Differentiation of human lymphocytes into nuclear vlimata by meiosis. The cytotoxic effect of calcium-activated neutral proteinase inhibitor

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Summary. Phytohaemagglutinin (PHA)-activated lymphocytes differentiated into nuclear vlimata (NVs) in vitro. Lymphocyte attachment was followed by formation and extrusion of cytoplasmic vesicles, nuclear elongation and fragmentation into NVs. NVs and cytoplasmic vesicles were detached and organized into large cell nodules in suspension. Immunocytochemistry showed that T-lymphocytes differentiated mainly to NVs while B-lymphocytes to buds. During differentiation there was a loss or gain of T-antigenicity by either mother or daughter cell. Cytogenetic analysis by chromosomal spreading and in situ techniques showed that NVs do carry chromosomes of hypodiploid or hypohaploid sets. NVs were the result of meiosis stimulated by PHA. A lymphocytic population exhibited diploidies attributed to mitosis or symmetrical meiosis and hypodiploidies, hypohaploidies attributed to meiosis. A comparison between NVs and spermatozoa was provided. The inhibitor of calcium-activated neutral proteinase (CANP-I) was cytotoxic to NVs without interfering with NV production. A model was proposed for the action of CANP-I which is a promising agent against autoimmune diseases.

Key words: Lymphocyte differentiation, Cytogenetics, Cytology, Nuclear vlima, Ca⁺⁺-activated neutral proteinase, CANP inhibitor, Immunocytochemistry, Meiosis

Introduction

Nuclear vlima (NV) is a new term defining a spermatozoo-like cell, consisting of a nuclear head and tail, carrying DNA, produced by incomplete, unequal and asymmetrical cell division. Nuclear vlimata are parasitic, common in human neoplasia, invading and dividing in the cytoplasm and/or nucleus of host cells *in*

vivo and in vitro (Logothetou-Rella, 1993a).

Human pre-activated peripheral lymphocytes also form NVs *in vitro*, closely associated with a specific extracellular matrix (ECM) which is produced by the interaction of malignant with normal cells. In addition Phytohaemagglutinin (PHA)-activated lymphocytes changed the fusiform type tumor nodule into the adenoid type (Logothetou-Rella, 1993b, 1994).

In this study an attempt is made to examine and correlate morphology with cytogenetics of PHAactivated lymphocytes and to investigate the effect of calcium-activated neutral proteinase inhibitor on NVs.

Materials and methods

Lymphocytic cell culture and cytogenetics

Lymphocytes were isolated from human blood in six healthy donors, by density gradient centrifugation (Boyum, 1968). Lymphocytes (5 x 10⁶) were seeded in a 25 cm² Costar flask, cultivated in 10 ml RPMI-1640, supplemented with 10% foetal bovine serum (Gibco), penicillin (100 U/ml, Gibco), streptomycin (100 μ g/ml, Gibco) and five drops of PHA, and incubated at 37 °C in a CO₂-humidified incubator. Each lymphocytic cell culture was designated by the initials of the donor. After four days of growth, lymphocytes were spun at 400 g for 10 min and refed with complete medium and three drops of PHA. Cytogenetic analysis was performed on the fifth day by the chromosomal spreading technique of Boue (1981), standard Giemsa and RhG banding. One hundred consecutive metaphases were studied in each case.

Chromosomal analysis was also performed *in situ*. Isolated lymphocytes, after five days of growth in complete medium with PHA, were spun at 400 g for 10 min. The cell pellet in one ml medium, was vortexed to break cell nodules and plated in a glass petri-dish, within a circle, drawn by a diamond and paraffin pencil. Lymphocytes were allowed to plate at 37 °C in a CO₂-humidified incubator for two hours. Then the medium was decanted carefully with a pasteur pipette without disturbing the attached lymphocytes. Having the petri

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dish on a dry 37 °C incubator rack, prewarmed KCl (0.075 M) was added drop by drop on the attached lymphocytes, within the marked circle, and incubated for 25 min. Then KCl was carefully removed and alcoholacetic acid (3:1) was added and incubated at room temperature for 45 min. Lymphocytes were dried, stained with 6% Giemsa for eight min, dried and used for chromosomal analysis. This method was also applied to 3-hour and 2-day-old lymphocytic cultures in complete medium containing PHA.

Cytology

Two million lymphocytes, per case, were seeded in a glass petri-dish (10 cm diameter) containing 6 ml complete medium and two drops of PHA. The cultures were then incubated at 37 °C in a CO₂-humidified incubator for two to three hours to allow lymphocytic attachment. These cultures were fixed in 50% ethanol and stained by Papanicolaou, PAS and PAS-diastase.

Another set of cultures were fixed in 4% formaldehyde in phosphate-buffered saline and stained with Feulgen.

Immunocytochemistry

Attached lymphocytes (2 hours after isolation) were fixed in cold acetone (~20 °C) for 10 min and immunostained (Hsu et al., 1981) using antiserum against B-cell antigen (1:50, Dako J 1725), T-cell antigen (1:40, Dako M742) and haematopoietic progenitor cell CD34 (1:20, Dako M824).

Detection of calcium-activated neutral proteinase (CANP)

CANP was detected by the cytotoxicity caused by the CANP-inhibitor (Sigma P-0787). The CANP-inhibitor (CANP-1) cytotoxicity was assayed by the liquid medium short term culture method (Chang et al., 1989), and by the continuous presence of the inhibitor in the culture medium.

a) Liquid medium short term culture

Seventy thousand resting or PHA-activated lymphocytes were treated with 4 U/ml CANP-I in complete RPMI-1640 with 25 mM hepes, at 37 °C for one hour. The cells were then washed twice with complete medium, spun at 400 g for 10 min, resuspended in 2 ml complete medium, vortexed and incubated at 37 °C in a CO_2 -humidified incubator for 4 days. Cytotoxicity assessment was performed using the dye exclusion method of 0.4% trypan blue. The degree of cytotoxicity was calculated according to the formula:

$$Cytotoxicity(\%) = \left(1 - \frac{\text{Number of viable cells in the experimental group}}{\text{Number of viable cells in the control group}}\right) \times 100$$

b) Continuous presence of CANP-I in the culture medium

Isolated resting lymphocytes were inoculated in conical polypropylene test tubes, each with 7 x 10^4 cells per 2 ml complete medium. Two sets of cell cultures each received 0.5 U/ml or 1 U/ml CANP-I and PHA (4 drops/10 ml medium), another complete medium with PHA and the control just the complete medium. Duplicate cell cultures were used in each case. All cell cultures were incubated at 37 °C in a CO₂-humidified incubator for four days. Cytotoxicity assessment was performed every day using the trypan blue exclusion method. At the end of the experiment CANP-I PHA treated, and control cells were plated and smeared for Papanicolaou stain.

Preliminary life span of lymphocytic NVs

Five-day-old lymphocytic cell cultures growing in complete medium with PHA were split into 4 flasks. Two flasks received complete medium while the other two received complete medium and 3 drops of PHA per 10 ml medium. Cultures were refed once a week and were examined for cell viability by trypan blue once a week. Exact counts were not made.

Effect of PHA on human spermatozoa

Human spermatozoa were processed by the swimming test in Earle's Balanced Salt Solution (EBSS) supplemented with pyruvate, foetal bovine serum and antibiotics (complete EBSS). Motile (100%) spermatozoa were collected and spun at 400 g for 5 min. The pellet was resuspended in 10 ml complete EBSS containing three drops of PHA, plated in a glass petridish, incubated at 37 °C in a CO₂-humidified incubator for 2 hours and then fixed in 50% ethanol and stained by Papanicolaou method.

Results

Cytology-Morphology

Phase contrast microscopical examination showed that PHA-activated lymphocytes started plating immediately upon incubation, reaching maximum plating efficiency in two and a half hours. A large portion, but not all, of the lymphocytes were plated. After three hours of incubation detachment of lymphocytes was initiated. In 24 hours lymphocytes formed cell nodules attached on the surface of the culture vessel and in 48 hours all round cell nodules of different sizes were floating in the medium.

Cytology showed lymphocytic attachment by a fairly thin, transparent foamy cytoplasm. Attachment was followed by immediate cytoplasmic vesiculation. Cytoplasmic vesicles formed were rejected away from the lymphocyte, which was still left attached, with very

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Cytology of mechanically-dispersed five-day-old lymphocytic nodules showed lymphocytic aggregation and support by the extruded cytoplasmic vesicles (Fig. 8).

Resting lymphocytes were also plated and formed NVs but failed to form cell nodules after 5 days of cultivation.

Five-day PHA-activated lymphocytes were PAS, PAS-diastase positive, embedded in PAS, PAS-diastase positive ECM.

Detection of CANP

The CANP-I cytotoxicity on resting and PHA preactivated lymphocytes by the liquid medium short term culture method is shown in Table 1. Viability and growth of resting lymphocytes are not affected by one-hour treatment with 4 U/ml CANP-I.

The CANP-I (4 U/ml for one hour) caused 98% cytotoxicity to six-day PHA pre-activated lymphocytes.

 Table 1. Sensitivity of resting and preactivated lymphocytes to CANPinhibitor by the liquid medium short term culture method.

	CANP-I CITOTOXICITY (%)
Donors' resting lymphocytes	
L	0
Т	0
N	0
G	0
A	0
6-day pre-activated lymphocytes	
L	98
Т	98
N	98
A	98
2-day pre-activated lymphocytes	
Т	43
G	35

Different donors' lymphocytes are designated as L, T, N, G and A.



Fig. 3. Different forms of NVs from different donors. Arrow points at a common lymphocytic type in all donors. Papanicolaou. x 1,000



Fig. 4. Different head size NVs and lymphocytic subsets. Papanicolaou. x 1,000 phase contrast.

little perinuclear cytoplasm. The cytoplasmic vesicles remained attached on the culture vessel surface for 24 hours (Fig. 1).

The lymphocytic nucleus was then elongated, pulling along very little or no cytoplasm, away from the mother cell. Mother and daughter cells remained connected for a while with a cytoplasmic or nuclear bridge which was eventually fragmented giving rise to one, two or three lymphocytic NVs, which were then detached and resuspended in the culture medium (Figs. 2-5). While mother and daughter cells were connected, the mother cell could be identified by the cytoplasmic vesicles near it and were usually of round and larger nucleus (Fig. 4). Some lymphocytes extruded nuclear fragments by budding. Buds were of different size and number per lymphocyte (Fig. 5). NVs were also of different nuclear head size and tail length. Although the majority of NVs within the lymphocytic culture of the same donor or among donors are not identical there was one repetitive morphology which was recognised within the lymphocytic culture of the same and different donors. This lymphocytic type is shown in Figure 3, consisting of foamy cytoplasm and triangular nucleus. During its division, mother cell cannot be distinguished from daughter cell and were connected by a nuclear bridge. Feulgen stain of this type of lymphocyte showed thin fibrous chromatin (Fig. 6).

The different, variable size and nuclear morphology of lymphocytic NVs is clearly shown by Feulgen stain. NVs consist of condensed DNA or fine fibrous chromatin or DNA organized into rings. The nuclear purple-coloured bridge of NV is apparent as well as the attached cytoplasmic vesicles of green colour (Figs. 6, 7). Cytoplasmic buds are extruded in the form of a bud with tail.

Detached or attached NVs can easily be identified, under phase contrast microscopy, by their spermatozoalike morphology.



Fig. 1. Attached PHA-activated lymphocytes, consisting of a large irregular nucleus and thin, transparent, foamy cytoplasm. Cytoplasmic vesiculation has been initiated. Papanicolaou. x 1,000

Fig. 2. NV formation. Mother and daughter cells are connected by an almost fragmented cytoplasmic bridge. Papanicolaou. x 1,000. Phase contrast. Insets: Attached and semidetached NVs. Papanicolaou. x 1,000 phase contrast





Cytotoxicity was less in 2-day PHA pre-activated lymphocytes.

Cytotoxicity caused by the continuous presence of CANP-I in the culture medium is shown in Figure 9. Cytotoxicity of CANP-I on resting lymphocytes appeared on the second day of treatment. Cytotoxicity on PHA activated lymphocytes appeared on the first day of treatment. Cytotoxicity was CANP-I dose dependent and more pronounced in the presence of PHA.

Cytology of PHA CANP-I treated, necrotized lymphocytes showed swollen, vacuolated cells containing highly condensed, pyknotic and shrunk nucleus (Fig. 10).

Immunocytochemistry

A portion of lymphocytic NVs with round morphology showed positive immunoreactivity to T-Ag (Fig. 11). T-Ag-negative NVs were produced by T-Ag-positive lymphocytes and T-Ag-positive NVs were formed by T-Ag-negative lymphocytes (Fig. 12).

Very few B-Ag-positive lymphocytes produced NVs. B-lymphocytes appeared mainly in the round form with attached buds. Budding was also observed in T-Agpositive lymphocytes (Fig. 12). Buds consisted of a small nuclear head and very short tail. Moreover non-T, non-B NVs were formed by non-T or non-B-lymphocytes. The particular lymphocytic type of Figure 3 is a non-B, non-T type. Lymphocytes and their NVs showed negative immunoreactivity to haematopoietic progenitor cell CD34.

Preliminary lifespan of lymphocytic NVs

Lymphocytic NVs in the absence of PHA failed to form cell nodules and were gradually necrotized thirteen days after lymphocytic culture initiation. On the contrary lymphocytic NVs in the presence of PHA formed cell nodules and complete lymphocytic death was accomplished in one month.



Fig. 5. Extrusion of nuclear material by multiple budding. Buds contain large to minute nuclear material. Papanicolaou. x 1,000

Fig. 6. The common lymphocytic type showing fibrous chromatin, foamy cytoplasm and nuclear bridge during NV formation. Feulgen. x 1,000



Cytogenetic analysis

In situ chromosomal analysis exhibited NVs containing condensed or intact chromosomes (Fig. 13). NVs after KCl treatment, although swollen, kept the head with tail morphology. Chromosomes, in most NVs, were contained in the head. Some NVs contained single chromosomes in a row along the tail (Fig. 14, 14b). A hypohaploid set of chromosomes observed nearby NVs of condensed nuclear material was considered belonging to a separate NV (Fig. 14a). Some metaphases exhibited identical chromosomes to those of human gametocyte metaphases such as 0 chromosomes and S chromatids (Fig. 14c,d). There were chromosomes (Chromosome 3) of identical texture and shape to those of human aneuploid parthenote embryo (Plachot et al., 1987). Different size of ring chromosomes was identified within the same lymphocytic population (Fig. 15). Hypohaploid set of chromosomes were often extruded by a lymphocytic nucleus (Fig. 15). Part of one chromosome was

located inside the lymphocytic nucleus and the other outside. More NV condensed heads extruding intact chromosomes are shown in Figure 16 insets. Single chromosomes of different texture, within the same microscopical field, are considered to belong to two different NVs (Fig. 16). Most of the spread countable in situ metaphases were hypodiploid and hypohaploid with fewer diploid (Fig. 17). Some metaphase chromosomes were short and thick (Fig. 17a) identical to those of normal human male and female pronuclei (Plachot et al., 1987). Intact single chromosome observed by a nucleus away from a metaphase was considered to belong to an NV (Fig. 17c). Some NVs contained fibrous chromatin (Fig. 18a,b) and more hypodiploid NVs are shown in Fig. 18. In conclusion, the in situ chromosomal analysis showed that NVs carry hypodiploid, hypohaploid and even single chromosomes. Hence, the numerical chromosomal aberrations of normal lymphocytes are not technically induced.



Fig. 7. NVs with condensed nuclear heads of different size. Arrow points to an NV containing small nuclei. Green cytoplasmic vesicles can be seen attached. Feulgen. x 1,000



Fig. 8. Five-day-old PHA-activated lymphocytic culture. Mechanically dispersed cell nodules show cytoplasmic vesicles holding the cells together. Papanicolaou. x 1,000

Complete chromosomal analysis and study was performed by the chromosomal spreading technique. Single, hypohaploid and hypodiploid sets of chromosomes were counted separately, as they belonged to different NVs shown by the *in situ* analysis. Single or double chromosomes near a hypodiploid or hypohaploid metaphase, of the same texture as the metaphase's chromosomes, were counted as part of the metaphase, in order to avoid artefacts of the chromosomal spreading technique. Chromosomal sets of one to ten chromosomes were counted as separate sets, when observed away from a metaphase by two microscopical fields of the objective 20. Detailed chromosomal analysis is shown in Table 2. Among donors, 28 to 49% of the metaphases are diploid,

Table 2. Numerica	l analysis oʻ	f lymphocyti	c metaphases
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DIPLOIDY (%)	HYPODIPLOIDY AND	HYPODIPLOID AND HYPOHAPLOID SETS OF				
	HYPOHAPLOIDY (%)	1-10 chromosomes	11-20 chromosomes	21-30 chromosomes	31-40 chromosomes	41-45 chromosomes
35	65	61	2	1	1	0
30	70	63	2	1	3	1
44	56	45	3	1	2	5
28	72	58	5	6	3	0
49	51	43	2	0	1	5
29	71	70	1	0	0	0
	DIPLOIDY (%) 35 30 44 28 49 29	DIPLOIDY (%) HYPODIPLOIDY AND HYPOHAPLOIDY (%) 35 65 30 70 44 56 28 72 49 51 29 71	DIPLOIDY (%) HYPODIPLOIDY AND HYPOHAPLOIDY (%) 1-10 chromosomes 35 65 61 30 70 63 44 56 45 28 72 58 49 51 43 29 71 70	DIPLOIDY (%) HYPODIPLOIDY AND HYPOHAPLOIDY (%) HYPODIPLOID 1-10 chromosomes HYPODIPLOID 1-20 chromosomes 35 65 61 2 30 70 63 2 44 56 45 3 28 72 58 5 49 51 43 2 29 71 70 1	DIPLOIDY (%) HYPODIPLOIDY AND HYPOHAPLOIDY (%) HYPODIPLOID AND HYPOHAPL chromosomes HYPODIPLOID AND HYPOHAPL 21-30 35 65 61 2 1 30 70 63 2 1 44 56 45 3 1 28 72 58 5 6 49 51 43 2 0 29 71 70 1 0	DIPLOIDY (%) HYPODIPLOIDY AND HYPOHAPLOIDY (%) HYPODIPLOID AND HYPOHAPLOID SETS OF chromosomes HYPODIPLOID AND HYPOHAPLOID SETS OF chromosomes 35 65 61 2 1-30 31-40 35 65 61 2 1 1 30 70 63 2 1 3 44 56 45 3 1 2 28 72 58 5 6 3 49 51 43 2 0 1 29 71 70 1 0 0

Different donors' lymphocytes are designated as L, T, E, S, N, A. Five female (F) and one male (M) were used.



Fig. 9. Effect of calcium-activated neutral proteinase inhibitor (CANP-I) on cell density of resting and activated lymphocytes.●: resting lymphocytes; ∆: resting lymphocytes+PHA; □: resting lymphocytes+1 U/ml CANP-I; ○: resting lymphocytes+PHA+0.5 U/ml CANP-I; ■: resting lymphocytes+PHA+1 U/ml CANP-I.



Fig. 10. Necrotized lymphocytes by CANP-I are swollen with vacuolated cytoplasm and pyknotic shrunk nucleus. Abundant cytoplasmic vesicles are apparent. Papanicolaou. x 1,000

and 51 to 72% are hypodiploid or hypohaploid sets of chromosomes. Among donors, 43 to 70% of chromosomal hypohaploidies consisted of one to ten chromosomal sets. Within the same donor there was not any repetitiveness of a particular hypodiploid or hypohaploid set of chromosomes. The repetitiveness of each chromosome in the hypohaploid sets of one to ten chromosomes for each donor, is shown in Table 3 and in sets of one to three chromosomes in Table 4. Sets of 1 to 3 chromosomes constituted 50-60% out of the total chromosomes counted in sets of 1 to 10, per donor, with the exception of the only male donor (N), where only 34% were sets of 1 to 3 chromosomes.

Haploid and hypohaploid sets of chromosomes are shown in Figures 19 and 20. Extrusion and release of chromosomes from a lymphocytic nucleus were also observed by the chromosomal spreading technique (Fig. 20). A representative diploid metaphase is shown in Figure 21. *In situ* and chromosomal spreading techniques



Fig. 11. Positive immunoreactivity, of 3-hour cultivated lymphocytes, to T-Ag. Round and budding lymphocytes show positivity to T-Ag. T-Ag. \times 1,000

were also applied to resting lymphocytes after 4 days of cultivation. No metaphases were observed. These cultures produced nuclear vlimata consisting of only pyknotic nuclear heads. NVs carrying chromosomes were not observed.

Identification of lymphocytic NVs in vivo

Lymphocytes were morphologically identified in a tissue section from human undifferentiated ovarian carcinoma (Fig. 22). They were observed in the form of different sized NVs inside the malignant cells, surrounded by a clear halo (Fig. 22 insets).

Effect of PHA on human spermatozoa

PHA caused immediate agglutination of spermatozoa into large clumps which were vibrating. Clumped spermatozoa kept their motility in the form of vibration (Fig. 23).



Fig. 12. Two formed NVs, one T-Ag negative and the other T-Ag positive. T-Ag. x 1,000. Insets: Gain or loss of T-antigenicity by either mother or daughter cell during NV formation. T-Ag. x 1,000

Table	3.	Repetitive	ness	of	each	chromosome	in	hypohaploid
chromosomal sets of 1 to 10 chromosomes.								

 Table 4. Repetitiveness of each chromosome in hypothaploid chromosomal sets of 1 to 3 chromosomes.

CHROMOSOME No.	DONOR					
	E	Т	Е	S	Ν	Α
X	6	6	6	7	1	7
Y	0	0	0	0	0	0
1	5	8	4	12	8	8
2	18	10	7	2	11	8
3	2	7	3	7	6	9
4	13	5	6	4	9	9
5	0	6	5	5	8	9
6	9	4	7	6	11	5
7	9	8	5	5	8	9
8	3	4	8	3	5	11
9	5	6	5	7	6	4
10	10	5	7	7	6	3
11	9	6	10	10	7	8
12	5	9	7	7	6	5
13	7	5	9	3	3	6
14	9	7	6	4	5	2
15	4	4	1	4	4	1
16	13	5	7	4	3	4
17	4	4	3	5	2	8
18	5	4	3	3	5	3
19	4	8	1	5	4	5
20	4	5	1	4	2	1
21	3	1	4	3	6	6
22	2	4	8	10	0	6
Total chromosomes	149	130	123	127	124	132

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CHROMOSOME No.	DONOR						
	L	Т	Е	S	N	Α	
Х	3	4	3	7	0	2	
Y	0	0	0	0	0	0	
1	3	5	2	9	4	6	
2	14	6	5	1	2	5	
3	1	7	2	3	1	3	
4	6	4	6	2	2	3	
5	0	4	3	3	4	7	
6	6	2	6	3	6	1	
7	4	7	2	2	1	3	
8	1	1	5	1	2	6	
9	1	2	3	5	1	1	
10	4	1	6	4	2	2	
11	4	2	5	7	3	5	
12	3	6	4	- 4	3	4	
13	4	2	4	3	1	4	
14	5	3	3	2	2	2	
15	2	2	1	3	1	1	
16	5	3	3	0	2	2	
17	2	2	1	2	0	4	
18	4	4	1	2	3	2	
19	2	4	0	2	1	3	
20	3	2	1	2	2	0	
21	0	1	2	2	0	2	
22	1	2	7	6	0	2	
Total chromosomes	78	76	75	75	43	70	



Fig. 13. In situ cytogenetic analysis showing different sized NVs carrying chromosomes or condensed nuclear head (arrow) wihin the same N lymphocytic population. Giemsa.

Differentiation of lymphocytes into NVs



Fig. 14. In situ cytogenetic analysis. Chromosomes are carried along the tail of one NV (short, thick arrow). Inset a: An NV with condensed nuclear head and a hypohaploid set of 4 chromosomes by its tail. Inset b: A semidetached NV carrying chromosomes along the tail. Insets c, d: Two metaphases exhibiting 0 chromosome and S chromatids in N lymphocytes (arrows). Giemsa.



Fig. 15. *In situ* cytogenetic analysis. meiotic extrusion of hypohaploid chromosomal set. Insets: 0 chromosomes in hypohaploid chromosomal sets and extrusion. Chromosome 3 is identical to that of human aneuploid parthenote embryo (arrows). Glemsa.



Fig. 17. In situ cytogenetic analysis. A 45-chromosome metaphase. Inset a: A diploid metaphase of short, thick chromosomes. Inset b: A diploid meiotic metaphase showing 0 chromosome (arrow). Inset c: A condensed nuclear head NV, a metaphase and an extruded chromosome in the same field. Inset d: A metaphase of 35 chromosomes. Giemsa.



Differentiation of lymphocytes into NVs

Fig. 18. In situ cytogenetic analysis showing hypodiploid NVs. Insets a, b: NVs containing fibrous chromatin. Giemsa x 1,200



Fig. 19. Cytogenetic analysis by chromosomal spreading technique. A haploid metaphase. Insets: Sets of one, two and three chromosomes away from other metaphases. RhG banding-Giemsa.

Discussion

This study shows that lymphocytes differentiation into NVs under the influence of PHA. Lymphocytes attach on the culture vessel surface, mainly for support, reject the cytoplasm in the form of cytoplasmic vesicles, and then nuclear division follows by extension of a nuclear and or cytoplasmic bridge that connects mother with daughter cell. Separation is accomplished by fragmentation. Lymphocytic NVs, like malignant NVs (Logothetou-Rella, 1993a), exhibit the morphology of a spermatozoo but differ in the nuclear head size and tail length. The smallest sized NV reaches that of a bud. Among the same or different lymphocytic population, there was only one common lymphocytic type and NV identified by its foamy transparent cytoplasm and triangular nucleus. Lymphocytic NVs and cytoplasmic vesicles, once formed, detach from the culture vessel surface, organize and form large cell nodules. NVs in the cell nodules are held together by the cytoplasmic vesicles enhanced by PHA. Long term survival of NVs is directly dependent upon anchorage offered by the cell nodule under the influence of PHA. NVs, in the absence of PHA, fail to form cell nodules and survive a short period of time, in vitro.

Immunocytochemistry showed that mainly Tlymphocytes and, to a lesser extent, B-lymphocytes form NVs. Buds were mainly formed by B-lymphocytes and less by T-lymphocytes. During T-lymphocyte differentiation into NVs, T-antigenicity was gained or lost from the daughter or mother cell or maintained by mother and daughter cells. Since all lymphocytes were negative for CD34, the lymphocytes undergoing differentiation are not myeloid stem cells.

In situ cytogenetic analysis showed a low percentage of diploid and a greater percentage of hypodiploid metaphases. The presence of 0 chromosomes, S chromatin, fibrous chromatin, the extrusion of chromosomes, and the hypodiploid and hypohaploid chromosomal sets documented that NVs are the result of meiosis of diploid lymphocytes. 0 chromosomes are distinctive at diakinesis and S chromatin at the second meiotic metaphase of an oocyte (Daniel, 1978). It is evident that diploid metaphases are the result of lymphocytes undergoing mitosis or symmetrical meiosis while hypodiploidies and hypohaploidies are the result of lymphocytes undergoing meiosis. Among tested donors, 30 to 49% of activated lymphocytes undergo symmetrical meiosis or mitosis while 51 to 71% undergo assymmetrical meiosis. NVs do carry chromosomes mostly in hypohaploid sets. All NVs, within the same lymphocytic population, are cyto-genetically different. There were no repetitive hypo-diploid or hypohaploid chromosomal sets within the same culture. Repetitiveness among donors, and within the same lymphocytic population, were only numerical. Chromosomes were mainly identified in the NV head and along the tail. Comparison of the in situ cytogenetic analysis with that of the chromosomal spreading technique documents that hypodiploidies and hypohaploidies are not a result of artefact, believed to occur by the technique, but are products of meiosis. In addition cytogenetics are well correlated with the morphology of lymphocytes. The fact that lymphocytic differentiation into NVs by meiosis is initiated and enhanced by PHA documents that PHA stimulates the process of meiosis.



FIg. 20. Chromosomal spreading technique also shows meiotic extrusion of chromosomes. Inset: A pair of chromosomes away from a metaphase. Giemsa. Lymphocytic NVs are sensitive to CANP-I. One-hour treatment of resting lymphocytes with CANP-I does not cause any cytotoxic effect. On the contrary, such treatment to PHA pre-activated lymphocytes causes 98% cytotoxicity. This difference is attributed to the difference in NV content between resting and preactivated lymphocytic cultures. Resting lymphocytic cultures do not contain any NVs and therefore are not affected. PHA pre-activated lymphocytes had been stimulated and had already produced NVs which are necrotized by one hour treatment with CANP-I. The lower percentage of CANP-I cytotoxicity shown by the 2-day pre-activated lymphocytes is attributed to necrotization of the already formed NVs, while the newly formed NVs or resting lymphocytes during the 4 day incubation are not affected. The longer lymphocytes are stimulated by PHA the more NVs are formed. CANP-I cytotoxicity to 6-day-old lymphocytes is almost 100% which means that by the sixth day of PHA stimulation all diploid lymphocytes have undergone meiosis and differentiated into hypodiploid and hypohaploid NVs. Cytotoxicity caused to resting lymphocytes, by the continuous presence of the CANP-I in the culture medium, is delayed by 24 hours when compared to PHA-activated lymphocytes. Again this different effect is explained in terms of NV production.

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3-3	4-8			
19	20			
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21	22		Y	

Fig. 21. A representative lymphocytic diploid metaphase by chromosomal spreading technique. RhG banding-Giemsa.

It takes 24 hours for resting lymphocytes to produce NVs sensitive to CANP-I. Stimulation of resting lymphocytes to produce NVs by the continuous presence of CANP-I cannot be excluded. In PHA-activated, CANP-I treated lymphocytes, NV production and necrotization may occur simultaneously since stimulation and inhibition co-exist. These results document that CANP-I inhibits the survival but does not inhibit the production of NVs. This effect is CANP-I dose dependent. Consequently survival of NVs is directly linked with the presence of Ca⁺⁺-activated neutral proteinase and CANP involvement is associated with meiosis.

CANP and CANP-I located intracellulary, of large molecular weight cannot pass through the cell membrane (Melloni et al., 1984). A mode of action of CANP-I is proposed as follows: cytoplasmic vesiculation and extrusion by lymphocytes results in the exit of CANP and its substrate (glycosaminoglycans) extracellularly. CANP-I, once in contact with the extracellular CANP- substrate, breaks down from 240-KDa to its tetramer subunits of 60-KDa. The inhibitor subunits can then pass through cellular and nuclear membrane to inactivate intra NV content of CANP. This is probably, why necrotized lymphocytes appear swollen and vacuolated after CANP-I treatment. Activation of both CANP and CANP-I are dependent on high Ca⁺⁺ concentration. Such elevated free intracellular calcium had already been demonstrated in PHA activated lymphocytes (Knapp et al., 1988).

Agglutinated spermatozoa in the presence of PHA, document the presence of PHA receptors on spermatozoa. Lymphocytic NVs and human spermatozoa exhibit the following similarities: both have the head with tail morphology; are haploid cells produced by meiosis; agglutinate by PHA; have short life span outside of the body; contain CANP (Logothetou-Rella, 1991), high Ca⁺⁺; and are parasites. Spermatozoa are considered parasites because their survival and propagation depends upon their invasion (penetration) into the host cell, «the oocyte». Lymphocytes have been observed intracellularly, in target cells *in vitro*, altering



Fig. 22. Histological picture of undifferentiated ovarian carcinoma. H-E. x 40. Insets: Intact lymphocytes and NVs are located intracellularly. H-E. x 1.000



Fig. 23. Agglutinated human spermatozoa by PHA. Papanicolaou. x 100. Inset. x 1,200

the phenotype of the target cells from fusiform to adenoid (Logothetou-Rella, 1993b). Phenotype though change is subsequent to genotype alteration. In that study lymphocytic degeneration occurred after or during genotype and phenotype alteration of target cells. In addition, intracellular location of lymphocytic NVs in malignant tissue sections strengthens their parasitic property. This lymphocytic property enables lymphocytes to involve into a new mechanism of cellular immunity which is successfully progressing in our laboratory.

In conclusion, lymphocytes differentiate to aneuploid NVs by meiosis which involves CANP. CANP inhibitors, besides being anticancer, antiviral and spermicidal agents (Logothetou-Rella, 1991), can also be the agents for autoimmune disease therapy.

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