Kinetics of division in PHA-stimulated pig lymphocytes[1]

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Summary. Our results indicate that pig lymphocytes in culture complete their 1st division at 24 h. At 36 h there are 9% of cells in 2nd division. 3rd mitosis appears at 48 h; and at 72 h there are cells engaged in the 4th division.

Short-term blood cultures are one of the most widespread experimental systems employed to analyzed the frequency and type of chromosome aberrations induced by physical and chemical agents. However, in order to obtain reproducible results it is necessary to take into account several variables which influence the chromosome aberration frequency.

One of the most important among these variables in the length of time that lymphocytes are maintained in culture. Cells with unstable chromosome aberrations usually have mechanical difficulties at division. Accordingly, after the 1st mitosis, cells may be lost, become poliploid, or survive but often with changes in the type or frequency of aberrations. Hence, it has been strongly recommended to employ the shortest possible culture time in order to restrict the analysis to only 1st division lymphocytes. In PHA-stimulated cultures of human lymphocytes, it has been well established that at 48 h practically all the dividing cells are 1st mitosis products (27). Conversely, results in species other than human beings are scanty or lacking.

Lately, we started in our laboratory a series of experiments aimed at determining the incidence of chromosome aberrations induced by X-rays in pig blood cultures. Since no data on the kinetics of pig lymphocyte division has thus far been published, we decided to analyze this problem in order to determine the best time to harvest the cultures. Results presented in this report will show that the time-sequence of pig lymphocyte division differs considerably from that established for human lymphocytes.

Material and methods. A total of 15 cultures were started with blood from 8 different animals (5 males and 3 females). Leukocyte cultures were initiated by adding 0.5 ml of total blood to 4.5 ml of culture medium consisting of 70% Parker 199 TC medium and 30% inactivated calf serum. The Parker 199 medium was enriched with 20 mg % of glutamine and 13 mg % of arginine. Each blood culture received 0.1 mg of phytohemagglutinin, 50 U/ml of penicillin, 50 µg/ml of streptomycin and 5 µg/ml of BrdU. Cultures were kept in complete darkness at 37.5 °C. Colchicine at a final concentration of 0.1 µg/ml was added during the last $1^1/_2$ h of culture. Harvestings were performed at 24, 36, 48 and 72 h of culture.

Cells were hypotonically shocked and fixed in 3:1 methanol-acetic acid. Chromosome spreads were prepared by air drying. Stain differentiation between BrdU uni-and bifilarly substituted chromatids was obtained by the method of Koremberg and Freedlander[4]. A total of 100 metaphases per culture were analyzed and the percentage of cells in 1st, 2nd, 3rd and 4th mitosis was obtained.

Results and discussion. The BrdU-Giemsa technique has proved to be an excellent method to identify the number of mitosis performed by the cell[5]. With this method, metaphases showing similar stain in both chromatids of all chromosomes are known to be in the 1st mitosis. After 2 rounds of replication in BrdU, the chromosomes show one chromatid more intensely stained than the other and a variable number of SCEs per cell. Metaphases showing approximately one half of the chromosomes with pale stain in both chromatids, and the other half with differential stain between sister chromatids, are considered to have gone through 3 rounds of replication in BrdU. Finally, cells in the 4th mitosis exhibit differential sister chromatid stain in about $^1/_4$ of the chromosomes.

The table furnishes data on the percentage of cells in 1st, 2nd, 3rd or 4th mitosis in each one of the harvesting times employed. This table shows an early wave of mitosis which appeared 24 h after starting the cultures. 12 h later, that is in 36-h-cultures, 9% of the cells are engaged in their 2nd mitosis, Moreover, in 48- and 72-h-cultures, the percentage of 2nd mitosis increases and cells start to appear in 3rd and 4th mitosis respectively. The division kinetics of PHA-stimulated human lymphocyte cultures is at the present time well-known. It comprises the following stages: a) DNA-synthesis starts approximately at 24 h, b) between 36 and 48 h the 1st wave of mitosis appears, c) after 72 h the number of dividing cells increases, appears a variable percentage of cells in 2nd mitosis appears and a small amount of cells in 3rd mitosis[3,5,6]. Accordingly, it is clear that pig lymphocytes are able to divide earlier and more frequently than human lymphocytes. This phenomenon probably arises by the combination of 2 events: the PHA-induced blastic transformation appears earlier and the length of the cell cycle is shorter in pig than in human lymphocytes.

Pig lymphocyte cultures have been employed on at least 2 occasions to analyze the type and frequency of chro-

Percentage of cells in 1st, 2nd, 3rd or 4th mitosis at different harvesting times of pig blood cultures

Time of harvesting (h)	Cultures	Metaphases analyzed	1st mitosis (%)	2nd mitosis (%)	3rd mitosis (%)	4th mitosis (%)
24	4	400	100	0	0	0
36	2	200	91	9	0	0
48	3	300	78	19	3	0
72	4	400	20	65.7	8	6.3

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mosome aberrations induced by X-rays and to establish comparisons with other species[7,8]. Moreover the chromosome action of radiation and chemical compounds has also been evaluated in leukocyte cultures of rabbits[9-11], mouse[7,12], marmoset[7], wallaby[7], Chinese hamster[7], kangaroo-rat[13], crab-eating monkey, beagle dog, cynomolgus monkey, slow loris, squirrel monkey, sheep and goat[8,13]. In rabbits and cows, several experiments were done in order to determine the appearance of the 1st wave of mitosis and the influence of the harvesting time in the yield of dicentrics[10,11]. However, the data from human beings were extrapolated for all the other species, and therefore 40–48 h harvesting times were employed in all these cases.

Our results show that PHA-stimulated pig lymphocytes are able to divide once, twice or even thrice in 40–48 h. Accordingly, to obtain only 1st mitosis, 24 h seems to be the best harvesting time for pig blood cultures. These findings stress the necessity of determining the division kinetics of cultured lymphocytes in all those species to be employed to test the chromosome action of chemical and physical agents. Such a precaution will avoid errors arising by the presence of cells in 2nd and 3rd mitosis in the sample of dividing cells scored.

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Occurrence of a new type of mosaicism in *Apis mellifera*[1]

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Summary. A mixture of tissue with male and female olfactory plates (sensilla placodea) was observed in the antennal segments number 8, 9 and 10 of a gynandromorphic honeybee obtained by radiation from a Co^{60} source.

Many different types of gynandromorphs were described in honeybees, including a specimen with a mixture of male and female tissue in the eye and in the abdomen[2-5]. There is genetic evidence which indicates that gynandromorphic honeybees usually originate from a zygote and one or more accessory sperms[6,7]. By induced increase and decrease of queen's oviposition rate, the gynandromorph production has been raised to 32.5% and lowered to 6.0%[8]. Mosaic females which developed from doubly fertilized binucleate eggs, have also been found[9]. In wasps, patterns of mosaicism in the antennae and legs of *Habrobracon juglandis* were described and abnormal polarity of the sensilla placodea was observed[10]. However, there are no references about tissue mixture in the same antennae or in the same segment of the flagellum in *Apis mellifera*. It is possible that no one has looked carefully for individual antennae in order to detect the phenomenon (Rothenbuhler, personal communication). The gynandromorph production in insects can be provoked by chilling the eggs or heat treat-

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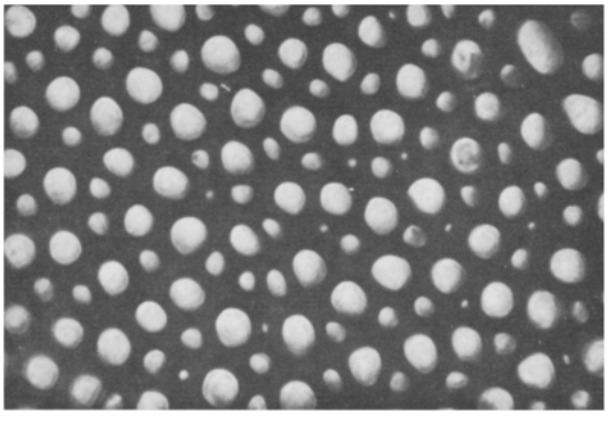

Fig. 1. Photograph of a segment of antennae of normal worker honeybees. Bigger round structures: olfactory plates. Intermediate and smaller round structures: sensorial hairs. × 500.

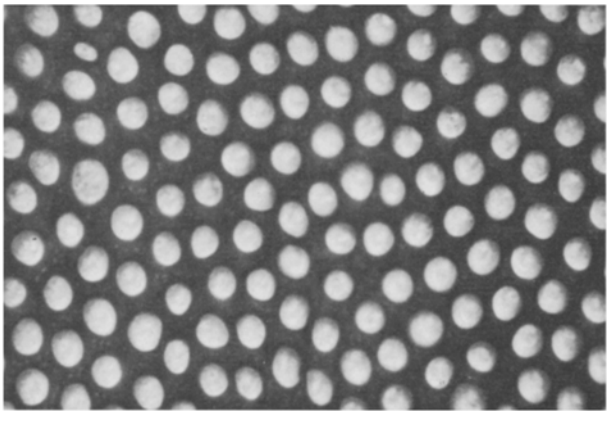

Fig. 2. Photograph of a segment of antennae of normal drone. Round structures: olfactory plates. × 500.