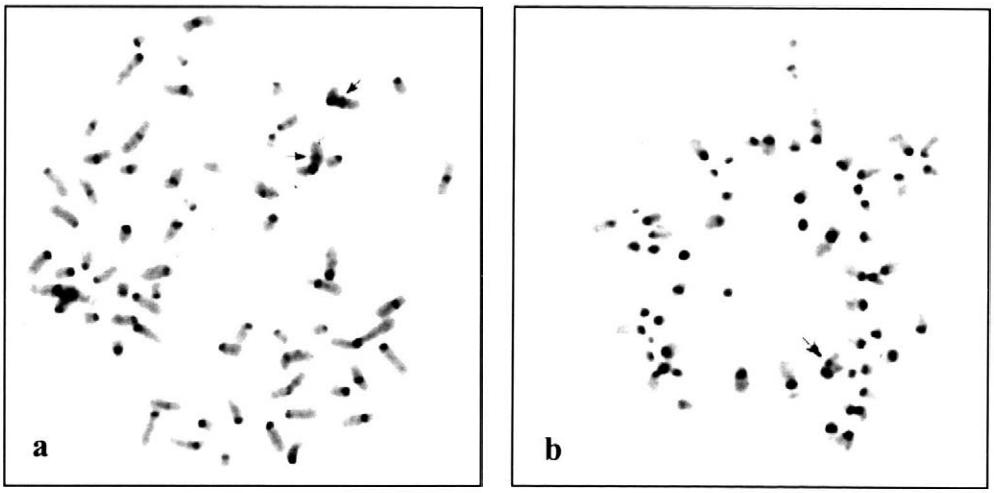
Blood samples were collected from mares of the Thoroughbred, half-bred, Arabian and Anglo-Arab mares from Polish studs. Standard lymphocyte culture technique was applied and the cytogenetic preparations were Giemsa stained and also C-banded.

At first, conventional Giemsa stained preparations were analysed. At least 50 metaphase spreads were observed and the diploid chromosome number was established. The C-banding technique facilitated the identification of sex chromosomes due to the presence of an interstitial C-band on the long arm of the bi-armed X chromosome and entire C-positive Y chromosome (Sumner 1982; Richer et al., 1990; Bowling et al., 1997).

RESULTS AND DISCUSSION

Among 24 mares, four (17%) demonstrated a mosaic karyotype 64,XX/63,X (Fig 1). Percentage of 63,X cells varied from 10 to 22%. All those mares were definitely sterile (Tab. I).

The presence of two cell lines (64,XX and 63,X) brings a question whether this status reflects mosaicism or chimerism. The XX/X syndrome in mares is commonly classified as mosaicism. However, the occurrence of chimerism may not be definitely excluded. Cytogenetic techniques are not sufficient to resolve this dilemma. To confirm that these cases were mosaic, blood typing and blood protein polymorphism studies (altogether 14–17 loci) were performed for two mares (Eroika and Martika) and their parents (Tab. II). Concordant results were obtained at all loci, thus we concluded that at least these two mares were mosaics. For another two mares data for both parents were not available.



1. C-banding of metaphase spreads originating from a mosaic 64,XX/63,X mare: (a)64,XX; (b) 63,X. The X chromosomes are indicated by arrows

I. List of four sterile mares with 64,XX/63,X karyotype

Mare (age in years)	Age* of mare's parents (years) Dam (foaling)		Sire	Breed	% of cells	
					64,XX	63,X
Eroika (7)	5	(2)	10	Half-bred	78	22
Kamfora (14)	6	(2)	13	Thoroughbred	90	10
Martika (9)	8	(3)	6	Anglo-Arab	86	14
Armenia (9)	21	(12)	13	Thoroughbred	86	14

*age of the parents at the time of foaling a monosomic offspring

An abnormal number of sex chromosomes has very often been found in infertile mares with developmentally retarded reproductive organs (Huges and Trommershausen-Smith, 1977; Trommershausen-Smith et al.,1979; Mäkinen et al., 1986; Long, 1988; Power, 1990). In most cases of mares subjected for examination due to infertility and gonadal dysgenesis, sex chromosome abnormalities were found. Among 12 mares examined by Huges and Trommershausen (1977), nine mares with 63,X karyotype, one with 63,X/64,XX, one

II. Paternity testing for two 64,XX/63,X mares

Name	Blood typing	Protein polymorphism
Eroika	A: adf/b; C:–/–; D: bcm/cgm; K: –/–; P: –/–; Q: abc/**	AL.-B; Es-I; Gc-FS; TF-DF1; Xk-K; PGD*; PGM*; PHI*
Mare's dam	A: adf/b; C: –/–; D: cgm/cgm; K: –/–; P: ac/**; Q: abc/**	AL.-AB; Es-I; Gc-FS; Hb-BI; PI-RU; TF-F1; Xk-K; PGD-FS; PGM-FS; PHI-I
Mare's sire	A: adf/**; C: a/**; D: bcm/cgm; K: –/–; P: ac/**; Q: abc/**	AL.-B; Es-IM; Gc-F; Hb-BIBII; PI-L; TF-DH2; Xk-K; PGD-FS; PGM-S; PHI-I
Martika	A: adf/**; C: a/**; D: cegimn/dkl; K: –/–; Q: abc/**	AL.-BI; Es-IS; Gc-F; Hb-BII; PI-IL; TF-D; Xk-K; PGD-FS; PGM-S; PHI-I
Mare's dam	A: –/–; C: a/**; D: delo/dkl; K: –/–; Q: abc/**	AL.-B, Es-IS; GC-F; Hb-BII, PI-IU; TF-DF2; Xk-K; PGD-FS; PGM-S; PHI-I
Mare's sire	A: adf/**; C: a/**; D: cegimn/cgm; K: –/–; P: ac/**; Q: abc/**	AL.-B; Es-I; Gc-F; Hb-BIBII; PI-L; TF-D; Xk-K; PGD-F; Cat-S; PGM-S; PHI-I

*not examined

**genotype was not established (no data about parents available)

with 63,X/64,XY, and one with 64,XY karyotype were found. Long (1988) reported that three out of 25 examined infertile mares (12%) showed chromosomal anomalies: one was monosomic – 63,X, another one was mosaic – 64,XX/63,X and the last one was sex-reversed – 64XY. It should however be mentioned that there are also rare reports showing fertile X monosomy mares (Halnan, 1985; Long, 1988).

In the last decade, cytogenetic evaluation of mares in Poland received a lot of interest. Bielański et al. (1980) described a phenotypically female foal with 64,XX/65,XXY chimerism. The first mare with the X monosomy was described by Sysa et al. (1989). A case of a trisomic horse 65,XXY was diagnosed by Kubień et al. (1993). Parada et al. (1996) analysed 244 mares with fertility disturbances. Among them, 78 animals were classified as sterile and nine (11.5%) of them showed an abnormal set of sex chromosomes: three of them displayed the X monosomy, four were mosaic – 64,XX/63,X and two were chimeric – 64,XX/64,XY. Jaszczak and Parada (1999) conducted an analysis of eight heterosexual twins. Leukocyte chimerism XX/XY was found in 4 pairs (7 horses). Two of these chimeric mares gave birth to foals. A similar case was described by Bugno et al. (1999). These results support earlier assumptions that chimeric XX/XY mares do not show severe fertility disturbances (Power, 1990).

The age of the mares' parents was recorded, to find out whether it might influence the meiotic non-disjunction leading to establishing the X monosomy syndrome in offspring (Tab. I). The average age of dams of the 64,XX/63,X mares was 10 years, but this was mainly influenced by the very old (21 years) dam of the mare Armenia. The average age of the mares' sires was quite similar (10.5 years). A similar investigation was carried out by Trommershausen-Smith et al. (1979), who described 17 cases of pure X mononsomy, four mosaics – 63,X/64,XX, three 63,X/64,XY and two sex reversed 64,XY. The age of the dams ranged from 3 to 13 years (with the mean of 8 years). The above results show that there are is clear relation between the incidence of X monosomy and the age of parents.

Our study shows that further investigation on the origin on X monosomy in mares is needed. It seems that a more sensitive microsatellite polymorphism analysis, as it was shown for cattle (Glowatzki-Mullis et al., 1995) or dog (Switonski et al., in press), should be applied to distinguish between mosaic and possible chimeric cases. It is also postulated to collect more data on the effect of parents' age on the incidence of the X monosomy.

Acknowledgements

We would like to thank Magdalena Stańdo for providing blood samples from the studied mares.

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A CASE OF XX/XY CELL POPULATIONS IN SHEEP

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ABSTRACT: A group of 50 ewe lambs was selected for reproduction. Micturition and sexual behaviour anomalies were detected in one animal. Peripheral blood cell cultures showed this individual is a $2n = 54$ XX/XY chimera. Both kinds of cells appear in very similar proportions (51% XX; 49% XY).

XX/XY chimera; genitourinary abnormalities; sheep

INTRODUCTION

Ovine genetic improvement programs are presently being developed in Aragón (Spain). Our laboratory participates in these programs by performing chromosomal abnormalities detection, genetic identification and paternity testing by means of protein markers and microsatellites, and production controls.

In this work, we are presenting the case of an individual showing chromosomal and physical abnormalities.

A group of 50 ewe lambs (70 days old) was selected for reproduction. Six month later, micturition and sexual behaviour anomalies were detected in this animal. This was the start point for further anatomical, functional and genetic analysis.

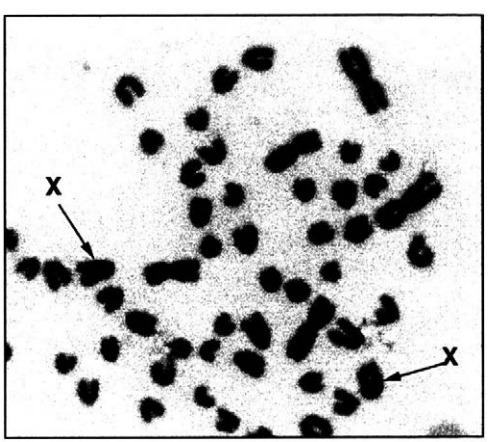
MATERIAL AND METHODS

Standard peripheral blood lymphocytes cultures were performed. Metaphase plates were Giemsa stained. Images were obtained by means of a CCD camera.

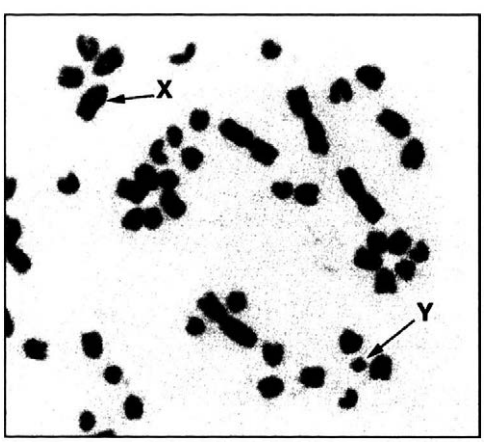
OBSERVATIONS AND RESULTS

Behaviour

Farm staff detected abnormal sexual behaviour in this individual. Even if it was classified as a female, it started trying the covering of the rest of females, showing a male behaviour. It refused any mating attempt by the males and even confronted them. During



1. XX metaphase



2. XY metaphase

the oestrum period, Bibi took a clear dominant position in the ewes group. At present, its work as estrum detector is deeply appreciated by farm staff.

Genitourinary abnormalities

At the moment of the first selection, genitourinary observation was superficial. This shallow control revealed no abnormalities at all. However, after detecting the formerly described sexual behaviour, a deeper examination was performed. At this moment, a clear protuberance was detected at clitoris area, close to the urethra distal portion (see Colour Plate, Fig. 3). Due to this conformation, micturition is quite unusual, giving an horizontal liquid flow.

In the first deep inspection, a single testicle was detected in cryptorchid position. Some months later, two atrophic testes (around 25% of normal size) were found in the inner of atrophic mammary glands. Their position is clearly extra-abdominal.

Chromosomes

Chromosomal analysis was performed when Bibi was about one year old. Peripheral blood cell cultures showed this individual is a $2n = 54$ XX/XY chimera (Figs. 1 and 2).

Both kinds of cells appear in very similar proportions (51% XX; 49% XY).

DISCUSSION

Several morphological and hormonal abnormalities have been reported by Bruere and McNab (1968), Smith et al. (1998), Vallenzasca and Galli (1992). In this papers, intersex, chimerism and sexual reverse are identified with different levels of abnormality in affected individuals.

Our results are widely concordant with those of Bosu and Basrur (1984) who detected an ovine intersex registered as a female at birth. That animal developed male-like appearance and behaviour and showed XX and XY cell populations too.

Further analysis are now under development, including hormones quantification, testicle biopsies and abdominal endoscopy to clarify the exact sexual organs morphology.

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X CHROMOSOME INACTIVATION STUDIES IN THE SWISS ERINGER BREED

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ABSTRACT: Investigations were carried out on cows of the Eringer breed because of their cowfighting behaviour. The observable interest in fighting between female animals is an integral element in the genetical diversity of the breed. It is known that the phenotype of female Eringer animals is masculine and that the fighting desire is also a characteristic feature of this breed when compared to other breeds. Cytogenetic examinations we carried out on Eringer cattle. Short term cultures from lymphocytes and long term cultures from fibroblasts were made. The metaphases prepared from the cell cultures, were banded and the karyograms were compared to the international standard. Special attention was given to the X chromosomes. In mammals the X chromosome is of special importance because of its variable genetic activity. Only one of the two X chromosomes is active in the somatic cells of females. The inactivated X chromosome could be identified due to a different R-banding structure. The differences between the active and the inactive X chromosomes between different animals were astonishingly clear and no artefacts were visible. This would indicate a varying activity of both X chromosomes despite their partial activation and inactivation. A phenotypical and behaviour specific variation in this breed may be related to the different inactivation effects.

X chromosome; cattle; R-banding

INTRODUCTION

The Eringer breed was developed from colour variants which are spread over the whole Alp area between Austria and Savoyen (Fellay, 1998). Today, this breed can practically only be found in Wallis, Switzerland. The animals, particulary the females have a vivacious and a fighting temperament, which manifests itself in dominance rituals. Due to these characteristics cowfighting championships are organised in autumn and spring. At the end of a long series of qualifying fights, the winning cow will obtain the prestigious "Queen" title, given by a jury (see Colour Plate, Fig. 2).

Until now these characteristics have no been examined in the field of heredity, however there is no doubt that this special property (aggressivity) is inherited among the Eringer breed (Preiswerk et al., 1986).

Normal Giemsa-stained chromosomes prepared from lymphocyte- and fibroblast cultures were used to count the chromosomes, identify the sex chromosomes and as a detection method for major chromosome abnormalities. Minor chromosome aberrations were detected using the R-banding technique and comparing the preparations to the internationally accepted standard karyotype.

Particular attention was given to the X chromosomes to see whether any X chromosome activation or X chromosome inactivation could be observed. Both X chromosomes are activated during the early cell divison phase (Hill and Yunis, 1967). The inactivation of one of the X chromosomes occurs during early embryogenesis and is conserved during later cell divisons in the animal. To compensate the dosis effect of the genes on the X chromosome in female animals, one of the X chromosomes is inactivated by heterochromatinisation. Hence both the female and the male animals have only one activated X chromosome in all cells.

The aim of this study was to investigate the cell division of autosomes and sex-chromosomes by means of mitotic chromosome preparations from specially selected animals with divergent phenotypes and behaviour appearances.

MATERIALS AND METHODS

Blood samples and skin biopsies of an Eringer bull were available from the Faculty of Veterinary Medicine, University of Zurich, Switzerland.

Two groups of Eringer cows were investigated during a fighting event in 1997. After the fights, blood samples were taken from the *vena jugularis* and used for lymphocyte cultures. Informations to certain parameters were gathered to the cows examined (Tab. I and II).

A third group of Eringer cows at a farm in Kriegstetten, Switzerland were used for similar investigations as above. The obtained parameters are shown in Tab. III.

I. Cow-fighting, Gampel (September 1997)

Start-number	Name	Circumference (cm)	Category	Place
26	Princesse	196	2	4
97	Melitta	185	4	1
158	Champion	187	5	4

II. Cow-fighting, Martigny (Oktober 1997)

Start-number	Name	Weight	Category	Place
9	Frimousse	727 kg	1	4
33	Tulipe	–	not participated	–
100	Lucifer	554 kg	3	not classified
127	Friponne	–	4	1
176	Vichy	–	5	1

III. Data from Eringer cows at a farm in Kriegstetten

Name	Date of Birth	Number	Milk production in the last year (kg)
Fany	1987	03 2918	3 045
Bambou	1992	C 2031	1 552
Charbon	1993	D 2660	2 647

The lymphocyte cultures were prepared as described by Hediger (1988). The fibroblast cultures were made according to Nett (1995). Giemsa-staining was applied to the mitotic preparations for all animals. In addition we performed R-banding. Only cells that had been treated with Bromdeoxyuridin (BrdU) during culturing could be stained by this method.

RESULTS

From the lymphocyte cultures prepared with blood from Eringer cattle, no visible aberrations were found, when the karyogramms were compared to the international standard (ISCNDA, 1990). Fibroblast cultures were prepared from the skin byopsies of 3 cows. This allowed us to compare the results obtained from the two different cell culturing methods. The chromosomes were carefully analysed with special attention given to the sex-chromosomes. Partial karyogramms of the sex-chromosomes from every animal are shown on Fig. 1. The activated X chromosome is in the first position and the inactivated X chromosome is at the second position. Certain differences were however observed in the R-banding between the standard and the Eringer cows (Tab. IV).

No differences were detected between the standard (ISCNDA, 1990) and the Eringer bull.

DISCUSSION

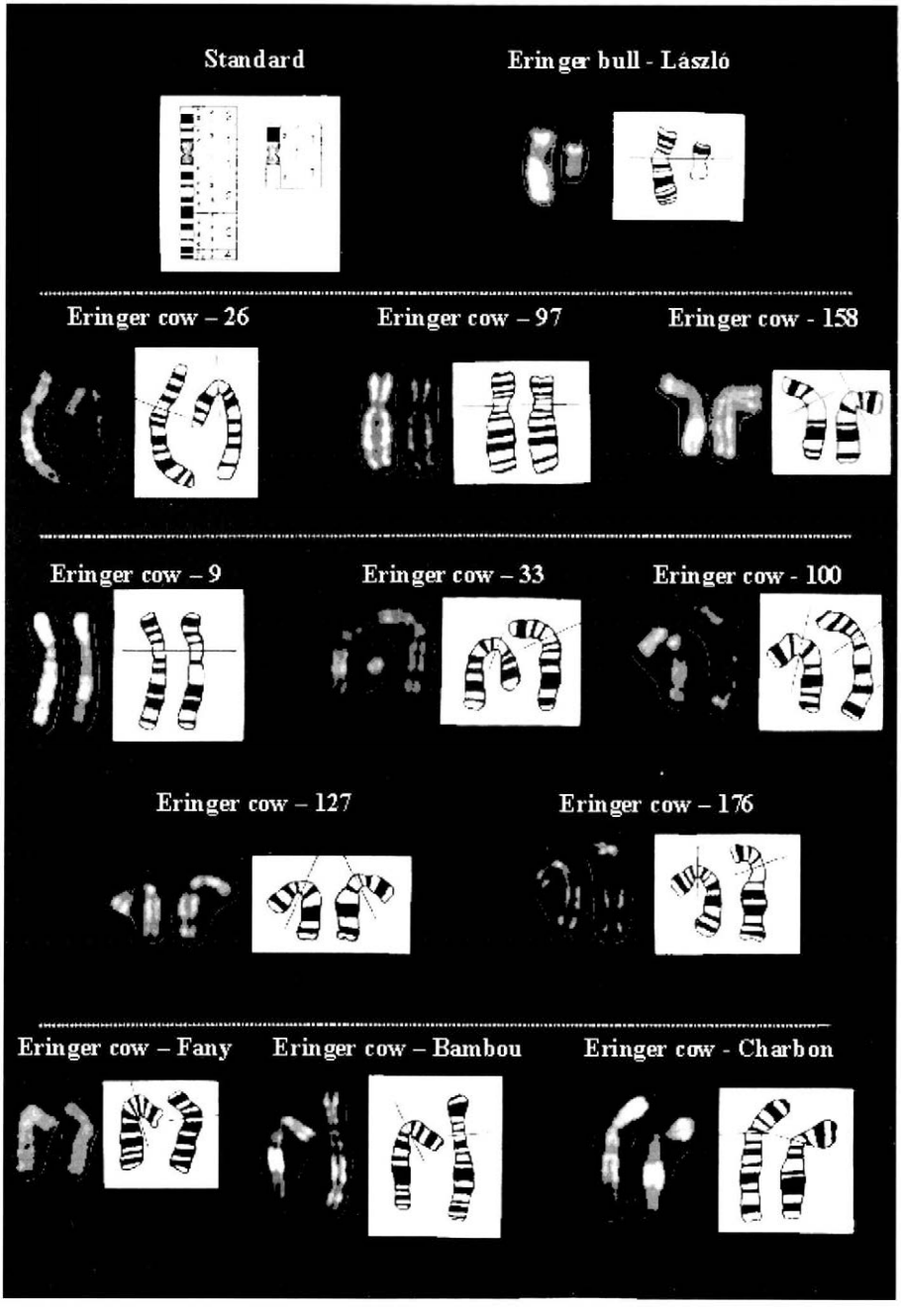
The corresponding X chromosome pairs of the R-banded sex chromosomes for the Eringer cows show differences. One possible explanation is the chromosome activation/inactivation theory. It has been known for a long time that the X-chromosome inactivation exists and it begins in the X-Inactivation-Centre (XIC region). A possible mechanism for the inactivation was suggested by Lyon (1998).

Male animals always show an activated X chromosome. In female animals the X chromosome inactivation occurs between the morula and blastocyte stages.

Although the banding patterns of the two X chromosomes often look very similar, the inactivated X chromosome can still be clearly recognized based of the intensity variations of the banding patterns. These intensity variations of the bands may however also be due to the R-banding technique. The variations could depend upon the concentration of the BrdU and at

IV. Chromosome regions without R-band on the activated and inactivated X chromosomes of different Eringer cows

Animal	Activated X chromosome	Inactivated X chromosome
Eringer cow – 26	–	q 41
Eringer cow – 97	q 33, q 41	q 33, q 41
Eringer cow – 158	p 12, q 23, q25, q35	p 12, q 21, q 23, q25, q35
Eringer cow – 9	q 31, q 41	q 23, q 31, q 41
Eringer cow – 33	q 35	q 31, q 35
Eringer cow – 100	p 14, q 23, q 25	q 23, q 31, q 35
Eringer cow – 127	q 23, q 25, q 33, q 35	q 23, q 25, q 33, q 35
Eringer cow – 176	q 33	q 21, q 31
Eringer cow – Fany	q 33	q 23, q 33, q35
Eringer cow – Bambou	–	–
Eringer cow – Charbon	q 35	q 25, q 35



1. Comparable R-banded sex chromosomes from different Eringer animals; the first chromosome is activated and the second inactivated

Figure 1: a) Simmental bull
c) Black Holstein Cow

b) Red Holstein Cow
d) Brown Swiss Cow

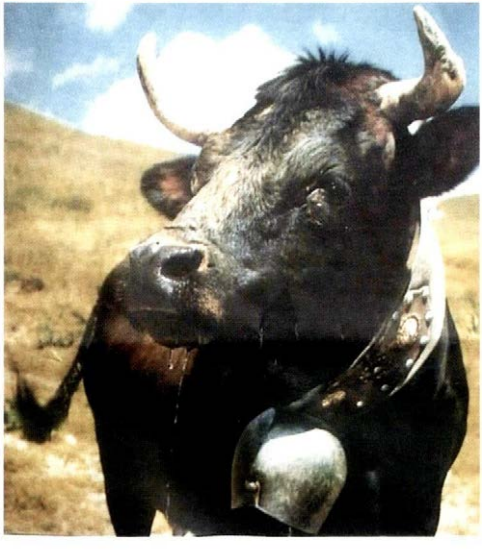


Figure 2: Eringer cow "Queen" (Melitta, 1997)

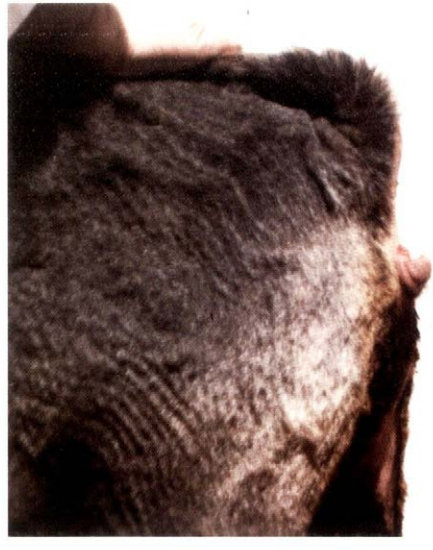


Figure 3: Clitoris protuberance

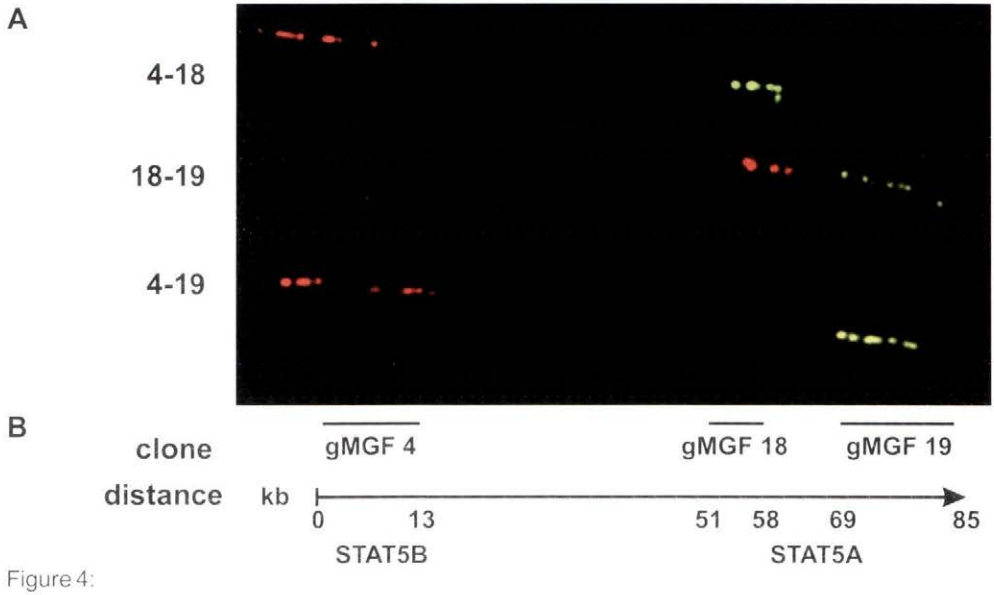


Figure 4:

A. Results of the fiber-FISH experiments with the following probe combinations and labels (in brackets): exp.1 probe mgf4(bio) - probe mgf18(dig); probe mgf18(bio) - probe mgf19(dig); probe mgf4(bio) - probe mgf19(dig);. Digoxygenine labeled probes were detected with FITC (green), biotin labeled probes with Texas red (red), respectively.

B. Physical organization of the bovine *STAT* locus as revealed by fiber FISH analysis. Hybridization probes are depicted by lines. The whole STAT locus as analyzed by 3 different clones covers about 85 kb of genomic DNA.

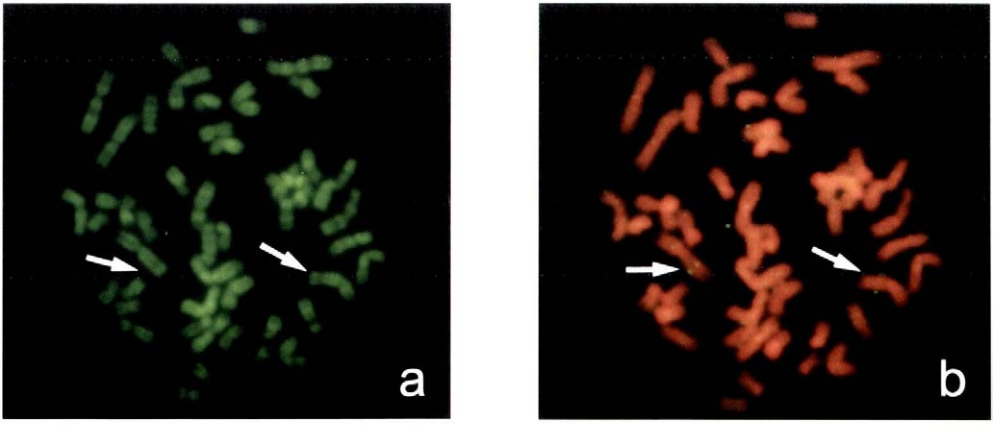


Figure 5:

Localisation of ZuBeCa4 microsatellite on the blue fox chromosomes: (a) QFQ banding, (b) FISH; chromosome pair No 4 is indicated by arrows

which stage of the cell cycle it was added. Differences in the morphological structure (banding pattern) between the chromosome of the investigated animal and the standard may also derive from mutations. A mutation on an X chromosome would probably affect the inactivation procedure. It could lead to one or more of the following effects:

- displacement of the inactivation to a different region
- additional inactivated regions
- activation of originally inactivated regions
- inactivation on both X chromosomes

Should a mutation lead to a different morphological structure, it is also probable that the expression of genes is also affected leading to phenotypical differences which may influence the behaviour of the animals.

Such an effect does not need to be a chromosome mutation; it could be only a point mutation. Since the process of inactivation is accidental, there is a 50% probability that either the maternal or the paternal X chromosome becames inactivated. In our case this probability showed no deviation and was also 50%.

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PRELIMINARY IDENTIFICATION OF THE FRAGILE X IN TWO CROSSBRED COWS

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ABSTRACT: Numerous chromosome breaks and gaps were observed in two crossbred cows (no. 177 BW x HF and no. 689 BW x Piedmontese). In the metaphase plates obtained from the cow no. 177 the breaks were observed in 17% of cells. In 7% of cells, we found an achromatic gap or break located near the middle of q arm, closer to the centromere, at a relative distance of 0.62 from the telomere of the p arm. Furthermore the breaks were visible in 18% of cells obtained from the cow no. 689 and in 5% of cells the breaks affected the X chromosome. In this case the gap or break was also localised near the middle of the q arm, but at a relative distance 0.82 from the telomere of the p arm. In the G-banded preparations two breakpoints in the X chromosome were localised: between positive and negative bands q2.3/q2.4 and in the negative G-band q3.1. Among the offspring of cow no. 177 (7 animals) two were stillborn and one died just after birth. The cow no. 689 delivered 5 calves, two out of which were stillborn. Furthermore, the interval between first and second pregnancy for this animal was long.

fragile X; cattle; cytogenetic analyse

INTRODUCTION

The X chromosome fragility in cattle has been described previously in several studies (El-Nahass et al., 1974; Genest and Guay, 1979; Uchida et al., 1986; Halnan, 1989; Llambi and Postiglioni, 1994). It was also demonstrated that the X chromosome instability causes reproductive disturbances (El-Nahass et al., 1974; Llambi and Postiglioni, 1994; Rincon et al., 1997).

The aim of the present study was to diagnose the nature of X chromosome fragility, to localise the break points and to determine the influence of this instability on the reproductive traits of cows.

MATERIAL AND METHODS

The blood samples for cytogenetic analyses were taken from two crossbred cows: no. 177 (BW x HF) and no. 689 (BW x Piedmontese), both born in 1990.

Lymphocytes were cultured in folic acid deficient RPMI and RPMI 1640 medium with folic acid concentration of 1 x 10^{-3} mg/ml. Both culture media were supplemented with 10% of foetal bovine serum, antibiotics and pokeweed mitogen. The slides were stained conventionally with Giemsa and GTG technique. The chromosome measurements were made with MultiScan computer system and the relative measurements are presented as a ratio of break point distance from the end of p arm to total length of X chromosome.

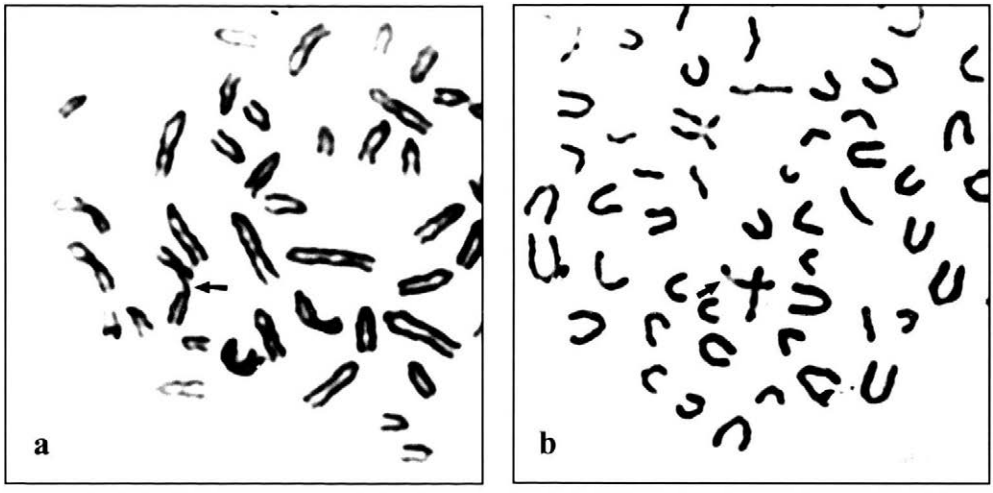
The complete report on the reproductive history of the two animals was included in the documentation.

RESULTS

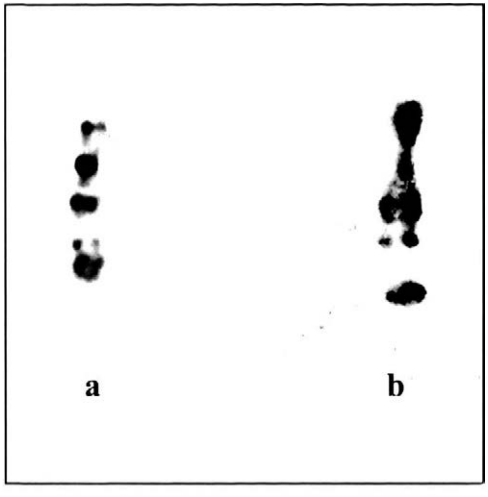
Cell preparations obtained after the culture in two different media were compared. We observed a low mitotic index in RPMI medium without folic acid and a very low frequency (about 1%) of X chromosome breaks. In the preparations obtained after the culture in conventional RPMI 1640 the mitotic index was suitable for analyses of 200 metaphase spreads per animal. The frequency of cells with gaps and breaks ranged from 17–18% and the percent of cells with fragile X chromosome ranged from 5 to 7% (Tab. I).

I. Frequency of cells with gaps and breaks in two crossbred cows

Cow no.	Number of analysed metaphase spreads	Total number (%) of cells with chromosome aberrations (gaps and breaks)	Number (%) of cells with fragile X chromosome
177	200	34 (17)	14 (7)
689	200	36 (18)	10 (5)



1. Metaphase plates of the cow No. 177 (a) and 689 (b). Arrows indicate breakpoints in the X chromosome



2. The G-banded X chromosome of the cow No. 177 (a) and 689 (b)

The mean relative distance of the break point measured in the X chromosome of the cow no. 177 was 0.62 and in X of the cow no. 689–0.82 (Fig. 1, Tab. II).

Comparing the break points on the G-banded X chromosomes with the ISCNDA (1990) standard we preliminarily localised them between positive and negative bands q2.3/q2.4 and in the negative G-band q3.1, respectively (Fig. 2).

Several reproductive traits of cows no. 177 and 689 are presented in Tab. III. Interestingly, the calving interval was long, ranging about 16.3 and 14.3 months and the proportion between live born and stillborn calves was 4/3 and 3/2 respectively, including abortion and early death.

II. Quantitative localisation of the break points in chromosome X

Cow no.	Relative break point distance measured in 10 metaphases	Mean relative distance	SD
177	0.61 0.62 0.66 0.60 0.63 0.62 0.60 0.64 0.59 0.61	0.62	0.02
689	0.76 0.82 0.77 0.88 0.85 0.84 0.86 0.87 0.75 0.76	0.82	0.05

DISCUSSION

Most of the fragile sites in chromosomes are revealed in response to the particular cell culture conditions. Fragility of X chromosome in humans is expressed under special culture conditions including cell culture in folic acid deficient medium (Sutherland and Hecht, 1985). On the contrary, the gaps and breaks in fragile X

III. Reproductive traits of cows with fragile X

Cow no.	Services/conception	Calving interval (in months)	Calves			
			Live born	Stillborn	Abortion	Early death
177	4.8	16.3	4	2		1
689	4.2	14.3	3	1	1	

chromosome in cattle are often visualised in the preparation obtained after routine culture conditions (Rincon et al., 1997). In our experiment both cases of the X fragility were observed also under normal culture conditions, so it can be concluded that two fragile sites on Xq do not represent common folate-sensitive fragile sites. On the other hand Uchida et al. (1986) found the achromatic gap in the q arm of X chromosome, closer to the centromere than that identified in humans. This gap was expressed in absence of folic acid. Possibly this site represents the folate-deficient FraX, is inherited and may cause the baldy calf syndrome, however, the achromatic gap on X chromosome was present also in healthy animals of the Holstein breed.

Llambi et al. (1999) tested if the fragile sites could be induced by aphidicolin and found that aphidicolin generated common fragile sites in cattle chromosomes including X fragility. The induction of X fragility by various culture conditions in the two cows under our investigation will be carried out in the future.

The results of cytogenetic investigation show that the breaks observed in X chromosome in cattle lead to reproductive disturbances, mainly long calving interval and reduced rates of non return for services after artificial insemination (El-Nahass et al., 1974; Llambi and Postiglioni, 1994; Rincon et al., 1997). These findings agree well with the reproductive traits observed in the two crossbred cows in the present study. Particularly the calving interval in these cows was very long in comparison to mean calving interval in the herd (about 12 months). However, we can not definitely conclude that the X chromosome instability causes the observed reproductive disturbances. This effect could be the result of a joint influence of the breaks in X chromosome and some other autosomes in which the loci determining reproductive traits are localised. In the cows under investigation we observed a high percentage of cells with chromosome breaks and gaps, which ranged from17–18%. The mean number of the cells with chromosome breaks and gaps in the herd was about 3–4%.

The identified break point q3.1 is in a location similar to the previously detected fragile site in this chromosome (Llambi and Postiglioni, 1994). Di Berardino et al. (1983) worked out the map of cattle chromosome fragile sites, induced by BrdU. The four sites were localised on the X-chromosome. Probably one of this sites is similar to our findings, showing the break point in the region q2.3./q2.4.

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ANALYSIS OF SYNAPTONEMAL COMPLEXES IN BULLS AND RAMS, CARRIERS OF XY/XX CHIMERISM

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ABSTRACT: The aim of this study was to investigate synaptonemal complexes in the bulls and rams originated from heterozygotic twins. We diagnosed dissociation of sex bivalents in the early stage of pachytene. Tissue samples used in this study were obtained from testes after castration of bulls and rams, carriers of the XY/XX chimerism. The analysis of 106 spermatocytes from two chimeric bulls and 175 spermatocytes from three chimeric rams was performed under the light microscope. The results obtained for chimeric animals and animals characterized by the normal karyotype were then compared. We found that the level of early dissociation of X-Y bivalent in chimeric bulls ranged from 2.0% to 5.6% (in the normal bulls – 3.1%). Furthermore, in the chimeric rams, frequency of the early dissociation was higher and ranged from 11.1% to 19.0% (in the normal rams – 3.4%). The early dissociation of sex bivalent could possibly be the cause of the lower fertility in males originating from dizygotic twinning.

synaptonemal complexes; XX/XX chimerism; bull; ram

INTRODUCTION

The correctness of meiosis and in consequence, of spermatogenesis is essential to animal breeding, reproduction, and development of progeny generations.

One of the best methods for evaluation of chromosome behaviour during meiosis is the analysis of protein structures appearing along homologous chromosomes (synaptonemal complexes) in pachytene.

Analysis of synaptonemal complexes has been used for estimation of pairing and segregation of the sex chromosomes in the male meiosis in several mammalian species (Gustavsson et al., 1983; Świtoński and Gustavsson, 1991; Villagomez, 1993; Dai et al., 1994; Słota, 1998).

The aim of the present study was to investigate synaptonemal complexes in the bulls and rams originated from heterozygotic twins.

MATERIAL AND METHODS

Tissue specimens used in this study were obtained from fragments of testis taken after castration from two bulls (A, B), three rams (E, F, G), all carriers of the XY/XX chimerism and two bulls (C, D), three rams (H, I, J) with normal karyotype.

Synaptonemal complexes analysis was carried out with the surface spreading technique (Counce and Meyer, 1973). The preparations were analyzed under the light microscope.

RESULTS

We analysed synaptonemal complexes in 206 spermatocyte cells from two chimeric bulls (60,XY/60,XX), and two bulls with normal karyotype (60,XY) (Tab. I).

In several cells we observed dissociation of the sex bivalents in the early stage of pachytne (Fig. 1). The level of early dissociation in the chmeric bulls ranged from 2.0% to 5.6% (in the bulls with normal karyotype – 3.1%).

I. Results of the analysis of the synaptonemal complexes in bulls

Bull	Karyotype	Number of spermato-cytes	Number of X-Y bivalents	Number of early dissociation of X-Y bivalent
A	60,XY/60,XX	50	49	1
B	60,XY/60,XX	56	53	3
C	60,XY	50	48	2
D	60,XY	50	49	1
Total		206	199	7

1. Early dissociation of X-Y bivalent in the spermatocyte of chimeric 60,XY/60,XX bull

The analysis of 325 spermatocyte cells (Tab. II) of three chimeric rams (54,XY/54,XX), and three rams with normal karyotype (54,XY) showed higher percentage of early dissociation (Figs. 2 and 3) in the chimeric rams (from 11.1% to 19.0%) in comparison to normal animals (3.4%).

II. Results of the analysis of the synaptonemal complexes in rams

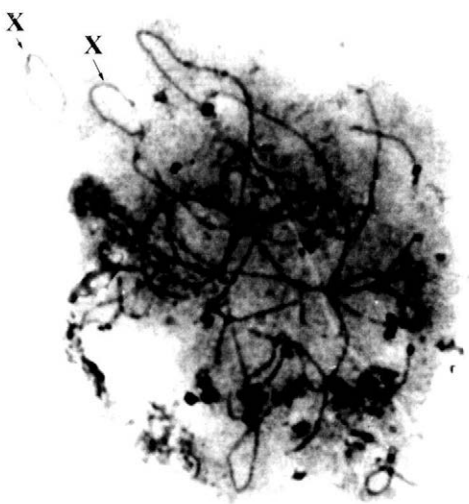
Ram	Karyotype	Number of spermatocytes	Number of X-Y bivalents	Early dissociation X-Y bivalent	Early dissociation X-X bivalent
E	54,XY/54,XX	75	65	9	1
F	54,XY/54,XX	50	42	8	–
G	54,XY/54,XX	50	45	5	–
H	54,XY	50	49	1	–
I	54,XY	50	48	2	–
J	54,XY	50	49	1	–
Total		325	298	26	1

DISCUSSION

Synaptonemal complexes are DNA-associated protein structures responsible for the pairing of homologous chromosomes during zygotene and pachytene stages of meiotic prophase I (Świtoński, 1995).

Behavior of the X-Y synaptonemal complex can be easily recognized on the background of sex chromosome morphology and pairing. Each substage of pachytene: early, mid and late displays different morphology of X-Y bivalent (Villagomez, 1993). It was shown that the appearance of X-Y bivalent is a useful tool for substaging of pachytene in cattle (Dollin et al., 1989) and sheep (Dai et al., 1994). The three essential substages are classified as follows: early pachytene – axes of X and Y chromosomes are not braided and the chromosomes are partly synapsed; mid pachytene – axes of sex chromosomes become braided and pairing can proceed beyond the pseudoautosomal region, alternatively additional pairing/association of free ends may occur; late

2. Early dissociation of X-Y bivalent in the spermatocyte of chimeric 54,XY/54,XX ram



3. Early dissociation of probable X-X bivalent in the spermatocyte of chimeric 54,XY/54,XX ram

pachytene – sex chromosomes become more braided or tangled and excrescences appear along axes (Świtoński and Stranzinger, 1998).

According to the morphology of the autosomal bivalents we found that the early dissociation of the sex bivalent took place in the mid pachytene stage.

In bulls and rams with normal karyotype the level of early dissociation of sex bivalent is about 3.6% (Świtoński et al., 1991) and 3.4% (Dai et al., 1994), respectively. These findings are in good agreement with our present results: dissociation of sex bivalent in bulls – 3.1% and in rams – 3.4%. However the level of early dissociation of X-Y in chimeric bulls observed by Switoński et al. (1991) was almost three times higher, than frequency of early dissociation diagnosed in our experiment. Furthermore, we observed also the difference between frequency of early dissociation of the sex bivalent in chimeric bulls and rams. The mean values ranged from 3.8% to 15.1% respectively.

Dissociation of the X–Y chromosome bivalent in diakinesis-metaphase I in mice may be resposible for some loss of spermatogonic cells (Krzanowska, 1989).The investigations performed in our laboratory showed the influence of chromosomal chimerism on the decrease of semen parameters of the bulls (Rejduch et al., 1998). On the other hand, Szatkowska and Świtoński (1996) did not observe any impact of chromosomal chimerism on reproductive traits in rams

In the mammals, changes in structure of chromosomes lead to pairing configurations other than correctly paired bivalents. Investigation of specific abnormalities of pairing process allows us to predict that the decrease of fertility in the carriers of various karyotype changes, due to the production of unbalanced gamets. Observation of the pairing chromosomes, by the study of the synaptonemal complexes, is an important diagnostic approach in searching for causes of decrease in fertility in domestic animals.

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LOCALISATION OF THREE CANINE MICROSATELLITES ON THE ARCTIC FOX (*ALOPEX LAGOPUS*) CHROMOSOMES

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ABSTRACT: The localisation of three canine-derived microsatellites on the arctic (blue) fox chromosomes by the use of Fluorescence In Situ Hybridisation (FISH) is presented in this paper. CanBern1 was assigned to the arctic fox chromosome 24q15, ZuBeCa4 to chromosome 4q14-16 and ZuBeCa11 to chromosome 11q11. These results are the first information about the physical localisation of canine-derived microsatellites in the blue fox genome.

microsatellites; dog; artic fox; genetic markers; FISH

INTRODUCTION

Chromosome studies of the blue fox were mainly focused on the analysis of extensive karyotype polymorphism, caused by the Robertsonian translocation. As a result of it three diploid chromosome numbers: 50, 49 and 48 were observed in populations of the blue fox (Makinen and Gustavsson, 1980; Świtoński, 1981; Møller et al., 1985).

In the Canidae family the most advanced marker genome map is that of the dog due to the outstanding progress of the DogMap project (DogMap Consortium, 1999) and similar projects. The cytogenetic map of the silver (red) fox genome, based on canine-derived cosmid probes containing microsatellites, was also recently advanced (Yang et al., 2000). In addition, comparative chromosome painting approach facilitated the identification of homologous chromosome fragments in the genomes of the human, dog, silver fox and blue fox (Yang et al., 1999; Graphodatsky et al., 2000).

In the present study the first localisation of canine-derived microsatellites in the blue fox genome is presented.

MATERIAL AND METHODS

Chromosome preparation

Peripheral blood samples were collected from normal, adult blue foxes and lymphocyte cultures were established. Chromosome slides were QFQ-banded prior to hybridisation. International nomenclature for the blue fox chromosomes was applied (Makinen et al., 1985b). Also the recent detailed description of G-banding patterns was considered (Graphodatsky et al., 2000).

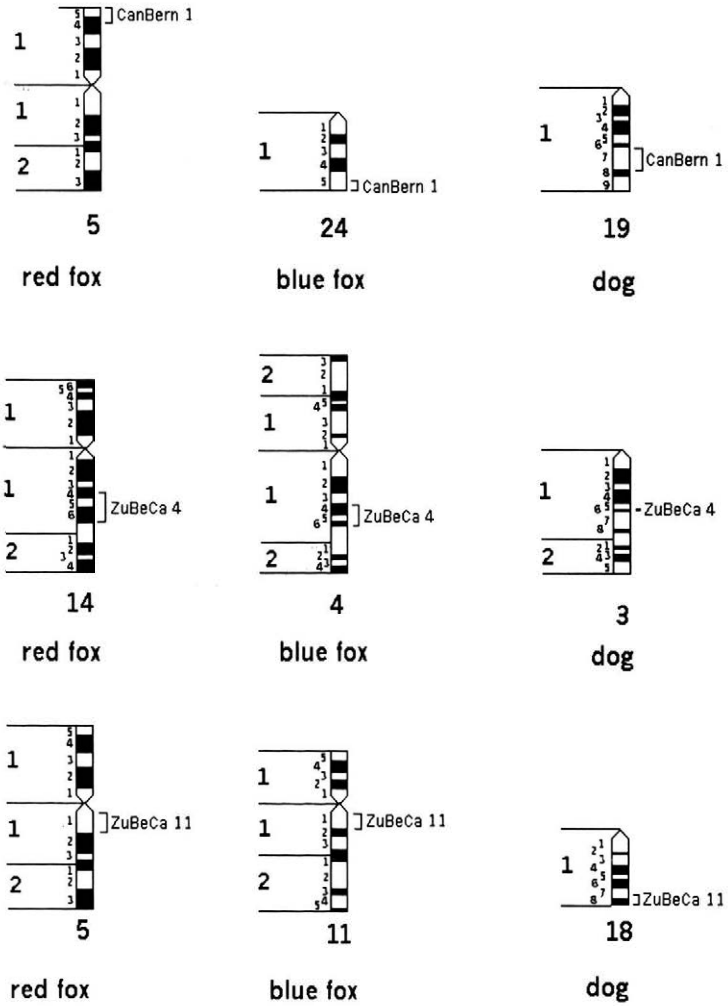
Labelling of the probes

Three canine cosmid probes, containing microsatellite sequences were used in this study (Yang et al., 2000). Cosmid DNA (400 ng) was labelled with biotin-16-dUTP (Boehringer Mannheim) using a nick translation kit (Boehringer Mannheim) following protocols recommended by the manufacturer. The reaction was stopped by incubation at 65 °C with 2 µl of 0.5M EDTA for 10 min.

Fluorescence in situ hybridisation

Slides were denatured in 70% formamide/2 x SSC for 2 min at 70 °C, plunged immediately into ice-cold 70% ethanol, dehydrated in an ethanol series (70, 80, 90 and 100%), and air-dried. Biotin-labelled cosmid probes (100 ng per slide) were denatured by heat at 70 °C for 10 min, pre-annealed for 10 min at 37 °C, applied onto slides and incubated at 37 °C overnight. Post-hybridisation washes were three 5-min incubations in 50% formamide/2 x SSC at 42 °C, followed by three 5-min incubations in 2 x SSC at 42 °C. For signal detection and for amplification a system of avidin-FITC and anti-avidin (Vector Laboratories) was used. Staining was performed with propidium iodide (200 ng/ml).

Image capture and processing were performed with a fluorescence microscope (Nikon E 600 Eclipse) equipped with a FITC filter, a CCD cooled camera, driven by computer aided software Lucia.



1. Comparative localiation of three microsatellites on the silver (red) fox, blue (arctic) fox and dog G-banded chromosomes. Details concerning the localisation are given in Table I. G-banding patterns follow international chromosome nomenclature for the silver (red) fox – Makinen et al. (1985a); blue (arctic) fox – Makinen et al. (1985b) and dog – Switonski et al. (1996)

RESULTS AND DISCUSSION

According to the standard karyotype of the blue fox (Mäkinen et al., 1985b), three microsatellites were assigned to the following chromosomes: 4q14–16 (ZuBeCa4) (see Colour Plate, Fig. 5a, b), 11q11 (ZuBeCa11) and 24q15 (CanBern1). All the three microsatellites, studied in this paper, were assigned to the dog and silver fox chromosomes (Yang et al., 2000). Unfortunately, in that paper the assignment of ZuBeCa4 on the red fox chromosomes was confusing since in a table the localisation was 14p23 while on a figure it was 14q23. This misleading information was probably the result of a printing mistake. Until now no such experiments were carried out on the blue fox chromosomes. Details concerning chromosome localisation of these three cosmids on the dog, red fox and blue fox chromosomes are given in Tab. I and are shown on Fig. 1.

The dog genome map can be successfully used to guide the assignment of the canine markers to the fox chromosomes. The application of comparative chromosome painting with the use of canine chromosome specific probes brought information about chromosome arms homology between the dog and silver fox karyotype (Yang et al., 1999) and also the dog and blue fox

I. Localisation of three microsatellites on the dog, red fox and blue fox chromosomes

Microsatellite	Dog *	Red fox*	Blue fox (this study)
CanBem1	19q17	5p22	24q15
ZuBeCa4	3q16	14q23	4q14-16
ZuBeCa11	18q18	5q12	11q11

* Yang et al. (2000)

karyotypes (Graphodatsky et al., 2000). Reciprocal chromosome painting and DAPI-banding comparison demonstrated that the remarkable karyotype differences between the red fox and the dog are due to 26 chromosomal fusion and 4 fission events (Yang et al., 1999). The comparative map between the blue fox, red fox and dog demonstrated that the genomes of these species consist of 42 highly conserved autosomal segments, with 34 segments each equivalent to a single dog chromosome (Graphodatsky et al., 2000).

The chromosome localisation of three microsatellites, presented in this study, is in agreement with results obtained from comparative chromosome painting studies by Graphodatsky et al. (2000). Further FISH experiments with canine-derived probes will allow more precise establishing of the blue fox genome map.

Our study shows that the development of the physical genome map of the dog brings an opportunity to develop the cytogenetic genome map, apart from that of the red fox, also of the blue fox. It is important to emphasize that the above species are farm animals and the annual world production of furs exceeds 4.5 mln items.

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SISTER-CHROMATID EXCHANGES INDUCED BY METAL CONTAINING EMISSION IN SHEEP PERIPHERAL LYMPHOCYTES AFTER *IN VIVO* AND *IN VITRO* EXPOSURE

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ABSTRACT: The effect of *in vivo* feeding and by standard *in vitro* assay of metal-containing emission originated from aluminum proceeding factory were tested to induce sister-chromatid exchanges (SCE) in sheep peripheral lymphocytes. Content of the emission tested, was performed by Al, Cd, As, Mn, Pb, Cu, Zn, Fe, Na, Ca, and Mg; majority of them as sulphide, sulphate, oxide or fluoride form. The experimental animals were given a daily dose of emission of either 0.75 g or 1.5 g per animal, for 1 year. The experiments were aimed to induce the chronic fluorosis. The occurrence of SCEs in the experimental group was higher (reaching the statistical significance at a dose 1.5 g/animal). The emission was found to reduce the proliferation index as well as to influence the mitotic index. The experiment was compared with sheep peripheral lymphocytes in the *in vitro* assay. The emission was dissolved and neutralised according to the standard method and analysed spectrophotometrically. Level of SCE was investigated at three concentrations of 30, 60 and 90 μg/ml, respectively with and without of S 9 mix metabolic activation. The lowest concentration used corresponds to daily oral intake for sheep grazing on contamined area. Results from both of *in vitro* assays were similar; the significant increasing of SCE started at the concentration of 60 μg/ml ($p < 0.01$) but more perspicuous results were seen without of S 9. No significant decrease was found in the induction of proliferation index. Indirect verification for genotoxic influence of the emission tested has been given by local veterinarians keeping evidence of developmental abnormalities. Total percentage of developmental defects in the district represented 9.58% all of the developmental disorders in cattle reported in the middle Slovakia territory at the given time.

pasture contaminants; genotoxicity; sister chromatid exchanges; sheep

INTRODUCTION

There are many genotoxic effects caused by environmental exposure to metals and non-metallic compounds. Almost all the metals and non-metallic compounds present in the emission tested have been reported to have some limited or positive genotoxic effects in different assays.

Aluminum (Al) is considered to be cytotoxic due its interference with the GTP-ase cycles (Exley and Birchall, 1993). It had also limited cancerogenic effect on chronically exposed workers in the aluminum reduction industry (Pearson et al., 1993). The majority of *in vivo* and *in vitro* cytogenetic assays performed to evaluate clastogenic potency of cadmium (Cd) yielded negative results (Léonard and Bernard, 1993); however, cadmium chloride induced DNA single-strand breaks, DNA protein cross-links, and chromosomal aberrations in V79 Chinese hamster cells (Ochi et al., 1984). Cadmium sulphate exhibited also a limited teratogenic effect inducing developmental abnormalities in the skeleton of mice (Murata et al., 1993).

Arsenic (As) is one of the few identified human carcinogens that has been shown to cause cancer in rodents (Moore et al., 1997). Its genotoxic effect is documented by numerous data about its clastogenic effect in different assays, e.g. SCE in human peripheral lymphocytes by an *in vitro* assay (Andersen et al., 1982); micronuclei (MN) in Chinese hamster ovary cells (Wang et al., 1997); chromosome aberrations (CA) in cultured human lymphocytes (Sweins, 1983). Lead (Pb), copper (Cu), and iron (Fe) as well as other metal (II) ions – zinc (Zn) and cobalt (Co) are also considered to be potentially genotoxic due to inhibition of nucleotide excision repair (Calsou et al., 1996).

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On the other hand, zinc (Zn) as well as magnesium (Mg) are known to act as protective agents against the genotoxic damage (Smith et al., 1994; Littlefield et al., 1994).

Fluoride (F^-) is not mutagenic in standard bacterial tests, but it produces chromosome aberrations and gene mutations in cultured mammalian cells (Zeiger et al., 1993). *In vivo* cytogenetic studies in mice did not confirm the clastogenicity of fluoride reported above (Zeiger et al., 1993).

Genotoxicity estimation in complex mixtures meets with serious problem because they might contain hundreds or thousands of chemicals, and many of them cannot be identified and quantified by current analytical capabilities (Houk and Waters, 1996). The present study will allow us to compare the effect of exposure to metal – containing emission on sheep peripheral lymphocytes after long term oral exposition with *in vitro* experiments carried out according to standard conditions.

MATERIAL AND METHODS

Emission

The industrial emission originated from aluminum reduction industry was collected and applied as a water mixture to the two groups of experimental animals consisting of five 2 years old sheep. The daily oral doses were 0.75 and 1.5 g per animal, i.e. 30 and 60 mg/kg of b. w., respectively. The one year long-termed experiment was aimed clinically, to induce the chronic fluorosis. Pollutants those used in the *in vitro* test were dissolved using the standard method neutralised and filtered. The concentration of dissolved metals and non-metallic compounds was analysed spectrophotometrically (Perkin-Elmer; Dept. of Internal Diseases, University of Veterinary Medicine in Košice). Percentual content of the emission is seen at Tab. I. The daily intake of emission by sheep grazing on contaminated area (30 mg/kg b. w.) as well as its arithmetic multiplies (60 and 90 mg/ml), were used in our *in vitro* experiments. The multiple next to the highest one used (120 mg/ml) was cytotoxic.

I. Composition of emission

Element	Per cent
Al	4.3
Cd	0.00475
As	0.00775
Mn	0.0626
Pb	0.1398
Cu	0.1398
Zn	0.036
Fe	1.54
Na	1.09
Ca	0.234
Mg	0.049
F	20.0

Each of elements was present in the ionic form as a sulphide, sulphate; or oxide chloride and fluoride.

Cultivation of cells

Heparinized peripheral blood samples (0.5 ml) from 5 different healthy donors in both of experimental *in vivo* exposures and control group were cultured in 5 ml of RPMI 1640 medium supplemented with L-glutamine, 15 μM/l HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum, antibiotics (penicillin 250 U/ml and streptomycin 250 μg/ml), and phytohaemagglutinin (PHA, 180 μg/mL, Welcome, Darford, England) at 38 °C 72 h. Bromdeoxyuridine (BrdUrd, Sigma) was added to all cultures at a final dose of 8 μg/ml 48 h before the harvest. To achieve cytostatic block, colchicine (Merck, Darmstadt, Germany) was added at a concentration of 5 μg/ml 2 h before the harvest.

The general approach for SCE estimation comprises *in vitro* assays with and without of metabolic activation. The experiments were carried out using heparinized blood samples from two healthy donors both of sexes, one year old, kept and fed under standard conditions. The samples collection and cultivation conditions, those they were described previously. The influence of different doses of emission in the experiment without metabolic activation took place lasted 24 h of cultivation. Metabolic activation in the *in vitro* assay was realised by freshly prepared S9 fraction according to the method of Maron and Ames (1983) (10% of the culture volume) applied to all control and experimental cultures. Cultures enriched with S9 mix were treated by the emission for 2 h, and then the cells were rewashed twice and replaced to the fresh culture medium. Mitomycin C (MMC, Sigma, St. Louis, MO, USA, 4 μM) and cyclophosphamide (CPA, Jenapharm, Ankerwerk, Rudolstadt, Germany, 4 μg/ml) were used as positive control agents in assays with absence and in presence of the metabolic activation (S9 mix).

Evaluation and statistic methods

Chromosome preparations were obtained by the standard method in both of experiments. Slides were stained with FPG technique to differentiate sister chromatides and cell cycles (Perry and Wolff, 1974) for the SCE delumination and cell cycle kinetics. Fifty differentially stained metaphases per donor and concentration were examined for the SCE and 100 metaphases for determination of M_1, M_2 and M_3, mitotic divisions. The proliferation index (*PI*) were calculated according to Lamberti et al. (1983). Statistical analysis of results was performed using a simple analysis of variance (ANOVA). Then Student's *t*-test was applied to evalua-

II. Effect of emission upon induction of SCEs and mitotic delay in sheep peripheral lymphocytes after chronic *in vivo* exposure

Group	SCE / cell	Mitotic cells (± SE)			(*PI* ± SE)
		M_1	M_2	M_{3+}	
Control	7.86 ± 0.45	65.25 ± 2.59	33.75 ± 2.28	1.0 ± 0.57	1.34 ± 0.0029
Emission (g/sheep)					
0.75	9.17 ± 0.44 [a]	78.5 ± 2.87*	20.5 ± 2.75	1.0 ± 0.57	1.225 ± 0.03[a]
1.5	9.28 ± 0.18*	76.7 ± 4. 1*	21.67 ± 3.84	0.66 ± 0.33	1.229 ± 0.043[a]

A total of fifteen animals were used for the control and experimental groups A total of 250 metaphases of each group were analysed for SCEs.

a – insignificant differences between treated and untreated groups (non parametric *t*-test was used for estimation of mitotic delay)

* – statistical significance $p < 0.05$ using Student's *t*-test and test to evaluate induced SCE and variability in the frequencies of M_1, M_2 and M_{3+}, respectively compared to the control

te the SCEs significance for the treated and untreated groups and χ^2 test for proliferation kinetics indices.

RESULTS

The results of cytogenetic studies in sheep peripheral lymphocytes after *in vivo* exposure are summarised in Tab. II. No significant increase in the frequency of SCEs was observed after administration of emissions at daily dose of 0.75 g/animal. When compared to the control, a dose of 1.5 g/animal caused a significant elevation in the mean of SCE ($p < 0.05$, Student's *t*-test), but the value of SCEs was not much higher than that with previous dose.

With both doses, the proportion of M_1, M_2 and M_{3+}, was significantly different from that seen in the control cultures ($p < 0.05$, χ^2 test). These results are reflected in the reduction of the *PI*; the difference of induced mitotic delay between the experimental and the control groups was not significant.

The frequencies of SCEs as well as proliferation kinetics induced by emission in the *in vitro* experiments both with and without of S9 mix metabolic activation are shown in Tab. III. A dose dependence of the induced changes was seen in both the assays. Positive clastogenic effect started at concentration of 60 μg/ml ($p < 0.01$) in both experiments, i.e. with and without metabolic activation. The highest concentrations tested (90 μg/ml) was also statistically significant. The results performed the level $p < 0.001$ according to the Student's *t*-test. More conspicuous increase in SCE frequency was observed in the latter assay, i. e. without metabolic activation.

A weak decrease in proliferation indices without of dose dependence and statistical significance were also observed (Tab. III). In both experiments, the proportion of M_1, M_2 and M_{3+} were not significantly different from those seen in control groups. The difference in induced mitotic delay between control and experimental groups reflected by *PI* was not thus verified.

DISCUSSION

Clinical observations after 10 month long-term fluoride intake from industrial emission revealed a slight effect on the haematopoetic system (anemia, eosinophilia and leucopenia). The next exposition to the emission caused statistical increasing of serum fluoride and aluminum level as well as accumulation of fluoride, aluminum, arsenic and cadmium in several organs (e.g. teeth, bone, liver, muscle, kidney and spleen) in groups of experimental sheep (Šiviková and Dianovský, 1995).

III. Frequency of SCEs and proliferation indices in sheep cultured peripheral lymphocytes exposed to the industrial emission with and without of S9 mix metabolic activation

Treatment	SCE/cell (mean ± SD)	*PI*	Treatment	SCE/cell (mean ± SD)	*PI*
Control (PBS)	6.72 ± 2.12	1.56	Control (PBS)	6.61 ± 1.42	1.54
Emission (μg/ml) 24 h (–S9)			Emission (μg/ml) 24 h (+ S9)		
30	6.52 ± 1.44 a	1.56 a	30	6.56 ± 1.54 a	1.52 a
60	7.65 ± 1.93 **	1.48 a	60	7.71 ± 1.77 **	1.54 a
90	9.00 ± 3.05 ***	1.50 a	90	8.66 ± 2.21 ***	1.46 a
Positive Control 0.4 μM, MMC	9.60 ± 3.05***	1.46 a	Positive Control 4μg/ml, cyclophosphamid	10.90 ± 3.25 ***	1.44 a

A total of 100 second – division metaphases of each group were analysed for SCE.

*, **, *** statistical significant data ($p < 0.05$, $p < 0.01$, $p < 0.001$: ANOVA, Student's *t*-test)

a – no statistical significance according to previous or c^2 test

PI – proliferation index

Human or animal peripheral lymphocytes are widely used to demonstrate clastogenic effects after exposure to mutagenic agents. Most lymphocytes populations are long-lived and it is believed that they accumulate DNA lesions during chronic exposure to genotoxic agents (Stetka et al., 1978). Induction of sister chromatid exchanges represents a sensitive cytogenetic endpoint for detection of genotoxic activity of environmental mutagens and carcinogens (Who, 1993).

Our results predicted the cytogenetic effects some of heavy metals ions (namely aluminum and fluoride) from an industrial emission that was fed to sheep for 1 year at doses of 0.75 g or 1.5 g/animal. Under condition of the present study no significant increase of SCE frequency in the first of concentration mentioned. The latter had a positive effect on SCE induction in comparison to the control ($p < 0.05$). Since only a small differences were found in the mean of SCEs/cell between both experimental groups. These data signify a statistically formal difference, insufficient to constitute the genotoxic hazard. They could indicate the greater sensitivity to cumulative genotoxic influences during long-term experiment because of lacking the relevant dose-related effect, on the other hand.

We have found dose-related SCE increases in both *in vitro* assays. On the basis of more conspicuous results in the assay without S9 metabolic activation we can conclude that the metabolic capacity of self-activating erythrocyte enzymes was sufficient for such type of experiments (Cantelli-Forti et al., 1988). On the other hand the 24-h exposure to the emitted material resulted in weak reduction of lymphocyte proliferation. The results were not statistically significant, and were not related to the dose used.

Various methods can be used to evaluate the *in vivo* and *in vitro* processes such as those described by Cardot and Beyssac (1993). We shall focus on the correlation which offers the greatest scope of matters studied, namely one–to-one relationship between the *in vivo* input and *in vitro* dissolution rates. This procedure has been used for a basic estimation of dose in the *in vitro* test. The basic dose 30mg/ml, exibiting a weak cytotoxic effect in the chronic oral experiment in sheep (Šiviková and Dianovský,1995) had no significant genotoxic or cytotoxic effect. The arithmetic increase of the doses tested led to a dose related response expressed in the SCE induction without any cytotoxic effect. This fact predicts the highest sensitivity for the *in vitro* assay. Because a linear relationship between the frequencies of SCE and gene mutations was suggested (Carrano et al., 1979), induced point mutations and the related changes might cause the genotoxic effect of the emission. Cumulative genotoxicity of industrial pollutants or other environmental factors is detectable in veterinary practice using different indirect criteria (Wurgler and Krammers, 1992). They include the resistance of insect (especially wormflies or ticks) to insecticides; bacterial resistance to currently used antibiotics; increased frequency of cancer in different animal species living in contaminated areas; reproductive errors or increase in developmental abnormalities in newborns. In spite of numerous resources dealing with cancerogenity, reproductive disturbances, mutagenic effect and other related changes of majority of metals and non-metallic compounds obtained in the emission, we did not confirm them because of incompleteness of data. Local veterinarians are obliged to keep evidence of developmental defects in newborns only of economically important species. According to reports given to respective authorities (Center for Genetic Prevention), the frequency of developmental defects in cattle (axial skeleton and body cavities, especially) was remarkably higher –14 in the affected district Žiar nad Hronom, in comparison to the adjacent districts – 6 in Zvolen, or 2 in Lučenec. Total percentage of developmental defects in the district represented 9.58% of all developmental disorders reported in cattle in the middle Slovakia territory at the given time.

In conclusion we can indicate the positive genotoxic effect of metal emission in cultured sheep peripheral lymphocytes, namely in *in vitro* assays.

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A DECREASE OF SISTER CHROMATID EXCHANGES INDUCED BY CARBON TETRACHLORIDE IN CULTURED SHEEP PERIPHERAL LYMPHOCYTES BY VITAMIN E AND SELENIUM

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ABSTRACT: The protective effect of vitamin E and selenium was studied for their possibility to decrease the sister chromatid exchanges (SCE) induced by carbon tetrachloride (CCl_4) in cultured sheep peripheral lymphocytes *in vitro*. Whole blood cultures of two healthy male lambs were treated with carbon tetrachloride at the concentrations of 2, 4, 8 and 16 μg/ml for the last 24 h of cultivation and subsequently with the same dose of CCl_4 and both nutrients vitamin E and selenium. After treatment of sheep peripheral lymphocytes with CCl_4 for 24 h, no dose-dependent increase of SCE was obtained. The statistically significant elevations in the mean of SCEs were seen at the concentrations of 4 and 8 μg/ml ($p < 0.001$ and $p < 0.01$, respectively) in both donors. No significant effects on SCE increases were recorded after the treatment with CCl_4 at the lowest and the highest dose. The later was also shown to reduce the proliferation index (*PI*) in both donors ($p < 0.001$ and 0.01). Vitamin E and selenium significantly inhibited CCl_4-induced SCEs at the concentration of 4 mg/ml in comparison to the corresponding non-protective dose ($p < 0.001$). No or a weak protective effect was achieved in the cultures with CCl_4 at the concentrations of 2 and 8 mg/ml supplemented with both nutrients in comparison to the cultures exposed to CCl_4 alone. No improvement of cell cycle kinetics was achieved.

carbon tetrachloride; sheep peripheral lymphocytes; sister chromatid exchanges; vitamin E; selenium

INTRODUCTION

Selenium and vitamin E are recognised as essential nutrients for humans as well as animals that have provided an essential mechanism for the control of peroxidation in tissues. At the early experiments the addition of vitamin E and/or selenium to the diet of experimental animals was studied to be shown a protective effect against a number pathological conditions (Fisher and Whanger, 1997). Most of the deficiency diseases in animals (e.g., polymyopathy, hepatic necrosis and exudative diathesis) are induced by dietary deficiencies of both the vitamin E and selenium, and supplements of either nutrient will offer satisfactory protection (Van Vleet and Watson, 1984).

As for the genotoxic or clastogenic effects of CCl_4 the micronucleus assay in vivo was widely employed. No increased frequency in micronuclei were obtained in mouse bone marrow and mouse peripheral lymphocytes in comparison to the controls after administration of a single intraperitoneal injection of carbon tetrachloride (Suzuki et al., 1997; Crebelli et al., 1999).

In contrary to *in vivo* assays, the positive clastogenic and aneugenic effects were found in metabolically competent human cell lines (Doherty et al., 1996) and in occupationally exposed persons, e. g. to a mixture of chlorinated solvents or carbon tetrachloride in combination with chlordecone (da Silva Augusto et al., 1997; Ikegwuonu and Mehendale, 1991).

Carbon tetrachloride is a known hepatotoxic agent commonly used for the study of liver damage in experimental animals (Di Renzo et al., 1982; David and Kroening, 1998). This substance was confirmed to cause a cancer in rodents after prolonged exposure to CCl_4 (Westbrook-Collins et al., 1990).

Most hepatotoxic effects of CCl_4 are attributed to its metabolism by cytochrome P450 (Raucy et al. 1993). Especially CYP2E1 has been demonstrated to be largely responsible for activation of CCl_4 to toxic metabolites, carbon dioxide and chloroform (Doherty et al., 1996).

Berger et al. (1986) reported at least two mechanisms by which CCl_4 induces injury in isolated hepatocytes. The first phase of damage occurs within several minu-

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tes of exposure to CCl_4 and the second is evident after 3 h, characterised by a marked increase in malondialdehyde formation, a fall in cellular glutation and substantial further cellular damage. These changes could be moderate due to the cytochrome P-450 inhibitors and antioxidants. Pre-treatment of experimental animals with vitamin E (α-tocopherol) was shown to be effective against carbon tetrachloride induced acute liver necrosis (Biasi et al., 1991; Odeleye et al., 1992; Parola et al., 1992).

In this paper we report the influence of antioxidants vitamin E and selenium on carbon tetrachloride induced sister chromatid exchanges (SCE) in the sheep peripheral lymphocytes *in vitro*.

MATERIAL AND METHODS

Carbon tetrachloride (99.8% – Microchem, Bratislava, Slovak Republic) was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and applied into all treated cultures with CCl_4 alone at the concentrations of 2, 4, 8, 16 μg/ml and, respectively with the same doses of CCl_4 plus nutrients. The top dose of CCl_4 was selected on a basis of the reduction in mitotic index (*MI*) > 50%. The final DMSO concentration in the treated and control cultures was 0.1%.

Vitamin E and selenium (as Tocopheryl acetate, 400 mg and sodium selenite, 12 mg, Bremer Pharma, GmbH, Bremerhaven, Germany), was dissolved in distilled water and applied into the control and treated cultures at a dose of 1 μg/ml. The dose of vitamin E and selenium (Se) was chosen after the producer recommendation and calculated according to Hopkinson et al. (1993) for *in vitro* conditions. Mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4 μM) was used as a positive control agent.

Experiments were carried out on two healthy male lambs' donors (Merino, 2–3 months old).

Whole blood cultures (0.5 ml,) were cultivated for 72 h at 38 °C in 5 ml of RPMI 1640 medium supplemented with L-glutamine, 15 μM HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum, antibiotics (penicillin 250 U/ml and streptomycin 250 μg/ml), and phytohaemagglutinin (PHA, 180 μg/ml, Welcome, Darford, England).

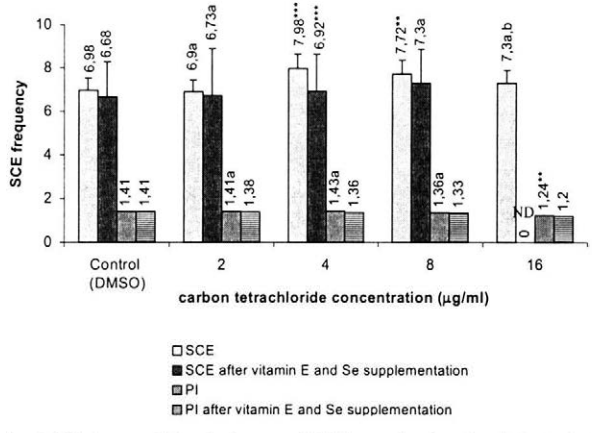
Lymphocyte cultures were exposed both to CCl_4 alone and to CCl_4, plus vitamin E and Se for the last 24 h. Bromodeoxyuridine (8 μg/ml, BrdUrd, Sigma, St. Louis, MO, USA) and colchicine (5 μg/ml, Merck, Darmstadt, Germany) were added to all cultures 24 h after initiation of division and 2 h before harvest, respectively. Slides were obtained by the standard cytogenetic method and stained with the FPG technique to differentiate cell cycles (Perry and Wolff, 1974).

Fifty differentially stained metaphases per donor and concentrations were examined for SCE, and 100 metaphases were analysed for determination of M_1, M_2 and M_{3+} mitotic divisions.

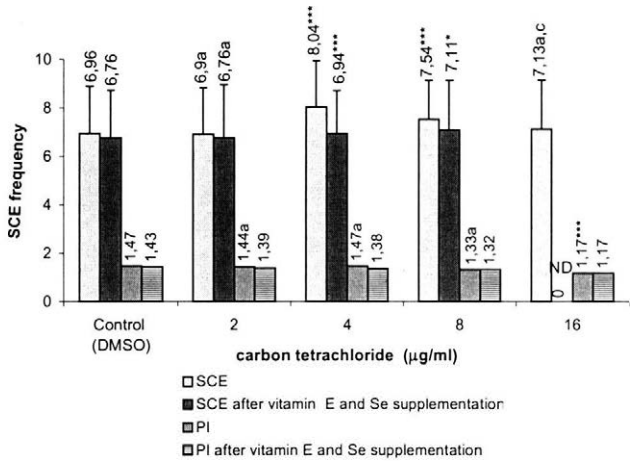
Statistical analysis of results was performed using simple analysis of variance (ANOVA), then the Student,s *t*-test was applied to evaluate SCE occurrence between treated and untreated groups and χ^2 test for estimation of the cell cycle delay was appropriated.

RESULTS

The data obtained in the SCE assays after the treatment with CCl_4 and simultaneously with CCl_4 plus vita-



1. Carbon tetrachloride induced SCE (means ± SD) and a decrease of SCE frequencies after vitamin E and selenium supplementation in donor 1
A total of 50 second – division metaphases of each group were analysedif it was possible.
*, **, *** statistically significant data ($P < 0.05$, $P < 0.01$, $P < 0.001$; ANOVA, Student's *t*-test for SCE and χ^2 test for *PI*)
a – no statistical significance, b – insufficient number of second metaphases – 25
ND – not done



2. Carbon tetrachloride induced SCE (means ± SD) and a decrease of SCE frequencies after vitamin E and selenium supplementation in donor 2

A total of 50 second – division metaphases of each group were analysed for SCEif it was possible.

*, **, *** statistically significant data ($P < 0.05$, $P < 0.01$, $P < 0.001$; ANOVA Student's t-test for SCE and χ^2 test for PI)

a – no statistical significance

c – insufficient number of second metaphases – 20

ND – not done

min E and Se in sheep peripheral lymphocytes are presented at the Figs. 1 and 2.

A 24 h exposure to carbon tetrachloride alone showed the elevations in the mean of SCEs with a statistical significance in both donors at the concentrations of 4 and 8 μg/ml, ($p < 0.01$ and $p < 0.001$, respectively; ANOVA and Student's t-test). The maximum frequency of exchanges was observed with a dose of 4 μg/ml. No significant enhancements in SCE induction were obtained after the treatment with the lowest and the highest doses (2 and 16 μg/ml, respectively, $p > 0.05$), but for the later concentration an insufficient number of second metaphases could be analysed (25 or 20 for each donor). This concentration was also reflected in the reduction of the *PI* in cultures of both donors (χ^2 test, $p < 0.001$ and $p < 0.01$).

Most expressive inhibition of CCl_4 induced SCE during the last 24 h was achieved in cultures with 4 μg/ml of carbon tetrachloride supplemented with vitamin E and Se; a decrease of SCE had a high statistical significance $p < 0.001$ in both donors. The lowest dose of carbon tetrachloride (2 μg/ml) with both nutrients caused no statistically significant decrease in induced SCE frequencies in comparison to the cultures with CCl_4 alone. Also a weak protective effect of vitamin E and Se was seen in cultures with a concurrent dose of 8 μg/ml CCl_4 only ($p < 0.05$), but no improvement in proliferation kinetics was observed. Vitamin E and selenium treatment alone caused no decrease of SCE in comparison to the corresponding controls. The mean frequencies of SCE after treatment with a positive control (0.4 mM, MMC) were 9.53 and 9.64, respectively in each donor.

DISCUSSION

Our results demonstrate that concurrent treatment of vitamin E and selenium with CCl_4 could reduce the SCE frequencies in comparison to the corresponding non-protective cultures.

SCE are very sensitive, although non-specific markers (Tucker and Preston, 1996), efficiently induced by a chemical substances that form covalent adducts to the DNA or otherwise interfere with the DNA metabolism or repair (Morimoto et al., 1985). The baseline SCE frequencies in peripheral lymphocytes vary among individuals. Besides the genetic factors, also culture conditions *in vitro* may affect the SCE frequency; namely BrdUrd concentration and serum used as well as time of sampling or cell cycle kinetics. Di Berardino et al. (1997) reported that the spontaneous SCE frequencies in the sheep peripheral lymphocytes were 4.08 to 7.82 SCE/cell if the BrdUrd concentration ranged from 0.1 to 5 μg/ml were used in the cultures. The mean level of SCE in young sheep donors included in our experiments was 6.96 and 6.98 in controls with DMSO and in the presence of BrdUrd at a dose of 8 μg/ml of medium. Taking into account also the environmental factors Holečková et al. (1993) did not described any significant differences in the elevation of chromosome

damage in the sheep peripheral lymphocytes associated with an age or food.

Positive clastogenic and aneugenic results after treatment with carbon tetrachloride was found in the human metabolically competent cell lines; MCL –5, which stably expresses cDNAs encoding human CYP1A1 and h2E1 cell line, containing a cDNA for CYP2E1 (Doherty et al., 1996). A significant higher frequency of micronuclei were observed in occupationally exposed persons to a mixture of chlorinated solvents (carbon tetrachloride and others) in comparison to the group that had not been exposed (da Silva Augusto et al., 1997). Carbon tetrachloride in combination with chlordecone was genotoxic using in the *vivo-in vitro* animal model and a batery of biochemical assays to measure the DNA repair in the rat hepatocytes (Ikegwuonu and Mehendale, 1991). In contrast to these findings, after treatment of human lymphocytes with different doses of carbon tetrachloride for 1/2 h with or without S9, no elevation in SCE induction and chromosome aberrations were shown (Garry et al., 1990).

Based upon our data of genotoxicity for 24 h a significant effect of carbon tetrachloride on SCE increases was found at a dose of 4 μg/ml ($p < 0.001$, Student's *t*-test). When compared to this dose, both higher concentrations tested (8 and 16 μg/ml) caused a decrease in the mean of SCEs. A decrease of SCE frequency correlated with the marked cell cycle delay in both donors ($p < 0.001$ or 0.01).

Positive protective effects of vitamin E and selenium in cultures during the last 24 h were reflected in a significant decrease of SCEs in comparison to a corresponding non-protective dose of 4 μg/ml CCl_4 ($p < 0.001$) in both donors. A weak decrease in induced SCE frequencies was obtained at the concentration of 8 μg/ml. The highest concentration could not be analysed, because no improvement of proliferation rates was achieved after the vitamin E and selenium supplementation.

Supplementation of rats with tocopherol analogs (d-α-tocopherol and tris salt of d-α-tocopheryl hemisuccinate) at either 6 or 18 h prevented CCl_4-induced increases in microsomal lipid peroxides (Tirmenstein et al., 1999). The later was more effective in a decrease in the CYP2E1 activity after 18 hours. These authors suggest that the loss of CYP2E1 activity 18 h after of d-α-tocopheryl hemisuccinate – administration has been caused by a decrease in the amount of CYP2E1 present in the cell rather than enzyme inhibition.

CCl_4 is highly soluble in cell lipids. Berger and Sozeri (1987) and Johnston and Kroening (1998) suggested that early hepatocyte death was independent of the metabolism of CCl_4, and might be related to the direct effects of CCl_4 on the intracellular membrane.

Fariss et al. (1993) demonstrated α-tocopheryl hemisuccinate as a powerful cytoprotective agent against CCl_4 hepatotoxicity *in vivo*. As to selenium, Ianas et al. (1995) described a global beneficial action of the Se preparation upon the organism in the CCl_4 exposed rats as well as a strong antioxidative effect confirming the essential role of Se in maintaining the cellular integrity. A protective effect of Se against cadmium genotoxicity in the Chinese hamster V79 cells was reported by Hurná et al. (1997). Similarly Piešová et al. (2000) described a positive effect of both the vitamin E and selenium in the decrease of micronuclei in sheep lymphocytes induced by CCl_4 *in vivo*.

Both vitamin E and selenium are included in the protection of CCl_4-induced lipid peroxidation, the later through its involvement in the biosynthesis of glutatione peroxidase, in a secondary antioxigenic role (Tappel, 1980; Hafeman and Hoekstra, 1977).

Our results showed that both nutrients have the ability to reduce the chromosome damage induced by CCl_4. Under the conditions of the present study a protective effect of vitamin E and selenium was found only partially at the selected concentrations. At the highest concentration rather cytotoxic than genotoxic effects were seen. Furthermore, no improvement in proliferation rates after nutrient supplementation has been observed. In conclusion we assume that just prolonged sampling times might be important to confirm the genotoxicity of the agent and the subsequent protective influence of both nutrients.

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CYTOGENETIC AND TUMOUR SUPPRESSOR GENE STUDIES ON FELINE SOFT TISSUE TUMOURS

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ABSTRACT: Soft tissue tumours represent a heterogeneous group of mesenchymal tumours including e.g. fibrosarcomas, malignant fibrous histiocytomas (MFH) lipomas and neurofibrosarcomas. Our cytogenetic analyses of such tumour types have led to the observation of recurrent types of numerical and structural chromosomal alterations including rearrangements like translocations. Despite the apparently nonrandom and recurrent observation of several anomalies, the detection of a strictly specific anomaly in fibrosarcomas and malignant fibrous histiocytomas remains often problematic and the karyotypic appearance is very heterogeneous. Lipomas tended to the occurence of recurrent simple rearrangements.The same patients were subjected to tumour suppressor gene analyses in the genes coding for p53, p21 WAF1, p27 kip1 and p16 MTS1/INK4. We observed mutations in p53, but not in p21 WAF1, p27 kip1 and p16 MTS1/INK4. The p53 mutations were five point mutations (two in exon 5, two in exon 7 and one in exon 8) in thirty investigated fibrosarcomas and one point mutation (in exon 7) in fifteen MFHs.

cytogenetics; tumour; suppressor; feline

INTRODUCTION

The presence of a wide spectrum of different benign and malign soft tissue tumours makes the reliable distinction between them often difficult. In the veterinary praxis, cats bearing soft tissue tumours, especially fibrosarcomas and/or malignant fibrous histiocytomas (MFHs) belong to the most common oncological patients. The vaccine-association in a minor subset of fibrsarcomas is widely discussed. The use of cytogenetic techniques in the analyses of soft tissue tumours in the domestic cat has led to the identification of chromosomal abnormalities associated with fibrosarcomas (Mayr et al., 1991, 1994, 1996c), malignant fibrous histiocytomas (Mayr et al., 1996b), lipomas (Mayr et al., 1996a) and neurofibroma (Kalat et al., 1990).

Molecular genetic methods have rapidly been developed in the last decades and applied to many field of veterinary research. In veterinary oncology, a panel of oncogenes and tumour suppressor genes was detected in a broad array of neoplasms. In the field of feline tumour suppressor genes, investigations in a series of neoplasms were initiated.

MATERIAL AND METHODS

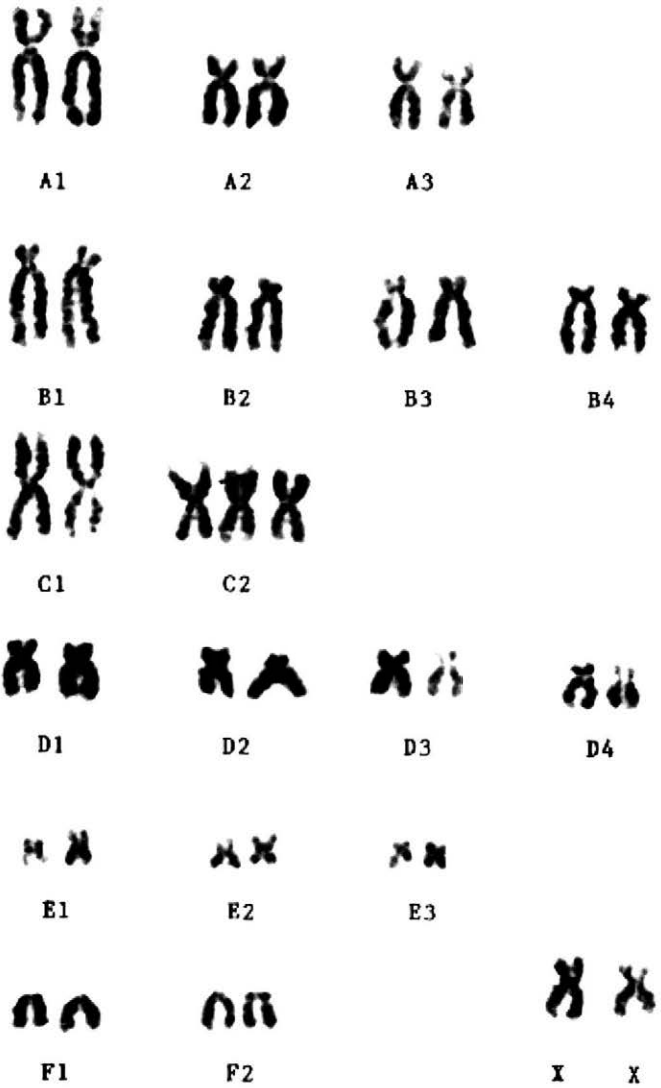
Material taken from 7 fibrosarcomas, 7 malignant fibrous histiocytomas (MFHs), 4 lipomas, 2 neurofibrosarcomas and 2 fibromas of 16 tumour bearing cats was analyzed cytogenetically. Molecular genetic studies involving tumour suppressor genes p53, p21 WAF1, p27 kip1 and p16 MTS1/INK4 was done in the same sixteen and in further patients. In summary, thirty fibrosarcomas, fifteen malignant fibrous histiocytomas, 4 lipomas, 2 neurofibrosarcomas and 3 fibromas were molecular genetically screened for the presence of alterations in the suppressor gene.

Cell culture

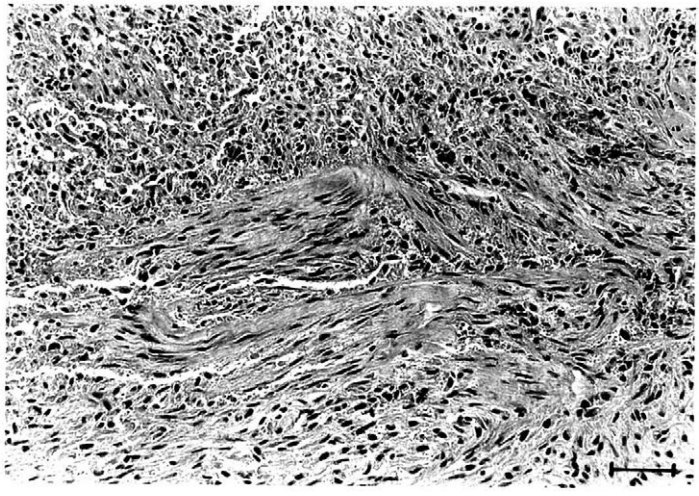
For cytogenetic studies, primary explant cell cultures were established by mincing the solid tissue into small fragments (< 1 mm^3). Fragments were transferred into sterile flasks containing 8 ml of RPMI 1640 medium, with L-glutamine, antibiotics (50 IU of penicillin and 50 µg of streptomycin/ml of medium) and 10% fetal bovine serum. Explants were cultured in 5% CO_2 in air for 10 days until harvesting. Cell metaphases were analyzed by use of trypsin-G-banding according to the method of Wang and Fedoroff (1972).

Extraction and amplification of DNA

For molecular genetic p53, p21 WAF1, p27 kip1 and p16 MTS1/INK4 analyses, DNA was extracted from the tumour tissue specimens, using standard techniques according to Müllenbach et al. (1989). Polymerase chain reaction (PCR) primers and PCR-conditions for the different tumour suppressor genes were described earlier (Okuda et al., 1994, 1997a, b). The products were analyzed by use of a 4% NuSieve/agarose gel du electrophoresis. The products were eluted from the Tris-borate-EDTA (TBE) gels, using a kit. The PCR products were sequenced, using a kit and an automated sequencer.



1. Pathohistology of a feline neurofibrosarcoma (bar represents 60 μm)



2. Trisomy 39, +C2 is present in this patient

RESULTS

Fibrosarcomas and malignant fibrous histiocytomas (MFHs) are rather common in our veterinary practices. However, the chromosome aberrations detected were remarkably heterogeneous. The chromosome aberrations were sometimes variable with diploid over hyperdiploid to near triploid or near tetraploid number. However, besides of this observation a nonrandom pattern of structural abnormalities involving deletions in the long arm A1q or B1q was present in several patients. Two patients had a deleted long arm E1q in a few MFHs. Other cases possessed a high percentage of trisomic cells, e.g. chromosome A2 or chromosome D1 in their tumour.

Recurrent translocations in the fibrosarcomas observed were t (D3q; E1q), t (A1q; B4p) or t (A1p; E1p). Further frequently recurrent changes were observed in the structure and/or the number of the F1-chromosome.

Neoplasms of the adipose tissue, also belongs to the group of soft tissue tumours and they showed distinct cytogenetic abnormalities. In our patients we observed altered F1-marker chromosomes and translocation t(B4; C1q) as aberrations in lipomas.

In the rare neurofibrosarcomatous neoplasms recurrent alterations were trisomies, e.g. of chromosome C2 (Fig. 1 and 2) or D2. These patients were also studied together with other patients for mutations in several tumour suppressor genes in 30 fibrosarcomas and 15 histiocytomas. However, we could not detect any mutation in the tumour suppressor p21 WAF, p27 kip1 and p16 MTS1/INK4. In contrast, five point mutations were present in the tumour suppressor p53.

DISCUSSION

The present study including soft tissue tumours clearly demonstrated the occurence of different aberrations in different patients and neoplasms. The study led to the detection of recurrent types of numerical and structural chromosomal aberrations. In principle, our data are in good correspondence with human data, where very few specific abnormalities have been found in fibrosarcomas, MFHs and related soft tissue tumours, but the karyotypic profiles are clearly nonrandom (Örndal et al., 1994; Heim and Mitelman, 1995; Sreekantaiah et al., 1998).

Molecular genetic studies performed will demonstrate the presence of novel genes, e.g. oncogenes and locally highly activated genes at the translocation junctions and their unique tumour-specific chimeric fusion products in the next future. As regards tumour suppressor gene mutations were detected in p53 only, but not in p21 WAF1, kp27 kip1 and p16 MTS1/INK4, this situation is similar to the rare occurence of these mutations in human.

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REVIEW ARTICLE

CHROMOSOME ABNORMALITIES IN HUMAN CANCER

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ABSTRACT: The karyotypic changes in malignant tumor cells are unevenly distributed throughout the human genome. Modern cancer cytogenetics showed that different chromosomal bands are preferentially involved in rearrangements in different neoplasms and specific aberrations were identified. Due to the availability of bone marrow cells first insights were done into pathogenesis of hematologic malignancies and were accompanied by elucidation of the role of chromosomal translocations and deletions. Deletions result very often in loss of a tumor suppressor genes whereas specific translocations and inversions lead to the two principal consequences: 1. new fusion gene encoding chimeric protein is created-mostly in myeloid disorders, 2. gene for the immunoglobulin or T-cell receptor is moved near to the proto-oncogene and enhaces its activity – mostly for lymphoid disorders. All three above mentioned rearrangements were later on proved in solid tumors as well. The breakpoints of many translocations specific for different hematologic and solid tumors have been cloned and serve as molecular markers for diagnosis. Cytogenetic analyses are part of the routine workout of the patients. A variety of molecular techniques are now available for wide genome screening of alterations in copy number, structure and expression of genes and DNA sequences. Molecular cytogenetics has special methods: fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), spectral karyotyping (SKY) and multicolor FISH (mFISH). Except for the basic research of human neoplasias all these methods are used routinely to monitor the effect of the treatment and follow the residual tumor cells after chemotherapy and/or bone marrow transplantation.

tumorous chromosome abnormalities; fluorescence *in situ*; hybridization

INTRODUCTION

The roots of oncocytogenetics can be found in the last years of the 19th century at the time when Arnold (1879) described mitotic errors in tumor cells. However, as a real founder of tumor cytogenetics is considered Boveri, who published in 1914 the first chromosomal hypothesis of origin of malignant tumors. Boveri suggested that tumor cells are originally normal cells and their abnormal behavior lies inside of the cells and not in their surrounding. The content of chromatin in tumor cells is abnormal and the normal cellular properties are lost. At that time the quality of the cytological techniques did not enabled to test this hypothesis and actually it took more than 40 years before the exact number of human chromosomes was established by Tjio and Levan (1956) and clinical cytogenetics became an important part of pre- and postnatal genetic counselling.

Oncocytogenetics is independent part of the medical genetics concerned with acquired chromosomal changes in benign and malignant tumor cells. Aberrant karyotype was found first in hematologic malignancies, and later on in solid tumors in more than 50% of specimens of bone marrow cells and/or peripheral lymphocytes studied by classical cytogenetic techniques. The proof of existence of pathological clone in examined sample is according to Heim and Mitelman (1995) the presence of two mitoses with the same chromosomal rearrangement or extra copy of the same chromosome, or three mitoses with the same chromosomal loss.

CHROMOSOMAL ABERRATIONS IN NEOPLASTIC TUMORS

The chromosomal changes can be numerical and/or structural ones and are specific for certain type or subtype of the tumor. Cytogenetic findings in different types of tumors serve not only to the basic research of pathogenesis of neoplastic process but are routinely used by clinicians to set up precise diagnosis of the patients, specially in hematologic diseases.

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1. Schematic representation of reciprocal translocation t(9;22) (q34;q11). The breakpoints are indicated by squares on individual chromosomes and the fused BCR/ABL gene is localized on Ph chromosome

Clinical applications of cytogenetic examinations are as follows:

1. Detection of numerical and structural chromosomal abnormalities
2. Identification of marker chromosomes
3. Monitoring of the effects of the therapy and detection of minimal residual disease
4. Detection of early relapse
5. Identification of the origin of bone marrow cells following bone marrow transplantation
6. Examination of the karyotypic pattern of non-dividing or interphase cells
7. Detection of gene amplification
8. Prognosis of the individual patients

Chromosomal translocations

Consistent and specific translocations and inversions are involved in tumor aetiology activating proto-oncogenes at or near the chromosomal breakpoints (Tab. I). Genes, localized at the specific breakpoints are studied by molecular biology methods. These genes are cloned and sequenced and their functions and products are studied from the point of view of their role in the pathogenesis of malignant disease. As an example can be quoted extensive research which was done by analysing the specific translocations in leukemias. The typical representative of specific translocation which is present in 95% of patients with chronic myeloid leukemia (CML) is so called Philadelphia chromosome (Ph-chromosome). It is reciprocal translocation between long arms of chromosome 9 and 22 – t(9;22)(q34;q11). Schematic representation of t(9;22) is in Fig. 1 with marked localization of the genes which are moved during the translocation event.

Two types of translocations were described in hematologic malignancies. The first one is leading to the occurrence of new, fused, hybrid gene coding product which differ somewhat from the original one, while the result of the second type is the displacement of the genes, mainly oncogenes, close to the immunoglobulin genes and enhancement of their functions. Both types of translocations play important role in malignant transformation of the cells (Rabbits, 1994). It is hypothesized that the same mechanisms exist in solid tumors and that there are not more then these two types of translocations. Translocation t(9;22)(q34;q11) belongs to the first type and new hybrid gene is coding the protein with different tyrosine kinase activity.

1. Breakpoints of the most frequent specific chromosomal translocations, affected genes and diseases with this type of chromosomal rearrangements

Fused genes – hematopaetic tumors

t(9;22) (q34;q11)	c-ABL/BCR	CML, ALL
t(15;17)(q21;q11)	PML/RARA	APL
t(8;21)(q22;q22)	AML1/ETO	AML-M2
t(4;11)(q21;q23)	MLL/AF4	ALL, pre-B ALL, AML
t(1;19)(q23;p13.3)	PBX1/E2A	Pre-B ALL
t(12;21)(p13;q22)	TEL/AML1	Children pre-B ALL
inv(16)(p13q22)	CBFβ/SMMHC	AMMoL

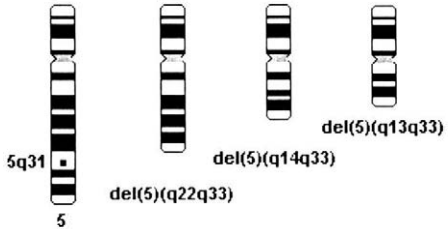
Fused genes – solid tumors

t(11;22)(q24;q12)	FL1/EWS	Ewing´s sarcoma
t(12;22)(q13;q12)	ERG/EWS	Ewing´s sarcoma
t(12;16)(q13;p11)	CHOP/FUS	Liposarcoma
t(2;13)(q35;q14)	PAX3/FKHR	Rhabdomyosarcoma

Non-fusions – hematopaetic tumors

t(8;14)(q24;q32)	c- MYC (8q24)	BL, BL-ALL
t(8;14)(q24;q11)	c-MYC (8q24)	T-ALL
t(8;12)(q24;q22))	c-MYC (8q24)	B-CLL, ALL
t(14;18)(q32;q21)	BCL-2 (18q21)	FL
t(11;14)(q13;q32)	BCL1/PRAD1	B-CLL and others

Disease nomenclature: CML – chronic myeloid leukemia, ALL – acute lymphocytic leukemia, APL - acute promyelocytic leukemia, AML-M2 – acute myeloid leukemia, AMMo4 – acute myelomonocytic leukemia, BL – Burkitt lymphoma, B-CLL – B-type chronic lymphocytic leukemia, FL – follicular lymphoma



2. Diagrammatic presentation of interstitial deletions of chromosome 5 found in patients with myelodysplastic syndrome

Chromosomal deletions

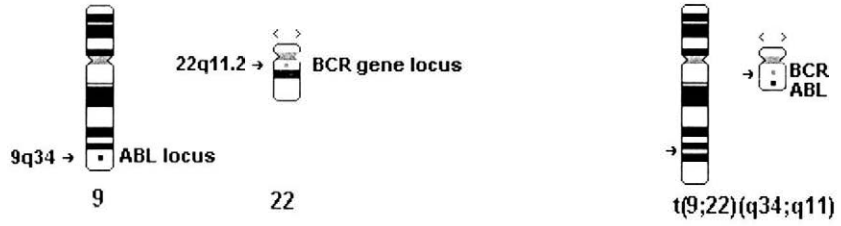
Deletions are specific acquired rearrangements which are connected with the loss of chromosomal material. Most of the deletions are interstitial and we can find them in the tissue of solid tumors, but they were described in hematologic malignancies as well. The first were cytogenetically and molecularly studied three solid tumors of childhood – retinoblastoma, neuroblastoma and Wilms tumor. Retinoblastoma tumors may develop sporadically or are hereditary and one or both eyes can be affected. Retinoblastoma has served as an example to elucidate the role of tumor suppressor genes in carcinogenesis. Retinoblastoma gene (Rb gene) which was cloned in 1986 was the first known tumor suppressor gene and its loss was proved in tumor tissue. Retinoblastoma became a model for extensive studies of tumorogenesis (Horsthemke, 1992; Heim and Mitelman, 1995). Since that time the loss of tumor suppressor genes was determined in many solid tumors and hematologic malignancies and the significance of deletions is studied on molecular level. As one of many examples the loss of p53 gene in solid and hematologic malignant tumors can be presented. For preleukemias, the interstitial deletion of long arms of chromosome 5 was described by van den Berghe in (1985) and 5q- syndrome has become a special clinical entity (Fig. 2). Many other malignancies with specific acquired deletions were described and these findings are routinely used for diagnostic work. But there is still necessary to perform cytogenetic studies in large cohort of patients with certain type of malignancy and specific acquired chromosomal deletions to elucidate significance of these changes for course of the disease and response to the therapy.

Numerical chromosomal changes

Numerical chromosomal aberrations i.e. gain or the loss of the whole chromosome are trisomies and monosomies which can be found in different tumors. By many cytogenetic studies it was proved that every numerical change may be found-even as the sole anomaly in almost every neoplastic disorder (van den Berghe, 1999). During the last years numerical chromosomal changes were found in benign tumors as for example trisomy 7 in tumors of the digestive tract (Heim and Mitelman, 1995) and consistent monosomies and trisomies were found in hematologic neoplasias. Pathogenetic significance of such abnormalities is totally unknown. Diagnostic and prognostic value of different aberrations, determined by classical chromosome banding techniques is now well established, mainly for hematologic tumors.

MOLECULAR CYTOGENETICS

Main progress in oncocytogenetics brought molecular cytogenetic techniques. Different modifications of fluorescence in situ hybridization (FISH) were introduced into routine practice and new, specific chromosomal changes were found. As an example we present the reciprocal translocation t(12;21)(p13;q22) which can be found in bone marrow cells of almost 30% of children with B-cell acute lymphocytic leukemia (B-ALL). This translocation is not detectable by G-banding, because involved parts of the short arms of chromosome 12 and long arms of chromosome 21 are approximately of the same size and color intensity after G-banding (Fig 3). Translocation t(12;21) was found using double color FISH method (Romana et al., 1995). Now the molecular basis of this translocation is known (fusion of TEL



3. Reciprocal translocation between chromosomes 12 and 21. Fused gene AML1/TEL is located on derivative chromosome 21

and AML1 genes) and all children with acute lymphocytic leukemia can be screened by PCR for presence of t(12;21). However, cytogenetic examinations at diagnosis and during the treatment are necessary as well, because additional chromosomal changes can be found. As one of the most frequent trisomy 21 is present in patients bone marrow cells. According to the results of extensive European study it seems that TEL/AM1 translocation, unlike many others, gives good prognosis to the patients. We participate in the prospective study in Czech Republic, where the treatment and course of the disease is evaluated in children which have t(12;21) as a single change in the karyotype in comparison to those who have additional complex chromosomal rearrangements (Zemanová et al., 1999).

Moreover, a variety of techniques are now available for wide genome screening of alterations in copy number, structure and expression of genes and DNA sequences. Molecular cytogenetics has special techniques of comparative genomic hybridization (CGH), spectral karyotyping (SKY) and multicolor FISH (mFISH) (Schrock et al., 1996; Speicher et al., 1996; Ried et al., 1997). SKY and mFISH are the newest modifications of the hybridization techniques. These techniques allow in one hybridization experiment to color all human chromosomes and identify chromosomal rearrangements. These both methods are above all suitable to identify even cryptic chromosomal translocations and insertions, which are not resolved by classical cytogenetics (Le Beau, 1996). Double color FISH, which is currently used in most laboratories, is suitable to confirm chromosomal changes which are suspected on the basis of conventional cytogenetic analyses. Small rearrangements, specially the reciprocal translocations and insertions, are not discovered by G-banding despite the fact that the quality of classical chromosomal preparations has improved tremendously during the last decade and practically reached the resolution limit of light microscopy. However, the identification of such changes is of great importance both in the analysis of somatic cells of patients with congenital syndromes as well as in malignant tumor cells.

In malignant tumors, where the acquired chromosomal aberrations were found in almost 75% of patients, we can expect to ascertain by mFISH new recurrent chromosomal changes which could play role for clinical and molecular biological diagnosis (Heim and Mitelman, 1995; Le Beau, 1996). Non-random chromosomal changes in malignancies often mirror events at the molecular level and provide entry points for gene identification strategy (Bishop, 1987; Ried et al., 1997; Gilliland, 1998) and contribute further to the understanding of malignant cell transformation.

As mFISH adds another level of resolution, the possibility of existence of malignant cells with normal karyotype at this level should be re-evaluated. So far only few patients with cancer and normal karyotype established by classical cytogenetics were examined. Beverloo et al. (1999) published results obtained by examination of 20 patients with acute myeloid leukemia (AML) and normal classical cytogenetic findings, that were also examined by SKY method of multicolor FISH. The authors did not find the unusual chromosomal changes in bone marrow cells of these patients, but discovered cryptic rearrangements, not identifiable by classical G-banding in patients with complex karyotype. Therefore they expect more chromosomal changes in patients with advanced malignancy. Their preliminary results support our findings in patients with advanced myelodysplastic syndrome and deletions of 5q (Michalová et al., 1999). The deletion of 5q31 is typical chromosomal change in patients with myelodysplastic syndromes (MDS). The clone with deletion can be found at the time of diagnosis and later, during the progression of the disease, further clonal evolution towards numerical and structural aberrations of chromosomes can be seen. During the last four years we concentrated on analyses of instability of deleted chromosome 5 in patients with advanced forms of MDS. We have found that deleted chromosome 5 is involved in many complex rearrangements with different partner chromosomes for translocated parts of 5q deleted chromosome

CONCLUSIONS

Cytogenetic research as well as clinical oncocytogenetics progressed remarkably during the last few years. The advances in generation of DNA probes, *in situ* hybridization technology, fluorescence microscopy and fast progress in digital and spectral imaging gave the chance to study precisely genetic rearrangements in malignant tumors. Many findings of previous research will be reviewed by molecular cytogenetic techniques. The application of new techniques in human tumors added significant amount to our knowledge of non-random tumor specific genetic changes and their significance in cancerogenesis. Clinical applications of cytogenetic results are routinely used to improve diagnostic and can help to devise therapeutic regimens.

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